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Fecal bacteria and short-chain fatty acids in irritable bowel syndrome: Relations to subtype

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Abstract

Background: The relationship between gut microbiota and irritable bowel syndrome (IBS) subtype is unclear. We aimed to explore whether differences in fecal bacteria composition and short-chain fatty acid (SCFA) levels were associated with subtypes and symptoms of IBS.

Methods: All participants delivered fecal samples and self-reports on IBS Symptom Severity Score (IBS-SSS), Bristol Stool Scale (BSS), and Gastrointestinal Symptom Rating Scale (GSRS). Fecal bacteria composition was assessed by the GA-map® Dysbiosis Test based on 16S rRNA sequences of bacterial species/groups. SCFAs were analyzed by vacuum distillation followed by gas chromatography.

Key Results: Sixty patients with IBS were included (mean age 38 years, 46 [77%] females): Twenty-one patients were classified as IBS-D (diarrhea), 31 IBS-M (mixed diarrhea and constipation), and eight IBS-C (constipation). Forty-two healthy controls (HCs) (mean age 35 years, 27 [64%] females) were included. Patients had a significantly higher relative frequency of dysbiosis, lower levels of Actinobacteria, and higher levels of *Bacilli* than HCs. Eight bacterial markers were significantly different across IBS subgroups and HCs, and 13 bacterial markers were weakly correlated with IBS symptoms. *Clostridia* and *Veillonella* spp. had a weak negative correlation with constipation scores (GSRS) and a weak positive correlation with loose stools (BSS). Diarrhea scores (GSRS) and looser stool (BSS) were weakly correlated with levels of total SCFAs, acetic and butyric acid. Levels of total SCFAs and acetic acid were weakly correlated with symptom severity (IBS-SSS).

Conclusions & Inferences: Patients with IBS had a different fecal bacteria composition compared to HCs, and alterations of SCFAs may contribute to the subtype.

KEYWORDS
brain-gut-microbiota axis, *clostridia*, constipation, diarrhea, IBS subgroups, IBS symptoms, *Veillonella* spp.

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1 | INTRODUCTION

Irritable bowel syndrome (IBS) is a disorder of brain-gut interaction[1] affecting about 4%–10% of the general population.[2] The diagnosis is based on clinical symptoms (recurrent abdominal pain for the last 3 months related to defecation and associated with a change in frequency or form of stool[1]) and exclusion of organic diseases. IBS is divided into four subgroups based on predominant stool pattern: IBS-D (diarrhea), IBS-M (mixed: alternating constipation and diarrhea), IBS-C (constipation), or IBS-U (unclassified).[1] The pathophysiology of IBS is complex and may involve dysbiosis, as defined by an imbalance in bacterial composition, low-grade inflammation, visceral hypersensitivity, impaired gut barrier integrity and function, diet, genetics, and epigenetics.[3] Gut bacteria deviating from the healthy state can result in transient or permanent dysbiosis, which is associated with several intestinal and extra-intestinal diseases including IBS and inflammatory bowel disease (IBD).[4] However, the search for a clinical disease-specific gut bacterial profile in patients with IBS has demonstrated to be challenging. Indeed, multiple inconsistent and contradictory results have been shown in case–control studies in IBS.[5,6] Reviews reveal that patients with IBS may both display a different fecal bacterial profile, or a decreased or not a different bacterial diversity (dysbiosis), compared to healthy controls (HCs). Some have observed increased levels of Firmicutes (phyla), reduced Bacteroidetes (phyla) and an increased Firmicutes/Bacteroidetes ratio, increased *Clostridia* and *Clostridiales* and decreased *Bacteroidea* and *Bacteroidales* in patients with IBS.[5] Others have found increased levels of *Enterobacteriaceae* (family), *Lactobacillaceae* (family) and *Bacteroides* (genus), and decreased levels of uncultured *Clostridiales* I, *Faecalibacterium* (genus, including *Faecalibacterium prausnitzii*) and *Bifidobacterium* (genus).[6] However, the microbiota diversity in IBS is either decreased or not different from HCs.[5,6]

Therapeutically manipulating dysbiosis through diet, prebiotics, probiotics, antibiotics, and fecal microbiota transplantation (FMT) has shown promising results in alleviating IBS symptoms.[7] Some studies have shown to reduce the severity of global IBS symptoms, abdominal pain, and increase quality of life (QoL) after FMT from healthy donors.[8] The mechanisms behind the interplay between gut bacteria and IBS symptoms are still unknown, but many explanations have been presented[9] including the role of short-chain fatty acids (SCFAs) in symptom generation of IBS.[10]

SCFAs, along with carbon dioxide (CO_2), methane (CH_4), and hydrogen (H_2), are products of fermentation by gut microbiota.[11] The major SCFAs (acetic, butyric, and propionic acid) have several central functions for colonic health as energy substrates for colonocytes, in maintaining intestinal barrier integrity and protection from intestinal inflammation, and importantly, they might directly or indirectly communicate via the brain–gut–microbiota axis.[12] Fecal SCFAs levels are found to differ between patients with IBS, across IBS subtypes, and HCs.[10,13,14] Treatment with FMT has been shown to increase SCFAs associated with a significant reduction in IBS symptom severity, and butyric acid is observed to be significantly inversely correlated with IBS symptom severity.[15] On the contrary, IBS patients with high acetic and propionic acid levels present significantly worse gastrointestinal symptoms, lower QoL, and more negative emotions in comparison with those with lower levels and HCs.[10]

Key points

- Patients with IBS had significantly higher relative frequency of dysbiosis, lower levels of Actinobacteria and higher levels of *Bacilli* compared to healthy controls.
- Eight bacterial markers were significantly different across IBS subgroups, and 13 bacterial markers were weakly correlated with IBS symptoms.
- *Clostridia* and *Veillonella* spp. had a weak negative correlation with constipation scores (GSRS) and a weak positive correlation with loose stools (BSS).
- Diarrhea scores (GSRS) and looser stool (BSS) were weakly correlated with levels of total SCFAs, acetic and butyric acid.
- Levels of total SCFAs and acetic acid were weakly correlated with symptom severity (IBS-SSS).

Overall, findings regarding fecal SCFAs and bacteria and its impact on symptoms in patients with IBS are inconsistent and their involvement in the pathophysiology is unclear. To address this, the multifaceted interdisciplinary Bergen BrainGut project was designed to stratify the complex interaction between the brain, gut, and microbiota in patients with IBS.[4] In this sub-study, we aimed to (i) investigate differences in fecal bacterial profile and fecal SCFAs in patients with IBS, IBS subtypes, and HCs; and (ii) investigate associations between fecal bacterial profiles and fecal SCFAs content with IBS symptoms.

2 | MATERIALS AND METHODS

2.1 | Participants and study design

Adult participants with IBS and HCs of both genders, aged 18–65 years, were consecutively recruited from April 2019 to June 2021 from the IBS outpatient clinic at Haukeland University Hospital, Bergen, Norway, and via media and posters at the University of Bergen.

All participants were screened for organic diseases by phone with a study nurse. Patients with IBS had to fulfill the Rome IV criteria for IBS and score >175 points corresponding to "moderate to severe symptoms" on the IBS-Severity Scoring System (IBS-SSS)[16] and underwent examinations such as ultrasonography, blood tests, sigmoidoscopy, and gastroscopy to exclude organic diseases.

Exclusion criteria included age <18 or >65 years, pregnancy, lactating, the presence of any organic disease such as celiac disease, IBD,

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Helicobacter pylori infection, diabetes, polycystic ovary syndrome, and neurological diseases such as Parkinson's disease, previously abdominal surgery except from appendectomy, treatment with systemic antibiotics the last 3 months and using medications affecting the gastrointestinal tract including treatment for depression and regularly use of analgesics. Participants following a vegan, vegetarian, or low FODMAP diet, or used probiotics within 3 weeks prior to inclusion, were also excluded. Lastly, participants were excluded if they had traveled outside Europe the last 3 weeks (due to risk of gastrointestinal infection), participated in any other simultaneous clinical study, or were living outside the Bergen area, or did not speak Norwegian. All patients with IBS who were avoiding any foods were informed to consume a typical Norwegian diet 3 weeks prior to participation in the study.

All participants gave written informed consent and were allowed to withdraw at any point without giving an explanation. The study was conducted according to the revised Declaration of Helsinki and approved by the Regional Committee for Medical and Health Research Ethics Southeast (reference number 2015/01621).

The design of this study has been described in detail in a previous publication.[17] In brief, eligible participants were included in an open, single-center, cross-sectional case–control characterization study. Fecal samples were collected and all questionnaires for symptom assessment were answered at one timepoint for all participants.

2.2 | Questionnaires for symptom assessment

All participants answered three questionnaires for symptom assessment. The Rome IV criteria were used as diagnostic criteria for IBS.[18]

IBS symptom severity was assessed using IBS Severity Scoring System (IBS-SSS).[16] The scale (possible range: 0–500) defines none (<75), mild (75 to <175), moderate (175–300), and severe (>300) symptoms.

The Gastrointestinal Symptom Rating Scale for IBS (GSRS-IBS) assessed gastrointestinal symptom severity for the last 7 days. All items were rated on a seven-point scale ranging from "1 = no discomfort at all" to "7 = very severe discomfort" and were clustered into five categories: pain, diarrhea, satiety, constipation, and bloating.[19]

Bristol Stool Scale (BSS) classified feces into seven consistency categories, with the highest scores[5–7] corresponding to loose stools and fast transit, while lower scores[1,2] represented hard stools and longer colonic transit time. Normal stool consistency was classified as number 3 and 4.[20]

2.3 | Fecal samples

Participants collected fecal samples at home using designated containers (Genetic Analysis [GA], Oslo, Norway). Samples were frozen immediately at −20°C and brought in a frozen state to the hospital at the next visit. Samples were stored in a −20°C freezer at the hospital until analysis.

2.4 | Analysis of fecal bacteria

An updated Luminex 200® method of the GA-map® Dysbiosis Test (Genetic Analysis Oslo, Norway) was used to assess fecal bacteria composition. This has been described in detail elsewhere.[4] In brief, the GA-map® Dysbiosis Test involves 48 bacterial markers detecting the 16S rRNA hypervariable regions V3-V9 for measurement of the relative abundance of bacteria according to the strength of fluorescent signal detection. In total, more than 300 species are covered. The test also algorithmically assesses fecal bacterial abundance and profile in comparison with a healthy population where a deviation in the gut bacteria composition from normobiosis is summarized in a Dysbiosis Index (DI) (0–5). DI ≤2 is characterized as normobiosis and DI of >2 is characterized as dysbiosis. The bacterial signal strength data are presented as median (IQ).

2.5 | Analysis of short-chain fatty acids

The method of SCFAs analysis has been described elsewhere in detail by Valeur et al.[21] In brief, 0.5 g of the fecal material was homogenized following the addition of water containing 3 mmol/L of 2-ethylbutyric acid (as internal standard) and 0.5 mmol/L of H_2SO_4: 2.5 mL of the homogenate was vacuum distilled. The distillate was analyzed by gas chromatography using a capillary column and quantified using internal standardization. Flame ionization detection was employed and the following SCFAs were analyzed: acetic, propionic, n-butyric, i-butyric, n-valeric, i-valeric, n-caproic, and i-caproic acids. The results were expressed in mmol/kg wet weight. Data are expressed as mean ± SD.

2.6 | Statistical analysis

Descriptive statistical analysis was performed using IBM SPSS Statistics for Windows, versions 25 and 29. R version 4.1.2 with stats with prcomp function was used to generate Principal component analysis (PCA) and qqplot 2 to generate figures. PCA was used to visualize the fecal bacteria data, showing the extent to which bacterial communities share branch length. Numerical variables were expressed by mean ± SD or median (inter-quartile range). For independent sample comparisons, the Mann–Whitney *U*-test was performed. To evaluate statistical differences for multiple groups the Kruskal–Wallis test with Dunn's post hoc test or Bonferroni adjustments for the alpha values was performed. For differences in dysbiosis index between HC and IBS, Fisher's exact test was used; and across IBS subgroups and HCs the chi-square and Fisher's exact tests with Bonferroni correction were used. Spearman's rho correlation analysis was used for correlation analysis, where $r = 0.0$–0.2 indicates a very weak correlation, $r = 0.2$–0.4 indicates a small, $r = 0.4$–0.6 indicates a moderate, and $r = 0.6$–0.8 indicate a strong correlation. $p < 0.05$ was considered statistically significant.

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3 | RESULTS

3.1 | Characteristics of participants

A total of 111 eligible participants were included in the study. However, one participant withdrew, one patient with IBS was excluded due to IBS-SSS < 175, and seven patients with IBS were excluded due to other diseases. Thus, a total number of 102 participants completed the first module of the study: 42 HCs and 60 patients with IBS (Figure 1). According to Rome IV phenotype definitions, 21 patients were classified as IBS-D, 31 patients as IBS-M, and eight patients as IBS-C.

3.2 | Gastrointestinal symptoms

All scores from IBS-SSS, GSRS, and BSS are presented in Table 1, and numbers for data included in the study are presented in Figure 1. Patients with IBS had moderate to severe symptoms (IBS-SSS > 175) and a significantly higher IBS symptom severity score compared to

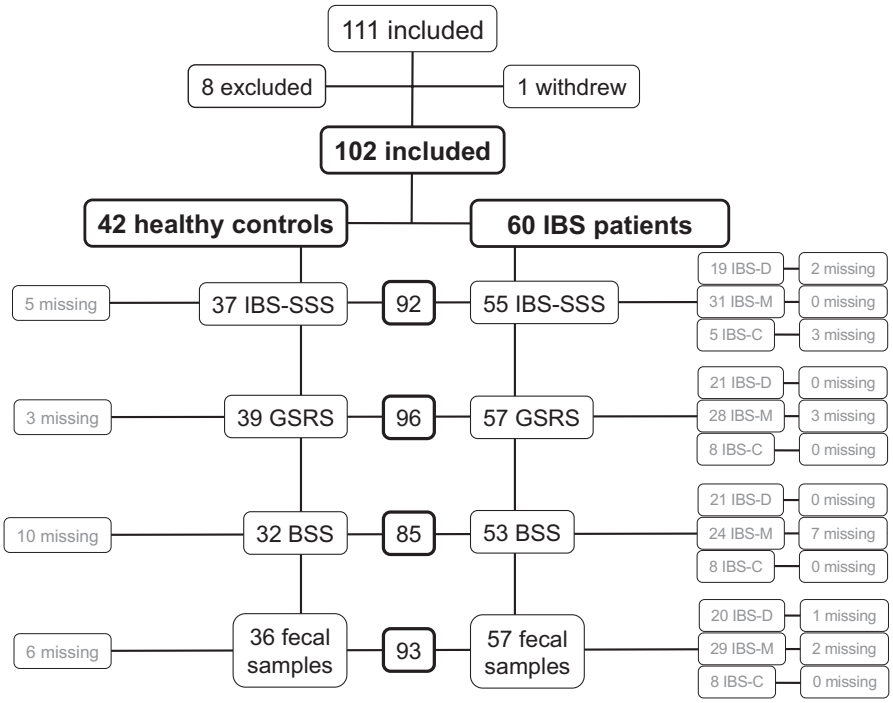

FIGURE 1 Numbers of participants and data included in the study. IBS: irritable bowel syndrome, IBS-D: IBS with predominant diarrhea, IBS-C: IBS with predominant constipation, IBS-M: IBS mixed–alternating constipation and diarrhea, IBS-SSS: IBS Symptom Severity Scale, GSRS: IBS Gastrointestinal Symptom Rating Scale, and BSS: Bristol Stool Scale. Data missing due to not delivered samples and incomplete filled out questionnaires.

TABLE 1 Characteristics of participants and scores (median [IQR]) of symptom questionnaires.

	HC	IBS	IBS-D	IBS-C	IBS-M
Total (*n*, %)	42 (41.2%)	60 (58.8%)	21 (35.0%)	8 (13.3%)	31 (51.7%)
Males (*n*, %)	15 (35.7%)	14 (23.3%)	8 (38.1%)	1 (12.5%)	5 (16.1%)
Females (*n*, %)	27 (64.3%)	46 (76.7%)	13 (61.9%)	7 (87.5%)	26 (83.9%)
Mean age ± SD	35.62 ± 12.3	38.0 ± 11.4	39.5 ± 12.1	42.1 ± 8.4	36.0 ± 11.4
IBS-SSS[a]	26 (9, 55)	258 (220, 325)	245 (211, 296)	283 (267, 342)	248 (220, 345)
GSRS[b]					
Abdominal pain	2.0 (2.0, 3.0)	8.0 (6.0, 9.0)	7.0 (6.0, 9.0)	8.0 (8.0, 8.75)	8.0 (6.5, 9.0)
Abdominal bloating	6.0 (4.0, 7.0)	13.0 (12.0, 16.5)	13.0 (10.5, 15.5)	14.5 (11.5, 16.0)	14.0 (12.0, 17.0)
Constipation	2.0 (2.0, 3.9)	6.0 (3.0, 8.0)	2.0 (2.0, 4.0)	8.5 (7.25, 12.5)	6.5 (5.0, 8.75)
Diarrhea	5.0 (4.0, 7.0)	15.0 (12.0, 17.0)	16.0 (12.0, 18.0)	11.0 (9.25, 16.0)	15.0 (12.0, 17.5)
Satiety	2.0 (2.0, 4.0)	4.0 (3.0, 7.0)	4.0 (3.0, 7.0)	4.5 (2.25, 6.0)	5.0 (3.0, 7.0)
BSS[c]	4.0 (2.0, 4.0)	4.0 (3.0, 6.0)	4.0 (3.0, 6.0)	3.5 (2.25, 4.75)	3.0 (2.23, 5.0)

[a]IBS Severity Scoring System.

[b]Gastrointestinal symptom Rating Scale.

[c]Bristol stool scale.

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HCs ($p < 0.001$). Patients with IBS also had significantly higher levels of abdominal pain, abdominal bloating, satiety, diarrhea, and constipation according to GSRS ($p < 0.001$ for all). There was no significant difference in symptoms and symptom severity across IBS subgroups, except from constipation (GSRS) being higher in IBS-C than in HCs, IBS-D, and IBS-M ($p < 0.014$ for all).

For stool consistency (BSS) there were no differences across IBS subgroups and the HC group except from IBS-D having a significantly higher median score (looser stool) in comparison to HC ($p = 0.043$). Two patients with IBS-D reported BSS = 1 and 2 (firm stool), and two IBS-C patients reported BSS = 5 and 6 (loose stool). Diarrhea (GSRS) correlated positively ($r = 0.180$, $p = 0.029$) and constipation correlated (nonsignificant) negatively ($r = -0.165$, $p = 0.055$) with the BSS score, with higher levels of self-reported diarrhea associated with looser stool and higher levels of self-reported constipation associated with harder stool. Abdominal bloating (GSRS) was significantly associated with abdominal pain (GSRS; $r = 0.754$, $p < 0.001$), with IBS patients having higher abdominal pain and bloating than HCs.

3.3 | Fecal bacteria

3.3.1 | Difference between the IBS and HC groups

The relative frequency of participants being dysbiotic was significantly higher in the IBS group (14/57; 24.6%) than in the HCs group (2/36; 5.6%), ($p = 0.023$) (Figure 2A). There were significant differences in dysbiosis frequency also when testing across the IBS subgroups and HCs ($p = 0.0003$), with a higher relative frequency of dysbiosis in IBS-D (8/20; 40%) and IBS-C (4/8; 50%) compared to HCs ($p = 0.002$ and $p = 0.007$, respectively, Fisher's exact test with Bonferroni adjusted alpha levels (0.05/6 = 0.0083); Figure 2B). No significant difference in dysbiosis frequency was observed between the IBS-M subgroup (2/29; 7%) and HC group, nor between the IBS-D and IBS-C subgroups (Figure 2B). Patients with IBS had a significantly lower bacterial signal strength of Actinobacteria (median 152.0 (50.5, 260.5), $p = 0.044$) and higher levels of *Bacilli* (median 116.0 (59.5, 215.0), $p = 0.038$) than HCs (median 209.0 (92.8, 429.2) and median 76.0 (54.4, 120.0), respectively) (Figure 3). Eight out of 48 bacterial markers were significantly different across IBS subgroups and HCs (Figure 4). PCA showed a similar bacterial profile for patients with IBS and HCs (Figure 5A) but regarding IBS subgroups the bacterial profile for IBS-D and IBS-C subgroups were different from the profiles in the HC group and the IBS-M subgroup (Figure 5B).

3.3.2 | Fecal bacteria in relation to IBS symptoms

Significant correlations between fecal bacteria and IBS symptoms (for both patients with IBS and HCs) are presented in Table 2. Higher levels of Firmicutes and *Actinomycetales* were associated with lower levels of abdominal pain (GSRS) ($r = -0.219$, $p = 0.038$ and $r = -0.249$, $p = 0.018$, respectively). Abdominal bloating (GSRS) was positively correlated with *Faecalibacterium prausnitzii* ($r = 0.217$, $p = 0.04$), and negatively correlated with Actinobacteria ($r = -0.227$, $p = 0.031$) and *Bifidobacterium* spp. ($r = -0.208$, $p = 0.05$). Lower levels of Firmicutes and higher levels of *Faecalibacterium prausnitzii* were associated with higher levels of satiety (GSRS; $r = -0.255$, $p = 0.015$ and $r = 0.230$, $p = 0.029$, respectively).

Diarrhea (GSRS) correlated positively with *Dorea* spp. ($r = 0.239$, $p = 0.023$) and *Proteobacteria* ($r = 0.229$, $p = 0.030$), and negatively with *Actinomycetales* ($r = -0.328$, $p = 0.002$) and Firmicutes ($r = -0.272$, $p = 0.009$). Constipation (GSRS) correlated negatively with Actinobacteria ($r = -0.221$, $p = 0.037$), *Clostridia* ($r = -0.226$, $p = 0.011$; Figure S1A), *Lachnospiraceae* ($r = -0.238$, $p = 0.024$) and *Veillonella* spp. ($r = -0.212$, $p = 0.045$; Figure S1C).

Looser stool consistency (BSS) was associated with higher levels of *Clostridia* ($r = 0.314$, $p = 0.004$; Figure S1B), *Dialister invisus* and *Megasphera micronuciformis* ($r = 0.234$, $p = 0.033$) and *Veillonella* spp. ($r = 0.312$, $p = 0.004$; Figure S1D), and associated with lower levels of *Bacteroides* spp. ($r = -0.252$, $p = 0.022$) and *Bacteroides* spp. & *Prevotella* spp. ($r = -0.328$, $p = 0.002$). No associations were found between IBS-SSS and any of the 48 bacterial markers (data not shown).

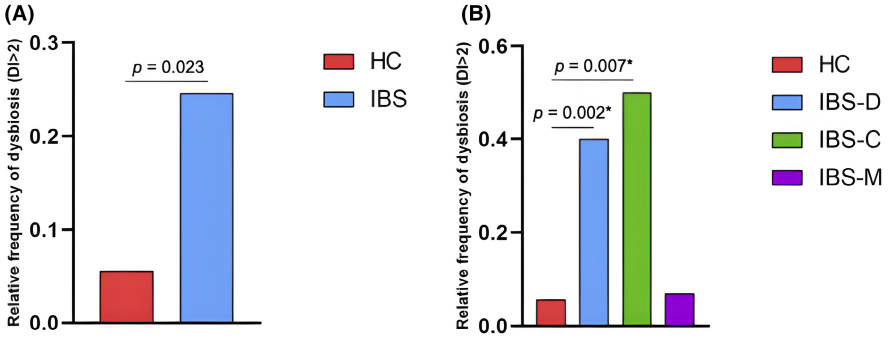

FIGURE 2 Relative frequencies of fecal dysbiosis (Dysbiosis Index, DI > 2) between (A) IBS (irritable bowel syndrome) and HCs (healthy controls) and (B) across IBS subgroups (IBS-D: IBS with predominant diarrhea, IBS-C: IBS with predominant constipation, and IBS-M: IBS mixed–alternating constipation and diarrhea) and HCs. *Statistically significant difference ($p < 0.0083$) after Bonferroni correction (0.05/6 = 0.0083).

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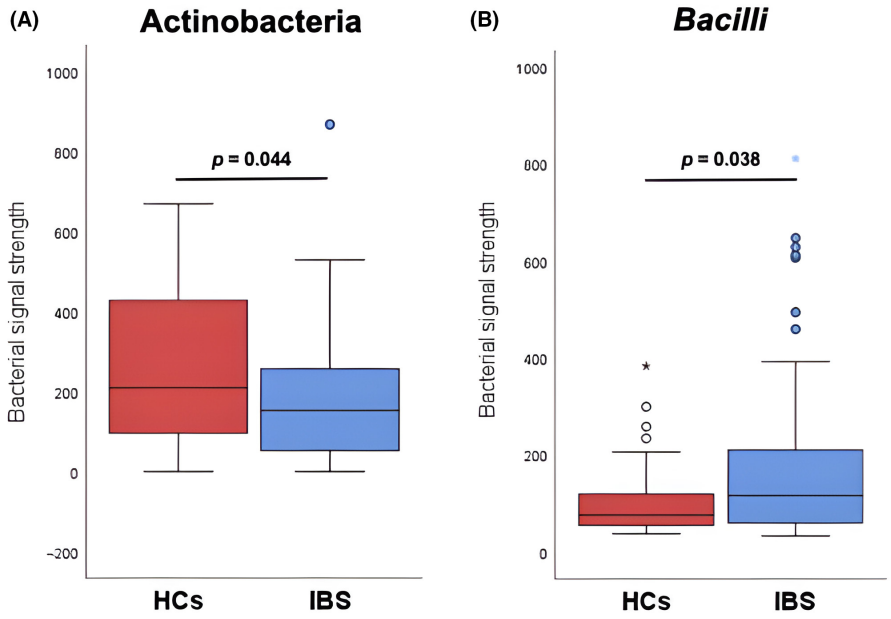

FIGURE 3 Boxplots showing bacterial signal strength of (A) Actinobacteria and (B) *Bacilli* between patients with IBS (irritable bowel syndrome) and HCs (healthy controls).

3.4 | Fecal short-chain fatty acids

3.4.1 | Difference between the IBS and HC groups

We found no significant difference in levels of fecal SCFAs (mmol/kg wet weight) between patients with IBS and HCs. In general, we observed tendencies for the total amount of SCFAs and the major SCFAs (acetic, propionic, and butyric acid) being higher in patients with IBS than in HCs (Figure S2A). The IBS-D subgroup displayed tendencies of higher levels and the IBS-C subgroup lower levels of the total amount of SCFAs and the major SCFAs, compared to HCs and the patients in the IBS-M subgroup (Figure S2B). However, these tendencies were not significant.

3.4.2 | Fecal short-chain fatty acid levels in relation to IBS symptoms

Correlations between SCFAs and IBS symptoms (for both patients with IBS and HCs) are reported in Table 2. Higher levels of total SCFAs and acetic acid were associated with higher symptom severity (IBS-SSS; $r=0.259$, $p=0.017$ and $r=0.238$, $p=0.029$, respectively; Figure 6A,D, respectively). There were no significant associations between fecal SCFAs and abdominal pain (GSRS), abdominal bloating (GSRS), and satiety (GSRS) (data not shown).

A higher level of diarrhea (GSRS) was associated with higher levels of total SCFAs ($r=0.222$, $p=0.036$; Figure 6B), acetic acid ($r=0.227$, $p=0.031$; Figure 6E) and butyric acid ($r=0.279$, $p=0.008$). A higher level of BSS (looser stool consistency) was associated with higher levels of total SCFAs ($r=0.229$, $p=0.037$; Figure 6C), acetic acid ($r=0.259$, $p=0.017$; Figure 6F) and butyric acid ($r=0.282$, $p=0.010$), and associated with lower levels of iso-butyric acid ($r=-0.244$, $p=0.026$) and iso-valeric acid ($r=-0.302$, $p=0.006$). No associations were found between constipation (GSRS) and SCFAs. However, scatterplots (not shown) indicated a trend (nonsignificant) towards a negative association between constipation and total amount of SCFAs ($r=-0.154$), acetic acid ($r=-0.140$) and butyric acid ($r=-0.163$). Correlation analysis (Spearman's rho) between SCFAs and relevant fecal bacterial markers is performed (Table S1).

4 | DISCUSSION

In the present study, we investigated the differences in fecal bacterial markers and SCFAs between HCs and patients with IBS and across IBS subgroups, and its correlations of fecal bacterial markers and SCFAs with IBS symptoms. The most prominent findings were the results showing increased levels of *Clostridia* and *Veillonella* spp. associated with loose stool, and reduced levels associated with constipation. Moreover, we found that higher levels of total SCFAs, acetic, and butyric acid were associated with diarrhea/loose stool, and total SCFAs and acetic acid were associated with increased symptom severity. Interestingly, correlation analyses indicated that acetic acid seemed to be a contributing factor for both symptom severity and looser stool in patients with IBS. We also found that *Bacilli* and Actinobacteria levels differed between patients with IBS and HCs, and eight out of the 48 bacterial markers (*Actinomycetales*, *Parabacteroides johnsonii*, *Bacilli*, *Faecalibacterium prausnitzii*, *Lactobacillus* spp. 2, *Streptococcus* spp., *Streptococcus* spp. 2, and *Enterobacteria*) differed across HCs and patients within IBS subgroups. No significant difference was observed in levels of SCFAs between the HC and the IBS group, nor between HCs and across IBS subgroups. Correlations with both bacterial markers and SCFAs with IBS symptoms and symptom severity were present.

Our results support evidence for a dysbiotic bacterial composition in patients with IBS compared to HCs. Although PCA revealed that the fecal bacterial profile in patients with IBS and HCs were mostly overlapping, two fecal bacterial markers separated IBS from HCs: patients with IBS showed higher signal strength

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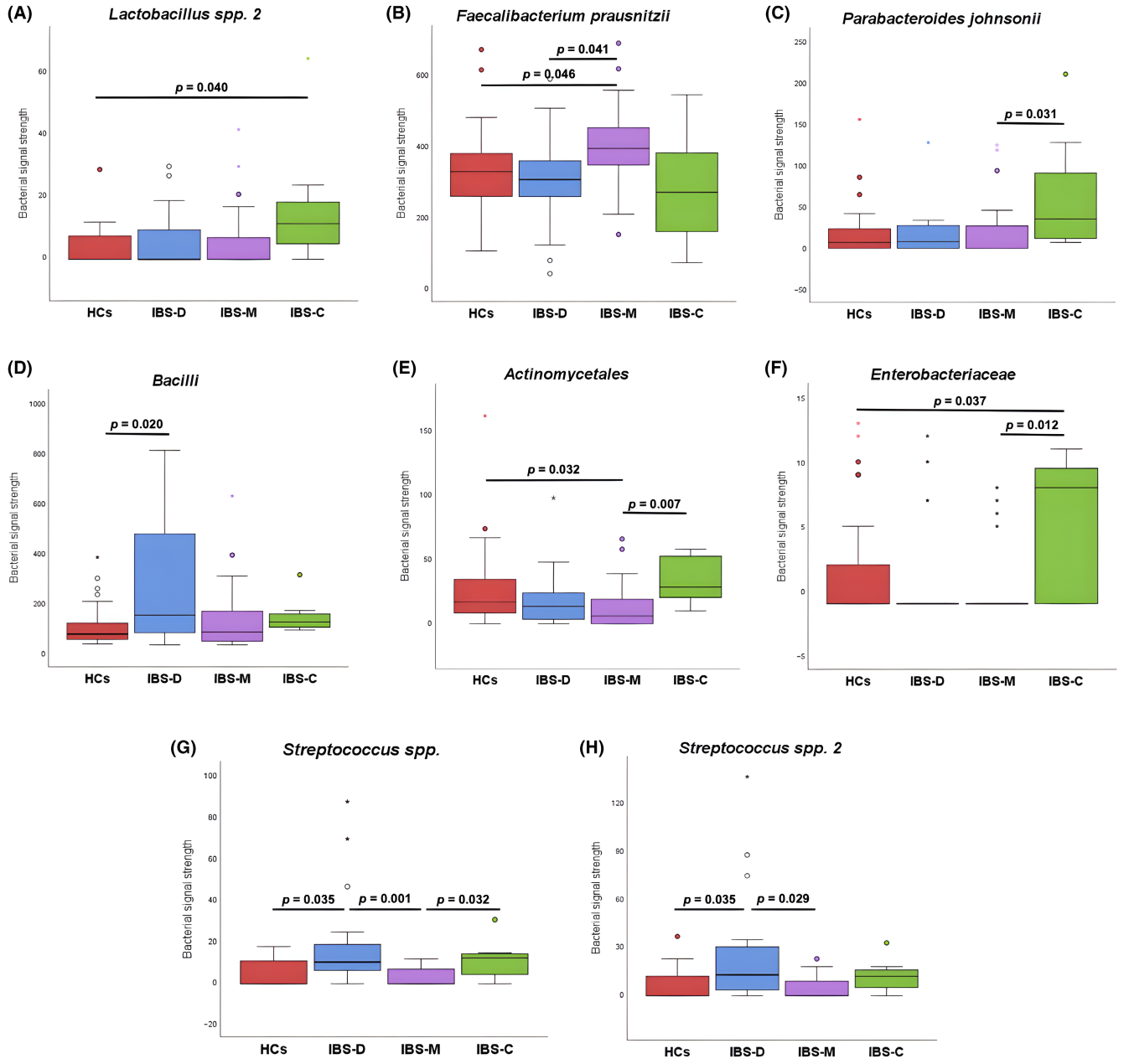

FIGURE 4 Boxplots (A–H) showing bacterial signal strength of the eight out of 48 bacterial markers that were significant different across HCs (healthy controls) and IBS (irritable bowel syndrome) subgroups (IBS-D: IBS with predominant diarrhea, IBS-C: IBS with predominant constipation, and IBS-M: IBS mixed—alternating constipation and diarrhea) using independent-Samples Kruskal Wallis Test with Dunn's post hoc.

of *Bacilli* (Firmicutes phyla) and lower signal strength of the phyla Actinobacteria than HCs. Unexpectedly, we did not observe any significant correlations with IBS symptoms for either Actinobacteria or *Bacilli*. Actinobacteria has previously been reported to contribute to the bacterial separation between IBS and HC groups with a 10% decreased level in patients with IBS compared to HCs.[22] An increase in Actinobacteria following FMT has been shown to be accompanied by a significant reduction in symptom severity score (IBS-SSS) in patients with IBS-D.[23] On the other side, Actinobacteria, *Firmicutes* (*Bacilli*), *Proteobacteria* (*Shigella/Escherichia*), and *Ruminococcus gnavus* were the predominant bacteria contributing to dysbiosis in the IBS cohort of Casén et al.[4] where all patients ($n = 109$) showed an increased signal. The literature is inconsistent regarding the level and the meaning of Actinobacteria. However, there is more evidence for patients with IBS having an increased ratio of Firmicute/Bacteroides at the phylum level.[5,22] The *Bacilli* class contains the genera *Bacillus* and *Lactobacillus* where its species are probiotics that have putative beneficial effects on the intestine[24,25] and has shown to improve symptoms in patients with IBS.[26] *Bacilli* and Proteobacteria have also been positively associated with the expression of the pro-inflammatory IL-6.[27] These findings make our results challenging to interpret.

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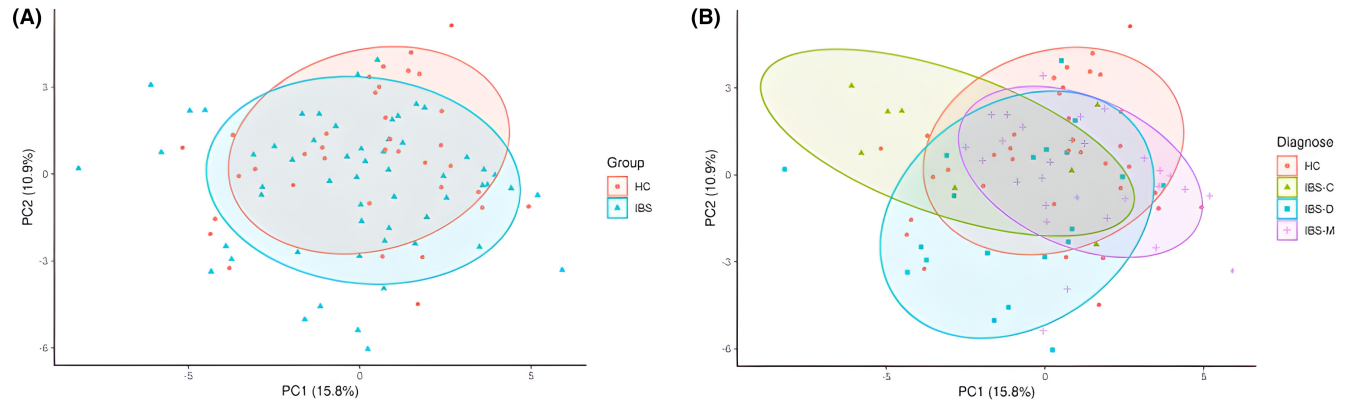

FIGURE 5 PCA (principal component analysis) for differences in fecal bacteria for (A) IBS (irritable bowel syndrome) and HCs (healthy controls). Red color (circle) representing HCs and blue (triangle) representing IBS; (B) for IBS subgroups (IBS-D: IBS with predominant diarrhea, IBS-C: IBS with predominant constipation, and IBS-M: IBS mixed—alternating constipation and diarrhea) and HCs. Red color (circle): HCs, green (triangle): IBS-C, blue (square): IBS-D and purple (cross): IBS-M.

From our results, it is clear that differences in gut bacterial composition between patients with IBS and HCs are characterized by the fact that IBS constitute a highly heterogeneous patient group. According to subgroups, the PCA showed a different bacterial profile for the IBS-D and IBS-C groups from the HC and IBS-M groups. It has been suggested that the difference in stool transit time might be an explanation for the difference in the profile of fecal microbiota and SCFAs between IBS-D and IBS-C[28,29] discussed below. Only two of the eight fecal bacterial markers that were different across the IBS subgroups and HCs were associated with gastrointestinal symptoms and bowel habits. For *F. prausnitzii*, similar observations have been described where *Faecalibacterium* spp. was increased in patients with IBS-M compared to HCs. This was interesting, as *F. prausnitzii* is termed as a gut health marker[22,30] and reduced signals have contributed to dysbiosis in patients with IBD.[4] The bacterial marker is also a highly fermentable bacteria contributing to the production of butyric acid and gasses[30,31] and is shown to be significantly increased in patients with functional abdominal bloating/distention compared to HCs.[32] Visceral hypersensitivity in patients with IBS and increased gas production contributes to distention[33] and might be a possible explanation for the positive correlation between *F. Prausnitzii* and abdominal bloating.

The only distinct fecal bacterial marker segregating the IBS-D from the IBS-C subgroup was the significant increase of *Enterobacteriaceae* in the IBS-C group. This bacterial marker is suggested to partly explain the increased dysbiosis in this group of IBS-C compared to HCs[34] and increased levels are found to be associated with elevated pain scores in patients with IBS.[22] However, the literature reveals inconsistent results about increased levels in the IBS-D or IBS-C subgroups in comparison to HCs.[35,36]

When investigating differences between the IBS-D and the IBS-C subgroups based on stool habits we found *Clostridia* and *Veillonella* spp. to be more abundant in loose stool (BSS) and less abundant in constipation (GSRS). *Clostridia* is a class in the Firmicutes phyla containing gut pathogens causing some of the most common clostridial infections of the gut[37] and it has been speculated that Clostridia-rich microbiota enhances bile acid excretion in patients with IBS-D leading to diarrhea.[38] Additionally, a spore-forming bacteria dominated by *Clostridia* Cluster IV and XIVa is shown to induce serotonin biosynthesis by enterochromaffin cells in the colon.[39] Intestinal serotonin dysregulation has been linked to intestinal dysfunction and has been proposed to contribute to the symptoms and stool habits of IBS.[40,41]

Both in HCs and those with constipation, supplementation with psyllium husk has been shown to increase the relative abundance of *Veillonella* along with increased levels of fecal acetic and propionic acid.[42] The difference in colonic water content, different intake of such oligosaccharides, or increased levels of acetic and propionic acids might contribute to the observation of increased levels of *Veillonella* associated with looser stool and lower levels of *Veillonella* associated with constipation in our study. However, we did not investigate the participants' diet intake in this sub-study. Hence, this will be investigated in another sub-study of our Bergen BrainGut project.[17] Others have shown that *Veillonellaceae* family is significantly higher in patients with IBS-D compared to HCs[43] and that *Veillonella* is significantly higher in patients with IBS[10] and IBS-C[44] compared to HCs. However, our findings support the associations between increased fecal *Clostridia* and *Veillonella* spp. and loose stools.

Proteobacteria (phyla) and *Dorea* spp. were associated with higher levels of diarrhea (GSRS), and *Bacilli*, *Streptococcus* spp., and *Streptococcus* spp. 2 was significantly higher in the IBS-D group compared to the HC group. In general, these bacterial markers seem to be associated with inflammation, excessive excreted bile acids, and/or intestinal serotonin dysregulation,[22,27,45] and some have previously been reported at higher abundances in patients with IBS-D,[38,46] in accordance with our results.

In our study, the IBS group had nonsignificant higher levels of total fecal SCFAs, acetic, propionic, and butyric acid compared to the HC group. However, fecal metabolites have previously been shown to be the main driver for separation between patients with IBS-D and IBS-C.[47] One systematic review and meta-analysis of fecal SCFAs in patients with IBS compared to HCs revealed that

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TABLE 2 Correlations between fecal bacterial markers and short-chain fatty acids (SCFAs) with irritable bowel syndrome (IBS) symptoms.

	IBS-SSS[a]	Abdominal pain (GSRS[b])	Abdominal bloating (GSRS[b])	Satiety (GSRS[b])	Diarrhea (GSRS[b])	Constipation (GSRS[b])	Loose stool (BSS[c])
Bacterial markers							
Firmicutes		$r=-0.219$, $p=0.038$		$r=-0.255$, $p=0.015$	$r=-0.272$, $p=0.009$		
Actinomycetales		$r=-0.249$, $p=0.018$			$r=-0.328$, $p=0.002$		
Faecalibacterium Prausnitzii			$r=0.217$, $p=0.04$	$r=0.230$, $p=0.029$			
Actinobacteria			$r=-0.227$, $p=0.031$			$r=-0.221$, $p=0.037$	
Bifidobacterim spp.			$r=-0.208$, $p=0.05$				
Dorea spp.					$r=0.239$, $p=0.023$		
Proteobacteria					$r=0.229$, $p=0.030$		
Clostridia						$r=-0.266$, $p=0.011$	$r=0.314$, $p=0.004$
Lachnospiraceae						$r=-0.238$, $p=0.024$	
Veillonella spp.						$r=-0.212$, $p=0.045$	$r=0.312$, $p=0.004$
Dialister invisus & *Megasphera micronuciformis*							$r=0.234$, $p=0.033$
Bacteroides spp.							$r=-0.252$, $p=0.022$
Bacteroides spp. & *Prevotella* spp.							$r=-0.328$, $p=0.002$
Short-chain fatty acids							
Total SCFAs	$r=0.259$, $p=0.017$				$r=0.222$, $p=0.036$		$r=0.229$, $p=0.037$
Acetic acid	$r=0.238$, $p=0.029$				$r=0.227$, $p=0.031$		$r=0.259$, $p=0.017$
Butyric acid					$r=0.279$, $p=0.008$		$r=0.282$, $p=0.010$
Iso-valeric acid							$r=-0.302$, $p=0.006$
Iso-butyric acid							$r=-0.244$, $p=0.026$

Note: Correlations between fecal bacterial markers and short-chain fatty acids (SCFAs) with irritable bowel syndrome (IBS) symptoms (for both patients with IBS and healthy controls). White for no significant association, purple for negative correlations and green for positive correlations, expressed by Spearman's rho correlation (r) and p-value. Table only presenting bacterial markers and SCFAs with significant findings.

[a]IBS Severity Scoring System.

[b]Gastrointestinal Symptom Rating Scale.

[c]Bristol Stool Scale.

higher fecal levels of propionate in the IBS group was the only significant difference between the IBS and HC group[48] but other studies have yielded conflicting results.[10,14,48,49] It has been suggested that differences in SCFAs between patients with IBS and HCs can only be found if subtypes are considered.[50]

The association between colon transit time and intestinal SCFAs in IBS subtypes may be explained by several mechanisms. In general, it is speculated whether reduced (faster) transit time increases the fecal content of SCFAs[51,52] or whether increased luminal SCFAs reduce transit time. Most theories conclude that faster transit time

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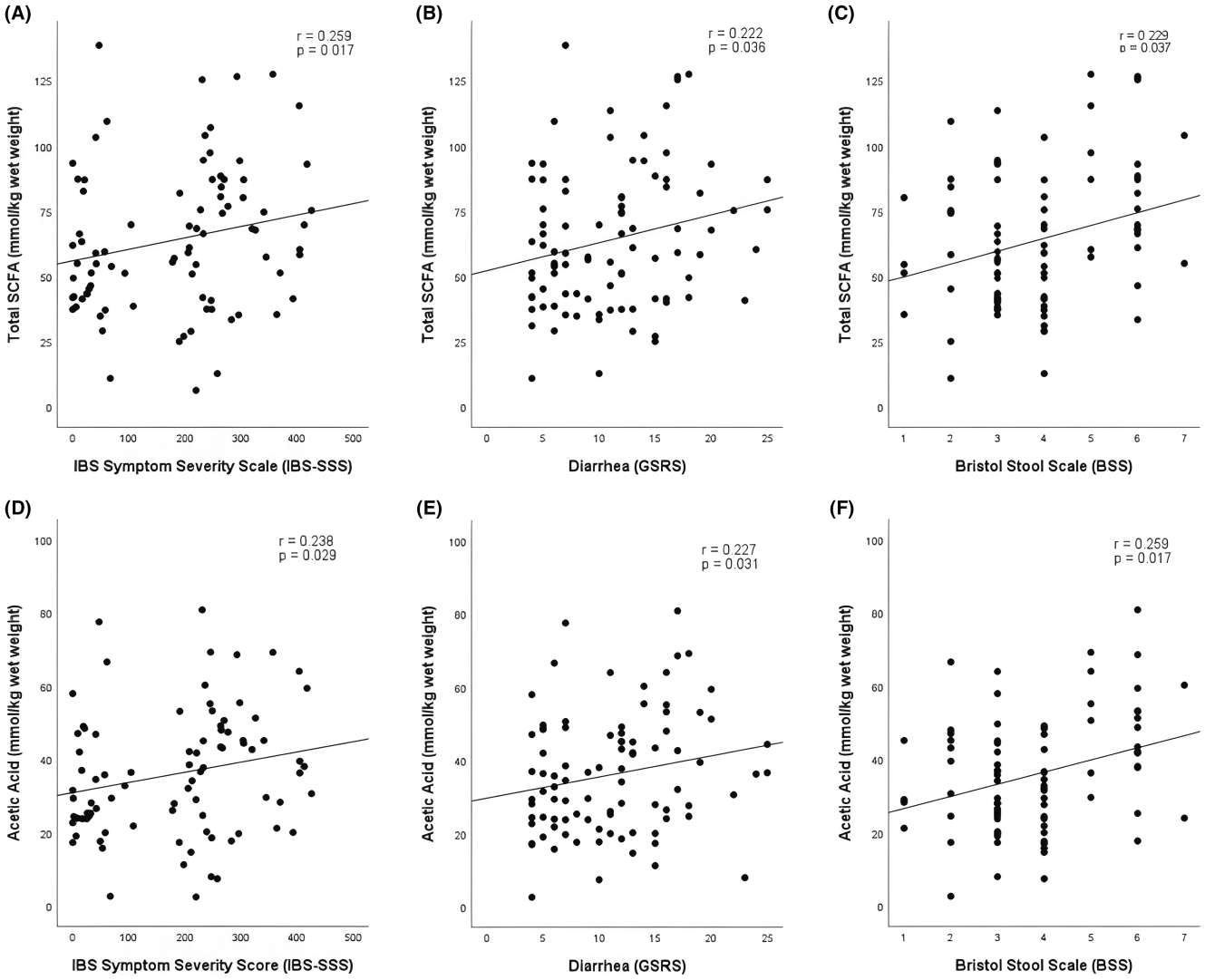

FIGURE 6 Scatter plots presenting correlations between total fecal SCFA (mmol/kg wet weight) and (A) IBS-SSS (IBS Symptom Severity Scale), (B) diarrhea (GSRS) and (C) Bristol Stool Scale (BSS); between acetic acid (mmol/kg wet weight) and D) IBS-SSS, (E) diarrhea (GSRS) and (F) Bristol Stool Scale (BSS). BSS scores 5–7 representing loose stools, BSS scores 1–2 representing hard stools and BSS 3–4 representing normal stool consistency. Correlation coefficient (R) and *p*-value are presented for each correlation.

is associated with diarrhea/loose stool and increased fecal levels of SCFAs.[20,29,49] A systematic review and meta-analysis support some of these findings with the observations of increased levels of butyric acid in patients IBS-D in comparison to HCs.[48] Other studies have observed increased fecal levels of acetic, propionic, butyric, and valeric acid in patients with IBS-D compared to patients with IBS-C.[49] Several studies have observed reduced levels of acetic,[50,53] propionic,[48,53] and butyric acids[48,53] in patients with IBS-C compared to HCs.

Interestingly, higher levels of total SCFAs and acetic acid were associated with both higher IBS symptom severity score (IBS-SSS) and diarrhea/loose stool (GSRS and BSS), and looser stool (BSS) was associated with higher levels of *Veillonella* spp. Tana et al.[10] found that patients with IBS had significantly higher fecal levels of acetic acid, propionic acid, and total organic acids and significantly higher levels of *Veillonella* spp. and *Lactobacillus* than HCs. The group of IBS patients with high levels of acetic and propionic acids had a significantly higher score of abdominal pain and bloating than IBS patients with lower levels. They also found a significant association between abdominal bloating and high levels of acetic acid, propionic acid, and total organic acids. Moreover, the IBS group with higher levels of *Veillonella* spp. and *Lactobacillus* displayed higher levels of total organic acids and acetic acid compared to the groups with low levels. *Veillonella* transforms lactic acid into acetic acid and a combination of *Veillonella* spp. and *Lactobacillus* produce acetic and propionic acid. This has been suggested to explain the increased levels of acetic and propionic acid in IBS.[10]

Tana et al.[10] discusses the pathogenesis and association between increased organic acids and increased abdominal pain and distention and finds the correlation likely due to visceral hypersensitivity in patients with IBS. Supporting this theory, neonatal rat studies have reported that infusion of 0.5% acetic acid in saline resulted in higher sensitivity of the colon to balloon distention compared to controls[10]

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and that high levels of SCFAs can induce powerful contractions in terminal ileum.[54] For this reason, it seems likely that higher levels of total SCFAs and acetic acid might play a role in increased gastrointestinal symptom severity in patients with IBS.

It should be noted that 95% of SCFAs are absorbed in the lumen and only 5% of SCFAs are excreted in the feces.[51] Hence, the fecal levels of SCFAs reveal information regarding non-absorbed SCFAs and do not reflect the in-situ production rates, absorption, and interaction with other biologically relevant molecular cell types.

4.1 | Strengths and limitations

The study has several strengths. To our knowledge, few studies have investigated the fecal bacterial profile and SCFAs and their correlation with symptom profile in patients with IBS. IBS is a complex disorder with no well discovered disease-specific markers and previous results are quite heterogeneous. Another strength of the study is that this is a sub-study of the large interdisciplinary Bergen BrainGut project which is a multifaceted study designed to stratify the complex interaction and dysfunction in the brain, the gut, and the microbiota in patients with IBS.[17] The method selected to analyze fecal bacterial markers, GA-map® Dysbiosis Test, is an affordable and easy method developed to identify dysbiosis in IBS and IBD.[4,55] Comparison of fecal bacterial markers across different methods might obscure results and contribute to inconclusive findings.

The study also has some limitations. First, and most importantly, this is a cross-sectional sub-study which will not reveal any causality. Optimally, fecal samples and dietary records should have been collected at several time points in a longitudinal study and the other variables abovementioned should have been controlled for. However, we intended to limit the geographical site and age by only including participants from the Bergen area speaking Norwegian aged between 18 and 65 years. Although dietary intake was not controlled for in this part of the study, we did not include candidates with dietary restrictions (e.g., vegan, vegetarian, following a low FODMAP diet, or using probiotics), who had been administered antibiotics within the last 3 months prior to inclusion, or regularly used medications affecting the gastrointestinal tract. Other factors known to impact gut bacteria and SCFA composition, for example, diet, BMI, and cultural norms are not controlled for in these analyses.[56,57]

Secondly, although the number of participants in the groups of IBS and HC and for IBS-D and IBS-M is satisfying, the number of participants with IBS-C was low, which affected the power of the statistical analyses.

5 | CONCLUSION

We have demonstrated that patients with IBS and across IBS subgroups have a different fecal bacterial profile than HCs, and observations indicate there are associations between fecal bacterial markers, gastrointestinal symptoms, and stool habits. The IBS group was characterized by significantly increased *Bacilli* and decreased Actinobacteria compared to the HC group. The most prominent observations regarding fecal bacterial markers were increased levels of *Clostridia* and *Veillonella* spp. being associated with diarrhea, and lower levels being associated with constipation. Levels of fecal SCFAs were associated with stool consistency and habits, and we saw a trend indicating different profiles between the groups of IBS-D and IBS-C. Total SCFAs and acetic acid were associated with both higher IBS symptom severity and diarrhea/loose stool. Altogether, these findings indicate that fecal bacterial markers and SCFAs might contribute to the symptom generation and stool habits in patients with IBS. However, further, longitudinal studies with a higher number of participants in the different IBS subgroups are needed to investigate the role of fecal bacterial profiles and its metabolites, including fecal SCFAs, in the pathophysiology of IBS.

AUTHOR CONTRIBUTIONS

E.S.T., E.M.R.H., E.K.S., I.B., G.A.L., T.H., B.B., collected the data, E.K.S., I.B., A.L., A.J.L., B.B., J.V. and G.A.L. designed the research study, B.B. coordinated the study, J.V. was responsible for SCFA analysis, E.S.T. performed the statistical analysis, E.S.T. drafted the manuscript and all contributed to review and editing. All authors have approved the final version of the article, including the authorship list.

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CONFLICT OF INTEREST STATEMENT

No competing interests declared.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Original article

Factors underlying the long-term efficacy of faecal microbiota transplantation for patients with irritable bowel syndrome

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ABSTRACT

The long-term effects of the transplant dose, its administration route and repeated faecal microbiota transplantation (FMT) on the outcomes of FMT for patients with irritable bowel syndrome (IBS) are unknown. This study included 171 patients (125 females and 46 males): 90 g of donor feces was administered into the large intestine (LI) in 58, into the small intestine (SI) in 57, and into the SI twice (repeated SI) in 56. The patients provided a fecal sample and completed five questionnaires at the baseline and at 2 years after FMT. Fecal bacteria and the dysbiosis index were analyzed using 16S rRNA gene PCR DNA amplification/probe. The response rates at 2 years after FMT were 47.2%, 80.9%, and 76.6% in the LI, SI, and repeated-SI groups, respectively. The response rate was significantly higher in the SI and repeated SI groups than in the LI group. IBS symptoms at 2 years after FMT were less severe in the SI and repeated-SI groups than in the LI group. Fluorescent signals of several bacteria were significantly correlated with IBS symptoms and fatigue after FMT. No long-term adverse events were observed. In conclusion, administering the transplant to the SI increased the long-term response rate and reduced IBS symptom severity compared with administering it to the LI, and led to the long-term colonization of beneficial bacteria. There was no long-term difference between one and two FMT procedures (www.clinicaltrials.gov: NCT04236843).

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1. Introduction

Irritable bowel syndrome (IBS) is a common gut–brain interaction disorder that affects 10% of the world population [1]. Its reported prevalence varies considerably between different geographical areas, being highest in South Europe, Africa, and South America (15–21%), lowest in South Asia and Middle East (7%), with in between in the Western world (Europe, North America, and Australia) (11.8 and 14%) [1]. The etiology of IBS is unknown, though several factors such as genetics, abnormal gut microbiome, and diet appear to be involved in its pathophysiology [1–7]. IBS is diagnosed based on symptom assessment (Rome IV criteria) and no effective treatment is currently available [1]. Although IBS does not increase mortality risk, it considerably reduces the quality of life of affected patients and their caregivers [1].

Fecal microbiota transplantation (FMT) is a promising intervention for IBS [8]. The efficacy of FMT differed considerably between different clinical trials most probably because of the difference in the protocols used and the difference in donor's stool processing, which may change the bacterial composition of faecal transplants [9–16]. In clinical trials in which FMT was the most effective for IBS, the protocol used comprised five favorable factors: careful donor selection, high transplant dose, direct freezing of the transplant until the FMT, careful handling of the transplant during its preparation, and administering the transplant to the small intestine (SI) [8,12,17,18].

Our recent clinical trial examined the effects of increasing the transplant dose, the route of the transplant administration, and repeated transplantation on the outcomes of the FMT [19]. The present study was a 2-year follow-up of the previous trial that

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aimed to determine the long-term effects of these three factors and possible long-term adverse events.

2. Material and methods

2.1. Study design

The study design has been described in detail previously [19]. In short, each patient provided a fecal sample and completed five questionnaires to assess their IBS symptoms, fatigue, and quality of life at the baseline and at 2 years after FMT. Polyethylene glycol and loperamide were allowed as rescue medications during the study.

2.2. Enrolment and randomization of patients

This study is a 2-years follow-up of patients included in our previous study [19], which included 186 patients (Fig. 1). These patients were randomized at a 1:1:1 ratio in blocks of six to receive 90-g fecal transplants from the donor administered into the cecum of the colon (large intestine [LI]), into the distal duodenum (SI), or into the distal duodenum twice at an interval of 1 week (repeated SI). Randomization was conducted by a nurse who was not involved in the trial [19]. Three patients dropped out and one was excluded because of pregnancy in the LI group by the follow-up 2 years after FMT. Four patients in the SI group dropped out and one was excluded due to the use of antibiotic treatment for sinusitis. Three patients in the repeated-SI group dropped out, and three were excluded due to pregnancy (n = 1), gastroenteritis obtained during travel abroad (n = 1), and breast cancer (n = 1) (Fig. 1). The investigation therefore finally involved 171 patients who fulfilled the Rome IV criteria for IBS: 58, 57, and 56 in the LI, SI, and repeated-SI groups, respectively. The baseline characteristics of the included patients are listed in Table 1.

The inclusion and exclusion criteria for patients were presented in detail in our previous report [19]. In summary, the inclusion criteria were being older than 18 years and having severe or moderate IBS symptoms (total score on the IBS Severity Scoring System [IBS-SSS] of ≥175). The exclusion criteria were the presence of a systemic disease, immune deficiency, treatment using immune-modulating medication, pregnancy or planning pregnancy, lactation, severe psychiatric disorders, alcohol or drug abuse, or the use of probiotics, antibiotics, or IBS medications within 8 weeks prior to the start of the study.

2.3. Donor

The donor was previously described in detail [12]. To summarize, he was screened according to the European guidelines for FMT donors [4,20], and vaccinated against COVID-19 and tested weekly for this disease during the period in which he donated his feces. He was a healthy Caucasian aged 40 years with a normal BMI and had been born via vaginal delivery and breastfed. He was a nonsmoker, was not regularly taking any medication, had only taken antibiotics a few times during his life, exercised regularly, and consumed a sport-specific diet that was richer in protein, fiber, minerals, and vitamins than the average diet [12]. He was not a first-degree relative of any recipient. He had high microbial diversity (normobiosis) [12]. His fecal bacteria profile and diversity were tested every 3 months and were stable over time (Supplementary Figs. 1–3).

2.4. Faecal sample collection, preparation, and administration

The collection, preparation, and administration of feces have been described in detail previously [12]. In brief, fecal samples from the donor and patients were immediately frozen and kept at −20 °C, and then delivered to the laboratory and stored at −80 °C. The feces were thawed for 2 days in a refrigerator at 4 °C, and a 90-g sample was manually mixed with 90 mL of cold sterile saline at 4 °C, and then filtered through a 110 cm × 10 cm nonwoven swab (OneMed, Helsinki, Finland). The fecal transplant was administered to the colon cecum after the bowel was prepared via the working channel of a colonoscope in the single-LI group, and to the distal duodenum after an overnight fast via the working channel of a gastroscope in the SI and repeated-SI groups. The working channels of the colonoscopes and gastroscopes were flushed with 30 mL of cold sterile saline at 4 °C after each transplantation procedure.

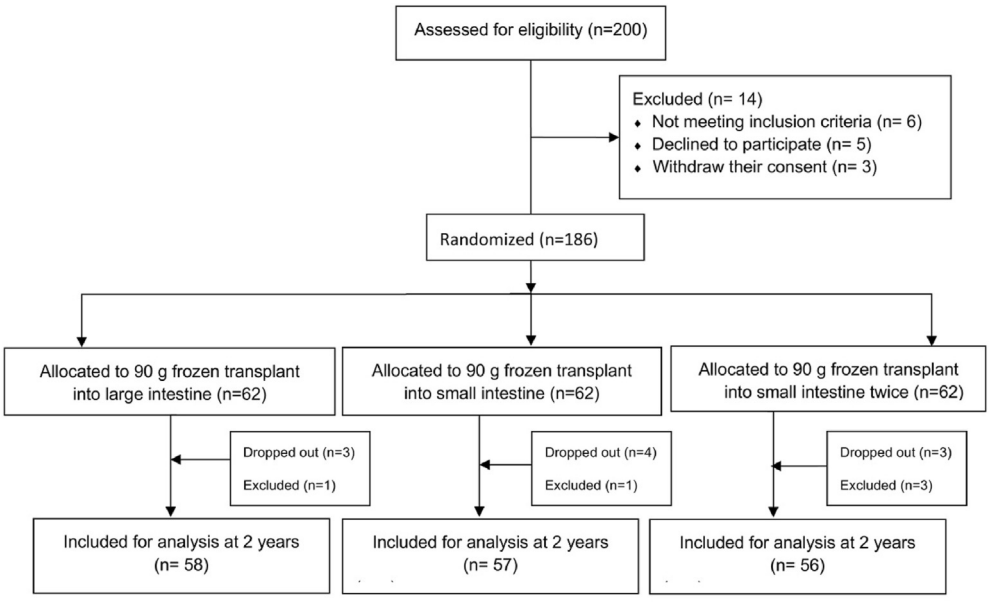

Fig. 1. CONSORT (Consolidated Standards of Reporting Trials) diagram presenting the enrolment, randomization, and follow-up of the patients.

Table 1
Characteristics of patients included in the trial.

	Overall	large intestine FMT	small intestine FMT	Repeated small intestine FMT	P values
Number	171	58	57	56	
Age, years	37.6 ± 13.0	40.0 ± 13.4	37.9 ± 13.4	34.5 ± 11.9	0.09
Sex, female/male	125/46	44/14	38/19	43/13	0.4
Body mass index (BMI)	24.6 ± 5.0	25.3 ± 5.7	24.9 ± 5.4	23.7 ± 2.5	0.6
IBS-D	66	22	22	22	
IBS-C	59	20	20	19	0.999
IBS-M	46	16	15	15	
Duration of IBS, years	23.2 ± 13.6	22.9 ± 13.6	25.1 ± 14.7	21.7 ± 12.2	0.6
PPI medication	22 (12.9%)	7 (12.1%)	9 (15.8%)	6 (10.7%)	0.7
Birth-control medication	47 (27.5%)	14 (24.1%)	14 (24.6%)	19 (33.9%)	0.4
Painkiller medication	18 (10.5%)	8 (13.8%)	7 (12.3%)	3 (5.3%)	0.3
Medication against asthma/allergies	39 (22.8%)	13 (22.4%)	14 (24.6%)	12 (21.4%)	0.4
Medication with levothyroxine	2 (1.2%)	0 (0%)	1 (1.8%)	1 (1.9%)	0.6
Anti-depression medication	26 (15.2%)	7 (12,1%)	8 (14.0%)	11 (19.6%)	0.5
Medication with heart/vascular drugs	9 (5.3%)	2 (3.5%)	4 (7.0%)	3 (5.4%)	0.7

Data are mean ± SD, n (%), except where indicated otherwise. PPI, proton-pump inhibitor.

2.5. Symptoms and quality-of-life assessment

The questionnaires used to assess symptoms were the IBS-SSS, Birmingham IBS Symptom Questionnaire, and Fatigue Assessment Scale (FAS) [21–23]. Responders were patients who exhibited a decrease in total IBS-SSS score of ≥50 points after FMT. Patients with total IBS-SSS scores of >300, 175–300, and 75–175 were considered to have severe, moderate, and mild IBS symptoms, respectively, while those with scores of <75 were considered to be in complete remission [21]. Quality of life was measured using the IBS Quality of Life (IBS-QoL) and Short-Form Nepean Dyspepsia Index (SF-NDI) questionnaires [24,25]. An increase in IBS-QoL score and a decrease in SF-NDI score indicated improved quality of life.

2.6. Bacterial analysis

Fecal bacteria compositions were analyzed using a predetermined bacteria-target approach as described in detail previously [5]. To summarize, this test uses 16S rRNA PCR DNA amplification covering variable regions V3–V9, followed by DNA probe hybridization of the predetermined 48 bacterial markers encompassing >300 bacteria with different taxonomic levels. The dysbiosis index (DI) was measured on a five-point scale from 1 (normal) to 5 (severe dysbiosis): 1 and 2 indicate normobiosis and 3–5 indicate dysbiosis [5].

2.7. Adverse events and medication

The patients were asked to use a diary to keep a record of their bowel habits and register any adverse events. They also kept a record of their use of rescue medication (polyethylene glycol and loperamide) or other medications. The patients continued using the same medications they used before the trial, which are listed in Table 1.

2.8. Outcomes

The primary endpoint was a ≥50-point reduction in total IBS-SSS score, and the secondary one was changes in the bacterial diversity as measured by DI and in the fecal bacterial profile.

2.9. Statistical analysis

Differences in response, complete remission, sex, IBS subtypes, proportion of patients with dysbiosis, and medication use were assessed using chi-square or Fisher's exact tests. Differences between the LI, SI, and repeated-SI groups in IBS-SSS, Birmingham IBS Symptom Questionnaire, FAS, IBS-QoL, and SF-NDI scores, DI, bacterial fluorescence signals, and IBS subtypes were analyzed using the Kruskal–Wallis test and a posttest with Dunn's multiple comparisons. The nonparametric Mann–Whitney test was used to identify differences between patients at the baseline and at 2 years after transplantation in IBS-SSS, Birmingham IBS Symptom Questionnaire, FAS, IBS-QoL, and SF-NDI scores, DI, bacterial fluorescence signals, sex, IBS subtypes, IBS symptom severity, and response. Scaled principal-components analysis was performed on log-transformed bacterial fluorescence signals, and the results were visualized using a different color for each group together with ellipses that included 80% of the samples within each group.

2.10. Ethics

The study was approved by the West Regional Committee for Medical and Health Research Ethics, Bergen, Norway (approval no. 2019/6841/REK vest). All subjects provided both oral and written consents before participating. This study is registered at www.clinicaltrials.gov: (NCT04236843).

3. Results

3.1. Patient responses to FMT

The response rates to FMT at 2 years after FMT were significantly higher in the SI and repeated-SI groups than in the LI group (Fig. 2A), with no significant difference between the SI and repeated-SI groups. The proportion of patients with IBS with severe symptoms decreased significantly in all groups at 2 years after FMT (Supplementary Table 1); however, this proportion was significantly lower in the SI and repeated-SI groups than in the LI group. The proportion of patients with IBS with mild symptoms and those with complete remission increased in all groups at 2 years after FMT. The proportion of patients with IBS and moderate symptoms at 2 years after FMT did not differ from that at the baseline in all groups. The SI and repeated-SI groups had a higher proportion of patients with IBS and mild symptoms and those in remission (though not significant) than in the LI group.

3.2. IBS symptoms, fatigue, and quality of life

The total IBS-SSS score and the scores for its four items decreased significantly in all groups at 2 years after FMT as compared to the baseline (Supplementary Table 2 and Fig. 2B). The

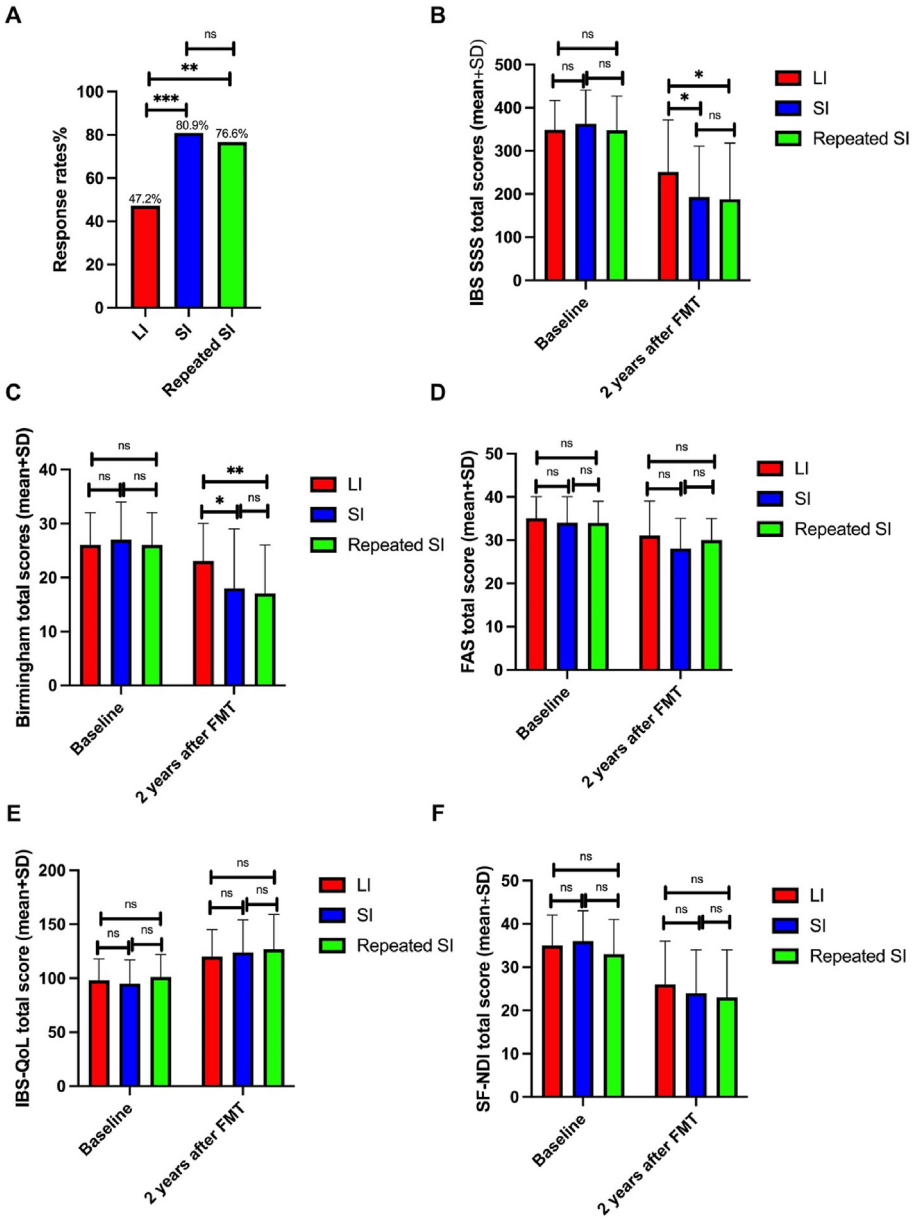

Fig. 2. Findings at the baseline and at 2 years after FMT in the LI, SI, and repeated-SI groups: FMT response rates (A), total IBS-SSS scores (B), total Birmingham IBS Symptom Questionnaire scores (C), total FAS scores (D), total IBS-QoL scores (E), and total SF-NDI scores. ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

total IBS-SSS scores in the SI and repeated-SI groups were significantly lower than those in the LI group at 2 years after FMT, with no significant difference between the SI and repeated-SI groups. Total scores on the Birmingham IBS Symptom Questionnaire decreased significantly in all groups at 2 years after FMT (Supplementary Table 3 and Fig. 2C). These total scores in the SI and repeated-SI groups were significantly lower than those in the LI group, with no significant difference between the SI and repeated-SI groups at 2 years after FMT. The total FAS scores and the scores for physical fatigue and mental health were decreased in all groups at 2 years after FMT (Supplementary Table 4 and Fig. 2D). There was no significant difference among the LI, SI, and repeated-SI groups regarding the total FAS scores or physical fatigue and mental health scores at 2 years after FMT.

The total IBS-QoL score and the scores in its eight domains increased significantly in all of the FMT-treated groups at 2 years after FMT (Supplementary Table 5). There was no significant difference among the LI, SI, and repeated-SI groups in the total IBS-QoL scores at 2 years after FMT (Fig. 2E). The total SF-NDI score and those for each of its five subclasses were decreased in all FMT-treated groups at 2 years after FMT (Supplementary Table 6). There was no significant difference among the LI, SI, and repeated-SI groups regarding those scores (Fig. 2F).

3.3. Bacterial analysis

The DIs decreased in all groups at 2 years after FMT (Supplementary Fig. 4). There was no significant difference in DI among the LI, SI, and repeated-SI groups ($p = 0.7$).

While the bacterial profiles of LI, SI, and repeated-SI groups overlapped at the baseline, the profiles of the SI and repeated-SI groups deviated from that of the LI group at 2 years after FMT (Supplementary Fig. 5 and Fig. 3). The fluorescence signals of 23, 22, and 25 bacterial markers were changed at 2 years after FMT in the

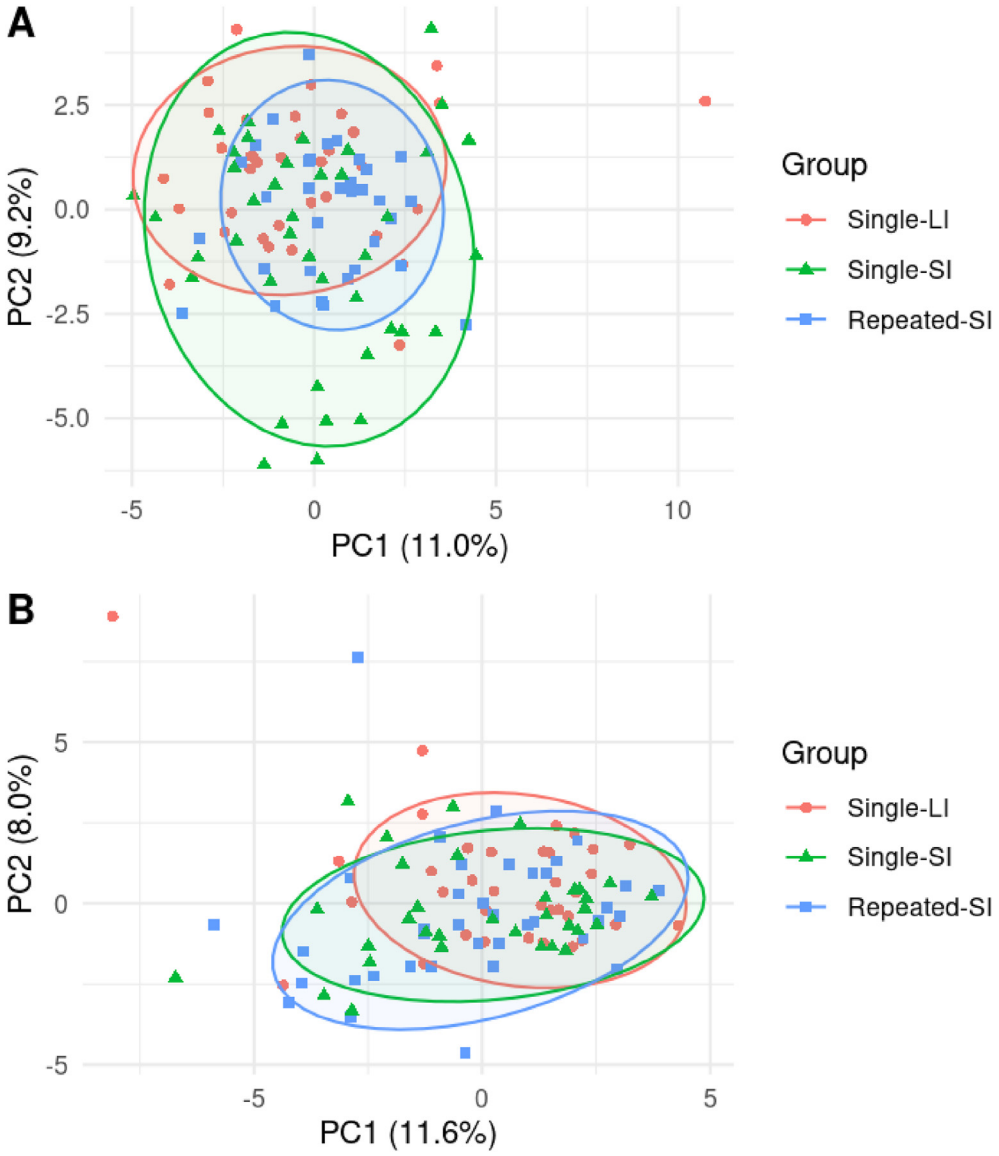

Fig. 3. Results of scaled PCA of the faecal bacterial profiles. The bacterial profiles of the LI, SI, and repeated-SI groups at the baseline (A) and at 2 years after FMT (B).

LI, SI, and repeated-SI groups, respectively (Supplementary Tables 7–9). Similar changes in 12 bacterial markers occurred in both the LI and SI groups (Supplementary Table 10). On the other hand, the fluorescence signals of 16 bacterial markers differed between the LI and SI groups (Supplementary Table 11), while only 5 differed between the SI and repeated-SI groups (Supplementary Table 12). The levels of two of these bacteria (*Bacteroides stercoris* and Proteobacteria spp.) were correlated with total IBS-SSS scores. While the fluorescence signals of these bacteria were decreased in the SI group, they were increased in the repeated-SI group (Supplementary Table 12). The remaining three bacteria (*Anaerobutyricum hallii*, *Phascolarctobacterium* spp., and Enterobacteriaceae spp.) were not correlated with total IBS-SSS scores.

3.4. Correlations

Among the 12 bacterial markers whose levels had changed similarly in the LI and SI groups at 2 years after FMT, 5 were inversely correlated and 4 were positively correlated with the total IBS-SSS scores (Figs. 4 and 5). The fluorescence signals of the bacterial markers that were inversely correlated with total IBS-SSS scores increased, and those that were positively correlated decreased in both the LI and SI groups at 2 years after FMT (Supplementary Table 10).

Among the 16 bacteria whose fluorescence signals differed between the LI and SI groups, 6 were inversely correlated and 7 were positively correlated with total IBS-SSS scores (Figs. 6 and 7). Four bacterial markers (*Alistipes* spp., *Bacteroides* and *Prevotella* spp. combined, *Bacteroides zoogleoformans*, and *Parabacteroides johnsonii*) that increased in both the LI and SI groups were inversely correlated with the total FAS scores (Supplementary Fig. 6A). The fluorescence signals of three bacterial markers (*Bacteroides fragilis*, *Bacteroides* spp., and Proteobacteria spp.) were positively correlated with total FAS scores, while two (*Holdemanella biformis* and *Lactobacillus* spp.) showed an inverse correlation (Supplementary Fig. 6B).

While the fluorescence signals of *Bacteroides stercoris* and Proteobacteria spp. decreased in the SI group at 2 years after FMT, those of the repeated-SI group decreased (Supplementary Table 12). These two bacterial markers were correlated with the total scores of both the IBS-SSS and FAS (Fig. 7, Supplementary Fig. 7B and C).

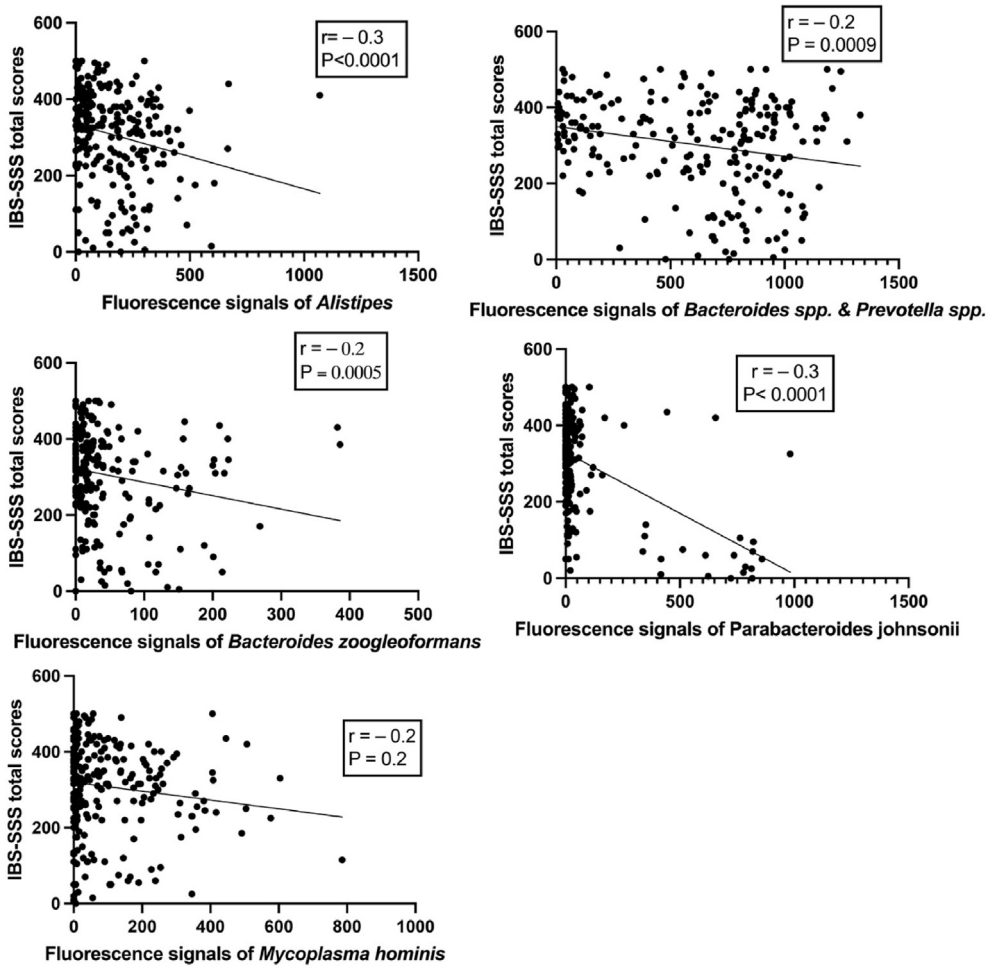

Fig. 4. Five bacterial markers whose fluorescence signals increased in both the LI and SI groups. The fluorescence signals of these bacterial markers were inversely correlated with total IBS-SSS scores.

3.5. Differences between IBS subtypes

There were no differences among the IBS-D, IBS-C, and IBS-M subtypes regarding the FMT response rates at 2 years after FMT (Supplementary Fig. 7A). The total scores of the IBS-SSS, Birmingham IBS Symptom Questionnaire, FAS, IBS-QoL, and SF-NDI did not differ among the IBS-D, IBS-C, and IBS-M at 2 years after FMT (Supplementary Fig. 7).

The DI did not differ among IBS-D, IBS-C, and IBS-M at the baseline or at 2 years after FMT (Supplementary Fig. 8). At the baseline, the fluorescence signal of *Alistipes onderdonkii* was higher in patients with IBS-M than in those with IBS-D and IBS-C (Supplementary Table 13). Patients with IBS-C had lower fluorescence signals of *Bacteroides fragilis* and *Dorea* spp., and higher signals of Firmicutes spp. than those with IBD and IBS-M (Supplementary Table 13). The fluorescence signal of *Ruminococcus gnavus* at 2 years after FMT was lower in patients with IBS-C than in those with IBS-D and IBS-M (Supplementary Table 14).

3.6. Sex differences

Responses to FMT did not differ between males and females in the LI, SI, and repeated-SI groups at 2 years after FMT (Supplementary Fig. 9A). The total scores of the IBS-SSS, Birmingham IBS Symptom Questionnaire, FAS, IBS-QoL, and SF-NDI did not differ between the sexes at 2 years after FMT (Supplementary Fig. 9).

The baseline DI was lower in females than in the males, while there was no significant difference in DI between the sexes at 2 years after FMT (Supplementary Fig. 10). At the baseline, while the fluorescence signals of *Alistipes* spp., *Ruminococcus albus*, *Ruminococcus bromii* combined, and *R. gnavus* were higher in females than in males, that of *H. biformis* was lower in females (Supplementary Table 15). At 2 years after FMT, the only bacterial marker with a sex-related difference in fluorescence signal was *A. hallii*, which was higher in females (Supplementary Table 15).

3.7. Differences between patients with severe and moderate IBS symptoms

The response to FMT at 2 years after FMT did not differ between patients with severe and moderate IBS symptoms (Supplementary Fig. 11A). At the baseline, the total scores of the IBS-SSS and Birmingham IBS Symptom Questionnaire were higher in patients with severe IBS symptoms than in those with moderate ones (Supplementary Fig. 11B and C). However, there was no difference between patients with severe and moderate IBS symptoms in the total scores of both the IBS-SSS and Birmingham IBS Symptom Questionnaire at 2 years after FMT.

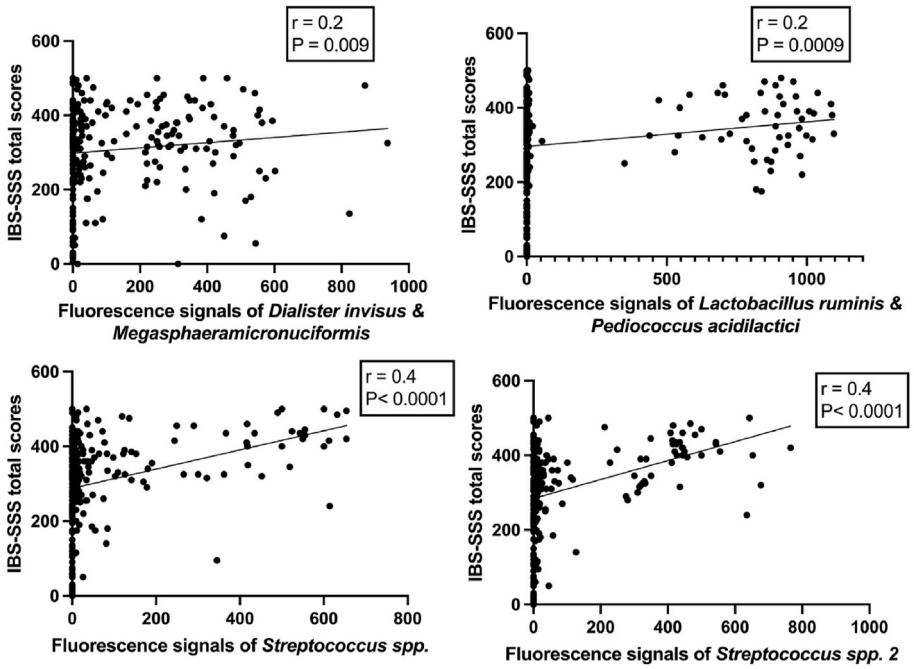

Fig. 5. Four bacterial markers whose fluorescence signals decreased in both LI and SI groups. The fluorescence signals of these bacterial markers were positively correlated with the total IBS-SSS scores.

FAS and IBS-QoL scores did not differ between patients with severe and moderate IBS symptoms either at the baseline or at 2 years after FMT (Supplementary Fig. 11D and E). The total SF-NDI scores were lower in patients with moderate IBS symptoms than in those with severe symptoms in the SI group, but not in the LI and repeated-SI groups, at 2 years after FMT (Supplementary Fig. 11F).

At the baseline, the DIs were higher in patients with severe IBS symptoms than in those with moderate symptoms in all treated groups (Supplementary Fig. 12A). The DI at 2 years after FMT did not differ between patients with severe and moderate IBS symptoms (Supplementary Fig. 12B).

The bacterial fluorescence signals of all examined bacterial markers did not differ between patients with severe and moderate IBS symptoms at the baseline in both the LI and SI groups (Supplementary Tables 16 and 18). In the LI group, the bacterial fluorescence signal of *Alistipes* spp. was higher and that of *Clostridium methylpentosum* was lower in patients with moderate IBS symptoms than in those with severe symptoms at 2 years after FMT (Supplementary Table 17). In the SI group, the bacterial fluorescence signals of *A. onderdonkii*, *Dialister invisus*, and *D. invisus* and *Megasphaera micronuciformis* combined were lower in patients with moderate IBS symptoms than in those with severe symptoms at 2 years after FMT (Supplementary Table 19). In the repeated-SI group, the bacterial fluorescence signals of *P. johnsonii* were higher in patients with moderate IBS symptoms than in those with severe symptoms at the baseline (Supplementary Table 20). The bacterial fluorescence signals of all examined bacterial markers in the repeated-SI group did not differ between patients with severe and moderate IBS symptoms at 2 years after FMT (Supplementary Table 21).

3.8. Difference between responders and nonresponders

The total scores of the IBS-SSS, Birmingham IBS Symptom Questionnaire, FAS, IBS-QoL, and SF-NDI did not differ between responders and nonresponders in all FMT-treated groups at the baseline (Supplementary Figs. 13 and 14). The total scores of the IBS-SSS and Birmingham IBS Symptom Questionnaire at 2 years after FMT were lower in responders than in nonresponders in all FMT-treated groups (Supplementary Fig. 13), while those of FAS were lower in responders than in nonresponders in both the SI and repeated-SI groups; those scores did not differ between responders and nonresponders in the LI group at 2 years after FMT. The total IBS-QoL scores at 2 years after FMT were higher in responders than nonresponders in all FMT-treated groups (Supplementary Fig. 14). The total SF-NDI scores at 2 years after FMT were lower in responders than in nonresponders in all FMT-treated groups (Supplementary Fig. 14).

The DIs did not differ between responders and nonresponders at either the baseline or at 2 years after FMT (Supplementary Fig. 14). The fluorescence signals of six bacteria, namely *Alistipes* spp., *Bacteroides pectinophilus*, *C. methylpentosum*, *Faecalibacterium prausnitzii*, *Streptococcus agalactiae* and *Eubacterium rectale* combined, and Firmicutes spp. were lower in nonresponders than in responders at the baseline (Supplementary Table 22). The fluorescence signals of Actinobacteria spp. were higher and those of *Alistipes* spp. were lower in nonresponders than in responders at 2 years after FMT (Supplementary Table 23).

4. Discussion

The FMT response rates at 3 months after FMT in our previous study did not differ between patients with IBS who received transplants from the donor into the LI or SI However, the FMT response rates at 1 year after FMT were significantly lower in patients with IBS who received the transplant into the LI than in those who received it into the SI [19]. The present study further found that the FMT response rates at 2 years after FMT were significantly higher in patients with IBS who received transplants from donor into the SI than those who received them into the LI. These observations indicate that administering donor transplants to the SI provides better long-term response rates than administering them into the LI. This conclusion is consistent with a recently published systematic review of randomized controlled trials on FMT in IBS demonstrating that delivery into the upper gut was effective [26].

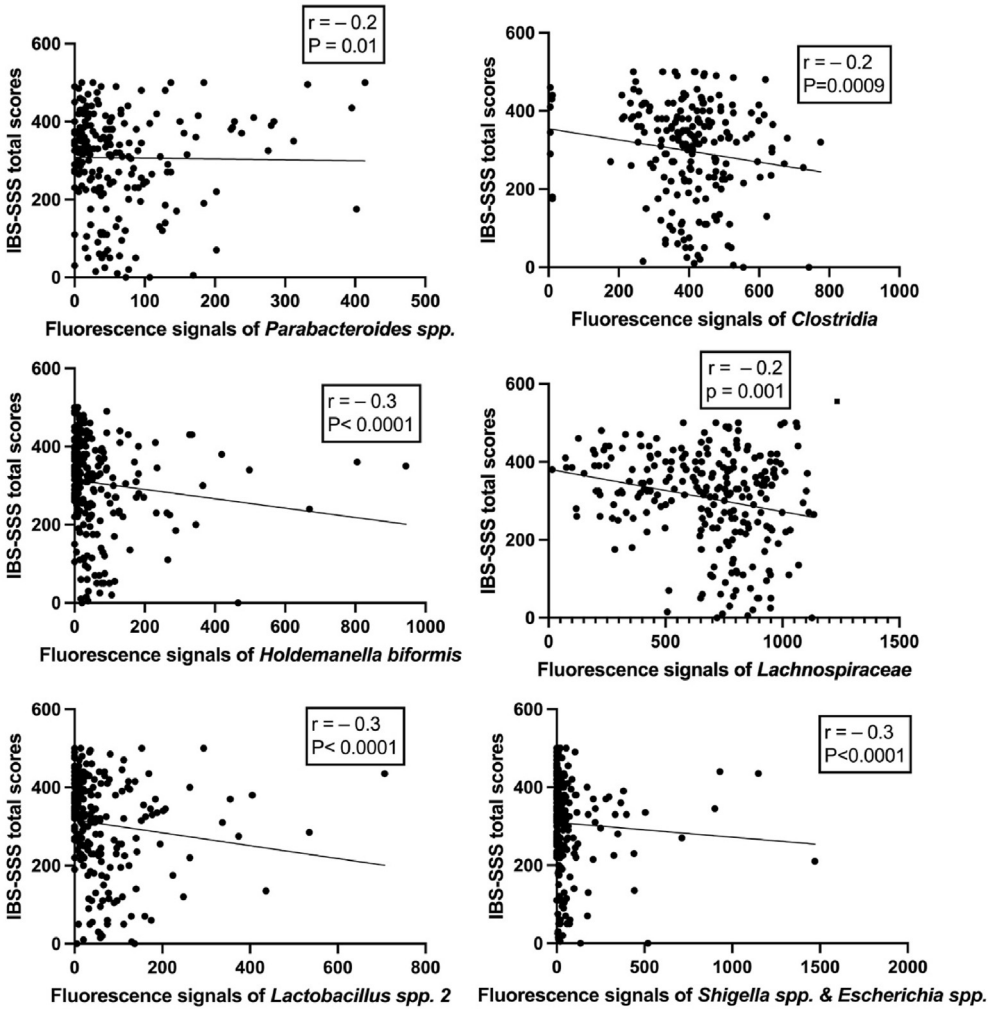

Fig. 6. Six bacterial markers whose fluorescence signals differed between the LI and SI groups. The fluorescence signals of these bacterial markers were inversely correlated with total IBS-SSS scores.

There was no difference between LI and SI groups in the reduction of IBS symptom severity at 1 years after FMT in our previous study [19]. The present study found that IBS symptoms at 2 years after FMT were significantly less severe in the SI group than in the LI group. These findings indicate that administering donor transplants to the SI results not only in higher FMT response rates but also greater long-term reduction of IBS symptom severity. Repeated FMT procedures reduced IBS symptom severity and increased the quality of life compared to a single FMT [19]. The observation in the present study of no differences between the SI and repeated-SI groups in the reduction of IBS symptom severity and in quality of life at 2 years after FMT suggests that there were no long-term differences between single and repeated FMT.

While patients with IBS in the SI group had a lower IBS symptom severity than those in the LI group, there was no intergroup difference in the quality of life. This could be explained by the finding that there was no difference in fatigue between the LI and SI groups. Fatigue seems to play a greater role in quality of life than IBS symptoms.

The present study found that IBS symptom severity measured by both IBS-SSS and Birmingham IBS Symptom Questionnaire scores were reduced significantly in both the LI and SI groups at 2 years after FMT. The levels of *Alistipes* spp., *Bacteroides* and *Prevotella* spp. combined, *Bacteroides zoogleoformans*, *P. johnsonii*, and *Mycoplasma hominis* increased in both groups at 2 years after FMT. The levels of these bacteria were inversely correlated with total IBS-SSS scores. Furthermore, the levels of *D. invisus*, *Lactobacillus ruminis* and *Pediococcus acidilactici* combined, *Streptococcus* spp., and *Streptococcus* spp. 2 were reduced in both groups at 2 years after FMT; these levels were correlated with the IBS-SSS scores. These changes in bacteria levels may explain the improvement in IBS symptoms in both the LI and SI groups at 2 years after FMT.

The reduction in IBS symptom severity at 2 years after FMT as quantified by both the IBS-SSS and Birmingham IBS Symptom Questionnaire scores was greater in the SI group than in the Li group. This could be explained by the difference in the bacterial changes in these groups at 2 years after FMT. Thus, whereas the levels of bacteria that were inversely correlated with total IBS-SSS scores (*H. biformis* and *Lactobacillus* spp.) increased in the SI group at 2 years after FMT, they decreased in the LI group. Moreover, the levels of bacteria that were positively correlated with total IBS-SSS scores (*Bacteroides fragilis*, *Bacteroides* spp., *Bacteroides stercoris*, *Coprobacillus cateniformis*, and *Streptococcus salivarius* subsp. *thermophilus* and *Streptococcus sanguinis* combined) were decreased in the SI group at 2 years after FMT, but were increased or unchanged in the LI group. These differences between the LI and SI groups in bacterial profiles could account for the greater reduction in IBS symptom severity in the SI group than in the LI group.

Chronic fatigue as measured by total FAS scores decreased significantly in both the LI and SI groups at 2 years after FMT

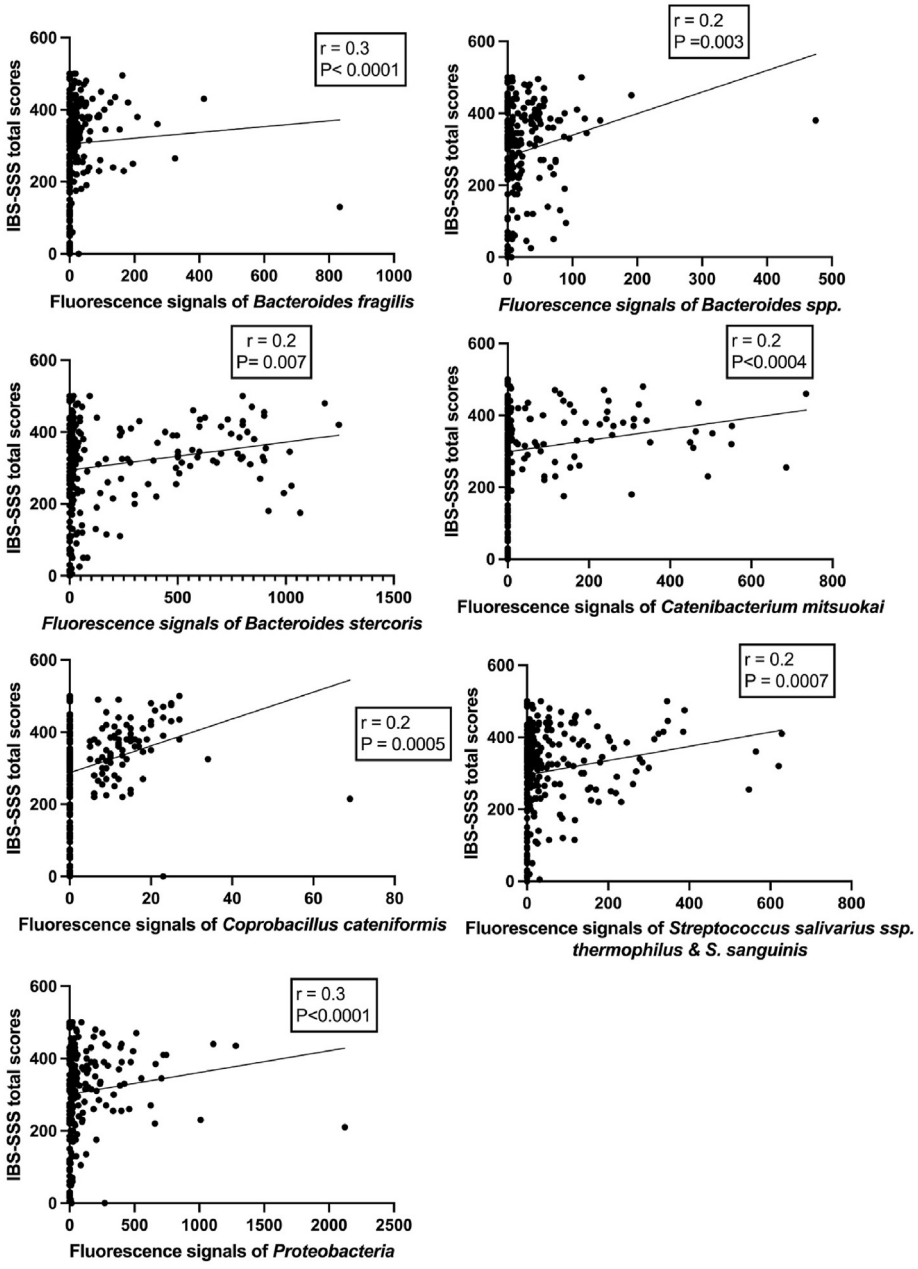

Fig. 7. The 7 bacterial markers correlated with total IBS-SSS scores among the 16 bacterial markers with fluorescence signals that differed between the LI and SI groups.

compared with the baseline values. This might be explained by the increases in both the LI and SI groups in the levels of four bacteria that were inversely correlated with total FAS scores: *Alistipes* spp., *Bacteroides* and *Prevotella* spp. combined, *Bacteroides zoogleoformans*, and *P. johnsonii.* However, two bacterial markers (*Bacteroides fragilis* and *Bacteroides* spp.) that were positively correlated with total FAS scores decreased in the SI group at 2 years after FMT, but were increased or unchanged in the LI group. Furthermore, *H. biformis* and *Lactobacillus* spp. were inversely correlated with total FAS scores and increased in the SI group at 2 years after FMT, but decreased in the LI group. These observations indicate that although administering the fecal transplant into either the LI or SI had the same improvement effect on chronic fatigue, administering into the SI led to long-term colonization of more-favorable bacteria associated with fatigue.

The present findings of no differences among IBS subtypes regarding FMT response rates or IBS symptoms, fatigue, and quality of life at the baseline and at 2 years after FMT were consistent with previously published observations [12,17,18].

Previous studies have found that FMT response rates were higher in females than males [10,27]. Furthermore, the response rates of patients with IBS with severe symptoms were higher than in those with moderate ones [28]. There was no difference in the response rates between females and males or between patients with IBS with severe or moderate IBS symptoms in the present study. One may conclude that the increase in the transplant dose from 60 g in the previous studies to 90 g in the present study underlies the difference between their findings [10,27,28].

The bacterial profile of responders to FMT after 2 years differed from that of nonresponders in six bacteria at the baseline. These differences disappeared at 2 years after FMT with the exception of *Alistipes* spp., which had lower levels in nonresponders than in responders. This finding was consistent with a previous

observation that the *Alistipes* spp. level is important for the response to FMT and its maintenance [29].

The main strengths of this study were that it included a relatively large cohort of patients with IBS comprising three subtypes, as well as female and male patients with IBS, which allowed subgroup analysis. However, the main limitations of this study were that it did not include the fourth IBS subtype, IBS-U, it did not investigate the entire bacterial composition of the intestinal, and it did not include a control placebo group.

5. Conclusions

At 2 years after faecal microbiota transplantation (FMT), administration the donor's faecal transplant to the small intestine had higher response rate and reduced IBS symptoms than that administrated to the large intestine, and led to long-term colonization of beneficial bacteria. FMT reduced fatigue and improved the quality of life at 2 years after FMT regardless the transplant administration. There was no long-term (2 years) difference between single and twice FMT. No long-term adverse events were reported.

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Data availability statement

Data presented in this study are available from the corresponding author on request.

CRediT authorship contribution statement

Magdy El-Salhy: Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Odd Helge Gilja:** Writing – review & editing, Data curation, Conceptualization. **Jan Gunnar Hatlebakk:** Writing – review & editing, Data curation, Conceptualization.

Declaration of competing interest

M.E.S and JGH have nothing to disclose. OHG has received speaker honoraria from Bracco, GE Healthcare, Takeda AS, Ferring AS, Allergan, and Janssen-Cilag. He has served as consultant for Bracco, GE Healthcare, Takeda and Samsung.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2024.105372.

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BRIEF REPORT

Treatment for Rheumatoid Arthritis Associated With Alterations in the Gastrointestinal Microbiota

Kristofer Andréasson,[1] Tor Olofsson,[1] Venu Lagishetty,[2] Zaid Alrawi,[1] Eline Klaassens,[3] Savanne Holster,[3] Roger Hesselstrand,[1] Jonathan P. Jacobs,[4] Johan K. Wallman,[1] and Elizabeth R. Volkmann[2]

Objective. Emerging research suggests that rheumatoid arthritis (RA) is associated with intestinal dysbiosis. This prospective pilot study evaluates changes in intestinal microbial composition in patients with RA initiating treatment with either methotrexate (MTX) or a tumor necrosis factor inhibitor (TNFi).

Methods. Consecutive patients, fulfilling the 2010 American College of Rheumatology/EULAR classification criteria for RA, who started treatment with either MTX or TNFi delivered a stool sample upon initiation of immunosuppression and 3 months later. A 16S ribosomal RNA gene-based validated microbiota test (GA-map Dysbiosis Index Score [DIS], Genetic Analysis, Oslo, Norway) was used to evaluate for the presence and degree of dysbiosis. Fecal levels of *Prevotella copri* (*P. copri*) were analyzed by custom-made quantitative polymerase chain reaction. Changes in microbial composition were analyzed in relation to changes in disease activity, as measured by the disease activity score based on 28-joint counts, using C-reactive protein.

Results. At baseline, dysbiosis was present in 33 of 50 (66%) participants and more common in participants with more than 2 years of disease duration ($P = 0.019$). At the 3-month follow-up, 27 of 50 (54%) were good treatment responders and the DIS had improved in 14 of 50 (28%). Participants initiating TNFi more often exhibited improvement in the DIS compared with those initiating MTX ($P = 0.031$). *P. copri* was identified in 32 of 50 (64%) at baseline. An improvement in disease activity score based on 28-joint counts, using C-reactive protein was associated with a simultaneous decrease in *P. copri* abundance ($r_s = 0.30$, $P = 0.036$).

Conclusion. This study affirms that dysbiosis is a feature of RA. Although patients were not randomized to MTX or TNFi, the findings suggest that specific therapies may differentially modulate the gastrointestinal microbiota in RA. The association between *P. copri* and treatment response requires further study.

INTRODUCTION

The etiology of rheumatoid arthritis (RA) is multifactorial. Although host genetics may contribute to disease susceptibility, environmental factors, such as smoking, may increase the likelihood of disease development.[1] Our current understanding of the key environmental factors associated with RA pathogenesis is limited, but recent studies suggest that alterations in the gastrointestinal (GI) microbiota are present early in the disease course.[2]

Prior studies have demonstrated an association between immune response to microbes in the intestine and synovial inflammation in patients with RA.[3] Cross-sectional studies have consistently reported the presence of intestinal dysbiosis in both early and established RA.[4–6] Furthermore, *Prevotella copri* (*P. copri*) has been associated with RA and RA development based on

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both epidemiologic studies and a well-defined molecular mimicry model.[2–5]

To date, few studies have explored the intestinal microbiota in relation to usage of immunomodulatory treatments in RA. Small, prospective studies have suggested that successful methotrexate (MTX) therapy in patients with RA may partially normalize the intestinal microbiota and that specific microbial alterations may predict treatment response to MTX.[4,7,8] To our knowledge, no studies have prospectively evaluated the impact of tumor necrosis factor inhibitors (TNFi) on intestinal dysbiosis in RA. However, in inflammatory bowel disease (IBD), TNFi administration has been associated with a reduction of intestinal dysbiosis that parallels improvements in disease activity with reduced intestinal inflammation.[9]

This prospective, observational pilot study investigated patients with RA initiating treatment with either MTX or TNFi at baseline and after 3 months. Our aims were to (1) examine the relationship between the baseline degree of intestinal dysbiosis and clinical characteristics of patients with RA and (2) evaluate changes in the degree of intestinal dysbiosis following treatment with MTX or TNFi. Finally (3), given the prior evidence linking *P. copri* to RA disease pathogenesis, we wanted to investigate *P. copri* abundance in relation to disease characteristics and treatment response. In an exploratory aim, this study also sought to explore the relationship between a candidate marker of intestinal inflammation (ie, fecal calprotectin) and intestinal dysbiosis in patients with RA.

MATERIALS AND METHODS

Patient eligibility. This study consecutively enrolled patients with RA fulfilling the 2010 American College of Rheumatology/EULAR classification criteria between February 2016 and March 2018. Eligible patients had to be initiated on therapy with either MTX or TNFi at the departments of Rheumatology in Lund, Sweden, and Karlskrona, Sweden.[10] Patients were included regardless of disease duration, and patients initiating TNFi were included irrespective of ongoing background disease-modifying antirheumatic drug (DMARD) therapy as long as it had been prescribed at a stable dosage for 3 months before the study start and remained unaltered during the study period. Treatment selection was based on physician discretion and typically reflected the national guidelines on the management of RA from the Swedish Society for Rheumatology.[11] In general, during this time period, these guidelines suggested MTX as the first line DMARD treatment for RA and TNFi as the first-line biologic DMARD therapy in refractory cases. Patients were excluded if they met any of the following exclusion criteria: a history of IBD, a history of diverticulitis, previous surgery of the GI tract (except appendectomies), a history of alcohol abuse, failure to comply with protocol, antibiotic use within the last 4 weeks, or ongoing biologic or targeted synthetic DMARD therapy. Patients were also excluded if the initiation of the study drug coincided with the initiation or cessation of another DMARD.

Disease activity and treatment response. Disease activity, measured as disease activity score based on 28-joint counts, using CRP (DAS28-CRP), was assessed at baseline and at 3 months follow-up, and the change in disease activity (ΔDAS28-CRP) between these two time points was used as a continuous measure of treatment response. Furthermore, participants were categorized as showing good, moderate, or no treatment response in accordance with EULAR response criteria.[12]

Patient reported measures. At baseline, GI symptoms were assessed using the validated Gastrointestinal Symptom Rating Scale (GSRS).[13] This Swedish rating scale is a self-administered questionnaire that includes 15 items rated on a 7-grade Likert scale. It encompasses the following five dimensions: reflux, abdominal pain, constipation, indigestion, and diarrhea. Usage of antibiotics, coffee, proton pump inhibitors, and smoking were assessed by a local questionnaire and alcohol consumption by the Alcohol Use Disorders Identification Test questionnaire.[14]

GI microbiota analysis. At baseline and at 3 months follow-up, participants delivered stool samples, collected at home, which were briefly stored in a refrigerator, and transported to a −80°C freezer within 12 hours of collection. The Dysbiosis Index Score (DIS) was measured with the GA-map Dysbiosis Test (Genetic Analysis), which quantifies intestinal microbial balance on a 5-grade ordinal scale, in which values of three or more denotes dysbiosis. This test analyzes the 16S ribosomal RNA (rRNA) sequence (V3-V9) by using 54 labeling probes aimed at 368 strains spread across 36 genera, 10 bacterial classes, and 6 phyla (Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Tenericutes, and Verrucomicrobia).[15] The GA-map Dysbiosis Test results demonstrate good agreement with the results of MiSeq Illumina high-throughput 16S rRNA amplicon sequencing in identifying intestinal dysbiosis.[15] The test has also been validated in relation to Scandinavian cohorts of healthy controls.[15–17] 17 All samples in the current study were analyzed simultaneously to minimize batch effects.

Fecal levels of *P. copri* were analyzed in triplicates with a custom-made quantitative polymerase chain reaction together with Baseclear at baseline and at 3 months (Leiden, The Netherlands) (Supplementary methods). The abundance of *P. copri* bacteria per gram of feces was calculated, and log 2 values (2logPcopri) were used for analysis. A two-fold change in *P. copri* levels was regarded as a significant increase or decrease of *P. copri* abundance.

Intestinal inflammation was studied by measurement of fecal calprotectin (F-calprotectin), which was analyzed with a polyclonal Elisa (Calpro AS). Levels of F-calprotectin greater than or equal to 50 μg/g were considered elevated.[18]

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Statistical analysis. To evaluate the relationships between the DIS or *P. copri* levels with clinical features, nonparametric testing, including the Mann-Whitney U-test and Spearman's rank correlation test, were used. The same nonparametric tests were used to evaluate how changes in the DIS and *P. copri* levels relate to therapy and treatment response. A *P* value less than 0.05 was considered statistically significant. Given the pilot nature of the study, a power calculation was not performed, and there were no corrections made for multiple hypothesis testing.

RESULTS

Patient characteristics. Sixty-four patients fulfilled the study inclusion criteria, of which 50 participants completed the study according to protocol. Out of those not completing the study (n = 14), a majority (n = 9) failed to deliver both stool samples, and another subgroup (n = 3) failed to complete 3 months of prescribed therapy.

Patient characteristics are shown in Table 1. At baseline, 29 of the 32 patients who initiated TNFi therapy were already prescribed conventional synthetic DMARD therapy (n = 29 of 32), in most cases MTX (n = 24 of 32). Among the included participants, there was a female predominance, and on average, participants had moderate RA disease activity at baseline. Disease duration was, as expected from the clinical treatment algorithm previously described, lower in participants starting MTX compared with TNFi (median 0.4 vs 6.4 years, respectively). The presence of anticitrullinated peptide antibodies and

Table 1. Baseline patient characteristics*

	All patients (N = 50)	MTX-start (n = 18)	TNFi-start (n = 32)
Age, y (SD)	58 (11)	59 (11)	58 (11)
Female, n (%)	39 (78)	14 (78)	25 (78)
ACPA positive, n (%)	38 (76)	11 (61)	27 (84)
RF positive, n (%)	34 (68)	11 (61)	23 (72)
HAQ, mean (SD)[a]	0.85 (0.47)	0.76 (0.51)	0.90 (0.45)
Disease duration, y, median, (IQR)	4.4 (0.7–12)	0.4 (0.3–1.1)	6.4 (3.1–17.3)
DAS28-CRP, mean (SD)	3.9 (1.2)	3.9 (0.8)	3.9 (1.4)
Daily coffee consumption, cups (SD)	4.3 (4.3)	3.9 (1.9)	4.5 (5.2)
Smoking, ever, n (%)[a]	34 (68)	10 (56)	24 (75)
Smoking, package y, mean (SD)[a]	13 (16)	12 (16)	14 (16)
Current smoker, n (%)	8 (16)	3 (17)	5 (13)
PPI usage, n (%)			
Daily	11 (22)	3 (17)	8 (25)
Sporadic	8 (16)	2 (11)	6 (19)
None	31 (62)	13 (72)	18 (56)
NSAID, n (%)			
Daily	12 (24)	4 (22)	8 (25)
Sporadic	10 (20)	4 (22)	6 (19)
None	28 (56)	10 (59)	18 (56)
Study drug			
MTX po, n (%)		16 (89)	N/A
MTX sc, n (%)		2 (11)	N/A
MTX weekly dose mg, mean (SD)		19 (2.7)	N/A
Etanercept		N/A	31 (97)
Infliximab		N/A	1 (3)
Ongoing treatment			
MTX, po, n (%)		N/A	16 (50)
MTX, sc, n (%)		N/A	8 (25)
MTX weekly dose mg, mean (SD)		N/A	14 (8)
Sulfasalazine, n (%)		0 (0)	6 (19)
Prednisolone, n (%)			
0 mg	27 (54)	11 (61)	16 (50)
1–5 mg	13 (26)	4 (22)	9 (28)
6–10 mg	7 (14)	3 (17)	7 (22)
>10 mg	0 (0)	0 (0)	0 (0)
BMI, mean (SD)[b]	26 (4.4)	27 (2.7)	26 (4.7)
F-calprotectin µg/g, median	25 (12–51)	27 (17–90)	22 (11–41)
Elevated (≥50 ug/g), n (%)	12 (24)	5 (28)	7 (22)

*ACPA, anticitrullinated peptide antibodies; BMI, body mass index; DAS28-CRP, disease activity score based on 28-joint counts; HAQ, health assessment questionnaire; IQR, interquartile range; MTX, methotrexate, N/A, not applicable; NSAID, nonsteroidal antiinflammatory drug; po, per os; PPI, proton pump inhibitor; RF, rheumatoid factor; sc, subcutaneous.
[a]Data missing = 3.
[b]Data missing = 6.

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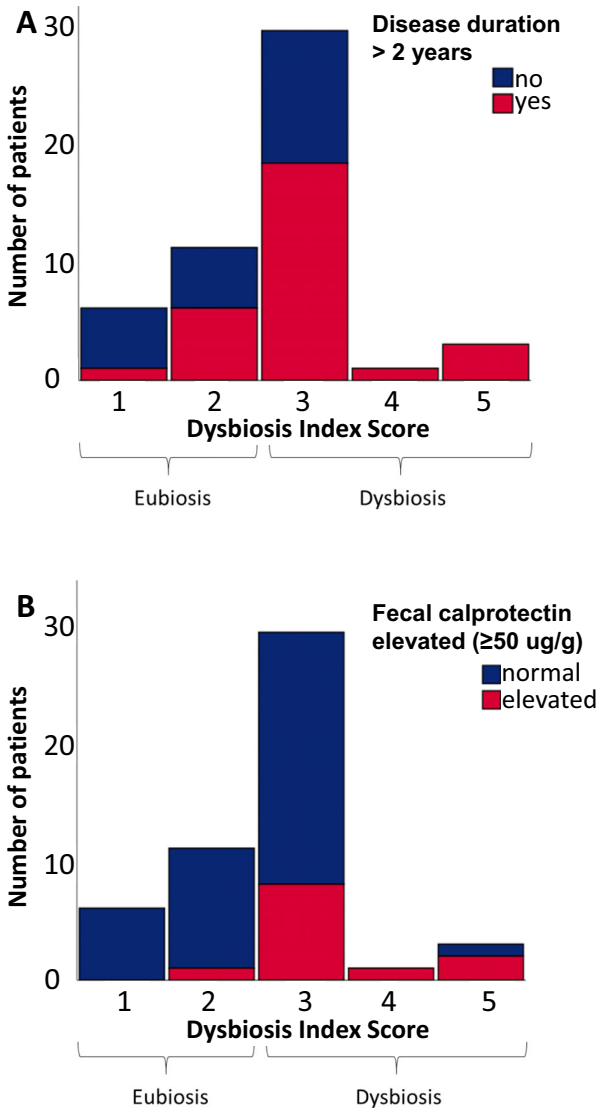

Figure 1. Histogram of the Dysbiosis Index Score at baseline. Each bar represents number of patients. A Dysbiosis Index Score of 3–5 represents intestinal dysbiosis and 1–2 eubiosis. In (A), patients with rheumatoid arthritis disease with a duration of more than 2 years are marked in red. In (B), patients with elevated fecal calprotectin, indicating intestinal inflammation, are marked in red.

rheumatoid factor (RF) were more common in participants initiating TNFi than MTX.

Intestinal dysbiosis in RA. At baseline, a DIS of greater than or equal to 3, indicating the presence of intestinal dysbiosis, was present in 33 out of 50 participants (Figure 1), with similar proportions in participants initiating MTX (11 of 18 [61%]) and TNFi (22 of 32 [69%]). The baseline DIS did not correlate with disease activity or disease duration (Table 2). However, when comparing participants with established (>2 years, n = 29) and early disease (≤2 years from first RA symptoms, n = 21), the DIS was higher in participants with established disease (mean 3.0 vs 2.3; $P = 0.019$) (Figure 1A).

Twelve patients (24%) exhibited elevated levels of F-calprotectin suggestive of intestinal inflammation. Increased F-calprotectin levels were associated with higher (worse) DIS ($r_s = 0.33$ $P = 0.018$), and the DIS was higher in participants with elevated F-calprotectin (mean 3.3 vs 2.5, $P = 0.006$) (Figure 1B).

GI symptoms, as assessed by the GSRS, were uncommon in this cohort (Supplementary Table 1). The DIS was not associated with the GSRS, body mass index, concomitant medications (nonsteroidal antiinflammatory drugs/prednisolone/proton pump inhibitor), serological profile, smoking, or alcohol habits (Table 2 and Supplementary Table 2).

Intestinal dysbiosis and inflammation at follow-up. At the 3-month follow-up, 54% of the study participants exhibited good EULAR-defined treatment response, whereas no treatment response occurred in 26% of the participants. Numerically, more participants who initiated MTX exhibited a good treatment response compared with TNFi (12 of 18 [67%] vs 15 of 32 [47%]).

At follow-up, 14 participants showed a decrease (improvement) of their DIS, whereas 11 exhibited an increase (worsening) of their DIS. For those initiating MTX, six (33%) experienced a worsening of their DIS at follow-up, and two (11%) experienced an improvement in their DIS. For participants initiating TNFi, 5 (15%) experienced worsening of their DIS, whereas 12 (38%) experienced an improvement in their DIS. There was a significant

Table 2. The Dysbiosis Index Score (a five-step semiquantitive measure of intestinal dysbiosis) and *P. copri* levels in relation to disease activity, disease duration, fecal calprotectin, and serology at baseline*

	Correlation with Dysbiosis Index Score, r_s	Correlation with *P. copri*, r_s
DAS28-CRP	0.11, $P = 0.479$	−0.04, $P = 0.780$
Disease duration	0.22, $P = 0.118$	0.11, $P = 0.445$
F-calprotectin	0.33, $P = 0.018$	−0.06, $P = 0.677$
Dysbiosis Index Score	N/A	−0.11, $P = 0.430$
	Median levels, (yes vs no)	
	Dysbiosis Index Score	*P. copri*, ng per g feces
RF-positivity	3.0 vs 3.0, $P = 0.304$	1.2e-3 vs 0.0, $P = 0.034$
ACPA-positivity	3.0 vs 3.0, $P = 0.789$	9.6e-5 vs 4.5e-5, $P = 0.340$

*ACPA, anticitrullinated peptide antibodies; DAS28-CRP, disease activity score based on 28-joint counts; N/A, not applicable; *P. copri*, *Prevotella copri*; RF, rheumatoid factor.

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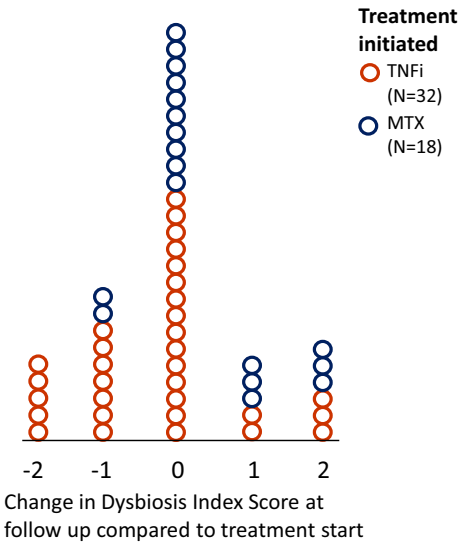

Figure 2. Change in intestinal microbial balance after initiation of TNFi or MTX. Dot-plot showing change in the Dysbiosis Index Score in patients starting treatment with either TNFi or MTX at 3 months follow-up. An improvement of the Dysbiosis Index Score was more common in patients starting TNFi than MTX. MTX, methotrexate; TNFi, tumor necrosis factor inhibitor.

difference between the mean change in DIS between participants initiating MTX and TNFi (0.28 vs –0.39, P = 0.031) at follow-up (Figure 2).

Changes in intestinal dysbiosis in relation to treatment response. Among participants achieving good EULAR-defined treatment response, seven exhibited a worsening of the DIS and five exhibited an improvement in the DIS. For those achieving no treatment response, three exhibited a worsening of the DIS and five exhibited an improvement (Figure 3).

***P. copri* in relation to disease characteristics and treatment response.** At baseline, *P. copri* was identified in measurable levels (above 1.0e-6 ng/g faces) in 32 of 50 (64%) participants, with similar proportions in participants initiating MTX (11 of 18 [61%]) and TNFi (21 of 32 [66%]), respectively. *P. copri* baseline levels were not significantly associated with disease activity, disease duration or early disease (as defined previously), F-calprotectin, or the DIS. *P. copri* was more prevalent among participants who were RF positive (Table 2).

After 3 months, a decrease in *P. copri* was observed in 19 participants, whereas 18 exhibited an increase in *P. copri*. Changes in *P. copri* levels were evenly distributed among MTX-starters and TNFi-starters, with 7 of 18 [39%] and 12 of 32 [38%] exhibiting a decrease in *P. copri* levels, respectively.

A decline in *P. copri* levels between treatment start and the 3-month follow-up was associated with an improvement in the EULAR-defined treatment response (r_s = 0.30, P = 0.034). This association with treatment response was evident in patients who were RF positive (Figure 4).

DISCUSSION

The present study affirmed that intestinal dysbiosis is present in a prospective cohort of consecutive patients with RA initiating

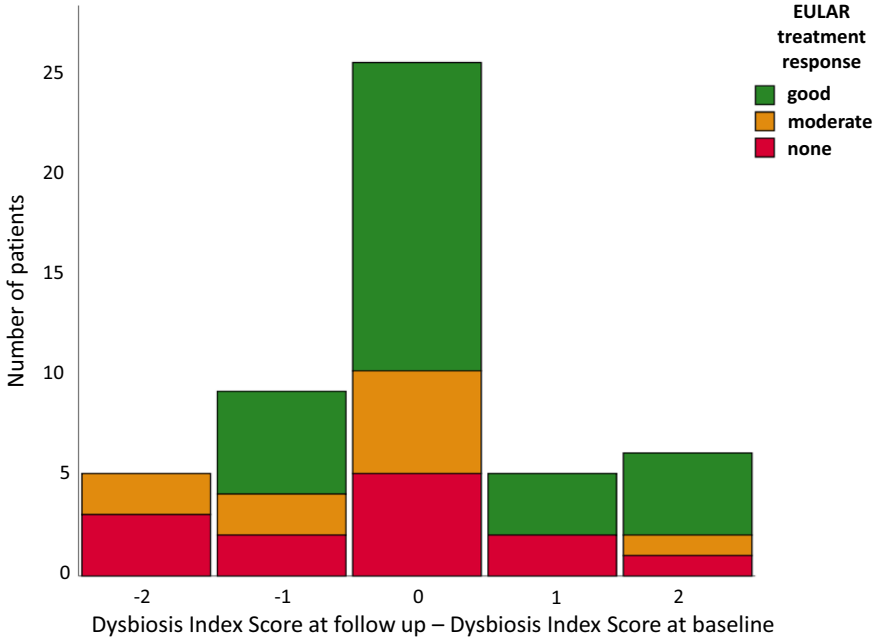

Figure 3. Histogram of the change in Dysbiosis Index Score at follow-up. Each bar represents number of patients. Good treatment response could be seen both in patients exhibiting an improvement (negative value on x-axis) and a worsening (positive value on x-axis) of the Dysbiosis Index Score at follow-up.

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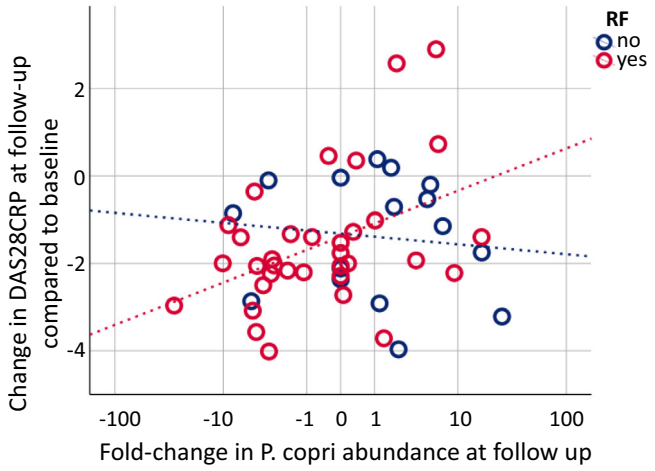

Figure 4. Change in disease activity in relation to change in *P. copri* abundance. At follow-up, 3 months after treatment start, change in disease activity is associated with change in *P. copri* abundance in patients with positive RF (r_s = 0.344, P = 0.046, n = 34) but not patients with negative RF (r_s = –0.17, P = 0.541, n = 16). The majority of patients with positive RF who experienced a positive response to their immunosuppressive therapy also exhibited a decline in P. copri levels (lower left quartile). DAS28-CRP, disease activity score based on 28-joint counts; *P. copri*, *Prevotella copri*; RF, rheumatoid factor.

therapy with either MTX or TNFi. This pilot study also found that participants initiating therapy with TNFi were more likely to have an improvement in their extent of intestinal dysbiosis compared with those initiating therapy with MTX. Although the impact of MTX on GI microbiota has been studied, to our knowledge, this is the first study to report longitudinal changes in the intestinal microbiota among patients with RA receiving TNFi.

The finding that changes in intestinal microbiota were observed in patients with RA who commenced on TNFi therapy is consistent with the results of studies in other patient populations, such as IBD and spondyloarthritis.[19] Although GI inflammation and dysbiosis are recognized disease features of IBD and spondyloarthritis, our understanding of GI-tract involvement in RA is still evolving. One cross-sectional study in RA suggested the possibility of normalization of the microbiota after starting treatment with TNFi,[20] but that study did not evaluate changes in intestinal microbiota over time. In our study, 31 out of 32 patients treated with TNFi received etanercept, which is a soluble TNF-receptor. In contrast to monoclonal antibodies, this TNFi has failed to show efficacy in IBD. Further studies are needed to elucidate how this TNFi may interact with the GI immune system and microbiota and if this differs from TNFi of monoclonal antibody type.

It is unclear whether the observed changes in intestinal microbiota in patients treated with TNFi therapy contribute to the pathobiology of RA and/or treatment response. However, the present study followed a significant relationship between reduction in *P. copri* abundance and response to therapy in both treatment groups. Accumulating evidence suggests that *P. copri* is directly involved in autoimmune reactions, leading to articular inflammation in RA.[21] A previous cross-sectional study reported a greater abundance of *P. copri* in untreated patients with RA compared with patients with RA receiving immunomodulatory treatment.[2] The interplay between intestinal microbes and the immune system in relation to systemic autoimmunity is an active area of research,[21–22] and future clinical trials in RA may consider evaluating the abundance of *P. copri* as a candidate biomarker of treatment response.

Although this study was not adequately powered to analyze treatment response in relation to dysbiosis as measured by the DIS, many participants who exhibited good treatment response did so without an improvement in the DIS (Figure 3). Although the reasons for this discrepancy are unknown, it is possible that further measurement of specific bacteria species could provide greater insight into how different therapies alter the intestinal microbiota in RA.[21,23] Furthermore, external factors (eg, age, sex) beyond the intestinal microbiota may modulate treatment response in RA and should be considered in future studies.

This study also demonstrated that *P. copri* was more prevalent in participants with positive RF, compared with participants with negative RF, which is a finding consistent with recent reports.[21,24] Future studies of larger magnitude are needed to evaluate whether *P. copri* can serve as a biomarker for RA treatment response in both patients with positive and negative RF.

The observed relationship between intestinal dysbiosis and elevated levels of F-calprotectin was intriguing and may reflect the dynamic relationship between dysbiosis and inflammation in the intestinal mucosa. Similar results have previously been reported in arthritis animal models.[25] Endoscopic studies involving biopsies may help to further elucidate the relationship between the intestinal immune system and microbiota in relation to RA development.

We were unable to confirm the findings of a previous study, which demonstrated partial resolution of intestinal dysbiosis following oral MTX therapy.[4] The present study population differed from that of the previous report in respect to geographic origin, disease activity, age, and disease duration. Specifically, the prior study included a majority of patients with high disease activity.

This study has notable limitations. First, although the microbial analyses of fecal samples were based on the Dysbiosis Index Score, which has shown comparable performance in identifying intestinal dysbiosis in relation to MiSeq Illumina high-throughput 16S rRNA amplicon sequencing, the DIS has limited power to identify novel bacterial strains and to provide comprehensive insight into all bacterial strains present in a sample. Based on the promising results of this pilot study, future analyses using high-throughput sequencing of the full metagenome are warranted.

Further limitations of this observational study include the fact that patients were not randomized to treatment, and therefore, confounding factors may contribute to the observed treatment arm differences. Twenty-four out of 32 patients initiating TNFi were on a stable dose of MTX at baseline and continued using this drug in unaltered dosage throughout the study period, which may have influenced group comparisons. Also, the number of included

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patients is limited, restricting further subgroup analyses. The strengths of this study include its prospective design, prespecified objectives and outcomes, as well as the inclusion of consecutive patients receiving two different therapeutic approaches.

In summary, the present study demonstrated alterations of the intestinal microbiota between treatment start and 3-month follow-up in patients with RA initiating MTX or TNFi. Reduction of *P. copri*, a bacterial species previously suggested to be of pathogenic importance in RA, paralleled observed improvements in RA disease activity. Future prospective studies in larger cohorts, encompassing analysis of the complete intestinal microbiome, are needed to validate these results and further explore the relevance of microbial changes induced by immunosuppression in RA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr Andréasson had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Andréasson, Olofsson, Alrawi, Wallman, Volkmann.

Acquisition of data. Andréasson, Olofsson, Alrawi, Klaassens, Holster, Wallman.

Analysis and interpretation of data. Andréasson, Olofsson, Lagishetty, Klaassens, Holster, Hesselstrand, Jacobs, Wallman, Volkmann.

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REVIEW

A Systematic Review: Fecal Bacterial Profile in Patients with Irritable Bowel Syndrome Analyzed with the GA-Map Dysbiosis Test Based on the 16S rRNA Gene of Bacterial Species or Groups

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Purpose: The diagnosis of irritable bowel syndrome (IBS) is based on symptom-based criteria due to lack of reliable disease-specific biomarkers. Gut microbiota is perturbed in IBS and when comparing different methods used to analyze gut microbiota, the results might be obscured. Therefore, in this systematic review we aimed to investigate the profile of fecal bacterial markers and dysbiosis index (DI) in patients with IBS and IBS subgroups compared to healthy controls (HCs) conducted by the same method (GA-map Dysbiosis Test based on16S rRNA sequencing).

Material and Method: We searched PubMed, EMBASE (Ovid) and Cochrane Library for case-control studies comparing fecal gut microbiota analyzed with the GA-map® Dysbiosis Test (Oslo, Norway) in patients with IBS and HCs. Our outcomes were the difference in fecal bacterial markers and DI in patients with IBS and IBS subgroups compared to HCs.

Results: The search identified 28 citations; five articles were included. Most studies evaluated fecal bacterial markers and DI in patients with diarrhea-predominant IBS (IBS-D). Results of fecal bacteria profile in IBS and IBS subgroups compared to HCs are inconsistent, however, two studies showed increased levels of *Ruminococcus gnavus* in IBS-D compared to HCs and results of DI indicated IBS and IBS subgroups (especially IBS-D) having higher DI compared to HCs.

Conclusion: This systematic review revealed inconsistent findings in respect to differences in bacterial markers between IBS and IBS subgroups with HCs in studies using the GA-map Dysbiosis Test based on 16S rRNA sequencing. However, the test is quite novel, and few studies have used the method so far. More research comparing fecal microbiota profile differences in IBS and IBS subgroups compared to HCs utilizing the same method of analysis is needed to give us further insight into the gut bacteria profile in IBS and the clinical consequences of intestinal dysbiosis.

Keywords: gut microbiota, dysbiosis, diagnostic test, *Ruminococcus gnavus*

Introduction

Irritable bowel syndrome (IBS) is a disorder of the brain-gut axis interaction[1] affecting 4–10% of the general population.[2] Today, the diagnosis is based on exclusion of organic diseases and fulfilling the Rome IV criteria[1] which is based on symptoms of recurrent abdominal pain at least one day per week associated with stool irregularities.[3] IBS is divided in four subtypes based on the predominant stool consistency: IBS with predominantly diarrhea (IBS-D), with predominantly constipation (IBS-C), with a mix of diarrhea and constipation (IBS-M) and unclassified subtype (IBS-U).[3] The symptoms are experienced from mild to severe and often comes with somatic and psychiatric comorbidities such as fibromyalgia, fatigue, depression and anxiety, and impacts the quality of life in these patients.[4–6] It also gives a profound burden on the healthcare system and the society.[6]

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The etiology of IBS is complex and multifactorial, including altered brain-gut-microbiota axis, visceral hypersensitivity, low-grade inflammation, intestinal dysbiosis, and impaired gut barrier functions and integrity.[1,7–9] Dysbiosis is an deviation from normobiosis and is defined as an imbalance in bacterial composition.[4] Today, the manipulation of gut microbiota is suggested to be a target for therapy of IBS[7] but the gut microbiota profile in IBS is still not fully understood as the literature reports inconsistent results. Several different methods are available and used for studying the gut microbiota and the costs are becoming more affordable.[7] However, when comparing the fecal microbiota profile across different methods, the results might be obscured.

Therefore, in this systematic review we aimed to investigate the profile of fecal bacterial markers and dysbiosis index (DI) in patients with IBS and IBS subgroups compared to healthy controls (HCs) conducted by the same method of analysis, GA-map Dysbiosis Test.[10] The test is based on 16S rRNA sequencing, is commercially available and is an easy to use method which aims to identify and characterize dysbiosis by determining deviation from normobiosis in fecal samples in IBS and IBD. Hopefully, this will give us further insight into the gut bacteria profile in IBS and the clinical consequences of intestinal dysbiosis.

Materials and Methods

Search Strategy

We performed a systematic review with relevant literature retrieved from the databases PubMed, EMBASE (Ovid) and Cochrane Library to identify case-control studies comparing fecal gut microbiota analyzed with the GA-map Dysbiosis Test in patients with IBS and HCs. The search terms used to identify potentially related publications included «Genetic analysis AND Dysbiosis test AND Irritable bowel syndrome», with results by year 2015 to January 2023. Two authors independently reviewed the studies retrieved by the search strategy and excluded trials based on titles, abstracts, and full-text analysis. We followed the checklist and flowchart of the Preferred Reporting of Systematic Reviews and Meta-Analyses (PRISMA) for this systematic review.

Study Selection

The inclusion criteria (Table 1) were studies investigating fecal microbiota in adult IBS patients compared with HCs, IBS patients classified according to the ROME II–IV criteria, GA-map Dysbiosis Test to analyze fecal bacterial samples, samples from stool, written in English, published from 2015 to January 2023 and articles published as a full article. The exclusion criteria were literature published before 2015, not written in English, fecal microbiota not analyzed with the GA-map Dysbiosis Test or not case-control studies.

Table 1 Inclusion Criteria for Articles Included in the Systematic Review

Study, First Author, Year	Case-Control (IBS vs HC)	GA-map Dysbiosis Test	Samples of Feces	Adult Population	Written in English	Article Published 2015–2023	Article Available as a Full Article
Ahluwalia et al, 2021[11]	*	*	*	*	*	*	*
Casén et al, 2015[10]	*	*	*	*	*	*	*
Vasapolli et al, 2021[12]	*	*	*	*	*	*	*
Iribarren et al, 2022[13]	*	*	*	*	*	*	*
Mazzawi et al, 2018[14]	*	*	*	*	*	*	*

Choice of Outcome

The primary outcome was differences in fecal bacterial markers and DI in patients with IBS compared to HCs. The secondary outcome was differences in bacterial markers and DI in subgroups of IBS compared to HCs. We classified the primary and secondary outcomes into three categories; significantly increased in IBS patients, significantly decreased in IBS patients and no significant (NS) difference between IBS patients and HCs. The DI was estimated as mean or median.

Fecal Bacteria and Dysbiosis Analysis

GA-map Dysbiosis Test (GA-map® Dysbiosis Test, Oslo, Norway)[10] was developed to identify and characterize dysbiosis by determining deviation from normobiosis in fecal samples in patients with IBS and IBD. It gives an algorithmically Dysbiosis Index (DI) from 1 to 5 where a DI value of 2 or lower being non-dysbiotic and 3–5 being dysbiotic. The higher DI above 2, the more it is considered to deviate from normobiosis and being dysbiotic. Transient luminal bacteria with no diagnostic values have the potential to obscure diagnostic results. Therefore, the probe set was based on intestinal microbiota observed in IBS and IBD in the literature and each probe was selected based on the ability to distinguish between IBS and IBD and HCs.[10] The technology uses 54 bacterial markers, based on the 16S rRNA sequences in seven variable regions (V3-V9) and measures relative abundance of bacteria according to strength of fluorescent signal detection. This provides a unique opportunity to investigate changes in gut microbiota profiles which potentially might be associated with GI-related disorders.

Eligibility Assessment and Data Extraction

To reduce the reporting error and bias in data manipulation, both authors independently reviewed the selected studies for complete analysis. One study author extracted the data and entered it into a spreadsheet, while the other study author evaluated the process. When there was a discrepancy between reviewers, we re-checked and discussed the data together to reach an agreement by consensus. The data included are presented in Table 2; distribution of age and sex, country in which the research was conducted, the size of the IBS and HC groups, IBS subtypes, details of intervention, multiple comparison correction, dysbiosis index and bacterial markers difference with a significant P value < 0.05.

Quality Assessment

We applied the Newcastle-Ottawa Scale (NOS) for assessing the quality of included case-control studies in this review (data presented in Table 3). The Newcastle-Ottawa Scale consists of 3 domains (maximum nine stars); selection (is the case definition adequate, representativeness of the cases, selection of controls, definition of controls); comparability (comparability of baseline characteristics); and exposure (ascertainment of exposure, same method of ascertainment for cases and controls, attrition rate). We decided that sex and age was the most important baseline characteristics to be described and compared in both groups.

Results

Study Selection and Characteristics

Overall, 28 citations were retrieved (Figure 1): six citations were duplicates and four citations were rejected based on their title. Within the 22 remaining studies, 13 were excluded based on the abstract or full-text review as nine studies had no healthy controls, two studies were conference abstracts and two papers included repetition of data. In total, five studies investigating fecal microbiota composition and/or DI were included in the systematic review.

Study populations across the studies were conducted from Sweden,[10,11,13] Norway,[10,14] Denmark,[10] Spain[10] and Germany.[12] All studies had case-control design, were published in the period of 2015 to January 2023 and fecal microbiota was assessed with the GA-map Dysbiosis Test in adults of both genders.

The Newcastle Ottawa Scale (Table 3) showed all relevant studies provided an adequate explanation in the definition and selection method for IBS patients and HCs with IBS classified according to Rome II–IV criteria by a physician, and HCs did not have any organic disease or gastrointestinal (GI) symptoms. All studies demonstrated comparable data of both sex and age in IBS and HCs, hence, one study had significantly higher median age in IBS compared to HCs[11] and as

Table 2 Characteristics of Included Studies in the Systematic Review

			IBS patients					Controls						
Study, first author, year	**Country**	**Type of specimen**	**IBS subtype**	**Age (years)**	**n participants**	**Female/ Males**	**DI[a]**	**Age (years)**	**n participants**	**Female/ Males**	**DI[a]**	**Microbiome assessment method**	**Multiple comparison correction**	**Bacterial markers difference with a significant P value < 0.05**
Ahluwalia et al, 2021[11]	Sweden	Stool	IBS, IBS-D, IBS-M, IBS-C, IBS-U	Median (min-max) 52 (24–70)	40	31/9	No	Median (min-max) 26 (19–54)	18	9/9	No	GA-map Dysbiosis Test	NR[b]	Yes
Casén et al, 2015[10]	Norway, Sweden, Denmark, Spain	Stool	IBS, IBS-D, IBS-C, IBS-M, IBS-U, IBS-A	Mean: 39	109	184/52	Yes	Mean (min-max) 41 (21–70)	43	187/110	Yes	GA-map Dysbiosis Test	NR[b]	Yes
Vasapolli et al, 2021[12]	Germany	Stool	IBS-D, IBS-C	Mean (SD) IBS-D: 43.5 (20.6) IBS-C: 50.6 (18.2)	IBS-D: 15 IBS-C: 13	IBS-D: 10/5, IBS-C: 10/3	Yes	Mean (SD) 54.8 (11.2)	22	12/10	Yes	GA-map Dysbiosis Test (Lx)	Yes	Yes
Iribarren et al, 2022[13]	Sweden	Stool	IBS-D	Median (range) 31 (26–66)	9	6/3	No	Median (range) 22 (20–36)	7	5/2	No	GA-map Dysbiosis Test	NR[b]	Yes
Mazzawi et al, 2018[14]	Norway	Stool	Mostly IBS-D	Mean (min-max) 32 (20–44)	13	4/9	Yes	Mean (min-max) 33 (20–42)	13	7/6	Yes	GA-map Dysbiosis Test	Yes	Yes

Notes: [a]Dysbiosis Index, [b]Not reported.

Table 3 Quality of Each Study Included by Newcastle Ottawa Scale (NOS)

	Selection				Comparability		Exposure		
Study, first author, year	**(1) Is the case definition adequate? (ROME criteria)**	**(2) Represen-tativeness of the cases**	**(3) Selection of controls**	**(4) Definition of controls**	**(5) Compara-bility of baseline characteristics 1 (sex)**	**(6) Compara-bility of baseline characteristics 2 (age)**	**(7) Ascertain-ment of exposure**	**(8) Same method of ascertainment for cases and controls?**	**(9) Non-response rate**
Ahluwalia et al, 2021[11]	*	*	*	*	*	IBS significantly older than HCs	*	*	Not relevant
Casén et al, 2015[10]	*	*	*	*	*	*	*	*	Not relevant
Vasapolli et al, 2021[12]	*	*	*	*	*	*	*	*	Not relevant
Iribarren et al, 2022[13]	*	*	NR[a]	*	*	*	*	*	Not relevant
Mazzawi et al, 2018[14]	*	*	*	*	*	*	*	*	Not relevant

Note: [a]Not reported.

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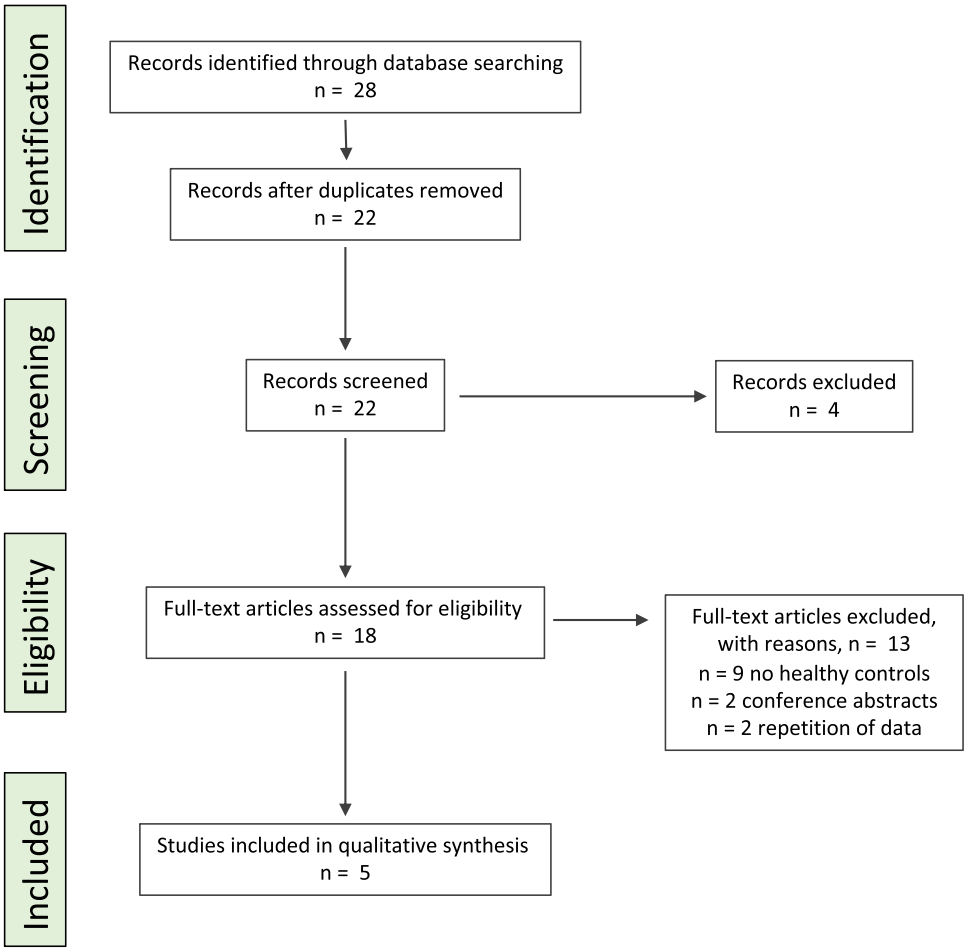

Figure 1 PRISMA flow diagram for depicting the literature search in PubMed (Medline) for this systematic review.

expected, all studies had an overrepresentation of females in both groups except from Ahluwalia et al 2021[11] having the same number for males and females in HCs, and Mazzawi et al 2018[14] having more males than females in IBS (Table 2). Two out of five articles reported multiple comparison correction[12,14] and all studies used a significant p-value < 0.05 for bacterial marker and/or DI difference.

In terms of IBS subtype, three out of five studies included both IBS-D and IBS-C[10–12] where two also included IBS-M.[10,11] The two remaining articles included only IBS-D.[13,14] One study did not investigate fecal microbiota composition between IBS and HCs nor across IBS subgroups but investigated DI.[10] One study investigated the DI for IBS,[10] three studies investigated the DI for IBS-D,[10,12,14] two studies investigated DI for IBS-C[10,12] and only one study investigated DI for IBS-M.[10] In respect to bacterial markers, one study investigated IBS compared to HCs,[11] three studies investigated IBS-D[12–14] one study investigated IBS-C[12] and no studies investigated the difference in bacterial markers in IBS-M compared to HCs.

Fecal Microbiota Composition

Findings for fecal microbiota are summarized in Table 4. Among five studies included in this review, the reported main findings are overall inconsistent. For differences between IBS and HCs, one study reported enrichment of *Acinetobacter junii* and *Akkermansia muciniphila* and decreased levels of *Clostridium* sp. in IBS compared to HCs. For differences between IBS subgroups and HCs only IBS-D was reported to have significant findings; two studies reported increased levels of *Ruminococcus gnavus*, one study reported enrichment of *Proteobacteria* and *Dorea* spp. and one study reported decreased levels of *Bacteroides pectinophilus*, Actinobacteria and *Bifidobacteria* compared to HCs.

Table 4 Differences in Bacterial Markers Between IBS and IBS Subgroups and Healthy Controls (HCs)

	Increased in IBS vs HCs				Decreased in IBS vs HCs				No difference in IBS vs HCs			
Study, first author, year	**IBS**	**IBS-D**	**IBS-M**	**IBS-C**	**IBS**	**IBS-D**	**IBS-M**	**IBS-C**	**IBS**	**IBS-D**	**IBS-M**	**IBS-C**
Ahluwalia et al, 2021[11]	*Acinetobacter junii; Akkermansia muciniphila*				*Clostridum sp.*							
Casén et al, 2015[10]	NR[a]											
Vasapolli et al, 2021[12]										NS[b]		NS[b]
Iribarren et al, 2022[13]		Proteobacteria *Pseudomonas spp.; Dorea spp.; Ruminococcus gnavus*				*Bacteroides pectinophilus*						
Mazzawi, 2018		*Ruminococcus gnavus*				*Actinobacteria; Bifidobacteria*						

[a]Not reported, [b]Non-significant.

Dysbiosis Index

Findings for DI are summarized in Table 5. The reported main findings indicate that IBS and IBS subgroups have higher levels of DI compared to HCs. For differences between IBS and HCs, one study found a significant increase in DI in IBS compared to HCs. For differences between IBS subgroups and HCs: two studies found IBS-D with increased[10,14] and one study with a non-significant increased[12] DI compared to HCs. In IBS-C one study found a significant and one study found a non-significant increase in DI compared to HCs. For the only study investigating IBS-M, the DI was significantly increased in comparison to HCs.

Table 5 Dysbiosis Index in IBS and IBS Subgroups Compared to Healthy Controls (HCs)

	Increased in IBS vs HCs				Decreased in IBS vs HCs				No difference in IBS vs HCs			
Study, first author, year	**IBS**	**IBS-D**	**IBS-M**	**IBS-C**	**IBS**	**IBS-D**	**IBS-M**	**IBS-C**	**IBS**	**IBS-D**	**IBS-M**	**IBS-C**
Ahluwalia, 2021	NR[a]											
Casén, 2015	73% (IBS) vs 16% (HCs) Mean: IBS: 2.98 HCs: 1.72	76% (IBS-D) vs 16% (HCs) Mean: IBS-D: 3.03 HCs: 1.72	67% (IBS-M) vs 16% (HCs) Mean: IBS-M: 3.33 HCs: 1.72	73% (IBS-C) vs 16% (HCs) Mean: IBS-C: 3.00 HCs: 1.72								
Vasapolli, 2021									NS[b]	NS[b]		NS[b]
Iribarren, 2022	NR[a]											
Mazzawi, 2018		Mean±SD: 4 ±0.5 (IBS) vs 2.6±0.2 (HCs)										

Notes: [a]Not reported, [b]Non-significant.

Abbreviations: IBS, irritable bowel syndrome; IBS-D, diarrhea-predominant IBS; IBS-M, IBS with mix of diarrhea and constipation; IBS-C, constipation-predominant IBS; HCs, healthy controls; DI, dysbiosis index; NOS, Newcastle Ottawa Scale; PRISMA, preferred reporting items for systematic reviews and meta-analysis; NR, not reported; NS, non-significant.

Discussion

To our knowledge, this is the first systematic review investigating differences in fecal bacterial profile and DI between IBS and IBS subgroups compared to HCs performed by using GA-map Dysbiosis Test based on the 16s rRNA gene of bacterial species or groups. Based on only five studies included the results was inconsistent. However, two studies showed an increase in *Ruminococcus gnavus* in IBS-D compared to HCs and results of DI indicated IBS and IBS subgroups (especially IBS-D) having higher DI compared to HCs.

Today, there are many methods used to investigate the gut microbiota profile in patients with IBS, and recently we have been able to increase the exploration due to progress in microbial analytic techniques.[4] Studies have shown that IBS have a different microbiota profile compared to HCs, but the existing literature is inconclusive.[15] There are many factors which makes it challenging to interpret the results of gut microbiota profile. Firstly, the gut microbiota is highly dynamic and is affected especially by diet, medications, ethnicity, and geographical deviations. Secondly, the samples can be taken at different sites such as in stool or mucosa, and thirdly, the results can be obscured when comparing findings across different methods.[15,16] All these factors might contribute to the inconclusive findings of gut microbiota in IBS and is needed to be taken into consideration when interpreting results of gut microbiota.

In the present review, only one study investigated the difference in fecal bacterial markers between IBS and HCs: increased levels of *Acinetobacter junii* (phyla Proteobacteria) and *Akkermansia muciniphila* (phyla Verrucomicrobiota) and decreased levels of *Clostridium* sp. (phyla Firmicutes) in IBS compared to HCs, and only one study compared the DI between the two groups: DI was significantly higher in IBS compared to HCs.

The observations of differences in bacterial composition are not similar to previous findings conducted with several different methods of bacterial analysis. A systematic review[17] investigating differences in gut microbiota in IBS compared to HCs conducted with 16S rRNA-targeted sequencing observed consistent findings which included increased level of the phyla Firmicutes and reduced level of the phyla Bacteroidetes and increased Firmicutes/Bacteroidetes ratio, higher levels of *Clostridia* and *Clostridiales* (phyla Firmicutes), and lower levels of *Bacteroidia* and *Bacteroidales* (phylum Bacteroidetes) in IBS. The studies were discrepant in results of changes in alpha-diversity (specie richness), observing both higher, lower and no difference between IBS and HCs using the Shannon index and Chao1 index. However, almost similar to our findings, the authors found a trend of IBS having lower alfa-diversity in comparison to HCs. A systematic review conducted by Pittayanon et al 2019[15] based on stool and colonic tissue microbiota analyzed with different methods found that the difference between IBS and HCs included increased levels of *Enterobacteriaceae* (family, phylum Proteobacteria), *Lactobacillaceae* (family, phylum Firmicutes) and *Bacteroides* (genus, phyla Bacteroidetes), and reduced levels of uncultured *Clostridiales* I (phyla Firmicutes), genus *Faecalibacterium* (including *Faecalibacterium prausnitzii*, phyla Firmicutes) and genus *Bifidobacterium* (phyla Actinobacteria) in IBS compared to HCs. One study showed increased levels and another no significant difference in the abundance of *Acinetobacter* (phylum Proteobacteria) in IBS compared to HCs. The diversity of microbiota was either decreased or not different in IBS compared to HCs. The author addressed that there were no two studies reporting the same differences in OTUs.[15] Another systematic review and meta-analysis of fecal microbiota profile in IBS compared to HCs using quantitative real-time PCR analysis found a reduced abundance of *Lactobacillus* (phyla Firmicutes), *Bifidobacterium* (phyla Actinobacteria) and *F. Prausnitzii* (phyla Firmicutes) in IBS compared to HCs.[18]

The abovementioned systematic reviews[15,17] reveal inconsistent results in the findings of *Clostridiales* and *Clostridia*. We observed decreased levels of *Clostridium* sp. (specie in the *Clostridia* class) in IBS. *Clostridia* class is observed to be at higher levels in IBS[19,20] and IBS-D[21] compared to HCs and has been speculated to be a part of the pathogenesis in IBS due to the contribution of overactive bile acid secretion[21] and dysregulation of gut serotonin production.[22,23] *Clostridia* class contains gut pathogens causing some of the most common clostridial infections in the gut,[24] such as *Clostridium difficile* infections which is a common cause of post-infectious IBS.[25] But some *Clostridium* species are commensals and might have potentials as probiotics.[26] Their cellular components and metabolites play a probiotic role primarily through strengthening the intestinal barrier and interacting with the immune system. Unique patterns of *Clostridium* species in gut can be shaped depending on our diets and physical state of body[26] and a balance of *Clostridium* spp. is needed to form a healthy gut. Therefore, both decreased and increased numbers might cause IBS symptoms.

Patients with IBS are a heterogenous group with different stool habits affecting the fecal and luminal microbiota.[27,28] This makes the comparison of gut microbiota in IBS across studies challenging as the relative frequency of each subgroup is different in all studies. We should take this into consideration and investigate all subgroups.

In respect to differences in IBS subgroups and HCs, IBS-D had significantly higher levels of Proteobacteria, *Pseudomonas* spp. (phyla Proteobacteria), *Dorea* spp. (phyla Firmicutes) and *Ruminococcus gnavus* (phyla Firmicutes) and significantly lower levels of *Bacteroides pectinophilus* (phyla Bacteroidetes), Actinobacteria and *Bifidobacteria* (phyla Actinobacteria) compared to HCs. *Ruminococcus gnavus* was found to be increased in IBS-D in two studies. Two studies also found DI to be significantly higher in IBS-D, hence, one study found a non-significant increase compared to HCs. DI in IBS-C and IBS-M was found to be significantly increased in comparison to HCs.

The findings of *Ruminococcus gnavus* being increased in IBS-D are directly in line with previous findings. The bacterial marker is a member of the *Clostridia* class (Firmicutes phyla) and it has been suggested to play a pathogenic role in IBS-D.[29] Monocolonization of germ-free mice with *Ruminococcus gnavus* induced IBS-D like symptoms, including increased GI transit and colonic secretion by stimulating the production of peripheral serotonin.[29] Serotonin (5-HT) has been shown to modulate gut motility and hypersensitivity functions.[30] The study of Rajilic-Stojanovic et al 2011[20] indicated that phylotypes related to *Ruminococcus gnavus* are significantly increased in patients with IBS and is positively associated with IBS symptoms. Increased levels of *Ruminococcus gnavus* contributed to the increased dysbiosis in patients with IBS in the study of Casén et al 2015,[10] along with increased levels of Proteobacteria (*Shigella/Escherichia*) and Actinobacteria. Our findings also reveal Proteobacteria (phylum) being increased in IBS-D according to Iribarren et al 2022.[13] An increasing amount of data identifies Proteobacteria as a possible microbial signature of disease as it includes pathogenic bacteria with potential inflammation causing mechanisms.[31,32] Increased levels in IBS[17,33] and IBS-D[31,34] are observed, and several studies has found the bacterial marker associated with IBS[17] and with individuals with high DI scores.[12] We also found *Dorea* spp. (Firmicutes phyla) at higher levels in IBS-D. Proteobacteria and *Dorea* spp. are observed to be increased in IBS-D together with excessive excreted bile acid[21] and higher levels of *Streptococcus* spp., *Dorea* spp. and *Ruminococcus* are reported to be associated with potentially harmful properties.[20] It has been proposed that *Dorea* spp. is associated with increased gas production and increased gut permeability, which is thought to contribute to IBS pathophysiology.[35] Altogether, our findings of increased levels of *Ruminococcus gnavus* and *Proteobacteria* in IBS-D seems to be supported by the literature.

Furthermore, we observed that Actinobacteria and *Bifidobacteria* (phylum Actinobacteria)[14] was decreased in IBS-D compared to HCs. A significant reduction in Actinobacteria has previously been observed in IBS[20] and IBS-D.[34] A systematic review showed that patients with IBS tend to have decreased levels of *Bifidobacteria*[15] and it is found a 1,5 decrease in *Bifidobacteria* in IBS compared to HCs and a negative association between *Bifidobacterium* spp. and abdominal pain in IBS.[20] Actinobacteria is one of the major phyla in the gut microbiota and is pivotal in the maintenance of gut homeostasis. Classes of this phylum are widely used as probiotics demonstrating beneficial effects in many pathological conditions,[36] especially *Bifidobacterium* is believed to exert positive health benefits on the host[37] and has been commercially exploited as probiotics.[38] These characteristics of Actinobacteria and *Bifidobacteria* might explain the observation of low levels of Actinobacteria in IBS-D. However, increased level of Actinobacteria was shown to contribute to the dysbiosis of IBS in the study of Casén et al 2015,[10] making the interpretation inconclusive.

Recent evidence suggests that dysbiosis might play a role in the pathogenesis of IBS[1,39] and a systematic review and meta-analysis[40] investigating gut microbial dysbiosis in patients with IBS concludes that IBS, including IBS-D and IBS-C, are characterized by gut microbiota dysbiosis. These findings are in line with the observations of dysbiosis in IBS, IBS-D, IBS-M, and IBS-C.

The above-mentioned studies have aimed to differentiate IBS and/or IBS subgroups from healthy controls based on fecal and/or gut microbiota composition but the results are inconclusive. When adding relevant variables (as Rome criteria, fecal transit time, gut microbiota metabolites and more), and using machine learning techniques the ability to distinguish IBS and/or IBS subgroups from healthy controls has been shown possible and might be a future method for diagnosing patients with IBS.[41–43]

Limitations

The similarities and differences in fecal bacterial profile across studies using the GA-map Dysbiosis Test in patients with IBS compared to HCs might be explained by several reasons; patients and cohorts have different ethnicities, geographical sites, and different diets. Studies suggest that people from the same ethnicity share the same number of gut microbiota and indicate that the ethnicity selects for specific gut taxa, and diet is reported to have profound effects on the gut microbiota composition.[16,44] Furthermore, although in the same group of patients, there are interindividual variations in the gut microbiota profile. Such variations are poorly understood, and appears to obscure the microbiota's associations with patients health and impacts the microbiota-based diagnostics of disease.[16] The use of both Rome II, III and IV criteria for participants included in this systematic review might affect the outcome. Rome IV criteria differs from Rome III criteria in different ways. The most distinct difference is the change in the criteria of "abdominal pain or discomfort" in Rome III (and Rome II) to only include "abdominal pain" in Rome IV.[45] Although Rome II, III and IV was used in the five studies included, three[11,12,14] studies and some of the participants of Casén et al[10] used Rome III criteria.

The GA-map Dysbiosis Test training and test cohort was included from Norway, Sweden, Denmark, and Spain, in an effort to achieve heterogeneity. Hence, they found a significantly different fecal bacterial profile in the Spanish cohort compared to the Scandinavian, and different bacterial markers contributing to the dysbiosis of the Spanish cohort.[10] The studies included in the present systematic review are conducted at different geographical sites and include cohorts with different ethnicities. Moreover, all participants in the studies were either told to obtain from a special diet or to have a normal Scandinavian diet without any major dietary change months prior to inclusion, or there were not reported any dietary inclusion or exclusion criteria. The diet at the time of inclusion naturally includes variation and were not reported to be controlled for in these studies. In respect to medications, as a minimum criterion, all studies did not allow the use of systemic antibiotics four weeks to six months prior to inclusion, and some studies excluded the use of immunosuppressive and/or glucocorticoids, or large doses of commercially available probiotics, and some, any other medications.

Additional limitations of this systematic review are the low number of studies (n = 5) included. Only one study investigated DI and one study investigated bacterial markers in IBS compared to HCs. Hence, three studies investigated the DI and fecal bacterial markers in IBS-D compared to HCs. In total there was a low volume of cases and controls. A higher number of females in respect to men was included in most of the studies. This was expected as IBS is reported to be more frequent in females.[46] Ahluwalia et al 2021[11] reported IBS patients to be significantly older than HCs.

Conclusion

This systematic review reveals inconsistent findings in respect to differences in bacterial markers between IBS and IBS subgroups with healthy controls in studies using the GA-map Dysbiosis Test. However, IBS-D was observed to have increased levels of *Ruminococcus gnavus* in two studies and IBS and IBS subgroups, especially IBS-D tend to have higher relative frequency of dysbiosis compared to HCs. The GA-map Dysbiosis Test is stated as a precise and easy in use method to identify dysbiosis in IBS and IBD. Comparing gut microbiota profiles in IBS patients across different ethnicities, geographical sites, sample sites, with different diets, medication use and with different methods will obscure the results. Hopefully, future research will take these aspects into account and investing fecal microbiota profile and dysbiotic differences in IBS and IBS subgroups compared to HCs. This will increase our understanding of gut microbiota's associations with IBS pathophysiology and the clinical consequences of intestinal dysbiosis.

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Prolonged Intake of Luvos Healing Earth does not alter the Composition of the Gut Microbiota in Patients with Diarrhea-predominant Irritable Bowel Syndrome and Healthy Controls

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ABSTRACT

Background & Aims: The mineral compound Luvos Healing Earth (LHE) is a commercially available remedy empirically used for a variety of gastrointestinal disorders. The aim of this study was to investigate the possible effect of prolonged LHE therapy on gut microbiota in healthy individuals and in patients with diarrhea-predominant irritable bowel syndrome (IBS-D).

Methods: In this prospective exploratory study, a total of 20 participants, including 12 healthy controls and 8 patients with IBS-D, received treatment with LHE (Magenfein Granulat, 1 sachet bid) for 6 weeks. Fecal samples were collected for microbiota analysis in the morning fasting state at regular intervals at 6 different timepoints: 2 weeks before starting therapy (Screen), and every 2 weeks during LHE therapy (V0-V3). Additionally, a follow-up visit was scheduled 4 weeks after the end of treatment (V4). Microbiota analysis was performed using the GA-map® Dysbiosis Test Lx v2. Dysbiosis Index, bacterial diversity, as well as the balance or imbalance of functionally important bacteria were assessed.

Results: The microbiota analysis revealed an overlap in gut microbiota profiles between healthy controls and patients with IBS-D. Bacterial communities were consistently stable during the entire treatment period, and no significant variations in composition were observed 4 weeks after the end of the therapeutic intervention. There was a remarkable stability of microbiota profiles over time within each individual and a high inter-individual variation. The majority of fecal samples exhibited profiles, reflecting an eubiotic state, with no significant changes in dysbiosis index, functional bacteria profiles, or bacterial diversity.

Conclusion: Our findings indicate intraindividual resilience of microbiota consortia during the entire study period. Prolonged intake of LHE does not cause significant alterations in fecal microbiota profiles in healthy controls and patients with IBS-D. Luvos Healing Earth does not affect the stability of gut microbial diversity and bacterial functions.

Key words: gut microbiota – mineral loess – healing earth – microbiota modulation – diarrhea – irritable bowel syndrome.

Abbreviations: DI: dysbiosis index; DLA-M: dietary lipid adsorbent-montmorillonite; IBS: irritable bowel syndrome; IBS-D: diarrhea-predominant IBS; LVE: Luvos Healing Earth; PCA: principal component analysis.

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INTRODUCTION

Healing clays are mineral compounds of various composition used since ancient times for therapeutic and cosmetic purposes [1]. The use of edible clays as a natural remedy to control gastrointestinal symptoms has gained increasing importance over the past years in the field of traditional medicine [2]. Luvos® Healing Earth (LHE) is a specific edible mineral compound that is derived from glacial loess, which is a sedimentary deposit of windblown particles resulting from the erosion and weathering of rocks during the last ice age [3]. It consists predominantly of silicate particles in the 20–60 µm size range (60-70%), carbonates (10-30%) and other minerals and trace elements, including calcium, potassium, iron, magnesium, copper and zinc [4]. Due to its physicochemical characteristics, LHE possesses high adsorbing and absorbing capacities and acid neutralization properties, which may contribute to its beneficial effects in the digestive tract.

Luvos Healing Earth is widely used as an over-the-counter medical device in Germany and in other Western countries for a

variety of gastrointestinal disorders, including gastroesophageal reflux disease [5], functional dyspepsia, diarrhea and diarrhea-predominant irritable bowel syndrome (IBS-D) [6, 7].

Recent studies indicate that certain natural clay minerals may have antimicrobial actions and, therefore, potentially impact the composition of various microbial populations [8]. For instance, the topical application of French green clays has been successfully used in the treatment of skin infections caused by *Mycobacterium ulcerans*, which leads to a necrotizing fasciitis known as Buruli ulcer [9]. *In vitro* studies have also demonstrated that specific mineral products exhibit intrinsic, heat-stable antibacterial activity against susceptible and resistant Gram-negative pathogens, including *Escherichia*, *Salmonella*, *Pseudomonas*, and Gram-positive *Staphylococcus* (PRSA/MRSA) [10, 11]. Other compounds, such as Jordan's red soil, which is rich in montmorillonite, kaolinite, illite, vermiculite, and palygorskite, can influence microbial interactions and induce the proliferation of certain bacterial strains, including Actinomycetes, *Bacillus* spp., and *Lysobacter* spp., capable of producing antibiotic substances to compete with other bacteria [12].

No important adverse effects associated with LHE therapy have been reported so far; however, the effect of a prolonged use of LHE on bacterial communities of the human gut microbiota is not known.

The aim of this study was to investigate variations in fecal microbiota composition of healthy individuals and patients with IBS-D before, during and after consumption of LHE.

METHODS

Study Participants

Patients with IBS-D and healthy controls were prospectively recruited at the Ludwig Maximilians University Hospital in Munich, Germany from May 2021 to May 2022.

Subjects were recruited within the ARENA project (DRKS-ID: DRKS00032274), a prospective pilot study focused on research on the impact of LHE consumption on the gut microbiota profiles of healthy individuals, patients with IBS-D, as well as dyspeptic patients with liver cirrhosis and concomitant minimal hepatic encephalopathy. The study protocol was reviewed and approved by the local ethics committee and government authorities, approval number 20-0880, and was conducted in accordance with the current Good Clinical Practice guidelines and the Declaration of Helsinki [13]. All recruited subjects provided their written informed consent for participation. Healthy subjects did not report any gastrointestinal disease, tumor disease, severe metabolic or cardiovascular disease requiring therapy, or neurodegenerative disease. Patients with IBS-D were diagnosed according to Rome IV criteria after an accurate anamnesis and clinical evaluation. Presence of a mixed form of IBS (IBS-M, with changing bowel habits including diarrhea and constipation) or constipation form of IBS (IBS-C) were exclusion criteria. Other exclusion criteria included: antibiotic treatment within 4 weeks prior to study inclusion or during study period, documented underlying allergy against LHE, hospitalization, planned bowel cleansing or major surgery during the study period, pregnancy and lactation status.

Study Design and Specimen Collection

Study subjects received treatment with Luvos® Magenfein Granulat (Heilerde-Gesellschaft Luvos Just GmbH & Co., Friedrichsdorf, Germany), 1 sachet bid over a period of 6 weeks. A 2-day long pause was observed after the first 3 weeks, according to the manufacturer recommendations. Participants were instructed to maintain a daily diary, recording their consumption of LHE together with clinical symptoms and changes in bowel habits. Fecal samples were collected from each participant at 6 different timepoints: 2 weeks before starting therapy (screening visit, Screen), on day 0 (visit 0, V0), during treatment on days 14, 28 and 42 (visits V1-V3), and 4 weeks after the end of treatment on a follow-up visit (day 71, visit V4), as illustrated in Fig 1.

Fecal samples were collected using a sterile stool sample collection kit (PT26.1; Carl Roth, Karlsruhe, Germany) containing RNA-Later (Ambion, Thermo Fisher Scientific, Waltham, MA) and kept in the refrigerator at 4°C (for a maximum of 8 h) until transfer to the laboratory. After aliquoting, the samples were stored at −80°C until further analysis.

Microbiota Analysis

Microbiota analysis was performed using the GA-map® Dysbiosis Test Lx v2 (Genetic Analysis, Oslo, Norway), which characterizes the microbiota composition, and assesses the degree of deviation from that of a reference healthy population, by detection of 48 bacterial markers (DNA probes) [14]. Each bacterial marker represents a preselected target in the bacterial 16S rRNA gene, detected at various taxonomic levels (e.g., species, family, phylum). In brief, the test utilizes fecal homogenization, and mechanical and enzymatic bacterial cell disruption to isolate bacterial genomic DNA. Subsequently, 16S rRNA hypervariable regions V3-V9 were amplified by polymerase chain reaction (PCR). The amplified bacterial DNA was used in a probe-labeling reaction (single nucleotide extension) before hybridization of the probe-set to solid-phase. Finally, the abundance of bacteria was assessed by the strength

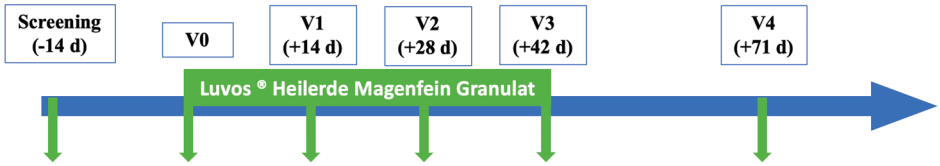

Fig. 1. Study design and sample collection schedule.

of fluorescent signal (probe intensity), detected and measured by a Luminex® L×200™ instrument (Luminex Corporation). The raw signal intensity measured of each bacterial marker was normalized using a hybridization control and a synthetic template, and background noise was removed prior to analysis. In total, the relative abundance of 48 bacteria markers targeting more than 300 bacteria species was measured.

The test algorithmically assesses fecal bacterial abundance and profile in comparison to a healthy reference population. A deviation in the microbiota from normobiosis is summarized using a dysbiosis index (DI) ranging from 1 to 5. Dysbiosis index of 1–2 indicates normobiosis, 3 indicates mild dysbiosis, and 4–5 indicates severe dysbiosis. Bacterial abundance is reported as a score within the range of −3 to +3 (0 indicates within the normal range) and is measured for all 48 bacteria markers for each sample analyzed (Supplementary file, Table S1). To estimate bacterial diversity normalized signal intensities from a set of 28 selected uncorrelated bacterial markers was utilized. Depending on the number of different species and their abundance, diversity was categorized as either expected ("balance") or lower than expected ("imbalance"). The assessment of key gut microbiota functions involved the evaluation of the abundance of selected bacterial markers consolidated into five functional bacteria profiles: butyrate-producing bacteria, gut mucosa-protective bacteria, gut health marker, gut barrier-protective and potentially harmful bacteria, and pro-inflammatory bacteria. Each profile represented a unique set of bacteria signatures linked to their specific functional properties. Based on the abundance scores of the selected markers, the profiles were categorized as either "balanced" or "imbalanced" (Supplementary file, Table S2 for summary).

Statistical Analyses

Descriptive statistical analysis was performed using IBM SPSS Statistics 21.0.0 (IBM Corporation, New York, N.Y., USA) and GraphPad Prism 7. Numerical variables were expressed as mean values ± standard deviation. The chi-square test was used for comparison of categorical data. Shapiro–Wilk test was used to assess the normality of the data. To evaluate statistical differences between two groups Mann-Whitney-U-Tests and Kruskal-Wallis tests were performed for non-normally distributed data. All tests were carried out two-sided with a level of significance set to 0.05.

Analysis of microbiota profiles was based on the normalized signal strengths from the 48 bacterial markers in the GA-map® Test, performed in R using the Stats package (version 4.0.5). Principal component analysis (PCA) with confidence ellipses was used to illustrate overall similarities and variations in the microbiota profile between samples (using scaled and log-transformed normalized signal strengths). Nonparametric statistical tests were used to assess differential expression of each bacterial marker, between and within groups. Mann-Whitney-U-test was used for comparing the groups comprising healthy or IBS-D. Wilcoxon signed-rank test (i.e., paired test) was used to compare timepoints within each of the groups. V0 was here compared with each of the remaining timepoints (Screen, V1, V2, V3 and V4). All p-values were corrected by applying Bonferroni correction. Differences were considered statistically significant if the adjusted p-value (p adj.) was <0.05.

RESULTS

Characteristics of the Study Cohort

A total of 20 subjects including 12 healthy individuals (male: 6, female: 6, mean age, 26.4±5.6 years) and 8 patients with IBS-D (male: 3, female: 5, mean age 29±10.8 years) were enrolled. No statistically significant differences were found in age distribution and gender proportions between groups. All recruited participants successfully completed the treatment with LHE according to the study protocol and provided fecal samples during the scheduled visits, except for two healthy subjects who did not provide a fecal specimen for V4. Four patients (50%) with IBS-D reported an improvement in bowel habits and/or gastrointestinal symptoms during the treatment period, whereas no changes in bowel habits were reported by the remaining patients with IBS-D and the healthy participants.

Gut Microbiota Composition during Study

The gut microbiota profiles of 118 fecal samples were analyzed using the GA-map® Dysbiosis Test Lx v2. Principal component analysis was used to visualize variation in signal strength data. The inter-individual comparison between the IBS-D and healthy groups PCA showed that the microbiota profiles were overlapping to a great degree both at baseline (V0) and at the end of LHE treatment (V3), (Fig. 2). No bacterial markers were found to display significant differences between healthy individuals and IBS-D groups (paired timepoints, p adj. >0.05).

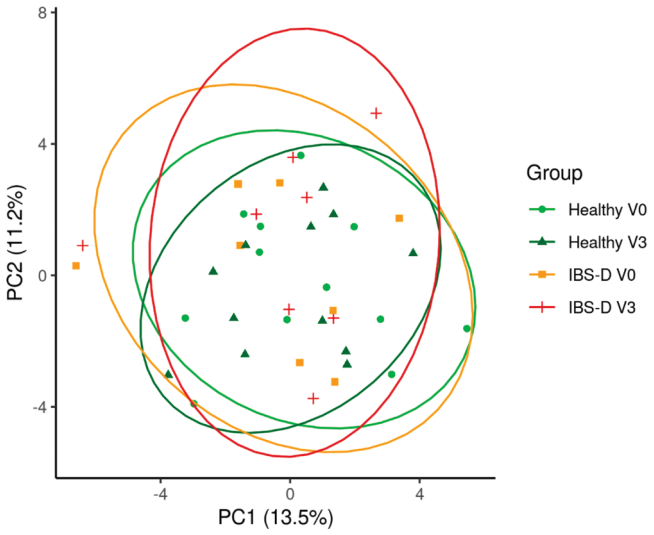

Fig. 2. PCA score plot for the between-groups comparison of healthy controls (n=12) and patients with IBS-D (n=8) at day 0/baseline (V0) and day 42/end of treatment (V3). The plot show 90% confidence ellipses and was generated using normalized signal strengths from the GA-map® Dysbiosis Test Lx.

The within-group analysis revealed a remarkable stability of bacterial communities over time. Microbiota profiles exhibited consistent similarity across different timepoints and covered a largely overlapping area in the PCA plot, demonstrating microbial stability prior to, during, and after LHE treatment in both healthy subjects and patients with IBS-D (Fig. 3 A-B). Overall, a notable inter-individual variation was observed.

The microbiota profiles showed high similarity within each individual and maintained their stability throughout the entire study period, including different timepoints, in both, healthy and IBS-D groups (see Fig. 3 C-D). No significant changes in the abundance (signal intensities) of single bacterial markers were observed in the intra-individual comparisons at different timepoints (V0 vs. Screen/V1/V2/V3/V4, p adj.>0.05).

Dysbiosis Index, Bacterial Diversity and Functional Bacteria Profiles

The large majority of fecal samples analyzed showed normobiotic bacterial profiles. Within the healthy group, the calculated DI indicated normobiosis (DI of 1-2) in 64 samples (91.4%), mild dysbiosis (DI of 3) in 4 samples (5.7%), and severe dysbiosis (DI of 4-5) in 2 samples (2.9%). For patients with IBS-D, the microbiota exhibited a DI of 1-2 in 42 samples (87.5%) and a DI of 3 in 6 samples (12.5%). No severe dysbiosis was observed in the IBS-D patient group. When comparing the different timepoints, no significant differences in the DI were observed for both patients with IBS-D and healthy controls (Fig. 4).

Most of the bacterial markers analyzed exhibited either no deviation or only a low degree of deviation from the normal abundances defined in the healthy reference population of the GA-map® Test [14] (Supplementary file, Fig. S3 and S4). There were a few exceptions that contributed to a high DI observed at certain timepoints among healthy individuals. For example, two healthy subjects had a DI of 4 or 5 at the Screen-timepoint, primarily due to elevated levels of *Bacteroides stercoris* or elevated levels of *Shigella* spp./*Escherichia* spp. and decreased levels of *Lachnospiraceae*, respectively.

Analysis of functional bacteria profiles revealed reduced abundances of butyrate-producing bacteria in 8.3-33.3% of healthy individuals and in 0-12.5% of patients with IBS-D (Fig. 5). Imbalances in the profiles of gut mucosa-protective bacteria, gut barrier-protective bacteria and potentially harmful bacteria were observed in 0-8.3% and 0-12.5% of healthy subjects and patients with IBS-D, respectively. Bacterial markers with pro-inflammatory functions were found to be more abundant only at the Screen-timepoint in 8.3% of healthy individuals. No imbalances in pro-inflammatory profiles were observed in patients with IBS-D, and no significant deviations in gut health profiles were observed in both groups. Bacterial diversity was normal in the IBS-D group, while a reduction was observed in 0-25% of healthy subjects, primarily in samples from the two subjects who presented with a high DI. Significant variations

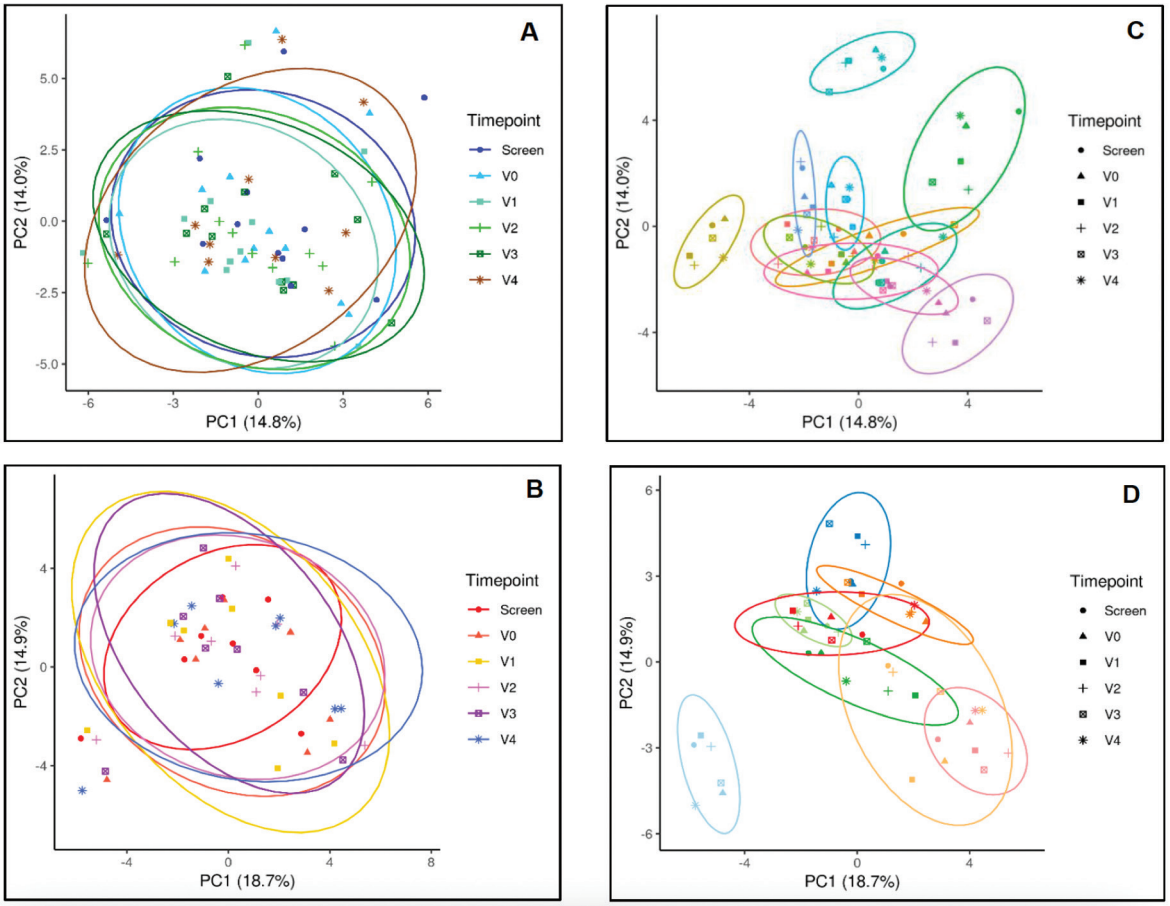

Fig. 3. PCA score plots for the within-groups comparison of healthy controls (n=12*) and patients with IBS-D (n=8). The plots are grouped by timepoints, for A) healthy group and B) IBS-D group, and by subjects, for C) healthy group and D) IBS-D group. Timepoints: -14 days (Screen), day 0/baseline (V0), day 14 (V1), day 28 (V2), day 42/end of treatment (V3), and day 71/4 weeks post-treatment (V4). *n=10 for timepoint 'V4'. The plots show 90% confidence ellipses and were generated using normalized signal strengths from the GA-map® Dysbiosis Test Lx.

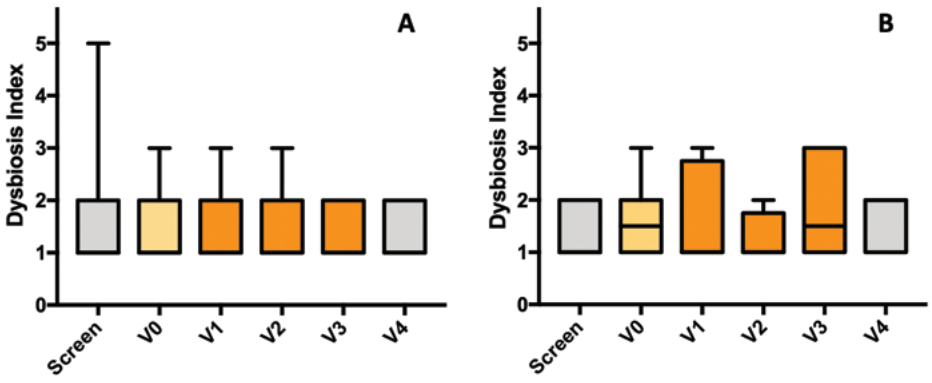

Fig. 4. Box plots showing the dysbiosis index (DI) in healthy subjects (A) and in patients with IBS-D (B) at different timepoints: -14 days (Screen), day 0/baseline (V0), day 14 (V1), day 28 (V2), day 42/ end of LHE-treatment (V3), and 4 weeks post-treatment (V4).

in functional bacteria profiles and bacterial diversity were not observed during or after LHE treatment.

DISCUSSION

To our knowledge this is the first study investigating the influence of a mineral compound therapy on human gut microbiota. The most remarkable finding in our analysis was the stability of microbial profiles observed during the entire study period. Regular use of LHE for 6 weeks was not associated with major changes in microbiome composition or alterations of functionally important bacterial markers. Microbial profiles showed a marked inter-individual variation, maintaining a consistent composition within the same individual across multiple timepoints (before, during, and after LHE exposure). This indicates that treatment with LHE does not result in significant perturbation of the gut microbiota.

The gut microbiota composition is highly sensitive to changes in exogenous factors, e.g. diet or medications, which may cause significant alterations in microbial diversity and function. Several studies have previously shown that different mineral clays may have antimicrobial effects [8]. However, only a small number of these studies have assessed the effect of mineral clays on gut microbiota, and such studies were conducted in animal models. Xia et al. [15] observed that dietary supplementation with copper bearing montmorillonite was associated with reduced incidence of diarrhea and decreased total viable counts of *Clostridium* spp. and *E. coli* in small intestine and proximal colon of weanling pigs as compared to controls. In experimental studies investigating high-fat diet-induced obesity, supplementation with clay mineral preparations, specifically Korean bentonite (Bgp35b-p) and Chinese dietary lipid adsorbent-montmorillonite (DLA-M), resulted in positive alterations in gut microbiome, shown in mouse models [16, 17]. The high-fat diet resulted in microbial dysbiosis, characterized by decreased *Bacteroidetes* and increased *Firmicutes* and *Proteobacteria* abundances. Conversely, the addition of bentonite Bgp35b-p was associated with a significant increase in the relative abundance of bacteria producing short-chain fatty acids (SCFA), such

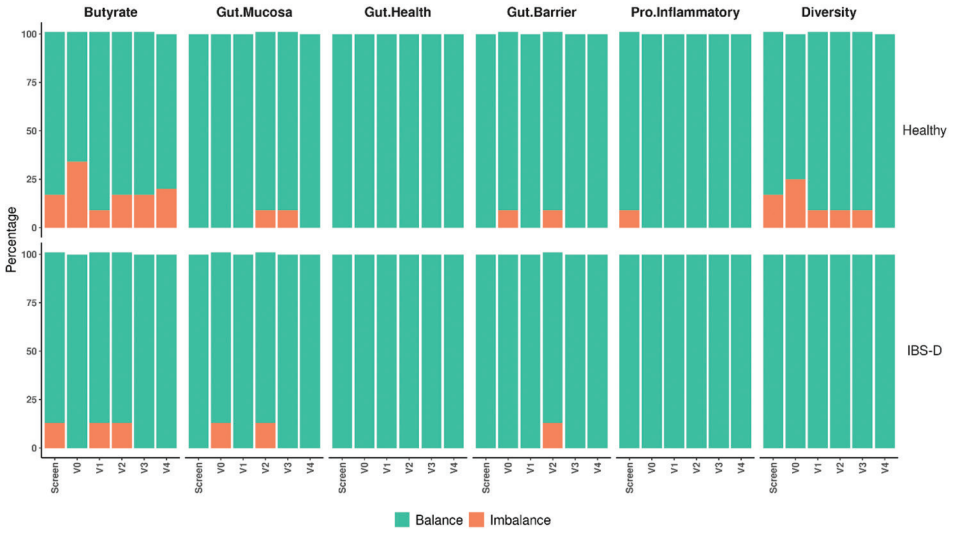

Fig. 5. Rates (percentage) of samples with „imbalance," marked in red, for each functional bacteria profile and diversity per timepoint, for healthy controls (n=12*) and patients with IBS-D (n=8). Timepoints: -14 days (screen), day 0/baseline (V0), day 14 (V1), day 28 (V2), day 42/end of treatment (V3), and 4 weeks post-treatment (V4). *n=10 for timepoint ‚V4'.

as *Ruminococcaceae*, and a decrease of *Clostridiaceae* and *Lachnospiraceae* [16]. Similarly, supplementation with DLA-M resulted in higher abundances of SCFA-producing *Blautia* bacteria and decreased levels of endotoxin-producing *Desulfovibrio* bacteria [17]. Our findings do not support an antibacterial activity of LHE, at least towards the bacterial markers selected in the GA-map® Dysbiosis Test Lx. Although the mechanisms how clay components affect bacteria are largely unknown, variations in composition and peculiar physicochemical characteristics (i.e. mineralogy, cation exchange capacity [8]) may explain different interactions with microorganisms and bacterial metabolites.

We observed a higher inter-individual variation than intra-individual variation in microbiota profiles among healthy subjects. These findings are in agreement with those of previous studies, indicating that gut microbiota composition is highly personalized as a result of continuous influences by multiple factors such as age, gender, diet, environment, lifestyle, diseases and medications [18-20]. Furthermore, bacterial assemblages exhibited remarkable stability over time within the same individual. This suggests that the inter-individual variability of gut microbiota is much stronger compared to the temporal variability, indicating that each individual possesses a unique and largely stable microbial signature over time [21, 22].

Irritable bowel syndrome is a common functional gastrointestinal disorder and alterations in microbiota composition and function are believed to play a significant role in its pathogenesis [23, 24]. While there is evidence supporting benefits of mineral clays in alleviating some IBS-related symptoms [6, 25], data on their impact on gut microbiota are currently lacking. In this study, analogously to the group of healthy subjects, microbiota profiles of patients with IBS-D were characterized by high inter-individual variability and an intra-individual stability over time. The comparison of microbiota profiles between patients with IBS-D and healthy controls revealed a large overlap, showing a certain degree of similarity in bacterial communities between the two groups. These findings are in line with a previous study conducted by our group, where we did not find significant differences between individuals with various IBS subtypes and healthy controls by employing the same methodology to compare the fecal microbiota [26]. Although an increasing number of studies highlights differences in microbial diversity and alteration in the relative abundance of specific bacteria in patients with IBS compared to healthy subjects, such findings often present conflicting results and reflect the heterogeneity of the IBS spectrum [27, 28]. Currently, no clear microbial signature that can reliably distinguish between patients with IBS and healthy individuals or discriminate different IBS subtypes has been reported.

One limitation of our study is associated with the methodology used to analyze the fecal microbiota. The GA-map® Dysbiosis Test Lx allows for the evaluation of a specific set of bacterial markers, targeting the most relevant gut bacteria in a standardized manner [14]. However, it is important to note that this analysis may not reveal potential variations in bacterial assemblages that are not covered by the predefined markers. Furthermore, the assessment of gut microbiota activity relies on functional prediction, which is based on 16S rRNA gene data and the relative abundances of bacteria included in the functional profiles.

Future studies should prioritize metabolomic analysis with direct measurements of bacterial products, such as short chain fatty acids SCFA, and comprehensive microbiota analysis encompassing all bacterial communities. This approach will provide a more precise evaluation of the impact of LHE on gut microbiota.

CONCLUSIONS

Our study indicates that short and prolonged use of LHE does not alter the composition of the microbiota in patients with IBS-D or in healthy individuals. These findings highlight the resilience properties characterizing the human gut microbiota. Additionally, our findings provide further strong support for the safety of LHE therapy in relation to gastrointestinal bacterial communities.

Conflicts of interest: P.M., M.S. are advisory board Luvos GmbH. K.G., K.H.K., C.C. are employees of Genetic Analysis AS. C.C. and K.H.K. own shares in Genetic Analysis AS. All other authors have no conflicts of interest to declare.

Authors' contribution: R.V., C.S., and P.M. designed the study. R.V., C.S., and P.M. obtained funding and supervised the study procedures. R.V., M.S., L.M., S.K. recruited suitable subjects, collected samples and registered data. K.G., K.H.K., C.C. performed laboratory workup, microbiota analysis, bioinformatic and statistical analyses. R.V., C.S., P.M., K.G., K.H.K. and C.C. interpreted the data. R.V., C.S. and P.M. drafted the manuscript. All authors critically revised the manuscript, approved the final version to be published, and agree to be accountable for all aspects of the work.

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IS MICROBIOTA CHARACTERIZATION A USEFUL TOOL IN CLINICAL PRACTICE? AN EXPLORATORY ANALYSIS OF PATIENTS FROM THE IBS – CONSTANT-CARE E-HEALTH MONITORING INITIATIVE

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Abstract – *Objective:* Intestinal microbiota are prominent in the etiology of irritable bowel syndrome (IBS). In this exploratory study, we investigated short- and long-term changes in the microbiota of IBS patients after intervening with a low-FODMAP diet (LFD) or 8-strains probiotics (Bifidobacterium, Lactobacillus and Streptococcus) using a standardized microbiota characterization test intended for routine use.
Materials and Methods: We analyzed data and fecal samples collected in a previous trial from non-comorbid IBS patients before treatment, and then again after four weeks and one year of treatment. Response to treatment was defined by a reduction in the IBS-SSS score, and the gut microbiota were characterized using the standardized and CE-marked GA-map® Dysbiosis Test Lx.
Results: Of the 25 responders to either treatment, two of the 22 with fecal samples available were dysbiotic at baseline, increasing to eight out of 19 after four weeks; after one year all responders providing a sample were normobiotic (n=15). After four weeks, the abundance of *Bacilli, Lactobacillus* spp. and *Streptococcus salivarius* ssp. *thermophilus* were temporarily increased in the 8-strains probiotics responder group ($p<.05$), while for LFD responders *Anaerobutyricum hallii* had decreased. There was a greater abundance of *Ruminococcus gnavus* at baseline in those responding to probiotics than in those responding to LFD.
Conclusions: In addition to improving IBS symptoms, sustained LFD or repeat 8-strains probiotics tended to temporarily alter the microbiota profile in responders. Microbiota characterization is a promising tool for monitoring IBS treatments; however, more extensive studies in treatment and monitoring are needed.

Keywords: Irritable bowel syndrome (IBS), Low-FODMAP diet (LFD), Probiotics, Microbiota, Dysbiosis.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a frequent, functional gastrointestinal (GI) condition that affects 5-10% of the population in the majority of European nations, the United States, and China[1]. Symptoms of IBS vary between patients but typically include intermittent abdominal pain associated with altered bowel habits and a low quality of life. The diagnosis is based on symptom assessment using the Rome Diagnostic Criteria[2,3]. Patients with IBS are divided into four categories based on their stool structure: constipation-predominant (IBS-C), diarrhea-predominant (IBS-D), mixed (IBS-M), and unclassified IBS (IBS-U)[4].

The majority of IBS types have an intermittent disease course that alternates between relapse and remission; a small number of patients have a chronic continuous or indolent course. A low-FODMAP (fermentable, oligosaccharides, disaccharides, monosaccharides, and polyols) diet (LFD) has been shown to improve the disease course of IBS and inflammatory bowel disease (IBD) with co-existent, long-term IBS[5].

Although the pathophysiology of IBS is still not fully understood, it is considered a multifactorial syndrome. In addition to genetic predisposition, visceral hypersensitivity, food intolerance, altered gut-brain axis, gut dysmotility, and impaired innate immunity[6], recent studies suggest that changes in intestinal microbiota play a prominent role[7,8]. This much is clear from the fact that IBS tends to occur more frequently following antibiotic use for gastroenteritis[9].

The gut microbiota is a complex ecosystem composed of trillions of microorganisms, many of which are essential for maintaining gastrointestinal homeostasis[6]. An imbalance in this ecosystem can lead to overgrowth of some bacteria, which can trigger inflammation. Several studies[10-12] have shown an association between changes in microbiota and low-grade inflammation. This association is believed to be caused by mucosal immunocyte infiltration that leads to increases in inflammation markers such as calprotectin. However, the mechanisms behind low-grade inflammation and how it influences the disease course of IBS, remain unclear.

There is evidence that dietary treatments (such as a regular meal pattern and reducing intake of alcohol, caffeine, fat and spicy foods) are effective at reducing risk and improving the disease course of IBS[13]. Some studies[14-16] have shown that at least 50% of patients can reduce their GI symptoms with dietary changes. Yet the widely varying response rates to dietary treatments in clinical trials, particularly among IBS patients, demonstrate that diverse responses to therapy are common[17]. Compliance is another factor that influences response rates.

As dysbiosis is linked to specific dietary habits, the modulation of gut microbiota by altering the diet is an attractive therapeutic option. Diet is an important and relatively fast-acting means for altering the intestinal ecosystem, including increasing microbiome diversity and promoting microbiota[1,18].

A LFD, and probiotics with colony-forming units (CFU) of sufficient proportions, have both been shown to improve IBS symptoms and reduce microbiota dysbiosis by altering the gut bacterial community[19-21], including *Bifidobacteria* and *Faecalibacterium prausnitzii*[22]. The latter is a butyrate-producing bacteria believed to improve intestinal barrier function[6,23]. Butyrate is one of the main short-chain fatty acids (SCFA) (besides acetate and propionate) produced by bacterial fermentation in the gut and is essential for gut permeability, immunological homeostasis, and intestinal secretory function[24,25].

Web-based home-monitoring approaches for the treatment and follow-up of patients with IBS and IBD with coexisting IBS can improve the disease course, patients' compliance, their quality of life, and generate financial benefits for the healthcare system treating them[26].

An earlier randomized control crossover trial[27] demonstrated that restricting short FODMAPs or supplementing with probiotics improved outcomes and quality of life in 34 IBS non-comorbid patients. However, the study could not correlate shotgun metagenomics-analyzed microbiota with IBS symptoms or responses. Therefore, we aimed to analyze fecal samples from patients participating in the previous study using the standardized 16s rRNA gene-based GA-map® Dysbiosis Test Lx to determine whether a response to the interventions affected the intestinal microbiota after four weeks and at one-year follow-up. The GA-map® Dysbiosis Test Lx calculates the dysbiosis index (DI), where DI 1-2 indicates normobiosis and DI 3-5 indicates mild to severe dysbiosis. The test can also measure several bacterial markers covering six phyla (*Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Tenericutes*, and *Verrucomicrobia*)[28].

MATERIALS AND METHODS

Study Population

We retrieved clinical data about IBS patients who had participated in a randomized crossover study to investigate the long-term effect of an LFD or probiotics supplements on their disease course and microbiota[27]. Patients were randomized to either LFD or 8-strains probiotics (Bifidobacterium, Lactobacillus and Streptococcus) for four weeks, and then evaluated for their response. Patients in the LFD arm were re-introduced to limited quantities of FODMAPs over four weeks and those who responded clinically were referred to a dietician who prepared a personalized meal plan according to their symptoms. Patients who had not responded after four weeks were transferred to the 8-strains probiotics study arm after a minimum two-week washout and evaluated again after four weeks. This study protocol resulted in five possible groups of participants: responder to LFD, responder to 8-strains probiotics, responder to LFD after crossover, responder to 8-strains probiotics after crossover, and non-responders. Responders were given a course of 8-strains probiotics or a short-duration LFD every time they experienced a flare-up and were followed up for a year via a home-monitoring web app, ibs.constant.care.com.

Each Bifidobacterium, Lactobacillus and Streptococcus sachet contains 450 billion live freeze-dried lactic acid bacteria: four strains of *Lactobacillus*, three strains of *Bifidobacterial* and one strain of *Streptococcus Thermophilus*. Bifidobacterium, Lactobacillus and Streptococcus will be referred as 8-strains probiotics in the text.

In the initial study, patients completed the IBS severity score system (IBS-SSS) and IBS-QoL questionnaires via a web application and provided stool samples at inclusion, at randomization, after four weeks and after one year of follow-up, as well as every time they experienced a flare-up and received treatment. Patients had simultaneously provided stool samples for future use as part of the study and these were analyzed for the present study (Feces biobank j.nr 2012- 58-0004/ local j.nr NOH-2015-017 with I suite nr 03719).

The initial study had 34 patients, three of whom withdrew before randomization and one of whom was excluded from the analysis as they were a non-responder to LFD and did not cross over to 8-strains probiotics. Another non-responder was excluded from the analysis due to antibiotic treatment during the follow-up period. This left 29 adult IBS patients without comorbidities, who were antibiotics-free during the study period, who fulfilled the Rome criteria and had been diagnosed with either IBS-D or IBS-M. Compliance with the diet or probiotics was confirmed orally by the investigator during the trial period. A detailed list of the participants can be found in the initial study article[27].

The Regional Research Ethical Committee of Denmark (jr nr H-22031187), Danish Data Protection Agency (J.nr. 2022-521-0199) and Capital Region Knowledge Center for Data Reviews (Jr nr P-2022-363) all approved the present study protocol. Written informed consent from the participants was obtained during the initial study.

Evaluation of Response to Either LFD or 8-Strains Probiotics

Clinical response was defined as a significant decrease in the IBS severity score system (IBS-SSS)[29]. The IBS-SSS questionnaire consists of five questions asking about frequency and severity of abdominal pain, presence of abdominal distention, dissatisfaction with bowel habits, and interference with QoL and each item is scored 0-100 on a visual analog scale (VAS), with a total score ranging from zero to 500. A score of 0-175 is considered mild IBS, 175-300 as moderate, and 300 or more as severe IBS.

Achieving complete remission of IBS is rare and flare-ups are common. We considered a reduction in IBS-SSS score to 175 or less (i.e., mild IBS) from a severe or moderate score to be remission or complete response (CR). If a patient's score was reduced from severe to moderate, we considered this a partial response (PR). Non-responders (NR) were defined as participants who did not benefit from either treatment and whose IBS-SSS was not meaningfully reduced.

Microbiota Characterization

The gut microbiota composition in stool samples was analyzed using the GA-map® Dysbiosis Test Lx (Genetic Analysis AS). The GA-map® technology utilizes a pre-selected multiplex targets approach based on DNA probe hybridization to bacterial 16S rRNA gene targets. The 48-plex DNA probe panel was established through systematic review of bacteria targets associated with IBD and IBS in peer-reviewed literature and selected based on their ability to distinguish between healthy controls and IBS patients. The assay includes an algorithm that calculates the degree of dysbiosis and the relative abundance of the target bacteria. Test results are automatically calculated using the integrated GA-map® Analyzer software and presented in a report easily interpretable by clinicians. With this high degree of standardization, the test was specifically developed for routine clinical use and is ideal for individualized clinical therapy and for following a disease course over time. The test is designed for home-sampling, and thus fits well with web-based home-monitoring approaches, e.g., IBS.Constant-Care e-health monitoring.

In brief, the test uses fecal homogenization and mechanical and chemical/enzymatic bacterial cell disruption to isolate bacterial genomic DNA. Then, 16S rRNA gene hyper-variable regions V3-V9 are amplified in a polymerase chain reaction (PCR) using the patented CoverAll primers. The amplified DNA is hybridized to a multiplex panel of DNA probes (probe-set) complementary to regions within the 16S amplicon specific to the target bacteria species. Hybridized probes are labelled with biotin through single nucleotide extension before hybridization to a solid phase (MagPlex® Microspheres, Luminex Corporation) and the addition of a detection fluorophore. Probe signal intensity corresponding to target abundance in the sample is measured by a Luminex® 200™ instrument (Luminex Corporation).

The GA-map® 48-plex probe-set targets bacterial species covering six phyla (Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Tenericutes, and Verrucomicrobia)[28]. The raw signal intensity for each probe measured by the Luminex 200 instrument is normalized using a hybridization control and a synthetic template. The normalized signals are then processed by the GA-map® algorithm, which converts the signal for each probe into a single integer, the Dysbiosis Index (DI) score, representing the degree of dysbiosis relative to an established normobiotic profile, where a DI of 1-2 indicates normobiosis, 3 indicates mild dysbiosis, and 4–5 indicates severe dysbiosis.

IBS-Quality of Life Questionnaire

In the initial study, IBS-QoL was measured in Constant Care. The IBS-QoL questionnaire consists of 34 items, each with a five-point response scale, resulting in a maximum score of 170. Scores were transformed to a scale of 0-100, where scores of 0-49 were indicated with a red color and a score of 50 or above was indicated in green, signifying a better QoL[27].

Statistical Analysis

Principal component analysis (PCA) was used to analyse variations in patients' microbiota between the study groups. We analysed scaled GA-map® signal intensity data using the R Stats package (version 4.0.5).

To determine differences in bacterial abundances, the Wilcoxon rank sum test (unpaired *t*-test) was applied to the GA-map® signal intensity data and $p<.05$ was considered significant. An unpaired *t*-test was used since not all patients provided samples at all three time points. Multiple testing was performed ($p.adj<0.1$) using the Bonferroni and Benjamini-Hochberg methods.

The correlation between DI and IBS-SSS questionnaire scores and QoL were analyzed using non-parametric Spearman's test and reported as correlation coefficients (R).

RESULTS

Clinical Response to Treatments

Patient characteristics and their responses to therapy are listed in Table 1.

TABLE 1. CLINICAL DATA OF 29 PATIENTS WITH IBS WHO WERE FOLLOWED UP FOR ONE YEAR AFTER RANDOMIZATION TO EITHER LFD OR 8-STRAINS PROBIOTICS VIA A HOME-MONITORING WEB APP. RESPONSE/NON-RESPONSE WAS ASSESSED FOUR WEEKS AFTER RANDOMIZATION.

Characteristic	Variables	Total, at baseline (n=29)	Responders - LFD group (n=12)	Responders - 8-strains probiotics group (n=13)	Non-responders (n=4)
Age in years, median (IQR)		43 (19-73)	32 (19-49)	49 (19-73)	44.5 (19-55)
Gender, n (%) of total 29 patients	Female	21 (72)	7 (24)	10 (34)	4 (14)
	Male	8 (28)	5 (17)	3 (10)	0 (0)
IBS-SSS, median (IQR)					
- At baseline		293 (59-434)	293.5 (59-434)	293 (155-423)	287.5 (217-382)
- At 4 weeks			136 (9-282)	131 (31-287)	283.5 (179-332)
- At 1 year			74 (22-190)	120 (40-302)	252 (219-352)
IBS-QoL, median (IQR)					
- At baseline		53 (10-92)	63 (18-92)	51.5 (10-90)	59.5 (38-84)
- At 4 weeks			84 (20-98)	77 (19-93)	57.5 (52-86)
- At 1 year			90 (55-95)	74 (35-95)	68 (38-82)

Twenty-five participants responded to one of the two treatments, six of whom responded after crossover. Four participants were non-responders to both treatments. Of the 25 responders (86%), 20 achieved remissions (mild IBS), while five had a partial response (moderate IBS) after 52 weeks.

Changes in Patients' Microbiota

Microbiota measurements were performed on a total of 66 samples from 29 patients, of which 26 were baseline samples, 23 were four-week samples and 17 were one-year samples.

PCA score plots based on the GA-map® signal intensity (**Supplementary Figure 1**) showed small differences in the overall microbiota of the samples at different time points, regardless of treatment and response status. There was no correlation between DI and IBS-SSS scores or QoL at any time.

Of the responders, two of the 22 were dysbiotic (DI>2) at baseline, increasing to eight of 19 after four weeks; the change was especially marked among the 8-strains probiotics responders, Figure 1. However, all responders who provided a sample after one year were found to be normobiotic (n=15). In contrast, among the non-responders (n=4) one patient was dysbiotic at baseline and after one year, and three were normobiotic at each time point. The changes in DI for each patient are shown in Figure 2.

Differences in bacterial abundance were observed in responders after four weeks compared to baseline and/or after one year ($p<.05$), as shown in Figure 3 and **Supplementary Table 1**. For instance, after four weeks *Faecalibacterium prausnitzii* was lower in abundance compared to year 1 and *Streptococcus salivarius* ssp. *thermophilus* was higher in abundance compared to baseline. More specifically, among 8-strains probiotics responders, but disregarding those who crossed over, the abundance of *Bacilli*, *Lactobacillus* spp. and *Streptococcus salivarius* ssp. *thermophilus* were higher after four weeks compared to baseline and/or year 1. Among LFD responders, again disregarding those who crossed over, *Anaerobutyricum hallii* was less abundant after four weeks compared to baseline. However, the changes were not significant when correcting for multiple testing. There were no significant changes in the groups "R to LFD after crossover" or "R to 8-strains probiotics after crossover."

Some bacterial markers were found to be differentially abundant ($p<.05$) between responders and non-responders, and between LFD and 8-strains probiotics responders (Figure 4 and **Supplementary Table 1**). At baseline, before the interventions, responders had a higher abundance of *Eubacterium siraeum*, and a lower abundance of *Veillonella spp*, than non-responders.

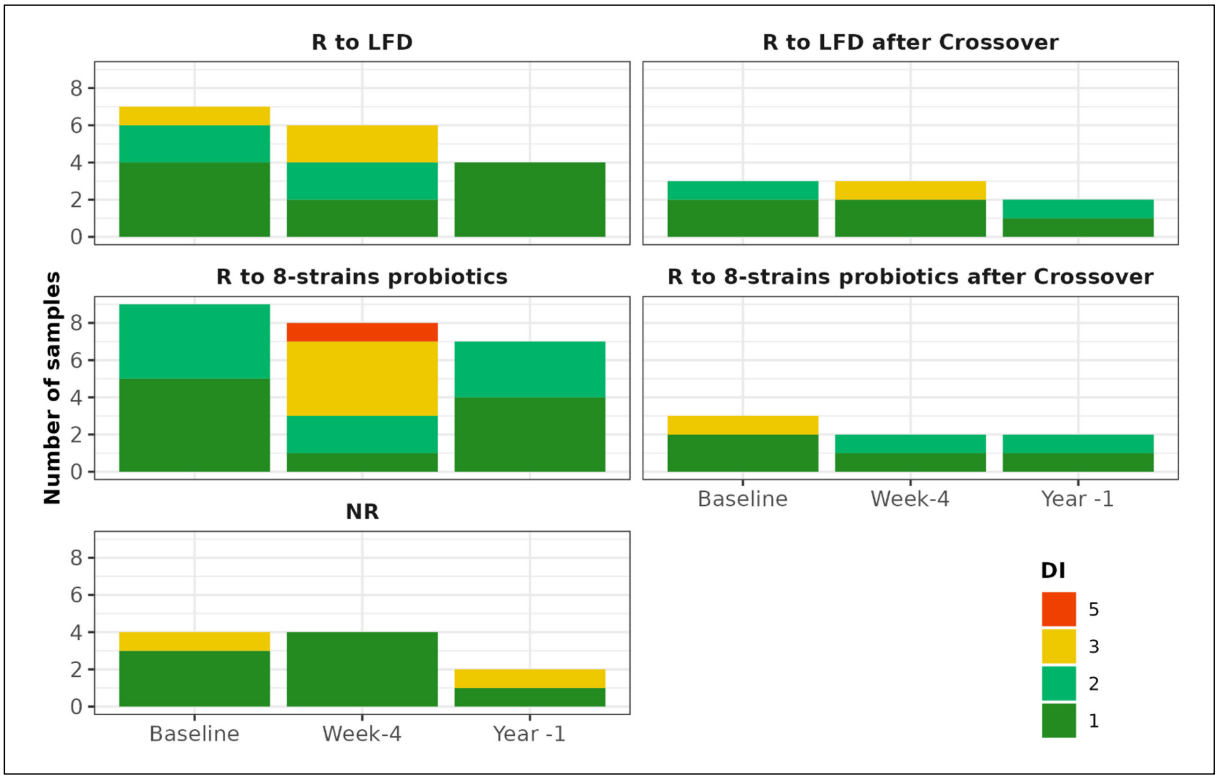

Figure 1. Histogram showing the Dysbiosis Index (DI) scores at the three-time points. The number of samples from baseline, week 4, and year 1, respectively, for each category are: responders to low-FODMAP diet (LFD): 7,6,4; responders to LFD after crossover 3,3,2; responders to 8-strains probiotics: 9,8,7; responders to 8-strains probiotics after crossover 3,2,2; non-responders (NR): 4,4,2. The Dysbiosis Index (DI) is scored between 1 and 5, where a DI of 1 or 2 signifies normobiosis (green), DI 3 signifies mild dysbiosis (yellow), and DI 4 or 5 signifies severe dysbiosis (red).

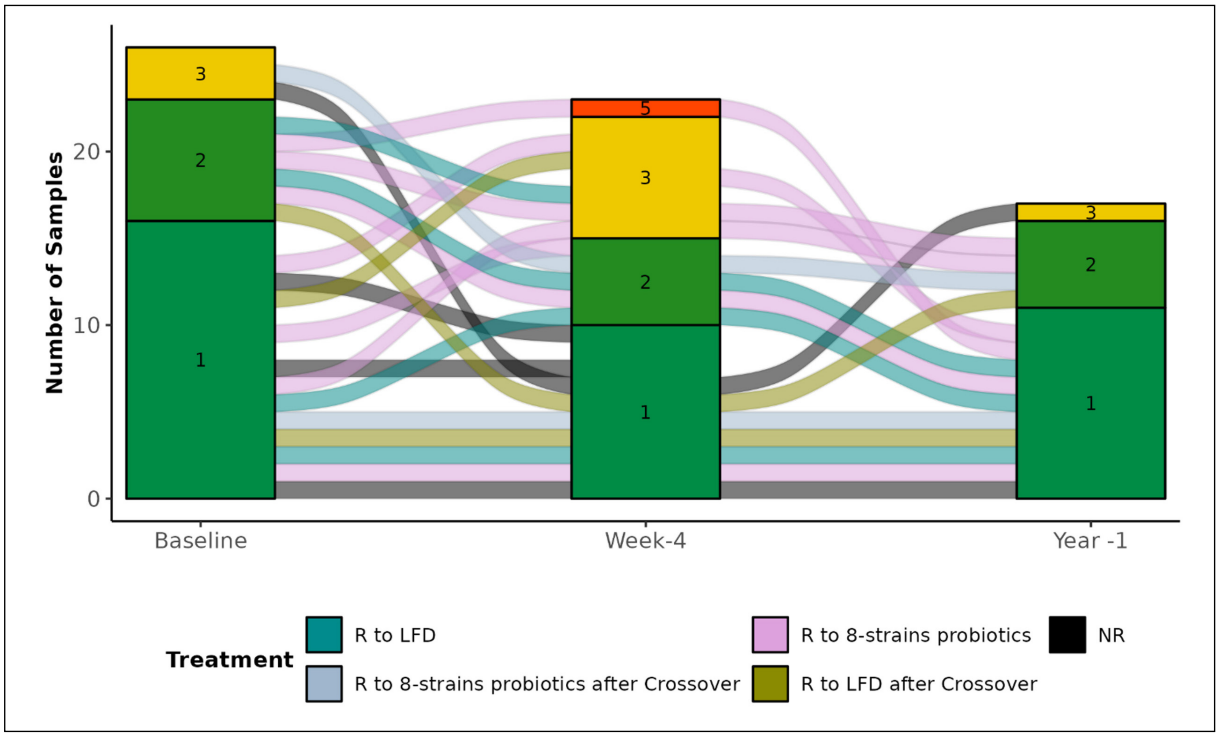

Figure 2. Sankey plot showing the change in Dysbiosis Index (DI) for each IBS patient. The sample count is shown on the *y*-axis. The colors of the lines indicate the treatment type.

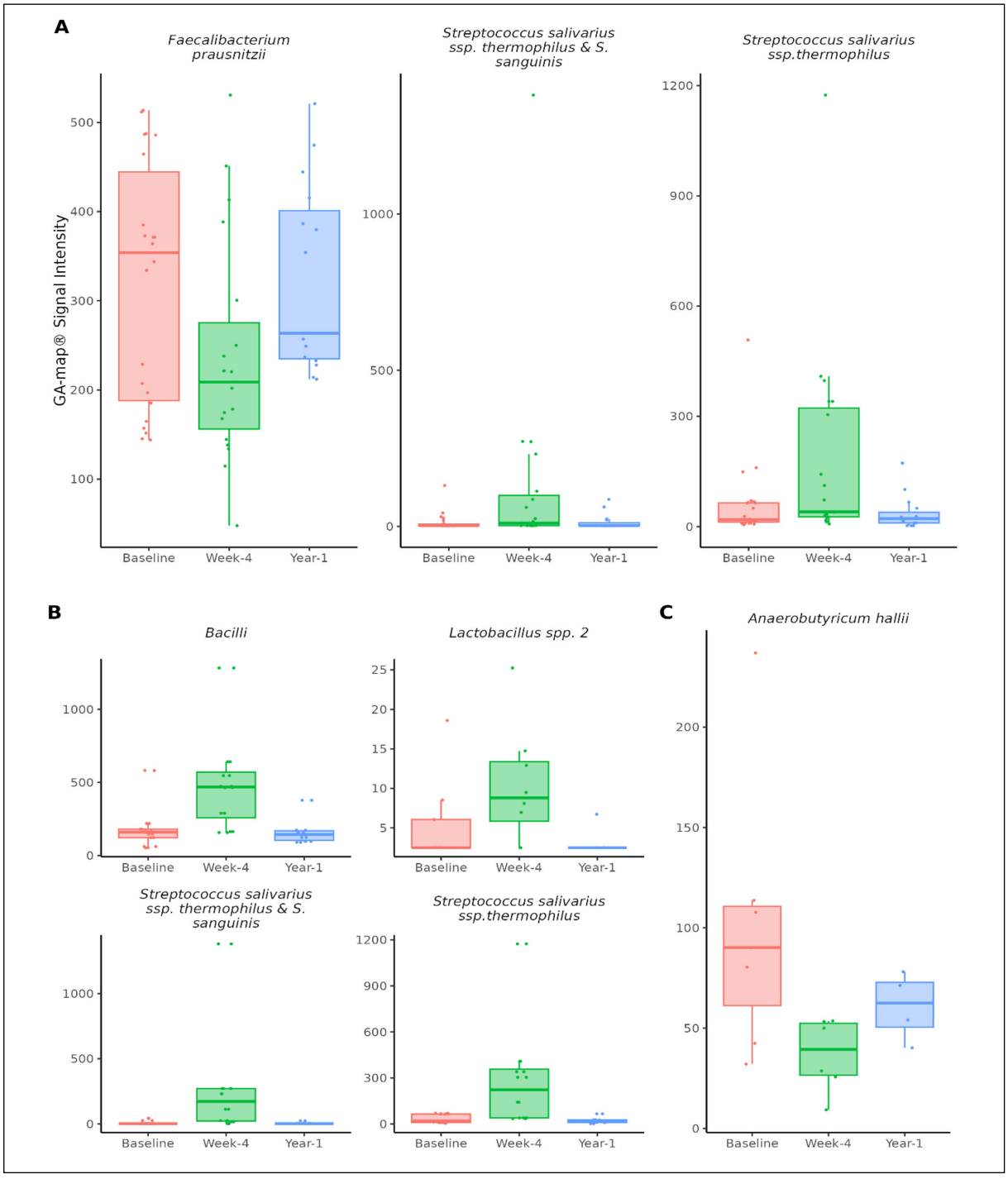

Figure 3. Box plot of bacterial markers with different abundances ($p<.05$) at various times among responders. The *y*-axis shows the GA-map® signal intensity for each of the bacterial markers. The horizontal line inside the box indicates the median value and dots represent the samples. A. Responders, baseline* (n=22) *vs.* week 4 (n=19); week 4 *vs.* year 1 (n=15). B. Responders to 8-strains probiotics, baseline* (n=9) *vs.* week 4 (n=8); week 4 *vs.* year 1 (n=7). C. Responders to LFD, baseline (n=7) *vs.* week 4 (n=6). **A) F. prausnitzii,* not significant (n.s.) for BL *vs.* week 4; B) *Lactobacillus spp.* 2, n.s. for BL *vs.* week 4. (No significant changes in the groups "R to LFD after crossover" or "R to 8-strains probiotics after crossover").

After one year, non-responders were found to have a greater abundance of *Eubacterium rectale*. Comparing probiotic and LFD responders at baseline in those who did not cross over, 8-strains probiotics responders had a higher abundance of *Ruminococcus gnavus*, while LFD responders had a greater abundance of *Catenibacterium mitsuokai*. However, these changes were not found to be significant after correcting for multiple testing.

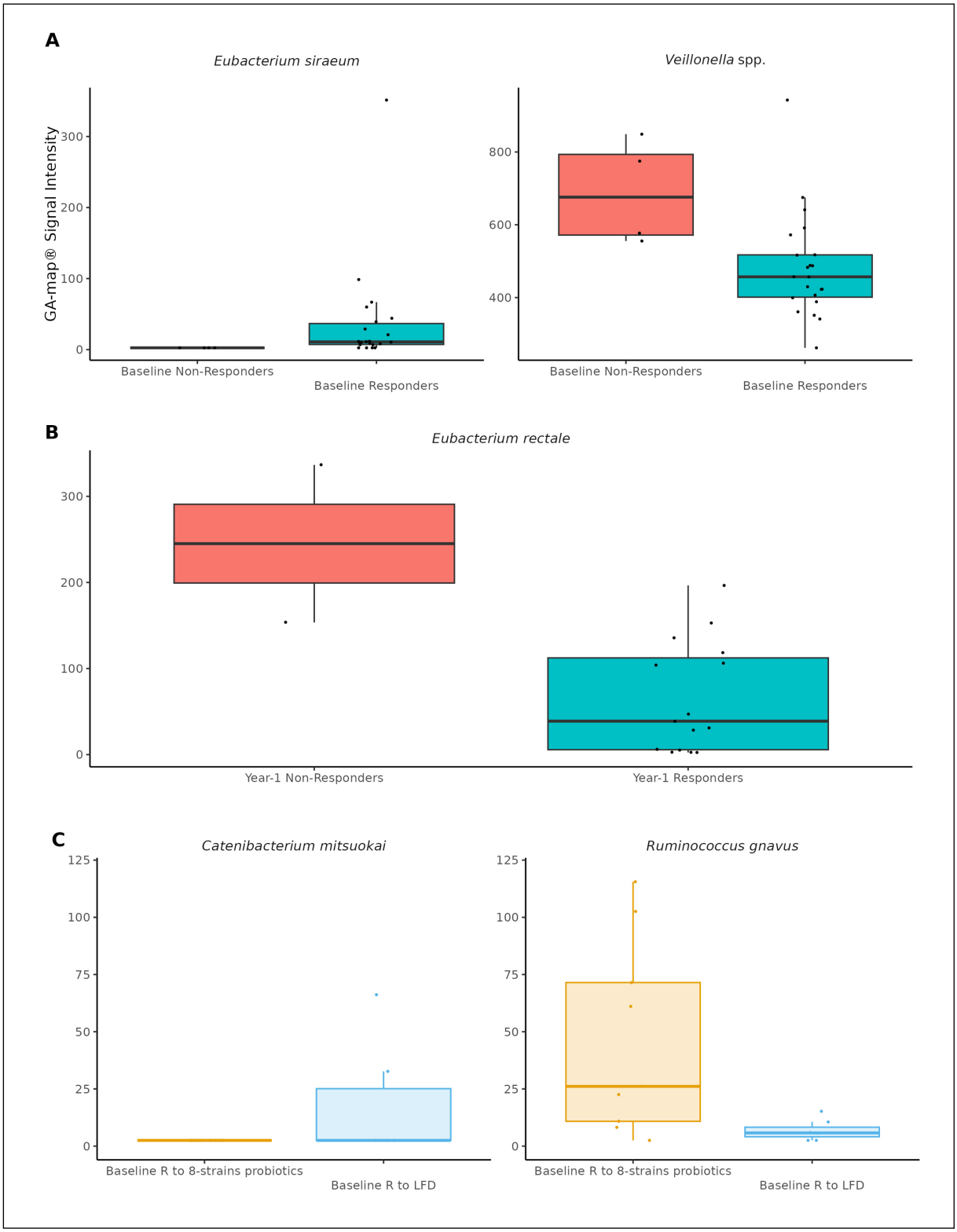

Figure 4. Box plot of bacterial markers with different abundances in responders and non-responders (p<.05). The *y*-axis shows the GA-map® signal intensity for each of the bacterial markers. The median value is indicated by the horizontal line inside the box and dots represent the samples. A. Responders (n=22) *vs.* non-responders (n=4), at baseline. B. Responders (n=15) *vs.* non-responders (n=2), at year 1. C. Responders to a low-FODMAP diet (R to LFD) (n=7) *vs.* responders to 8-strains probiotics (R to 8-strains probiotics) (n=9), at baseline.

DISCUSSION

Our study reports the short- and long-term effects on the intestinal microbiota of non-comorbid IBS patients after a four-week, FODMAP-restricted diet followed by a personalized re-introduction plan and/or 8-strains probiotics supplementation. Three of the study participants were dysbiotic before the intervention, defined as having a DI of 3 or higher; two of these were responders and one was a non-responder.

Clinical response was reported in 86% of patients following either treatment, which includes several patients who underwent multiple, short-term treatments for flare-ups during this year-long study.

Despite improvements in symptoms and normobiosis at the end of the study period, no significant changes in bacterial abundances were observed between baseline and one year later. However, an increased Dysbiosis Index in the short term was observed in eight of 19 responders to either treatment. This might be explained, at least for LFD patients, by the elimination of the prebiotic effect of restricting fructans and galacto-oligosaccharides as part of a low-FODMAP diet, which was previously shown to lower the abundance of beneficial bacterial in the short term, especially *Bifidobacterium*[30]. Two other studies have reported similar findings[31,32]. In a later, randomized clinical trial, Staudacher et al[15] concluded that co-administration of probiotics to patients on a low-FODMAP diet can help in restoring *Bifidobacterium*. However, after four weeks of treatment the bifidobacterial abundance in our patients had not changed. The abundance of butyrate- producing *Anaerobutyricum hallii* decreased between baseline and week 4, which possibly contributed to the dysbiosis we observed.

Patients in the 8-strains probiotics study arm experiencing short-term dysbiosis after four weeks can be explained by the effect of probiotics on the bacterial balance in the gut, as is apparent in the increased abundance of certain species after the intervention (Figure 4). When introduced to the intestine, probiotics have been associated with a short-term exacerbation of symptoms such as abdominal pain and bloating, although these effects can vary considerably[33]. Probiotics alter resident microbial communities either directly, by increasing acidity in the gut environment through producing lactate and short-chain fatty acids (SCFA), or indirectly, by interacting with the gut epithelium through Immunoglobulin A (IgA) or mucin stimulation, leading to transient, increased growth of the resident bacteria and dysbiosis, before restoring the microbiota[34]. However, there is no evidence that short-term use of probiotics can cause dysbiosis. On the contrary, other studies found the gut microbiome to have benefited after both four and eight weeks of treatment with 8-strains probiotics, although not significantly.

Comparing the microbiota of responders to non-responders at baseline, responders had a significantly lower abundance of *Veillonella spp*, which are producers of SCFA[35]. However, responders had a greater abundance of *E. siraeum* than non-responders did at baseline. *E. siraeum* is an acetate-producing bacteria[36]; acetate is a SCFA produced by colonic bacteria, known for its local and systemic anti-inflammatory properties and for improving gut barrier integrity[37]. *Catenibacterium mitsuokai* was significantly higher at baseline in LFD responders than in 8-strains probiotics responders. This species produces lactic and acetic acids[38], which have been associated with health benefits; however, a study of the microbiota of mice transplanted with human feces showed an overgrowth of *Catenibacterium mitsuokai* when they were first fed a low-fat, plant polysaccharide diet, before they were switched to a Western diet[39].

There was a greater abundance of *R. gnavus* in 8-strains probiotics responders than in LFD responders at baseline. Several studies[40,41] have shown that *R. gnavus* has a proinflammatory effect in IBS and IBD. It has been linked to mucin degradation and can cause epithelial barrier dysfunction and increased gut permeability, as well as slow colon transit in IBS. Moreover, a study from Harvard demonstrated that in Crohn's disease patients *R. gnavus* plays a role in the production of proinflammatory polysaccharide, which induces TNF-α secretion[42].

After one-year, non-responders had a greater abundance of the *Firmicutes* subtype, *Eubacterium rectale*, than responders. Although *E. rectale* is butyrate-producing, it has also been linked to obesity and metabolic disease[43]. A review of the correlation between gut microbiota composition and obesity revealed a greater abundance of *E. rectale* in obese individuals from enhanced host energy harvest from a given diet[44]. The authors of a different study suggested *E. rectale* might cause inflammation and cancer[45].

Interestingly, the abundance of another *Firmicutes* subtype, the butyrate producing *Faecalibacterium prausnitzii*[46], increased in responders between week 4 and year 1 (Figure 3A). Given its essential role for colonocytes in modulating intestinal barrier and enteric motility, and its anti-inflammatory properties in the intestine, enhanced butyrate production is of great importance to gut health[47,48]. Re-introducing fibers after four weeks of FODMAP restriction, or short-term 8-strains

probiotics, was enough to increase *F. prausnitzii* in the long term, along with personalized diet plans and treatments throughout the year, as needed. Therefore, patients with fewer butyrate-producing bacteria may benefit from a diet high in fiber and fiber supplements, such as psyllium husk, as husk has prebiotic properties and can increase gut microbial diversity, as well as optimize SCFA production in the colon[49].

Wilson et al[50] recommend only short-term FODMAP restriction, as long-term restriction is associated with lower butyrate concentration and a reduction of beneficial actinobacteria. A low-FODMAP diet contains less fiber than a regular diet and can cause short-term dysbiosis; it should be restricted in duration, and this was why a four-week treatment was used in the initial study. Furthermore, studies have shown that FODMAP restriction can increase the risk of gastrointestinal, mainly colorectal, cancers by causing dysbiosis and inflammation[51,52]. This is consistent with the known risk of colorectal cancer among patients with IBD[53,54]. An Italian study observed dysbiosis in the microbiota of seven IBD patients with colorectal cancer and 10 patients with sporadic (colorectal) cancer compared to 10 healthy controls[55]. However, IBS patients appear to have a lower risk of developing colorectal cancer than healthy controls[56].

The strengths of this study, in addition to its participants being free of comorbidities, include its duration, with stool samples analyzed at multiple time points (before the interventions, after four weeks, and after a year), allowing us to assess both short- and long-term changes in participants' microbiota. Another strength was the use of the eHealth web-app, Constant Care IBS, to follow symptoms and IBS-SSS closely, providing us with more detailed data. Finally, the GA-map® Dysbiosis Test Lx offers a standardized and CE-marked analysis of microbiota composition using 16sRNA gene amplification and subsequent DNA hybridization which allowed us to measure 48 bacterial markers at different taxonomic levels, covering six phyla (*Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Tenericutes*, and *Verrucomicrobia*). The standardized test is especially suitable for routine clinical use and for use in studies, for instance when following patients over time. This is the first test to improve the reproducibility of microbiome measurements at a relatively low cost and with results available in less than a week. The GA-map® Dysbiosis Test Lx offers reliable, long-term tracking of changes in the microbiome and can be used by clinicians on an everyday basis to guide the treatment of patients.

The main limitation of this study was its small sample size and the few number of patients who provided stool samples at all three time points. Due to the low number of non-responders in the study, comparisons between responders and non-responders should be interpreted with caution.

A limitation of the GA-map® test is the use of preselected bacteria targets. Although covering many bacteria across major phyla, the test is limited to bacteria detectable at predefined taxonomic levels. As with all methods based on PCR amplifications of bacterial DNA, the GA-map® test can also be biased by non-homogenous amplification of DNA from different bacteria. Furthermore, our test results were compared to the microbiota of a healthy reference population of European subjects, and this might not cover all variations found in healthy subjects.

CONCLUSIONS

In addition to improving symptoms, interventions with LFD and 8-strains probiotics were associated with changes in responders' microbiota. These changes occurred between baseline and week 4 during treatment; after one year, all of the responders who had provided a sample were normobiotic. LFD and 8-strains probiotics supplementation resulted in improved quality of life in IBS patients. More studies, with larger patient cohorts and strict treatment protocols, are needed to document the effects we have described. Furthermore, concerted efforts at the national and global levels should be made to establish standards for microbiota profiling in clinical laboratories, to better inform the development of new modalities for treating IBS.

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Conflicts of Interest

Marwah Al-sheikh, Dorit Vedel Ankersen and Mette Bennedsen declare no conflict of interest.
Christina Casén is an employee of Genetic Analysis AS and owns stocks and shares in Genetic Analysis AS.
Kristin Gravdal and Graceline Tina Kirubakaran are employees of Genetic Analysis AS.
Johan Burisch reports personal fees from AbbVie, grants and personal fees from Janssen-Cilag, personal fees from Celgene, grants and personal fees from MSD, personal fees from Pfizer, grants and personal fees from Takeda, grants and personal fees from Tillots Pharma, personal fees from Samsung Bioepis, grants and personal fees from Bristol Myers Squibb, grants from Novo Nordisk, personal fees from Pharmacosmos, personal fees from Ferring, personal fees from Galapagos, outside the submitted work.
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Ethics Statement and Informed Consent

The Regional Research Ethical Committee of Denmark (jr nr H-22031187), Danish Data Protection Agency (J.nr. 2022-521-0199) and Capital Region Knowledge Center for Data Reviews (Jr nr P-2022-363) all approved the present study protocol. Written informed consent from the participants was obtained during the initial study.

Data Availability Statement

The datasets generated during and/or analyzed during the current study are not publicly available, as the patients did not consent to share pseudonymized data but are available from the corresponding author on reasonable request.

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RESEARCH

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Exploring the gut microbiota in patients with pre-diabetes and treatment naïve diabetes type 2 - a pilot study

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Abstract

Background Compared to their healthy counterparts, patients with type 2 diabetes (T2D) can exhibit an altered gut microbiota composition, correlated with detrimental outcomes, including reduced insulin sensitivity, dyslipidemia, and increased markers of inflammation. However, a typical T2D microbiota profile is not established. The aim of this pilot study was to explore the gut microbiota and bacteria associated with prediabetes (pre-T2D) patients, and treatment naïve T2D patients, compared to healthy subjects.

Methods Fecal samples were collected from patients and healthy subjects (from Norway). The bacterial genomic DNA was extracted, and the microbiota analyzed utilizing the bacterial 16S rRNA gene. To secure a broad coverage of potential T2D associated bacteria, two technologies were used: The GA-map® 131-plex, utilizing 131 DNA probes complementary to pre-selected bacterial targets (covering the 16S regions V3-V9), and the LUMI-Seq™ platform, a full-length 16S sequencing technology (V1-V9). Variations in the gut microbiota between groups were explored using multivariate methods, differential bacterial abundance was estimated, and microbiota signatures discriminating the groups were assessed using classification models.

Results In total, 24 pre-T2D patients, 18 T2D patients, and 52 healthy subjects were recruited. From the LUMI-Seq™ analysis, 10 and 9 bacterial taxa were differentially abundant between pre-T2D and healthy, and T2D and healthy, respectively. From the GA-map® 131-plex analysis, 10 bacterial markers were differentially abundant when comparing pre-T2D and healthy. Several of the bacteria were short-chain fatty acid (SCFA) producers or typical opportunistic bacteria. Bacteria with similar function or associated properties also contributed to the separation of pre-T2D and T2D from healthy as found by classification models. However, limited overlap was found for specific bacterial genera and species.

Conclusions This pilot study revealed that differences in the abundance of SCFA producing bacteria, and an increase in typical opportunistic bacteria, may contribute to the variations in the microbiota separating the pre-T2D and T2D patients from healthy subjects. However, further efforts in investigating the relationship between gut microbiota, diabetes, and associated factors such as BMI, are needed for developing specific diabetes microbiota signatures.

Keywords Type 2 diabetes, Prediabetes, Bacterial profiling, Microbiota signatures, 16S rRNA bacterial gene

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Background

Type 2 diabetes (T2D) is a significant global health challenge, constituting over 90% of diabetes cases worldwide, and with more than 700 million adults estimated to be impacted by 2045 [1]. Often there is an extended pre-diagnostic period, and a large proportion of people with T2D are thought to be undiagnosed. T2D pathophysiology involves gradually rising blood glucose levels (hyperglycemia) due to increasing insulin resistance and/or decreasing beta cell function, and is strongly associated with overweight and obesity and/or central adiposity [1, 2]. Prediabetic individuals have blood glucose levels higher than normal but below the threshold for T2D, are often overweight, and have an elevated risk of T2D and cardiovascular disease.

Gut microbiota plays a pivotal role in metabolism, immunomodulation and overall human health, and disruptions to the balance of this community are associated with many diseases, including inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), and metabolic diseases such as T2D [3]. Abundant evidence in both animal models and humans with T2D point to an altered or dysbiotic gut microbiota composition as compared to that found in healthy individuals [4–9]. Individuals with an unbalanced gut microbiota composition may exhibit a plethora of detrimental health outcomes: higher BMI, increased fat mass, reduced insulin sensitivity, dyslipidemia, and an increased inflammatory state [10]. It has been proposed that the intestinal bacteria and the metabolites they produce play a role in inducing a harmful chronic low-grade inflammatory state and subsequent development of insulin resistance [3, 9]. However, so far, no typical microbiota profile for T2D has been identified.

Over the last decade, the most common approach for microbiota profiling has been to target the nine hypervariable regions (V1–V9) of the bacterial 16S rRNA gene. This relatively short (~1,500 bp) gene region provides phylogenetic signatures on different taxonomic levels. The hypervariable regions are surrounded by highly conserved sequences, which are used for primer design.

The aim of this pilot study was to explore the gut microbiota and bacteria associated with pre-T2D patients, and newly diagnosed, treatment naïve T2D patients, compared to healthy subjects. To secure a broad coverage of potential T2D associated bacteria, two technologies were used: The GA-map® technology platform 131-plex (GA-map® 131-plex), utilizing 131 DNA probes complementary to pre-selected bacterial targets (covering the 16S rRNA gene regions V3-V9), and the Long 16S using Unique Molecular Identifiers – Sequencing (LUMI-Seq™) platform, a full-length 16S sequencing technology (V1-V9).

Methods

Study population and sample collection

Adult patients diagnosed with either pre-T2D ($n=24$) or T2D ($n=18$) were recruited, and native fecal samples collected, by three diabetes clinics in Norway (Østerås, Sandnes and Tananger). In addition, healthy adult subjects ($n=52$) were recruited, and native fecal samples collected by Oslo Metropolitan University (OsloMet) in Oslo, Norway. The study was approved by the regional Norwegian ethical committee (REC South-East, Norway), and informed consent was obtained from all participants. All samples and information were de-identified before analysis. The samples collected by the clinics were sent non-frozen to Genetic Analysis (GA), Oslo, Norway by mail and frozen upon delivery. The samples collected by OsloMet were frozen upon delivery to the university, before transferal to GA (frozen, on dry ice). All fecal samples were frozen (between -40 °C and -80 °C) within 5 days after collection before further processing.

A case record form was completed for all participants. The T2D patients were all newly diagnosed and treatment-naïve, except for one patient (> 5 months since the last dose of metformin). Criteria for the inclusion of T2D patients included analysis of blood glucose levels, specifically, hemoglobin A1c (HbA1c) ≥ 6.5%. Inclusion criteria for the pre-T2D patients included HbA1c of 6.0–6.4%. Criteria for the inclusion of healthy subjects included no history of diabetes or pre-diabetes, and HbA1c < 6.0%. Exclusion criteria for all groups included recent use of antibiotics (last 4 weeks), and a positive fecal calprotectin (F-cal) test (> 200 mg/kg). See Table 1 for characteristics of the study population. Samples from 86 subjects (16 T2D, 22 pre-T2D and 48 healthy) were analyzed with LUMI-Seq™ and included in the downstream data analysis (4 healthy, 2 pre-T2D and 2 T2D excluded after sequencing due to low taxa counts). Samples from 78 subjects (18 T2D, 22 pre-T2D and 38 healthy) were analyzed with the GA-map® 131-plex, after exclusions (11 healthy subjects excluded due to lack of sufficient number of wells in the plate setup, and additional 5 subjects (2 pre-T2D, 3 healthy) due to F-cal > 200 mg/kg).

Sample processing and analysis

Total bacterial genomic DNA extraction was performed by GA, using a protocol previously described [11]. Briefly, the extraction was performed by fecal homogenization (using stirring rod) and mechanical cell lysis (FastPrep-96™, MP Biomedicals), followed by chemical/enzymatic heat lysis and automated DNA extraction using a MagMAX™ Express-96 or KingFisher™ Flex (Thermo Fisher Scientific) in combination with the mag™ maxi reagent kit (LGC Genomics GmbH). After extraction, DNA samples were aliquoted, and aliquots were

Table 1 Study population – main characteristics

Characteristics	Type 2 Diabetes[a,b]		Pre-Type 2 Diabetes[a,b]		Healthy[a,b]	
	LUMI-Seq (*n* = 16)	131-plex (*n* = 18)	LUMI-Seq (*n* = 22)	131-plex (*n* = 22)	LUMI-Seq (*n* = 48)	131-plex (*n* = 38)
Male/female (*n*)	13/3	14/4	12/10	13/9	12/36	8/30
Age, median	64 (54–72)	64 (53–71)	67 (53–74)	66 (53–74)	31 (26–50)	40 (26–51)
BMI, median	28 (26–32)	28 (26–33)	30 (27–34)	30 (26–34)	23 (21–25)	23 (22–25)
HbA1c, %, median	6.8 (6.6–7.2)	6.8 (6.6–7.6)	6.1 (6.1–6.2)	6.1 (6.0–6.2)	5.2 (5.0–5.5)	5.2 (5.0–5.5)
F-cal, mg/kg, median	31 (12–68)	31 (13–61)	50 (32–119)	46 (32–89)	27 (15–55)	23 (14–45)

The characteristics of the participants in each of the study groups, analyzed and included in the LUMI-Seq™ and GA-map® 131-plex data analysis (the 25th and 75th percentiles are shown in parentheses)

[a] Samples from 16 T2D, 22 pre-T2D and 48 healthy were analyzed with LUMI-Seq™

[b] Samples from 18 T2D, 22 pre-T2D and 38 healthy were analyzed with the GA-map® 131-plex

shipped to BIOASTER in Lyon, France. The DNA samples were analyzed using the LUMI-Seq™ platform (BIOASTER, France) and by the GA-map® 131-plex (Genetic Analysis, Norway) (Fig. 1).

GA-map® 131-plex (GA-map® Technology Platform 131-plex)

The GA-map® 131-plex utilizes a pre-targeted approach, based on DNA probe hybridization to bacterial 16S rRNA gene targets, to identify and characterize bacterial profiles from fecal samples. This research-only panel of DNA probes (bacterial markers) was established to cover major bacterial observations made from the literature relating to the microbiota in healthy, IBS and IBD [11]. Disruptions to the regular community of bacteria have also been associated with other conditions, such as diabetes type 2 [3]. Each bacterial marker was designed to identify a specific bacterial species or group (e.g., phylum, class, genus), based on their 16S rRNA gene sequence [11]. As such, a large number of bacteria are detected at different taxonomic levels. The bacterial markers were intensively tested *in-silico* (target detection, non-target exclusion, cross-labelling, self-labelling, and cross hybridization), and against bacterial DNA from a selection of culturable bacterial species in vitro.

The same laboratory procedures as described for the standardized and CE-marked GA-map® technology

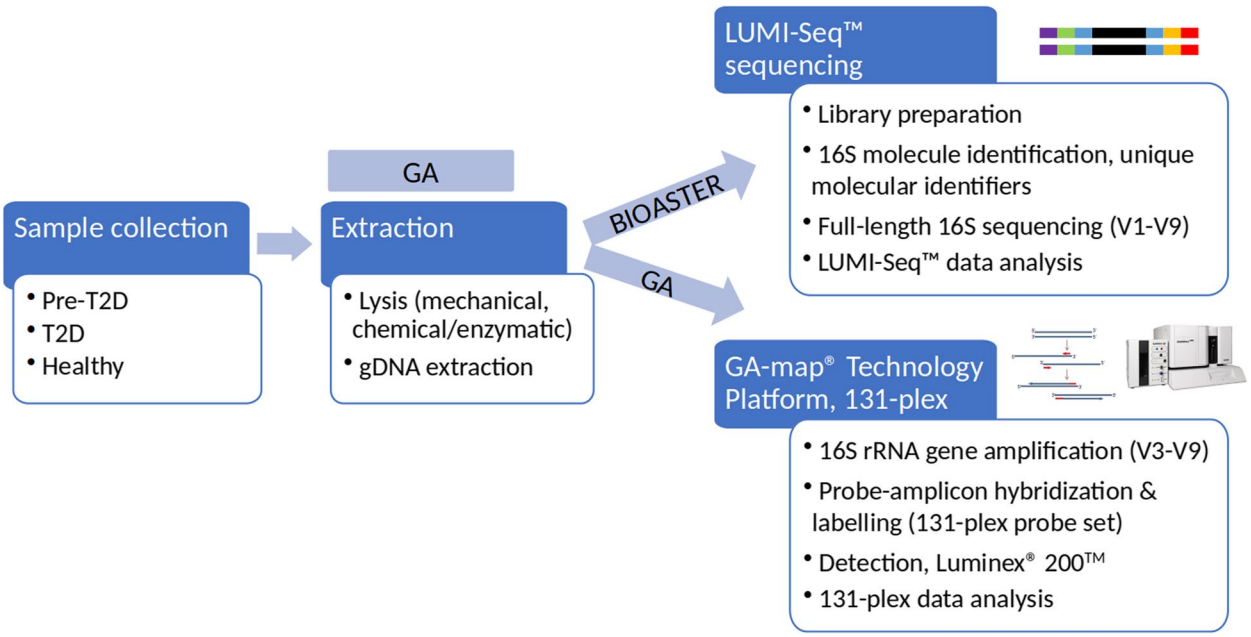

Fig. 1 Study workflow. Starting with collection of fecal samples, and genomic DNA extraction at Genetic Analysis (GA), through analysis of the DNA samples on the two different platforms: LUMI-Seq™ (Long 16S using Unique Molecular Identifiers – Sequencing) at BIOASTER and the GA-map® Technology Platform 131-plex at GA

platform Dysbiosis Test were followed [11], with a few modifications (Fig. 1). Briefly (after DNA extraction), the 16S rRNA gene hypervariable regions V3-V9 are amplified by the polymerase chain reaction (PCR) using a universal primer pair [11]. The amplified DNA is hybridized to a 131-plex panel of DNA probes, complementary to regions within the amplicon specific for the targeted bacteria. Hybridized probes are labeled with biotin through single nucleotide extension before hybridization of the probe-set and solid-phase (carboxylated magnetic beads), as well as addition of a detection fluorophore. After washing of the samples, the fluorescent signal (probe signal intensity), corresponding to the abundance of target bacteria in the sample, is detected and quantified using a Luminex® 200™ instrument (Luminex Corp., Austin, TX, USA).

LUMI-Seq™ platform (Long 16S using Unique Molecular Identifiers—Sequencing)

Synthetic-long read sequencing is now emerging in the microbiome space as a methodology for generation of reliable quality long fragments from Illumina short reads [12–14]. In that context, BIOASTER recently developed LUMI-Seq™ to recover thousands of full-length 16S sequences from complex samples [15]. For this study, the standard LUMI-Seq™ workflow was followed (Fig. 1), in which each 16S molecule within each sample was first barcoded using unique molecular identifiers (UMI) for their tracking during the entire workflow. Then, the molecules were amplified to make multiple copies and to increase the signal. The PCR products were then fragmented while keeping the UMI information on all pieces. The fragments were sequenced using the MiSeq™ platform (Illumina, San Diego, California), in 2×200 bp. Preprocessing of the raw data was performed to remove low-quality ends of the reads with fastp [16]. Read pairs sharing the same UMI and the same sample barcode were grouped together in silico to make long accurate consensus sequences. Assembly was performed with SPAdes [17]. The reconstruction of full-length 16S sequences was performed using V-Revcomp [18] and V-Xtractor [19]. On average, 4,812 full-length 16S sequences were reconstructed per sample (range: 1,124–10,511). Based on the UMI redundancy, the LUMI-Seq™ error rate was assessed at 0.0047%.

Statistical analysis

GA-map® 131-plex data analysis

To account for variable signal levels, the raw signal data (fluorescent intensities) was normalized using a hybridization control, as previously described [11], and background noise was subtracted. Variations in the bacterial profiles between and within the groups (pre-T2D, T2D and healthy) were explored using the non-parametric multivariate methods principal component analysis (PCA) and permutational multivariate analysis of variance (PerMANOVA), using Euclidean and Bray–Curtis methods, respectively. Possible confounding effects on the data due to the clinical variables (e.g., age, BMI, F-cal) were also explored, using the above-mentioned methods. The non-parametric Wilcoxon Rank Sum Test with Benjamini–Hochberg correction (Stats R package [20]) was used to determine significant difference (adjusted $p < 0.1$) in abundance of the bacterial markers.

Microbiota signatures for separation of pre-T2D or T2D and healthy subjects were calculated using the caret (classification and regression training) R-package [20]. To evaluate the robustness and performance of the classification models, a tenfold cross-validation was performed. 90% of the cohort was used for model training and 10% for model testing. A parameter, "importance", ranging from 0 (no contribution) to 100 (maximum possible contribution for training of the model), was reported for each bacterial marker forming the basis for the microbiota signatures. The best performing model, multi-step adaptive elastic-net (MSA-ENet) [21], was chosen. A ROC curve was built, presenting the mean value of the area under the curve (AUC).

LUMI-Seq™ data analysis

After obtaining full-length 16S sequences, QIIME scripts (version 2019.7.0) were used for collapse of 100% identical sequences [22]. Each unique sequence was then assigned to a taxonomy by mapping to a custom 16S database made by BIOASTER, as well as the widely used SILVA reference database. However, due to the lower number of sequences assigned down to the species level, the BIOASTER database was chosen for the downstream analyses (76% with the 16S database vs. 60% with SILVA). Taxa with a total count (summed over all samples) lower than five counts were removed. After normalization of taxa counts, eight samples (four healthy, two pre-T2D and two T2D) showed lower counts and were thus discarded from the analysis to avoid interpretation bias.

Principal coordinates analysis (PCoA) and PerMANOVA, using the Bray–Curtis method, was used to explore variations in bacterial profiles between and within the groups (pre-T2D, T2D and healthy subjects). The above-mentioned methods were also used to explore any confounding effects on the data due to the clinical variables (e.g., age, sex, BMI). Differential abundance analyses were conducted with the DESeq2 package from Bioconductor [23, 24]. Based on a Wald test, taxa with an absolute log-fold change larger than a 0.5 threshold and an adjusted p-value lower than 0.05 were considered as differentially abundant.

Microbiota signatures for the separation of pre-T2D or T2D and healthy subjects were calculated using one of the most commonly used classifiers, Random Forest [25], in a fivefold cross-validation. The sample assignation was repeated until the three groups were equally distributed in the 5-fold range based on the Fisher's exact test. As the majority of the taxa and genes were not associated with the groups, a univariate selection (Welch test, p-value < 1% or 10%) was performed to reduce the dimensionality. The cross-validation procedure was repeated 300 times.

Results

Multivariate analysis of the bacterial profiles

For the GA-map® 131-plex data, the PCA score plot (Fig. 2), indicates the largest differences in the bacterial profiles of the pre-T2D group as compared to the healthy group. Additionally, PerMANOVA analysis revealed significant impact of BMI (p: 0.001, R^2: 0.93) on the data, while the parameters clinical groups (p: 0.001, R^2: 0.063), age (p: 0.8, R^2: 0.56), sex (p: 0.038, R^2: 0.022) and F-cal (p: 0.106, R^2: 0.67), had little to no significant impact. For the LUMI-Seq™ data, PCoA revealed little discrimination of samples according to the clinical groups (data not shown). Further, PerMANOVA analysis showed no significant impact on the data of the parameters clinical groups (p: 0.168, R^2: 0.03), age (p: 0.297, R^2: 0.01), sex (p: 0.23, R^2: 0.01) and BMI (p:0.053, R^2: 0.02), tested as one PerMANOVA, without interaction.

Differential abundance analysis and microbiota signatures

As found by the combined results from the GA-map® 131-plex and LUMI-Seq™ data analysis, mainly bacteria from the phyla Bacillota (Firmicutes), were differentially abundant in pre-T2D and T2D compared to the healthy group (Tables 2 and 3), also confirmed by classification modelling (Figs. 3A&B and 4A&B). Note that from the GA-map® 131-plex analysis, no significant abundance differences were found when comparing the T2D group and the healthy group.

For instance, according to the GA-map® 131-plex data analysis (Table 2), the Clostridia *Faecalibacterium prausnitzii* and *Roseburia intestinalis*, major producers of the SCFA butyrate [26], and the Negativicutes *Veilonella* spp. and *Dialister (D. invisus)*, that can produce SCFAs such as propionate and acetate [27, 28], were more abundant (adjusted p < 0.1) in healthy than pre-T2D. From the classification modeling, the butyrate producing *R. intestinalis* and *F. prausnitzii* [26] were also among the top ten contributors for the separation of T2D and healthy (Fig. 3B), while the Bacteroidota *Parabacteroides johnsonii*, producer of the SCFAs succinate and acetate [29], contributed to the separation of pre-T2D and healthy (Fig. 3A). Based on the LUMI-Seq™ classification modeling, *F.*

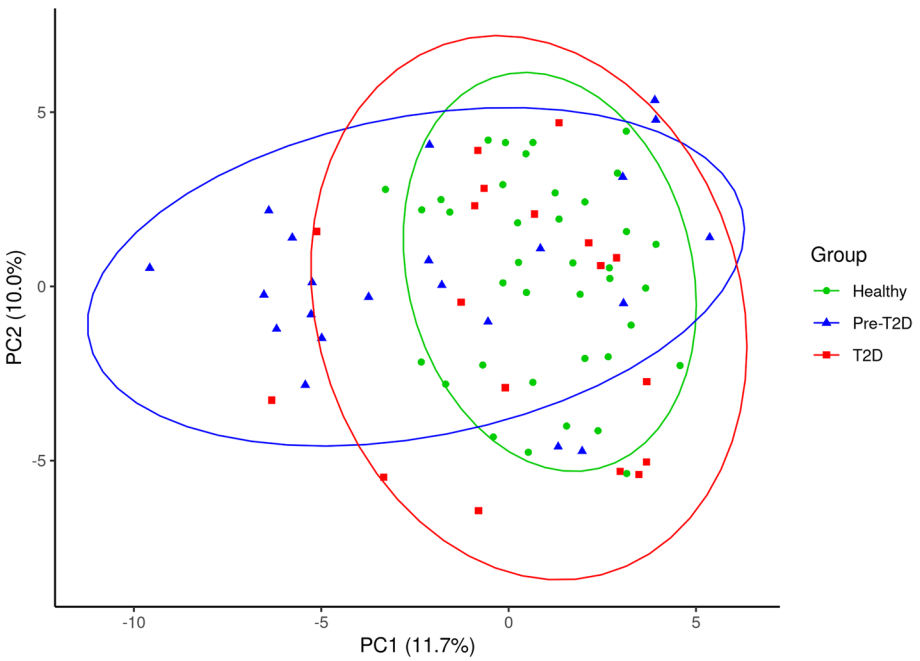

Fig. 2 Principal Component Analysis (PCA) of GA-map® 131-plex data. The PCA score plot illustrates the similarities and variations of the groups healthy (n = 38), pre-T2D (n = 22) and T2D (n = 18), based on scaled and log-transformed normalized signal strength data. 90% confidence ellipses are shown for each of the groups

Table 2 Differentially abundant bacteria – pre-T2D vs. healthy, GA-map® 131-plex

Groups	Phylum	Class	Bacterial marker	I/D[a]	*p* adj
Pre-T2D vs. healthy	Bacillota (Firmicutes)	Clostridia	*Dorea* spp.	I	9.30E-02
			Roseburia sp.	D	9.30E-02
			Roseburia intestinalis	D	9.30E-02
			Faecalibacterium prausnitzii	D	3.90E-02
		Negativicutes	*Dialister invisus*	D	9.30E-02
			Veillonella spp.	D	8.20E-02
	Pseudomonadota (Proteobacteria)	(Phylum marker)	Pseudomonadota	I	2.00E-02
		Gammaproteobacteria	Enterobacterales	I	3.20E-02
			Shigella spp. & *Escherichia* spp.	I	6.90E-02
		Betaproteobacteria	*Sutterella wadsworthensis*	D	9.30E-02

Bacterial markers with significantly different abundance (adjusted $p < 0.1$) when comparing the groups pre-T2D ($n = 22$) and healthy ($n = 38$), based on the GA-map® 131-plex data

[a] Increased (I) or decreased (D) abundance, in pre-T2D (vs. healthy)

Table 3 Differentially abundant bacteria — pre-T2D and T2D vs. healthy, LUMI-Seq™

Groups	Phylum	Class	Bacterial taxa	Log2Fold Change[a]	*p* adj
Pre-T2D vs. healthy	Bacillota (Firmicutes)	Bacilli	Bacilli	1.84	2.42E-04
			Lactobacillales	1.90	2.42E-04
			Streptococcaceae	1.76	4.03E-04
			Streptococcus	1.92	2.42E-04
		Clostridia	*Eisenbergiella*	1.86	1.20E-03
			Eisenbergiella massiliensis	1.43	1.25E-02
			Neglecta	1.35	4.03E-04
			Neglecta timonensis	1.35	4.03E-04
			Oscillibacter valericigenes	-0.77	3.26E-02
	Bacteroidota (Bacteroidetes)	Bacteroidia	*Bacteroides coprocola*	-23.36	2.90E-17
T2D vs. healthy	Bacillota (Firmicutes)	Clostridia	*Dorea*	1.16	1.22E-02
			Dorea formicigenerans	0.89	3.14E-02
			Dorea longicatena	1.03	2.34E-02
			Anaerotignum	-0.99	3.14E-02
		Erysipelotrichia	Erysipelotrichia	1.05	1.91E-02
			Erysipelotrichales	1.05	1.91E-02
			Erysipelotrichaceae	1.05	1.91E-02
			Turicibacter	0.71	3.56E-02
			Turicibacter sanguinis	0.71	3.56E-02

Bacterial taxa with significantly different abundance (adjusted $p < 0.05$) when comparing the groups pre-T2D ($n = 22$) and healthy ($n = 48$), or T2D ($n = 16$) and healthy ($n = 48$), based on the LUMI-Seq™ data

[a] Increased or decreased abundance, in pre-T2D (vs. healthy), or in T2D (vs. healthy)

prausnitzii and another major butyrate producer, *Agathobacter rectalis* (*Eubacterium rectale*) [26], and the Negativicutes *Phascolarctobacterium* and *Dialister*, that can produce e.g., propionate and acetate [30, 28], were among the top ten contributors for separation of pre-T2D and healthy (Fig. 4A). The butyrate producers *Roseburia* and *Roseburia faecis* [26] also contributed to the separation of T2D and healthy (Fig. 4B).

Oppositely, e.g., the Clostridia *Dorea* spp., gas- and SCFA-producing bacteria [31], was more abundant in pre-T2D according to the GA-map® 131-plex analysis (Table 2), and, based on classification modeling, *Dorea* spp. contributed to the separation of pre-T2D and healthy (Fig. 3A). Similarly, as found from the LUMI-Seq™ analysis, the gas- and SCFA-producing *Dorea formicigenerans* and *D. longicatena* [31] were more abundant (adjusted

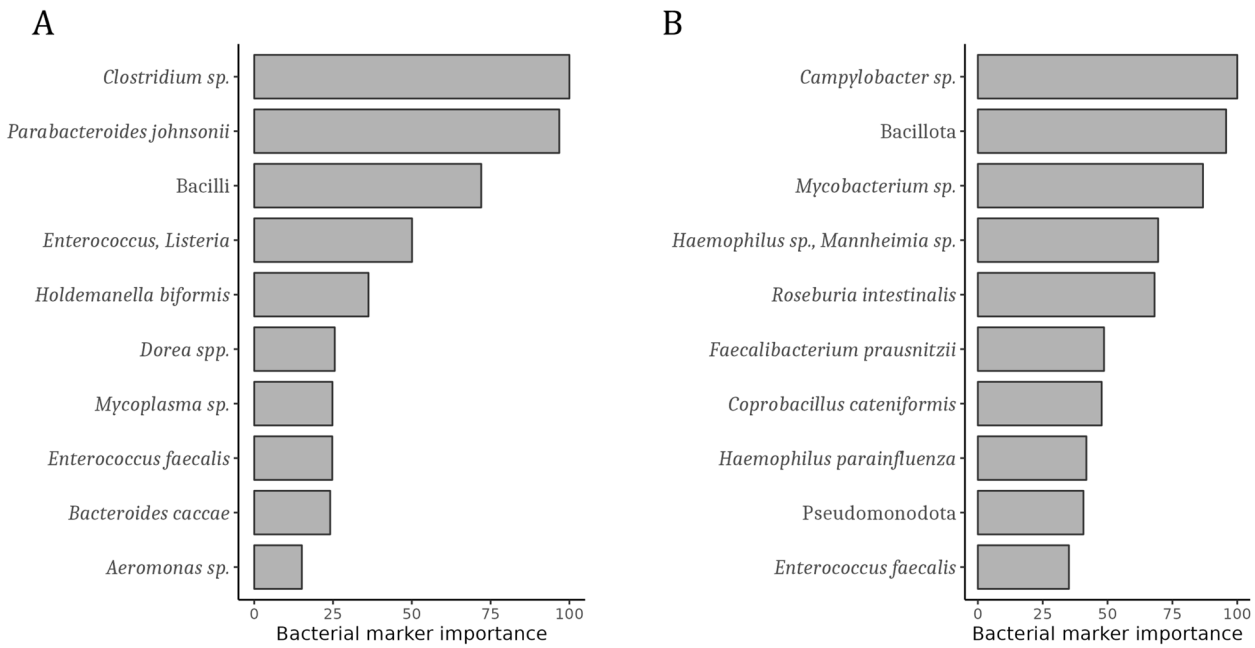

Fig. 3 GA-map® 131-plex classification. The 10 most discriminant bacterial markers for separation of the groups **A**) pre-T2D ($n=22$) and healthy ($n=38$), or **B**) T2D ($n=18$) and healthy ($n=38$), based on microbiota signatures. Measured by 'Bacterial marker importance' as computed by MSA-ENet classifier

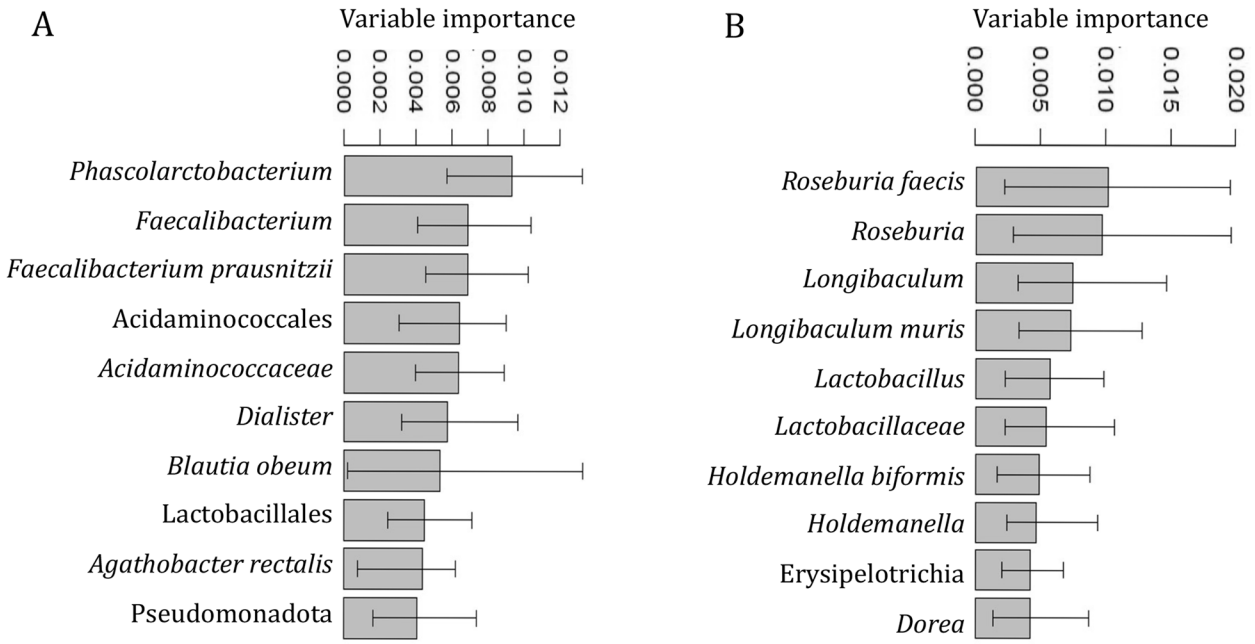

Fig. 4 LUMI-Seq™ classification. The 10 most discriminant bacterial taxa for separation of the groups **A**) pre-T2D ($n=22$) and healthy ($n=48$), or **B**) T2D ($n=16$) and healthy ($n=48$), based on microbiota signatures. Measured by 'Variable importance' as computed by Random Forest classifier

$p<0.05$) in T2D (Table 3), and *Dorea* was among the top ten contributors for the separation of T2D and healthy according to classification modeling (Fig. 4B).

Moreover, as found by the LUMI-Seq™ analysis (Table 3), *Erysipelotrichaceae*, possible SCFA producers also associated with metabolic disorders and inflammation [32, 33], and *Turicibacter sanguinis*

(Erysipelotrichia class), a lactate producing bacteria [34], were more abundant in T2D. According to classification modeling, the possible SCFA producing Erysipelotrichia [32], including *Holdemanella biformis* (*Eubacterium biforme*), contributed to the separation of T2D and healthy (Fig. 4B). *H. biformis*, that can produce SCFAs such as propionate and butyrate [35], also contributed to the separation of pre-T2D and healthy based on the GA-map® 131-plex classification modeling (Fig. 3A).

Further, based on the LUMI-Seq™ analysis (Table 3), Bacilli, including the lactic acid producing *Streptococcus* (Lactobacillales order), numerous being opportunistic [36], as well as *Eisenbergiella massiliensis* (Clostridia class), a potential SCFA producer that may be associated with obesity [37, 38], and *Neglecta timonensis* (Clostridia class), possibly associated with T2D [39], were more abundant in pre-T2D than in healthy. According to classification modeling, Lactobacillales, that may be both commensal and opportunistic [40], was among the top ten contributors for separation of pre-T2D and healthy (Fig. 4A), and the lactate producing *Lactobacillus* [41] for the separation of T2D and healthy (Fig. 4B). From the GA-map® 131-plex classification modeling, Bacilli (including *Enterococcus faecalis*), a diverse bacterial class containing both commensal and opportunistic bacteria [40], contributed to the separation of pre-T2D and healthy (Fig. 3A), and *E. faecalis* (Lactobacillales order), as well as Bacillota, a highly diverse and abundant group of gut bacteria [40], to the separation of T2D and healthy (Fig. 3B).

Additionally, the opportunistic pro-inflammatory bacteria Pseudomonadota (Proteobacteria) and *Shigella* spp./*Escherichia* spp. (Gammaproteobacteria class) [42] were more abundant in pre-T2D according to the GA-map® 131-plex analysis (Table 2). Based on classification modeling, the Gammaproteobacteria *Aeromonas* sp., and the Bacillota *Clostridium* sp., a potential pathogen [40], were among the top ten contributors for separation of pre-T2D and healthy (Fig. 3A), while Pseudomonadota, *Campylobacter* sp. (Epsilonproteobacteria), typical pathogenic bacteria [43], and the Gammaproteobacteria *Haemophilus* sp./*Mannheimia* sp., contributed to the separation of T2D and healthy (Fig. 3B). Pseudomonadota are also found by the LUMI-Seq™ classification to be among the top ten contributors for the separation of pre-T2D and healthy (Fig. 4A).

For the GA-map® 131-plex classification modeling, area under the curve (AUC) values of 0.88 or 0.77 were achieved for the separation of pre-T2D and healthy subjects, or T2D and healthy subjects, respectively. For the LUMI-Seq™ classification modeling, AUC values of 0.78 or 0.64 were achieved for the separation of pre-T2D and healthy subjects, or T2D and healthy subjects, respectively.

Discussion

The primary aim of this pilot study was to explore the gut microbiota and associated bacteria in pre-T2D and treatment naïve T2D patients, compared to healthy subjects. LUMI-Seq™ and the GA-map® 131-plex represent two methods which can be used for bacterial profiling and to identify bacterial biomarkers. The results represent a preliminary discovery of possible diabetes specific bacterial patterns.

Differences in the abundance of SCFA producing bacteria in the phylum Bacillota (Firmicutes) between healthy subjects and pre-T2D and T2D patients were revealed. SCFA producing Bacillota were also among the top ten contributors for the separation of pre-T2D and T2D from healthy using classification models. For example, the SCFA producing bacteria *F. prausnitzii* and *Roseburia* were found to be less abundant, and the gas- and SCFA-producing *Dorea* more abundant, in pre-T2D and/or T2D, and were also among the ten most discriminative bacteria separating the groups from healthy. Additionally, SCFA producing bacteria such *A. rectalis (Eubacterium rectale)* and *H. biformis* (*Eubacterium biforme*), were indicated by classification models to contribute to this separation.

Further, also typical opportunistic bacteria contributed to the differentiation between the groups. For instance, bacteria from the Bacilli class, such as the opportunistic *Streptococcus*, and typical opportunistic, pro-inflammatory bacteria from the phylum Pseudomonadota (Proteobacteria) were increased in pre-T2D, and as found by classification models, contributed to the separation of pre-T2D and T2D from healthy.

Roseburia, F. prausnitzii and *A. rectalis* are major SCFA producing bacteria, particularly of butyrate, which have been negatively associated with T2D and/or pre-T2D [44–47]. Oppositely, *Dorea*, and bacteria from the Bacilli class and phylum Pseudomonadota have been found increased in T2D and/or pre-T2D [5, 6, 44, 45, 48]. Interestingly, *Dorea* may promote inflammation, and increased abundance of *Dorea* in T2D patients has also been negatively correlated with the abundance of butyrate-producing bacteria [49, 50].

Bacteria and their microbial products can impact the development of T2D by various and connected mechanisms, for instance, by affecting gut permeability, inflammatory regulation, and glucose metabolism (reviewed in [4, 8]). Butyrate producing bacteria and the metabolite butyrate are important for promoting anti-inflammatory properties, and maintaining regular gut functions, and may also improve insulin resistance and glucose

tolerance [51, 52]. In contrast, factors such as bacterial-derived lipopolysaccharide (LPSs), e.g., coming from Pseudomonadota, as well as the increased abundance of opportunistic bacteria in itself, can promote inflammation, and may contribute to the induction of a low-grade inflammatory state and insulin resistance [53, 54].

A strength of this study is that DNA was extracted by the standardized GA-map® method, and DNA samples split, before analysis by the two analysis platforms, as the choice of extraction method may influence the end results [55, 56]. Thus, the use of one extraction method enables easier comparisons of the downstream results. Also, the combination of mechanical and chemical lysis (as utilized by the GA-map® method), has been shown to enhance the extraction of both Gram-negative and -positive bacteria, and to increase bacterial DNA yields [55, 56].

Another strength is the inclusion of treatment naïve T2D patients, as it has been shown that the use of the common diabetes drug Metformin may affect the gut microbiota [57, 58]. Including prediabetic patients and treatment naïve T2D patients may make it more straightforward to understand the connections between the disease development and gut microbiota—by avoiding the effect of treatment or prolonged disease. Further, participants included in this study had not used antibiotics recently, also known to influence the gut bacterial composition [59, 60].

Even though the same criteria for age and BMI were used for the inclusion of patients and healthy subjects, the healthy subjects were younger and had lower BMI (and included more females). Also, the pre-T2D group had a slightly higher median F-cal, and the LUMI-Seq™ analysis included 5 subjects that should have been excluded due to F-cal > 200. While PerMANOVA of the GA-map® 131-plex data, showed that BMI had a significant effect on the data, F-cal levels did not. This is perhaps not surprising, as diabetes is strongly associated with higher BMI/overweight [1, 2].

The GA-map® 131-plex detects 131 DNA probes representing pre-selected 16S rRNA bacterial targets, while the LUMI-Seq™ platform entails full length 16S rRNA sequencing. Differences in the targeted 16S regions for the GA-map® 131-plex and LUMI-Seq™ (V3-V9 vs. V1-V9, respectively), as well as the selected targets and the pre-determined taxonomic levels of the GA-map® method, may lead to differences in the phylogenetic resolution. This may be one reason for the two method's limited overlap in genera and species of potential T2D associated bacteria. For instance, *Turicibacter sanguinis*, found elevated in T2D by LUMI-Seq™, cannot be detected directly by the GA-map® 131 plex – however, may be covered by the broad Bacillota marker. Another limitation may be the use of different statistical methods, chosen to fit the dataset in question. For instance, for the GA-map® 131-plex data, bacterial abundances were considered as significantly different if $p.\text{adj.} < 0.1$, as a limit of 0.05 gave limited results.

It is critical for researchers to take into consideration the strengths and limitations of different platforms and choose a system appropriate for their experimental design. The GA-map® platform offers the advantage of a standardized method, utilizing a pre-selected target approach, allowing for a reduced assay turn-around time and less resource-demanding data analysis. At the level of genera, the GA-map® technology exhibit strong correlation to MiSeq amplicon sequencing [11]. Even though the LUMI-Seq™ follows similar protocols as the standard Illumina sequencing, it is difficult to assess differences due to technical variations since no comparative study has been performed. However, the low error rate and the high number of sequences assigned to the species level in this study illustrates that the LUMI-Seq™ technology constitutes a robust approach for microbiota profiling studies.

This pilot study focused on a limited number of Scandinavian (Norwegian) participants only, and so the results and interpretation should be taken with caution. The recruitment and inclusion of treatment naïve T2D patients is especially challenging due to, following standard guidelines, the limited time between diagnosis and start of treatment. The lower number of pre-T2D and T2D patients may have affected the outcome and can be one explanation for the pre-T2D group seemingly having the most distinct microbiota composition. Differences in diet may be another factor affecting the results, as no detailed description of the diet was recorded. To strengthen the foundation for developing bacterial signatures for Type 2 diabetes, future studies should be larger, international, multi-site studies, to account for variation in inter-individual microbiota. Possible confounding factors that ought to be controlled closely include medication-use, diet, and lifestyle [59–63].

Multiple studies have provided compelling evidence of an altered state in gut microbiota composition in pre-T2D and T2D individuals as compared to healthy subjects, with a strong correlation to insulin resistance and β-cell dysfunction, detected even prior to glucose abnormalities in these individuals [46, 64–66]. The implication of an altered gut microbiota composition in diabetic and prediabetic patients was also supported by this study.

Conclusions

This pilot study revealed that differences in the abundance of short chain fatty acid (SCFA) producing bacteria, and an increase in typical inflammation-associated

or potentially pro-inflammatory or opportunistic bacteria, may contribute to the variations in the microbiota separating the pre-T2D and T2D patients from the healthy subjects. However, further efforts in investigating the relationship between gut microbiota, diabetes, and associated factors such as BMI, are needed for developing specific diabetes microbiota signatures.

Abbreviations

T2D	Type 2 Diabetes
BMI	Body Mass Index
SCFA	Short Chain Fatty Acids
IBS	Irritable Bowel Syndrome
IBD	Inflammatory Bowel Disease
Pre-T2D	Pre-Type 2 Diabetes
HbA1c	Hemoglobin A1c
F-cal	Fecal Calprotectin
PCR	Polymerase Chain Reaction
UMI	Unique Molecular Identifier
PCA	Principal Component Analysis
PCoA	Principal Coordinates Analysis
PerMANOVA	Permutational Multivariate Analysis of Variance
MSA-ENet	Multi-Step Adaptive Elastic-Net
LPS	Lipopolysaccharides
OsloMet	Oslo Metropolitan University
GA	Genetic Analysis
LUMI-Seq™	Long 16S using Unique Molecular Identifiers Sequencing
GA-map® 131-plex	GA-map® Technology platform 131-plex

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Authors' contributions
CC, KrG and KG conceived and designed the technical and clinical studies. GTK and KHK analyzed the GA-map® data, AS and PL analyzed the LUMI-Seq™ data. KrG administered sample collection and clinical data recording. All authors contributed to writing the manuscript and approved the final submitted version. Guarantor of the article: Christina Casèn, Genetic Analysis AS, Oslo, Norway.

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Availability of data and materials
The sequence datasets (16S rRNA sequences) generated and/or analysed during the current study are available in DDBJ/EMBL/GenBank under the accession KHUV00000000. The version described in this paper is the first version, KHUV01000000. The other datasets are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The study was approved by the Norwegian Regional Committee for Medical and Health Research Ethics South East (REC South East), PB 1130, Blindern, 0318 Oslo, Norway (application reference number 2018/652), and performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants. All methods were carried out in accordance with relevant guidelines and regulations.

Consent for publication
Not applicable.

Competing interests
Authors from GA (CC, KrG, KG, GTK and KHK) are employees of Genetic Analysis AS. CC and KHK are shareholders in Genetic Analysis AS. Authors from BIOASTER (PL, AS) declare no competing interests.

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Randomised controlled trial: effects of gluten-free diet on symptoms and the gut microenvironment in irritable bowel syndrome

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Summary

Background: A gluten-free diet reduces symptoms in some patients with irritable bowel syndrome (IBS) through unclear mechanisms.

Aims: To assess the effects of gluten-free versus gluten-containing diet on symptoms and the gut microenvironment, and to identify predictors of response to the gluten-free diet in IBS

Methods: Twenty patients with IBS and 18 healthy controls (HC) followed a gluten-free diet during two 14-day intervention periods where they sprinkled either gluten (14 g/day) or rice flour powder over their meals. Primary outcomes included effects of the interventions on IBS symptoms (IBS-SSS) and bowel habits. Secondary outcomes included effects of gluten-free diet on faecal microbiota and metabolite profile.

Results: IBS symptoms improved during the gluten-free ($p = 0.02$), but not the gluten-containing period, with no difference between the interventions. IBS patients reported fewer loose stools during the gluten-free intervention ($p = 0.01$). Patients with IBS and HC presented distinct metabolite profiles based on the effects of the gluten-free diet ($p < 0.001$). True responders (reduced IBS-SSS by ≥50 solely after gluten-free period) and non-responders were discriminated based on the effects of the gluten-free diet on the microbiota ($p < 0.01$) and metabolite profiles ($p < 0.001$). The response to the gluten-free diet could be predicted by the metabolite profile before the intervention ($p < 0.001$).

Conclusions: A gluten-free diet may influence symptoms in a subset of patients with IBS, with a particular effect on bowel habits. A gluten-free diet seems to impact the gut microenvironment. Responsiveness to the gluten-free diet may be predicted by the metabolite profile. Clinicaltrials.gov: NCT03869359.

The Handling Editor for this article was Professor Peter Gibson, and it was accepted for publication after full peer-review.

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1 | INTRODUCTION

The majority of irritable bowel syndrome (IBS) patients relate their symptoms to intake of certain foods.[1] The gut microenvironment, where microbiota, food components and the nervous system interact, is suggested to play a key role in gastrointestinal (GI) symptom generation in a subset of IBS patients.[2] Currently, dietary treatments focus on excluding specific food components, for example, gluten. Somewhat conflicting and heterogeneous results have emerged when assessing the gluten-free diet in IBS.[3] Therefore, the effects of gluten on GI symptoms in IBS patients still remain unclear.

Although a recent study has shown that exclusion diets have an effect on the gut microbiota in IBS,[4] it has not been investigated whether a gluten-free diet influence the microbiota composition differently in IBS patients and healthy controls (HC). Furthermore, gut microbiota metabolism utilising food components results in a large variety of metabolites,[5] which are suggested to be of importance for gut function and play a role in visceral hypersensitivity in IBS.[5] The effects of the gluten-free diet on the gut metabolite composition have not been determined in IBS patients.

This study investigated the hypothesis that a gluten-free diet can reduce GI symptoms in a subset of IBS patients through alterations in the gut microenvironment. Therefore, the primary aim of the study was to assess and compare the efficacy of the gluten-free and gluten-containing diets in terms of effects on GI symptoms in IBS patients. Secondary aims were to identify the putative link between gut microenvironment and the diets´ effect on GI symptoms, and to identify potential predictors of clinical response to the gluten-free diet.

2 | MATERIALS AND METHODS

2.1 | Participants

Adult sex- and age-matched IBS patients and HC were recruited by public advertisement in university buildings and university-related social media of the University of Gothenburg, Sweden. The IBS diagnosis (Rome IV) was confirmed by experienced gastroenterologists (M.S. and H.T.) after initial assessment by a trained medical doctor (J.A.). The same data were collected for the whole study population. Exclusion criteria were specific allergy or intolerance to food, severe cardiovascular, hepatic, neurological or psychiatric diseases, other GI disease, diabetes, compliance to a specific diet (including gluten-free, vegan, low-carb high-fat, and low fermentable oligo-, di-, monosaccharides and polyols [FODMAP] diet), GI surgery (except for appendectomy or cholecystectomy), use of antibiotics within 1 month before inclusion, and pregnant or lactating females. Participants that already identified gluten as a trigger of their GI symptoms were also excluded. Baseline laboratory tests, including faecal calprotectin, tissue transglutaminase immunoglobulin (Ig) A antibodies, haemoglobin, leucocytes, thrombocytes, C-reactive protein, sodium, potassium, calcium, albumin, e-glomerular filtration rate, creatinine, alkaline phosphatase, alanine transaminase, aspartate aminotransferase, thyroxine, thyroid stimulating hormone, haemoglobin A1c, fasting glucose, cholesterol (total), high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglycerides were done to rule out any severe concomitant disease. In addition, the participants reported usage of IBS medication (e.g., anti-diarrhoeal medication, laxatives and neuromodulators) and probiotics. All participants signed informed consent at the screening visit and before any study related procedures. The study was approved by the regional Ethical Review Board in Gothenburg (Dnr 627-18) in August 2018, and was carried out at Sahlgrenska University Hospital, Gothenburg, Sweden between October 2018 and November 2019, according to the declaration of Helsinki. All authors had access to the study data and reviewed and approved the final manuscript. Clinicaltrials.gov: NCT03869359.

2.2 | Study design

IBS patients and HC were challenged with gluten (14 g/day) and rice flour, both for 2 weeks, while adhering to a strict gluten-free diet in this single-centre, double-blind, randomised, placebo-controlled, cross-over trial (Figure 1). A wash-out period of at least 2 weeks separated the interventions. The participants were on their habitual (gluten-containing) diet during the screening and wash-out periods. Randomisation was done by drawing concealed envelops by a nurse not involved in the study. The randomisation code was broken after the analyses of the primary outcomes.

2.3 | Interventions

The participants were carefully instructed to follow a gluten-free diet during the interventions, and in order to increase compliance they obtained gluten-free meal boxes (Table S1) and lists of gluten-free foods they were allowed to eat. The participants sprinkled powder sachets (gluten and rice flour) over their meals during the interventions. Detailed information of the gluten-free diet and a list of gluten-free breakfast options were provided, and the participants recorded all their dietary intake in food diaries. The participants received two meal boxes per day. The participants obtained plastic sachets with vital gluten protein 14 g/day (per 100 g; energy 375 kcal, protein 78.0 g, carbohydrates 7.0 g, fat 1.4 g, fibre 0.5 g, Real Foods) and rice flour 14 g/day, which was low in FODMAPs (per 100 g; energy 375 kcal, protein 7.7 g, carbohydrates 78.0 g, fat 3.6 g, fibre 0.4 g, Doves Farm Foods Ltd). The gluten dose of 14 g/day was chosen in order to be similar to the gluten intake in the general population (10 g/day) and Swedish IBS patients (11 g/day).[6,7] The participants were instructed to sprinkle the powder sachets over their meal boxes (twice per day) during the different diet periods. Although the plastic sachets were identical (except marked with 'A' or 'B'), the contents differed in colour. To assess

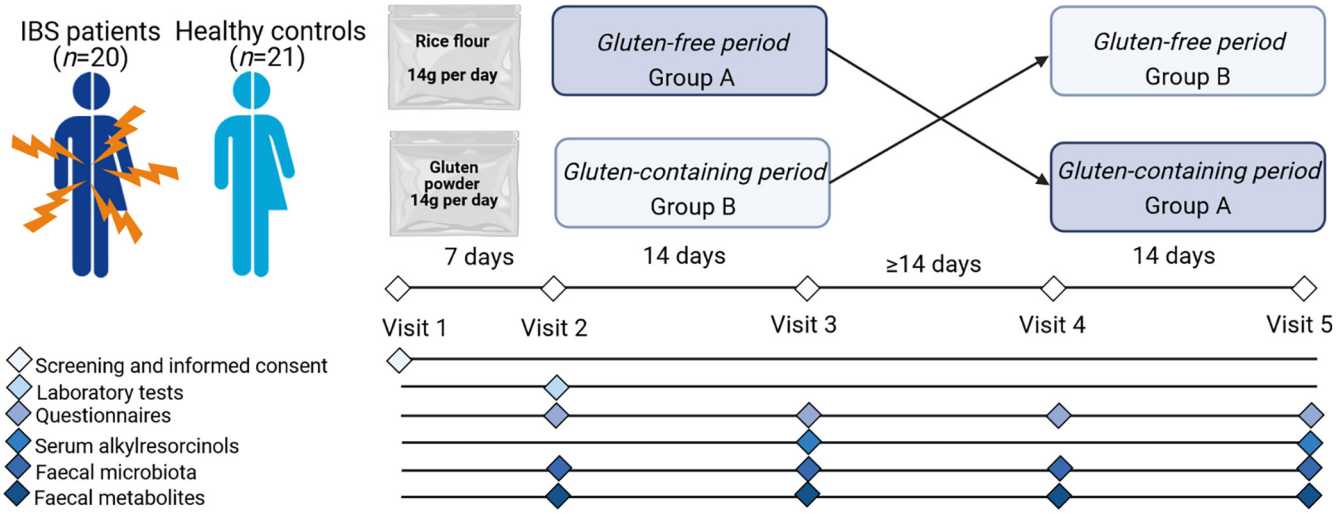

FIGURE 1 Study design, randomised double-blind placebo-controlled crossover trial

adequate blinding, the participants were asked after each intervention whether they thought they received gluten, placebo, or if they could not guess. After both interventions, serum samples were taken and serum alkylresorcinols were analysed using liquid chromatography/mass-spectrometry (LC–MS) (Food and Nutrition Science, Chalmers Technical University, Gothenburg, Sweden) for assessment of compliance to both gluten-free and gluten-containing interventions. Alkylresorcinols are long-chain phenolic lipids present in gluten-containing grains, and both epidemiological and intervention studies have shown the potential of alkylresorcinols as a biomarker for both gluten intake and a gluten-free diet.[7–9]

2.4 | IBS symptom severity

Before and after the interventions, the participants reported GI symptom severity, using the IBS-Severity Scoring System (IBS-SSS).[10] The IBS-SSS incorporates typical IBS symptoms in five domains scored 0–100 including intensity and frequency of abdominal pain, intensity of abdominal bloating, bowel habit dissatisfaction, and the daily life interference of IBS in general (maximum aggregated score of 500).

2.5 | Bowel habits

Bowel habits were documented daily using a stool diary based on the Bristol Stool Form (BSF) scale,[11] which depicts stool types 1–7; 1–2 hard stools, 6–7 loose stools. The data were used to assess the difference in proportions of hard stools, loose stools, and normal stools between the interventions, and to categorise IBS patients into IBS subtypes according to the Rome IV criteria: IBS with constipation (IBS-C), IBS with diarrhoea (IBS-D), and IBS with mixed bowel habits (IBS-M) or unclassified IBS (IBS-U).[12]

2.6 | Anxiety, depression and somatic symptoms

Assessments of anxiety and depression were done using the Hospital Anxiety and Depression Scale (HADS),[13] and somatic symptoms were assessed with the Patient Health Questionnaire (PHQ)-12,[14] which assesses non-GI somatic symptoms. For both HADS and PHQ-12 (assessed before and after both interventions), higher scores indicate more severe symptoms.

2.7 | Faecal microbiota

Faecal samples (online supporting information) were analysed for microbiota profiles by the commercially available GA-map Dysbiosis Test (Genetic Analysis AS).[15] The test provides faecal bacterial profiles (16S rRNA analysis), using 48 DNA probes targeting ≥300 bacteria on different taxonomic levels assessed as probe signal intensity. To assess the effects of the interventions, the β-diversity was estimated by use of the Bray–Curtis dissimilarity index.

2.8 | Faecal metabolites

Extracted faecal supernatants were used for untargeted metabolomics, performed by LC–MS (Food and Nutrition Science, Chalmers Technical University, Gothenburg, Sweden). A dataset of >13,000 faecal metabolites was obtained after an analytical workflow ('Notame') for untargeted metabolic profiling approaches, utilising LC–MS analysis (online supporting information).[16]

2.9 | Data analyses and statistics

The first step was to assess the difference after the interventions for the primary outcomes. Here, linear mixed models with unstructured

covariance pattern were used with intervention, period and randomisation sequence as fixed factors, and study participant as random factor. Randomisation sequence had no significant effect in any model, therefore, this variable was excluded from all models. Outcomes are presented for intervention. For the primary outcomes, the interventions were also analysed separately (before versus after each intervention period) with linear mixed models. A decrease of ≥50 points in IBS-SSS defined a responder to treatment, and our definition of a true responder was an IBS patient that solely responded to the gluten-free intervention. All others were defined as non-responders. According to this definition, responders solely to the inactive treatment (i.e., gluten-containing intervention) could also be identified. These patients were defined as placebo-responders. *The second step* was to compare the effects of the interventions on the secondary outcomes within groups. Here, both linear mixed models and multivariate analyses, including principal component analysis (PCA) and orthogonal partial least squared discriminant analysis (OPLS-DA) were used. IBS patients, HC, true responders, and non-responders, were assessed separately. *The third step* was to assess effects of the gluten-free diet while comparing the groups, that is IBS versus HC and true-responders versus non-responders, using multivariate analyses. Finally, *the fourth step* was to assess predictors of response to the gluten-free diet, where data of true responders and non-responders were compared before the gluten-free intervention using multivariate analyses (online supporting information). Data are presented as mean ± standard deviation (SD), frequency (%) or β coefficients with 95% confidence interval (CI). The microbiota and metabolites analyses were also done separately for placebo-responders. Linear mixed models and univariate analyses were performed using IBM SPSS Statistics, Version 27.0 (IBM). Estimations of Bray–Curtis dissimilarity and PCAs were performed in RStudio (R version 4.0.3) using the vegan and pca3d packages, and OPLS-DAs were performed using SIMCA Software Version 16.0.2 (Umetrics AB). $p \leq 0.05$, and when appropriate after the false discovery rate (FDR) correction ($q < 0.05$), were considered as statistical significant in all analyses.

2.10 | Sample size calculation

The sample size calculation was based on the difference in IBS-SSS within the same study subject between the diets using paired samples power test ($n = [t_{n\text{-}1\ \alpha/2} + t_{n\text{-}1,\ \beta}]^2/d^2$). A total of 18 pairs ($n$) was needed to achieve a power of 80% (β) and a level of significance of 0.05 (α), for detecting a mean of the differences of 50 (t) in IBS-SSS between the pairs, assuming the SD of the differences to be 70 (d = delta/SD).

3 | RESULTS

3.1 | Participants

Twenty IBS patients and 21 HC were eligible for randomisation to the interventions. All IBS patients and 18 HC completed the study (flow chart: Figure S1). Baseline characteristics are presented in Table 1. None of the participants used IBS medication and one IBS patient used probiotics. The participants had the following outcome on the question whether they thought they had received gluten, placebo or if they did not know: IBS patients: correct answer, 35%; wrong answer, 20%; did not know, 45%; and HC: correct answer, 33%; wrong answer, 11%; did not know, 56%. Higher levels of serum alkylresorcinols were found after the gluten-containing intervention compared with after the gluten-free intervention (Table S2), which indicated good dietary compliance.

TABLE 1 Baseline characteristics

	IBS patients ($n = 20$)	Healthy controls ($n = 21$)
Age, years, mean ± SD	25.4 ± 3.2	24.6 ± 3.6
Females, %	90	90
BMI, kg/m^2, mean ± SD	24.6 ± 6.6	23.6 ± 2.8
Subtype (%)		
IBS-C	25	NA
IBS-D	40	
IBS-M	30	
IBS-U	5	
IBS duration, years, mean ± SD	9.8 ± 5.6	NA
Anti-tTG antibodies positivity (%)	0	0
Faecal calprotectin, mean ± SD	22 ± 38	22 ± 11
IBS-SSS total, mean ± SD	334 ± 63	11 ± 16
BSF hard stools, mean % ± SD	15.6 ± 21.2	6.6 ± 9.5
BSF loose stools, mean % ± SD	22.3 ± 24.0	3.2 ± 5.5
BSF normal stools, mean % ± SD	62.1 ± 24.2	90.2 ± 11.6
HADS anxiety, mean ± SD	7 ± 5	4 ± 3
HADS depression, mean ± SD	3 ± 4	1 ± 1
PHQ-12 somatic symptoms, mean ± SD	8 ± 4	3 ± 2

Abbreviations: BMI, body mass index; BSF, Bristol stool form; HADS, Hospital Anxiety and Depression Scale; IBS, irritable bowel syndrome; IBS-C, IBS with predominant constipation; IBS-D, IBS with predominant diarrhoea; IBS-M, IBS with mixed bowel habits; IBS-SSS, IBS severity scoring system; IBS-U, IBS with unspecified bowel habits; PHQ, Patient Health Questionnaire; tTG, tissue transglutaminase.

3.2 | Step 1: Primary endpoint; comparison of interventions in IBS patients

3.2.1 | IBS symptom severity

The effects on IBS symptom severity in IBS patients did not differ between the interventions. However, the overall IBS symptom severity, abdominal pain intensity, and daily life interference improved after the gluten-free period, but no change in severity of IBS symptoms

was found after the gluten-containing period (Table 2). The flow chart of responses to the interventions and individual changes in IBS-SSS are presented in Figure 2A,B. Six IBS patients were defined as true responders to the gluten-free diet, and the remaining 14 IBS patients were defined as non-responders. Of these non-responders, five IBS patients were also defined as placebo-responders.

3.2.2 | Bowel habits

The proportion of reported loose stools differed between the interventions. The IBS patients reported fewer loose stools during the gluten-free intervention, compared with the gluten-containing intervention (19.2%±17.9% vs 27.4%±20.9%, $p = 0.01$, $q < 0.05$). No differences were observed between the gluten-free versus gluten-containing interventions in proportions of reported hard stools (17.0%±18.6% vs 12.7%±20.8%, $p = 0.38$) or normal stools (63.8%±17.3% vs 59.9%±25.2%, $p = 0.47$). True responders included IBS-D ($n = 4$), IBS-M ($n = 1$) and IBS-U ($n = 1$) subtypes, and non-responders included IBS-C ($n = 4$), IBS-D ($n = 5$) and IBS-M ($n = 5$) subtypes ($p = 0.14$).

3.3 | Step 2: Secondary endpoints; comparison of interventions within groups

3.3.1 | IBS symptoms and bowel habits in HCs

IBS symptoms did not differ between the interventions in HC, and the numerical score remained very low compared to the IBS patients (Table 2). No differences were observed between the gluten-free vs. gluten-containing interventions in proportions of hard stools (8.8%±12.2% vs 9.6%±13.2%, $p = 0.68$), loose stools (3.0%±4.9% vs 4.6%±6.5%, $p = 0.41$), or normal stools (88.2%±12.9% vs 85.8%±13.6%, $p = 0.46$).

3.3.2 | Anxiety, depression and somatic symptoms

The change in anxiety, depression and somatic symptom severity did not differ between the interventions in IBS patients, HC or non-responders. However, in true responders, the symptoms of anxiety and depression were more reduced after the gluten-free compared with the gluten-containing period (Table S3).

3.3.3 | Faecal microbiota

The Bray–Curtis dissimilarity indices did not differ between the interventions analysing all IBS patients ($\beta = 0.002$ [−0.031, 0.036], $p = 0.63$), non-responders ($\beta = 0.012$ [−0.040, 0.063], $p = 0.88$), HC ($\beta = -0.003$ [−0.025, 0.018], $p = 0.75$), or placebo-responders ($\beta = -0.078$ [−0.364, 0.207], $p = 0.97$). In true-responders, the Bray–Curtis dissimilarity indices after the gluten-free period were lower compared to after the gluten-containing period ($\beta = -0.030$ [−0.041, −0.020], $p < 0.001$, $q < 0.05$). PCA (Figure S2) on the change of faecal microbiota composition showed no distinct separation between the interventions and only poor OPLS-DA models were obtained (Table S4).

TABLE 2 Severity of gastrointestinal symptoms in gluten-free and gluten-containing diet interventions

	Gluten-free diet			Gluten-containing diet			
	Before	After	*p* within[a]	Before	After	*p* within[a]	*p* between[a]
IBS ($n = 20$)							
IBS-SSS total score	315±63	262±111	0.02[b]	313±87	281±88	0.06	0.31
Abdominal pain intensity	59±18	45±29	0.02[b]	62±19	56±19	0.11	0.07
Abdominal pain frequency	54±26	50±28	0.42	60±33	52±27	0.14	0.78
Abdominal bloating	62±32	52±34	0.16	60±24	54±36	0.45	0.68
Bowel habit dissatisfaction	66±21	57±26	0.22	62±28	55±26	0.40	0.78
Daily life interference	73±18	63±27	0.04	70±20	64±22	0.07	0.85
HC ($n = 21$)							
IBS-SSS total score	12±15	9±12	0.10	9±12	9±11	0.47	0.81
Abdominal pain intensity	2±5	0±1	0.18	0±0	1±3	0.32	0.47
Abdominal pain frequency	1±5	1±5	0.32	0±0	1±5	0.32	1.00
Abdominal bloating	2±6	1±3	0.07	2±6	1±3	0.59	0.54
Bowel habit dissatisfaction	5±7	6±9	0.66	5±6	6±7	0.52	0.66
Daily life interference	1±1	0±1	0.06	1±3	1±1	0.31	0.62

Note: Data are shown as mean±SD or frequencies (%), assessed with linear mixed models. *p* values are main effect for intervention.

Abbreviation: IBS-SSS, irritable bowel syndrome severity scoring system.

[a]Comparisons were made intention to treat (IBS, $n = 20$; HC $n = 21$).

[b]$q < 0.05$ (false discovery rate correction).

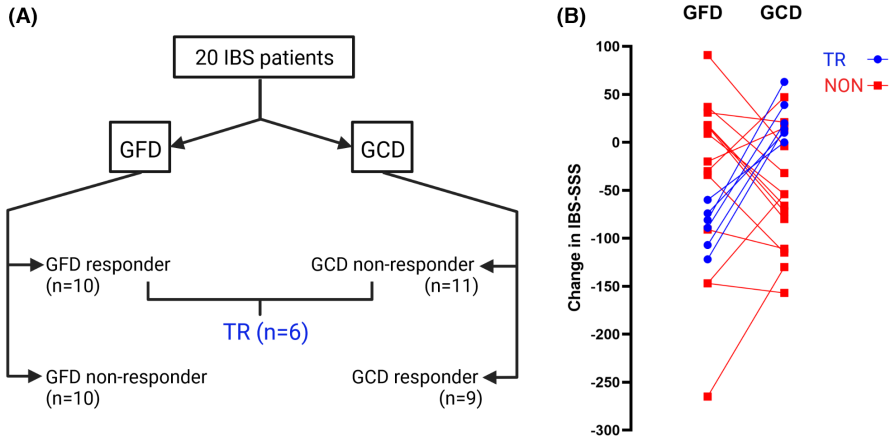

FIGURE 2 Flow chart of responses to both gluten-free diet (GFD) and gluten-containing diet (GCD). (A) Six IBS patients responded to the gluten-free intervention and did not respond to the gluten-containing intervention and were identified as true responders (TR), and all others ($n = 14$) were identified as non-responders (NON). (B) individual changes in IBS-SSS during both GFD and GCD.

3.3.4 | Faecal metabolites

PCA (Figure S3) on the change of faecal metabolites showed no distinct separation between the interventions for any of the study groups (i.e., all IBS patients, HC, true responders, non-responders, or placebo-responders), and no OPLS-DA models could be obtained.

3.4 | Step 3: Secondary endpoints; comparison of gluten-free diet between groups

3.4.1 | Anxiety, depression and somatic symptoms

The changes in anxiety, depression and severity of somatic symptoms based on the effects of the gluten-free diet intervention did not differ between IBS patients versus HC or true responders versus non-responders (Table S5).

3.4.2 | Faecal microbiota

PCA of change in faecal microbiota based on the effects of the gluten-free intervention did not separate IBS patients and HC (Figure 3A), and no OPLS-DA model could be obtained. However, for true responders versus non-responders the PCA indicated that the groups could be separated (Figure 3B). After selection of the most discriminant *X*-variables, the OPLS-DA model revealed that the effects of the gluten-free intervention on change in faecal microbiota differed in true responders versus non-responders (*X*-variables, $n = 16$). The model had good robustness ($R^2Y = 0.71$) and good predictive ability ($Q^2 = 0.49$) to separate true responders and non-responders ($p = 0.003$; Figure 3C). The taxonomic data of the model are presented in Figure S4. PCA of change in faecal microbiota based on the effects of the gluten-free intervention showed no distinct separation between placebo-responders and the other patients (Figure S5), and the OPLS-DA model was poor ($R^2Y = 0.60$; $Q^2 = 0.28$; $\Delta R^2Y\, Q^2 > 0.3$).

3.4.3 | Faecal metabolites

PCA on change in faecal metabolites of IBS patients versus HC indicated a separation between the groups based on the effects of the gluten-free intervention (Figure 4A). However, PCA showed no distinct separation of true responders and non-responders (Figure 4C). After selection of the most discriminant *X*-variables, OPLS-DA models revealed that the effects of the gluten-free intervention on change in faecal metabolites differed between IBS and HC (*X*-variables, $n = 29$), as well as between true responders and non-responders (*X*-variables, $n = 24$; Figure 4B–D). The models had good robustness (IBS versus HC, $R^2Y = 0.73$; true responders versus non-responders $R^2Y = 0.82$) and good predictive ability (IBS versus HC, $Q^2 = 0.66$; true responders versus non-responders $Q^2 = 0.79$) to separate IBS versus HC and true responders versus non-responders ($p < 0.001$ for both). PCA of change in faecal metabolites based on the effects of the gluten-free intervention showed no distinct separation between placebo-responders and the other patients (Figure S5), and no OPLS-DA model could be obtained.

3.5 | Step 4: Secondary endpoint; prediction of response to the gluten-free diet in IBS

PCA on faecal microbiota showed no evidence of separation between true responders and non-responders before the gluten-free intervention, and the OPLS-DA model had poor robustness ($R^2Y = 0.39$) and poor predictive ability ($Q^2 = 0.12$; Figure 5A,B). However, for faecal metabolites, the PCA indicated that true responders and non-responders could be separated based on their

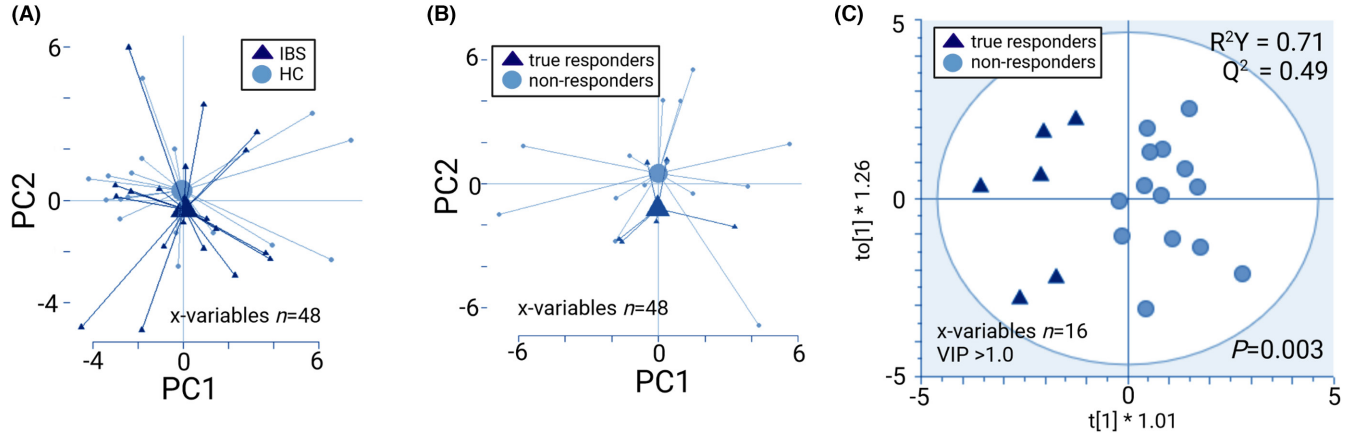

FIGURE 3 Effects of a 2-week gluten-free diet on fold changes (after/before gluten-free intervention) in faecal microbiota (GA-map™ dysbiosis test), shown as PCA scatterplots with centroids (A and B) and OPLS-DA scatterplot (C) Permutation test for (C) showed no overfitting. IBS ($n = 20$), HC ($n = 18$), true responders ($n = 6$) and non-responders ($n = 14$).

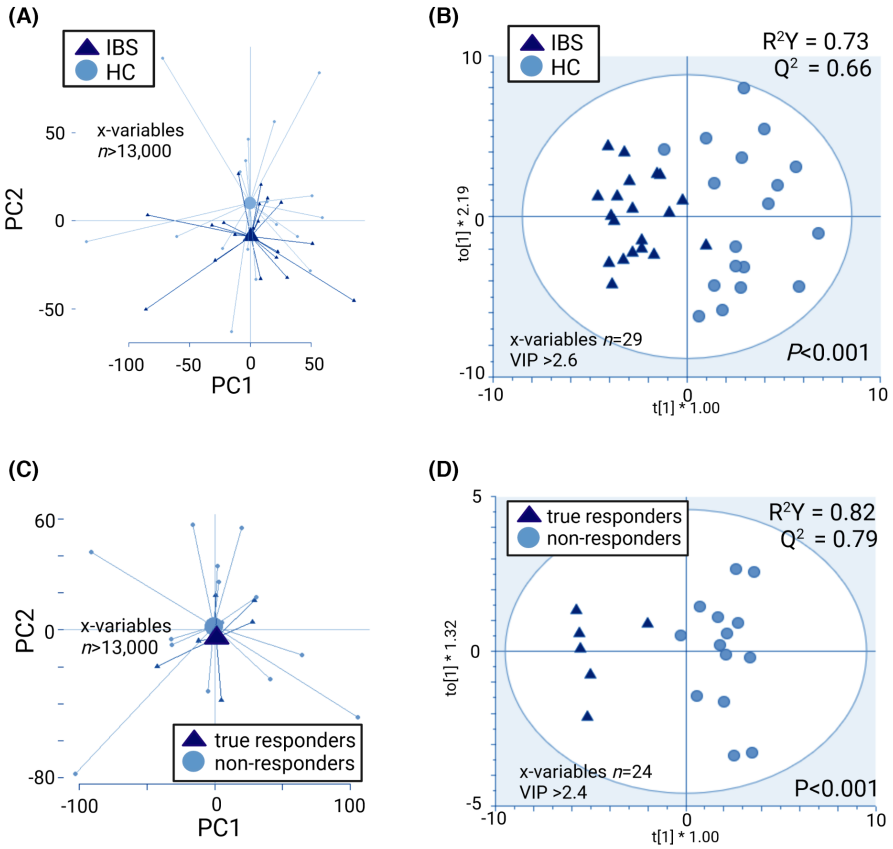

FIGURE 4 Effects of a 2-week gluten-free diet on fold changes (after/before gluten-free intervention) in metabolites of faecal supernatants (LC–MS), shown as PCA scatterplots with centroids (A and C) and OPLS-DA scatterplots (B and D). The R^2Y value determines goodness of the fit and the Q^2 value represents the predictive ability of the model; $R^2Y \geq 0.5$ and $Q^2 \geq 0.4$ are considered satisfactory. Permutation tests for (B) and (D) showed no overfitting. IBS ($n = 20$), HC ($n = 18$), true responders ($n = 6$) and non-responders ($n = 14$).

faecal metabolites profile before the gluten-free intervention (Figure 5C). An OPLS-DA revealed that the faecal metabolites of true responders and non-responders differed before the intervention, after selection of the 21 most discriminant variables. The model had a good robustness ($R^2Y = 0.81$) and high predictive ability ($Q^2 = 0.71$) ($p < 0.001$) (Figure 5D). PCA on faecal microbiota and metabolites showed no distinct separation between placebo-responders and the other patients before the gluten-free intervention (Figure S5), and no OPLS-DA models could be obtained.

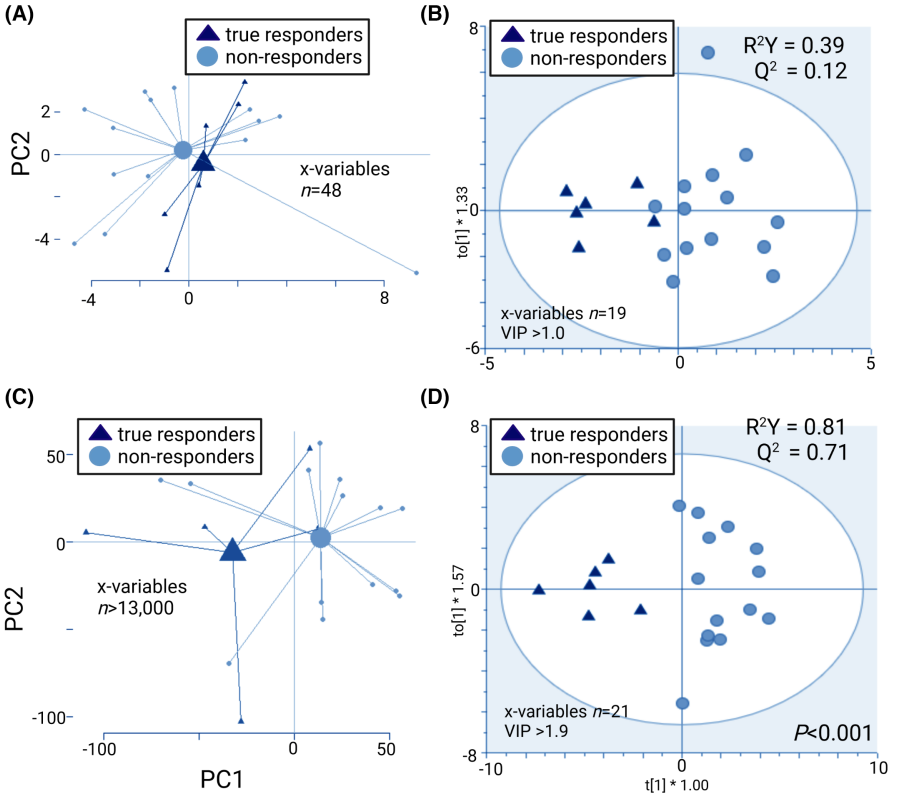

FIGURE 5 Prediction of response to gluten-free diet in IBS, assessed before the gluten-free intervention. Faecal microbiota profiles (GA-map™ dysbiosis test) shown as PCA scatterplot with centroids (A), and OPLS-DA scatterplot (B). Metabolite profiles of faecal supernatants (LC–MS) shown as PCA scatterplot with centroids (C), and OPLS-DA scatterplot (D). The R^2 value determines goodness of the fit and the Q^2 value represents the predictive ability of the model; $R^2Y \geq 0.5$ and $Q^2 \geq 0.4$ are considered satisfactory. Permutation test for (D) showed no overfitting. True responders ($n = 6$) and non-responders ($n = 14$).

4 | DISCUSSION

This study indicates that gluten might influence symptoms positively in a small subset of IBS patients, with a particular effect on bowel habits. More specifically, although the effect of the gluten-free versus the gluten-containing diets on overall IBS symptoms did not differ significantly between the groups, the effect on bowel habits differed between the interventions, with fewer reports of loose stools during the gluten-free diet. However, the gluten-free diet improved GI symptoms in a subset of IBS patients, and also had different effects on the faecal metabolite profiles of IBS patients and HC. Moreover, the gluten-free diet had distinct effects on changes of the faecal microbiota and metabolites profiles in IBS patients who responded favourably to gluten reduction, and the clinical response to a gluten-free diet may be predicted by the faecal metabolite profile before the intervention.

Previous studies assessing the gluten-free diet demonstrated heterogeneous findings, and there is yet insufficient evidence to recommend a gluten-free diet in IBS.[3] The current study shows that a beneficial effect of the gluten-free diet was only seen in a subset of IBS patients, which could be an explanation for the previous heterogeneous findings. Our data show that the true responders have similar reductions of IBS symptoms to the gluten-free intervention without a placebo response to the gluten-containing intervention, whereas the non-responders constitute a group with heterogeneous IBS symptom responses to both interventions. Regarding the effect on bowel habits, a minor difference between the interventions was observed, with fewer reported loose stools during the gluten-free intervention. Furthermore, we found that the majority of the true responders were IBS-D patients, and all IBS-C patients were non-responders. Hence, it could be hypothesised that a gluten-free diet is more effective in IBS-D, which is in line with results from a previous open label study.[17] Among the true responders, the effects of the gluten-free diet were not restricted to IBS symptoms and bowel habits, as they also reported reduced anxiety and depression after the gluten-free, but not after the gluten-containing intervention. These results are in in line with a recent large open label study, showing decreased levels of anxiety and depression after a gluten-free diet in IBS patients.[18] However, the mechanisms by which the gluten-free diet may improve anxiety and depression remain to be determined.

Gluten is the storage protein of wheat grains, and wheat also contains several other components that could aggravate GI symptoms, for example amylase-trypsin inhibitors (ATIs), and fructan, which is included in the FODMAP concept.[3] In this study, the participants were challenged with vital gluten, which is prepared by a wet milling process. Due to this process, the starch, including fructan, and the majority of ATIs are washed away.[19] During both interventions, the FODMAP content was stable and similar to the reported FODMAP intake in IBS.[20] The participants were challenged with moderate dosages of gluten (14 g/day), which was chosen in order to be similar to the actual gluten intake in the general population (10 g/day) and Swedish IBS patients (11 g/day),[6,7] and it was not expected to aggravate GI symptoms to an extent that would risk unblinding or reduce compliance.

We hypothesised that reducing GI symptoms by a gluten-free diet could be linked to the gut microenvironment in IBS patients.

However, no changes in β-diversity, that is variation of microbial abundances between the interventions, were observed in IBS patients and HC, or non-responders. However, in true responders the β-diversity was more similar after the gluten-free intervention compared with gluten-containing intervention. Still, this result should be interpreted with caution because the difference between the diets in β-diversity was, although statistical significant, only minor. Furthermore, within-group comparisons of changes in faecal microbiota composition showed no differences between the interventions. An explanation for this could be the duration of the interventions, which were relatively short. Individual microbiota composition is known to be stable over time and it may not be expected to change substantially after an intervention of 2 weeks.[21]

We have also demonstrated that the gluten-free intervention influenced faecal microbiota and metabolites differently in true responders and non-responders. These novel findings suggest that gut microbiota composition and metabolism are linked to the response to the gluten-free diet in IBS. In addition, no differences were found analysing placebo-responders versus the other patients. The different effects of the gluten-free diet on faecal metabolites profiles in IBS and HC, suggest that gut microbial metabolism may react differently to the gluten-free diet in health and disease.

We investigated if faecal microbiota and metabolite profiles could predict the response to gluten-free diet in IBS patients. Although true responders and non-responders could not be separated based on the faecal microbiota profile, they were separated based on their faecal metabolites profile before the intervention. Similar results were found assessing the faecal microbiota profile in IBS, regarding the response to a low FODMAP diet,[22] and recently, a study found that anti-gliadin immunoglobulin G antibodies may predict the response to the gluten-free diet.[18] Investigating mechanisms that could be involved in prediction of treatments in IBS are of major importance for moving towards a more individualised treatment approach in IBS. However, these studies,[18,22] and also the current study, have small sample sizes and larger studies are needed to validate these findings.

Although the use of serum alkylresorcinols as a biomarker for a gluten-free diet remains to be validated in larger studies, several studies have shown its potential.[7–9] The findings in our sample that serum alkylresorcinols were lower after the gluten-free intervention compared with the gluten-containing intervention support this, and indicates good compliance to the interventions, which we consider as a strength. Another strength of this study is the adequate blinding of the participants and investigators, and confounding factors were minimised by using the randomised crossover design,[23] and the strict in- and exclusion criteria. Nocebo and/or placebo responses, which are common in IBS studies,[23] were further minimised due to our definition of 'true responders'. This definition takes both interventions into account, and reduces the risk of patients with placebo responses in the true responders group. Furthermore, we investigated HCs as a comparator group, and the interventions did not induce any change in primary outcomes in this group. Exclusively validated questionnaires were used, and outcomes were predefined before initiation of the study.

Limitations of this study include the small sample size. Therefore, the results of the secondary outcomes should be interpreted with caution, also taking into account that the IBS patients were divided into true responders and non-responders for some analyses. Also the large proportion of females and the relatively young age of the cohort could be seen as a limitation of the study. Therefore, our results are representative of young females, but still warrants generalisation to an older population. Further, concerning the faecal microbiota, the GA map Dysbiosis Test[15] analyses a pre-identified set of bacterial taxa, and 16S gut microbiota sequencing might provide a more detailed understanding of the gut microbiota composition. Still, our chosen method provides absolute abundance of the determined bacteria taxa with high confidence and throughput.

In conclusion, our study indicates that a gluten-free diet may affect IBS symptoms in general, and bowel habits in particular in a subset of IBS patients. The gluten-free diet has distinct effect on the gut microenvironment in IBS patients who respond favourably to gluten reduction. Moreover, it seems to be possible to predict the response to the gluten-free diet by analysing the faecal metabolites profile. These findings suggest that the gut microenvironment may be of importance in the clinical response to the gluten-free diet in IBS, and future studies should aim to further assess these factors in relation to clinical response to the gluten-free diet.

AUTHOR CONTRIBUTIONS

Joost P. Algera: Study concept and design; study visits; acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content. **Maria K. Magnusson:** Analysis and interpretation of data; critical revision of the manuscript for important intellectual content. **Lena Öhman:** Critical revision of the manuscript for important intellectual content. **Stine Storsrud:** Study concept and design; critical revision of the manuscript for important intellectual content. **Magnus Simrén:** Study concept and design; obtained funding; critical revision of the manuscript for important intellectual content; study supervision. **Hans Törnblom:** Study concept and design; obtained funding; critical revision of the manuscript for important intellectual content; study supervision. All authors approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information will be found online in the Supporting Information section.

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Lipids in Health and Disease

RESEARCH

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Replacing saturated fatty acids with polyunsaturated fatty acids increases the abundance of Lachnospiraceae and is associated with reduced total cholesterol levels—a randomized controlled trial in healthy individuals

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Abstract

Background: Improving dietary fat quality strongly affects serum cholesterol levels and hence the risk of cardiovascular diseases (CVDs). Recent studies have identified dietary fat as a potential modulator of the gut microbiota, a central regulator of host metabolism including lipid metabolism. We have previously shown a significant reduction in total cholesterol levels after replacing saturated fatty acids (SFAs) with polyunsaturated fatty acids (PUFAs). The aim of the present study was to investigate the effect of dietary fat quality on gut microbiota, short-chain fatty acids (SCFAs), and bile acids in healthy individuals. In addition, to investigate how changes in gut microbiota correlate with blood lipids, bile acids, and fatty acids.

Methods: Seventeen participants completed a randomized, controlled dietary crossover study. The participants received products with SFAs (control) or PUFAs in random order for three days. Fecal samples for gut microbiota analyses and fasting blood samples (lipids, fatty acids, and bile acids) were measured before and after the three-day intervention.

Results: Of a panel of 40 bacteria, *Lachnospiraceae* and *Bifidobacterium* spp. were significantly increased after intervention with PUFAs compared with SFAs. Interestingly, changes in *Lachnospiraceae,* as well as *Phascolarlactobacterium* sp. and *Eubacterium hallii,* was also found to be negatively correlated with changes in total cholesterol levels after replacing the intake of SFAs with PUFAs for three days. No significant differences in SCFAs or bile acids were found after the intervention.

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Conclusion: Replacing SFAs with PUFAs increased the abundance of the gut microbiota family of *Lachnospiraceae* and *Bifidobacterium* spp. Furthermore, the reduction in total cholesterol after improving dietary fat quality correlated with changes in the gut microbiota family *Lachnospiraceae*. Future studies are needed to reveal whether *Lachnospiraceae* may be targeted to reduce total cholesterol levels.

Trial registration: The study was registered at Clinical Trials (https://clinicaltrials.gov/, registration identification number: NCT03658681).

Keywords: Dietary fat, Polyunsaturated fatty acids, Total cholesterol, Gut microbiota, Lachnospiraceae, Randomized controlled trial

Background

Improving dietary fat quality by exchanging the intake of saturated fatty acids (SFAs) with polyunsaturated fatty acids (PUFAs) has been shown to reduce serum cholesterol levels [1–4] and thereby the risk of cardiovascular diseases (CVDs) [3, 5]. Emerging evidence suggests that gut microbiota play a significant role in human metabolic regulation [6]. The gut microbiota and its host are in a symbiotic relationship by a joint utilization of consumed nutrients, and the human gut microbiota with its fermentation products are hypothesized to play a major role in host energy and substrate metabolism [7–9]. Hence, dietary habits affect both the abundance and composition of gut microbiota and host health [10–12]. Diet is one of the most important factors affecting the gut microbiota, and changes in diet can alter the gut microbiota within one to three days [13–16]. Recent studies have suggested that dietary fat is a potential modulator of the human gut microbiota composition [15, 17–20] and that both total dietary fat and fat quality may shape the gut microbiota [21]. The complex interplay between diet and the gut microbiota leads to the growth of specialist microbes that produce metabolites, such as short-chain fatty acids (SCFAs), influencing host metabolism and cholesterol regulation [17, 22–24]. The type of fat seems to elicit distinct effects on gut microbiota. While high-fat diets containing SFAs have been shown to decrease bacterial diversity [25], unsaturated fat is reported to increase the total bacterial count [26]. A correlation between omega-3 (n3) fatty acids and gut microbiota [27, 28], including a positive correlation with bacteria of the *Lachnospiraceae* family [28, 29], has been reported. In a recent study in mice, an omega-6 (n6) high-fat diet induced changes in gut microbiota, including a reduction in *Lachnospiraceae* [30]. However, only a few studies have investigated the effect of fat quality on gut microbiota and the impact on human metabolic regulation.

Bile acids are produced from cholesterol in the liver and facilitate digestion and absorption of lipids. Bile acids may also have hormonal or signaling functions by binding to several receptors and thereby play a crucial role in metabolic regulation [31]. It is well known that gut microbiota can oxidize and degrade bile acids. Even though gut microbiota may affect metabolic regulation by conversion of bile acids and the interaction between gut microbiota and bile acid metabolism is an emerging topic, there are few clinical studies investigating the interaction between gut microbiota, bile acid metabolism, and risk of CVD [32].

In a recent publication, it was reported that exchanging intake of SFAs with PUFAs effectively reduced total cholesterol levels after only three days in healthy individuals [1]. Whether this effect could be related to changes in the gut microbiota is not known.

The aim of the present study was to investigate the effect of dietary fat quality on gut microbiota, SCFAs, and bile acids in healthy individuals and to explore the relationship between gut bacteria and blood lipids, bile acids, and fatty acids.

Methods

Subjects and study design

The results from the present study were obtained from the same participants and set of samples as previously described [1]. Healthy volunteers (aged 18–65 years) with body mass index (BMI) between 18.5–27 kg/m^2 were recruited from advertisements on Facebook, Oslo Metropolitan University (OsloMet) website, and from the student mass and employees at OsloMet to participate in a randomized controlled crossover study as described previously [1]. The study was performed between April 2018 and January 2019 at OsloMet. Inclusion and exclusion criteria have been described previously [1]. Briefly, the exclusion criteria were fasting blood glucose values $\geq$ 6.1 mmol/L, any food allergies or intolerances or chronic metabolic diseases (e.g., diabetes, CVD, and cancer), including the use of medication, intestinal diseases, including inflammatory bowel disease, celiac disease, and irritable bowel disease. Furthermore, those with high sensitivity C-reactive protein (hsCRP) > 10 mg/L, treated with antibiotics the previous three months and during the study, donating blood the previous two months or during the study, were pregnant or lactating, planned weight reduction and/or had $\geq$ 5% weight change the previous

three months, used any hormonal treatment (except from oral contraception), tobacco, or had a high alcohol consumption (> 40 g/day) were excluded. Seventy-two volunteers were screened for participation, and 20 participants were willing to participate in the dietary crossover study, while 17 completed the study. Participants lost during follow-up and those included in the study are outlined in the flow chart (Fig. 1).

After a one-week run-in period, the participants were randomized (1:1 allocation ratio) and received either SFA products (muffins with butter-based spread and butter-based spread) (control) or PUFA products (muffins with margarine and margarine) for three consecutive days before crossing over to the other intervention, separated by a 1.5-week washout period (Fig. 2). In the run-in and washout periods, the participants received SFA (control) products. The study products (muffins and margarine or butter-based spread) replaced fat-containing products in their habitual diet. The fatty acid composition of the study products is given in Gaundal et al. [1]. The SFA products contributed 1085.5 kcal, 78.5 g carbohydrate, 13.4 g protein, 79.5 g total fat, 29.9 g SFAs, 33.4 g monounsaturated fatty acids (MUFAs) and 10.2 g PUFAs per day. The PUFA products contributed 985.8 kcal, 74.4 g carbohydrate, 13.9 g protein, 69.8 g total fat, 2.4 g SFAs, 30.9 g MUFAs and 26.4 g PUFAs per day [1].

The participants were advised to maintain their habitual diet and physical activity level throughout the study period, except for the dietary restrictions: limit their intake of dietary fats and products rich in beta-glucan, not taking dietary supplements (fish oil, probiotics, etc.) and antibiotics. Details about the study design, description of participants, and study products are described elsewhere [1].

All participants signed a written informed consent form prior to participation. The study was approved by the Regional Committees for Medical and Health Research

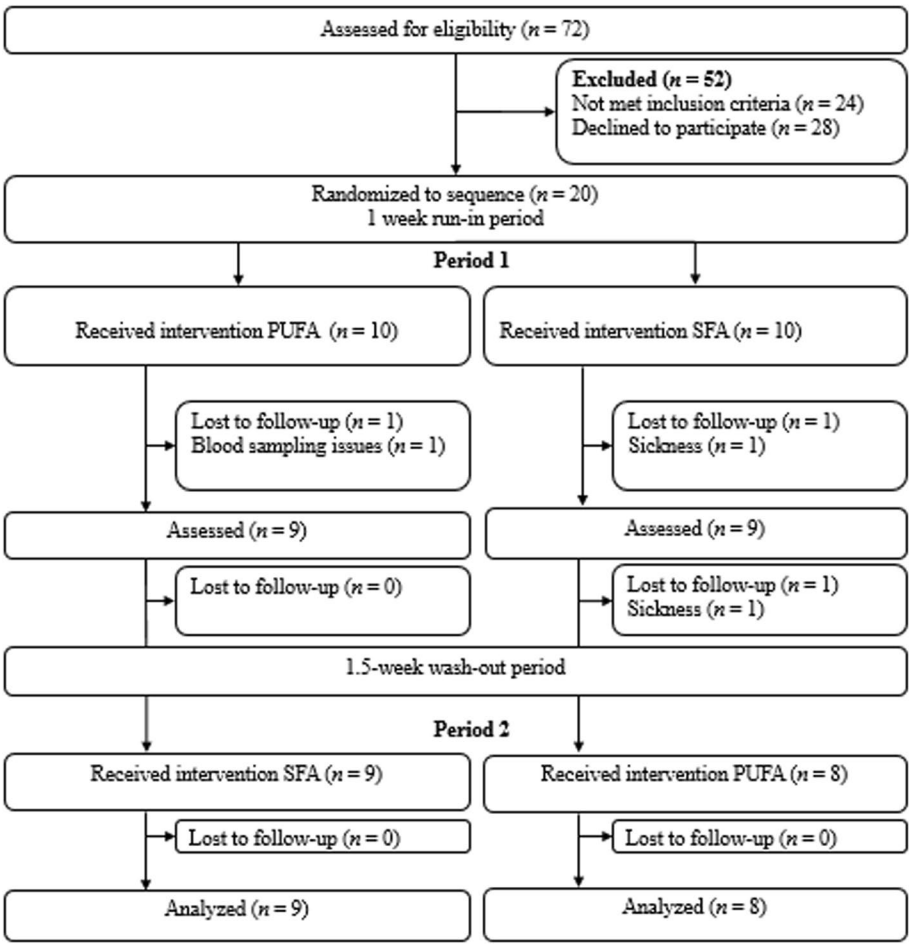

Fig. 1 Flow chart of the participants included in the dietary crossover study ($n = 17$) [1]. Abbreviations: n: number; PUFAs: polyunsaturated fatty acids; SFAs: saturated fatty acids

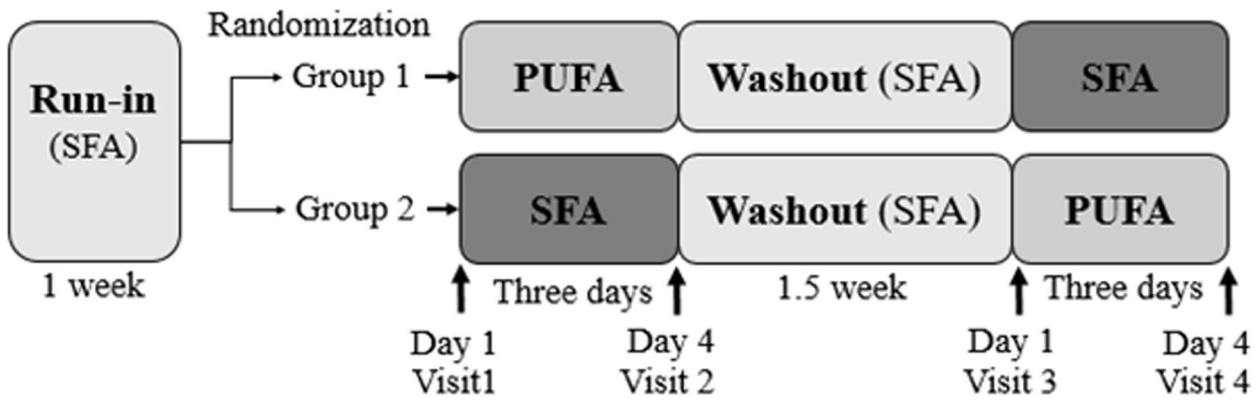

Fig. 2 Study design of the double-blind, randomized, controlled crossover study. Described previously [1]. Seventeen healthy volunteers (group 1: $n=9$; group 2: $n=8$) received daily PUFA products (two muffins and 20 g margarine spread) or SFA products (two muffins and 20 g butter-based spread) for three consecutive days, separated by a 1.5-week washout period. The participants received SFA products in the run-in and washout periods. Fasted blood was measured for glucose, insulin, triglyceride, NEFAs, fatty acid profiles, SCFAs, bile acids, and total cholesterol at each visit. In addition, fecal samples were collected for gut microbiota analyses at each visit. Abbreviations: NEFAs: nonesterified fatty acids, PUFAs: polyunsaturated fatty acids, SCFAs: short-chain fatty acids, SFAs: saturated fatty acids

Ethics (2018/104) and conducted according to the Declaration of Helsinki guidelines. The crossover study was registered at Clinical Trials (http://clinicaltrials.gov/, registration identification number: NCT03658681).

Blood sampling and laboratory analyses

Blood samples were collected after an overnight fast (≥ 12 h) at each visit in the crossover study [1]. Fasting triglyceride and total cholesterol were measured in serum by a routine laboratory (Fürst Medical Laboratory) within 24 h. Furthermore, nonesterified fatty acids (NEFAs), fatty acid profiles and the SCFAs acetate, propionate and butyrate were measured in EDTA plasma in a commercial laboratory (Vitas Analytical Service, Oslo, Norway). NEFAs were measured using an enzymatic colorimetric assay with acyl-CoA oxidase and MEHA as color reagents. The total plasma (EDTA) fatty acid profile was measured with GC flame ionization detection. An internal standard (triheptadecanoin) was added, and the samples were methylated with 3 N HCl in methanol. Fatty acid methyl esters were extracted with hexane, and then samples were neutralized with 3 N KOH in water. After mixing and centrifuging, the hexane phase was injected into the GC flame ionizing detection. Analysis was performed on a 7890A GC with a split/splitless injector, a 7683B automatic liquid sampler and flame ionization detection (Agilent Technologies). Separations were performed on an SP-2380 (30 m × 0·25 mm i.d. × 0·25 µm film thickness) column from Supelco. The concentration of the individual fatty acids was measured as µg fatty acid/ml plasma and presented as a percentage of total fatty acids.

Nine bile acids, cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA), glycocholic acid (GCA), glycodeoxycholic acid (GDCA), and glycochenodeoxycholic acid (GCDCA), were measured in plasma using LC–MS/MS with a sample volume of 50 µL and a single deuterium-labeled isotope as an internal standard. The plasma was initially treated with aqueous formic acid [1%] prior to protein precipitation with acetonitrile. The extracts were loaded onto a Hybrid SPE Phospholipid 96-well plate, and the effluent was dried in a vacuum centrifuge concentrator. LC–MS/MS was performed on the reconstituted residues using a Shimadzu LC-20ADXR LC system coupled to a Sciex QTRAP5500 mass spectrometer with a Turbo V ion source and a TurboIonspray probe. Separation of the analytes was achieved using a Phenomenex Kinetex 2.6 µm Biphenyl 100A 100 × 4.6 mm column with an aqueous solution of ammonium acetate [5 mM] and acetic acid [2.1 mM] and an acetonitrile gradient mobile phase. Negative mode multiple reaction monitoring was used for detection. Linear calibration curves of the peak area ratios of the analyte and internal standard in aqueous methanol [50%] were used for quantification.

Fecal collection and gut microbiota analyses

The participants received a sample collection kit (GA-map™ Dysbiosis Test, Genetic Analysis AS) for fecal collection and were instructed to sample the stool from three different places and place it in the sampling tubes. The samples were kept at room temperature for a maximum of three days before they were stored at -80 °C at

OsloMet. The participants were instructed to sample the stool before (day 1) and after (day 4) each intervention, as close to the visits as possible. Fecal samples were collected at each visit. All samples were collectively sent to Genetic Analysis AS (GA) for microbiota analyses after the study was completed. The GA-map™ Dysbiosis Test is a commercially available genome-based test using fecal samples for analyses of gut bacteria, described in detail elsewhere [33]. The test comprises 48 DNA probes targeting ≥ 300 bacteria on different taxonomic levels, thus allowing mapping of the intestinal microbiota profile for a selected set of bacteria. To characterize and identify bacteria present, probes targeting seven variable regions (V3—V9) of the 16S rRNA gene were used. Human fecal sample homogenization and mechanical and enzymatic bacterial cell disruption were utilized to isolate and bind total bacterial genomic DNA to magnetic beads. The hypervariable regions V3—V9 of the 16S rRNA were further amplified by polymerase chain reaction (PCR). To determine bacterial DNA labeling, single nucleotide extension and hybridization to a complementary DNA strand were coupled to beads. The abundance of bacteria was assessed by the strength of the fluorescent signal (probe intensity) and measured by a Luminex 200 (Luminex Corporation). Of the 48 bacteria, 40 were detected in more than 50% of the participants at each timepoint and included in the analyses.

Statistical analyses

The primary aim of the crossover study was to investigate the effect of fat quality on glycemic regulation, and the sample size was based on literature on fat and glycemic regulation as described previously [1], whereas secondary outcomes were fasting blood lipids, fatty acids, SCFAs, bile acids, and gut microbiota. Hence, the nature of the present study is explorative, and therefore correction for multiple testing has not been performed.

For the gut microbiota analyses, the relative abundance number was logarithmically transformed with base 2 (Log2) to reduce the skewness of the original data. The log2 ratio was obtained by calculating the ratio before and after intervention with SFAs or PUFAs. A paired t test was used to assess differences between the two interventions based on the log2 ratio. In addition, a paired t test was also used to calculate differences within each group (SFAs and PUFAs) based on the log2 values before and after intake of SFAs and PUFAs.

For the variables of SCFAs and bile acids, a paired sample comparison (Wilcoxon signed rank test) was used to assess differences between the two interventions (SFA and PUFA) and differences within each group. SCFA levels were reported as both absolute values and as a percent of the total SCFA level.

Associations between the change in blood parameters (lipids, fatty acids, bile acids) and the change in gut microbiota were assessed with Pearson correlation. $P < 0.05$ was regarded as statistically significant for all statistical analyses. Statistical analyses were performed with IBM SPSS statistics (version 27), and figures were designed with GraphPad Prism 8 for Windows (version 8.0.0.).

Results

The participants were healthy and normal weight, with fasting glucose, hemoglobin A1c (HbA1c), triglyceride, total cholesterol, and diastolic and systolic blood pressure within the normal range (Table 1). Their baseline characteristics have previously been shown [1].

Whether intake of different fat qualities could affect the gut microbiota was further addressed. Of the 40 gut bacteria analysed, the abundance of *Bifidobacterium* spp. and *Lachnospiraceae* was significantly increased after intake of PUFAs compared to SFAs ($P = 0.029$ and $P = 0.013$, respectively) (Table 2). Furthermore, there was a tendency toward differences in the abundance of Actinobacteria, *Dialister invisus*, *Dialister invisus* & *Megasphaera micronuciformis, Eubacterium hallii* and *Phascolactobacterium* sp. between the interventions (Table 2).

The microbial metabolites SCFAs acetate, propionate and butyrate were measured in blood before and after intake of SFAs and PUFAs. There were no significant

Table 1 Baseline characteristics of the participants, as previously shown [1]

		Median	25^{th}—75^{th} percentiles
Male/female (*n*)	6/11		
Age (years)		28.0	25.0—46.0
BMI (kg/m^2)		22.8	22.0—25.0
HbA1c (%)[a]		5.2	5.0—5.4
HbA1c (mmol/mol)[a]		33.0	31.2—35.5
Glucose (mmol/L)		5.1	4.9—5.4
Insulin (pmol/L)		51.0	31.0—60.0
Triglyceride (mmol/L)		0.9	0.6—1.4
Total cholesterol (mmol/L)		4.9	4.4—5.4
hsCRP (mg/L)		0.9	0.3—1.4
Systolic blood pressure (mmHg)[a]		123	113—136
Diastolic blood pressure (mmHg)[a]		71	66—75
Body fat percent (%)		24.4	12.5—33.9
FFM (kg)		47.8	45.3—69.3

[a] HbA1c, systolic and diastolic blood pressure measured at screening. Variables are measured fasted

FFM Fat free mass, *HbA1c* Hemoglobin A1c, *hsCRP* High sensitivity C-reactive protein

Table 2 Differences in bacterial abundance after a three-day intervention with SFA and PUFA

	SFA			PUFA			
Gut bacteria	**Day 1**	**Day 4**	**p^1**	**Day 1**	**Day 4**	**p^1**	**p^2**
Actinobacteria (A)	7.9 (1.1)	7.4 (1.1)	*0.087*	7.5 (1.2)	7.7 (1.3)	ns	*0.063*
Actinomycetales (A)	4.0 (1.1)	4.0 (1.0)	ns	4.4 (1.3)	4.2 (1.0)	ns	ns
Bifidobacterium spp. (A)	7.1 (1.3)	6.6 (1.3)	*0.091*	6.6 (1.3)	7.0 (1.4)	ns	**0.029**
Alistipes (B)	7.1 (1.8)	7.3 (1.8)	ns	7.2 (1.8)	7.5 (1.8)	**0.006**	ns
Alistipes onderdonkii (B)	4.2 (1.9)	4.3 (1.9)	ns	4.2 (1.9)	4.5 (2.1)	ns	ns
Bacteroides fragilis (B)	4.0 (2.0)	4.2 (2.1)	ns	3.8 (1.8)	4.1 (2.2)	ns	ns
Bacteroides pectinophilus (B)	3.6 (0.6)	3.5 (0.5)	ns	3.7 (0.7)	3.6 (0.7)	ns	ns
Bacteroides spp. (B)	5.3 (1.6)	5.4 (1.6)	ns	5.3 (1.5)	5.4 (1.5)	ns	ns
Bacteroides spp. & *Prevotella* spp. (B)	9.9 (0.4)	10.0 (0.3)	ns	9.9 (0.4)	9.9 (0.3)	ns	ns
Bacteroides stercoris (B)	3.8 (1.7)	3.9 (1.5)	ns	3.8 (1.5)	3.8 (1.4)	ns	ns
Bacteroides zoogleoformans (B)	3.7 (1.5)	4.1 (1.6)	*0.086*	3.9 (1.5)	3.9 (1.4)	ns	ns
Parabacteroides johnsonii (B)	3.9 (1.8)	4.0 (1.6)	ns	3.9 (1.8)	4.3 (1.8)	**0.014**	ns
Parabacteroides spp. (B)	5.5 (1.5)	5.6 (1.5)	ns	5.5 (1.5)	5.8 (1.6)	ns	ns
Bacilli (F)	7.2 (0.4)	7.1 (0.7)	ns	7.1 (0.8)	7.1 (0.5)	ns	ns
Clostridia (F)	8.9 (0.2)	9.0 (0.3)	ns	8.9 (0.3)	9.0 (0.2)	ns	ns
Clostridium methylpentosum (F)	4.1 (0.8)	4.2 (0.8)	ns	4.1 (0.8)	4.4 (0.9)	*0.082*	ns
Coprobacillus cateniformis (F)	2.9 (0.9)	2.8 (0.5)	ns	3.0 (1.0)	2.9 (1.2)	ns	ns
Dialister invisus (F)	7.1 (2.3)	7.3 (2.4)	ns	7.5 (2.3)	7.3 (2.2)	ns	*0.073*
Dialister invisus & *Megasphaera micronuciformis* (F)	5.6 (2.6)	5.8 (2.6)	**0.005**	5.9 (2.8)	5.8 (2.6)	ns	*0.067*
Dorea spp. (F)	4.5 (1.0)	4.5 (0.9)	ns	4.5 (1.2)	4.7 (0.9)	ns	ns
Eubacterium hallii (F)	6.6 (0.8)	6.3 (0.9)	*0.064*	6.3 (0.7)	6.5 (0.9)	ns	*0.095*
Eubacterium biforme (F)	5.0 (2.4)	4.9 (2.2)	ns	4.8 (2.2)	4.7 (2.2)	ns	ns
Eubacterium rectale (F)	6.2 (2.2)	6.2 (2.1)	ns	6.2 (2.0)	6.4 (2.0)	ns	ns
Eubacterium siraeum (F)	4.4 (1.1)	4.1 (1.1)	**0.043**	4.7 (0.8)	4.7 (1.2)	ns	ns
Firmicutes (F)	9.4 (0.4)	9.4 (0.6)	ns	9.5 (0.5)	9.4 (0.5)	ns	ns
Faecalibacterium prausnitzii (F)	8.8 (0.3)	8.7 (0.5)	ns	8.7 (0.3)	8.9 (0.3)	ns	ns
Lachnospiraceae (F)	9.8 (0.2)	9.8 (0.2)	ns	9.7 (0.3)	9.8 (0.2)	**0.009**	**0.013**
Lactobacillus spp. (F)	5.7 (2.3)	5.7 (2.0)	ns	5.5 (2.1)	5.5 (2.0)	ns	ns
Lactobacillus spp. 2 (F)	3.5 (1.0)	3.5 (1.2)	ns	3.2 (1.1)	3.4 (1.3)	ns	ns
Phascolarctobacterium sp. (F)	4.2 (1.9)	4.2 (1.8)	ns	4.1 (1.8)	4.2 (1.9)	*0.082*	*0.069*
Ruminococcus albus & *Ruminococcus bromii* (F)	4.9 (1.9)	5.0 (2.0)	ns	5.1 (1.9)	5.4 (2.0)	ns	ns
Ruminococcus gnavus (F)	4.0 (1.2)	4.0 (1.3)	ns	3.9 (1.1)	4.0 (1.1)	ns	ns
Streptococcus agalactiae & *Eubacterium rectale* (F)	5.9 (1.7)	5.9 (1.6)	ns	5.7 (1.7)	5.9 (1.5)	*0.065*	ns
Streptococcus salivarius ssp. thermophilus & *S. sanguinis* (F)	2.8 (0.4)	2.8 (0.5)	ns	3.4 (1.6)	3.3 (1.1)	ns	ns
Streptococcus salivarius ssp. thermophilus (F)	4.6 (1.4)	4.6 (1.4)	ns	4.7 (1.6)	4.8 (1.5)	ns	ns
Streptococcus spp. 2 (F)	3.5 (0.9)	3.4 (1.0)	ns	3.3 (0.8)	3.3 (0.8)	ns	ns
Veillonella spp. (F)	9.2 (0.4)	9.2 (0.4)	ns	9.2 (0.5)	9.2 (0.4)	ns	ns
Firmicutes various (F/T/B)	7.7 (1.5)	7.5 (1.4)	ns	7.4 (1.5)	7.6 (1.5)	ns	ns
Proteobacteria (P)	5.9 (0.3)	6.0 (0.8)	ns	6.0 (0.5)	6.0 (0.5)	ns	ns
Mycoplasma hominis (T)	6.3 (2.3)	6.3 (2.2)	ns	6.1 (2.2)	5.9 (2.3)	ns	ns

The values are log2 transformed and given as mean (SD). *P*-values indicate differences within (p^1) or between (p^2) SFA and PUFA interventions calculated with t-test

A Actinobacteria, *B* Bacteroidetes, *F* Firmicutes, *ns* Non-significant, *P* Proteobacteria, *PUFA* Polyunsaturated fatty acids, *SFA* Saturated fatty acids, *T* Tenericutes

differences in total SCFA levels or relative levels of acetate, propionate, or butyrate between the interventions (Fig. 3). However, intake of PUFAs for three consecutive days significantly increased the relative level of butyrate ($P=0.015$) (Fig. 3).

We have previously shown that total cholesterol levels significantly decreased after intervention with

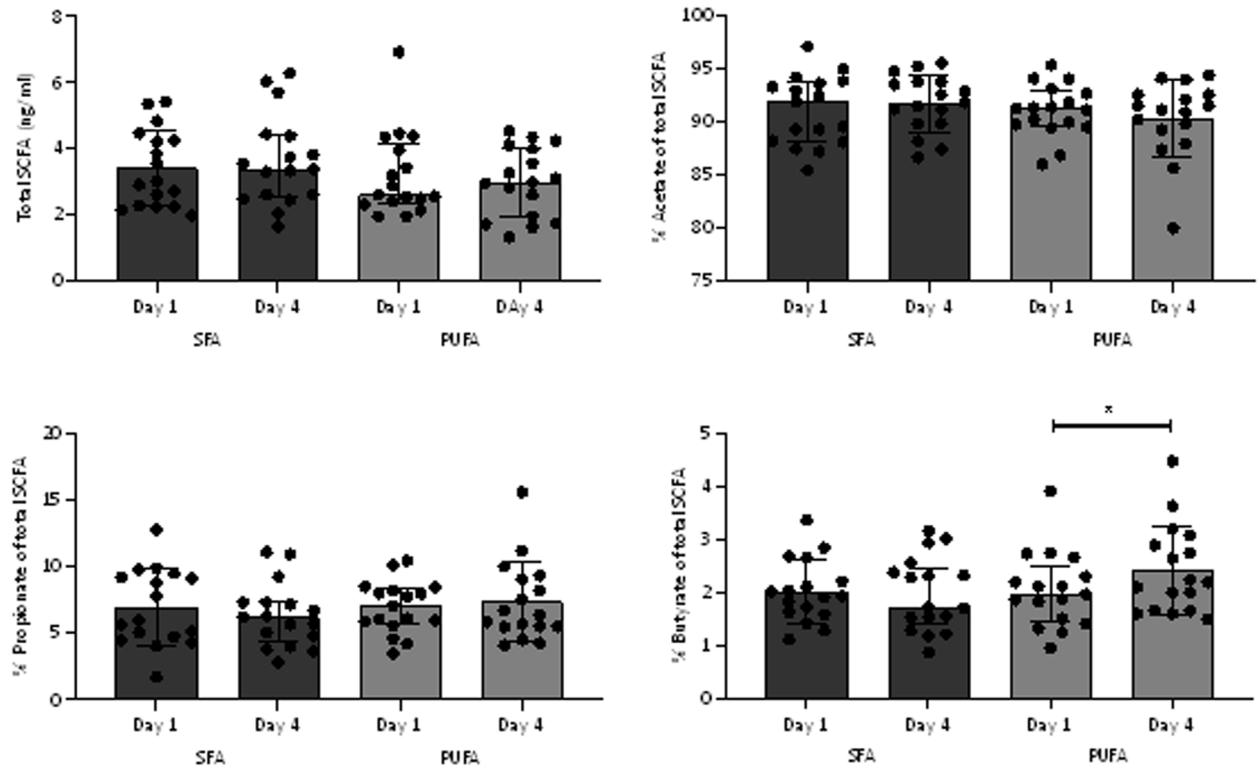

Fig. 3 SCFAs before and after intake of SFAs or PUFAs. Total SCFAs and relative levels of acetate, propionate, and butyrate before (day 1) and after (day 4) intake of SFAs and PUFAs in healthy individuals ($n = 17$) for three consecutive days. Data are shown as the median, and bars indicate the 25th—75th percentiles. Within- and between-group differences were analyzed with the Wilcoxon signed rank test. * indicates $P \leq 0.05$. Abbreviations: PUFAs: polyunsaturated fatty acids, SCFAs: short-chain fatty acids, SFAs: saturated fatty acids

PUFAs compared with SFAs, whereas no significant effect on triglycerides and NEFAs was found [1]. As bile acids are synthesized from cholesterol, we wanted to investigate the effect of dietary fat on circulating levels of bile acids. Of the nine bile acids measured in plasma, no significant changes between the interventions were detected. Within interventions, however, changes were evident. Within the SFA intervention, TCA, GCA, and GCDCA decreased significantly ($P = 0.039$, $P = 0.013$, $P = 0.028$, respectively), while GDCA decreased and

Table 3 Differences in bile acids after a three-day intervention with SFA and PUFA

	SFA			PUFA			
Bile acid (nM)	**Day 1**	**Day 4**	**p^1**	**Day 1**	**Day 4**	**p^1**	**p^2**
Cholic acid (CA)	27.6 (20.2–111.2)	26.2 (12.2–299.0)	0.723	84.5 (14.2–239.4)	24.0 (8.7–83.5)	0.193	0.102
Deoxycholic acid (DCA)	244.9 (117.5–496.6)	275.0 (143.2–404.8)	0.332	253.0 (170.2–539.0)	207.7 (161.8–311.6)	0.113	0.124
Chenodeoxycholic acid (CDCA)	116.0 (61.9–194.9)	65.0 (35.9–284.6)	0.653	135.9 (65.3–315.7)	82.7 (17.0–216.4)	0.193	*0.068*
Taurocholic acid (TCA)	3.6 (1.5–7.8)	2.3 (0.6–4.1)	**0.039**	3.4 (1.9–14.5)	2.7 (1.4–8.3)	0.237	0.868
Taurodeoxycholic acid (TDCA)	26.9 (11.2–46.6)	18.9 (6.5–38.7)	0.177	25.6 (11.4–72.0)	17.7 (14.0–33.7)	0.193	0.586
Taurochenodeoxycholic acid (TCDCA)	46.6 (24.6–99.7)	43.1 (18.1–64.9)	*0.062*	60.3 (34.2–101.3)	54.6 (19.5–90.2)	0.163	0.831
Glycocholic acid (GCA)	47.4 (33.2–99.0)	32.3 (17.2–72.1)	**0.013**	58.3 (27.6–112.3)	53.8 (15.7–90.1)	0.287	0.869
Glycodeoxycholic acid (GDCA)	153.4 (76.4–236.8)	99.0 (50.5–205.8)	0.124	162.0 (74.2–285.3)	121.6 (62.9–165.5)	**0.017**	0.210
Glycochenodeoxycholic acid (GCDCA)	549.2 (219.6–724.7)	300.6 (222.3–491.1)	**0.028**	442.4 (248.6–763.0)	443.8 (108.8–585.9)	**0.025**	0.653

Data is given as median (25^{th}—75^{th} percentiles). *P*-values indicate differences within (p^1) or between (p^2) SFA and PUFA interventions calculated with Wilcoxon signed rank test

nM Nanomolar, *PUFA* Polyunsaturated fatty acids, *SFA* Saturated fatty acids

GCDCA increased within the PUFA intervention ($P=0.017$, $P=0.025$, respectively) (Table 3).

The gut bacteria that significantly differed or tended to differ after intervention with SFAs compared to PUFAs were thereafter correlated with the other variables. Changes in gut microbiota were associated with changes in total cholesterol levels, triglycerides, bile acids, and fatty acids (Fig. 4). We found a significant, negative correlation between the change in total cholesterol and the change in abundance of *Lachnospiraceae* (r: -0.511, $P=0.002$), *Phascolarctobacterium* sp. (r: -0.452, $P=0.007$), and *Eubacterium hallii* (r: -0.397, $P=0.020$) (Figs. 4 and 5). There were also significant negative correlations between changes in NEFAs and changes in the abundance of Actinobacteria (r: -0.397, $P=0.020$), *Eubacterium hallii* (r: -0.348, $P=0.044$), and *Bifidobacterium* spp. (r: -0.379, $P=0.027$) and between changes in triglycerides and *Bifidobacterium* spp. (r: -0.346, $P=0.045$) (Fig. 4). Interestingly, we found that three bacteria (*Eubacterium hallii, Lachnospiraceae,* and *Phascolarctobacterium* sp.) correlated negatively with total cholesterol and positively with several of the bile acids. However, a significant positive correlation was only found between changes in the bile acid GCDCA and *Eubacterium hallii* (r: 0.343, $P=0.047$) (Fig. 4). We also investigated correlations between the gut bacteria and fatty acids in blood. The change in *Lachnospiraceae* after intervention was negatively correlated with the change in eicosapentaenoic acid (EPA) (C20:5 n3) (r: -0.384, $P=0.025$), but no other significant correlations were observed between the gut bacteria and fatty acids in blood (Fig. 4). Correlations between gender and age and the different variables were investigated. Age correlated significantly with *Eubacterium hallii* (r: 0.353, $P=0.040$), *Eubacterium biforme* (r: 0.364, $P=0.034$), *Eubacterium rectale* (r: -0.361, $P=0.036$) and 20:4 n6 (r: 0.349, $P=0.043$), while sex correlated significantly with *Bacilli* (r: -0.348, $P=0.043$), *Actinomycetales* (r: -0.390, $P=0.022$), *Shigella* spp. & *Echerichia* spp. (r: -0.346, $P=0.045$), butyrate (r: 0.357, $P=0.038$), and the bile acids GCA (r: -0.409, $P=0.016$) and GCDCA (r: -0.408, $P=0.017$).

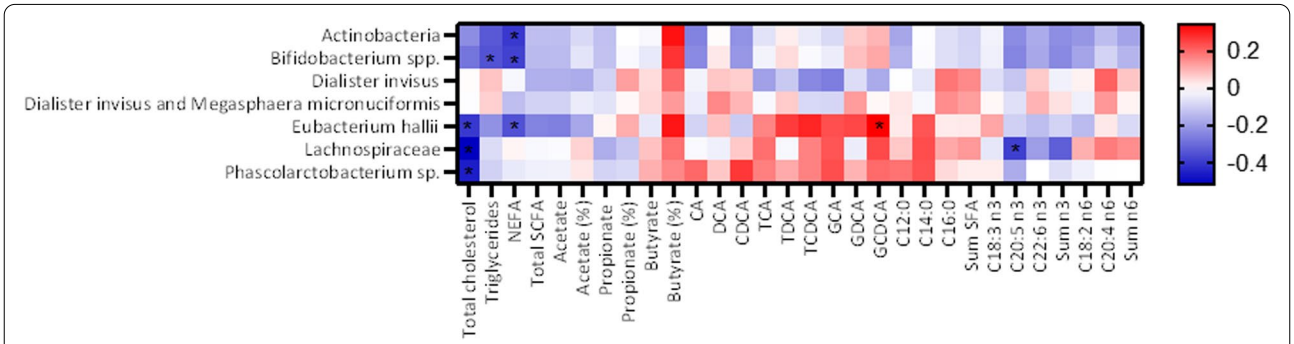

Fig. 4 Relationship between changes in gut bacteria and total cholesterol, triglycerides, NEFAs, SCFAs, bile acids, and fatty acid profiles after intervention with SFAs and PUFAs. Correlation analysis was performed with Pearson correlation. * indicates $P \leq 0.05$. Abbreviations: C12:0: lauric acid, C14:0: myristic acid, C16:0: palmitic acid, C18:3 n3: alpha-linolenic acid, C20:5 n3: eicosapentaenoic acid (EPA), C22:6 n3: docosahexaenoic acid (DHA), C18:2 n6: linoleic acid, C20:4 n6: arachidonic acid, CA: cholic acid, CDCA: chenodeoxycholic acid, DCA: deoxycholic acid, GCA: glycocholic acid, GDCA: glycodeoxycholic acid, GCDCA: glycochenodeoxycholic acid, n3: omega-3, n6: omega-6, NEFAs: nonesterified fatty acids, PUFAs: polyunsaturated fatty acids, SCFAs: short-chain fatty acids, SFAs: short-chain fatty acids, TCA: taurocholic acid, TDCA: taurodeoxycholic acid, TCDCA: taurochenodeoxycholic acid

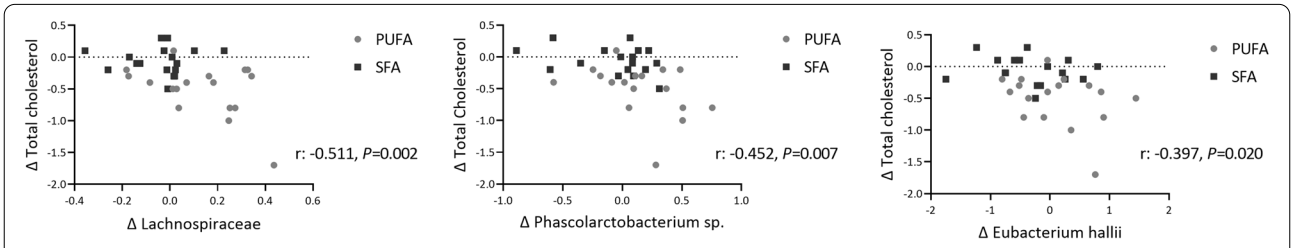

Fig. 5 Relationship between the change in abundance of bacteria and total cholesterol level after interventions with SFAs and PUFAs. Correlation analysis was performed with Pearson correlation, and * indicates $P \leq 0.05$. Abbreviations: PUFAs: polyunsaturated fatty acids, SFAs: saturated fatty acids

Discussion

In this study, the effect of different fat qualities (SFAs vs. PUFAs) on the human gut microbiota, SCFAs, and bile acids in healthy individuals was examined, in addition to the relationship between changes in gut bacteria and blood total cholesterol, triglycerides, NEFAs, bile acids, and fatty acid profiles. In a recent study, Gaundal et al. showed that replacing the intake of SFAs with PUFAs reduced total cholesterol levels by 8% after only three days in healthy individuals [1]. Accompanied by the reduced total cholesterol level, the present study demonstrates a shift in specific gut bacteria after replacing intake of SFAs with PUFAs for three days and identified specific bacteria associated with cholesterol levels in healthy individuals.

The abundance of *Lachnospiraceae* and *Bifidobacterium* spp. increased after three days following intake of PUFAs compared to SFAs, and the change in *Lachnospiraceae* was inversely associated with the change in total cholesterol levels. This is in line with a recent study showing that intake of PUFAs increased the abundance of *Lachnospira* and *Bifidobacterium* species in healthy individuals [34]. Findings from an animal study also showed similar results, where *Bifidobacterium* was negatively correlated with total cholesterol levels in hamsters [35]. Furthermore, oral administration of *Bifidobacterium* strains has been shown to elicit beneficial health effects, including lowering of total cholesterol levels both in mice [36] and humans [37, 38] and increasing the abundance of *Lachnospiraceae* [37]. Tindall and colleagues recently showed that members of the *Lachnospiraceae* family were inversely related to CVD risk factors, including total cholesterol, following a 6-week diet containing walnuts rich in PUFAs [39]. Consistent with these findings, the abundance of *Lachnospiraceae* was inversely associated with LDL cholesterol in patients with hyperlipidemia treated with rosuvastatin, a third-generation statin drug [40]. After dividing the patients into two groups based on their efficacy for rosuvastatin, those with high efficacy displayed a higher relative abundance of *Lachnospiraceae* compared to those with lower efficacy [40]. Taken together, the present results and findings from intervention trials might indicate that *Lachnospiraceae* and *Bifidobacterium* may act as cholesterol reducing agents.

Bile acids are formed from cholesterol in the liver, and increased synthesis and excretion of bile acids may affect cholesterol levels. This is the mechanism for the cholesterol reducing effect of bile acid binding resins. Even though a positive correlation between three bacteria and several bile acids was observed in this study, there

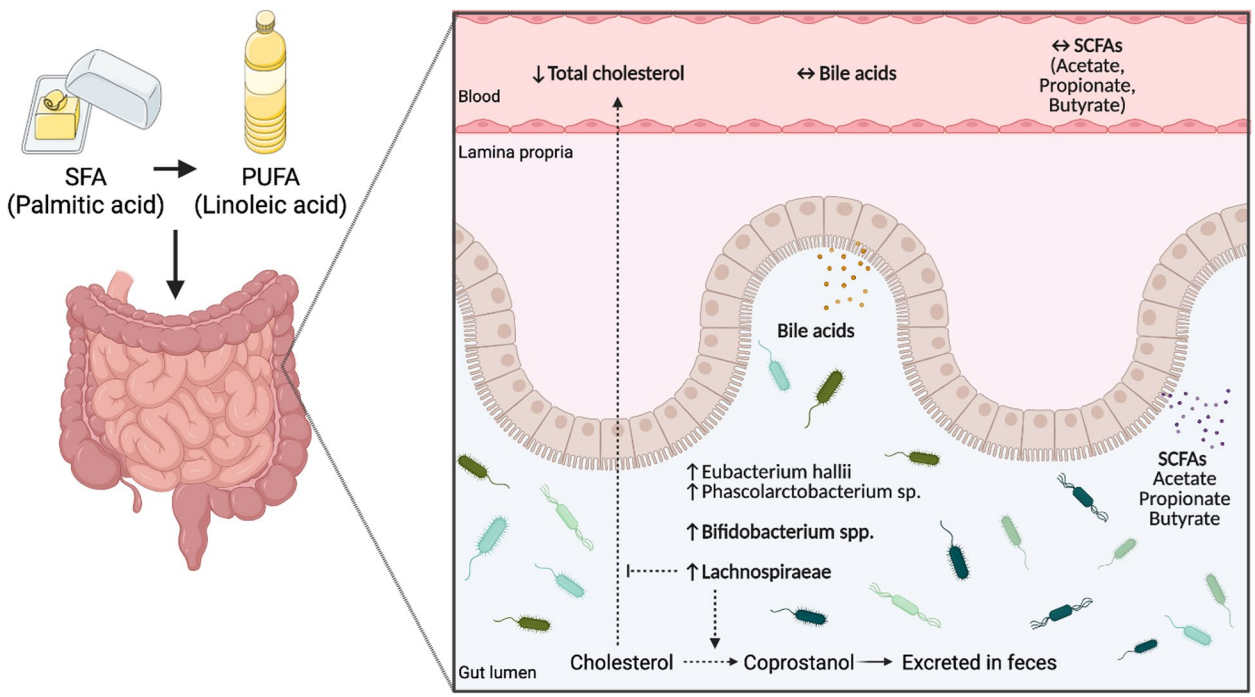

Fig. 6 A graphic illustration of the hypothetical mechanisms related to fat quality, gut microbiota, and circulating cholesterol as outlined in the discussion. Replacing SFAs with PUFAs for three days did not affect the levels of bile acids and SCFAs in the circulation. However, gut bacteria may convert cholesterol in the intestine to coprostanol, which is secreted in feces, and hence the circulating levels of cholesterol will decrease. The figure was created with BioRender.com. Abbreviations: PUFAs: polyunsaturated fatty acids, SCFAs: short-chain fatty acids, SFAs: saturated fatty acids

was no significant effect on bile acids between interventions; hence, the cholesterol-lowering effect previously observed [1] may not be explained by changes in bile acids. Another explanation for the reduced cholesterol levels might be related to the bacterial conversion of cholesterol to coprostanol, a nonabsorbable sterol [41] (Fig. 6). Approximately 1 g of cholesterol originating from diet and bile enters the colon each day and interferes with colonic bacteria that reduce cholesterol to coprostanol [23]. Coprostanol is poorly absorbed by human intestinal cells and is subsequently excreted via feces [42]. Even though intestinal cholesterol conversion was discovered more than a century ago [41], only limited knowledge about cholesterol metabolism by the gut microbiota exists. In an early study, Wilson found that rats fed palmitic acid (C16:0) or oleic acid (C18:0) had decreased coprostanol formation, while rats fed n6 linoleic acid (C18:2n6) had increased formation of coprostanol from cholesterol [43]. This is in line with the present study, where the exchange of fatty acids was mainly from the SFA palmitic acid to linoleic acid [1]. Linoleic acid is well recognized to decrease cholesterol levels [2, 44, 45], but whether this effect might partially be caused by gut microbiota is still to be elucidated. Other studies also suggest that gut microbes play an important role in regulating serum cholesterol levels. In one study, they showed an inverse relationship between serum cholesterol levels and coprostanol and cholesterol levels in feces [42], and in a recent study by Kenny et al., they identified microbial cholesterol dehydrogenase enzymes and found an association between cholesterol-metabolizing bacteria and reduced serum cholesterol levels [46]. In the present study, an inverse relationship between *Lachnospiraceae*, *Eubacterium hallii* and *Phascolarctobacterium* sp. and total cholesterol levels are demonstrated after replacing intake of SFAs with PUFAs for three days. Interestingly, conversion of cholesterol to coprostanol has previously been shown after only three days [47]; however, the rate of this conversion in humans seems to be highly variable [23, 48]. In line with the present results, most of the cholesterol-reducing bacteria involve members of *Eubacterium*, but strains of *Bifidobacterium* and bacteria belonging to the *Lachnospiraceae* family are also reported to reduce cholesterol to coprostanol [23, 49, 50]. These findings suggest that specific gut bacteria may influence cholesterol metabolism via conversion of cholesterol to coprostanol, but whether this is the case in this study is not known. Therefore, more studies are needed to further elucidate the gut bacterial mechanisms involved in cholesterol regulation.

The current findings indicate that fat quality elicits different microbial changes, irrespective of the total fat amount. This is in line with a study by Caesar and colleagues, who showed that mice fed lard and fish oil had different effects on the gut microbiota composition and diversity [21]. Interestingly, the same group reported an interaction between dietary lipids and the gut microbiota in the regulation of hepatic cholesterol metabolism [51]. They showed that in mice fed lard, but not fish oil, the gut microbiota increased hepatic levels of cholesterol and cholesteryl esters. Furthermore, a recent systematic review of randomized controlled trials and observational studies showed that a high fat intake, and in particular a high intake of SFAs, had unfavorable effects on human gut microbiota richness and diversity [52]. In the present study, the fatty acid composition of the study products differed mainly in exchanging palmitic acid with linoleic acid, while n3 fatty acids did not differ between the interventions [1]. There were no correlations between gut bacteria and n6 fatty acids, but there was a negative correlation between EPA and *Lachnospiraceae*. Selmin et al. found a reduction in *Lachnospiraceae* after a n6 high-fat diet in mice [30]. Others have shown a positive correlation between circulating levels of docosahexaenoic acid (DHA) (C22:6n3) and *Lachnospiraceae* [29] and an increase in several butyrate-producing bacteria in the *Lachnospiraceae* family after intervention with a n3 fatty acid-rich diet in adult males [27]. The *Lachnospiraceae* family consists of several butyrate-producing bacteria [53]. Even though a significant increase in circulating relative butyrate levels within the PUFA intervention was observed in this study, there were no differences in total SCFAs or butyrate between the interventions or a correlation with *Lachnospiraceae.* SCFAs are well-known fermentation products from fiber and have been shown to have positive health effects [8]. However, the present study does not indicate that fat and fat quality affect SCFA fermentation.

Taken together, the effect of total fat intake and fat quality on gut microbiota and *Lachnospiraceae* is not clear. Nevertheless, fat quality may be a promising factor affecting host metabolism by altering the composition and function of the gut microbiota, but robust clinical studies are warranted to investigate this further.

Study strengths and limitations

The randomized controlled crossover design strengthens the findings in the present work, as it corrects for any biological and interindividual differences between participants. This is important when investigating gut microbiota that are known to have large interindividual variation. However, there are some limitations to the study. First, due to the explorative nature of the study, we did not correct for multiple testing. Second, targeted analysis of the gut microbiota, including a panel of 48 bacteria, was performed (in which 40 bacteria were included in statistical

analyses), and thus, from this study, it is not known whether other bacteria not included in this panel may impact cholesterol metabolism. The most common bacteria found in the human gut were measured, and specific bacteria associated with cholesterol levels were identified, in line with other studies. However, the *Lachnospiraceae* family consists of several bacterial species, and there is a need to further investigate which of the bacteria in this family may impact cholesterol levels. Third, even though diet has been shown to affect gut microbiota within one to three days, the short-term duration of the present study (three days per intervention) does not reflect the long-term effects. Fourth, the participants in the present study were normal weight, healthy adults (median age of 28 years); thus, the results cannot be generalized to the population as a whole.

Conclusion and perspectives

In this study, the abundance of the gut bacterial families *Lachnospiraceae* and *Bifidobacterium* spp. was increased after replacing SFAs with PUFAs for three days in healthy individuals. Furthermore, the increase in *Lachnospiraceae* was associated with reduced total cholesterol levels. Whether the reduction in cholesterol levels is linked to linoleic acid-driven coprostanol formation by gut microbiota needs to be elucidated. Future robust studies are needed to investigate whether fat quality may be a promising factor affecting circulating cholesterol levels in the host by altering the composition and function of the gut microbiota. Dietary manipulation of the gut microbiota might be important for lipid-lowering prevention and treatment in the future.

Abbreviations

A: Actinobacteria; B: Bacteroidetes; CA: Cholic acid; CDCA: Chenodeoxycholic acid; CVD: Cardiovascular diseases; DCA: Deoxycholic acid; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; F: Firmicutes; FFM: Fat free mass; GCA : Glycocholic acid; GDCA: Glycodeoxycholic acid; GCDCA: Glycochenodeoxycholic acid; HbA1c: Hemoglobin A1c; hsCRP: High sensitivity C reactive protein; n: Number; n3: Omega 3; n6: Omega 6; NEFA: Nonesterified fatty acid; ns: Nonsignificant; P: Proteobacteria; PUFA: Polyunsaturated fatty acid; RCT: Randomized controlled trial; SCFA: Short-chain fatty acid; SFA: Saturated fatty acid; T: Tenericutes; TCA: Taurocholic acid; TDCA: Taurodeoxycholic acid; TCDCA: Taurochenodeoxycholic acid.

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Authors' contributions

VHTH, LG, KBH, SMU and MCWM formulated the research questions and designed the study; VHTH, LG and MCWM conducted the study; MGB provided study products; NB conducted the bile acid analyses; VHTH and LG performed statistical analyses and prepared the figures; VHTH, LG, IR, NB, MGB, TG, KR, KBH, SMU and MCWM interpreted the data; VHTH and LG drafted the article; VHTH, LG and MCWM had primary responsibility for the final content; all authors read and approved the final version of the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Regional Committees for Medical and Health Research Ethics (2018/104). Written informed consent was obtained from all subjects. The study was registered at Clinical Trials (https://clinicaltrials.gov/, registration identification number: NCT03658681).

Consent for publication

Not applicable.

Competing interests

LG, NB, TG and IR report nothing to declare. VHTH has been employed at Mills AS and reports grants from Mills AS. She does not own any stocks in the company, and the work performed in this paper was done after she left the company. MGB is employed at Mills AS. She does not own any stocks in the company. She did not have a role in the design of the study and analysis of the data and was not involved in the statistical analysis. KR reports personal fees from MedXplore, Amgen, Mills AS, The Norwegian Medical Association, The Norwegian Directorate of Health, Sanofi, Takeda, Chiesi, Bayer and MSD, outside the submitted work. KBH reports grants from Tine SA, Mills AS, Olympic Seafood, Amgen, Sanofi, Kaneka and personal fees from Amgen, Sanofi, Pronova, outside the submitted work. SMU has received research grants from Tine DA, Mills AS, and Olympic Seafood, none of which are related to the content of this manuscript. MCWM is involved in projects with research grants from Tine SA and Olympic Seafood and has received research funds from Mills AS, none of which are related to the content of this manuscript.

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ORIGINAL ARTICLE

Gut microbiota is associated with dietary intake and metabolic markers in healthy individuals

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Popular scientific summary

- Identifying gut bacteria associated with both diet and metabolic risk markers may be a potential strategy in dietary disease prevention.
- We explored the relationship between a panel of gut bacteria, diet, and metabolic and anthropometric markers in healthy adults.
- *Bacteroides stercoris* was associated with higher intake of healthy foods and lower diastolic blood pressure.
- Further studies are needed to address whether these findings are causally linked and whether targeting these bacteria can prevent metabolic diseases.

Abstract

***Background*:** Metabolic diseases have been related to gut microbiota, and new knowledge indicates that diet impacts host metabolism through the gut microbiota. Identifying specific gut bacteria associated with both diet and metabolic risk markers may be a potential strategy for future dietary disease prevention. However, studies investigating the association between the gut microbiota, diet, and metabolic markers in healthy individuals are scarce.

***Objective*:** We explored the relationship between a panel of gut bacteria, dietary intake, and metabolic and anthropometric markers in healthy adults.

***Design*:** Forty-nine volunteers were included in this cross-sectional study. Measures of glucose, serum triglyceride, total cholesterol, hemoglobin A1c (HbA1c), blood pressure (BP), and body mass index (BMI) were collected after an overnight fast, in addition to fecal samples for gut microbiota analyzes using a targeted approach with a panel of 48 bacterial DNA probes and assessment of dietary intake by a Food Frequency Questionnaire (FFQ). Correlations between gut bacteria, dietary intake, and metabolic and anthropometric markers were assessed by Pearson's correlation. Gut bacteria varying according to dietary intake and metabolic markers were assessed by a linear regression model and adjusted for age, sex, and BMI.

***Results*:** Of the 48 gut bacteria measured, 24 and 16 bacteria correlated significantly with dietary intake and metabolic and/or anthropometric markers, respectively. Gut bacteria including *Alistipes*, *Lactobacillus* spp., and *Bacteroides stercoris* differed according to the intake of the food components, fiber, sodium, saturated fatty acids, and dietary indices, and metabolic markers (BP and total cholesterol) after adjustments. Notably, *Bacteroides stercoris* correlated positively with the intake of fiber, grain products, and vegetables, and higher *Bacteroides stercoris* abundance was associated with higher adherence to Healthy Nordic Food Index (HNFI) and lower diastolic BP after adjustment.

Conclusion: Our findings highlight the relationship between the gut microbiota, diet, and metabolic markers in healthy individuals. Further investigations are needed to address whether these findings are causally linked and whether targeting these gut bacteria can prevent metabolic diseases.

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Keywords: *gut microbiota*; *metabolic markers*; *diet*; *healthy*; *humans*; *dietary fiber*; *vegetables*; *dietary fat*; *blood pressure*; *cholesterol*

To access the supplementary material, please visit the article landing page

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New knowledge has pointed out gut microbiota as a mediator of dietary impact on host metabolism (1). The gut microbiota possesses important functions for the host, such as fermentation of dietary fibers and extraction of nutrients, synthesis of certain vitamins, improvement of gut integrity and the protection against pathogens, and the regulation of host immune system and host signaling pathways (2). It can therefore be regarded as a metabolically active organ complementing the host metabolism through its traits (3, 4). In healthy individuals, the gut microbiota comprises a wide range of bacteria belonging to the two major phyla: Firmicutes and Bacteroidetes (5–7). Changes in the gut microbiota composition may disrupt normal functions, and a wide spectrum of diseases, including obesity, type 2 diabetes (T2D), and cardiovascular diseases (CVDs) are associated with gut microbiota dysbiosis (4, 5, 8, 9). Dysbiosis is often referred to as general changes in the gut microbiota composition (10). For example, individuals with lower microbiota gene content are characterized by higher insulin resistance, adiposity, and dyslipidemia than those with higher bacterial richness (11). Furthermore, a promising role of the gut microbiota affecting human metabolism has been shown after microbiota transplant, in which fecal transplantation from lean donors to recipients with metabolic syndrome showed increased gut microbiota diversity and butyrate-producing bacteria, together with improvements in insulin sensitivity (12). The gut microbiota can therefore be an attractive future target for the prevention of metabolic diseases (4).

Both genetic and environmental factors shape the gut microbiota, in which the latter seem to dominate (13–15). Diet is one of the most important factors influencing the gut microbiota composition and function (16–18). The supply of dietary compounds such as non-digestible carbohydrates, protein, and fat strongly impacts the formation of microbiota-derived metabolites (19). A large body of evidence suggest that the beneficial effect of dietary fiber is mediated by the microbial formation of short chain fatty acids (SCFAs), mainly acetate, propionate, and butyrate (20). SCFAs have been shown to regulate metabolic processes, including glucose and lipid metabolism, and are implicated in the regulation of blood pressure (BP) (4, 9, 18, 19, 21). Furthermore, the capability of dietary fats to alter host metabolism through the gut microbiota composition has also been shown (22, 23). Diet-derived free fatty acids may have antimicrobial effects or be used as substrates for the formation of microbiota-derived metabolites, which are involved in inflammatory processes and the regulation of glucose and lipid homeostasis (22, 23). The type of fat also seems to have distinct effects on host metabolism through the gut microbiota. Studies in mice show that dietary fat quality affects inflammation and insulin sensitivity through effects via the gut microbiota composition (24, 25).

Even though studies have shown associations between diet and gut microbiota (26–31), studies investigating metabolic markers associated with both gut microbiota and diet in healthy adults are scarce (32–35). Identifying specific gut bacteria associated with both diet and metabolic markers may be valuable and could represent a potential strategy for the prevention of disease. The aim of this study was therefore to explore the relationship between a panel of gut bacteria, dietary intake, and metabolic and anthropometric markers in a cross-sectional study in healthy individuals.

Materials and methods

Subjects and study design

Healthy participants originally recruited to a randomized controlled trial (RCT) (36) were included in this exploratory cross-sectional study. The participants who met to a screening visit prior to the randomized trial performed at Oslo Metropolitan University (OsloMet) were included in this study. Healthy volunteers (aged 18–65 years) with body mass index (BMI) between 18.5 and 27.0 kg/m^2 were recruited from advertisement and from the student mass and employees at OsloMet between April 2018 and January 2019. Seventy-two volunteers were assessed for eligibility. The exclusion criteria were fasting blood glucose values ≥6.1 mmol/L, any food allergies, intolerances, chronic metabolic diseases (e.g. diabetes, CVD, and cancer), or intestinal diseases, including inflammatory bowel disease (IBD), celiac disease, and irritable bowel syndrom (IBS). Those treated with antibiotics the previous 3 months, who were blood donors the previous 2 months, who were pregnant or lactating, who had ≥5% weight change the previous 3 months, who used any hormonal treatment (except from oral contraception) and tobacco,

or who had a high alcohol consume (>40 g/day) were excluded. All participants signed a written informed consent form prior to participation. The study was approved by the Regional Committees for Medical and Health Research Ethics (2018/104) and conducted according to the Declaration of Helsinki guidelines. The study was registered in clinicaltrials.gov (NCT03658681).

Blood sampling and clinical assessment

Blood sampling and clinical assessment were performed after an overnight fast (≥12 h). Fasting glucose was measured in finger prick capillary blood samples using a HemoCue Glucose 201 Analyser and Micro cuvettes (HemoCue, USA). Fasting triglyceride and total cholesterol were measured in serum and sent to a routine laboratory (Fürst Medical Laboratory) within 24 h. EDTA (Ethylene-Diamine-Tetra-Acetic acid) tubes with whole blood were kept in room temperature for ≤24 h before analyzation of hemoglobin A1c (HbA1c) (Fürst Medical Laboratory). BP was measured twice with an automated BP monitor (ri-champion©, Riester, Germany) after sitting still for at least 15 min. Participants were asked to relax during the measurements. The mean value from both measurements was used in the statistical analyses. Body weight and composition were measured after an overnight fast (≥12 h) using Bioimpedance analyzers (BC-418 Segmental Body Composition Analyser and InBody 720). One kg was subtracted compensating for clothing. Height was measured using a wall-mounted stadiometer (Acumed).

Habitual dietary intake and dietary patterns

Forty-eight of the 49 participants included in this study completed a validated food frequency questionnaire (FFQ), reporting their habitual dietary intake the previous 12 months (37). The FFQ consisted of 270 food items and included questions about frequency of intake (from several times a day to never) and portions size based on household units (slices, glasses, cups, pieces, spoons and teaspoons) (37). Food groups included in the FFQ consisted of categories such as grain products; bread and vegetables; fruits and berries; nuts, olives, and seeds; meat, blood, and offal; fish and shellfish; cheese; and butter, margarine, and oil. Information about the consumption of these food groups and intake of specific foods within these categories (for example, intake of apples within the food group fruits and berries) was used to calculate adherence to dietary indices and to assess correlation with the gut microbiota and metabolic and anthropometric markers.

The dietary indices, the Healthy Nordic Food Index (HNFI) and the Healthy Diet Score (HDS), were used to assess correlation between adherence to the indices and the gut microbiota. The HNFI includes six food groups (fish, cabbage, apple and pears, root vegetables, rye bread, and oatmeal) (38) (Supplementary Table 1). Rye bread was replaced with whole grain bread because information on rye bread specifically was not included in the FFQ, similar to previous studies (39, 40). Thus, in this study, the six food groups included in the modified HNFI were fish (cod, pollack, salmon, trout, herring, mackerel, and shellfish), cabbage (cabbage, cauliflower, broccoli, Chinese cabbage, and Brussels sprouts), apple and pears, root vegetables (carrot and rutabaga), oatmeal, and whole grain bread (>50% whole meal flour). One point was given for intake equal to or above the sex-specific median intake for each food group, and zero points were given for intake below the median. The maximum total score was six points. The total score was summarized for each participant, and a high score (4–6 points) indicates high adherence to the HNFI (38–40).

The HDS reflects adherence to the Norwegian dietary recommendations (41). In this study, the food groups included in the HDS were whole grain, vegetables, fruits and berries, low fat milk, fish, beans and lentils, vegetable oils (monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs)), red/processed meats, salt, sugar, and saturated fatty acids (SFAs). Each participant was given scores (0, 5, and 10 points) for the intake of food groups according to the Norwegian dietary recommendation and summed into an HDS (Supplementary Table 2). A high score (65–120 points) indicates high adherence to the recommendation, with a maximum score of 120 points.

Findings from a population-based study in Norway show that intakes of dietary fiber, SFA, salt, and sodium are not according to the recommendations, where the intake of fiber is less than recommended and intakes of SFA, sodium, and salt are higher than recommended (42). We therefore assessed whether the intake of these dietary components according to the recommendations was associated with the gut microbiota. Participants were divided into groups based on the average recommendations of fiber set by NNR (Nordic Nutrition Recommendations) (43) (</≥30 g/day, n = 19/29). In addition, participants were divided into groups based on recommended intake of SFA (≤/>10 E%/day, n = 9/39), salt (≤/>5.0 g/day, n = 13/35), and sodium (≤/> 2.3 g/day, n = 23/25). Participants were also divided into groups based on high or low adherence to the indices HNFI (0–3/4–6 points, n = 30/18) and HDS (0–60/65–120 points, n = 10/38).

Fecal collection and gut microbiota analyses

The participants were provided with a fecal sample collection kit for home use (GA-map™ Dysbiosis Test, Genetic Analysis AS, Oslo, Norway). Participants were instructed to sample the stool from three different places and place it in the included tubes. Samples were kept in room temperature for maximum 3 days according to the manufacturers protocol. The GA-map™ Dysbiosis Test

has shown stability in room temperature up to 5 days (44). All samples were stored at −80°C at OsloMet before they were collectively sent to Genetic Analysis AS (GA) for microbiota analyses after this study was completed.

The GA-map™ Dysbiosis Test is a commercially available genome-based test using fecal samples for analyses of gut bacteria associated with dysbiosis, described in detail elsewhere (44). In brief, the test was developed to identify and characterize bacterial groups that were able to distinguish patients with IBS and IBD from healthy controls. The test comprises 48 DNA probes targeting ≥300 bacteria on different taxonomic levels. Probes targeting seven variable regions (V3–V9) of the 16S rRNA gene were used to characterize and identify bacteria present, thus allowing mapping of the intestinal microbiota profile for a selected set of bacteria. Human fecal sample homogenization, and mechanical and enzymatic bacterial cell disruption were utilized to isolate and bind total bacterial genomic DNA to magnetic beads. The hypervariable regions V3–V9 of the 16S rRNA were further amplified by polymerase chain reaction. Single nucleotide extension and hybridization to a complementary DNA strand coupled to beads determined bacterial DNA labeling. To assess the abundance of bacteria, the strength of fluorescent signal (probe intensity) was detected and measured by Luminex 200 (Luminex Corporation). Probes are listed in Supplementary Table 3.

Statistical analysis

The current cross-sectional study is part of a randomized controlled dietary crossover study, with the primary aim to investigate the effect of fat quality on glycemic regulation, as described previously (36). Thus, this study is an exploratory study investigating the relationship between a panel of gut bacteria, dietary intake, and metabolic and anthropometric markers in healthy individuals. Gut bacteria were log2-transformed before analysis. Correlations between gut bacteria, dietary variables, and metabolic and anthropometric markers were assessed with Pearson's correlation. Only bacteria showing statistically significant correlations with correlation coefficients ≥0.3 with either dietary intake or anthropometric and/or metabolic markers are presented. Differences in daily intake of dietary nutrients and dietary index score between males and females were assessed by independent sample *t*-test. Participants were divided into groups based on whether the intake was in accordance with the dietary recommendations or not. Differences between log2-transformed gut bacteria based on whether the dietary intake (fiber, sodium, salt, and SFA [E%], and adherence to HNFI and HDS) and metabolic markers (systolic and diastolic BPs and total cholesterol) were below or above the recommended level were thereafter assessed by a linear regression model, hereafter called unadjusted model. Further adjustments for age, sex, and BMI were performed in adjusted models. $P < 0.05$ was regarded as statistically significant. Statistical analyses were performed using IBM SPSS statistic (version 25), and figures were designed using GraphPad Prism 8 for Windows (version 8.0.0.).

Results

Seventy-two volunteers were assessed for eligibility, and 49 participants (12 males and 37 females) were included in this cross-sectional study. The mean age was 35.6 years (standard deviation [SD] 13.1 years), and measures of BMI, HbA1c, fasting glucose, triglyceride, total cholesterol, and diastolic and systolic BP were within the normal range (Table 1).

Data on dietary intake were collected by an FFQ reflecting dietary intake the past 12 months (Table 2). Mean daily intake of protein, total fat, MUFA, PUFA, and carbohydrates was 16.0 E%, 36.8 E%, 14.4 E%, 6.7 E%, and 41.4 E%, respectively. The intake of protein, total fat, MUFA, PUFA, and carbohydrates was within the recommended levels, whereas the intake of SFA was higher than recommended (12.5 E%) (45). The intake of fiber, 41.7 and 39.7 g/day for males and females, respectively, was higher than the recommended minimum daily intake, that is, 35 and 25 g/day, respectively (Table 2). Significant gender differences between dietary intake were found only for the intake of starch in g ($P = 0.005$) and E% ($P = 0.014$). For the HNFI and HDS indices, 18 (37.5%) and 38 (79.2%) participants had high adherence to the indices, respectively.

The abundance of the 48 gut bacteria probes included in this study represents bacteria belonging to the phyla Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, Tenericutes, and Verrucomicrobia (Supplementary Table 3). Twenty-four bacteria significantly correlated (correlation coefficient ≥ 0.3) to one or more dietary

Table 1. Characteristics of the participants

Anthropometric and biochemical variables		Mean	SD
Female, *n* (%)	37 (75.5)		
Age (years)		35.6	13.1
BMI (kg/m^2)		22.8	2.2
HbA1c (%)		5.2	0.3
HbA1c (mmol/mol)		33.9	3.4
Glucose (mmol/L)		5.1	0.4
Triglyceride (mmol/L)		0.9	0.4
Total cholesterol (mmol/L)		4.8	0.9
Systolic blood pressure (mmHg)[1]		122.4	15.3
Diastolic blood pressure (mmHg)[1]		71.7	10.7

Variables are measured fasted.
[1]Expressed as mean values based on two measurements.

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Table 2. Daily intake of nutrients and dietary index score assessed by FFQ

Dietary variables	Total (n = 48)		Male (n = 12)		Female (n = 36)	
	Mean g or score[1] (SD)	Mean E% (SD)	Mean g or score[1] (SD)	Mean E% (SD)	Mean g or score[1] (SD)	Mean E% (SD)
kJ	11301.6 (5576.6)		11964.0 (4364.9)		11080.9 (5964.2)	
kcal	2701.2 (1332.8)		2859.5 (1043.2)		2648.4 (1425.5)	
Protein	104.3 (41.7)	16.0 (2.5)	108.7 (36.7)	15.5 (2.3)	102.8 (43.7)	16.2 (2.6)
Alcohol	10.6 (10.0)	2.8 (2.5)	13.2 (12.7)	3.1 (6.1)	9.7 (9.0)	2.8 (2.5)
Total fat	112.6 (67.8)	36.8 (7.3)	113.1 (48.7)	35.3 (2.7)	112.5 (73.7)	37.3 (7.7)
SFA	37.8 (22.7)	12.5 (3.4)	39.9 (24.8)	12.0 (3.8)	37.2 (22.2)	12.6 (3.4)
MUFA	44.4 (29.1)	14.4 (3.3)	42.7 (17.1)	13.5 (2.9)	45.0 (32.3)	14.8 (3.4)
PUFA	20.8 (15.1)	6.7 (2.0)	20.5 (7.6)	6.7 (2.0)	20.9 (16.9)	6.7 (2.0)
Trans-fat	1.0 (0.7)	0.3 (0.2)	1.2 (1.1)	0.4 (0.2)	0.9 (0.6)	0.3 (0.1)
Cholesterol (mg)	310.3 (142.2)		317.3 (174.2)		307.9 (132.9)	
Carbohydrates	278.4 (147.5)	41.4 (7.2)	306.8 (114.1)	43.1 (5.4)	268.9 (157.3)	40.8 (7.7)
Fiber	40.2 (25.8)	2.9 (0.6)	41.7 (18.4)	2.9 (0.7)	39.7 (28.0)	2.9 (0.6)
Starch*	133.0 (61.3)	20.3 (5.3)	170.2 (70.8)	23.9 (5.3)	120.6 (53.3)	19.0 (4.8)
Mono- and disaccharides	126.8 (92.0)	18.3 (6.1)	119.9 (51.6)	16.8 (5.0)	129.1 (102.5)	18.9 (6.4)
Sugar	37.0 (34.1)	5.5 (3.2)	35.1 (22.2)	5.1 (2.7)	37.7 (37.5)	5.6 (3.3)
Salt	6.8 (2.8)		7.0 (3.0)		6.7 (2.8)	
HNFI	3.1 (1.3)		3.2 (1.2)		3.0 (1.3)	
HDS	73.8 (13.9)		70.0 (13.1)		75.0 (14.0)	

[1]Mean score of the adapted version of Healthy Nordic Food Index (HNFI) and Healthy Diet Score (HDS). n = 48.
*Significant difference in dietary intakes between males and females, assessed by independent sample t-test, $P < 0.05$.

variables as assessed with Pearson's correlation. These bacteria and their correlations to dietary variables are illustrated in Fig. 1. Eleven bacteria were related to one or more macronutrients, 12 bacteria were related to one or more micronutrients, and 16 bacteria were related to one or more food groups or dietary indices (Fig. 1).

As shown in Fig. 1, the bacteria correlating with the most dietary variables were *Bacteroides stercoris*, *Clostridia*, and *Streptococcus* spp., showing correlations with 12, 10, and eight dietary variables, respectively. The strongest positive correlation was between *Streptococcus* spp. and the intake of bread with <50% whole flour (r: 0.655, $P < 0.001$), whereas the strongest negative correlation was between *Bacteroides* spp. & *Prevotella* spp. and the intake of sugar (E%) (r: −0.488, $P < 0.001$). The intake of fiber and fiber-rich foods such as grain products and vegetables, and micronutrients commonly found in these food groups (tocopherol, thiamine, niacin, vitamin B6, folate, and phosphorus) correlated positively with *Bacteroides stercoris* (Fig. 1).

Correlations between gut bacteria and metabolic and anthropometric markers were thereafter investigated. Sixteen gut bacteria showed a statistically significant correlation with metabolic and/or anthropometric markers (correlation coefficients ≥ 0.3), as outlined in Fig. 2. Of these, two bacteria from the Bacteroidetes phylum (*Alistipes onderdonkii* and *Parabacteroides* spp.) and nine bacteria from the Firmicutes phylum (*Lachnospiraceae*, *Bacilli*, *Ruminococcus albus* & *R. bromii*, *Lactobacillus* spp., *Eubacterium biforme*, *Eubacterium rectale*, *Streptococcus salivarius* spp. *thermophilus* & *S. sanguinis*, and *Dialister invisus* & *Megasphaera micronuciformis*) correlated significantly to metabolic and/or anthropometric markers. In addition, Actinobacteria and *Bifidobacterium* spp. belonging to the Actinobacteria phylum, and *Akkermansia muciniphila* and *Enterobacteriaceae* from the phyla Verrucomicrobia and Proteobacteria, respectively, correlated to metabolic and/or anthropometric markers. The strongest positive correlation was between *Alistipes onderdonkii* and body fat (%) (r: 0.428, $P = 0.002$), and the strongest negative correlation was between *Lactobacillus* spp. and diastolic BP (r: −0.411, $P = 0.003$). Furthermore, BP correlated with several gut bacteria showing a positive correlation with Actinobacteria and *Bifidobacterium* spp. and a negative correlation with *Parabacteroides* spp., *Bacilli, Eubacterium biforme*, and *Lactobacillus* spp. The abundance of *Lachnospiraceae*, *Bacilli*, *Alistipes onderdonkii*, *Dialister invisus*, and *Megasphaera micronuciformis* were significantly correlated to blood lipids (total cholesterol and/or triglyceride) (Fig. 2).

Based on the correlation analysis shown in Figs. 1 and 2, significant correlations between gut bacteria and both dietary variables and metabolic and anthropometric

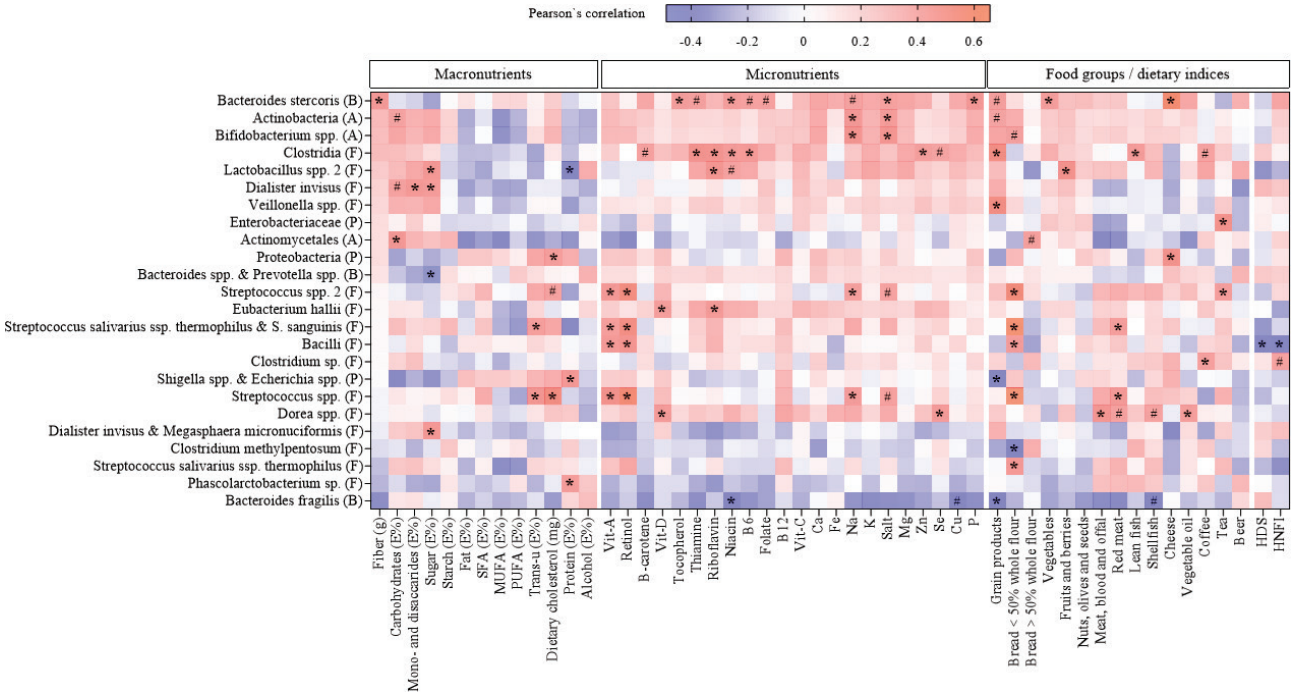

Fig. 1. Heat map of Pearson's coefficient between gut bacteria and dietary intake of macronutrients, micronutrients, and food groups. The bacterial taxa are sorted from negative (blue) to positive (red) correlations in relation to the intake of fiber (g) assessed by Pearson's correlation. Significant correlations are marked by * (correlation coefficient ≥ 0.3) or # (correlation coefficient < 0.3). Phylum is indicated within parentheses; A, Actinobacteria; B, Bacteroidetes; F, Firmicutes; P, Proteobacteria; Ca, calcium; Cu, copper; E%, percentage of total energy intake; Fe, iron; HDS, Healthy Diet Score; HNFI, Healthy Nordic Food Index; K, potassium; Mg, magnesium; MUFA, monounsaturated fatty acids; Na, sodium; P, phosphorus; PUFA, polyunsaturated fatty acids; Se, selenium; SFA, saturated fatty acids; Vit, vitamin; Zn, zinc.

markers are summarized and illustrated in a correlation map in Fig. 3. These gut bacteria are color coded according to their representative phyla: Actinobacteria, Firmicutes, Bacteroidetes, and Proteobacteria. The abundance of *Bacteroides stercoris* correlated positively with the intake of healthy food groups or components such as fiber, grain products, and vegetables, and negatively with diastolic BP. Furthermore, the abundance of *Bacilli* correlated positively with total cholesterol and the intake of bread with <50% whole flour, and negatively with systolic and diastolic BP and the indices HDS and HNFI. The abundance of *Alistipes onderdonkii* correlated positively with total cholesterol, age, and body fat (%), and negatively with adherence to HNFI (Fig. 3).

As shown in Fig. 3, *Bifidobacterium* spp. and *Streptococcus salivarius* spp. *thermophilus* showed only positive correlations with metabolic and/or anthropometric markers and dietary components (systolic BP, BMI, intake of bread with <50% whole flour, and micronutrients [Ca, Na, and salt]; and fasting glucose, intake of trans fat [E%], intake of bread with <50% whole flour, red meat, and micronutrients [vitamin A and retinol], respectively). *Bacteroides* spp. and *Prevotella* spp. showed only negative correlations with metabolic markers and dietary components (systolic and diastolic BP and intake of sugar [E%], respectively) (Fig. 3).

To investigate if the abundance of gut bacteria differed in relation to the intake of food components, especially linked to health effects, participants were divided into groups based on whether the intake was below or above the recommended intake. Participants were divided based on the average recommended intake of fiber, irrespective of gender (</≥30 g/day) (n = 19/29), and the recommended intake of sodium (≤/> 2.3 g/day) (n = 23/25), salt (≤/> 5.0 g/day) (n = 13/35), and SFA (≤/>10 E%/day) (n = 9/39), in addition to high or low adherence to HNFI and HDS indices (0–3/4–6 points (n = 30/18), and 0–60/65–120 points (n = 10/38), respectively). Gut bacteria showing a significant difference in abundance between groups in the linear regression analyses are shown in Table 3.

The intake of fiber (≥30 g/day) was significantly associated with lower abundance of *Alistipes*, whereas higher intake of sodium (>2.3 g/day) was significantly associated with higher abundance of *Bacteroides stercoris* after adjustment for age, sex, and BMI (Table 3). Furthermore, the intake of SFA ≥10 E%/day was significantly associated with increased abundance of *Ruminococcus gnavus* and *Phascolarctobacterium* sp., and lower abundance of *Actinomycetales* and *Mycoplasma hominis* after adjusting for age, sex, and BMI (Table 3). The indices HNFI and HDS were further examined. A high adherence to

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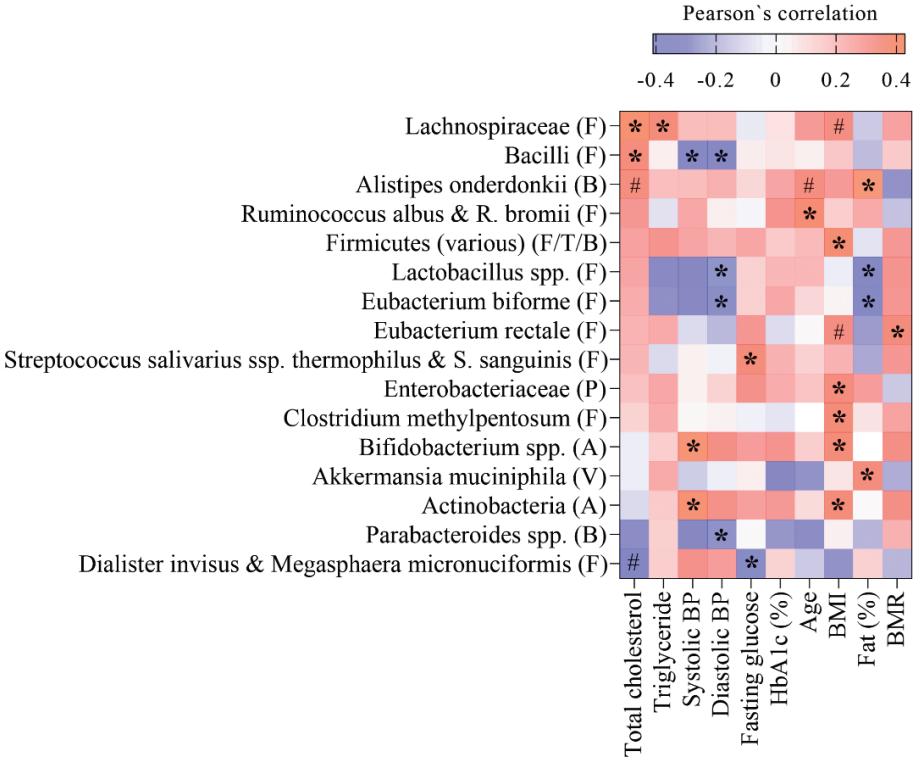

Fig. 2. Heat map of Parsons's coefficient between gut bacterial taxa and metabolic and anthropometric markers. The bacterial taxa are sorted from negative (blue) to positive (red) correlation toward total cholesterol levels, assessed by Pearson's correlation. Significant correlations are marked by * (correlation coefficient ≥ 0.3) or # (correlation coefficient < 0.3). Phylum is indicated within parentheses; A, Actinobacteria; B, Bacteroidetes; F, Firmicutes; P, Proteobacteria; T, Tenericutes; V, Verrucomicrobia; BMI, body mass index; BMR, basal metabolic rate; BP, blood pressure; HbA1c, hemoglobin A1c.

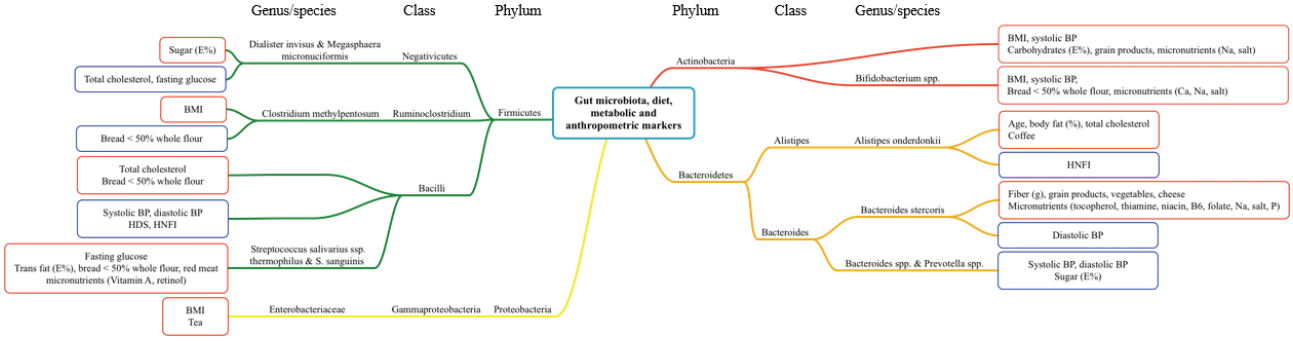

Fig. 3. Correlation map between gut bacteria, dietary intake, and metabolic and anthropometric markers. The gut bacteria are grouped according to representative phyla, indicated by different colors (red: Actinobacteria; orange: Bacteroidetes; green: Firmicutes; yellow: Proteobacteria). Correlation between the gut bacteria with one or more dietary variables, metabolic and/or anthropometric markers is illustrated by red boxes (positive correlation) or blue boxes (negative correlation). BMI, body mass index; BMR, basal metabolic rate; BP, blood pressure; E%, percentage of total energy intake; HDS, healthy diet score; HNFI, Healthy Nordic food index.

HNFI (≥4 points) was significantly associated with higher abundance of *Bacteroides stercoris* and lower abundance of *Bacilli*, *Lactobacillus* spp., *Eubacterium biforme*, and *Streptococcus salivarius* spp. *thermophilus*, while a higher adherence to the HDS (≥65 points) was associated with lower abundance of *Lactobacillus* spp. 2 after adjusting for age, sex, and BMI (Table 3).

Differences in the abundance of gut bacteria between participants stratified according to their systolic BP (</≥120 mmHg) (n = 25/24), diastolic BP (</≥80 mmHg) (n = 39/10), and total cholesterol levels (</≥5.0 mmol/L) (n = 29/20) were thereafter investigated. Gut bacteria showing a significant difference in abundance between groups in the linear regression analyses are shown in

Table 3. Gut bacteria associated with the intake of fiber, sodium, salt, SFA (E%), and adherence to HNFI and HDS indices[1]

Gut bacteria	Unadjusted values		Adjusted values			
	B	95% CI	*B*	95% CI	*P*[♯]	*P*[♯]
	Fiber intake (≥ 30 g/d) (*n* = 29)					
(B) *Alistipes*	−0.306	−1.697, −0.068	−0.301	−1.730, −0.008	**0.034**	**0.048**
	Sodium intake (> 2.3 g/d) (*n* = 25)					
(B) *Bacteroides fragilis*	−0.297	−2.672, −0.063	−0.250	−2.495, 0.198	**0.040**	0.093
(B) *Bacteroides stercoris*	0.367	0.325, 2.303	0.329	0.118, 2.241	**0.010**	**0.030**
	Salt intake (> 5.0 g/d) (*n* = 35)					
(P) *Proteobacteria*	0.314	0.087, 1.595	0.288	−0.044, 1.585	**0.030**	0.063
	SFA intake (≥ 10 E%/d) (*n* = 20)					
(A) *Actinomycetales*	−0.385	−1.928, −0.325	−0.404	−2.079, −0.283	**0.007**	**0.011**
(F) *Firmicutes*	−0.300	−0.766, −0.022	−0.319	−0.839, 0.001	**0.038**	**0.050**
(F) *Ruminococcus gnavus*	0.204	−0.264. 1.520	0.238	0.050, 1.966	0.163	**0.040**
(F) *Phascolarctobacterium* sp.	0.246	−0.202, 2.620	0.331	0.056, 3.187	0.091	**0.043**
(T) *Mycoplasma hominis*	−0.221	−2.175, 0.289	−0.327	−2.716, −0.076	0.130	**0.040**
	High adherence to HNFI (≥ 4 points) (*n* = 18)					
(B) *Bacteroides stercoris*	0.333	0.198, 2.266	0.317	0.114, 2.229	**0.021**	**0.031**
(F) *Bacilli*	−0.338	−1.056, −0.182	−0.420	−1.123, −0.219	**0.006**	**0.005**
(F) Lactobacillus spp.	−0.260	−2.481, 0.122	−0.332	−2.721, −0.291	**0.075**	**0.016**
(F) *Eubacterium biforme*	−0.276	−2.639, 0.041	−0.345	−2.905, −0.335	0.057	**0.015**
(F) *Streptococcus salivarius* spp. *thermophilus*	−0.294	−1.556, −0.029	−0.329	−1.673, −0.098	**0.042**	**0.028**
	High adherence to HDS (≥ 65 points) (*n* = 38)					
(F) *Lactobacillus* spp. 2	−0.383	−1.680, −0.278	−0.337	−1.618, −0.107	**0.007**	**0.026**

[1]Gut bacteria values were log2-transformed before analysis.
♯*P* for unadjusted values assessed by a linear regression model.
♯*P* for values adjusted for age, sex, and BMI, assessed by a linear regression model.
Phyla are indicated within parentheses; (A), Actinobacteria; (B), Bacteroidetes; (F), Firmicutes; (P), Proteobacteria; (T), Tenericutes
The level of significance was set at $P < 0.05$ and are indicated in bold italic.

Table 4. A higher systolic BP (≥120 mmHg) was associated with lower abundance of *Lactobacillus* spp. after adjusting for age, sex, and BMI (Table 4). A higher diastolic BP (≥80 mmHg) was associated with lower abundance of *Bacteroides stercoris*, *Bacteroides* spp., *Bacilli*, *Eubacterium biforme*, *Eubacterium rectale*, *Lactobacillus* spp., and *Streptococcus* spp. 2, and higher abundance of *Dialister invisus* and *Megasphaera micronuciformis*, after adjusting for age, sex, and BMI. A higher total cholesterol level (≥5.0 mmol/L) was associated with higher abundance of *Ruminococcus albus* and *Ruminococcus bromii*, but this association was no longer significant after adjusting for age, sex, and BMI (Table 4).

Discussion

In this study, we explored the relationship between a panel of gut bacteria commonly found in the human gut, dietary intake, and metabolic and anthropometric markers in healthy adults. Of the 48 gut bacteria analyzed in this study, 24 bacteria were shown to correlate with dietary intake and 16 bacteria correlated with metabolic and/or anthropometric markers. Several of the gut bacteria correlated with both dietary intake and metabolic and/or anthropometric markers. Furthermore, we show that specific gut bacteria differed in relation to the intake of food components such as fiber, sodium, SFA, and healthy food indices, and between participants stratified according to BP and total cholesterol.

The abundance of *Bacteroides stercoris* was positively associated with higher adherence to the HNFI index and negatively associated with a higher diastolic BP after adjusting for age, sex, and BMI. Moreover, *Bacteroides stercoris* correlated positively with the intake of healthy foods and food components, including fiber, grain products, and vegetables. Dietary carbohydrates provide important substrates for microbial metabolism in the human gut. Many members of the *Bacteroides* genus are enriched with genes encoding carbohydrate-active enzymes and are involved in the breakdown of complex carbohydrates from the diet (46–48). As such, *Bacteroides* members are the predominant organisms involved in carbohydrate metabolism and are considered generalists due to their capacity to switch between host and diet-derived energy sources (19). Furthermore, *Bacteroides*

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Table 4. Gut bacteria associated with metabolic markers[1]

Gut bacteria	Unadjusted values		Adjusted values			
	B	95% CI	*B*	95% CI	*P*ǂ	*P*♯
Systolic BP (≥ 120 mmHg) (*n* = 24)						
(A) *Actinobacteria*	0.285	0.014, 2.028	0.238	−0.196, 1.903	**0.047**	0.108
(A) *Bifidobacterium*	0.305	0.097, 2.201	0.251	−0.152, 2.045	**0.033**	0.090
(F) *Lactobacillus* spp.	−0.229	−2.269, 0.251	−0.309	−2.583, −0.140	0.114	**0.030**
Diastolic BP (≥ 80 mmHg) (*n* = 10)						
(B) *Bacteroides stercoris*	−0.300	−2.570, −0.087	−0.320	−2.718, −0.123	**0.036**	**0.033**
(B) *Bacteroides* spp. and *Prevotella* spp.	−0.288	−1.259, −0.015	−0.225	−1.163, 0.166	**0.045**	0.138
(B) *Bacteroides* spp.	−0.279	−2.348, 0.010	−0.335	−2.635, −0.166	0.052	**0.027**
(F) *Bacilli*	−0.350	−1.286, −0.155	−0.387	−1.404, −0.189	**0.014**	**0.011**
(F) *Dialister invisus* and *Megasphaera micronuciformis*	0.330	0.363, 4.138	0.384	0.644, 4.584	**0.020**	**0.010**
(F) *Eubacterium biforme*	−0.345	−3.487, −0.389	−0.313	−3.361, −0.157	**0.015**	**0.032**
(F) *Eubacterium rectale*	−0.325	−2.661, −0.209	−0.357	−2.814, −0.343	**0.023**	**0.013**
(F) *Lactobacillus* spp.	−0.378	−3.553, −0.580	−0.348	−3.400, −0.407	**0.007**	**0.014**
(F) *Streptococcus* spp. 2	−0.294	−1.217, −0.029	−0.305	−1.284, −0.007	**0.040**	**0.048**
Total cholesterol (≥ 5.0 mmol/L) (*n* = 20)						
(F) *Ruminococcus albus* and *R. bromii*	0.374	0.433, 2.739	0.245	−0.579, 2.659	**0.008**	0.202

[1]Gut bacteria values were log-transformed before analysis.
ǂ*P* for unadjusted values assessed by a linear regression model.
♯*P* for values adjusted for age, sex, and BMI, assessed by a linear regression model.
Phyla are indicated within parentheses; A, Actinobacteria; B, Bacteroidetes; F, Firmicutes.
The level of significance was set at $P < 0.05$ and are indicated in bold italic.

stercoris has been identified as part of a common set of microbial species referred to as a common bacterial core, which are largely shared between individuals (7). Interestingly, *Bacteroides stercoris* has been postulated as a keystone species of the human gut microbiome influencing the microbial community structure including the growth of butyrate-producing bacteria (49).

Higher abundance of *Bacteroides stercoris* in healthy individuals compared with patients with IBD has been shown (50), suggesting a potential beneficial impact of the presence of *Bacteroides stercoris* (51). Taken together, these findings indicate a potential protective role of *Bacteroides stercoris* on human health. However, these studies cannot establish causality and the role of *Bacteroides stercoris* for health and disease needs to be further elucidated in intervention studies.

A higher intake of fiber was associated with lower abundance of *Alistipes* after adjusting for age, sex, and BMI in this study. Interestingly, diets low in fiber and high in fats have shown to increase the abundance of *Alistipes* (52, 53). Whether these differences are related to metabolic regulation by diet needs further investigation, as the impact of *Alistipes* has been reported as both protective and detrimental on CVD (54). In addition to *Alistipes*, members of the Proteobacteria phylum are reported to increase with the presence of fat, indicating that these members utilize dietary fats for growth (52). Here, we show that Proteobacteria abundance correlated positively with the intake of dietary cholesterol and cheese. A high-cholesterol diet was recently shown to increase Proteobacteria abundance in zebrafish (55), and high-fat feeding resulted in higher abundance of Proteobacteria in mice (56–58). Furthermore, Proteobacteria has been reported to increase in low-grade inflammation, a common feature of metabolic diseases (59). *Enterobacteriaceae*, a member of the Proteobacteria phylum, correlated positively with BMI in this study. In line with our results, *Enterobacteriaceae* has been associated with obesity (60), while weight-loss has been shown to reduce the abundance of *Enterobacteriaceae* (61–63). Taken together, these findings may indicate that dietary components such as fiber and fat modulate the gut microbiota composition, which in turn may impact host metabolic regulation.

In addition to *Bacteroides stercoris*, we show that a lower abundance of bacteria belonging to the Firmicutes phylum, specifically *Bacilli*, *Eubacterium biforme*, *Eubacterium rectale*, *Streptococcus* spp. 2, and *Lactobacillus* spp., was associated with higher diastolic BP after adjusting for age, sex, and BMI. Moreover, a higher systolic BP was also associated with lower abundance of *Lactobacillus* spp. after the same adjustments. The relationship between *Lactobacillus* abundance and BP has also been reported by others. Palmu and colleagues recently demonstrated a strong negative correlation

between certain *Lactobacillus* species and BP, and sodium intake in a Finish cohort including 6,953 participants (64). In a study by Wilck et al., a high salt diet was shown to increase BP and reduce the abundance of *Lactobacillus* species in mice (65). In this study, the administration of *Lactobacillus* species to mice resulted in a reduced systolic BP and normalization of diastolic BP. The authors further tested this effect in a pilot study in healthy males receiving 6 g sodium for 14 days, which led to a higher BP and a loss of *Lactobacillus* species (65). The relationship between *Lactobacillus* and BP was also evaluated in a meta-analysis of RCTs including 702 individuals (66). The effect of probiotic fermented milk, commonly adding *Lactobacillus* species for the fermentation purpose, reduced systolic and diastolic BP with 3.10 and 1.09 mmHg, respectively, compared with placebo, and the effect on systolic BP was suggested to be even more effective in hypertensive individuals (66). Another meta-analysis of RCTs with 543 participants also showed an effect of probiotic consumption on BP (67). The intake of probiotics, including probiotics from dairy, reduced systolic BP with 3.56 mmHg and diastolic BP with 2.38 mmHg compared with control groups. Interestingly, this effect was found to be similar to the effect of salt reduction and resistance training (67). Furthermore, pre-hypertensive and hypertensive individuals displayed a reduced gut microbiota diversity and richness compared with healthy controls (68). Furthermore, transplantation of fecal bacteria from the hypertensive donors to germ-free mice increased BP and reduced microbiota diversity in hypertensive mice compared with control mice (68). These findings may, therefore, indicate that the gut microbiota, in particular the abundance of *Lactobacillus*, may be involved in the regulation of BP.

A positive correlation between *Lachnospiraceae* and fasting total cholesterol and triglyceride levels was found in this study. The gut microbiota has been reported to play an important role in the variation of blood lipid levels in humans (69). A positive correlation between cholesterol levels and *Lachnospiraceae* has been shown previously (70), while others show an inverse relationship (34, 71, 72). *Lachnospiraceae* is a heterogenic family comprised of taxa reported as both potentially beneficial and harmful (73, 74). For example, differences in *Lachnospiraceae* members between healthy controls and patients with T2D and metabolic syndrome have been shown (75, 76), and different members have been related to either higher or lower lifetime CVD risk (77). The heterogeneity of *Lachnospiraceae* emphasizes these discrepancies, and investigating gut microbes in lower taxonomic levels may give more insight to the potential role of *Lachnospiraceae* in host lipid metabolism.

In this study, we demonstrate associations between a panel of gut bacteria, dietary intake, and metabolic and anthropometric markers in healthy adults. This study is explorative by nature, and the results obtained may be used to further generate new hypothesis. Hence, we did not control for multiple testing. In this study, the sample size, the inclusion of relatively young adults (mean age 35.6 years), and mostly women (75.5%) in a Norwegian population residing in the Oslo area represent limitations. In addition, dietary data were collected by a FFQ and showed a high fiber intake (41.7 g/day for males and 39.7 g/day for females). In comparison, findings from the Norwegian Women and cancer cohort, also using FFQ data, reported fiber intakes of 20 g/day (78). Hence, our findings may not be generalized to other populations in different geographical regions as the gut microbiota has been shown to differ among age, gender, and geographical regions (79–81). The findings in this study may, however, highlight the relationship between a high fiber intake and the gut microbiota.

Using a targeted method to detect only 48 bacteria may be criticized, as other bacteria than those analyzed may have an impact on metabolic regulation. However, the method provides a rapid, high-throughput analysis of human fecal samples allowing researchers to assess fecal microbiota composition without advanced expertise. Here, we explored a panel of bacteria commonly found in the human gut, shown to differ between healthy controls and patients with IBD and IBS, characterized as dysbiotic (44). Although no clear consensus about the definition of dysbiosis in the literature currently exist, dysbiosis often refers to as general changes in the gut microbiota composition, an imbalance in composition, or as changes in specific taxa in that composition (10). Here, we further investigate the associations of the selected set of gut bacteria related to dysbiosis with diet and metabolic markers in healthy adults. Identifying potentially harmful or beneficial bacteria by targeted methods may, in the future, be useful to predict disease risk and/or be used as a therapeutic potential for treating disorders. However, the lack of measures of microbial metabolites such as SCFAs limits our possibility to investigate the functionality of the gut microbiota and its relationship with dietary intake and metabolic and anthropometric markers. A complete characterization of the gut microbiota and measures of microbial metabolites are encouraged in future studies. Our findings may provide useful information about the association between a panel of gut bacteria, dietary intake, and metabolic and anthropometric markers in healthy adults. The cross-sectional design does not allow us to establish causality, and we encourage more and larger randomized controlled studies to further investigate this relationship.

Conclusion

In this study, we demonstrate associations between a panel of gut bacteria, dietary intake, and metabolic and

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anthropometric markers in healthy individuals. Of specially interest was the abundance of *Bacteroides stercoris*, which correlated positively with the intake of fiber. Further adjustments for age, sex, and BMI showed that *Bacteroides stercoris* was positively associated with higher adherence to the HNFI index and negatively associated with higher diastolic BP. These findings may indicate a role for the gut microbiota in metabolic regulation through diet, or that the gut microbiota may reflect a healthy versus unhealthy lifestyle. Whether we can modify the gut microbiota by diet and consequently impact metabolic status remains to be elucidated.

Data described in the manuscript will be made available upon request pending application and approval.

Conflict of interest and funding

Funding for this research was provided by OsloMet, University of Oslo, Genetic Analysis, and Mills AS. Mills AS had no role in the analysis of the data. L.G., I.R., and T.G. report nothing to declare. M.G.B. is employed at Mills AS. She does not own any stocks in the company. K.R. reports research grants and/or personal fees from Akcea, Amgen, Sanofi, and Sunnovion, and none of which are related to the content of this manuscript. K.B.H. reports grants from Tine SA, Mills AS, Olympic Seafood, Amgen, Sanofi, and Kaneka, and personal fees from Amgen, Sanofi, and Pronova, outside the submitted work. S.M.U. has received research grants from Tine DA, Mills AS, and Olympic Seafood, and none of which are related to the content of this manuscript. M.C.W.M. is involved in projects with research grants from Tine SA and Olympic Seafood and has received research fund from Mills AS, and none of which are related to the content of this manuscript. V.H.T.-H. reports research grants from Mesterbakeren, Det Glutenfrie Verksted, and Norsk cøliakiforening, and none of which are related to the content of this manuscript. She has been employed at Mills AS. She does not own any stocks in the company, and the work performed in this paper was done after she left the company.

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Authors' contributions

The author contributions were as follows: L.G., M.C.W.M., I.R., T.G., K.R., K.B.H., S.M.U., and V.H.T.H. designed the research; L.G., M.C.W.M., and V.H.T.H. conducted the research; L.G., M.C.W.M., and V.H.T.H. analyzed data and performed statistical analysis; L.G., M.C.W.M., and V.H.T.H. wrote the manuscript; L.G., M.C.W.M., I.R., T.G., M.G.B., K.R., K.B.H., S.M.U., and V.H.T.H. provided critical editorial comments; L.G., M.C.W.M., and V.H.T.H. had primary responsibility for final content; and all authors read and approved the final manuscript.

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Variability in the Pre-Analytical Stages Influences Microbiome Laboratory Analyses

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Abstract: Introduction: There are numerous confounding variables in the pre-analytical steps in the analysis of gut microbial composition that affect data consistency and reproducibility. This study compared two DNA extraction methods from the same faecal samples to analyse differences in microbial composition. Methods: DNA was extracted from 20 faecal samples using either (A) chemical/enzymatic heat lysis (lysis buffer, proteinase K, 95 °C + 70 °C) or (B) mechanical and chemical/enzymatic heat lysis (bead-beating, lysis buffer, proteinase K, 65 °C). Gut microbiota was mapped through the 16S rRNA gene (V3–V9) using a set of pre-selected DNA probes targeting >300 bacteria on different taxonomic levels. Apart from the pre-analytical DNA extraction technique, all other parameters including microbial analysis remained the same. Bacterial abundance and deviations in the microbiome were compared between the two methods. Results: Significant variation in bacterial abundance was seen between the different DNA extraction techniques, with a higher yield of species noted in the combined mechanical and heat lysis technique (B). The five predominant bacteria seen in both (A) and (B) were *Bacteroidota* spp. and *Prevotella* spp. (p = NS), followed by *Bacillota* ($p = 0.005$), *Lachhnospiraceae* ($p = 0.0001$), *Veillonella* spp. ($p < 0.0001$) and *Clostridioides* ($p < 0.0001$). Conclusion: As microbial testing becomes more easily and commercially accessible, a unified international consensus for optimal sampling and DNA isolation procedures must be implemented for robustness and reproducibility of the results.

Keywords: microbiome; pre-analysis; DNA; 16S rRNA

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1. Introduction

An estimated 500–1000 species of bacteria exist in the human body at any one time with each bacterial strain accompanied by a genome consisting of thousands of genes [1]. The human gastrointestinal tract alone consists of 10–100 trillion symbiotic microbial cells that is encompassed by a single individual [2]. Traditionally, the intestinal microbiota composition has been evaluated using breath-testing techniques, small-bowel culture techniques, in situ hybridisation and culture-independent techniques. One of the most common approaches for microbiota profiling is targeting the 16S rRNA gene by using high-throughput next-generation sequencing (NGS) or probe-based analysis [3]. Although culturing techniques have improved in recent years with targeted phenotypic culturing, the majority of species cannot be cultured with standard methods [3,4]. The decreasing costs and increasing speed of DNA sequencing have prompted researchers to instead embark on small-subunit (16S) ribosomal RNA (rRNA) gene-sequence-based surveys

of bacterial communities that reside on or in the human body [2]. NGS technologies have evolved over the past 15 years, becoming increasingly cost-effective and leading to substantial improvements in quality and yield [5], revolutionising genomics research by deciphering the complex microbial community from faecal samples which can be performed even in small laboratories [6]. The development of culture-independent DNA sequencing techniques has also allowed us to better understand the functionality of the microbiome, such as metagenomics, metabolomics, and meta-transcriptomics [7]. The variable regions (V1–V9) within the bacterial 16S rRNA genes show unique sequence diversity which can be utilised to determine bacterial taxa, sometimes at the species level [8]. Consequently, 16S rRNA sequencing has become the preferred method for studying microbial diversity [3].

Despite the recent advances in 16S rRNA sequencing, there are numerous host and environmental factors that can influence the gut microbiome. This includes patient factors, such as polypharmacy, history of antibiotic/pre- and probiotic use, age, gender, ethnicity, surgical history/comorbidities, smoking, geography and diet [9,10]. Whilst it may be difficult and unfeasible to standardise every one of these factors, optimising faecal collection, transportation, storage and DNA extraction techniques becomes essential to attain high-quality metagenomic DNA for an unbiased microbial analysis [9,11–13]. These numerous differences can all have variable impact on downstream results, making it difficult to directly compare results from different studies/patient cohorts/laboratories. As a result, there has been a lack of standardisation such that determining the causal role of the microbiota composition on disease pathogenesis has remained elusive. The primary aim of this study is to compare the pre-analytical steps, including handling of the samples pre-treatment and DNA extraction methods from the same human faecal samples between two different laboratory techniques and determine what, if any, differences can be seen in microbial composition. The outcome may aid in the standardisation of microbiome detection methods using 16S rRNA.

2. Materials and Methods

2.1. Ethical Approval and Good Clinical Practice

The study was performed in accordance with the recommendations guiding physicians in biomedical research involving human subjects, adopted by the 18th World Medical Assembly, Helsinki, Finland 1964, amended at Edinburgh in 2000. The study was conducted in accordance with the International Conference on Harmonisation Good Clinical Practice (ICH GCP) guidelines. Patient information was anonymised and any collection of patient data was in compliance of the Data Protection Act 1998. The study underwent full ethical approval by London—Stanmore Research Ethics Committee. REC ref: 16/LO/1325. All authors had access to the study data and reviewed and approved the final manuscript.

2.2. Human Samples

Faecal samples were collected from the Royal Wolverhampton NHS Trust, Wolverhampton, UK. Samples were collected from patients referred to gastroenterology for symptoms of diarrhoea. In total, 13 patients had confirmed bile acid diarrhoea (BAD) with a positive SeHCAT scan (<12% retention), of which 6 patients had a previous cholecystectomy, 3 had idiopathic BAD and 4 had a terminal ileal resection secondary to Crohn's disease. The remaining 7 patients were diagnosed with functional diarrhoea and consisted of 1 patient with a previous cholecystectomy, 4 with no previous comorbidities and 2 with a terminal ileal resection secondary to Crohn's disease (disease in remission). The faecal samples were collected prior to patients starting treatment for BAD and were excluded from the study if they received antibiotics within four weeks of the initial trial participation. Patients were given a large container and advised to store their first morning defecation into the tub. They were then advised to store the container in their fridge or freezer (if possible) or in a cold room such as the garage as the next possible option. The amount of stool obtained varied between patients and was collected from their home, office of work or hospital and each sample was then split into three universal tubes. An equal amount of stool was placed

into each tube with no extra preservative added and stored in a −80 °C freezer on the same day of collection. The time variability between collection and storage was up to 8 h. A sample from one universal tube was used for DNA extraction in the Wolverhampton (WLV) laboratory (Method A), and the same sample from another tube was sent on dry ice to another laboratory for extraction, Genetic Analysis AS, Oslo, Norway (GA MAP) (Method B). For both laboratories, a single cycle of freeze/thaw occurred prior to DNA extraction and quantification.

2.3. DNA Extraction at Wolverhampton Laboratory (Method A)

Samples were analysed according to the manufacturer's instruction using the commercially available QIAamp Fast DNA Stool Mini Kit (Qiagen, Manchester, UK). Briefly, Faecal samples were mixed with InhibitEX Buffer and homogenised by vortexing. Samples were incubated at 95 °C for 30 min. Samples were lysed with Protease K and Buffer AL prior to re-incubating at 70 °C for 10 min. The lysed samples were mixed with 100% ethanol and lysates, which was then loaded onto the QIAamp spin column. To remove any remaining impurities, the samples underwent two wash steps with buffers AW1 and AW2 and centrifuged. DNA was then eluted in low-salt buffer ATE. Purity and concentration of DNA were determined using the NanoDrop™ 2000/2000c spectrophotometers (ThermoFisher, Manchester, UK) and an A260/280 ratio of 2.0 was accepted as 'pure' for DNA and A260/230 in the range of 0.9–1.2 considered free of other contaminants. A detailed protocol is available in the Supplementary Files.

2.4. DNA Extraction at GA Laboratory (Method B)

Faecal samples were extracted following the GA-map® methods already published in detail in Casén et al. [3]. Briefly, each faecal sample was first homogenised before mixed with buffer (S.T.A.R., Roche, Basel, Switzerland). They were then transferred to Lysing Matrix E tubes (MP Biomedicals Inc., Santa Ana, CA, USA) and mechanically lysed in a FastPrep-96™ (MP Biomedicals Inc.). Lysed samples were centrifuged and incubated at 65 °C with lysis buffer and Protease K. Each protease-treated faecal sample was then used to extract total genomic DNA according to mag™ maxi kit instructions (LGC Genomics, Berlin, Germany), adjusted for a KingFisher™ Flex 96/MagMAX™ Express-96 DNA extraction robot (Life Technologies, Waltham, MA, USA). This extraction method was extensively tested and validated in cooperation with the Norwegian University of Life Science (NMBU). The detailed protocol is provided in the Supplementary Files.

2.5. The GA-Map® Technology Platform (GA-Map® Dysbiosis Test)

WLV and GA MAP samples, extracted at the Wolverhampton or GA laboratory, respectively, were further analysed using the GA-map® Dysbiosis Test Lx (Genetic Analysis AS, Oslo, Norway). The GA-map® technology uses a pre-selected set of DNA probes (bacterial markers) and the variable regions of the bacterial 16S rRNA gene (V3 to V9) to map the intestinal microbiota and identify a bacterial profile.

Briefly, the polymerase chain reaction (PCR) was used to amplify ~1200 base pair fragments of the 16S rRNA gene (V3–V9), followed by a reaction clean-up as described previously [14]. The PCR template was then used in a probe labelling reaction (single-nucleotide extension), before hybridisation of the probe- and bead-set, fluorophore addition (Streptavidin-phycoerythrin) and detection, with the following modifications: a probe-set of 50 probes (48 bacterial target probes, a hybridisation control and universal control) was labelled, and hybridised to the GA-map® bead set. The hybridisation signal was detected and quantified using a Luminex® 200™ instrument (Luminex Corp., Austin, TX, USA). Each of the probes in the GA-map® test were designed to target a bacterial species or group based on their 16S rRNA sequence (V3–V9). Due to technical limitation in the Luminex instrument platform, six probes (probe no. 1, 15, 16, 26, 30, 49) were removed. The software then identified and quantified median signals, bead count and flags. Raw

data was normalised before it was exported for further analysis. Additional information on the microbiome data analysis is provided in the Supplementary Files.

2.6. GA-Map® Dysbiosis Test Analysis

This test was developed using a normal healthy cohort (n = 211) to build a normobiotic reference profile. This was then validated using an independent cohort of healthy individuals (n = 43) and patients with irritable bowel syndrome (n = 109) and inflammatory bowel disease (n = 135) [15]. This test is CE marked and suitable for use in a routine, clinical setting and in clinical research. This standardised protocol enables comparison of results between laboratories and between research studies, as well as for following patients over time. Based on the resulting normalised signal strengths, an algorithm compares the bacterial profile of the sample with that of a normal reference cohort and calculates the Dysbiosis Index (DI), ranging from 1 to 5. A DI value above 2 indicates a microbiota profile that differs from the normal reference population, and the higher the DI the further the bacterial profile deviates from the reference. Therefore, the higher the DI above 2, the more the sample is considered to deviate from normobiosis. Bacterial abundance is also calculated and is reported on a scale from −3 to +3 with 0 being the average level in the normal reference population. If the selected bacteria are reported to be less than 0, there is reduced abundance of that bacteria relative to the reference population, and if above 0, there is a greater abundance relative to the reference population.

2.7. Statistical Analysis

Data was analysed using Prism (Graphpad Version 9.2.0) and Microsoft Excel 2019 Version 16.55 (Microsoft, Redmond, WA, USA). Categorical data was expressed as the number of subjects (and percentage) and the mean, as appropriate. A test for association between the two techniques was performed using an independent *t*-test. The Mann–Whitney U test was used for testing DI values. All tests were two-sided and a p-value < 0.05 was considered to be statistically significant.

3. Results

A total of 20 patients equating to 40 samples were analysed (20 from method A and 20 from method B). The full details on patient demographics can be found in the Supplementary Files. The mean age of the patient cohort was 51.2 years with 60% being female. The median DNA concentration and interquartile range obtained from Method A was 16.3 ng/uL (IQR 12.9–20.9 ng/uL). The median DNA concentration from Method B was 17.8 ng/uL (14.9–19.6 ng/uL).

In total, 5 samples from method A and 1 sample from method B did not pass the GA-map® Dysbiosis Test quality control requirements. Although method A is not validated for use with the Dysbiosis test, all the samples were still analysed for its microbiota.

Figure 1 shows the DI scores between analysis of method A and method B samples (p = NS). With method A, 3 patients (15%) were classified as normobiotic (DI scale of 1 + 2), 7 patients (35%) with mild dysbiosis (DI scale of 3), and 10 patients (50%) with severe dysbiosis (DI scale of 4 + 5). With method B, 3 patients (15%) were classified as normobiotic (DI scale of 1 + 2), 9 patients (45%) with mild dysbiosis (DI scale of 3), and 8 patients (40%) showed severe dysbiosis (DI scale of 4 + 5).

As can be seen in Figure 2, the predominant bacteria contributing to dysbiosis in both method A and B sample cohorts were *Bacteroidota* spp. and *Prevotella* spp. showing a normalised median platform signal strength of 565 and 735, respectively, p = NS; followed by *Bacillota* (362 vs. 614, p = 0.005), *Lachnospiraceae* (460 vs. 814, p = 0.0001), *Veillonella* spp. (182 vs. 570, p < 0.0001), and *Clostridioides* (152 vs. 439, p < 0.0001). The overall average abundance of bacterial composition for method A was 7.54 (95% confidence interval 0.0–21.5) and method B was 16.5 (95%CI 6.4–59.7), p < 0.0001. The differences in bacterial abundance as well as the full microbiota profile of each patient was compared between method A and method B and can be found in the Supplementary Files.

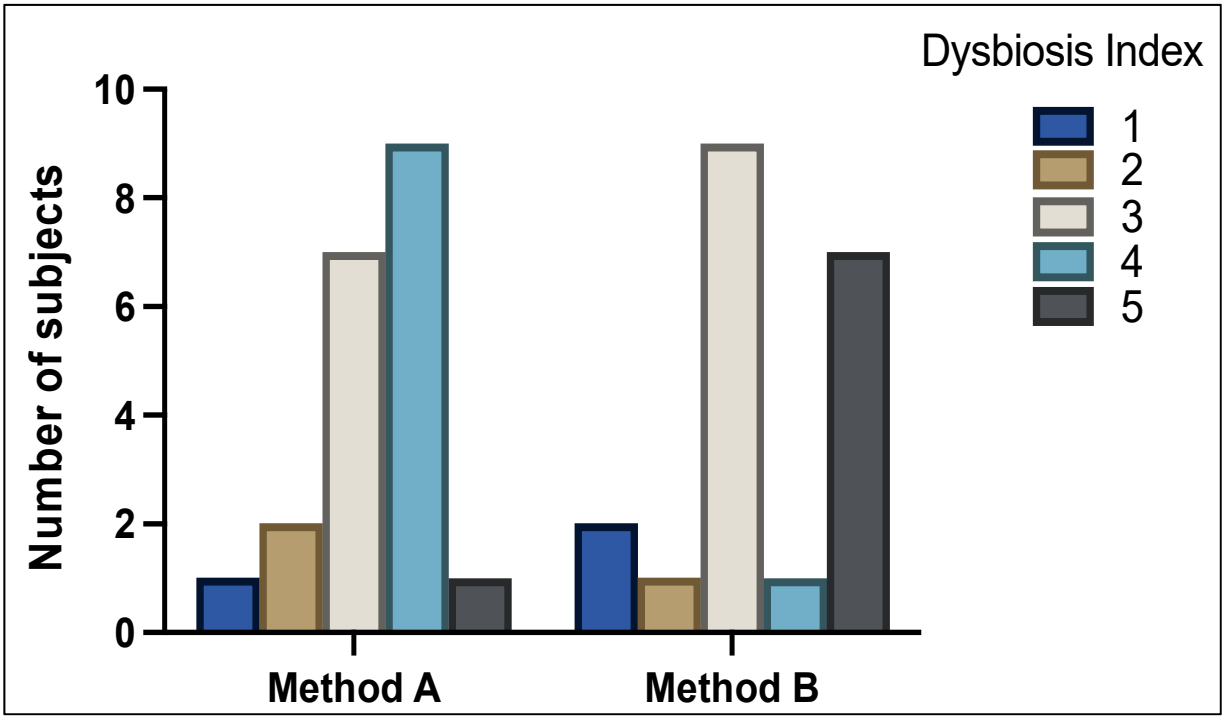


Figure 1. Distribution of Dysbiosis Index (DI) scores 1–5 as determined by GA-map® Dysbiosis test. High variability of DI can be seen between method A and method B.

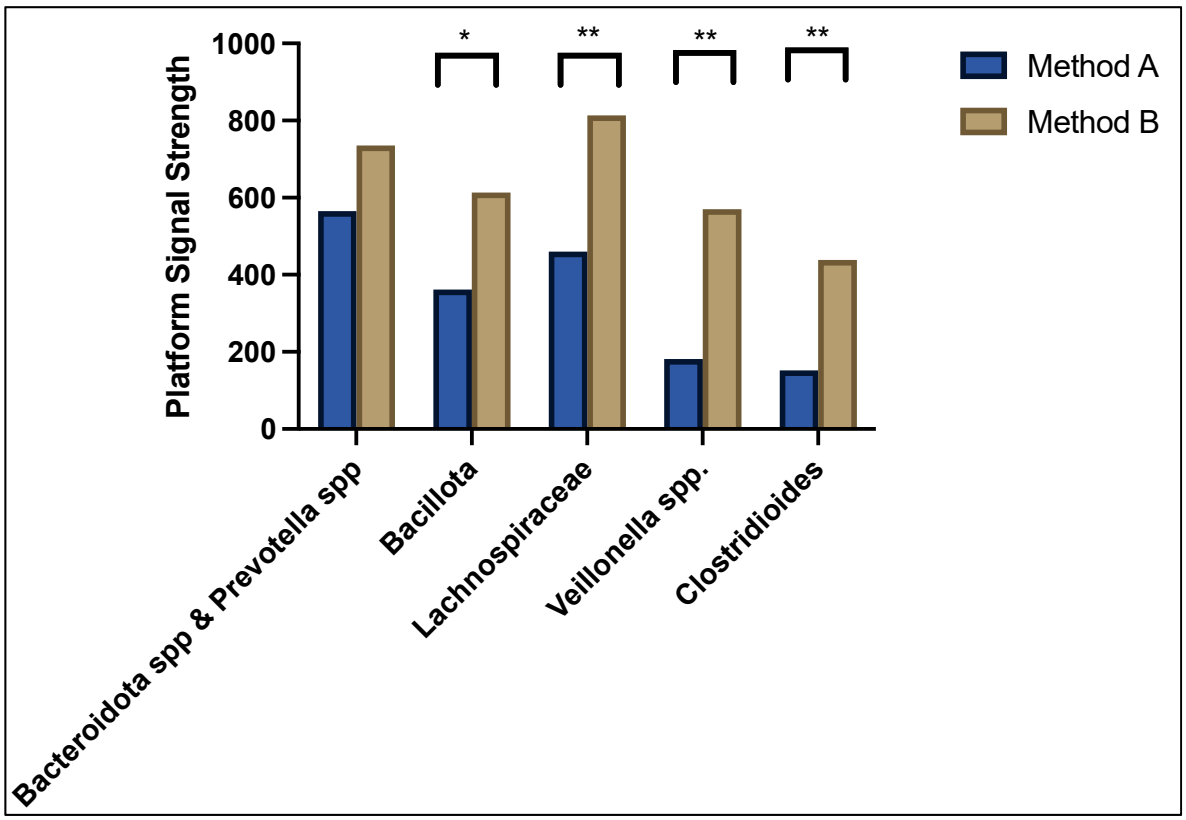


Figure 2. Median signal strength for top five predominant bacteria contributing to dysbiosis. * denotes $p < 0.05$ and ** denotes $p < 0.001$.

There are six phyla detected with the GA-map® platform: *Actinomycetota, Bacteroidota, Bacillota, Pseudomonadota, Tenericutes* and *Verrucomicrobiota*. Out of the 48 bacterial probes used, 19 (39.6%) demonstrated statistically significant differences between methods A and B; 17/19 (89.5%) bacteria that had significant differences in abundance between the two methods were Gram positive bacteria.

In the *Actinomycetota* phylum (Figure 3A), there was reduced abundance seen of *Actinomycetales* and *Actinomycetota* in both the method A and B samples; however, there was a significant difference in abundance variation in the former ($p = 0.03$). Method A samples demonstrated a reduced abundance in *Bifidobacteria* as compared to the sample from method B ($p = 0.0047$).

The Bacteroidota phylum (Figure 3B) demonstrated generally reduced abundance in the species *Alistepes*, *B pectinophilus*, *Bacteroidota* spp., *Bacteroidota* spp. and *Prevotella* spp., *B zoogleformans* and *Parabacteroides* spp., and a greater abundance in *Alistepes onderdonkii, B fragilis*, *B stercoris* and *Parabacteroides jonsonii*. Between the two methods, the results demonstrated generally similar abundance levels.

The *Bacillota* phylum (Figure 3C) had variable abundance, but there were notable differences seen between A and B. Specifically, method B samples consistently demonstrated a greater abundance in *Bacilli, Clostridia*, *Dialister invisus* and *Megasphaera micronuciformis (D/M), Lactobacillus* spp., *Streptococcus agalactiae* and *Eubacterium rectale (S/E), Streptococcus salivarius ssp thermophilus (SST),* and *Bacillota* (various), whilst the method A samples demonstrated a reduced abundance in these bacteria. In contrast, method A demonstrated a higher reduced abundance of *Veillonella*, *Streptococcus salivarius ssp thermophilus*, *Streptococcus agalactiae* and *Eubacterium rectale*, *Lactobacillus* spp., *Lachnospiraceae*, *Faecalibacterium prausnitzii, Anaerobutyricum halii, Dorea* spp., *Clostridioides methypentosum, Clostridioides, Bacilli* and *Bacillota*. Apart from *Bacillota* (various), these differences were statistically significant.

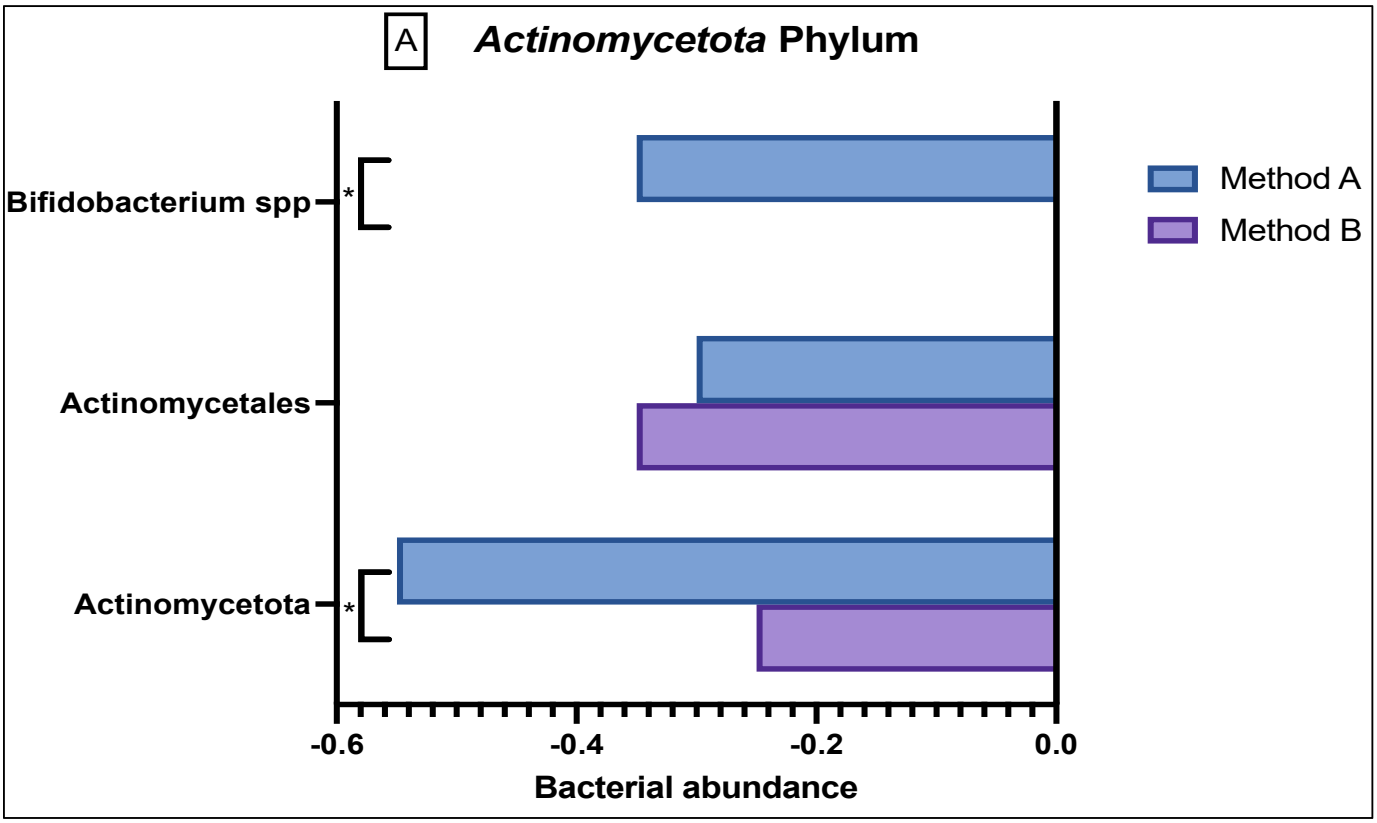
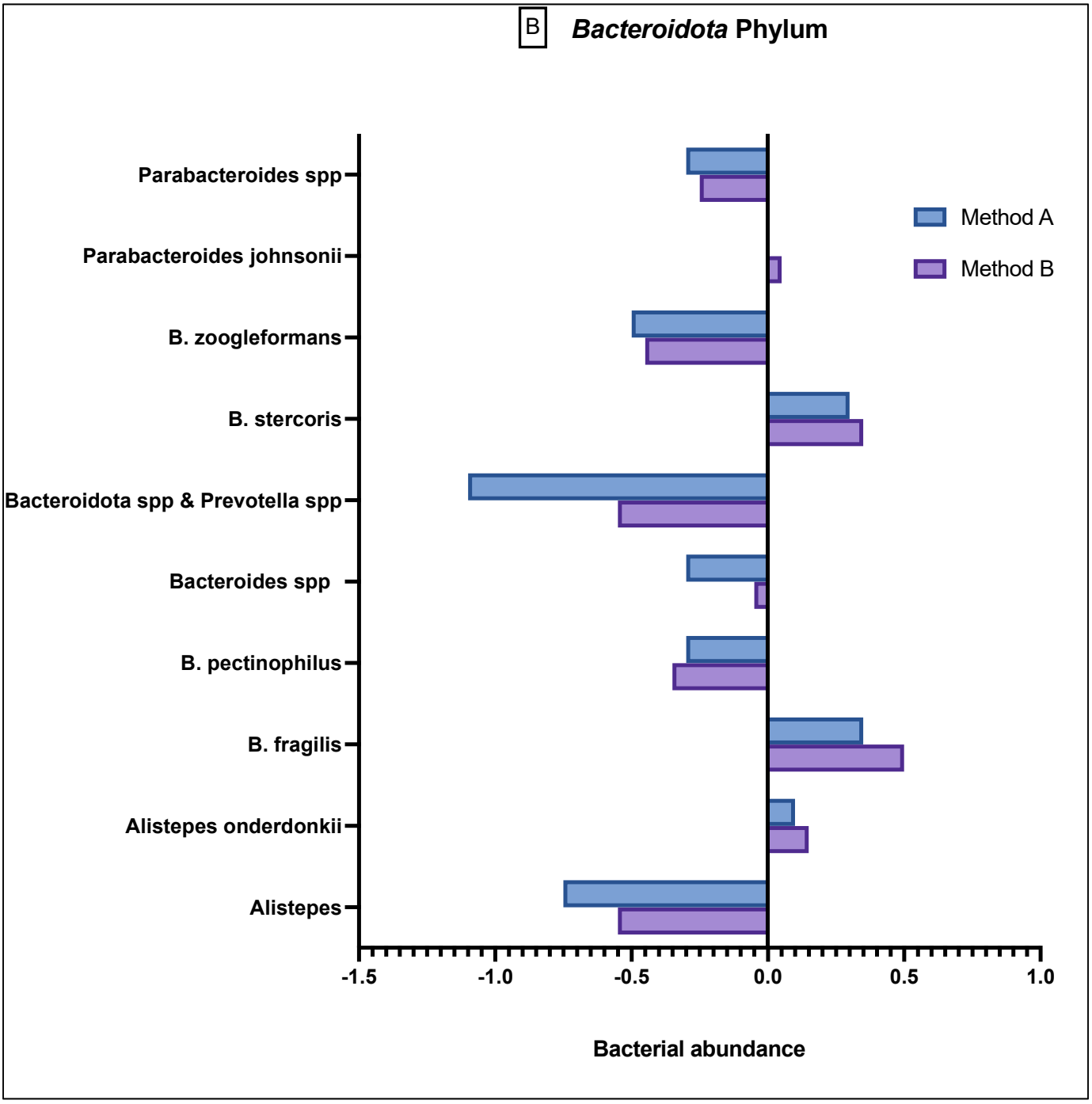


Figure 3. *Cont.*



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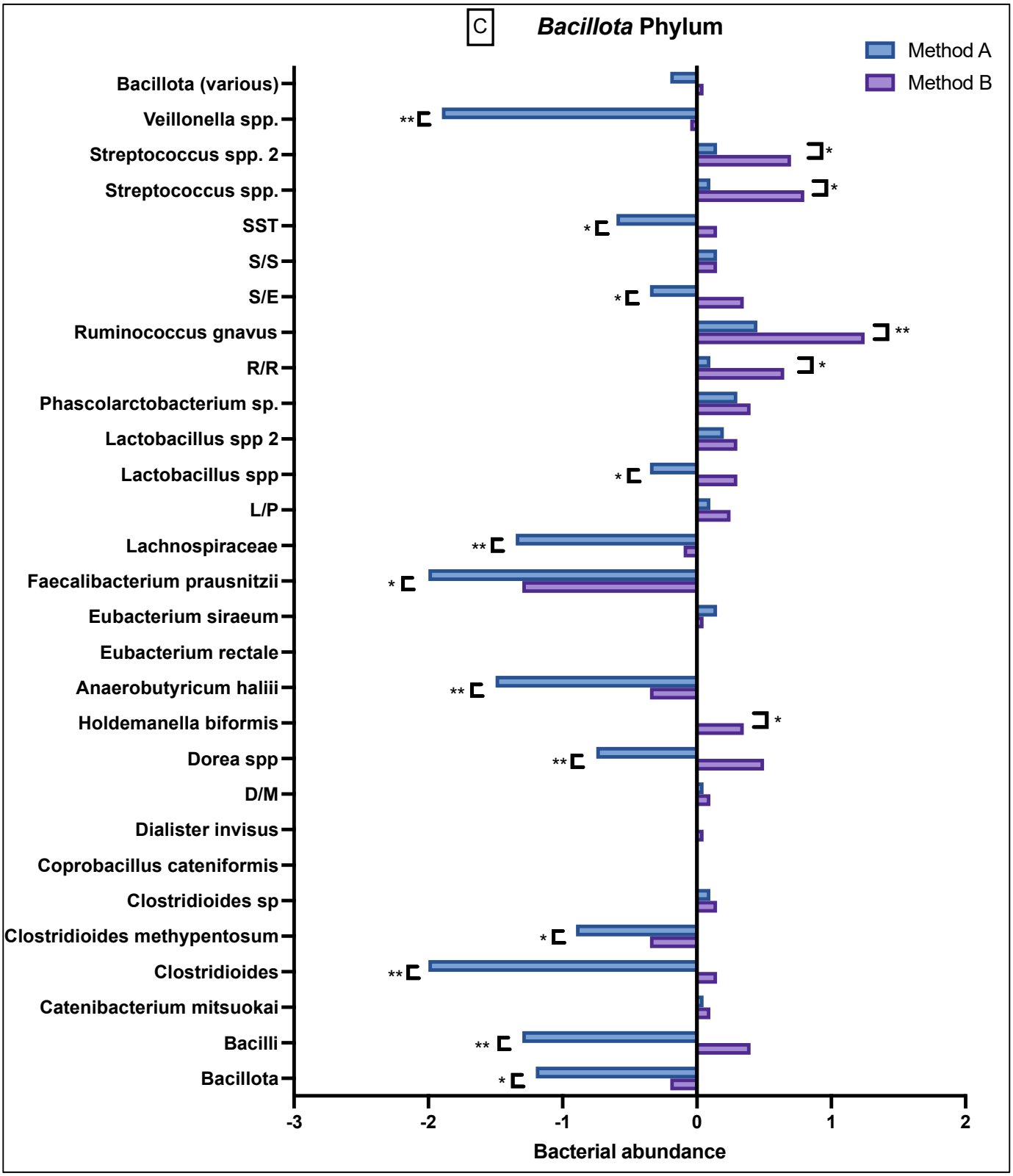

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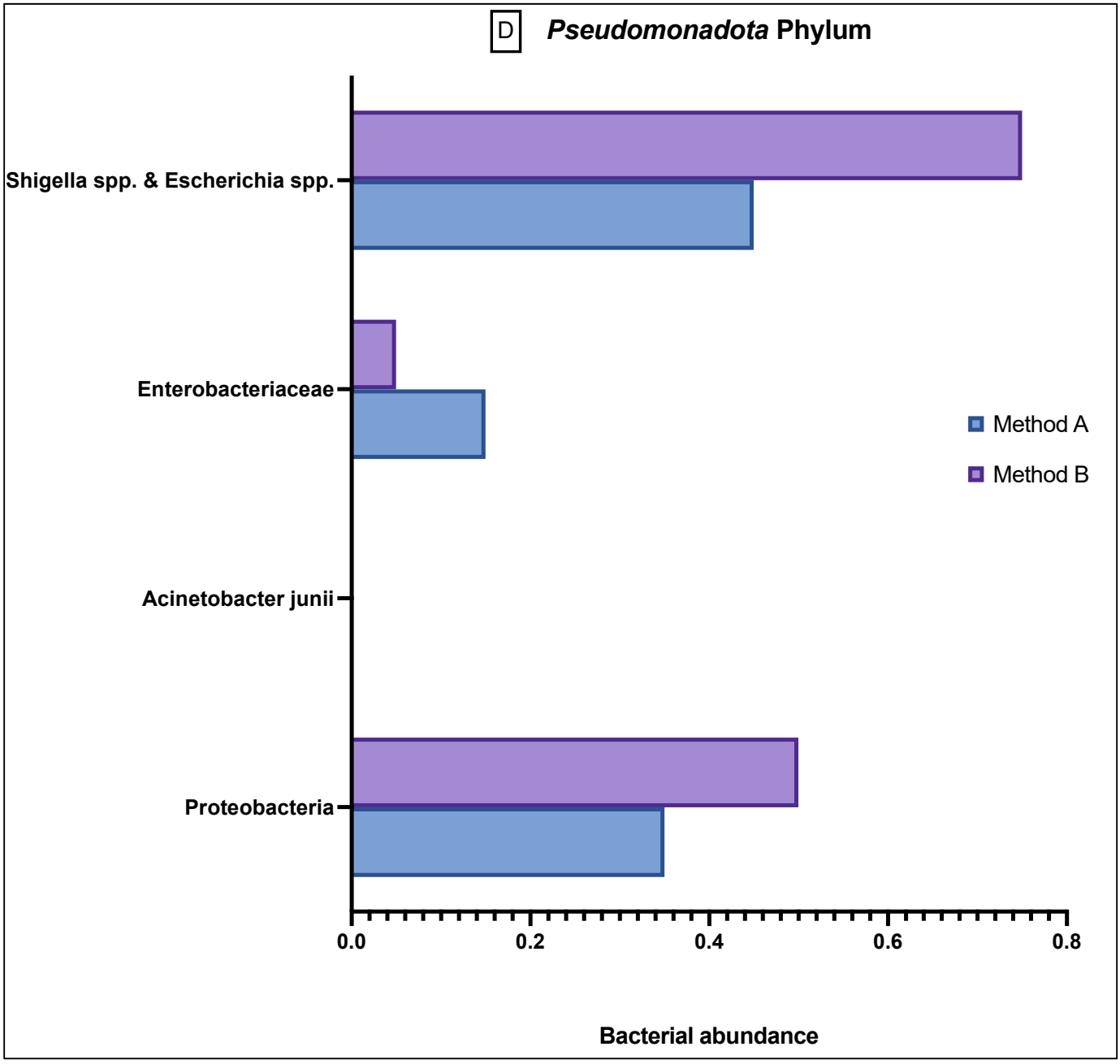

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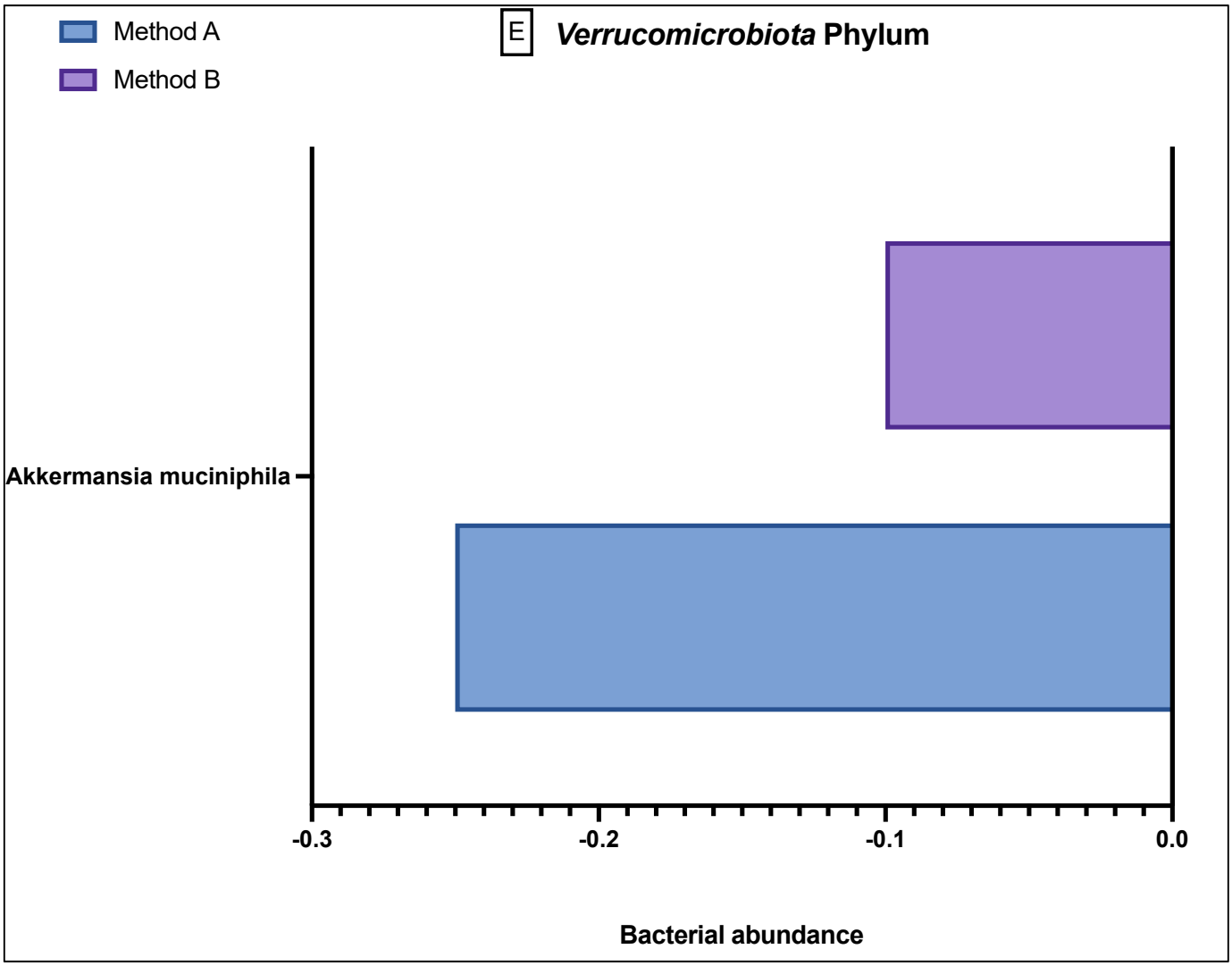

Figure 3. Comparison of bacterial abundance between method A and B samples as determined by the GA-map® Dysbiosis test. Relative bacterial abundance is shown on a scale from ±3 in deviation from the GA-map® normal reference range (0). Bacterial species are broken into their phyla: (**A**) *Actinomycetota*, (**B**) *Bacteroidota*, (**C**) *Bacillota*, (**D**) *Pseudomonadota*, (**E**) *Verrucomicrobiota*. The sixth phyla, *Tenericutes*, did not show any difference between the two methods and did not deviate from the normal reference range. D/M: *Dialister invisus* and *Megasphaera micronuciformis*, L/P: *Lactobacillus rumminus* and *Pediococcus acidolactici*, R/R: *Ruminococcus albus* and *R bromii*, S/E: *Streptococcus agalactiae* and *Eubacterium rectale*, S/S: *Streptococcus salivarius ssp thermophilus* and *Streptococcus sanguinis*, SST: *Streptococcus salivarius ssp thermophilus.* * denotes $p < 0.05$ and ** denotes $p < 0.001$.

The *Pseudomonadota* phylum (Figure 3D) generally showed a greater abundance than the relative population in both A and B samples, and although there was still variation between the different bacteria, the results were not statistically significant.

The *Verrucomicrobiota* phylum (Figure 3E) demonstrated only one prominent bacterium, *Akkermansia muciniphilia*, which showed a greater reduced abundance in the method A samples as compared to method B. The final phylum, *Tenericutes*, which had one relevant bacterial species, *Mycoplasma hominis*, is not shown in Figure 3 because in all 40 samples, this bacterium was found to be in the reference population range, with no variation in abundance in either the method A or B samples.

4. Discussion

This study demonstrated significant variation in bacterial abundance in 19/48 bacterial probes due to methodological differences in faecal DNA extraction, with the majority of the differences seen in Gram-positive bacteria. The primary difference in DNA extraction between the method A and B samples was the combined use of mechanical and chemical/enzymatic heat lysis in the latter whilst only using chemical/enzymatic heat lysis in the former. There are two main DNA extraction methods, which are mechanical lysis/bead-beating and chemical lysis. Whilst bead-beating is considered to produce superior DNA yields, bacterial diversity, Gram-positive bacteria, spores and fungi [16,17], vigorous bead-beating should be avoided due to risk of shearing the nucleic acids [18,19] which can lead to the formation of chimeric molecules during PCR amplification [20,21]. Whilst emulsion PCR may help to prevent chimera formation, this was not used in our study and future work using this technique should be considered. Although recent studies have shown that chemical lysis is not inferior to mechanical lysis in rumen samples and from human saliva [16,22], human gut samples have consistently shown that mechanical lysis or a combination of enzymatic and mechanical disruption results in higher degrees of microbial diversity and have the greatest effect on gut microbiome composition [17,23–25]. Furthermore, our results demonstrated that between the two methods, there was considerable variation in relative abundances within Gram-positive bacteria but similar abundance levels in the Gram-negative Bacteroidota phylum. It is well known that due to their thick, rigid cell walls, Gram-positive microorganisms are resistant to cell lysis, which can be overcome through the use of bead-beating [26]. Thus, these findings corroborate previous studies confirming that a combination technique is of greater use in detecting and isolating Gram-positive bacteria [17,22,27].

Besides the use of bead-beating or mechanical lysis, another difference between the two protocols includes the initial step of homogenising, or thoroughly mixing of the stool prior to subsampling. Previous studies have demonstrated that there can be high variability of gut microbes within a single faecal sample [28] and homogenisation can reduce within-stool heterogeneity [12,29]. Hsieh et al. also demonstrated that homogenised stools were more similar to each other than samples that were not homogenised, with a general increase seen in phyla-containing Gram-positive bacteria [29]. Whilst this mixing step was performed in method B, it was not a part of the DNA extraction protocol from method A, highlighting again the need to standardise the sample treatment in the pre-analytical steps.

A confounding factor that could have affected the results and interpretation of this study is the handling of the faecal samples after collection. Changes in temperature and humidity can alter or contaminate the samples [30], as can transit conditions and duration of travel. The microbial composition is unstable from the point of sample collection and thus immediate freezing at −80 °C is advised to conserve microbial diversity, which can be significantly altered by dry storage at 4 °C [31]. Whilst our samples were stored at −80 °C immediately after collection, there were delays in receiving the patients' samples from the time of defecation by up to 8 h. Some studies have reported that optimal freezing time should be completed within 15 min of defecation [12] whilst others suggest within 2 h [32], although the feasibility of doing this is questionable. Furthermore, multiple studies have shown that repeated temperature fluctuations or dramatic temperature changes are a major stress for bacteria, leading to DNA aggravation and degradation [33–35]. It is, therefore, particularly important to avoid repeated freeze–thaw cycles. Whilst we attempted to minimise the number of freeze–thaw cycles, transportation from the hospital to the lab and from the lab to Norway was unavoidable and was done with the use of dry ice. The transfers from the hospital to the lab was a short journey of 10 minutes and samples were kept on dry ice and were immediately stored in a −80 °C freezer on arrival. The samples were also kept on dry ice to ensure that the temperature was consistent during the period of travel between the countries. Therefore, we maintained minimal (to none) freeze–thaw cycles, indicating that our microbial results were a true outcome rather than possible variability from sample storage and transport.

The technique used to map bacterial targets by GA-map® with the use of DNA probes are pre-determined and pre-defined on certain taxonomic levels; therefore, it is difficult to comment whether there was variation seen between the two methods in extracting different bacterial species and demonstrating greater microbial diversity. The use of NGS may have provided a more comprehensive analysis, allowing for a broader range of microbial groups on each taxonomic level. However, probe-based analysis has several advantages including an easily understandable, semi-quantitative score of dysbiosis (based on bacterial abundance and profile within a sample), a standardised protocol which enables comparison of results between laboratories and between research projects, and the test is CE marked making it suitable for use in a routine clinical setting as well as in clinical research. The primary aim of our study was to demonstrate variability in results when using different pre-analytical techniques and whether we used NGS or probe-based analysis should not change our final results as there was clear variability in bacterial abundance seen between the two pre-analytical protocols. The general low signal strengths and low abundance levels in method A, which used only chemical/enzymatic heat lysis indicates that the bacterial target extraction was not (as) successful as method B, which used the combination of mechanical and chemical/enzymatic heat lysis techniques.

Ultimately, the major limitation to this study is the small sample size. In spite of this, our study still demonstrated significant variability in the microbial composition when all parameters apart from the pre-analytical DNA extraction technique remained the same. Whilst it may be difficult to regulate inter-individual variables such as age, gender and other environmental factors, the pre-analytical inter-assay methodology needs to be standardised to reduce variability in microbial analysis results. Several different extraction methods have been published and proven to work well to identify the microbiome using faecal samples. However, standardisation and the validation of methods is the most important to ensure comparable results over time and between different laboratories and studies. As microbial testing becomes more easily and commercially accessible, developing a unified international standard and consensus for the pre-analytical stages will become essential to ensure robustness and reproducibility of the results. Otherwise, there will always remain a fundamental bias generated from certain DNA extraction protocols when comparing microbiota datasets. As shown in our study, differences in extraction methods affects bacterial abundance and this should be taken into consideration when interpreting study findings reported in the literature.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes13061069/s1, Supplementary Method A: DNA extraction at Wolverhampton laboratory; Supplementary Method B: DNA extraction at GA laboratory; Figure S1: Comparison of platform signal strength between Method A and B across all 48 bacterial probes; Figure S2: The following twenty heat graphs display the differences in bacterial abundance (demonstrated by platform signal strength) of each patient's microbial profile between Method A and Method B; Table S1: List of the bacterial targets of the 48 probes in the GA-map®Dysbiosis Test Lx; Table S2: Patient demographics of the twenty patients with bile acid diarrhoea.

Author Contributions: A.K. conducted the study, recruited patients, collected stool samples and analysed samples in the laboratory (method A). A.K. analysed the results and wrote the main manuscript. K.G. also analysed the results in the laboratory (method B). J.P.S., H.S. and K.G. provided critical revisions to the manuscript. M.J.B. and H.O.A.-H. were the principal investigators and oversaw the whole study. They provided critical revisions to the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki 1964, (amended at Edinburgh in 2000) and the International Conference on Harmonisation Good Clinical Practice (ICH GCP). The study underwent full ethical approval by

London- Stanmore Research Ethics Committee; REC ref: 16/LO/1325, approved on 13 July 2016 (with amendment approval on 13 January 2020).

Informed Consent Statement: Informed consent was obtained from all subjected involved in the study.

Data Availability Statement: Requests for any data, analytic methods and study materials will be considered and made available upon request to the corresponding author. Individual participant data will not be shared.

Conflicts of Interest: J.P.S. has received speaker fees for Takeda, Janssen and Abbvie outside of the submitted work. He has also received an unrestricted research grant from Tillotts Pharma outside of the submitted work. M.J.B. has received grants and travel expenses from Vifor International and Tillotts Pharma, outside of the submitted work. H.S. has received travel and conference expenses from Tillotts Pharma, Norgine, MSD, Abbvie and Janssen outside of the submitted work. A.K., K.G. & H.O.A.-H. declare no competing interests.

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Efficacy of Fecal Microbiota Transplantation for Patients With Irritable Bowel Syndrome at 3 Years After Transplantation

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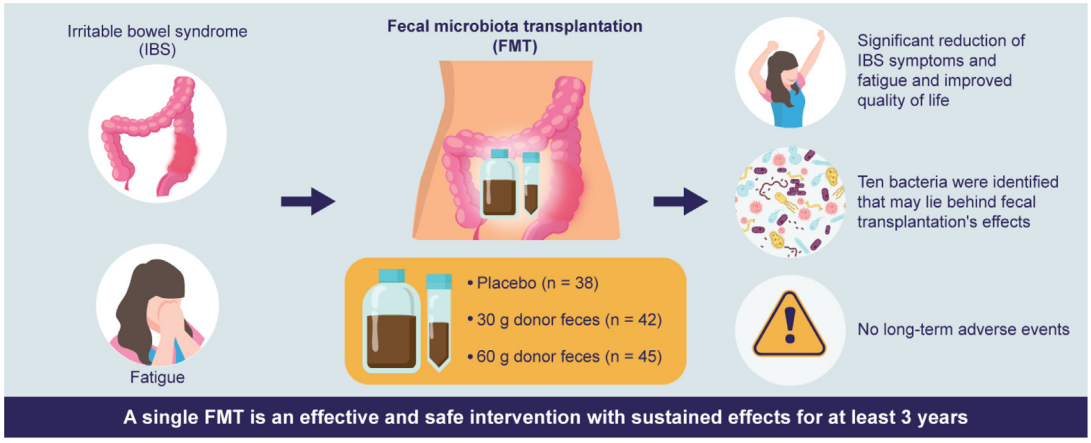

See editorial on page 815.

BACKGROUND & AIMS: The long-term efficacy and possible adverse events of fecal microbiota transplantation (FMT) for irritable bowel syndrome (IBS) are unknown. This study performed a 3-year follow-up of the patients in our previous clinical trial to clarify these aspects. **METHODS:** This study included 125 patients (104 females, and 21 males): 38 in a placebo group, 42 who received 30 g of donor feces, and 45 who received 60 g of donor feces. Feces was administered to the duodenum. The patients provided a fecal sample and completed 5 questionnaires at baseline and at 2 and 3 years after FMT. Fecal bacteria and dysbiosis index were analyzed using 16S ribosomal RNA gene polymerase chain reaction DNA amplification/probe hybridization covering the V3 to V9 regions. **RESULTS:** Response rates were 26.3%, 69.1%, and 77.8% in the placebo, 30-g, and 60-g groups, respectively, at 2 years after FMT, and 27.0%, 64.9%, and 71.8%, respectively, at 3 years after FMT. The response rates were significantly higher in the 30-g and 60-g groups than in the placebo group. Patients in the 30-g and 60-g groups had significantly fewer IBS symptoms and fatigue, and a greater quality of life both at 2 and 3 years after FMT. The dysbiosis index decreased only in the active treatment groups at 2 and 3 years after FMT. Fluorescent signals of 10 bacteria had significant correlations with IBS symptoms and fatigue after FMT in the 30-g and 60-g groups. No long-term adverse events were recorded. **CONCLUSIONS:** FMT performed according to our protocol resulted in high response rates and long-standing effects with only few mild self-limited adverse events. This study was registered at www.clinicaltrials.gov (NCT03822299)

Keywords: *Alistipes*; *Eubacterium biforme*; *Faecalibacterium prausnitzii*; Fatigue; Retransplantation.

The intestinal bacterial composition is considered to play a pivotal role in irritable bowel syndrome (IBS) pathophysiology.[1] Fecal microbiota transplantation (FMT) might be a promising treatment for IBS, and this has been investigated in 7 randomized controlled trials (RCTs).[2] In 4

Abbreviations used in this paper: **DI, dysbiosis index; FAS, Fatigue Assessment Scale; FMT, fecal microbiota transplantation; IBS, irritable bowel syndrome; IBS-C, constipation-predominant IBS; IBS-D, diarrhea-predominant IBS; IBS-M, mixed-diarrhea-and-constipation IBS; IBS-QOL, IBS-Quality of Life; IBS-SSS, IBS Severity Scoring System; RCT, randomized controlled trial; SF-NDI, Short-Form Nepean Dyspepsia Index.**

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WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Fecal microbiota transplantation is a promising treatment for patients with irritable bowel syndrome with unknown long-term effects and adverse events.

NEW FINDINGS

This study applied a safe fecal microbiota transplantation protocol to irritable bowel syndrome with a high response rate and long-standing effects. The study identified 10 bacteria that are associated with irritable bowel syndrome symptoms and fatigue.

LIMITATIONS

The study investigated a targeted selection of the intestinal bacterial contents, and did not include the IBS-U irritable bowel syndrome subtype.

IMPACT

A successful fecal microbiota transplantation protocol for irritable bowel syndrome and identifying the bacteria that may lie behind its effects facilitates the clinical use of the fecal microbiota transplantation, and the identified bacteria as probiotics.

of these, FMT reduced symptoms and improved the quality of life of patients with IBS, whereas no effects were indicated in the other 3.[2] The difference in these results was likely because of differences in the protocols used, the selected donors, the cohort of treated patients, the fecal transplant dose, and the route by which the transplant was administratered.[2]

The longest observation time of the previously mentioned RCTs was 1 year, and hence the long-term efficacy and long-term adverse events of FMT for IBS are unknown.[2] Its long-term safety raised some concerns after patients who received FMT for a *Clostridium difficile* infection were followed up, bringing about concerns of weight gain, development or worsening of inflammatory bowel diseases, cancer, autoimmune diseases, allergies, and neurological diseases.[3]

The present study was a 3-year follow-up of the patients with IBS who received FMT in our previous RCT, aiming to determine the long-term efficacy and possible long-term side effects of FMT.[4] Both female and male patients with IBS were included in our previous RCT.[4]

Material and Methods

Study Design

The study design has previously been described in detail.[4] To summarize, patients provided a fecal sample and completed 5 questionnaires to assess their symptoms and quality of life at baseline and at 2 and 3 years after FMT.

Patients

This study initially included 165 patients (Figure 1).These patients were randomized into placebo (own feces), 30 g of donor feces, or 60 g of donor feces groups as described in detail for our previous study.[4] In the placebo group, 10 patients were excluded, as they received a second FMT with a donor transplant, and 7 patients dropped out 2 years after FMT. In the 30-g group, 11 were excluded due to pregnancy (n = 1) and due to retransplantation with 60 g of donor feces at 3 to 4 months after the first FMT (n = 10), and 2 patients dropped out at 2 years after FMT. In the 60-g group, 6 patients were excluded due to pregnancy (n = 2), breast cancer diagnosis (n = 1), pyelonephritis (n = 1), gastroenteritis caught overseas (n = 1), and after receiving abdominal surgery (n = 1), and 4 patients dropped out 2 years after FMT (Figure 1). Therefore, 125 patients were finally investigated in this study: 38 in the placebo group, 42 in the 30-g group, and 45 in the 60-g group. The characteristics of included patients at baseline are listed in Table 1.

The patient inclusion and exclusion criteria have previously been presented in detail.[4] To summarize, the inclusion criteria were being 18 to 85 years old and having a total score on the IBS Severity Scoring System (IBS-SSS) of $\geq$175. The exclusion criteria were the presence of a systemic disease; immune deficiency; being treated by immune-modulating medication such as Methotrexate, Azathioprine, Ciclosporin, tumor necrosis factor α inhibitors, and steroids; pregnancy or planning pregnancy; lactating; severe psychiatric disorders; alcohol or drug abuse; or use of probiotics, antibiotics, or IBS medications within 8 weeks before the start of the study.

Donor

The donor has previously been described in detail.[4] To summarize, he was a healthy male aged 36 years with a normal body mass index who was born via vaginal delivery, breastfed, a nonsmoker, was not taking any medication, was only treated a few times with antibiotics, exercised regularly, and consumed a sport-specific diet that was richer in protein, fiber, minerals, and vitamins than the average diet.[4] He was also not a first-degree relative of any recipient. He had high microbial diversity (normobiosis), and his fecal bacteria composition was different from that of 254 healthy subjects for 14 of the 48 tested bacterial markers.[4] His fecal bacteria composition was tested every 3 months and appeared to be stable over the period in which he donated his feces.[4] Fecal samples were taken from the donor at the baseline, and 2 and 3 years.

Fecal Sample Collection, Preparation, and Administration

The collection, preparation, and administration of feces has previously been described.[4] To summarize, fecal samples from the donor and patients were immediately frozen and kept at −20°C until they were delivered to the laboratory, where they were stored at −80°C. The patients were randomized into 3 groups: 30 g of own feces (placebo group), 30 g of donor feces, and 60 g of donor feces. The feces were thawed for 2 days at 4°C, manually mixed with 40 mL of sterile saline, filtered through a 110 cm × 10 cm nonwoven swab (OneMed, Helsinki, Finland), and administered to the distal duodenum via the working channel of a gastroscope.

Symptoms and Quality-of-Life Assessment

Symptoms were assessed using the IBS-SSS and the Birmingham IBS Symptom Questionnaire, and fatigue was

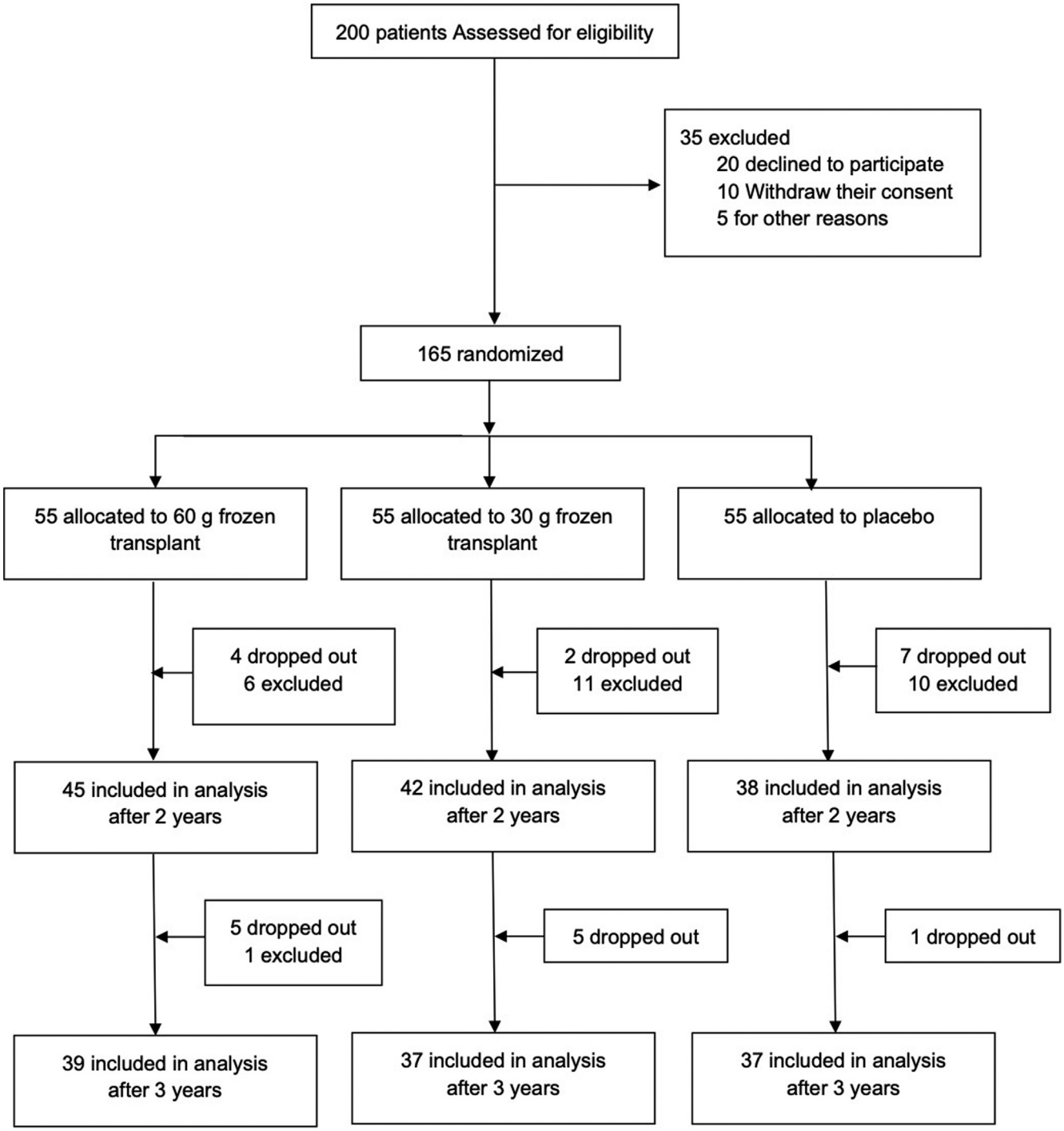

Figure 1. Enrollment flowchart and randomization of the patients.

measured using the Fatigue Assessment Scale (FAS) questionnaire.[5–7] Responders were patients who exhibited a reduction of $\geq$50 points on the IBS-SSS after FMT, whereas those with an IBS-SSS total score of $\leq$75 were considered in complete remissions.[5] Quality of life was measured using the IBS-Quality of Life (IBS-QOL) and Short-Form Nepean Dyspepsia Index (SF-NDI) questionnaires.[8,9] An increase in IBS-QOL, and a decrease in SF-NDI score indicated a quality-of-life improvement.

Bacterial Analysis

The fecal bacteria composition were analyzed using the GA-map Dysbiosis Test, a method using a predetermined targets approach, as described in detail previously.[10] To summarize, this test uses 16S ribosomal RNA polymerase chain reaction DNA amplification covering the variable regions V3 to V9 , followed by DNA probe hybridization of the predetermined 48 bacterial markers covering >300 bacteria at different taxonomic levels. The dysbiosis index (DI) was measured on a 5-

Table 1. Patient Characteristics at Baseline

	Overall	Placebo	30-g FMT	60-g FMT	*P*
Number	125	38	42	45	
Age, *y*	40.3 ± 13.2	42.3 ± 14.7	38.5 ± 12.4	39.7 ± 13.2	.6
Sex, female/male	104/21	34/4	31/9	37/8	.2
IBS-D	47	12	17	18	
IBS-C	46	16	15	15	.9
IBS-M	32	10	10	12	
Patients with severe IBS symptoms,[a] %	62	55	67	62	.6
IBS-SSS score	314.9 ± 76.3	300.3 ± 81.6	322.8 ± 64.1	315.3 ± 81.1	.5
Birmingham IBS-S score	25.1 ± 8.1	23.3 ± 8.1	26.7 ± 5.1	25.4 ± 6.8	.050
FAS score	31.1 ± 4.9	31.5 ± 4.5	30.8 ± 5.0	31.2 ± 4.5	.634
IBS-QOL score	113.5 ± 21.8	117.8 ± 20.2	109.8 ± 22.3	113.4 ± 22.4	.117
SF-NDI score	29.9 ± 7.2	29.1 ± 6.2	30.1 ± 7.2	30.5 ± 8.3	.728
DI	3.2 ± 1.1	3.1 ± 1.2	3.4 ± 1.0	3.2 ± 1.1	.743
Patients with dysbiosis	77.3%	74.4%	78.8%	78.6%	.735
PPI medication	58 (46.4)	21 (38.2)	19 (45.2)	18 (32.7)	.810
Birth control medication	81 (64.8)	25 (45.5)	26 (61.9)	30 (54.5)	.601
Antimigraine medication	12 (9.6)	3 (5.5)	5 (11.9)	4 (7.3)	.764
Medication against asthma/allergies	18 (14.4)	6 (10.9)	7 (16.7)	5 (9.1)	.829
Medication with levothyroxine	3 (2.4)	1 (1.8)	0 (0)	2 (3.6)	.361
Medication with heart/vascular drugs	6 (4.8)	3 (5.5)	2 (4.8)	1 (1.8)	.595

NOTE. Data are mean ± SD or n (%) values except where indicated otherwise.
PPI, proton-pump inhibitor.
[a]IBS-SSS total score of ≥300.

point scale from 1 (normal) to 5 (severe dysbiosis), where values of 1 and 2 indicate normobiosis, and those of 3 to 5 indicate dysbiosis.[10]

Retransplantation of Relapsed Patients

Ten patients who responded to FMT at 3 months after transplantation and relapsed 3 years after FMT, 5 from the 30-g group and 5 from the 60-g group received another transplant of 90 g of donor feces using the same methods. These patients comprised 7 women and 3 men aged 41.0 ± 17.7 years (mean ± SD). Diarrhea-predominant IBS (IBS-D), constipation-predominant IBS (IBS-C), and mixed-diarrhea-and-constipation IBS (IBS-M) were classified in 3, 4, and 3 patients, respectively. These patients completed the IBS-SSS, Birmingham IBS Symptom Questionnaire, FAS, IBS-QOL, and SF-NDI, and delivered a fecal sample both before and 3 months after retransplantation.

Outcomes

The primary endpoint was a reduction in the IBS-SSS total score of ≥50 points at 3 months following transplantation. The secondary endpoint was a change in the DI and intestinal bacterial profile.

Adverse Events and Medication

The patients were asked to record bowel habits and register any adverse events in a diary. They were also asked to continue use and record their consumption of rescue medications (polyethylene glycol and loperamide), which was permitted during the previous randomized, double-blind, and placebo-controlled study. They were also asked to record the use of other IBS medications. The patients continued using their medications, which are listed in Table 1.

Statistical Analysis

The differences in response, complete remission, sex, IBS subtypes, dysbiosis, and medication use were assessed using Fisher's exact test. Differences between patients at baseline and at 2 and 3 years after transplantation in IBS-SSS, Birmingham IBS Symptom Questionnaire, FAS, IBS-QOL, and SF-NDI scores, DI, bacterial fluorescence signals, and IBS subtypes were analyzed using the Kruskal-Wallis test and a posttest of Dunn's multiple comparisons. The nonparametric Mann-Whitney test was used to identify differences between responders and nonresponders, patients with moderate and severe IBS symptoms, and women and men. Scaled principal-components analysis of log-transformed bacterial fluorescence signals was

performed, with the results visualized using a different color for each group, together with ellipses that included 80% of the samples within each group. Targeted Maximum Likelihood Estimation was performed to test the effect of the dropouts and excluded patients on the outcome.

Ethics

The study was approved by the West Regional Committee for Medical and Health Research Ethics, Bergen, Norway (approval no. 2017/1197/REK vest). All subjects provided both oral and written consent before participating. This study was registered at www.clinicaltrials.gov (NCT03822299).

Results

Patients, Donor, and FMT Response

Three years after FMT, 11 patients had dropped out: 1 in the placebo group, 5 in the 30-g group, and 5 in the 60-g group. One patient from the 60-g group was excluded because of pregnancy (Figure 1). Targeted Maximum Likelihood Estimation showed that the dropouts and excluded patients did not affect the outcome of the study regarding symptoms, quality of life and DI (Supplementary Material).

The donor had a DI of 1 at baseline and at 2 and 3 years after FMT, indicating that bacterial abundance remained healthy (normal) over the follow-up period. The bacterial profile of the donor was stable throughout the observation period (Supplementary Figure 1). The abundance of 15 bacterial markers deviated from that of healthy subjects during this period (Supplementary Figure 2).

The response rates of the 30-g and 60-g groups were significantly higher than in the placebo group at 2 and 3 years after FMT (Figure 2). Figure 2 illustrates the complete remission rates of patients at 2 and 3 years after FMT for all groups.

Symptoms and Quality of Life

The total IBS-SSS scores in the 30-g and 60-g groups were significantly lower than those in the placebo group at both 2 and 3 years after FMT (Figure 3 and Supplementary Table 1). The scores for the 4 items in the IBS-SSS—abdominal pain, abdominal distension, bowel-habits dissatisfaction, and quality-of-life interference—were also significantly reduced compared with the placebo group at 2 and 3 years after FMT (Supplementary Table 1). The total scores on the Birmingham IBS Symptom Questionnaire in the 30-g and 60-g groups were significantly lower than those in the placebo group at 2 and 3 years after FMT (Figure 3 and Supplementary Table 2). The total FAS scores in the 30-g and 60-g groups were significantly lower than those in the placebo group at 2 and 3 years after FMT (Figure 3 and Supplementary Table 3).

The total IBS-QOL scores were significantly higher in the 30-g and 60-g groups than in the placebo group at 2 and 3 years after FMT (Figure 3 and Supplementary Table 4). The total SF-NDI scores were significantly lower in the 30-g and 60-g groups than in the placebo group at 2 and 3 years after FMT (Figure 3 and Supplementary Table 5).

Bacterial Analysis

DIs decreased significantly 2 and 3 years after FMT in the 30-g and 60-g groups, but not in the placebo group (Supplementary Figure 3). DI was positively correlated with the total IBS-SSS and FAS scores (Supplementary Figure 4). The proportion of patients with dysbiosis decreased significantly at 2 and 3 years after FMT in all groups (Supplementary Figure 5).

The fecal bacterial profiles of the placebo, 30-g, and 60-g groups changed over time (Figure 4). Although the fecal bacterial profiles were similar in all groups at baseline, it differed significantly at 2 and 3 years after FMT (Figure 4).

The fluorescence signals of 19 bacterial markers changed between baseline and 2 years after FMT in the placebo group (Supplementary Table 6). The fluorescence signals of 9 bacterial markers decreased, whereas the remaining 10 increased. The fluorescence signals of 22 bacterial markers changed between baseline and 3 years after FMT. The fluorescence signals of 10 bacterial markers decreased and those of the remaining 12 increased (Supplementary Table 6).

The fluorescence signals of 26 bacteria markers changed at both 2 and 3 years after FMT in the 30-g group (Supplementary Table 7). The fluorescence signals of 15 and 18 bacteria markers increased at 2 and 3 years after FMT, respectively, and those of 11 and 8 bacteria markers decreased at 2 and 3 years after FMT, respectively. Among these bacteria markers, 11 and 10 changed and did not change in the placebo group at 2 and 3 years after FMT, respectively (Supplementary Table 7).

The fluorescence signals of 27 and 24 bacteria markers changed at 2 and 3 years after FMT, respectively, in the 60-g group (Supplementary Table 8). Of these, the fluorescence signals of 15 and 13 increased at 2 and 3 years after FMT, respectively, and those of 12 and 11 decreased at 2 and 3 years after FMT, respectively. Of the bacteria markers that changed at 2 and 3 years after FMT, 12 and 11 did not change in the placebo group. The fluorescence signals of 7 bacteria markers increased at both 2 and 3 years after FMT. The fluorescence signals of 5 and 4 bacteria markers decreased at 2 and 3 years after FMT, respectively (Supplementary Table 8).

Correlations

Of the bacteria markers whose fluorescence signals changed in the 30-g and 60-g groups but not in the placebo group at both 2 and 3 years after FMT, 9 were significantly correlated with the total IBS-SSS scores (Figure 5). Six of these bacteria markers whose fluorescence signals increased after FMT were negatively correlated with the total IBS-SSS scores: *Alistipes*, *Bacteroides* spp, *Prevotella* spp, *Parabacteroides johnsonii*, *Firmicutes*, *Eubacterium biforme*, and *Faecalibacterium prausnitzii*. The remaining 3 bacteria markers, whose fluorescence signals decreased after FMT, were correlated positively with the total IBS-SSS scores: *Coprobacillus cateniformis*, *Streptococcus salivarius* spp *thermophilus*, and Enterobacteriaceae (Figure 5).

Ten bacteria markers whose fluorescence signals changed in the 30-g and 60-g groups after FMT, but not

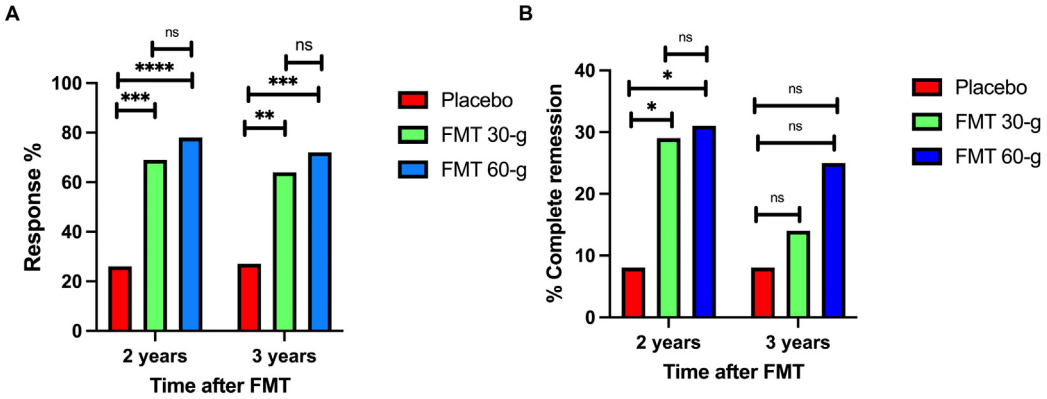

Figure 2. Response rates (*A*) and complete remission rates (*B*) in the placebo, 30-g, and 60-g groups at 2 and 3 years after FMT. ns, not significant; *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$.

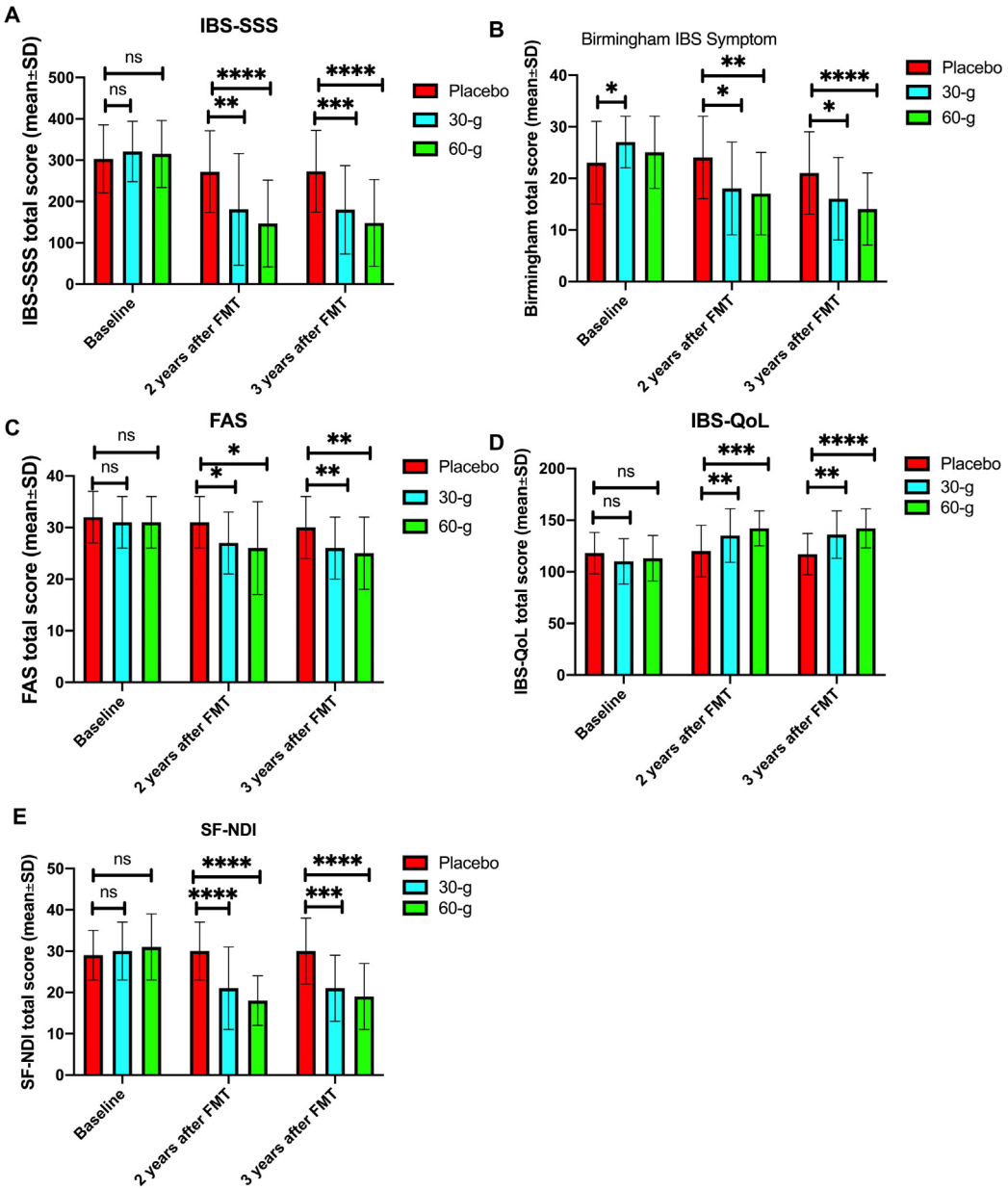

Figure 3. Total scores on the IBS-SSS (*A*), Birmingham IBS Symptom Questionnaire (*B*), FAS (*C*), IBS-QOL (*D*), and SF-NDI (*E*) in the placebo, 30-g, and 60-g groups at baseline and at 2 and 3 years after FMT. ns, not significant; *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$.

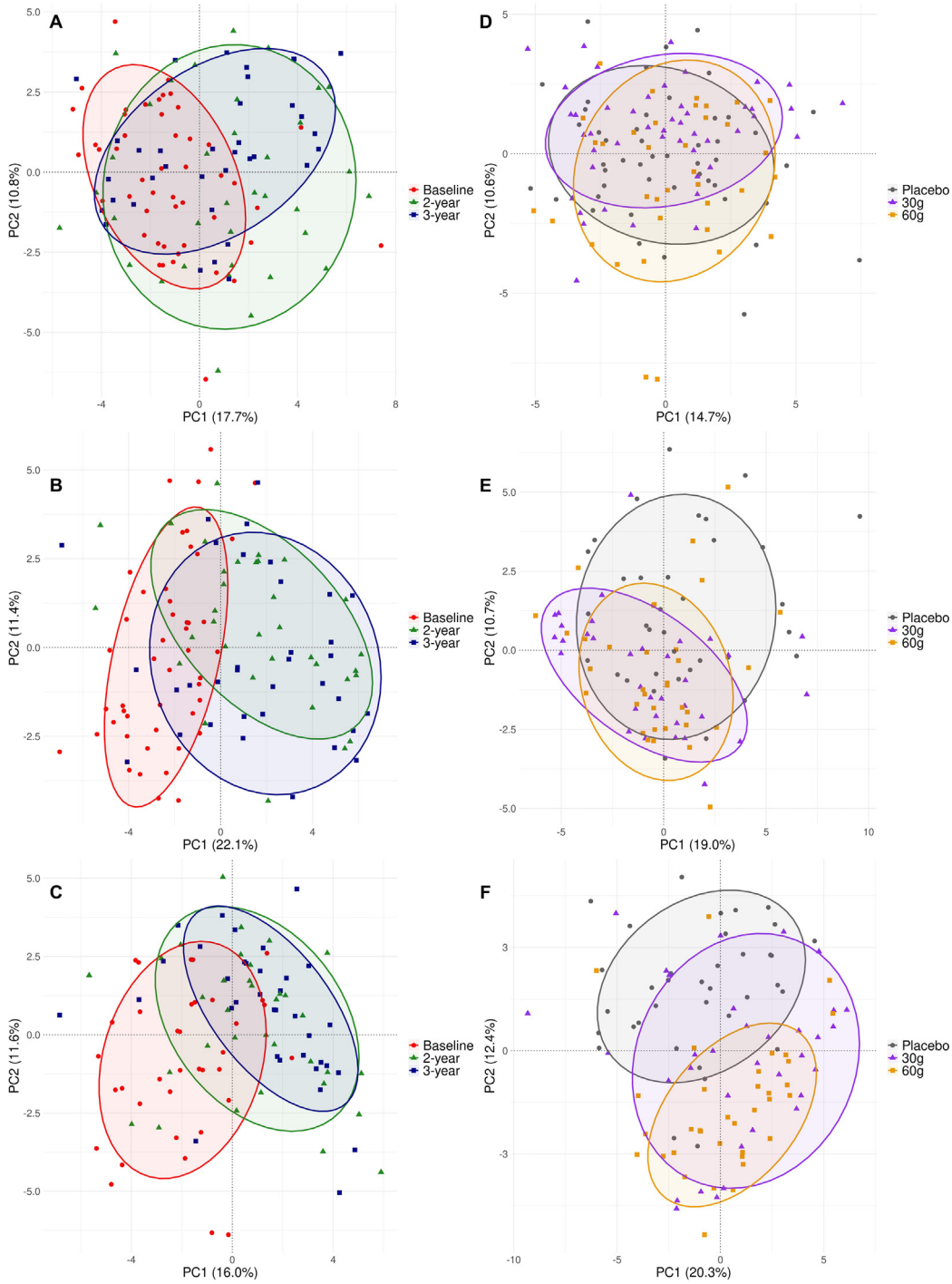

Figure 4. Results of the scaled principal-components analysis of the fecal bacterial profiles. The bacterial profiles of the placebo group (*A*), 30-g group (*B*), and 60-g group (*C*) changed over time. The changes in the 30-g and 60-g groups differed from those in the placebo group. At baseline, the bacterial profiles of the placebo, 30-g, and 60-g groups overlapped (*D*). Two (*E*) and 3 (*F*) years after FMT, the bacterial profiles of the 30-g and 60-g groups differed markedly from those of the placebo group.

in the placebo group, were significantly correlated with the total FAS scores (Figure 6). Seven bacteria markers whose fluorescence signals increased after FMT were correlated negatively with the total FAS scores. The other 3 whose fluorescence signals decreased after FMT were correlated positively with the total FAS scores (Figure 6).

Differences Between Responders and Nonresponders

Responders had significantly lower total scores on the IBS-SSS, Birmingham IBS Symptom Questionnaire, FAS, and SF-NDI, and higher total IBS-QOL scores than did non-responders at both 2 and 3 years after FMT (Supplementary Figure 6).

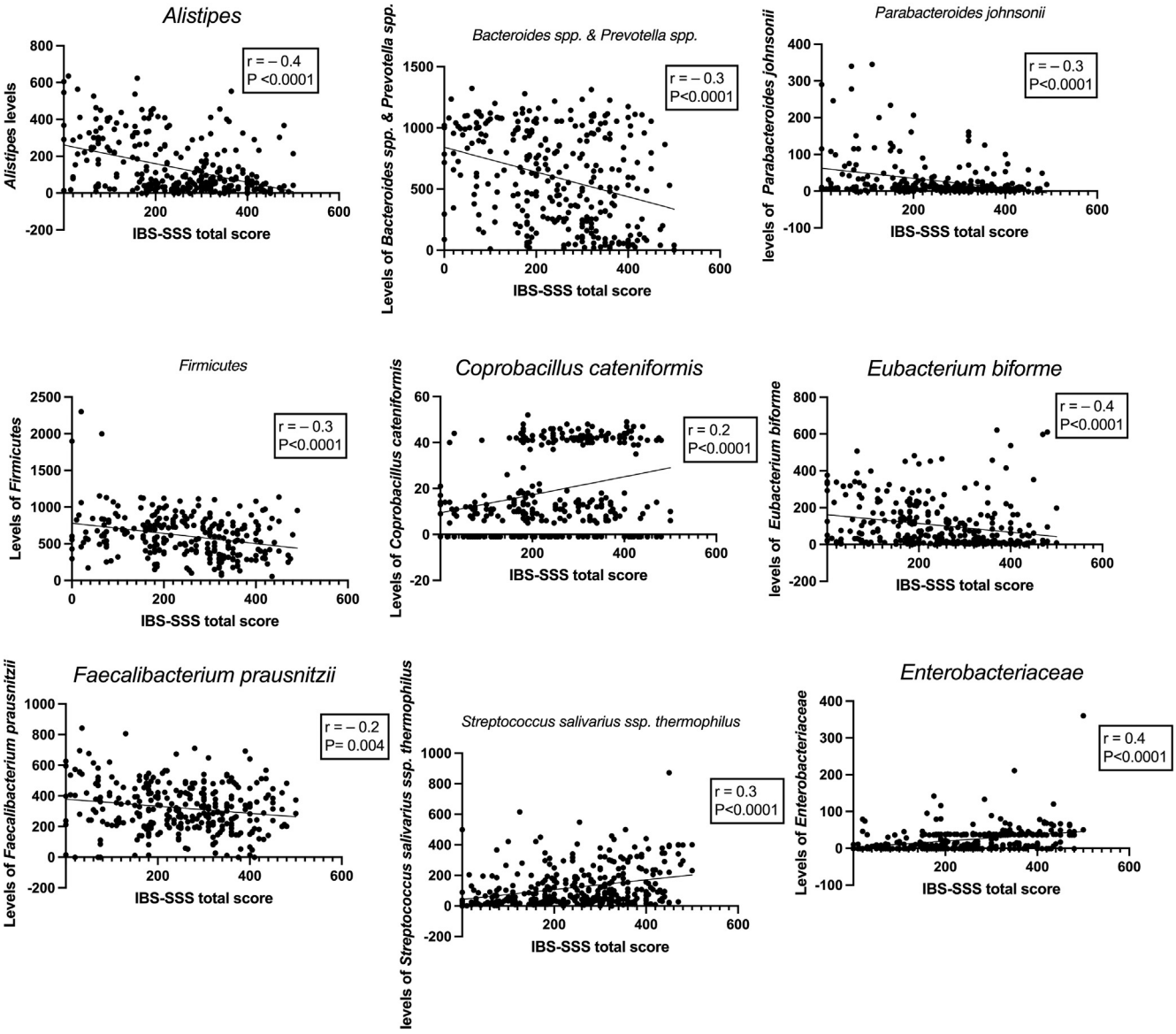

Figure 5. Fluorescence signals of 9 bacteria that changed significantly in both the 30-g and 60-g groups at 2 and 3 years after FMT correlated with the total IBS-SSS score.

The proportions of patients with dysbiosis among responders were 84%, 30%, and 37% at baseline and at 2 and 3 years after FMT, respectively; the corresponding values among nonresponders were 72%, 41%, and 39%. There was no significant difference between the proportion of patients with dysbiosis among responders and nonresponders at baseline and at 2 and 3 years after FMT (P = .2, .2, and >.999, respectively). There were no differences in DIs between responders and nonresponders at baseline, but the DI of responders was significantly lower than that of nonresponders at 2 and 3 years after FMT (Supplementary Figure 7).

The bacterial profiles of responders and nonresponders were similar at baseline but differed at 2 and 3 years after FMT (Supplementary Figure 8). At baseline, the fluorescence signals of *Lactobacillus* spp were 45.8 ± 87.6 and 60.4 ± 95.2 among responders and nonresponders, respectively. The fluorescence signal of *Lactobacillus* spp was significantly lower in responders than in nonresponders (P = .04). The fluorescence signals of 10 and 7 bacteria were significantly higher in responders than in nonresponders at 2 and 3 years after FMT, respectively (Supplementary Tables 9 and 10).

Differences Between Patients With Moderate and Severe IBS Symptoms

The proportion of patients with severe IBS symptoms in the 30-g and 60-g groups, but not in the placebo group, was significantly reduced at 2 and 3 years after FMT (Supplementary Table 11). The response rates of patients with severe and moderate IBS symptoms were 74% and 56%, respectively, at 2 years after FMT; the corresponding values for 3 years after FMT were 72% and 50%. Patients

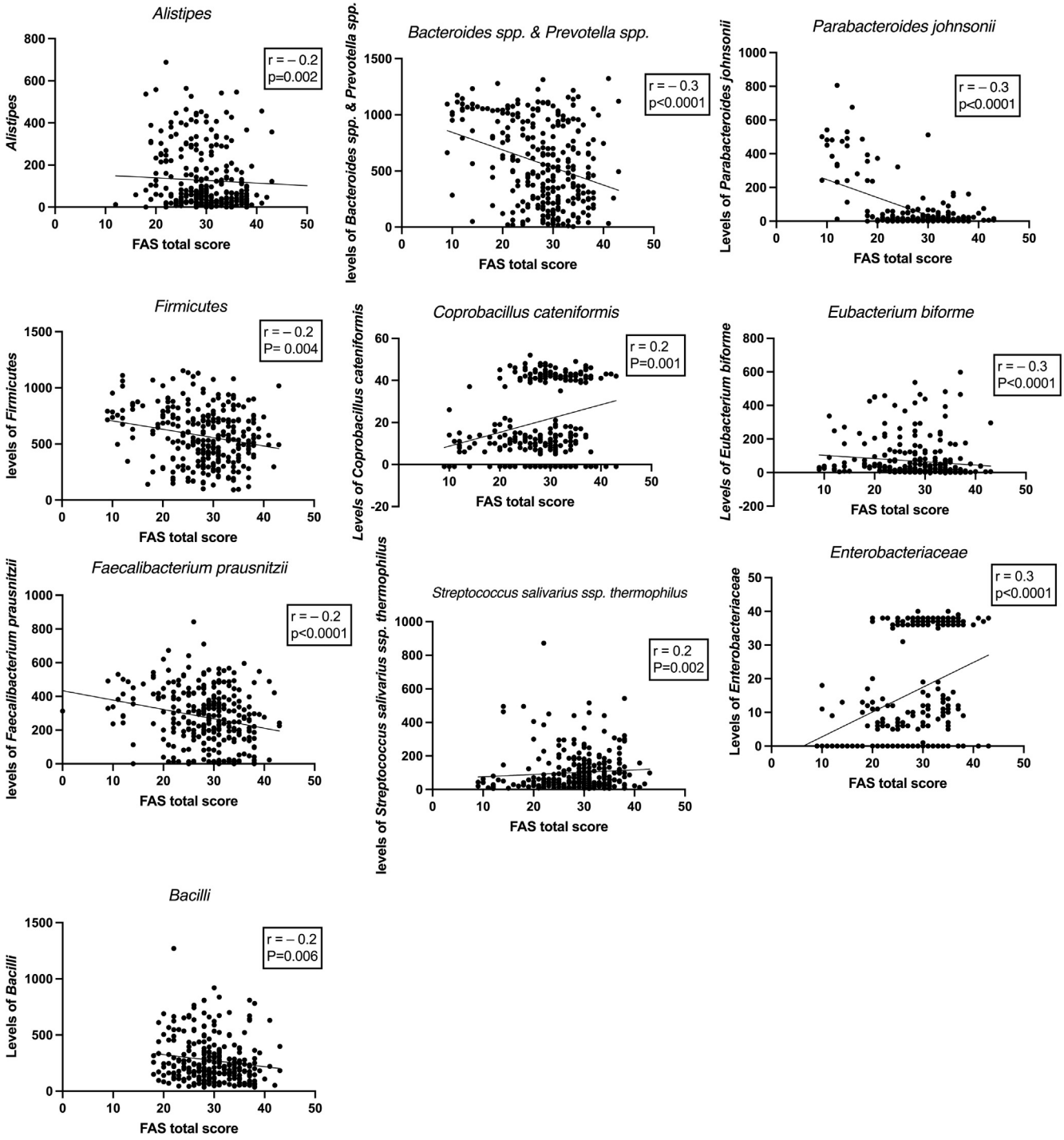

Figure 6. Among the bacteria whose fluorescence signals changed significantly at 2 and 3 years after FMT in the 30-g and 60-g groups, 10 were correlated with the total FAS score.

with severe IBS symptoms had significantly higher response rates both 2 and 3 years after FMT (P = .02 and .01, respectively).

The total IBS-SSS and Birmingham IBS Symptom Questionnaire scores were higher in patients with severe IBS symptoms than in those with moderate IBS symptoms at baseline. The difference between these scores decreased over time between 2 and 3 years after FMT, and did not differ at 3 years after FMT (Supplementary Figure 9). The total scores on the IBS-QOL and SF-NDI were lower and higher, respectively, in patients with severe IBS symptoms than in those with moderate IBS symptoms at baseline. However, there was no difference between the scores of patients with severe and moderate symptoms at 2 and 3 years after FMT (Supplementary Figure 9). Total FAS scores did not differ between

patients with severe and moderate IBS symptoms at all observation intervals.

Although the DIs of patients with severe and moderate IBS symptoms did not differ at baseline, that of patients with severe IBS symptoms was lower than that of patients with moderate IBS symptoms at 2 and 3 years after FMT (Supplementary Figure 10).

The fecal bacterial profiles of patients with moderate and severe IBS symptoms exhibited a varied overlap over time after FMT (Supplementary Figure 11). At baseline, the fluorescence signals of *Clostridia* in patients with moderate and severe IBS symptoms were 411.6 ± 56.9 and 385.6 ± 41.4, respectively. The fluorescence signals of *Clostridia* were significantly higher in patients with moderate IBS symptoms than in patients with severe IBS symptoms ($P = .01$). The fluorescence signals of *Firmicutes* in patients with moderate and severe IBS symptoms were 576.1 ± 267.6 and 735.0 ± 268.2, respectively. Patients with severe IBS symptoms had a significantly higher fluorescence signal for *Firmicutes* than did patients with moderate IBS symptoms ($P = .01$). The fluorescence signals of *Eubacterium siraeum* and *Lactobacillus* spp were 310.1 ± 153.8 and 20.8 ± 24.3 in patients with moderate IBS symptoms, respectively; the corresponding values for patients with severe IBS symptoms were 402.6 ± 146.3 and 11.1 ± 15.9. Patients with severe IBS symptoms had a significantly higher fluorescence signal for *Eubacterium siraeum*, and lower signals of *Lactobacillus* spp than did patients with moderate IBS symptoms ($P = .01$, and .03, respectively). There were no significant differences in the remaining tested bacteria between patients with moderate and severe IBS symptoms at baseline and at 2 and 3 years after FMT.

Sex Differences

Women had higher response rates than men at both 2 and 3 years after FMT (Supplementary Figure 12). There were no differences between the complete remission rates of women and men at both 2 and 3 years after FMT (Supplementary Figure 12).

The total IBS-SSS, Birmingham IBS Symptom Questionnaire, FAS, IBS-QOL, and SF-NDI scores did not differ between women and men at baseline and at 2 and 3 years after FMT (Supplementary Figure 13).

There was no significant difference between the DIs of women and men at baseline and at 2 and 3 years after FMT (Supplementary Figure 14). The fecal bacterial profile of women and men overlapped at all time points after FMT (Supplementary Figure 15). The fluorescence signals of 11 bacterial markers differed significantly between female and male patients with IBS at baseline (Supplementary Table 12). The fluorescence signals of 4 bacteria differed significantly between female and male patients with IBS at 2 years after FMT (Supplementary Table 13). The levels of 5 bacteria differed significantly between female and male patients with IBS at 3 years after FMT, including bacteria that had already changed from baseline (Supplementary Table 14).

Differences Between IBS Subtypes

The response rates in patients with IBS-D and IBS-M were significantly higher than in those with IBS-C at 2 years after FMT (Supplementary Figure 16). The response rates of patients with IBS-D did not differ significantly from those of patients with IBS-C and patients with IBS-M at 3 years after FMT. The complete remission rate of IBS-D did not differ significantly from those of IBS-C and IBS-M at 2 and 3 years after FMT (Supplementary Figure 16).

The total IBS-SSS, Birmingham IBS Symptom Questionnaire, FAS, IBS-QOL, and SF-NDI scores did not differ between patients with IBS-D, IBS-C, and IBS-M at baseline and at 2 and 3 years after FMT (Supplementary Figure 17).

DIs did not differ between patients with IBS-D, IBS-C, and IBS-M at baseline and at 2 and 3 years after FMT (Supplementary Figure 18)

The fecal bacteria profiles of the IBS subtypes changed over time and exhibited different overlaps at baseline and at 2 and 3 years after FMT (Supplementary Figure 19). The fluorescence signals of *Streptococcus* spp at baseline were 30.1 ± 38.4, 62.3 ± 15.7, and 69.4 ± 24.6 in IBS-D, IBS-C, and IBS-M, respectively, and here were significantly lower in IBS-D than in IBS-C and IBS-M ($P = .0002$ and $<.0001$, respectively). The fluorescence signals of *Veillonella* spp were 325.6 ± 81.3, 297.5 ± 66.1, and 260.7 ± 74.1 in IBS-D, IBS-C, and IBS-M, respectively, and hence were significantly lower in IBS-M than in IBS-D ($P = .01$). With the exception of *Alistipes*, there was no significant difference between the IBS subtypes regarding the fluorescence signals of all tested bacteria at 2 and 3 years after FMT. At 3 years after FMT, the fluorescence signals of *Alistipes* were 279.7 ± 173.9, 298.9 ± 153.5, and 158.5 ± 106.3 in IBS-D, IBS-C, and IBS-M, respectively, and hence were significantly lower in IBS-M than in IBS-D and IBS-C ($P = .02$ and .01, respectively).

Retransplantation of Relapsed Patients

Eight of the 10 retransplanted patients (80%) responded to FMT at 3 months after retransplantation. IBS symptoms were significantly reduced at 3 months after retransplantation as assessed by IBS-SSS and the Birmingham IBS Symptom Questionnaire (Supplementary Figure 20). However, the total FAS, IBS-QOL, and SF-NDI scores did not differ significantly between before and after retransplantation (Supplementary Figure 20).

The DIs before and 3 months after retransplantation were 2.4 ± 0.9 and 1.4 ± 0.7, respectively, and hence were significantly lower at 3 months after FMT than before retransplantation ($P = .03$). The proportions of patients with IBS with dysbiosis were 50% and 10% before and 3 months after retransplantation, respectively, with no significant difference between before and after retransplantation. Fecal bacterial profiles changed considerably at 3 months after retransplantation (Supplementary Figure 21). The levels of 5 bacteria changed significantly 3 months after retransplantation (Supplementary Table 15).

Adverse Events Diet and Medication

Mild intermittent abdominal pain, diarrhea, and constipation were reported within the first 2 days after FMT (Supplementary Table 16), but no other adverse events were reported during the follow-up period. Two patients who developed diverticulitis at 2 and 3 months after FMT did not experience any new diverticulitis attacks during the follow-up period.[4] The patients consumed average Norwegian diet before and after FMT.

Among the patients in the placebo group, 32 regularly used medication to counteract IBS symptoms. These included loperamide, polyethylene glycol, and bulking agents (psyllium or ispaghula). The remaining 6 patients used linaclotide (n = 4) or prucalopride (n = 2).

In the 30-g group, 2 patients used loperamide once and 1 used polyethylene glycol twice. Three patients used loperamide, another 3 used polyethylene glycol, and 6 used loperamide and polyethylene glycol regularly.

In the 60-g group, 4 patients used loperamide, 2 used polyethylene glycol, and another 4 used both loperamide and polyethylene glycol regularly.

Discussion

The present study found that most patients with IBS maintained their response at 3 years after receiving FMT. Furthermore, those who received FMT had significantly reduced abdominal symptoms and fatigue, and an increased quality of life at 2 and 3 years after FMT. Except for the placebo group, the proportion of FMT-treated patients with severe IBS symptoms was also significantly lower at 2 and 3 years after FMT. It showed further that patients who relapsed within 3 years subsequently responded to FMT on retransplantation, which is consistent with earlier observations.[11] It is therefore reasonable to conclude that the FMT protocol for IBS as used in the present study resulted in high response rates and had durable long-term effects.[4]

The protocol used in our study differs in several aspects from those used in the 6 other RCTs of FMT on patients with IBS.[2] In the present study, donor selection was based on clinical criteria and a specific fecal microbial signature.[4] The clinical criteria were based on factors that are considered to positively or negatively affect intestinal microbiota and on the intestinal bacterial profile and diversity of the donor, which were compared with those of healthy subjects to identify possible deviations.[2] Moreover, the bacterial profile of the donor was stable over time, a criterium that was found to be associated with an effective donor.[4,11] In our protocol, fecal samples from both the donor and patients were immediately frozen, delivered frozen, and were kept frozen until transplantation was performed. Fecal samples were thawed at 4°C, mixed, and filtered manually. The human intestine is dominated by strictly anaerobic bacteria, and freezing and keeping them at 4°C for a short period protects their viability and growth.[12,13] Freezing the transplant from the donor and the fecal samples from the patients could have preserved the bacteria within the fecal samples, while keeping and transporting fecal samples in normal air could damage these bacteria. The findings of the present study support this assumption, in that 6 of the 10 bacteria that appeared to affect IBS symptoms and fatigue are anaerobic: *Alistipes*, *Parabacteroides johnsonii*, *Bacteroides* spp, *Prevotella* spp, *Eubacterium biforme*, *Faecalibacterium prausnitzii*, and *Coprobacillus cateniformis*.[14–18] Mixing the transplant from the donor manually without using mixing devices may have improved the preservation of its bacterial content. Finally, the transplant was administered into the small intestine. Whether administering the fecal transplant to the small or large intestine is more effective is yet to be determined, but in the 2 RCTs with the most-positive outcomes, the fecal transplant was administered to the small intestine.[4,11]

The findings that the bacterial profiles and proportions of patients with dysbiosis in both the placebo and FMT-treated groups changed over time indicate that intestinal microbiota is a dynamic time-varying process. Microbiota dysbiosis is believed to be an important contributing factor to IBS etiology.[19] However, its relevance in IBS has recently been questioned.[20,21] It seems that the degree of dysbiosis, rather than its presence or absence, is the clinically relevant factor. This assumption is supported by our finding that DIs decreased in FMT-treated patients but not in placebo patients, and that the proportion of patients with dysbiosis did not differ between the placebo and FMT groups. This assumption is also supported by the observation that DI was positively correlated with the total IBS-SSS and FAS scores.

In the active treatment groups, 9 bacteria whose fluorescence signals changed after FMT were correlated with the total IBS-SSS and FAS scores. One bacterium whose fluorescence signals changed in the active treatment group was also correlated with total FAS scores. The 7 bacteria with increased fluorescence signals were negatively correlated with the total IBS-SSS and FAS scores. The remaining 3 bacteria with decreased fluorescence signals were positively correlated with the total IBS-SSS and FAS scores. The importance of these 10 bacteria in improving IBS symptoms and fatigue is supported by the observation that fluorescence signals of 6 of these bacteria were higher in responders than in nonresponders. Furthermore, in patients with IBS, *Alistipes*, *Faecalibacterium prausnitzii*, and *Parabacteroides* levels have been reported to decrease, whereas Enterobacteriaceae levels increase.[22–25]

The roles that the previously mentioned bacteria play in IBS symptom manifestation and fatigue are yet to be determined. However, *Alistipes* appears to play a significant role in several diseases, such as depression, anxiety, chronic fatigue syndrome, autism, cirrhosis, and aging.[14] *Faecalibacterium prausnitzii* is the most important butyrate producer in the human colon, and *Eubacterium biforme* produces large amounts of butyric acid.[16,17,26–28] Notably, within the same cohort, fecal butyric acid levels were significantly increased at 1 month and 1 year after FMT in both the 30-g and 60-g groups.[29,30] Butyrate provides colonic epithelial cells with energy, affects the immune response, modulates colonic hypersensitivity, and decreases intestine-cell permeability and intestinal motility.[2]

The authors of a recently published RCT on FMT for patients with refractory IBS concluded that the successful

application of FMT is subset-dependent.[11] The present study found that all categories of patients with IBS benefited from FMT, but differences were detected between these categories. Female patients have been reported to respond better than male patients at 3 months after FMT.[11,31] The present study found that at 2 and 3 years after FMT, women still responded better than men. It is difficult to determine whether this difference is caused by psychosocial or biological factors. Patients with severe IBS symptoms had higher FMT response rates and lower DIs than those with moderate IBS symptoms in the present study. Our previous RCT found no differences in FMT response between the IBS subtypes at 3 months after FMT.[4] The observation that patients with IBS-C had a lower FMT response rate than those with IBS-D and IBS-M at 2 years after FMT is difficult to interpret because there were no differences between the IBS subtypes at 3 years after FMT.

Concerns have been raised about the safety of FMT for IBS, considering that IBS is a benign condition.[32–34] Discussion on the safety issues of FMT as an intervention for gastrointestinal and nongastrointestinal diseases was prompted by a report of 2 patients who experienced serious adverse events after FMT for indications other than IBS, which resulted in 1 fatality.[35,36] Moreover, some concerns on the long-term side effects in patients who received FMT to treat *Clostridium difficile* infection have also been discussed.[3] The short-term adverse events observed after FMT in patients with IBS were mild and self-limiting gastrointestinal symptoms.[2] The present finding that the FMT-treated patients with IBS had no further adverse events after a 3-year follow-up period indicates that FMT is a safe intervention. However, it should be remembered that the patients included in this study did not have a systemic disease or immune deficiency.

Major strengths of this study are that it included a relatively large IBS patient cohort comprising 3 IBS subtypes and used a single well-defined donor. However, limitations of this study are that it did not include the fourth IBS subtype, IBS-U, and it only investigated a part of the intestinal bacterial contents.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2022.06.020.

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Christina Casen, PhD (Formal analysis: Equal; Investigation: Equal; Writing – review & editing: Equal).
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Conflict of Interest
The authors disclose no conflicts.

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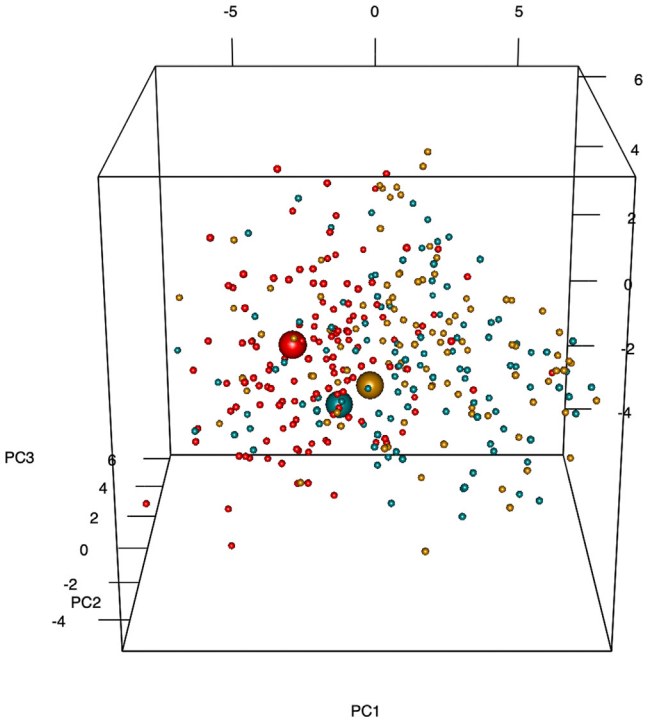
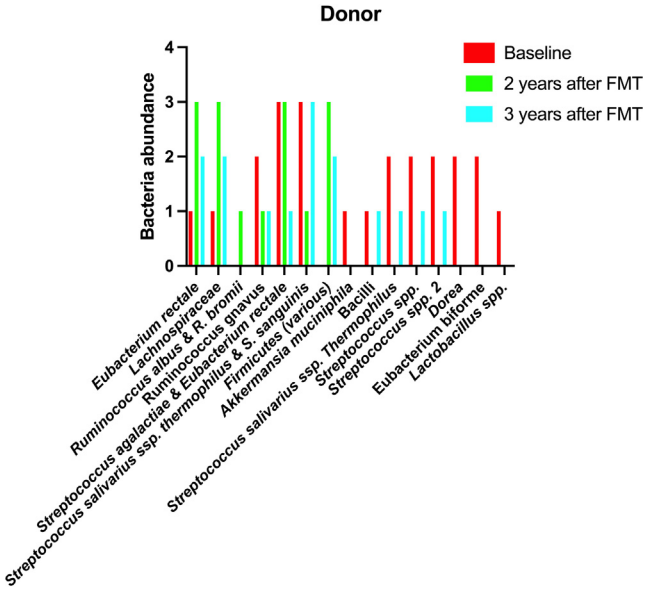
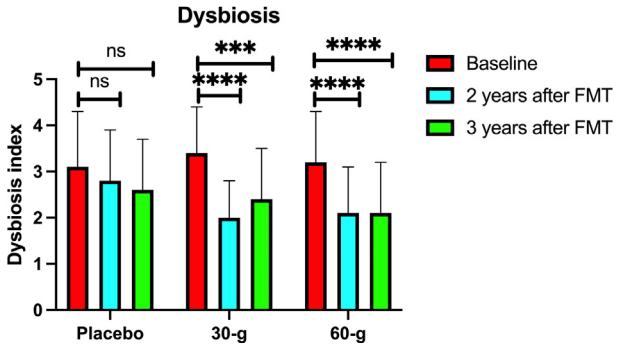
Data Availability
Data presented in this study are available from the corresponding author on request.



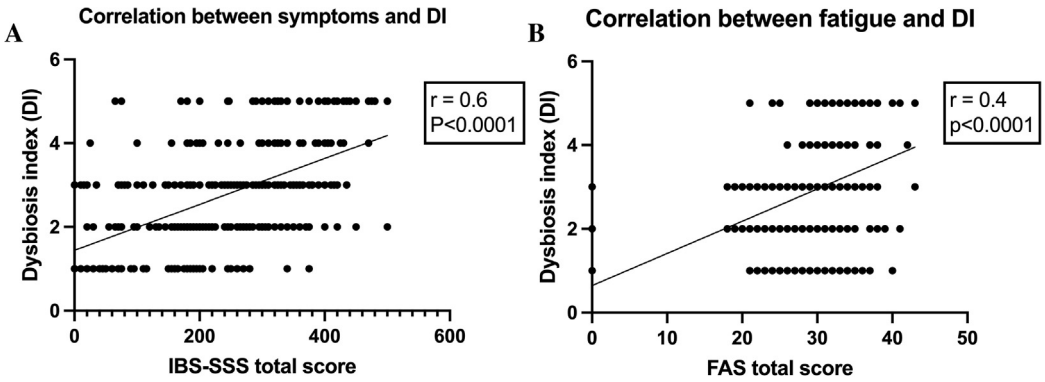
Supplementary Figure 1. Scaled principal-components analysis plot of fecal samples from the donor and patients at baseline (*red*) and at 2 years (*yellow*) and 3 years (*turquoise*) after FMT. The donor and patient samples are represented by *large* and *small circles*, respectively. The donor samples are closely grouped and were very similar over the follow-up period to those at baseline.



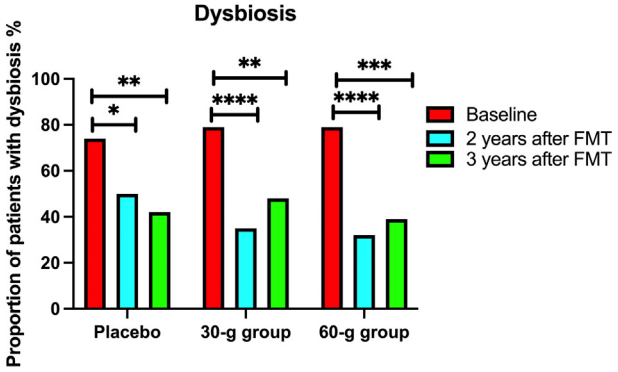
Supplementary Figure 2. Fecal bacterial profiles of the donor at baseline and at 2 and 3 years after FMT. The fecal bacterial profile of the donor deviated from that of healthy subjects for 15 of the 48 bacterial markers. The level of a healthy subject was 0.



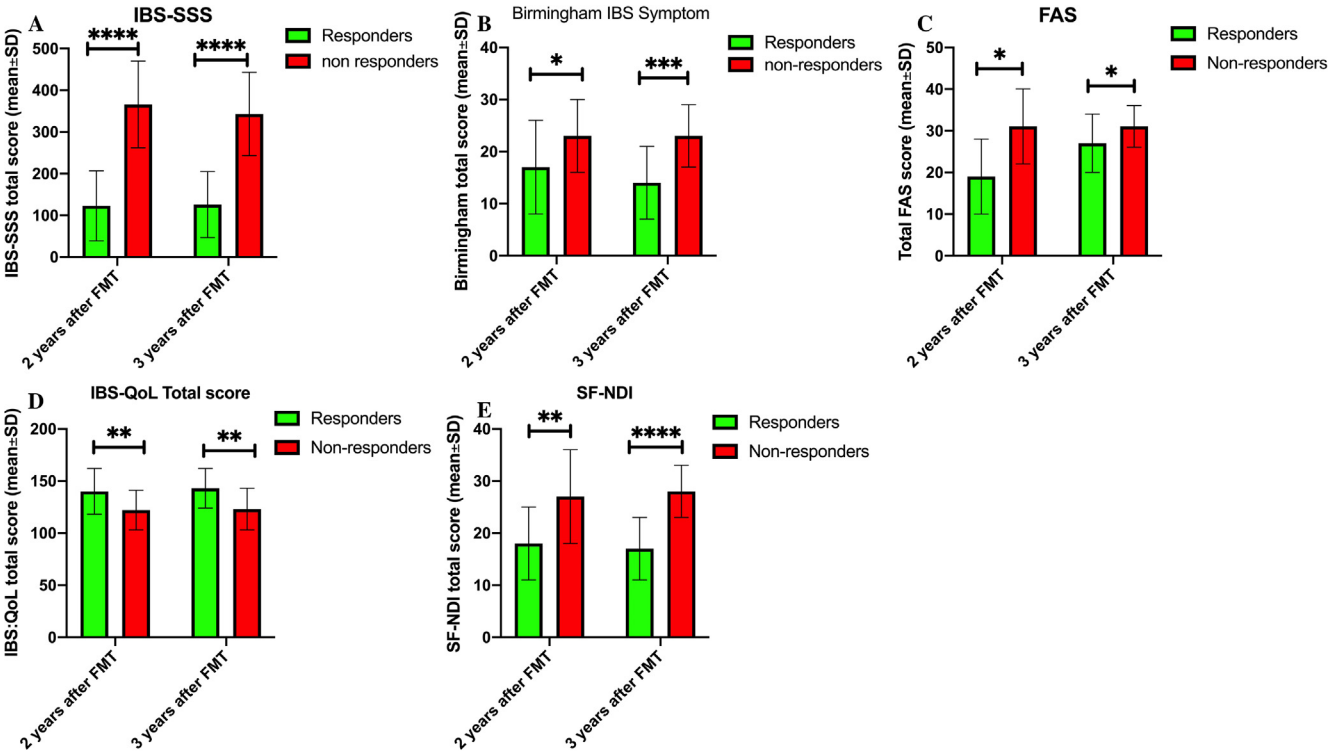
Supplementary Figure 3. DIs of the placebo, 30-g, and 60-g groups at baseline and at 2 and 3 years after FMT. ns, not significant; ***$P < .001$; ****$P < .0001$.



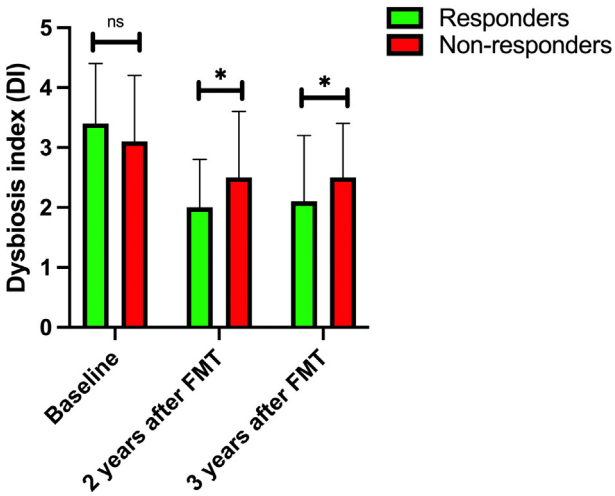
Supplementary Figure 4. Correlations between DI and total IBS-SSS (*A*) and FAS scores (*B*).



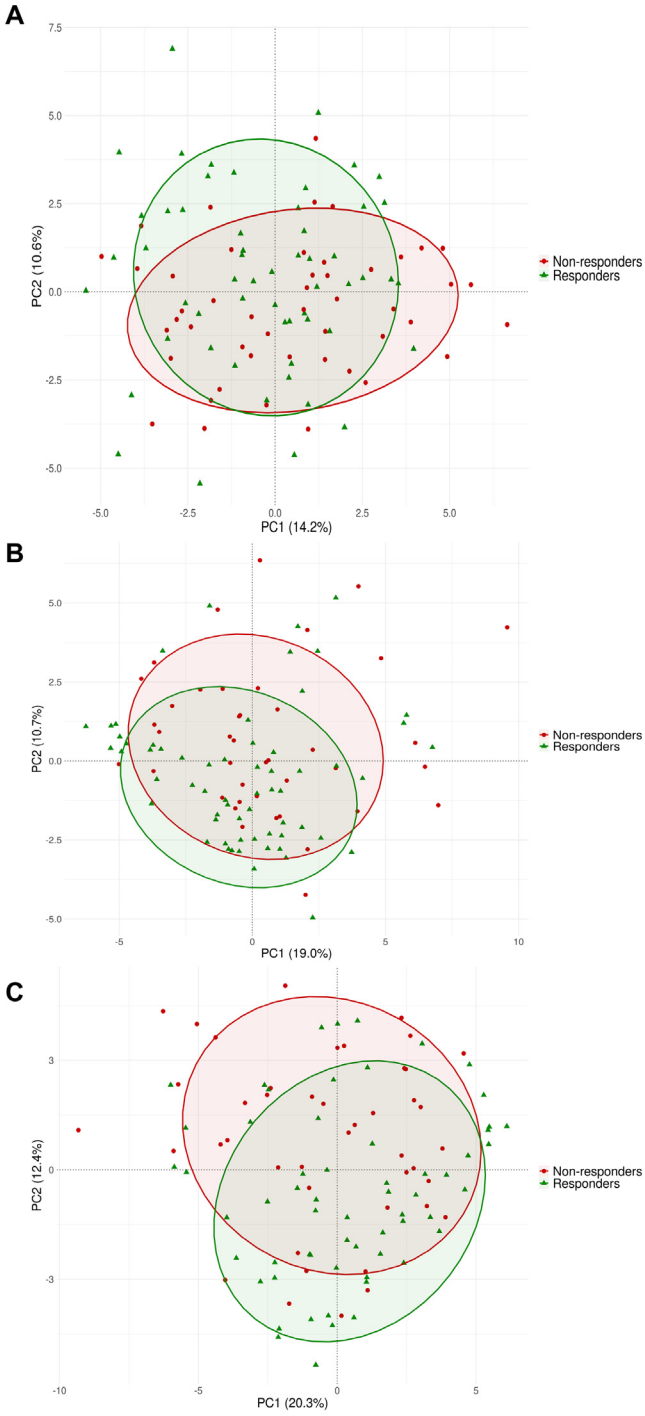
Supplementary Figure 5. Proportions of patients with dysbiosis in the placebo, 30-g, and 60-g groups at baseline and at 2 and 3 years after FMT. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$.



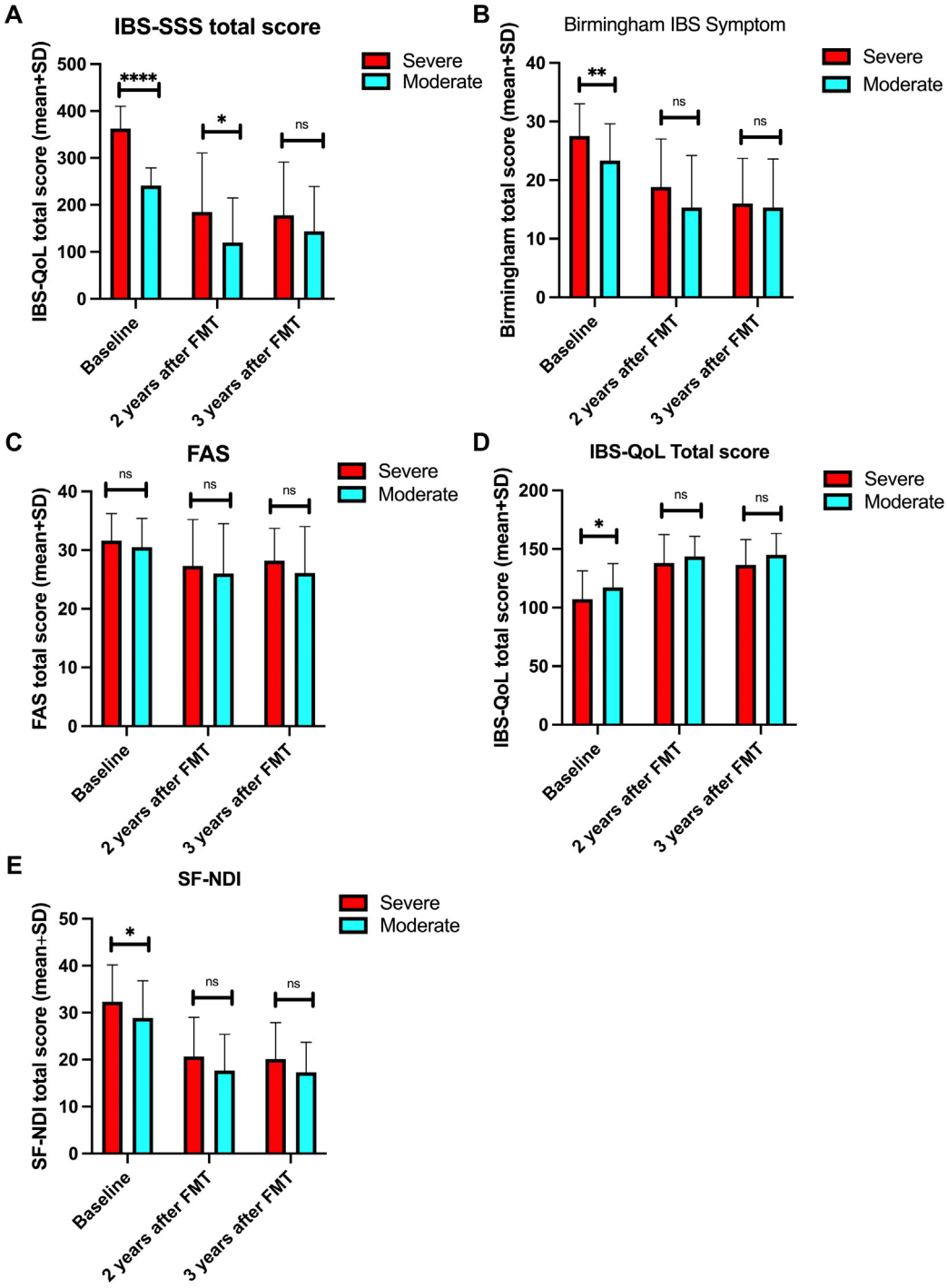
Supplementary Figure 6. Differences between responders and nonresponders in total scores on the IBS-SSS (*A*), Birmingham IBS Symptom Questionnaire (*B*), FAS (*C*), IBS-QOL (*D*), and SF-NDI (*E*). $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$.



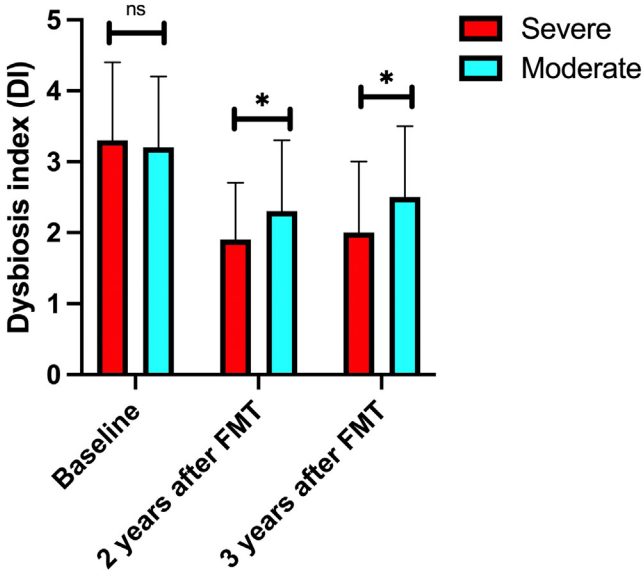
Supplementary Figure 7. DIs of responders and non-responders at baseline and at 2 and 3 years after FMT. ns, not significant; $^{*}P < .05$.



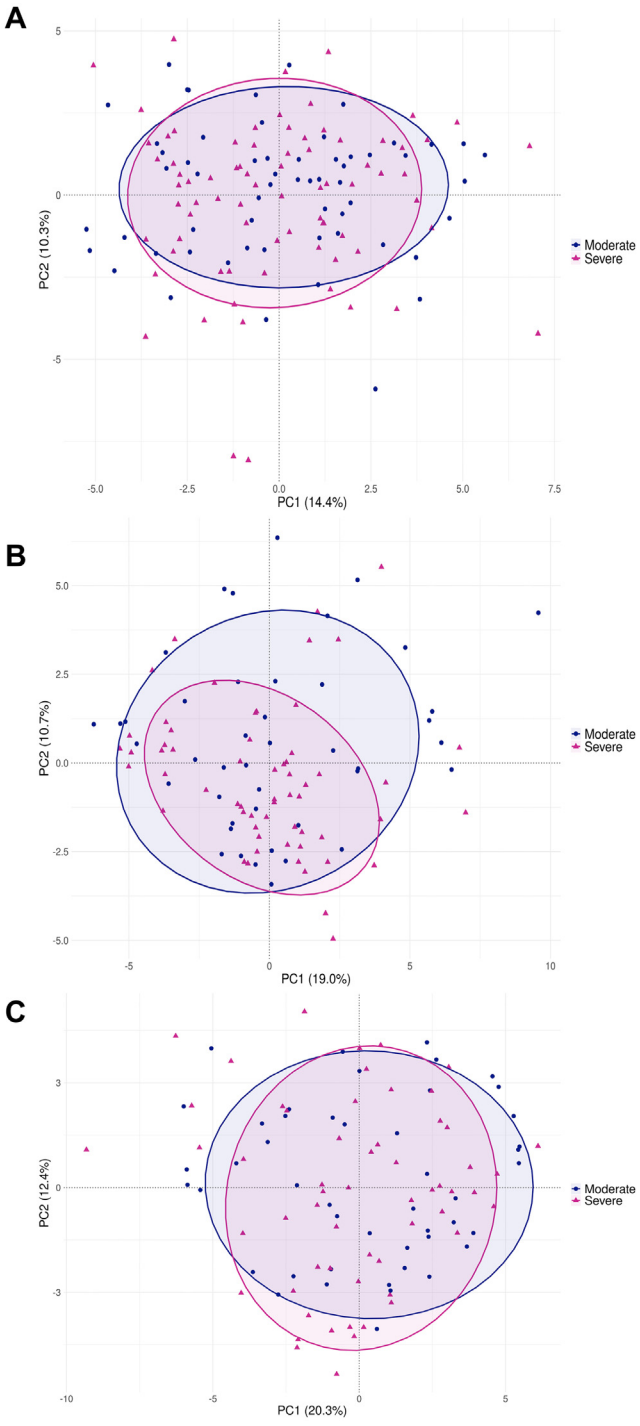
Supplementary Figure 8. Scaled PCA plot of the fecal bacterial profiles of the responders and nonresponders at baseline (*A*) and at 2 years (*B*) and 3 years (*C*) after FMT. The overlap between responders and nonresponders changed at 2 and 3 years after FMT.



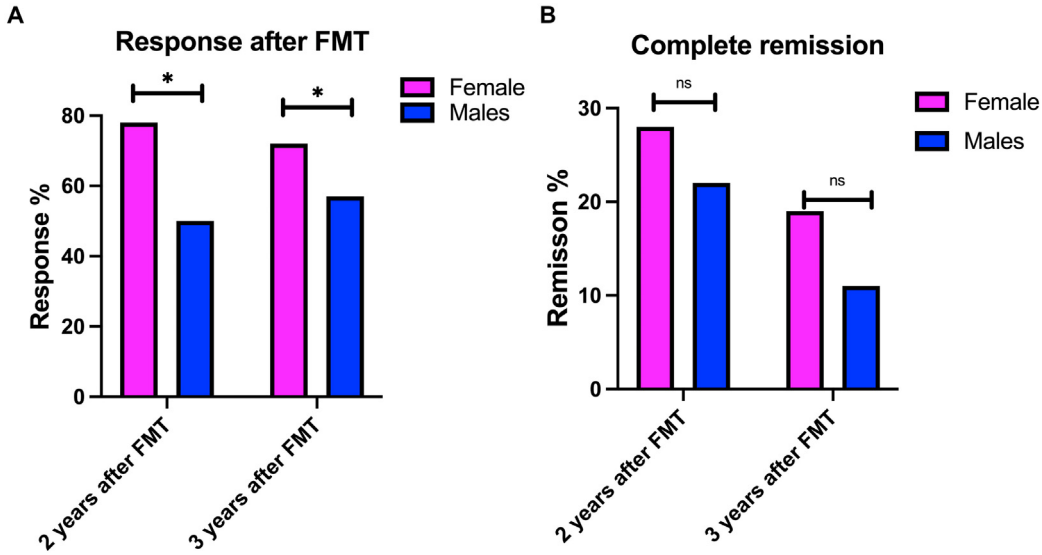
Supplementary Figure 9. Total scores on the IBS-SSS (*A*), Birmingham IBS Symptom Questionnaire (*B*), FAS (*C*), IBS-QOL (*D*), and SF-NDI (*E*). ns, not significant; *$P < .05$; **$P < .001$; ****$P < .0001$.



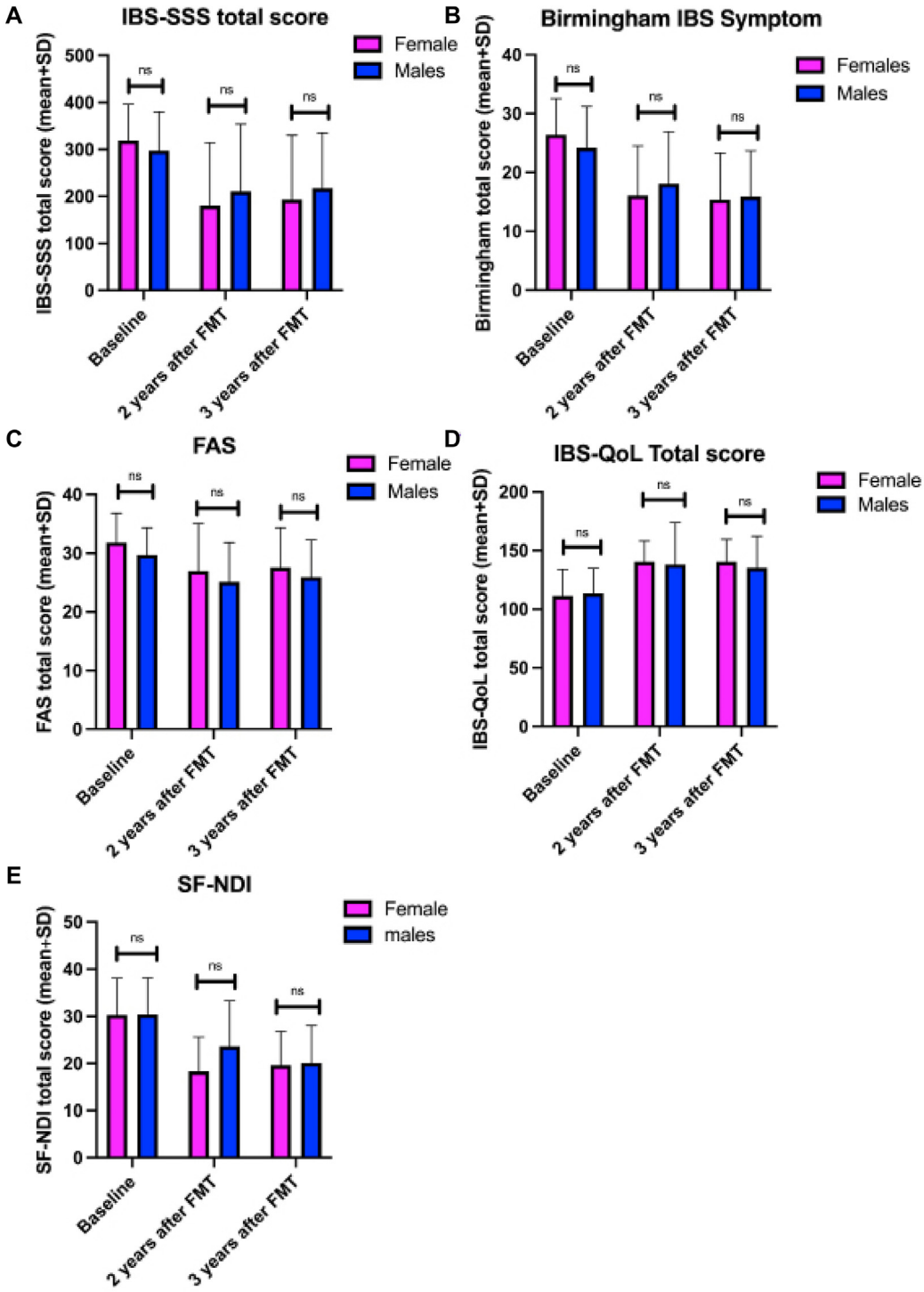
Supplementary Figure 10. DIs of patients with moderate (IBS-SSS total score of ≥175) and severe (IBS-SSS total score of ≥300) IBS symptoms at baseline and at 2 and 3 years after FMT. ns, not significant; *$P < .05$.



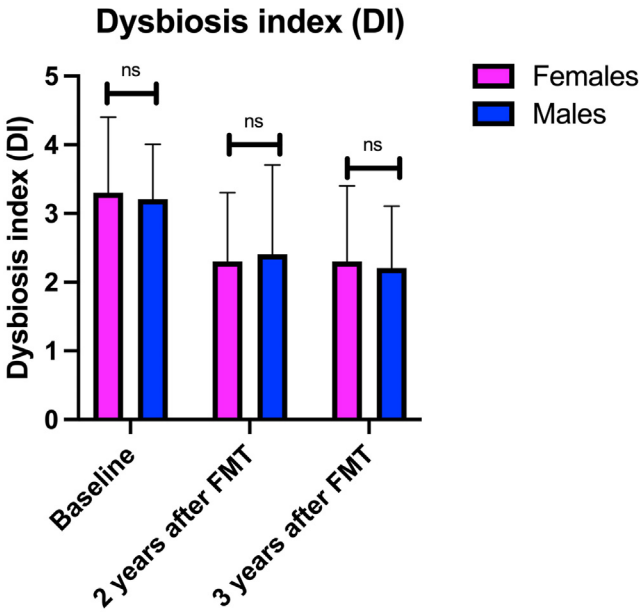
Supplementary Figure 11. Scaled principal-components analysis plot of patients with IBS with moderate and severe symptoms at baseline (*A*) and at 2 years (*B*) and 3 years (*C*) after FMT.



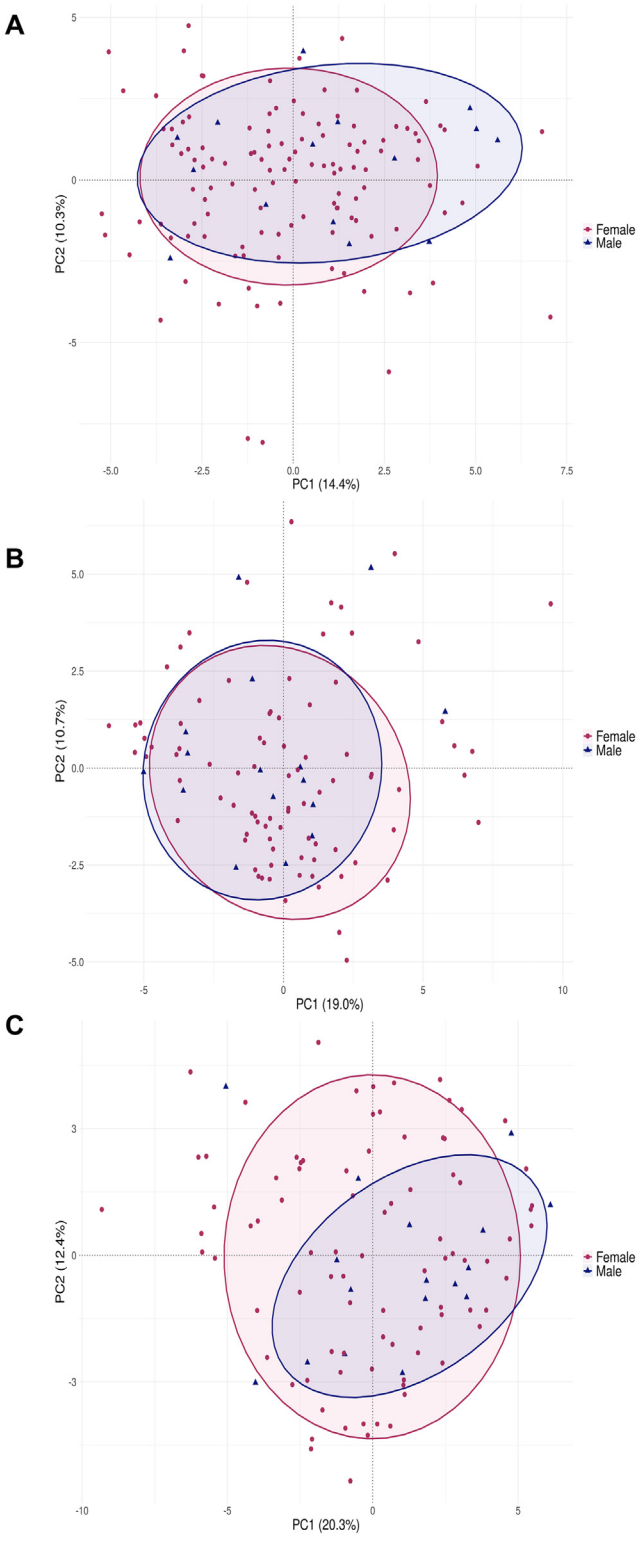
Supplementary Figure 12. FMT response rates (*A*) and complete remission rates (*B*) of female and male patients with IBS. ns, not significant; *$P < .05$.



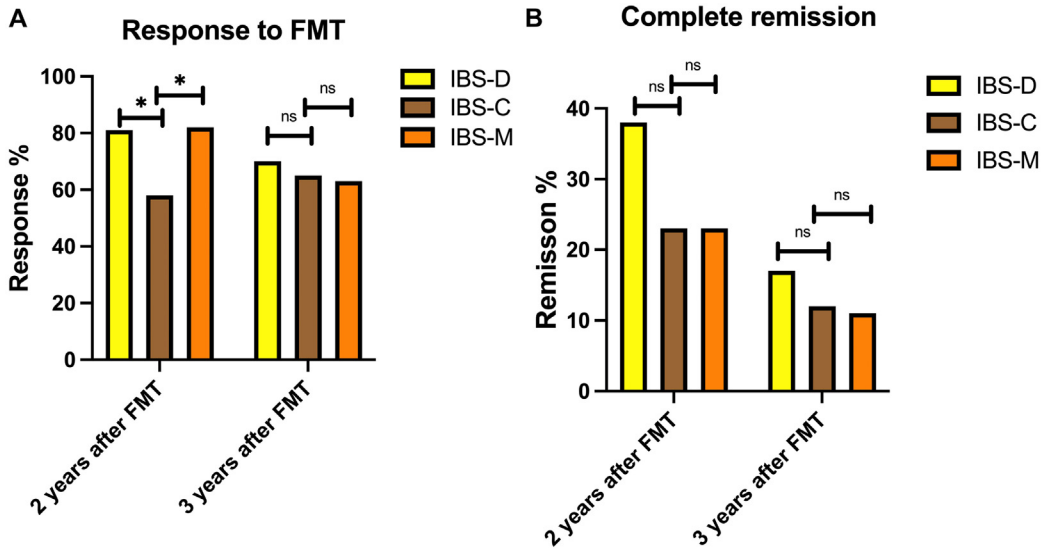
Supplementary Figure 13. Total scores on the IBS-SSS (*A*), Birmingham IBS Symptom Questionnaire (*B*), FAS (*C*), IBS-QOL (*D*), and SF-NDI (*E*) for female and male patients with IBS at baseline and at 2 and 3 years after FMT. ns, not significant.



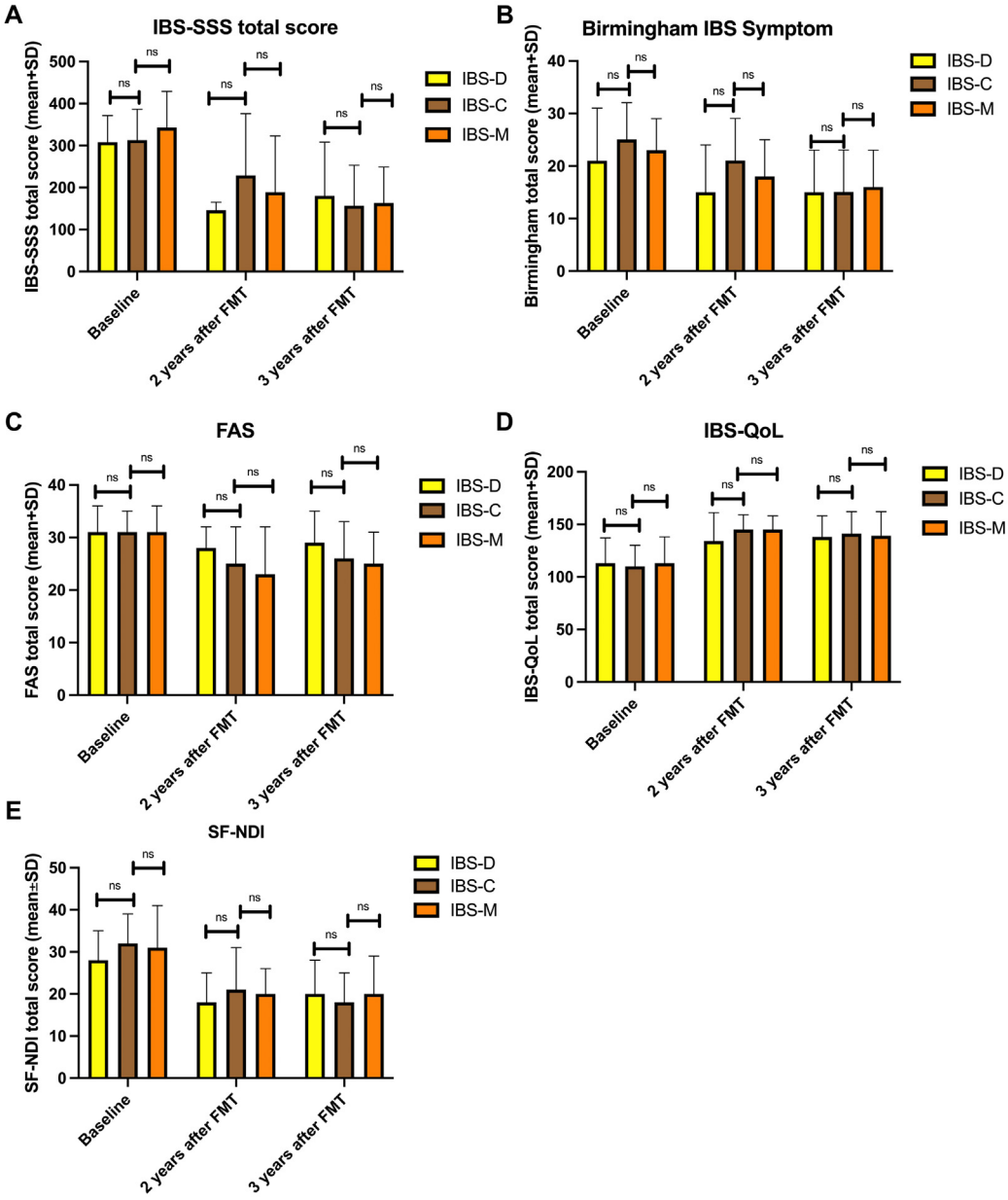
Supplementary Figure 14. DIs of female and male patients at baseline and at 2 and 3 years after FMT. ns, not significant.



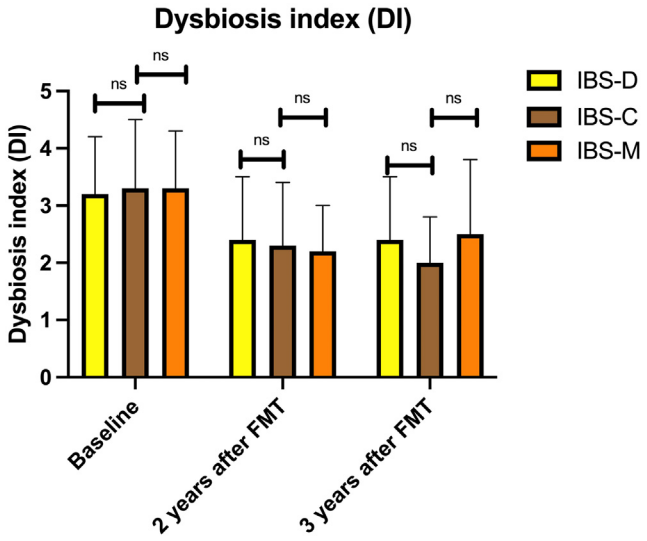
Supplementary Figure 15. Scaled principal-components analysis plot of the fecal bacteria profiles of female and male patients at baseline (*A*) and at 2 years (*B*) and 3 years (*C*) after FMT. Although the bacterial profile of female and male patients changed over time, they mostly overlapped.



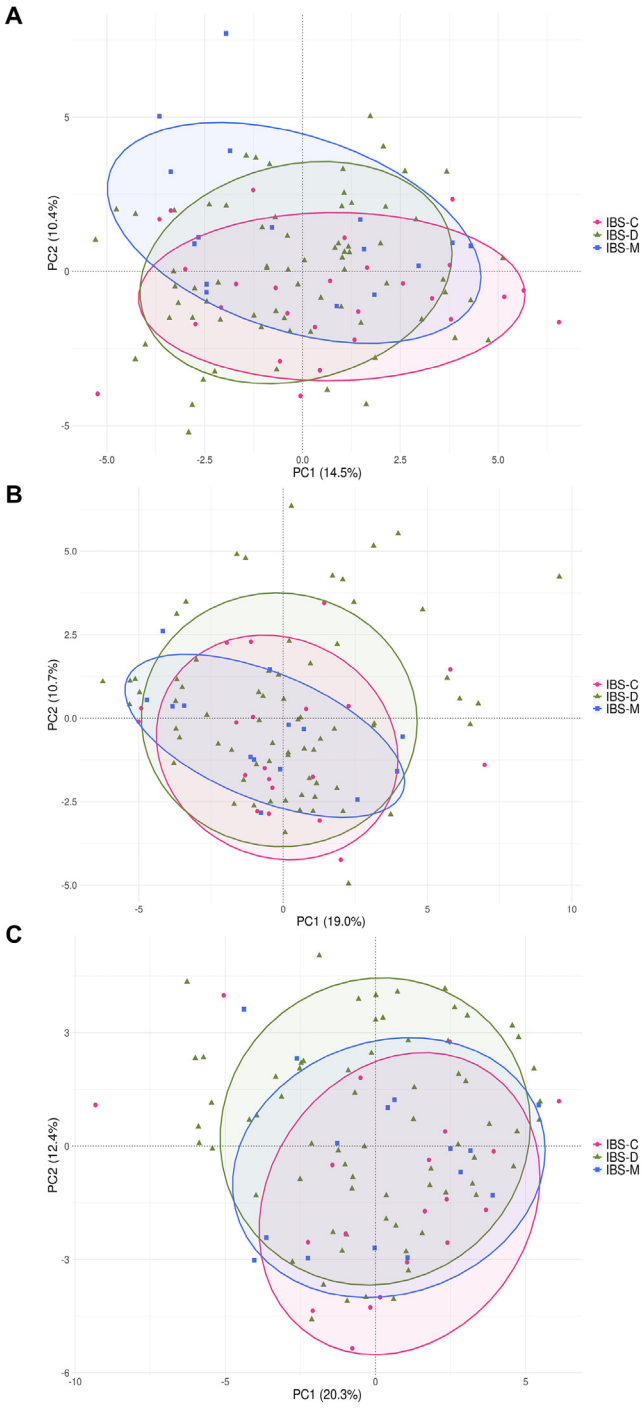
Supplementary Figure 16. FMT response rates (*A*) and complete remission rates (*B*) of patients with IBS-D, IBS-C, and IBS-M at 2 and 3 years after FMT. ns, not significant; *$P < .05$.



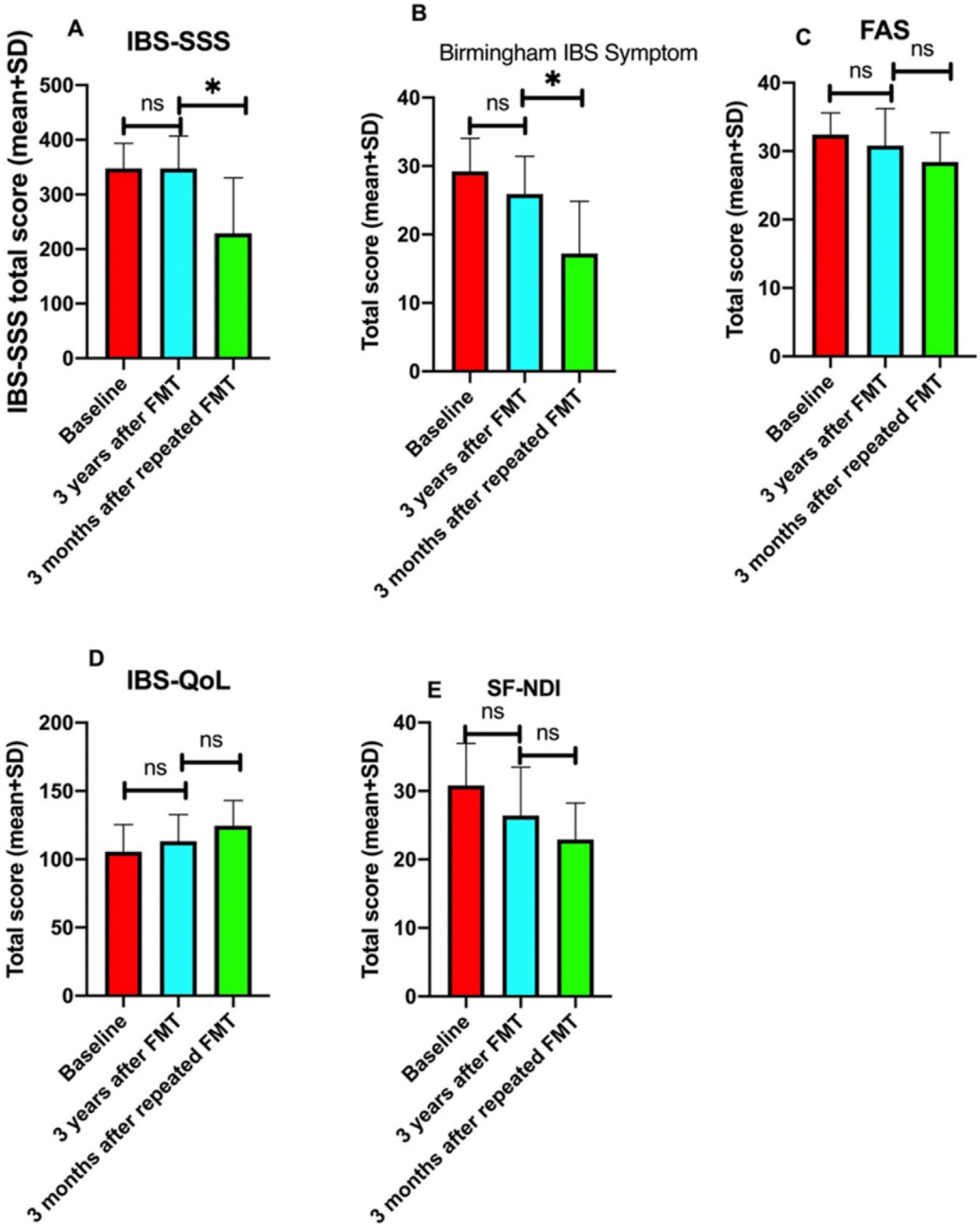
Supplementary Figure 17. Total scores on the IBS-SSS (*A*), Birmingham IBS Symptom Questionnaire (*B*), FAS (*C*), IBS-QOL (*D*), and SF-NDI (*E*) for IBS subtypes at baseline and at 2 and 3 years after FMT. ns, not significant.



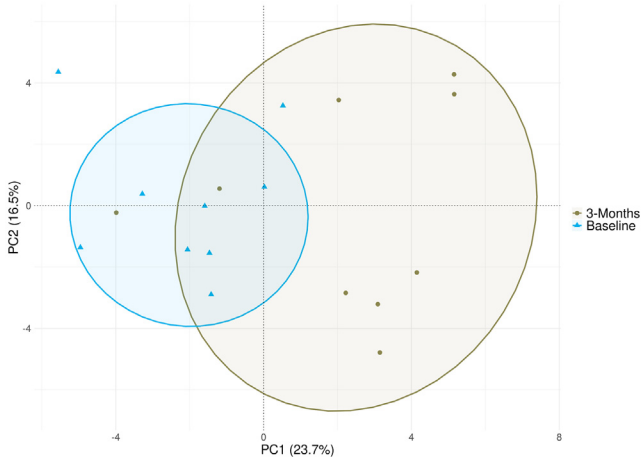
Supplementary Figure 18. DIs for IBS subtypes at baseline and at 2 and 3 years after FMT. ns, not significant.



Supplementary Figure 19. Scaled PCA plot of fecal bacteria profiles of IBS-C, IBS-D, and IBS-M at baseline (*A*) and at 2 years (*B*) and 3 years (*C*) after FMT.



Supplementary Figure 20. Effects of retransplantation on patients with IBS who responded to FMT at 3 months, but relapsed after 3 years on the total scores on the IBS-SSS (*A*), Birmingham IBS Symptom Questionnaire (*B*), FAS (*C*), IBS-QOL (*D*), and SF-NDI (*E*). ns, not significant; *$P < .05$.



Supplementary Figure 21. Scaled principal-components analysis plot of the fecal bacteria profile before and after retransplantation of the patients with IBS who responded to FMT after 3 months, but relapsed after 3 years.

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ORIGINAL ARTICLE

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Fecal luminal factors from patients with irritable bowel syndrome induce distinct gene expression of colonoids

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Abstract

Background: Alteration of the host-microbiota cross talk at the intestinal barrier may participate in the pathophysiology of irritable bowel syndrome (IBS). Therefore, we aimed to determine effects of fecal luminal factors from IBS patients on the colonic epithelium using colonoids.

Methods: Colon-derived organoid monolayers, colonoids, generated from a healthy subject, underwent stimulation with fecal supernatants from healthy subjects and IBS patients with predominant diarrhea, phosphate-buffered saline (PBS), or lipopolysaccharide (LPS). Cytokines in cell cultures and fecal LPS were measured by ELISA and mRNA gene expression of monolayers was analyzed using Qiagen RT^2 Profiler PCR Arrays. The fecal microbiota profile was determined by the GA-map™ dysbiosis test and the fecal metabolite profile was analyzed by untargeted liquid chromatography/mass spectrometry.

Key results: Colonoid monolayers stimulated with fecal supernatants from healthy subjects (n = 7), PBS (n = 4) or LPS (n = 3) presented distinct gene expression profiles, with some overlap ($R^2Y = 0.70$, $Q^2 = 0.43$). Addition of fecal supernatants from healthy subjects and IBS patients (n = 9) gave rise to different gene expression profiles of the colonoid monolayers ($R^2Y = 0.79$, $Q^2 = 0.64$). Genes (n = 22) related to immune response (CD1D, TLR5) and barrier integrity (CLDN15, DSC2) contributed to the separation. Levels of proinflammatory cytokines in colonoid monolayer cultures were comparable when stimulated with fecal supernatants from either donor types. Fecal microbiota and metabolite profiles, but not LPS content, differed between the study groups.

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Conclusions: Fecal luminal factors from IBS patients induce a distinct colonic epithelial gene expression, potentially reflecting the disease pathophysiology. The culture of colonoids from healthy subjects with fecal supernatants from IBS patients may facilitate the exploration of IBS related intestinal micro-environmental and barrier interactions.

KEYWORDS
colonoids, epithelial barrier, host-microbiota cross talk, intestinal microenvironment, irritable bowel syndrome

1 | INTRODUCTION

The intestinal epithelial barrier is composed of a single layer of cells, which allows the absorption of microbial and dietary metabolites and limits the access of harmful antigens and commensal bacteria to the underlying tissues.[1,2] Therefore, the loss of homeostatic host-microbiota cross talk may compromise the integrity of the intestinal epithelial barrier and lead to gastrointestinal diseases such as irritable bowel syndrome (IBS).[1,3] Indeed, there are reports of an impaired intestinal epithelial barrier,[4,5] altered mucosal expression of antibacterial genes[6] and imbalance of microbiota composition[7,8] in at least subgroups of patients with IBS. Furthermore, it has been proposed that IBS patients with diarrhea have an altered fecal metabolite profile compared to healthy subjects.[9-11]

Hitherto, functional and structural properties of the intestinal epithelial barrier of patients with IBS have been explored using cell lines or tissue samples. Stimulation with plasma, soluble mediators from colonic biopsies as well as luminal proteases from patients with IBS negatively influenced the integrity of the colon cancer derived Caco-2 cell line.[12–14] Further, intestinal barrier permeability in IBS has been functionally addressed ex vivo by Ussing chamber experiments and characterized by the expression of adhesion proteins in short-term cultures of intestinal biopsies.[13,15] However, the often used Caco-2 cell line, an immortalized line originally derived from a single donor, provide a limited and simplistic physiological representation of the gut,[16] whereas access to primary intestinal epithelial cells and biopsies may be limited, and when available only suitable for short-term experiments.[17]

During recent years, intestinal organoids have evolved as an attractive alternative for studying physiology, cell-cell or microbe-cell interactions. Compared to traditional in vitro methods, intestinal organoids provide a cell culture system more accurately mimicking the intestinal epithelium[18,19] with long-term viability.[20] Organoids generated from tissue-resident stem cells under specific culture conditions resemble the organ from which they have been derived and after which they are named.[21] Organoids from colonic tissue, termed as colonoids, grow as spheres with the apical surface facing inwards[19] and can be seeded as monolayers for stimulation assays.[18,19] Traditionally, organoids are developed from the study subject (patients/healthy) of interest.[22] However, the impact of the local intestinal microenvironment for intestinal epithelial barrier configuration and function is lost in this set-up. Hence, model systems to specifically study interactions between the local intestinal microenvironment and the epithelium, providing possibilities to better understand the complex pathophysiology of IBS, is much warranted.

Key points

- Cross talk between the luminal factors and the epithelial layer likely participates in the pathogenesis of irritable bowel syndrome (IBS). It may be explored in vitro using human colon crypt-derived organoid monolayers and fecal supernatants.
- Fecal luminal factors from healthy subjects and IBS induce distinct gene expression profiles of colon crypt-derived organoid monolayers.
- Here, we present a unique model of colonoid monolayers to evaluate IBS-specific regulation of colonic epithelium, which may provide improved understanding of the pathophysiology of IBS.

We hypothesized that supernatants from fecal samples, containing microbial ligands, metabolites, and other luminal factors, provide stimuli similar to that present in the intestinal lumen, which will reproduce the intestinal microenvironment of the donor who provided the fecal material. Therefore, with the aim to identify IBS-specific regulation of colonic epithelium, we determined the effects of fecal luminal factors from IBS patients in colonoids established from a healthy donor.

2 | MATERIAL AND METHODS

2.1 | Study subjects and sample collection

Patients with IBS with predominant diarrhea and high severity of symptoms, based on the Bristol Stool Form scale[23] and IBS Severity Scoring System (IBS-SSS),[24] respectively, were selected among the baseline data of participants from a previously reported intervention study.[25] Patients were diagnosed according to the Rome IV

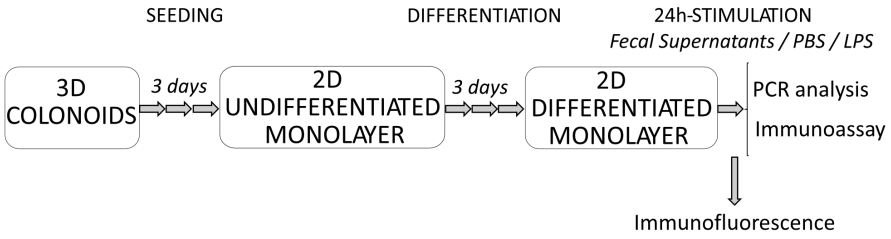


FIGURE 1 Schematic diagram of the experimental setup. Three-dimensional (3D) colonoids generated from biopsies from a healthy subject were seeded as monolayers on transwell membranes and grown for 3 days until confluence. After 3 days of differentiation, colonoid monolayers were fixed for immunofluorescence staining or stimulated with fecal supernatants for 24 h. Stimulation with phosphate-buffered saline (PBS) and lipopolysaccharide (LPS) were used as negative and positive controls, respectively. After stimulation, RNA was isolated for gene expression analysis and culture supernatants collected for detection of cytokines. 3D, 3-dimensional; 2D, 2-dimensional; RNA, ribonucleic acids

criteria.[26] The healthy subjects were randomly selected from a previous study[8] and had no previous or current history of gastrointestinal diseases.[8] All study participants fulfilled the inclusion criteria described in Data S1. Prior to visiting the clinic, study subjects collected fecal samples at home and kept them in the freezer until transportation. Samples were then stored on site at −80°C until preparation of fecal supernatants. Further, a healthy subject provided sigmoid colonic biopsies (25–35 cm proximal from the anus), without prior bowel preparation, using standard biopsy forceps (Olympus, 3.3 mm). All subjects gave written informed consent prior to participation in the corresponding studies.[8,25] The protocols were approved by the Regional Ethical Review Board at the University of Gothenburg (Reg. No. 266–16, 18 April 2016; Reg. No. 548–16, 4 July 2016; and Reg. No. 988–14, 9 February 2015).

2.2 | Preparation of fecal supernatants

Feces were weighed and dissolved in two weight volumes of ice-cold phosphate-buffered saline (PBS), prior centrifugation for 10 min at 1,600 ***g***. The liquid phase was then ultra-centrifuged at 35,000 ***g*** for 2 h at 4°C. The collected fecal supernatant was stored at −80°C until use.

2.3 | Establishment of human colonoid cultures

Colonic crypts were isolated from sigmoidal colonic biopsies from a healthy subject following protocols established elsewhere[27] with minor modifications. Briefly, first crypts and later colonoids, were maintained embedded in 40 µl solid Matrigel® Matrix (Corning®) containing 1 µM Jagged-1 peptide (AnaSpec), in 24-well plates (Nuclon™ Delta Surface, Thermo Fisher Scientific) and were cultured in expansion medium as described previously.[27] The expansion medium was complete medium (Advanced Dulbecco's modified Eagle medium (DMEM)/Ham's F-12, 100 U penicillin/streptomycin, 10 mM HEPES and 0.2 mM GlutaMAX (purchased from Gibco®, Life Technologies™) supplemented with 1× B27 supplement, 1× N2 supplement, 50 ng/ml human epidermal growth factor (purchased from Gibco®), 1 µg/ml [Leu-15] Gastrin (AnaSpec), 500 nM A 83–01, 10 µM SB202190, 10 nM prostaglandin E2 and 1mM N-acetylcysteine (from Sigma-Aldrich), 100 µg/ml Primocin (InvivoGen), 10 µM CHIR99021 (Sigma-Aldrich) and 10 µM Y-27632 dihydrochloride (Tocris).[27] In addition, the media contained 50% Wnt3A conditioned medium, 20% R-spondin conditioned medium and 10% Noggin conditioned medium. The cell lines used to supply Wnt3A and Noggin were kind gifts from Professor Hans Clevers (Hubrecht Institute); the Cultrex® R-spondin1 cell line was purchased from Trevigen, Inc. The expansion medium, without CHIR99021 and Y-27632,[27] was replaced every 2–3 days. Colonoids were maintained at 37°C, 5% CO_2. When denser colonoid cultures had been established, colonoids were passaged every 7 days. All passages were performed by using Corning Cell Recovery Solution (Corning®) and dissolving the Matrigel on ice similar to reference,[27] without using an orbital shaker. The dissolved matrigel was collected into basal media (DMEM/Ham's F-12 with 1× Glutamax, 10 mM HEPES and 10% heat-inactivated fetal bovine serum (FBS)). The colonoid structures were disrupted by pipetting and approximately 50 colonoids were seeded in each well. After each passage, cells were cultured in expansion media as described above.[27]

2.4 | Establishment of colonoid monolayers

Colonoid monolayers were established from colonoid cultures harvested after 7 days. To establish a monolayer, approximately 200 colonoids were completely disaggregated with the help of a 27-gauge needle. Approximately 100 µl of cell suspension in IntestiCult™ Organoid Growth medium (Human) (Stemcell™ Technologies) with 10 µM Y-27632 and 100 µg/ml Primocin (InvivoGen) were seeded on permeable polyester filter insert (Corning Transwell, pore size 0.4 µM) coated with 10 µg/ml human collagen IV (Sigma-Aldrich) solution, in 24-well plates.[27] An additional 50 and 600 µl of IntestiCult™ Organoid Growth medium with Y-27632 and Primocin was added to the transwell filter and the well, respectively, and kept in culture for 3 days. Then, expansion medium was substituted with differentiation medium, where 50% v/v IntestiCult (Component B) was substituted by DMEM/Ham's F-12. The differentiation medium was

replaced with fresh differentiation medium after 2 days and colonoid monolayers were then kept in culture for one additional day to reach 3 days of differentiation.

2.5 | Stimulation of colonoid monolayers with fecal supernatants

The experimental design is schematically shown in Figure 1. Differentiated colonoid monolayers were stimulated for 24 h at 37°C with fecal supernatant from either healthy subjects or IBS patients to both transwell compartments at a concentration of 1:100. For negative controls, an equal volume of PBS was added to generate non-stimulated monolayers. Colonoid monolayers stimulated with lipopolysaccharide (LPS, InvivoGen), diluted in DMEM/Ham's F-12 and added to a final concentration of 100 ng/ml,[28] were used as proinflammatory, positive controls.

2.6 | Immunofluorescence and imaging

Triple immunofluorescence staining was performed against muc-2, phospho-ezrin or wheat germ agglutinin, in combination with phalloidin and Hoechst, in filter inserts in a 24-well plate as previously described.[18] Rabbit anti-muc2 IgG (dilution 1:400), rabbit anti-phospho-Ezrin IgG (dilution 1:200) were used as primary antibodies (Abcam), while the secondary antibody was anti-rabbit Alexa 488 (dilution 1:200, Life Technologies). Probes included Phalloidin-647 (dilution 1:500, Abcam) and wheat germ agglutinin-488 (dilution 1:500, Thermo Fisher Scientific). Hoechst 33342 (dilution 1:10,000, Thermo Fisher Scientific) was used to stain the nucleus of cells at a final step. Washes with PBS buffer followed every step described.[18] Finally, stained filter inserts were mounted with ProLong Gold Antifade Mountant (Thermo Fisher Scientific) on a glass slide. The preparations were kept sealed at 4°C until visualization. Images of stained monolayers were acquired using 20× objective and the same acquisition settings on a LSM 700 inverted confocal microscope (Carl Zeiss) using Zen 2012 SP5 (Black edition. Version 14.0.3.201) imaging software. Confocal images were processed using the software Zen 3.0 2019 (Blue version) and Fiji (ImageJ, Version 1.52p). Changes in brightness/contrast and reduction of background noise were applied to emphasize the qualitative analysis of the images. Images of the human healthy colonoids during culture were taken with Leica DM IL LED Inverted Microscope with ICC50 HD Camera (Type: 11 090 137 001) using 4× objective.

2.7 | Gene expression analysis

The material from 2 to 3 monolayers, which underwent the same stimulus, was pooled prior to RNA extraction. Briefly, total RNA was isolated with the Reliaprep RNA Cell Miniprep System (Promega) following the manufacturer's instructions. For reverse-transcription of RNA into complementary DNA synthesis (cDNA), RT^2 First Strand Kit (Qiagen) was used. Following manufacturer's instructions, both cDNA and RT^2 SYBR® Green qPCR Mastermix (Qiagen) were used on a custom RT^2 Profiler PCR array (Qiagen) targeting 86 genes related to antibacterial and inflammatory response, and epithelial barrier integrity. PCR arrays were analyzed in a QuantStudio 12K Real-Time PCR System (Applied Biosystems™, Life Technologies). CT values were removed if *undetermined*; CT > 36 was adjusted to CT = 38. Genes were excluded from analysis if >60% of samples had missing or very high CT values (n = 11). Gene expression was calculated as $2^{-\Delta CT}$ ($2^{-(\text{CT Target gene}-\text{CT Housekeeping gene})}$ and the average of the housekeeping genes ACTB, B2M, GAPDH, HPRT1 and RPLP0 was used for normalization. A complete list of the genes targeted in the custom array is shown in Data S1.

2.8 | Measurement of cytokines and LPS

Levels of IL-1β, TNF-α and IL-8 in culture supernatants were measured using V-PLEX custom human biomarker plate from MSD® Multi-Spot Assay System (Meso Scale Diagnostic) following the manufacturer's instructions. Culture supernatants obtained from both transwell compartments were evaluated. LPS levels were measured in fecal supernatants using the LPS ELISA kit (Aviva System Biology), following the manufacturer's instructions.

2.9 | Untargeted liquid chromatography-mass spectrometry analysis

The luminal factors of fecal supernatants were analyzed at Chalmers Mass Spectrometry Infrastructure using a non-targeted liquid chromatography–mass spectrometry (LC-MS) approach. Briefly, the analyses were carried out using a reversed-phase chromatography (RP) and hydrophilic interaction chromatography (HILIC) with positive and negative electrospray ionization polarities, as described elsewhere.[29] The samples of each study group were analyzed in separate batches, which included its own quality control samples. The analytical workflow named "notame", described in,[30] was used to pre-process the acquired data and included drift correction within and between batches, data imputation using *missForest* R package and clustering of features to remove weak and repeated features.[30] Log_{10} transformation was applied prior between-batch-correction to reduce possible batch effects caused by the instrument.

2.10 | Fecal microbiota DNA analysis

The microbiota profile was determined at Genetic Analysis AS using the GA-map™ dysbiosis test. In short, total bacterial genomic DNA is extracted with magnetic beads from homogenized and mechanically disrupted fecal samples. PCR is used to amplify the 16S rRNA gene (V3-V9 regions) followed by probe labeling. The DNA probes, labeled

with nucleotides, hybridize to complementary probes coupled to magnetic beads, that allow signal detection by BioCode 1000A 128-Plex Analyzer (Applied BioCode).[31] Fifty-four DNA probes allow targeting ≥300 bacteria belonging to different taxonomic levels. This test generates as a result a bacterial profile based on fecal bacterial abundance (or probe signal intensity).[31]

2.11 | Data analysis

Multivariate data analyses were used to evaluate the relationship between the different study groups (Y-variables) and gene expression (X-variables). Principal Component Analysis (PCA) was performed to recognize clustering in the observations based on the gene expression (X-variables). Orthogonal Partial Least-Squares-Discriminant Analyses (OPLS-DA) were performed to visualize and identify correlations between selected Y-variables and X-variables. R (version 3.6.2) and SIMCA® software (version 15.0.2, MKS Umetrics AB) were used for these purposes. The quality of the OPLS-DA was determined by the parameters of R^2Y and Q^2, indicating discrimination and predictability, respectively. R^2Y values ≥0.5 correspond to good fit (max. $R^2Y = 1$). Q^2 values ≥0.4, but no more than 0.3 away than R^2Y, are considered adequate for biological variables. Univariate analyses were applied to identify differences between specific variables. The statistical tests performed (parametric or non-parametric) were based on the distribution of the data determined by Shapiro-Wilk tests and histograms. $p < 0.05$ were considered statistically significant. More details can be found in Data S1.

3 | RESULTS

3.1 | Demographic information

In total, 7 healthy subjects (5 females) and 9 IBS patients (6 females) with predominant diarrhea provided fecal samples from which supernatants were prepared. The IBS patients were older than the healthy subjects (31 (26–66) vs. 22 (20–36) years), $p < 0.05$), but did not differ with regards to sex (healthy: 5 females, 2 males vs. IBS: 6 females, 3 males) or body mass index (25.4 (3.7) vs. 21.6 (2.5) kg/m^2). For colonoid culture, a healthy 32-year-old female with body mass index of 22.0 kg/m^2 provided colonic biopsies.

3.2 | Establishment and characterization of colonoid monolayers

The cultures of colonoids comprised spheres with relatively thin walls and a dark core ("lumen") containing debris. Colonoids in varying sizes were located in different focal planes throughout the extracellular matrix that supported their growth (Figure 2A). The colonoid structures were established as a monolayer comprising polarized, organized, and differentiated intestinal epithelial cells expressing phospho-ezrin and displaying a glycocalyx (Figure 2B,C). Filamentous actin staining identified epithelial cells tightly connected by junctional complexes (Figure 2B–D). Moreover, the differentiated colonoid monolayers contained dispersed mature mucin-producing goblet cells (Figure 2D).

3.3 | Addition of fecal supernatants from healthy subjects modifies gene expression profile of colonoid monolayers

The addition of fecal supernatants from healthy subjects, LPS or PBS to the monolayers resulted in distinct clusters, although with some overlap, based on gene expression (Figure 3A). The three different culture conditions gave rise to distinct gene expression profiles and 27 out of the 75 genes contributed to the separation between the groups (Figure 3B,C). The expression of CCL20, DSC2 and ICAM1 was lower in monolayers stimulated with fecal supernatants from healthy subjects compared to LPS-stimulated monolayers (Figure 3C, Table S1). In contrast, the expression of PECAM1 was higher in monolayers stimulated with fecal supernatants from healthy subjects compared to LPS-stimulated monolayers. Further, the expression of PVRL1 was higher in monolayers stimulated with fecal supernatants from healthy subjects compared to PBS-stimulated monolayers (Figure 3C, Table S1).

3.4 | Fecal supernatants from IBS patients induce a distinct gene expression of colonoid monolayers

Next, we compared the effects of fecal supernatants from healthy subjects and patients with IBS on gene expression of colonoid monolayers. Addition of fecal supernatants from the two study groups to colonoid monolayers induced distinct gene expression clusters with only minor overlap (Figure 4A). In total, 22 out of the 75 genes were differently regulated by the addition of fecal supernatants from the two study groups (Figure 4B). Genes related to immune response such as CD1D, IRF7, TNFSF13, IRF5, TLR9, LYZ, ICAM1 and CX3CL1 showed higher expression in colonoid monolayers stimulated with fecal supernatants from IBS patients, while TLR5 expression was higher in colonoid monolayers stimulated with fecal supernatants from healthy subjects (Figure 4C). Also, the addition of fecal supernatants from IBS patients upregulated the gene expression of the pro-inflammatory cytokines IL-1β and TNF-α and enhanced the expression of genes related to maintenance of epithelial integrity, such as DSC2, CLDN15 and TJP2 (Figure 4C). The different gene regulation of the colonoid monolayers was not explained by the fecal supernatants' content of LPS, which was similar in both study groups (healthy subjects; 0.12 (0.01–0.55) vs. IBS patients; 0.15 (0.02–0.89) mg/ml), $p > 0.05$), nor influenced by age or sex (Figure S1). Moreover, the secreted levels of IL-1β, TNF-α or IL-8 did not differ in colonoid monolayers stimulated with fecal supernatants

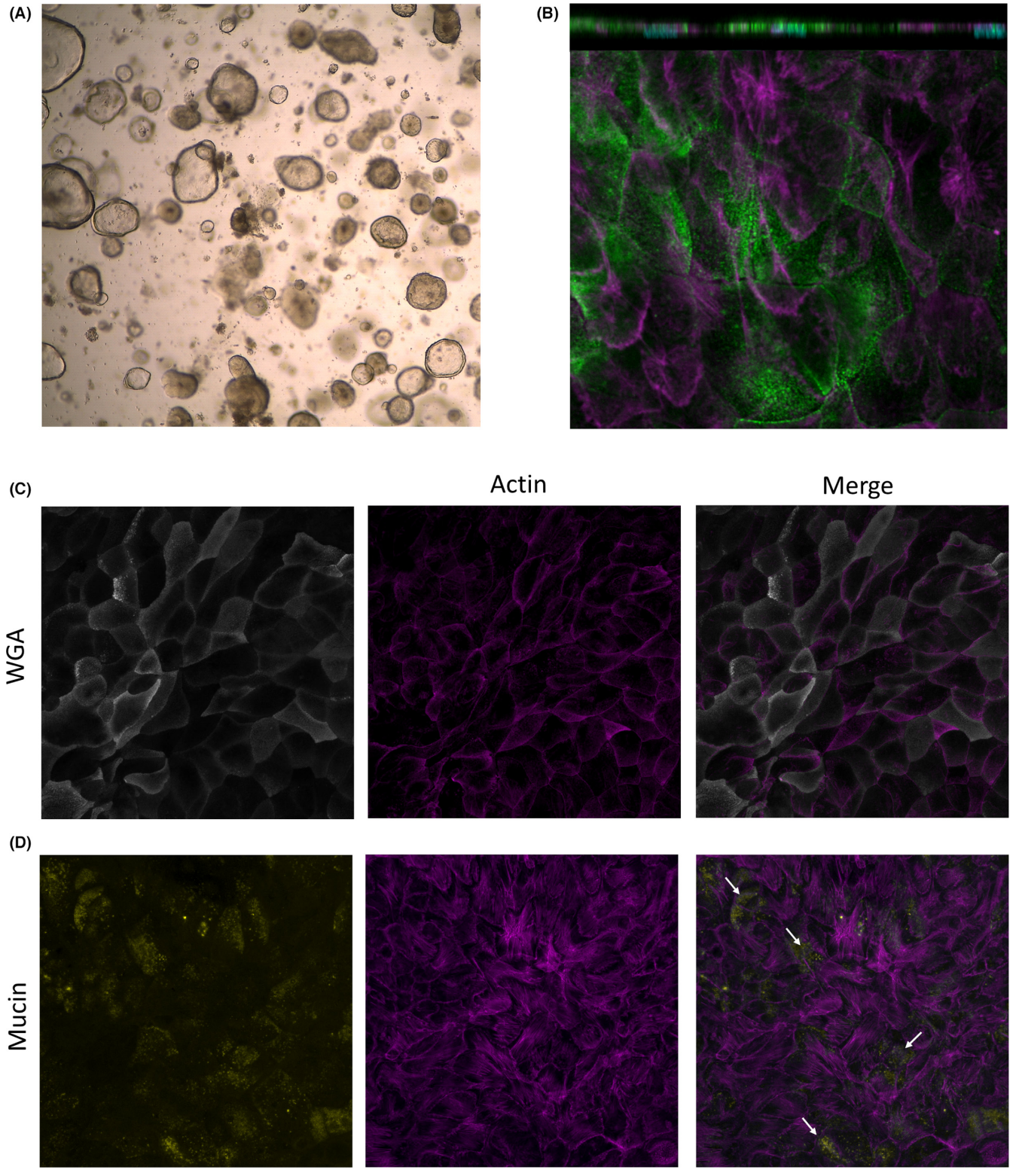


FIGURE 2 Characterization of differentiated colonoid monolayers. Colonoids obtained from colonic biopsies from a healthy subject were seeded as 2D-monolayers and differentiated. (A) Image from light microscopy of BME-suspended 3D colonoids; 4× magnification. (B) Polarized apical surface of cells defined by phospho-ezrin (green) in differentiated monolayers. Orthogonal view (XZ projection), top panel. Top view (XY projection), bottom panel. (C) Glycocalyx-rich cell membrane (wheat germ agglutinin, WGA in gray) and (D) mucin-producing goblet cells (mucin in yellow) in differentiated colonoid monolayers. Mature microvilli and cell membrane borders on the apical surface were identified by actin expression (magenta). Nuclei are shown in cyan. Images were acquired with (A) Leica DM IL LED Inverted Microscope with ICC50 HD Camera and (B–D) LSM700 inverted confocal microscope; 20× magnification. BME, basement membrane extract

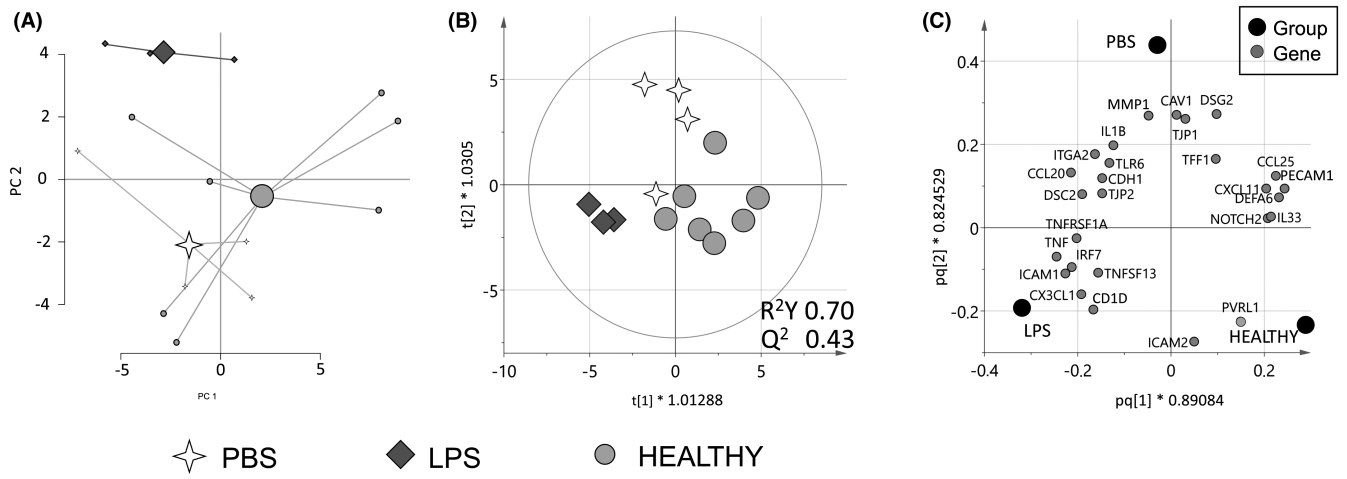
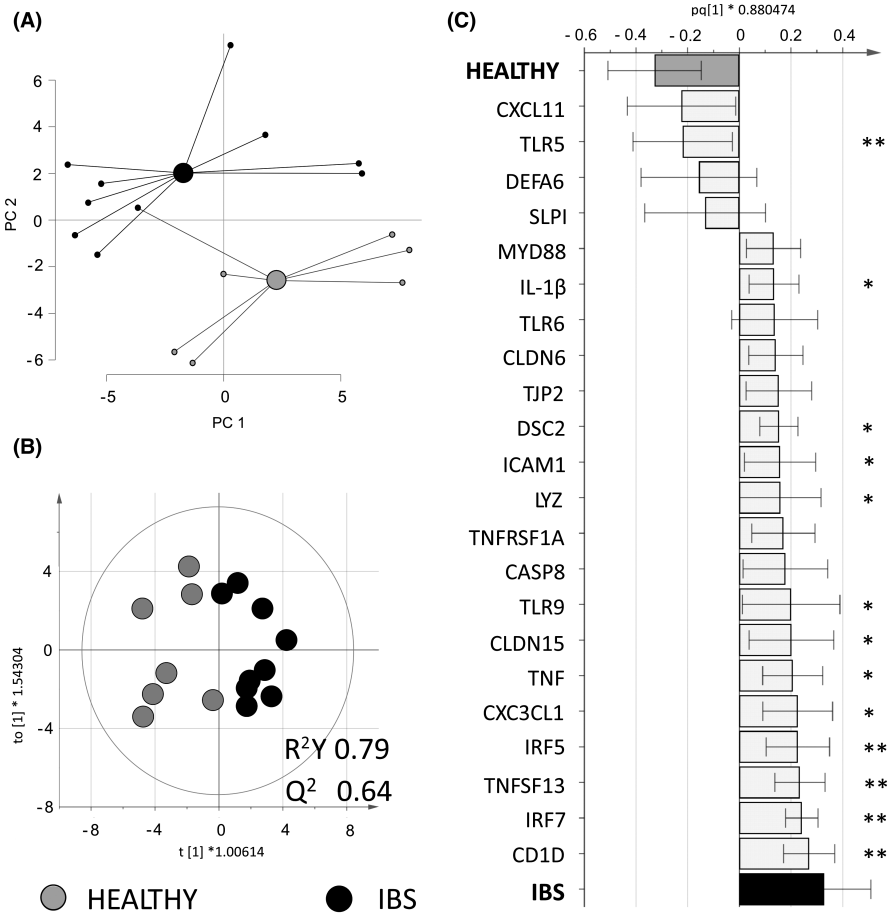


FIGURE 3 Gene expression of colonoid monolayers stimulated with fecal supernatants from healthy subjects or control stimuli. Colonoid monolayers were stimulated for 24 h with fecal supernatants from healthy subjects ($n = 7$, light gray dots), LPS ($n = 3$, dark gray diamonds) or PBS ($n = 4$, white 4-point stars). Gene expression was analyzed by PCR arrays for genes involved in antibacterial and inflammatory response and barrier function. (A) A principal component analysis (PCA) plot based on 75 expressed genes (11 unique genes excluded as they were below detection limit). (B) Score scatter plot and (C) loading scatter plot from an orthogonal partial least squares-discriminant analysis (OPLS-DA) using a variable influence on projection (VIP) cut-off >1.1. HEALTHY, colonoid monolayers stimulated with fecal supernatants from healthy subjects; LPS, lipopolysaccharide-stimulated colonoid monolayers; PBS, PBS-stimulated colonoid monolayers

FIGURE 4 Gene expression of colonoid monolayers stimulated with fecal supernatants from healthy subjects or IBS patients. Colonoid monolayers were stimulated for 24 h with fecal supernatants from healthy subjects ($n = 7$ gray dots) or IBS patients ($n = 9$ black dots). Gene expression was analyzed by PCR arrays for genes involved in antibacterial and inflammatory response and barrier function. (A) A principal component analysis (PCA) based on 75 genes (11 unique genes excluded as they were below detection limit). (B) Score scatter plot and (C) loading column plot from an orthogonal partial least squares-discriminant analysis (OPLS-DA) using a VIP cut-off >1.15. HEALTHY, colonoid monolayers stimulated with fecal supernatants from healthy subjects; IBS, colonoid monolayers stimulated with fecal supernatants from IBS patients. Between group-comparisons were tested with Mann-Whitney *U* test; asterisks identify statistically significant *p* values: $^{*}p < 0.05$; $^{**}p < 0.01$



from healthy subjects and IBS patients in either the apical or basal compartments (Figure 5). Overall, fecal supernatants caused a lower secretion of cytokines when compared to the control stimuli, that is, PBS and LPS, and cytokines were detected at very low levels in the basolateral compartment compared to the apical compartment (Figure 5).

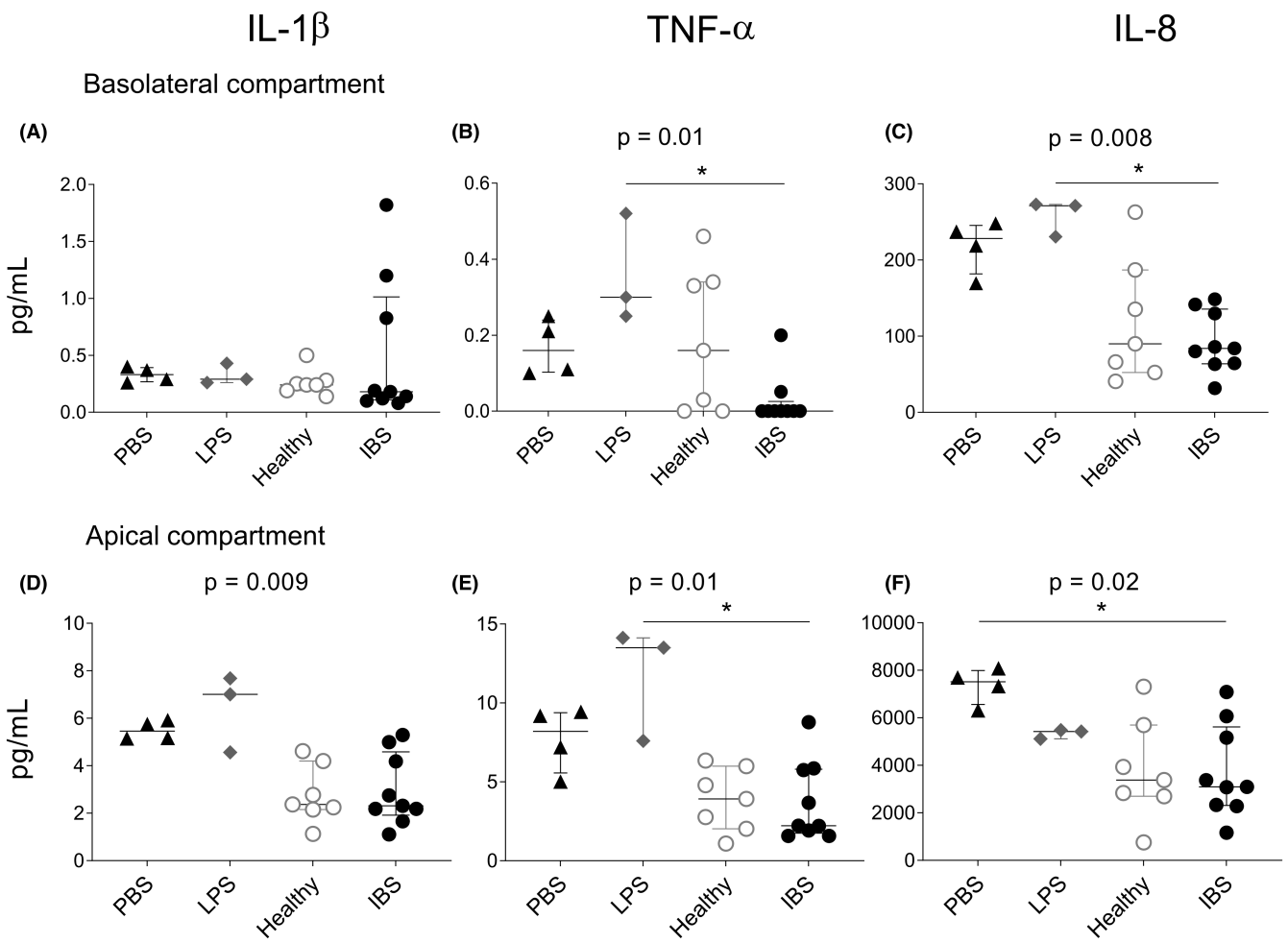


FIGURE 5 Cytokine secretion from colonoid monolayers stimulated fecal supernatants from healthy subjects or IBS patients or control stimuli. Colonoid monolayers were stimulated for 24 h with PBS ($n = 4$, black triangles), LPS ($n = 3$, gray diamonds), fecal supernatants from healthy subjects ($n = 7$ gray dots) or IBS patients ($n = 9$ black dots). Cytokine levels in culture supernatants were analyzed by MSD® Multi-Spot Assay system. (A–C) Levels in the basolateral compartment and (D–F) in the apical compartment of IL-1β (A,D), TNF-α (B,E) and IL-8 (C,F). PBS, PBS-stimulated colonoid monolayers. HEALTHY, colonoid monolayers stimulated with fecal supernatants from healthy subjects; IBS, colonoid monolayers stimulated with fecal supernatants from IBS patients; LPS, lipopolysaccharide-stimulated colonoid monolayers; PBS, phosphate-buffered saline-stimulated colonoid monolayers. Cytokine concentrations (pg/ml) are shown as median (interquartile range) of two independent stimulation experiments. Between group-comparisons were performed using the Kruskal–Wallis test with Dunn's correction; asterisks identify statistically significant p values: $^*p < 0.05$

3.5 | The fecal microbiota and metabolite profiles distinguish healthy subjects and IBS patients

The microbiota profile of the fecal samples used for preparing fecal supernatants differed between healthy subjects and IBS patients (Figure 6A,B). The bacterial taxa contributing most to the separation were Proteobacteria, *Pseudomonas* spp., *Dorea* spp. and *Ruminococcus gnavus* ($p < 0.05$), and were more abundant in IBS patients, whereas *Bacteroides pectinophilus* was more abundant in healthy subjects ($p < 0.05$). Additionally, the fecal supernatants from healthy subjects and IBS patients, respectively, presented distinct profiles based on the 9699 spectral features detected in an untargeted LC-MS analysis (Figure 6C). Two hundred of the detected compounds contributed to the separation between the two groups (Figure 6D). Among the top five metabolites in either direction contributing most to the separation, only xanthine has been annotated and was more abundant in fecal supernatants from IBS patients compared with healthy subjects. However, neither metabolite nor microbiota profiles were influenced by the age or sex of the donors (Figure S2).

4 | DISCUSSION

In this work we describe that components of fecal supernatants, a proxy for the intestinal microenvironment, regulate gene expression of colonoids. Hence, the microbiota and metabolite profiles of fecal supernatants from healthy subjects and IBS patients with predominant diarrhea differed substantially and induced distinct regulation of colonoid gene expression. This suggests that our experimental set-up, adding patient-derived fecal supernatants to colonoids

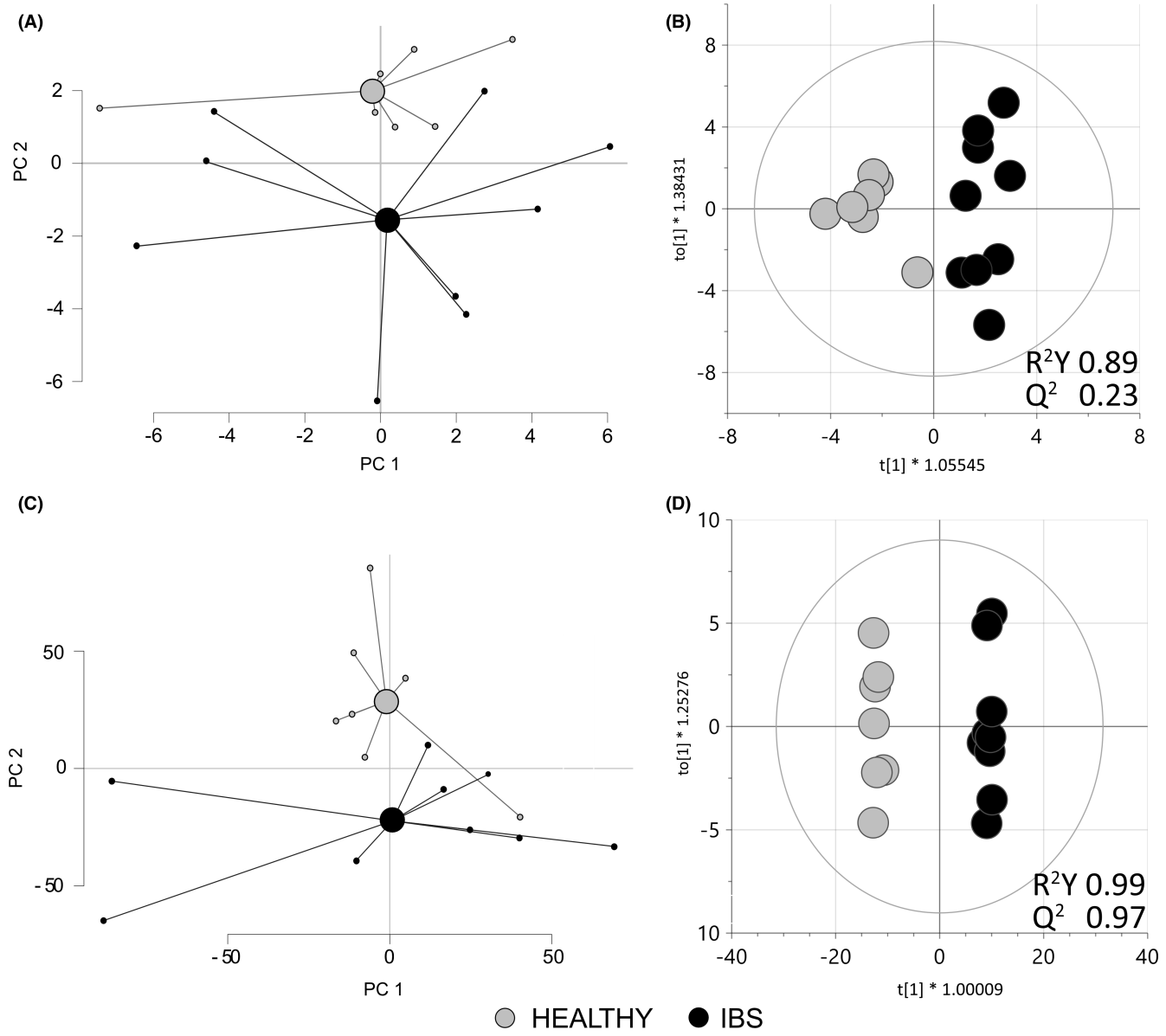


FIGURE 6 Fecal microbiota and metabolite profiles from healthy subjects and IBS patients. Fecal samples were analyzed by GA-map™ dysbiosis test while fecal supernatants were analyzed by untargeted liquid chromatography/mass spectrometry analysis from healthy subjects ($n = 7$ gray dots) or IBS patients ($n = 9$ black dots). (A) A principal component analysis (PCA) based on the 54 bacterial taxa determined in fecal samples. (B) Score scatter plot from an orthogonal partial least squares-discriminant analysis (OPLS-DA) that shows the distinct fecal microbiota profile between fecal samples from healthy subjects and IBS patients. (C) A PCA based on the 9699 spectral features detected in fecal supernatants. (D) Score scatter plot from an OPLS-DA using a VIP cut-off >1.73 that included the 200 most discriminatory spectral features differentiating the fecal supernatants of healthy subjects and IBS patients. HEALTHY, fecal supernatants from healthy subjects; IBS, fecal supernatants from IBS patients

established from a healthy donor, has the potential to facilitate studies of intestinal barrier cross talk and explore the impact of the intestinal microenvironment on the pathophysiology of IBS.

Although the recently developed approach of establishing colonoids from intestinal tissue from the individual of interest to create subject-specific intestinal epithelium has provided a multicellular model system, the importance of luminal factors has been overlooked.[32] In this study, we therefore exposed colonoids established from a healthy subject to patient-derived fecal supernatants, to determine effects of luminal factors on the colonic epithelium, potentially involved in IBS pathophysiology. Similar to the commonly used CaCo-2 cell platform that comes from a patient with colorectal adenocarcinoma, a single healthy donor of colonic tissue was used for this study. Whereas subject-specific organoids are costly concerning both time and money, collection of fecal samples are non-invasive and easy to obtain and allow us to investigate the importance of the intestinal microenvironment in IBS versus health. The colonoid monolayers in our study presented with similar features, that is, polarization and specialized cells as mucin-producing goblet cells, to those previously described in the literature.[27,33–35] The addition of

fecal supernatants from IBS patients and healthy subjects induced different gene regulation of the colonoid monolayers, suggesting that the fecal supernatants reproduce the intestinal microenvironment of the donor who provided the fecal material. Importantly, sex or age of donors of fecal supernatants did not influence our results.

The vast majority of genes explored in our study, related to antibacterial response, inflammation and cell junctions, were more highly expressed by the colonoid monolayers stimulated with fecal supernatants from IBS patients compared to healthy subjects. The pro-inflammatory cytokines IL-1β[36] and TNF-α,[37] the receptor TLR9 sensing unmethylated CpG dinucleotides of microbial DNA,[38] the antibacterial enzyme lysozyme[39] and transcription factors for type 1 interferons IRF5 and IRF7[38] reflect activation of the immune response and were all expressed to a higher degree in IBS patients. Interestingly, fecal supernatants from IBS patients also induced higher gene expression of CD1D, which encodes an MHC class I-like molecule presenting bacterial lipid antigens to natural killer T cells (NKT cells). Activated NKT cells rapidly secrete a plethora of cytokines aiming to direct other immune cells to fight infections.[40] Also, gene expression of TNFSF13 (known as APRIL),[41] a cytokine supporting class switching to IgA, was higher in colonoid monolayers stimulated with fecal supernatants from IBS patients. Altogether, this suggests that fecal supernatants from IBS patients induce immune activity of epithelial cells. While an impaired barrier function is considered to be central for IBS pathophysiology, it is somewhat surprising to note a higher gene expression of TJP2, DSC2 and CLDN15, known to be involved in maintaining epithelial integrity,[42] in colonoid monolayers stimulated with fecal supernatants from IBS patients. Our findings are not yet corroborated by other studies, potentially due to the fact that only a few previous studies have addressed the effects of luminal factors on intestinal epithelium, represented by cell lines, organoids or biopsies, in relation to IBS.[13,14,43] Still, soluble mediators from colonic biopsies from patients decreased the gene expression of ZO-1, but not occludin, both important components of tight junctions, and induced increased the permeability in Caco-2 cultures.[14] Further, fecal supernatants from IBS patients increased the paracellular permeability, assessed by FITC-Dextran flux, in colonoid cultures.[43] In parallel, the increase of paracellular permeability in enteroid cultures following stimulation with the proinflammatory stimuli IFN-γ corresponded with a decrease of the gene expression of the tight junctions ZO-1 and occludin in the same study.[43] Similarly, another study provided evidence of impaired barrier function when adding fecal supernatants from IBS patients to intestinal biopsies mounted in Ussing chambers.[13] In the above mentioned studies, cell function and gene expression was dependent on the type of stimuli[14,43] as well as concentration and time,[43] indicating the complexity of studying regulation of barrier function. Hence, the contradictory results may depend on different culture conditions, including concentration of fecal supernatants and time of culture, but also on the different cell systems or tissues. Furthermore, the increased gene expression of junctional complexes recorded in our study may reflect an attempt to regulate expression of tight junction proteins needed to maintain epithelial integrity under IBS-like conditions. Whether addition of fecal supernatants from IBS patients and healthy subjects, respectively, gives rise to functional differences related to epithelial integrity of the colonoids in our model system remains to be further elucidated. Still, our results support the notion that fecal supernatants from IBS patients have a distinct effect on the intestinal epithelium, regulating genes maintaining epithelial barrier integrity.

In line with a previous study,[14] fecal supernatants were added to both the apical and basolateral side of the colonoid monolayer to rule out potential effects of not fully confluent monolayers and ensure similar conditions during the stimulations of all the parallel colonoid cultures. Further, it is well-described that the intestinal epithelial barrier is permeable and certain luminal factors can be transported through the barrier (e.g., intra- or paracellularly)[44] and might reach the basolateral side. Similar to previous studies, we identified a polarized response of the epithelial cells,[18,45,46] and pro-inflammatory cytokines were primarily secreted in the apical compartment of the cell cultures. Thus, despite the lack of transepithelial electrical resistance (TEER) measurement, the polarized response of the epithelial cells shows that the monolayers were indeed functional and differentiated and not disrupted by the addition of fecal supernatant or LPS on both sides. There were no differences in the secretion of pro-inflammatory cytokines between colonoid monolayers stimulated with fecal supernatants from healthy subjects and IBS patients. Interestingly, fecal supernatants had a suppressive effect on cytokine secretion of colonoid monolayers as compared to PBS (negative control) and LPS (positive control). This is in line with a previous study of our group, where macrophages showed reduced cytokine production after conditioning with fecal supernatants as compared to untreated cells.[47] Altogether, our results sustain the concept of the intestinal microenvironment educating the host to stay unresponsive to the load of bacterial stimuli constantly present in the gut to maintain homeostasis.[47]

The fecal microbiota profiles differed between IBS patients and healthy subjects. The taxa Proteobacteria, *Pseudomonas* spp., *Dorea* spp. and *Bacteroides pectinophilus* were found to be linked to IBS patients in our study, have previously been associated with IBS-D and symptoms, including intestinal permeability.[48–54] Further, the mucin degrader *Ruminoccocus gnavus* has been associated to IBS severity.[55,56] An altered microbiota composition may result in altered metabolite composition, reflecting the overall function and metabolism of the bacterial community. Indeed, similar to previous reports by us and others, in this study IBS patients presented with distinct fecal metabolite profiles, which were, however, not influenced by age or sex.[9–11] Analyzing the fecal supernatants with LC-MS allowed us to determine the presence of almost 10,000 spectral features, of which 200 were found significant for separation of IBS from the healthy controls. The possibility to identify compounds from untargeted LC-MS analysis is, however, resource intensive and the ability to, with reasonable certainty, annotate the top metabolites driving the separation between study groups was limited with the available resources in this project. The only unique annotated top metabolite, the purine-derived metabolite xanthine, previously demonstrated to

promote intestinal barrier development,[57] was found in higher levels in IBS patients, similar to a previous study by our group.[11] Besides, although the vast majority of metabolite compounds have yet to be annotated, it is still more likely that the combination of compounds rather than the presence or absence of specific compounds give rise to the IBS- specific gene regulation of colonoid monolayers. IBS patients have demonstrated higher concentration of fecal proteases,[13] which may cause alterations of the epithelial permeability in Caco-2 cells[12] and humanized mice.[13] Additionally, LPS content of fecal samples has been reported to be higher in IBS patients than in healthy subjects.[58] However, the different effect on gene regulation of colonoid monolayers cultured with fecal supernatants from IBS patients and healthy subjects seen in our study was not driven by LPS, as the level of LPS did not differ between the two groups. Altogether, our data strongly implies that the gene expression profile of colonoid monolayers cultured in the presence of fecal supernatants derived from IBS patients is driven by the content of disease specific luminal factors, with low likelihood of influence of sex hormones or age.

This study has several weaknesses and strengths. The described results originate from experiments using colonoids established from one unique donor and a limited number of study subjects providing fecal supernatants. The time the colonoid monolayers were exposed to fecal supernatants was optimized for detecting regulation of gene expression, which might have jeopardized the possibility to optimally determine secreted cytokines. Also, future experiments to evaluate response to apical exposure alone will clarify potential confounding effect of double-side stimulation. In addition, factors such as concentration of fecal supernatants, group size or the cytokines measured may have influenced the lack of differences in cytokine secretion. Additionally, this study did not investigate the potential effect of luminal factors on barrier permeability, and analyzing epithelial permeability, measuring for example the TEER, could further have clarified the effect of fecal supernatants on epithelial barrier function, not only gene expression. Moreover, comparing the gene expression of fecal supernatant stimulated organoids to that of colonic mucosal biopsies from IBS and healthy donors could have provided additional validity to our in vitro model. Nevertheless, the study subjects providing fecal samples were all well clinically characterized, and the study design enabled a relatively broad analytical approach while exploring effects on gene regulation using custom made gene arrays. Moreover, the establishment of the microbiota and metabolite profiles of the fecal samples used to prepare supernatants strongly supported the distinct effect of fecal components on gene regulation of the colonoid monolayers, despite the fact that the metabolite composition could not be deciphered in any detail. Even so, this unique study can be regarded as a pilot study proposing an innovative experimental setup for investigating cross talk at the intestinal epithelial barrier, although generalization of results should be cautiously done until further support is available.

In conclusion, the stimulation of polarized and differentiated colonoid monolayers with fecal supernatants from healthy subjects and IBS patients with predominant diarrhea induced distinct gene profiles, potentially reflecting the intestinal microenvironment of the donor providing fecal material. Thus, we propose that the model described in this study may be used to study interactions between components of the intestinal environment and the epithelial layer in more depth, providing improved understanding of the pathophysiology of IBS.

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CONFLICT OF INTEREST

CI, SN, JS, SI, OS and MKM do not have conflict of interest to declare. HT has served as advisory board member for Almirall, Allergan, and Shire. MS has received unrestricted research grants from Danone, Ferring Pharmaceuticals and Glycom (now DSM); served as advisory board member for Almirall, Allergan, Albireo, AstraZeneca, Danone, Nestlé, Glycom (now part of DSM), Menarini, and Shire, as well as a speaker for AlfaSigma, Allergan, Almirall, Alimentary Health, Biocodex, Kyowa Kirin, Menarini, Tillotts, Takeda and Shire. LÖ has received a financial support for research by Genetic Analysis AS, Biocodex, Danone Research and AstraZeneca and served as Consultant/Advisory Board member for Genetic Analysis AS, and as a speaker for Biocodex, Ferring Pharmaceuticals, Takeda, AbbVie, and Meda.

AUTHOR CONTRIBUTIONS

Guarantor of article: Lena Öhman, PhD. CI carried out the experiments, processed biological data, performed analysis and interpreted, prepared visualizations of the data and drafted the manuscript. SN conceptualized the experimental design, established colonoid cultures, carried out the experiments, and drafted parts of the manuscript. JS provided the healthy study subjects and provided intellectual and scientific input on the final version of the manuscript. SI carried out the experiments. OS performed metabolomic analysis of samples and provided intellectual and scientific input on metabolomic statistical analysis. HT collected the study subject materials and provided intellectual and scientific input on the final version of the manuscript. MKM conceptualized the experimental design, processed and interpreted biological data and critically reviewed the

manuscript. MS provided intellectual and scientific input, critically reviewed the manuscript and obtained funding. LÖ conceived the original idea, conceptualized the exploratory study design, interpreted the data, drafted the manuscript and obtained funding. CI and SN shared first authorship. All authors have approved the final draft submitted.

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SUPPORTING INFORMATION

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The fecal microbiota transplantation response differs between patients with severe and moderate irritable bowel symptoms

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ABSTRACT

Objectives: Fecal microbiota transplantation (FMT) is a promising intervention for patients with irritable bowel syndrome (IBS). The present study aimed to identify any differences in FMT response between patients with severe and moderate IBS symptoms.

Materials and method: The study included the 164 patients who participated in our previous study, of which 96 (58.5%) and 68 (41.5%) had severe (S-IBS-S) and moderate (Mo-IBS-S) IBS, respectively. The patients were randomly divided into a placebo group (own feces) and 30-g and 60-g (donor feces) FMT groups. Patients completed three questionnaires that assessed their symptoms and quality of life at baseline and at 2 weeks, 1 month, and 3 months after FMT, and provided fecal samples before and 1 month after FMT. The fecal bacteria were analyzed using the 16S rRNA gene in PCR DNA amplification covering the V3–V9 variable genes.

Results: Response rates of the placebo group did not differ between S-IBS-S and Mo-IBS-S patients at 2 weeks, 1 month and 3 months after FMT. The response rates in the active treatment group were higher in S-IBS-S patients than in Mo-IBS-S patients at each observation time. FMT reduced abdominal symptoms and fatigue and improved the quality of life in patients with both severe and moderate IBS. Patients with S-IBS-S had higher levels of *Eubacterium siraeum*, and lower levels of *Eubacterium rectale* than Mo-IBS-S, *after FMT*.

Conclusion: Patients with S-IBS-S have a higher response rate to FMT and a marked improvement in fatigue and in quality of life compared with those with Mo-IBS-S.

The clinical trial registration number is NCT03822299 and is available at www.clinicaltrials.gov.

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Introduction

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder that considerably reduces the quality of life [1,2]. Clinical IBS treatment is directed merely at symptom relief [3]. The gut microbiota and bacterial diversity significantly affect IBS pathophysiology [4]. The outcomes of fecal microbiota transplantation (FMT) in treating IBS differ considerably in different studies from highly beneficial to no effect at all [5]. These mixed results are caused by differences in the selection criteria of the donor and the FMT protocol used as well as the cohort of patients included [5]. These differences explain why pooling all randomized clinical trials showed that FMT is not superior to placebo [6]. However, with careful donor selection and using the appropriate protocol, FMT reduces abdominal symptoms and fatigue and improves the quality of life of IBS patients [5,7].

FMT for IBS has been investigated in seven randomized controlled trials (RCTs) mostly involving patients with moderate-to-severe IBS symptoms [5]. In a recent study with a successful outcome, the patients included were those with refractory IBS patients with severe bloating who had failed to respond to at least three conventional therapies for IBS [8]. This study raised the question as to which category of IBS patients should FMT used as an intervention [8].

The present study aimed to determine the differences between patients with severe IBS symptoms (S-IBS-S) and moderate IBS symptoms (Mo-IBS-S) regarding FMT response, symptom reduction, quality-of-life improvement, dysbiosis and bacteria profile for the same patient cohort included in our previous RCT [9].

Materials and methods

Study design and patient randomization

The study design has been described in detail elsewhere [9]. To summarize, patients completed three questionnaires that assessed their symptoms and quality of life at baseline and at 2 weeks, 1 month and 3 months after FMT. They also provided fecal samples for bacterial analysis both before and 1 month after FMT. The patients were randomly divided at a

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Table 1. Characteristics of the patients in the placebo and active treated groups.

	Overall	Placebo	Active treated group
Number	164	55	109
Age, years	39.9 ± 9.0	41.2 ± 13.7	39.3 ± 12.4
Sex, female/male	133/32	47/8	86/23
IBS duration, years	15.5 ± 7.9	15.6 ± 8.0	17.3 ± 8.9
Severe symptoms*	96 (58.5%)	32 (58%)	64 (59%)
Moderate symptoms**	68 (41.5%)	23 (42%)	45 (41%)
IBS-D	63	21	42
IBS-C	62	22	40
IBS-M	39	12	27

Data are *n*, *n* (%) or mean ± SD values.
*IBS-SSS total score of ≥300; **IBS-SSS total score between 175 and 300.

1:1:1 ratio into a placebo (30-g own feces) group and 30-g and 60-g (donor feces) FMT groups.[9] The patients who received 30 g or 60 g of donor feces were combined and described as the active treatment group in order to increase the sample size and thereby reduce the probability of type-II statistical errors.

Patients

The present study included 164 patients who had participated in our previous RCT [9]. Table 1 lists the characteristics of these patients. The details of these patients have previously been described in detail [9]. To summarize, outpatients from Stord Hospital who fulfilled the Rome IV criteria for an IBS diagnosis were included. The inclusion criteria were being older than 18 years and experiencing moderate-to-severe IBS symptoms, as indicated by an IBS Severity Scoring System (IBS-SSS) score of ≥175. The exclusion criteria were pregnancy or planning pregnancy, lactating, the presence of a systemic disease, immune deficiency or having received immune-modulating medication, psychiatric illness, excessive alcohol consumption, or drug abuse. Patients who took probiotics, antibiotics, or IBS medications within 8 weeks before the study were also excluded [9].

Donor

The donor used in this study has previously been described in detail [9]. To summarize, he was screened according to the European guidelines for FMT donors [10]. He was a healthy young nonsmoking male, was not taking any medication, and had a normal BMI. He had been born via vaginal delivery, breastfed and had undertaken only a few courses of antibiotics during his life. He regularly exercised and took sport-specific dietary supplements, meaning his diet was richer in protein, fiber, minerals and vitamins than average. Bacterial analysis of his feces revealed normal bacteria diversity (normobiosis) [9].

Collection, preparation and administration of fecal samples

Fecal samples from the donor and patients were immediately frozen and stored at −20 °C until delivery to the laboratory, where they were stored at −80 °C. The FMT process has previously been described in detail [9]. To summarize, fecal samples were thawed for two days at 4 °C, mixed manually (i.e., without using a mechanical device) with 40 ml of sterile saline, and filtered before being administered. The transplant was administered to the distal duodenum via the working channel of a gastroscope [9].

Symptom and quality-of-life assessments

IBS symptoms were assessed using the IBS-SSS questionnaire. This questionnaire contains five questions, each with a maximum score of 100 using a visual analog scale. Total scores between 75 and 175 were considered indicative of mild IBS severity (Mi-IBS-S), 175–300 were Mo-IBS-S, and >300 were S-IBS-S [11]. The response was defined as a decrease in the total IBS-SSS score after FMT of ≥50 points.

Fatigue was assessed using the Fatigue Assessment Scale (FAS) questionnaire [12]. The questionnaire comprises 10 questions with 5-point-scale answers varying from never to always. Five of these questions measured physical fatigue and the other five measured mental fatigue.

Quality of life was measured using the IBS Quality of Life Scale (IBS-QoL) questionnaire [13–15]. This questionnaire contains 34 items concerning the physical and psychological functioning of IBS patients in 8 domains: dysphoria, activity interference, body image, health concerns, food avoidance, social reactions, sexual function and impact on relationships.

Microbiome analysis and dysbiosis index

The fecal bacteria profile and dysbiosis were determined using a method utilizing a pre-determined targets (PDT) approach [16]. The test uses the *16S rRNA* gene in PCR DNA amplification covering V3–V9 variable genes, and probe labeling with single nucleotide extension and signal detection using the BioCode 1000 A 128-plex Analyzer (Applied BioCode, Santa Fe Springs, CA, USA) [16]. The 48 bacterial markers used covered different taxonomic levels of >300 bacteria [17]. A dysbiosis index (DI) was measured on a 5-point scale from 1 to 5, where DI values of 1 and 2 indicate normobiosis, while those of 3–5 indicate dysbiosis [16].

Statistical analysis

Differences in response and dysbiosis proportion between S-IBS-S and Mo-IBS-S patients in the placebo and active treatment groups were analyzed using Fisher's exact test. The nonparametric Kruskal–Wallis's test with Dunn's multiple comparisons test as a post-test was used in analyzing the difference between S-IBS-S and Mo-IBS-S patients in their scores for the IBS-SSS, FAS and IBS-QoL. The Mann–Whitney *U*-test was used to analyze the differences between in fecal bacteria fluorescent signals. A nonparametric Spearman's test was used for correlation between bacterial fluorescent signals and IBS-SSS total score. These analyses were performed using GraphPad Prism 8 (La Jolla, CA, USA).

Ethics

The West Regional Committee for Medical and Health Research Ethics, Bergen, Norway approved the present study (approval no. 2017/1197/REK vest). All subjects provided both oral and written consent before participation. The study was registered at www.clinicaltrials.gov (NCT03822299) and www.cristin.no (ID657402).

Results

Response to FMT

The response rates of the placebo group did not significantly differ between S-IBS-S and Mo-IBS-S patients at 2 weeks, 1 month and 3 months after FMT. The response rates in the active treatment group were significantly higher in S-IBS-S patients than in Mo-IBS-S patients at each observation time

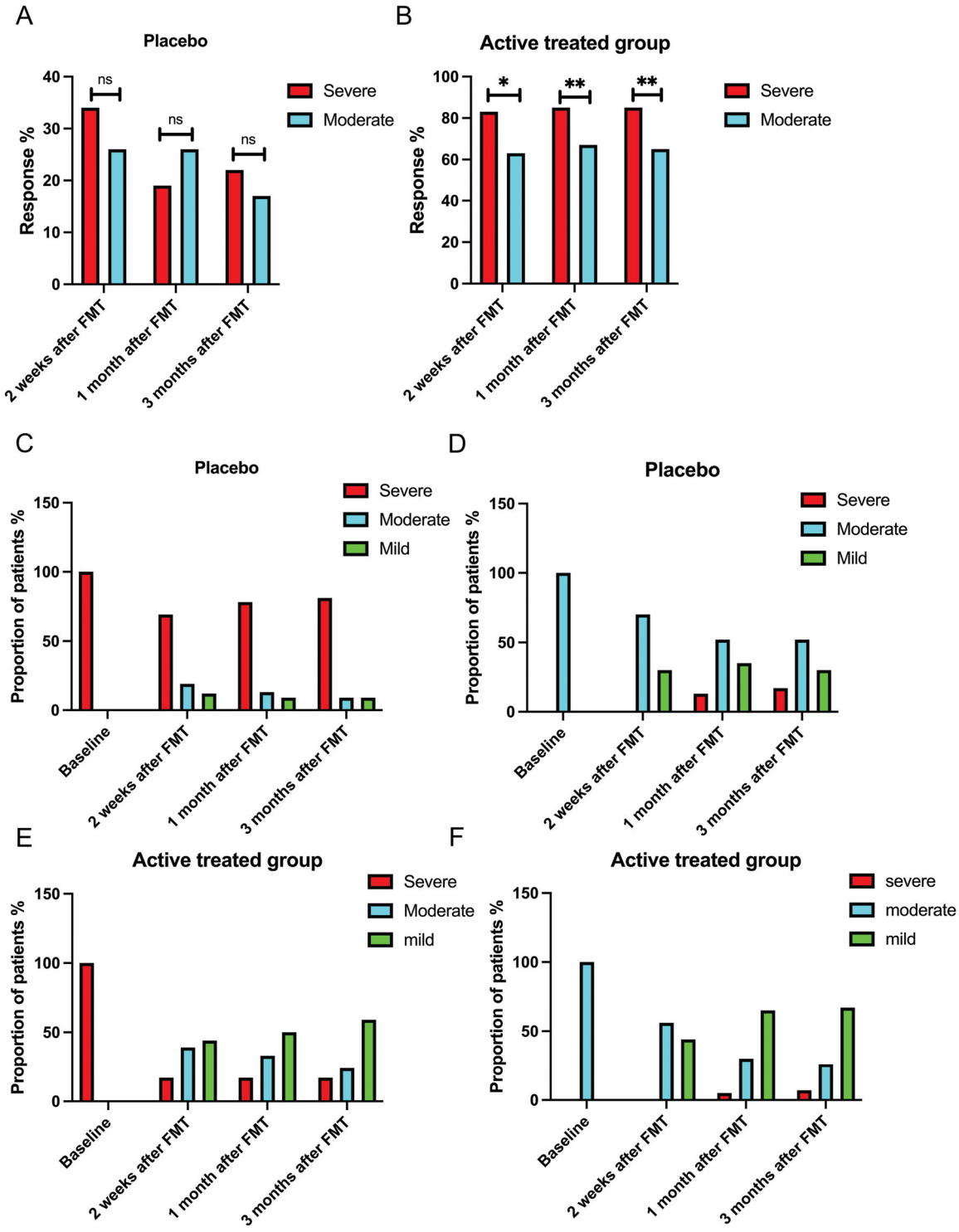

Figure 1. Fecal microbiota transplantation (FMT) response rates in patients with severe and moderate irritable bowel syndrome (IBS) symptoms at 2 weeks, 1 month and 3 months after FMT in the placebo (A) and active treatment (B) groups. Changes in IBS symptom severity over time in the placebo (C, D) and active treatment group (E, F). ns, not significant; *adjusted $p < .05$; **adjusted $p < .01$.

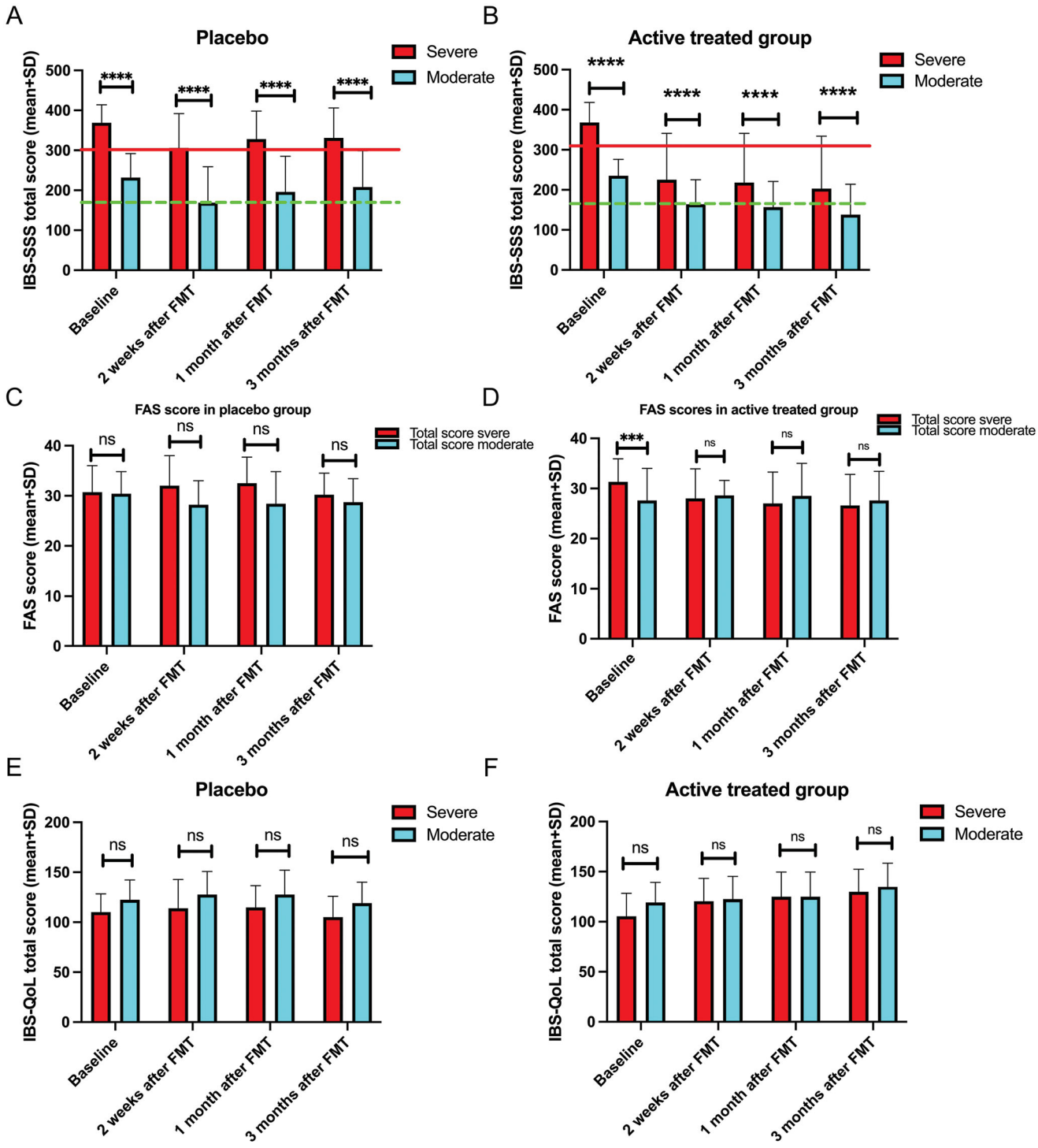

Figure 2. Total scores of IBS-SSS (A, B), of FAS (C, D), of IBS-QoL (E, F). at baseline and at 2 weeks, 1 month and 3 months after FMT. In (A) and (B), values above and below the red line indicate severe and moderate IBS symptoms, respectively. Values above and below the green line indicate moderate and mild IBS symptoms, respectively. ns, not significant; ***adjusted $p < .001$; ****adjusted $p < .0001$.

(Figure 1). In the placebo group, a small proportion of the S-IBS-S patients converted to Mo-IBS-S or Mi-IBS-S at 2 weeks, 1 month and 3 months after FMT. Similarly, a small proportion of patients with Mo-IBS-S converted to S-IBS-S or Mi-IBS-S (Figure 1). Conversely, a large proportion of either S-IBS-S or Mo-IBS-S patients experienced lower degrees of symptom severity at 2 weeks, 1 month and 3 months after FMT (Figure 1).

Symptoms and quality of life

The total IBS-SSS scores of the active treatment group decreased significantly after FMT at each observation interval, whereas those of the placebo group did not. The placebo and active treatment groups had higher total IBS-SSS scores among the S-IBS-S than the Mo-IBS-S patients at each observation interval (Figure 2). However, the difference in

Table 2. IBS-SSS total scores and scores for the four items of the scale in patients with severe and moderate IBS symptoms in placebo and active-treated groups at different intervals following FMT.

Time	Group		Total score	1	2	3	4
0	Placebo	Overall	315.2 ± 77.1	107.5 ± 46.1	55.5 ± 24.7	76.1 ± 20.7	75.4 ± 19.8
		Severe	368.9 ± 44.8	128.9 ± 40.3	70.3 ± 19.4	83.9 ± 18.0	84.2 ± 12.9
		Moderate	240.5 ± 41.5	77.8 ± 36.6	34.8 ± 14.3	65.2 ± 19.7	63.0 ± 21.4
	Active treated group	Overall	314.9 ± 83.2	108.6 ± 44.0	52.8 ± 25.6	77.1 ± 18.3	75.4 ± 19.5
		Severe	368.7 ± 51.7	124.0 ± 37.1	61.5 ± 24.0	81.8 ± 15.4	78.0 ± 18.9
		Moderate	241.5 ± 57.9	87.4 ± 44.3	41.0 ± 23.2	70.6 ± 20.1	71.8 ± 20.0
2 weeks	Placebo	Overall	278.7 ± 124.7	88.1 ± 57.0	51.2 ± 30.5	62.6 ± 28.5	64.5 ± 27.5
		Severe	383.9 ± 55.0	133.3 ± 40.0	77.0 ± 16.7	84.8 ± 19.4	85.0 ± 10.9
		Moderate	177.2 ± 80.4	44.5 ± 30.5	26.3 ± 16.5	51.1 ± 26.1	44.6 ± 23.7
	Active treated group	Overall	204.3 ± 98.9****	71.5 ± 47.3****	33.8 ± 25.3****	48.4 ± 26.2****	51.7 ± 25.6****
		Severe	214.3 ± 109.8****	75.8 ± 51.0****	36.6 ± 27.1****	50.7 ± 27.2****	54.6 ± 27.1****
		Moderate	188.3 ± 77.2***	64.8 ± 40.3**	29.3 ± 21.8**	44.8 ± 24.4****	46.9 ± 22.5****
1 month	Placebo	Overall	299.5 ± 106.1	102.0 ± 51.2	53.5 ± 27.4	70.3 ± 25.1	69.1 ± 26.3
		Severe	369.4 ± 48.3	132.8 ± 36.5	70.9 ± 20.0	80.8 ± 19.0	83.0 ± 13.5
		Moderate	202.4 ± 86.0	60.9 ± 34.4	29.1 ± 15.3	55.7 ± 25.5	49.8 ± 27.7
	Active treated group	Overall	200.0 ± 104.0****	73.2 ± 49.6****	35.3 ± 23.7****	44.8 ± 28.9****	48.6 ± 27.9****
		Severe	212.6 ± 108.9****	79.1 ± 50.6****	39.2 ± 24.5****	45.7 ± 30.0****	51.9 ± 28.8****
		Moderate	180.7 ± 94.0****	64.2 ± 47.0**	29.2 ± 21.2***	43.5 ± 27.4****	43.6 ± 26.0****
3 months	Placebo	Overall	307.0 ± 87.1	112 ± 69.7	56.8 ± 31.8	73.1 ± 22.3	71.7 ± 22.0
		Severe	363.8 ± 49.3	136.4 ± 41.6	70.5 ± 19.6	80.9 ± 18.2	82.7 ± 11.8
		Moderate	228.1 ± 63.3	71.1 ± 36.1	32.8 ± 17.0	62.0 ± 22.9	56.5 ± 24.0
	Active treated group	Overall	175.5 ± 113.6****	62.9 ± 48.4****	30.4 ± 24.2****	39.5 ± 27.3****	42.2 ± 28.6****
		Severe	190.4 ± 118.8****	69.2 ± 49.1****	32.9 ± 25.8****	42.0 ± 28.5****	45.5 ± 29.5****
		Moderate	152.9 ± 102.4****	53.5 ± 46.4***	26.5 ± 21.4***	35.8 ± 25.3****	37.2 ± 26.9***

Severe, patients with severe IBS symptoms; Moderate, patients with moderate IBS symptoms. IBS-SSS items: 1, abdominal pain; 2, abdominal distension; 3, dissatisfaction with bowel habits; 4, interference with quality of life. Data are mean ± SD values.
**Adjusted $p < .01$; ***adjusted $p < .001$; ****adjusted $p < .0001$ compared to baseline values.

the total IBS-SSS score between S-IBS-S and Mo-IBS-S was smaller in the active treatment group at 2 weeks, 1 month and 3 months after FMT (Figure 2). Notably, this suggests that the means of the total IBS-SSS scores of the S-IBS-S in the active treatment group were in the region of Mo-IBS-S after FMT at all of the observation intervals. Similarly, the means of the total IBS-SSS scores for Mo-IBS-S patients in the active treatment group were lower than those for Mi-IBS-S patients (Figure 2, Table 2).

The total FAS scores in both S-IBS-S and Mo-IBS patients decreased significantly compared to the baseline in the active treatment group at 2 weeks, 1 month and 3 months after FMT (Figure 2, Table 3).

The total IBS-QoL scores increased in both S-IBS-S and Mo-IBS-S patients in the active treatment group 2 weeks after FMT (Table 4). The total IBS-QoL scores increased in S-IBS-S patients, but not in Mo-IBS-S patients in the active treatment group 1 month after FMT. The total IBS-QoL scores increased in both S-IBS-S and Mo-IBS-S patients at 3 months after FMT (Table 4). There were no changes in the total IBS-QoL scores in the placebo group at any observation interval. Mo-IBS-S patients had higher total IBS-QoL scores than S-IBS-S patients at all observation intervals in the placebo group (Figure 2). In the actively treated group, the total IBS-QoL scores were higher in Mo-IBS-S patients than in S-IBS-S patients at the baseline, but there were no differences after FMT at any observation interval (Figure 2).

Bacterial analysis

DIs in the active treatment group in both S-IBS-S and Mo-IBS-S patients decreased 1 month after FMT, whereas they did not in the placebo group (Figure 3). DIs did not differ between patients with S-IBS-S and Mo-IBS-S at either the baseline or 1 month after FMT in both the placebo and active treatment groups (Figure 3). The fluorescence signals for all investigated bacteria did not differ between S-IBS-S and Mo-IBS-S patients in the placebo group at either the baseline or 1 month after FMT. In the active treatment group, Mo-IBS-S patients had higher fluorescence signals of *Clostridia* species than did S-IBS-S patients at the baseline. The fluorescence signals of *Eubacterium siraeum*, and *Shigella* spp., were significantly higher in S-IBS-S than Mo-IBS-S patients, and that of was lower in S-IBS-S than Mo-IBS-S patients 1 month after FMT (Figure 3). In Mo-IBS-S the fluorescence signals of *Eubacterium rectale*, *E. siraeum* and *Shigella* spp. at 1 month after FMT did not differ from those at the baseline (Table 5). The fluorescence signals of *E. siraeum* significantly decreased 1 month after FMT, but not those of *E. rectale*, and *Shigella* spp. (Table 5). Whereas *E. rectale* correlated negatively with IBS-SSS total scores, *E. siraeum*, correlated positively with IBS-SSS total scores. *Shigella* spp. did not correlate with IBS-SSS total scores (Figure 4).

Discussion

FMT as a treatment for IBS patients was investigated in seven randomized controlled trials (RCTs) [8,9,18–22]. four of which showed a positive effect [8,9,18,22] while the other three showed no effect [19–21]. It is challenging to compare these RCTs as they differed considerably in the criteria used to select the donors and patients, in the dose of the fecal transplant used and in the FMT protocols [5].

There are some safety concerns when using FMT as an IBS treatment. These concerns emerged when two patients had serious adverse events in the form of infectious complications (with one fatality) after FMT for indications other than IBS [23,24]. However, long-term follow-ups of patients

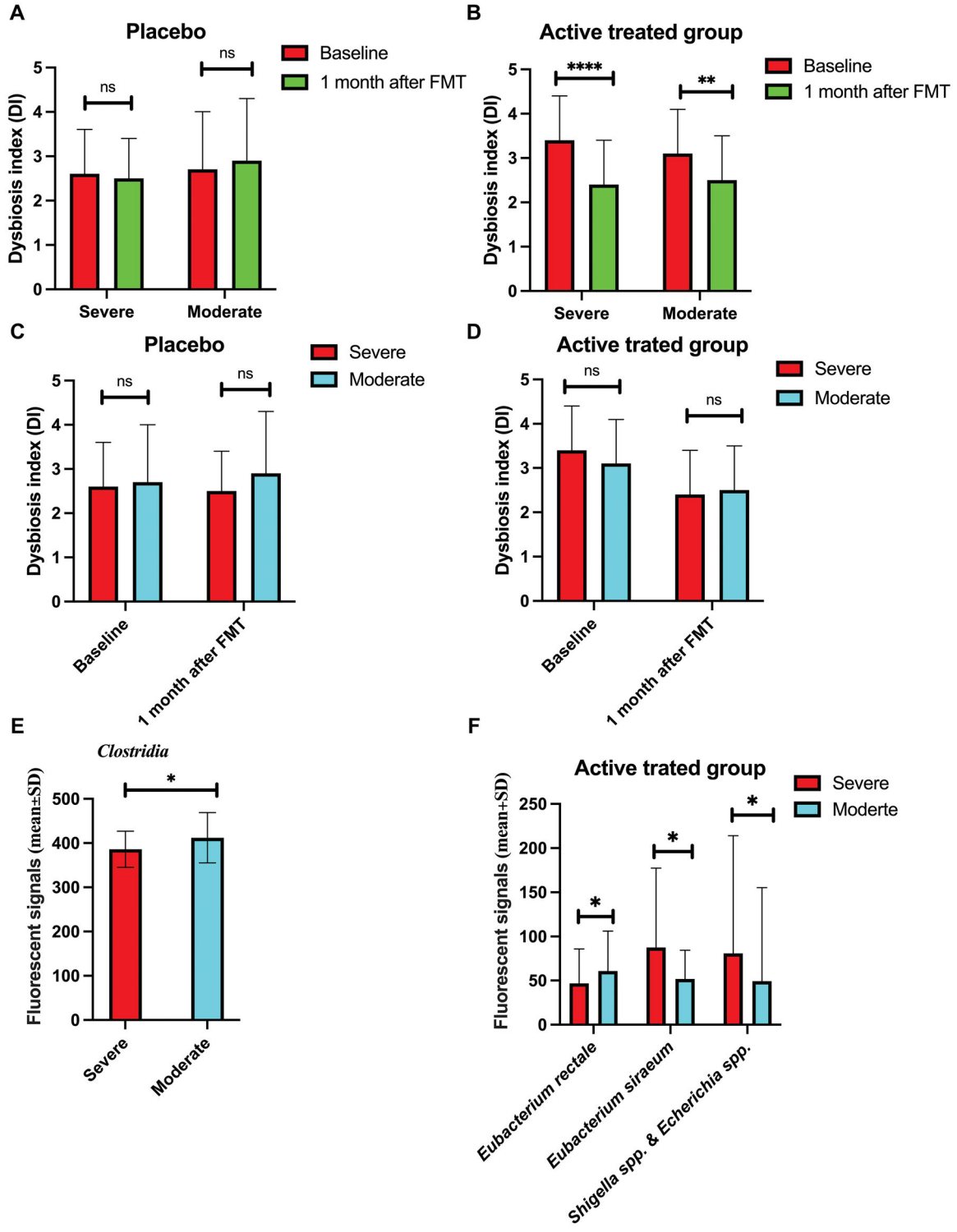

Figure 3. The Dysbiosis index (DI) of patients with severe and moderate IBS symptoms at the baseline and 1 month after FMT in the placebo (A) and active treatment (B) groups.Comparison between DIs in patients with severe and moderate IBS symptoms at the baseline and 1 month after FMT in the placebo (C) and active treatment (D) groups. The bacteria whose fluorescence signals differed between patients with severe and moderate IBS symptoms belonging to the active treatment group at the baseline (E) and 1 month after FMT (F). ns, not significant; *adjusted $p < .05$; **adjusted $p < .01$; ****adjusted $p < .0001$.

receiving FMT because of a *Clostridium difficile* infection concluded despite the development of a number of new conditions post-FMT, no clustering of diseases was associated with FMT [25]. Furthermore, recently published studies showed that FMT is safe when the international rigorous guidelines for selecting and testing the donor were applied [26–28]. IBS has been considered a benign gastrointestinal condition that is not life-threatening and using FMT as an intervention for this condition is not justified due to possible complications [29–31]. The adverse events of FMT in IBS patients are mild and self-limited, in the form of mild abdominal pain, diarrhea, or constipation [5]. To minimize risks from FMT for IBS, it

Table 3. FAS total scores and scores for physical fatigue and mental fatigue in patients with severe and moderate IBS symptoms in placebo and active-treated groups at baseline, 2 weeks, 1 month and 3 months after FMT.

Time	Group		Total score	Physical fatigue	Mental fatigue
0	Placebo	Overall	30.6 ± 4.9	15.8 ± 2.7	14.8 ± 2.6
		Severe	30.7 ± 5.3	15.8 ± 2.8	14.9 ± 2.7
		Moderate	30.4 ± 4.4	15.8 ± 2.5	14.6 ± 2.6
	Active treated group	Overall	31.3 ± 5.0	15.9 ± 2.9	15.5 ± 2.7
		Severe	31.3 ± 4.6	16.1 ± 2.8	15.3 ± 2.5
		Moderate	27.6 ± 6.4	14.4 ± 3.6	13.3 ± 3.1
2 weeks	Placebo	Overall	24.7 ± 9.9	15.5 ± 3.3	15.2 ± 3.1
		Severe	32.0 ± 6.0	16.8 ± 3.2	15.2 ± 3.1
		Moderate	28.2 ± 4.8	14.6 ± 2.6	13.7 ± 2.4
	Active treated group	Overall	28.2 ± 5.7****	14.5 ± 3.1**	13.9 ± 3.1***
		Severe	28.0 ± 5.9**	14.4 ± 3.1**	13.7 ± 3.3**
		Moderate	28.6 ± 3.0*	14.7 ± 3.0	14.2 ± 3.0*
1 month	Placebo	Overall	30.8 ± 6.0	16.2 ± 2.9	14.7 ± 3.4
		Severe	32.5 ± 5.2	17.1 ± 2.5	15.5 ± 3.0
		Moderate	28.4 ± 6.4	14.9 ± 3.0	13.5 ± 3.6
	Active treated group	Overall	27.6 ± 6.4****	14.4 ± 3.6**	13.3 ± 3.1****
		Severe	27.0 ± 6.3***	14.1 ± 3.5	13.0 ± 3.1**
		Moderate	28.5 ± 6.5*	14.8 ± 3.7**	13.8 ± 3.1***
3 months	Placebo	Overall	29.8 ± 4.6	15.2 ± 2.6	14.5 ± 2.7
		Severe	30.2 ± 4.3	15.8 ± 2.4	14.7 ± 2.6
		Moderate	28.7 ± 4.7	14.2 ± 2.5	14.3 ± 2.8
	Active treated group	Overall	27.0 ± 6.0****	13.7 ± 3.4****	13.3 ± 3.0****
		Severe	26.6 ± 6.2****	13.7 ± 3.4****	13.0 ± 3.1****
		Moderate	27.6 ± 5.8**	13.6 ± 3.5**	13.6 ± 3.0**

Data are mean ± SD values.
*Adjusted $p < .05$; **adjusted $p < .01$; ***adjusted $p < .001$; ****adjusted $p < .0001$ compared to baseline values.

Table 4. IBS-QoL total scores and scores in the eight domains of the scale in placebo and active treated groups.

Time	Group		Total score	1	2	3	4	5	6	7	8
0	Placebo	Overall	117.8 ± 19.7	27.3 ± 5.8	23.6 ± 5.8	11.9 ± 2.7	10.6 ± 2.6	7.2 ± 2.6	15.0 ± 3.1	7.8 ± 1.7	11.2 ± 2.5
		Severe	110.0 ± 18.4	26.5 ± 6.2	22.6 ± 5.2	11.2 ± 2.5	10.8 ± 2.8	6.8 ± 2.5	14.6 ± 3.2	7.6 ± 1.8	10.8 ± 2.6
		Moderate	122.4 ± 19.9	28.4 ± 5.1	25.1 ± 6.3	13.0 ± 2.6	10.4 ± 2.5	7.8 ± 2.7	15.4 ± 3.0	8.2 ± 1.5	11.7 ± 2.3
	Active treated group	Overall	111 ± 22.6	26.8 ± 6.8	20.7 ± 5.8	11.4 ± 3.1	10.4 ± 2.7	6.3 ± 2.8	13.8 ± 3.4	7.6 ± 1.9	10.8 ± 2.7
		Severe	105.4 ± 22.6	25.7 ± 7.1	19.7 ± 5.5	10.3 ± 2.8	10.1 ± 3.1	5.7 ± 2.3	13.2 ± 3.3	7.1 ± 2.0	10.4 ± 2.7
		Moderate	119.3 ± 20.0†	28.4 ± 6.1	22.0 ± 5.9	12.9 ± 2.9	10.9 ± 2.1	7.1 ± 3.3	14.7 ± 3.5	8.2 ± 1.5	11.3 ± 2.6
2 weeks	Placebo	Overall	122.4 ± 28.1	29.1 ± 6.5	23.0 ± 4.9	12.3 ± 3.33	11.3 ± 2.7	8.0 ± 3.4	14.7 ± 4.6	7.6 ± 2.6	11.1 ± 3.5
		Severe	113.9 ± 28.9	28.4 ± 7.0	22.4 ± 5.6	13.0 ± 3.6	11.1 ± 3.3	7.7 ± 3.7	14.3 ± 4.8	7.2 ± 2.8	10.7 ± 3.8
		Moderate	127.8 ± 23.0	30.2 ± 5.9	23.7 ± 3.6	13.7 ± 3.7	11.6 ± 1.7	8.4 ± 3.0	15.7 ± 4.3	8.3 ± 2.2	12.3 ± 2.4
	Active treated group	Overall	121.2 ± 24.2***	29.9 ± 6.7***	21.9 ± 6.0*	13.1 ± 3.5***	11.6 ± 2.9**	7.7 ± 3.0***	14.9 ± 3.5*	7.9 ± 1.9	11.3 ± 2.4
		Severe	120.4 ± 25.4***	29.8 ± 6.9***	21.2 ± 6.4*	13.1 ± 3.5****	12.0 ± 2.9***	7.6 ± 2.9****	14.8 ± 3.4**	7.8 ± 2.1	11.0 ± 2.6
		Moderate	122.4 ± 22.8	29.9 ± 6.7	22.7 ± 5.3ω	13.2 ± 3.6	11.2 ± 2.9	7.8 ± 3.1	15.1 ± 3.7	8.1 ± 1.6	11.7 ± 2.1
1 month	Placebo	Overall	122.9 ± 25.4	29.2 ± 7.7	23.1 ± 4.8	12.7 ± 4.2	12.3 ± 2.4	8.6 ± 2.9	14.7 ± 3.4	7.0 ± 2.3	11.4 ± 2.8
		Severe	114.8 ± 21.7	28.8 ± 6.8	21.1 ± 4.2	11.7 ± 4.2	12.4 ± 2.5	8.2 ± 3.2	14.4 ± 3.5	6.8 ± 2.3	11.0 ± 2.8
		Moderate	127.7 ± 24.4	30.0 ± 7.3	24.0 ± 5.1	13.9 ± 3.9	12.2 ± 2.3	9.1 ± 2.4	15.1 ± 3.1	7.3 ± 2.2	11.8 ± 2.7
	Active treated group	Overall	124.7 ± 24.8****	30.3 ± 6.9****	22.4 ± 5.0**	13.6 ± 3.6****	11.9 ± 2.5****	7.9 ± 3.2***	15.5 ± 3.5***	8.0 ± 1.8	11.6 ± 2.6*
		Severe	124.8 ± 25.2****	30.7 ± 7.0****	22.5 ± 4.9***	13.5 ± 3.3***	12.1 ± 2.5***	7.9 ± 3.1****ω	15.2 ± 3.7***	8.0 ± 2.1*	11.3 ± 2.7
		Moderate	124.5 ± 24.7	29.8 ± 6.8	22.2 ± 5.0	13.7 ± 4.0	11.6 ± 2.3*	8.0 ± 3.2ω	15.8 ± 3.3	8.1 ± 1.5	12.0 ± 2.3
3 months	Placebo	Overall	113.0 ± 24.3	27.2 ± 6.3	21.2 ± 4.6	12.1 ± 3.0	12.8 ± 6.3	7.1 ± 3.0	13.6 ± 3.8	7.3 ± 2.3	10.4 ± 2.5
		Severe	105.1 ± 21.3	23.8 ± 6.6	19.2 ± 4.1	11.4 ± 3.1	11.3 ± 4.6	6.7 ± 32.7	12.7 ± 4.1	6.8 ± 2.3	10.1 ± 2.6
		Moderate	119.1 ± 21.0	28.9 ± 5.5	22.3 ± 4.3	13.0 ± 2.8	14.1 ± 6.8	7.8 ± 3.2	14.7 ± 3.1	8.0 ± 2.1	10.9 ± 2.2
	Active treated group	Overall	131.7 ± 23.2****	32.3 ± 6.8****	24.1 ± 4.9****	14.0 ± 3.2****	12.4 ± 2.6****	9.2 ± 3.4****	15.9 ± 3.1****	8.2 ± 1.6*	12.4 ± 2.2****
		Severe	129.6 ± 22.8****	31.8 ± 6.6****	23.5 ± 4.6****	13.8 ± 3.3****	12.0 ± 2.7***	9.2 ± 3.5****	15.7 ± 3.1****	8.0 ± 1.6*	12.2 ± 2.3****
		Moderate	134.7 ± 23.7****	34.0 ± 7.0****	24.8 ± 5.3**	14.9 ± 3.0****	12.8 ± 2.3****	9.2 ± 3.3**	16.4 ± 3.0*	8.6 ± 1.5	12.6 ± 2.2*

IBS-QoL domains: 1, dysphoria; 2, interference with daily activities; 3, body image; 4, health worries; 5, food avoidance; 6, social reaction; 7, sexual function; 8, impact on relations.
*Adjusted $p < .05$; **adjusted $p < .01$; ***adjusted $p < .001$; ****adjusted $p < .0001$ compared to baseline values. †$p < .05$ compared to patients with severe IBS symptoms.

has been proposed that donor screening should include testing for extended-spectrum-beta-lactase-producing *Escherichia coli* and SARS-CoV-2, and FMT for IBS patients should be restricted to those without systemic disease, immune deficiency, immune-modulating medication treatment, or severe illness [31]. A recently published RCT involving refractory IBS patients with a successful outcome indicated that not all patients will benefit from FMT treatment and that the success of FMT is dependent on the IBS subset [8].

The present study showed that FMT reduces abdominal symptoms and fatigue and improves the quality of life in both S-IBS-S and Mo-IBS-S patients. FMT is therefore beneficial for both groups of IBS patients, but its effects differed between these two groups. The response rates were higher in S-IBS-S patients than in Mo-IBS-S patients at all observation intervals. Moreover, Fatigue that was higher in S-IBS-S than that of Mo-IBS-S patients at baseline decreased to the same level of MO-IBS-S after FMT. Furthermore, the quality of

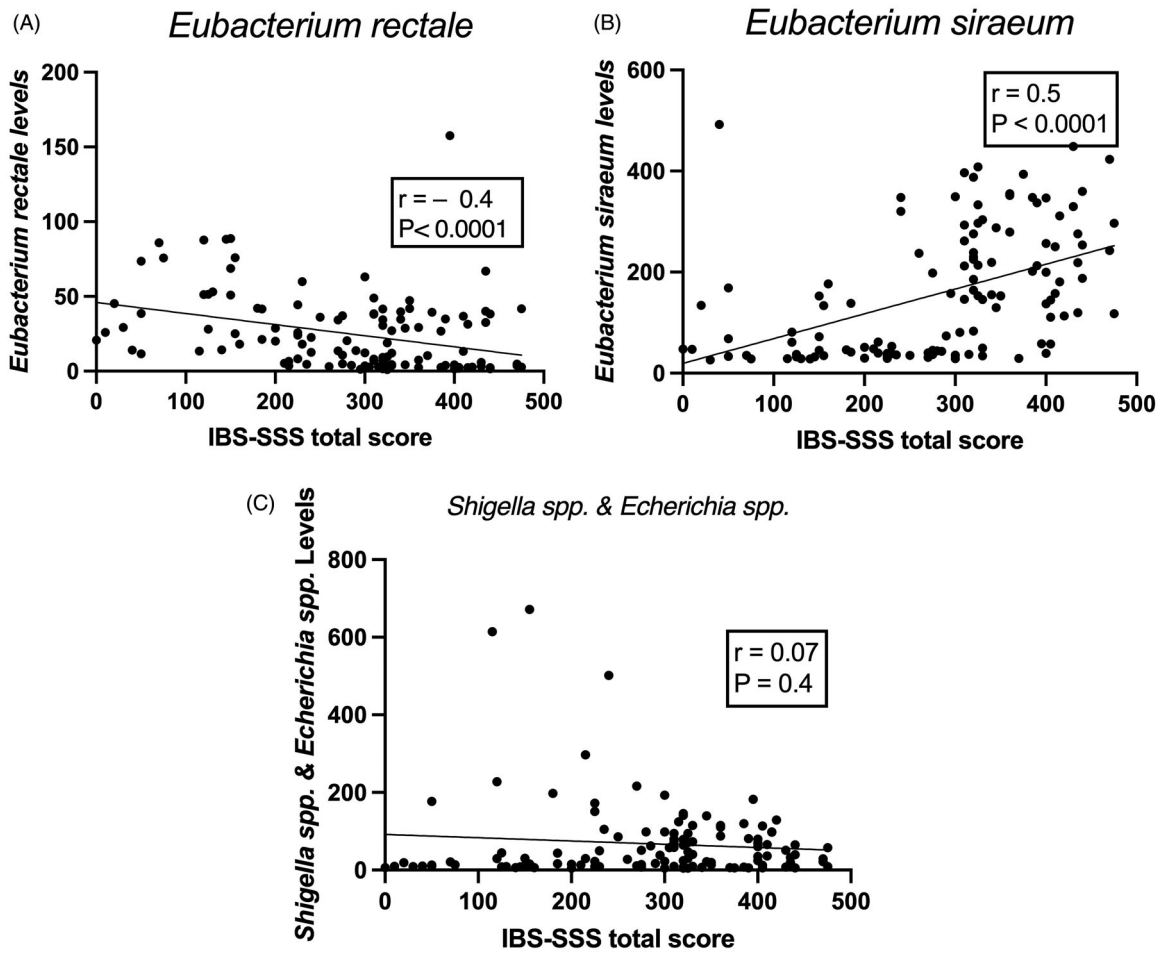

Figure 4. Correlation between IBS-SSS total score, and *Eubacterium rectale* (A), *Eubacterium siraeum* (B) and *Shigella* spp. (C).

Table 5. The fluorescence signals of *Eubacterium rectale*, *Eubacterium siraeum* and *Shigella* spp. at baseline and 1 month after FMT in IBS patients with moderate IBS symptoms (Mo-IBS-S) and patients with severe IBS Symptoms (S-IBS-S).

	Mo-IBS-S			S-IBS-S		
Bacteria	Baseline	1 month after FMT	*p*-Values	Baseline	1 month after FMT	*p*-Values
Eubacterium rectale	48.9 ± 40.1	76.6 ± 104.3	.6	62.7 ± 63.1	48.8 ± 39.4	.06
Eubacterium siraeum	56.2 ± 39.6	60.1 ± 59.9	.7	248.0 ± 92.1	83.0 ± 87.7	<.0001
Shigella spp.	53,7 ± 123,5	49.3 ± 108.1	.5	58.0 ± 46.0	81.1 ± 134.0	.4

Values expressed as mean ± SD.

life of S-IBS-S patients, which was lower than that of Mo-IBS-S patients, increased to the same level as that of Mo-IBS-S patients after the FMT intervention.

S-IBS-S patients had lower levels of *E. rectale*, and higher levels of *E. siraeum* than Mo-IBS-S patients at 1 month after FMT. It is worthy of note that the levels of *E. siraeum* decreased in S-IBS-S patients 1 month after FMT. *Eubacterium rectale* and *E. siraeum* are both anaerobic rod-shaped, Gram-positive bacteria belonging to the *Eubacteriaceae* family. *Eubacterium rectale* produces butyrate and *E. siraeum* produces acetic acid during carbohydrate fermentation [32,33]. Butyrate is a modulator of colonic hypersensitivity, and butyrate intake decreases abdominal pain in IBS patients [34–36]. Butyrate is also used as an energy source for epithelial cells in the colon. Furthermore, butyrate decreases intestinal cell permeability and intestinal motility, and modulates the immune response [37,38]. Recent research has shown that butyric acid levels are inversely correlated with the total scores for both the IBS-SSS and FAS [39]. On the other hand, acetic acid has been indicated to induce visceral hypersensitivity in rodents [40]. It is therefore tempting to speculate that the lower levels of *E. rectale* and the higher levels of *E. siraeum* in S-IBS-S patients after FMT with the subsequent decrease of butyrate and increase in acetic acid production explains why the total IBS-SSS score was higher in S-IBS-S patients than in Mo-IBS-S patients in the present study. In support of this assumption are the present findings that *E. rectale* correlated negatively with IBS-SSS total score, and *E. siraeum* correlated positively with IBS-SSS total score.

The strengths of this study are that it included a relatively large cohort of IBS patients, comprising three IBS subtypes and used a single well-defined donor. Thus, allowing a

subgroup analysis. limitations of this study are that it did not include the fourth IBS subtype, IBS-U, predominated by females and it only investigated some of the intestinal bacterial contents.

In conclusion, FMT reduced abdominal symptoms and fatigue and improved the quality of life in both S-IBS-S and Mo-IBS-S patients. However, S-IBS-S patients have a higher response rate to FMT and a marked improvement in fatigue and in quality of life compared with Mo-IBS-S patients. Although FMT seems to be more beneficial for S-IBS-S patients, FMT trials should also include Mo-IBS-S patients.

Author contributions

M.E.S. designed the study, obtained the funding, administered the study, recruited the patients, performed FMT, collected, analyzed and interpreted the data, and drafted the manuscript. T.M., T.H. and J.G.H. contributed to the design of the study, to data analysis and critically revised the manuscript for important intellectual content.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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ORIGINAL ARTICLE

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Irritable bowel syndrome patients who are not likely to respond to fecal microbiota transplantation

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Abstract

Background: Fecal microbiota transplantation (FMT) interventions have recently been advocated to not succeed in every irritable bowel syndrome (IBS) patient, since the outcome of FMT varies with the IBS subset. This study investigated the factors potentially affecting FMT response using the same patient cohort used in our previous study.

Methods: This study included 109 patients who received allogenic FMT. Patients completed five questionnaires that assessed their symptoms and quality of life at baseline and at 2 weeks, 1 month, and 3 months after FMT. Patients also provided fecal samples at baseline and 1 month after FMT. The fecal bacterial profile and dysbiosis index (DI) were determined using 16S rRNA gene PCR DNA amplification covering variable genes V3–V9. Response to FMT was defined as a decrease of ≥50 points in the total IBS-SSS score after FMT.

Results: An IBS patient's response or nonresponse to FMT was not determined by age, IBS duration, IBS subtype, IBS symptoms, fatigue, quality of life, or DI. There were more male nonresponders than responders, and the fluorescence signals of *Alistipes* were lower in nonresponders than in responders.

Conclusions: We concluded that IBS patients who are male and/or have low fecal *Alistipes* levels are most likely to not respond to FMT treatment. Whether low fecal *Alistipes* levels could be used as a marker for predicting the outcome of FMT remains to be determined.

www.clinicaltrials.gov (NCT03822299).

KEYWORDS
Alistipes, fatigue, IBS subtypes, nonresponders, responders, sex

1 | INTRODUCTION

In addition to bacteria, the gut microbiota includes fungi, archaea, viruses, and protozoans.[1,2] Although bacteria have been detected in the esophagus and stomach, most gut bacteria are located in the intestine.[1,2] The intestine contains more than 2000 bacteria species that mostly belong to the phyla Firmicutes and Bacteroides.[1–4] Gut microbiota are important for health, and disturbing their composition is associated with several gastrointestinal and nongastrointestinal disorders/diseases.[1,2]

The intestinal bacteria of irritable bowel syndrome (IBS) patients deviate from those of healthy subjects, with these patients also having a lower bacterial diversity (dysbiosis).[5–10] This deviation is believed to be involved in the pathophysiology of this disorder.[11]

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Fecal microbiota transplantation (FMT) has been performed on IBS patients in seven randomized controlled trials with varying outcomes.[12] The successful outcome of FMT seems to depend on the donor and the FMT protocol.[12]

FMT intervention has recently been suggested to not succeed in every IBS patient, with the outcomes varying with the IBS subset.[13] Another IBS intervention, low-FODMAP (fermentable oligo-, di-, mono-saccharides, and polyols)-diet, which reportedly improves the symptoms and quality of life in about 50%–75% of IBS patients,[14] identified a subset that is likely to respond to this intervention.[15] The subtyping selection was based on microbiota profiles.

This study investigated which factors could affect the FMT responses in the same IBS patient cohort that was used in our previous randomized, double-blind, placebo-controlled study.[16]

Key points

- It has been suggested that the outcomes of FMT in IBS patients vary depending on the IBS subset.
- The present study showed that females are more likely to respond to FMT than males. Age, IBS duration, IBS subtype, IBS symptoms, fatigue, quality of life, or DI did not differ between responders and nonresponders.
- Nonresponders to FMT had lower fecal levels of *Alistipes* than responders at the baseline. Further studies are needed to establish whether fecal levels of *Alistipes* could be used as a marker to identify the IBS subset that is likely to respond to FMT.

2 | MATERIALS AND METHODS

2.1 | Study design

The design of this study has previously been described in detail.[16] To summarize, patients completed five questionnaires that assessed their symptoms and quality of life at baseline and at 2 weeks, 1 month, and 3 months after FMT. Patients also provided fecal samples at baseline and 1 month after FMT. The patients were randomized at a 1:1:1 ratio into placebo (own feces), 30-g (donor feces), and 60-g (donor feces) groups.[16] Fecal samples were immediately frozen and stored at −80°C. The FMT process has previously been described in detail.[16] To summarize, the transplant was mixed manually with sterile saline and was administered to the distal duodenum via a gastroscope.[16]

2.2 | Patients and donor

This study included the 109 patients who received allogenic FMT (30 g and 60 g of donor feces) according to our previous study.[16] Table 1 lists the characteristics of these patients, who are described in detail elsewhere.[16] The inclusion criteria were an age between 18 and 75 years and having moderate-to-severe IBS symptoms, as indicated by an IBS Severity Scoring System (IBS-SSS) score of ≥175. The exclusion criteria were those who were pregnant or planning pregnancy, lactating, had a systemic disease, had immune deficiency, were receiving treatment via immune-modulating medication, had a psychiatric illness, excessively consumed alcohol, or abused drugs. Patients who took probiotics, antibiotics, or IBS medications within 8 weeks prior to the study were also excluded.[16]

The donor used in this study has also been previously described in detail.[16] To summarize, he was screened according to the European guidelines for FMT donors.[7,17]

He was a healthy male aged 36 years who fulfilled the clinical criteria of a superdonor.[12,16]

2.3 | Symptom and quality-of-life assessments

This study used the IBS-SSS, Birmingham IBS Symptom, Fatigue Assessment Scale (FAS), IBS Quality of Life Scale (IBS-QoL), and the Short-Form Nepean Dyspepsia Index (SF-NDI) questionnaires.[18–24] FMT response was defined as a decrease of ≥50 points in the total IBS-SSS score after FMT.

2.4 | Microbiome analysis and dysbiosis index

The fecal bacteria profile and dysbiosis index (DI) were determined using 16S rRNA gene PCR DNA amplification covering variable genes V3–V9. Probe labeling was achieved by single-nucleotide extension and signal detection using the BioCode 1000A 128-Plex analyzer (Applied BioCode).[8] The 48 employed bacterial markers detected bacteria within 5 phyla (Firmicutes, Proteobacteria, Bacteroidetes, Tenericutes, and Verrucomicrobia) and assessed >300 bacteria at different taxonomic levels.[8,9] DI was measured on a 5-point scale from 1 to 5, where DIs of 1 and 2 were normobiosis, and 3–5 were dysbiosis.[8]

2.5 | Statistical analysis

Differences between females and males and with IBS subtypes were analyzed using the χ^2 and Fisher's exact tests. Differences in the total scores on the IBS-SSS, Birmingham IBS Symptom, FAS, IBS-QoL, SF-NDI, DI, and in fluorescence signals of fecal bacteria between responders and nonresponders were analyzed using the Mann–Whitney test. These analyses were performed using GraphPad Prism (version 8.0).

2.6 | Ethics

The study was approved by the Western Regional Committee for Ethics, Bergen, Norway (approval no. 2017/1197/REK vest). All subjects provided both oral and written consents prior to participation. The study was registered at www.clinicaltrials.gov (NCT03822299).

3 | RESULTS

3.1 | Response to FMT

The response rates in males were significantly lower than in females ($p = 0.006$). Whereas, in responders, the proportion of females was significantly higher than males, in nonresponders the proportion of males was significantly higher than females (Figure 1). There were no differences between responders and nonresponders in IBS subtypes, age, or IBS duration (Figure 1).

3.2 | Symptoms and quality of life

The total IBS-SSS and Birmingham IBS Symptom scores did not differ between responders and nonresponders at baseline, but decreased among responders 3 months after FMT (Figure 2). The total FAS scores and the scores for its two subitems did not differ between responders and nonresponders at baseline and at 3 months after FMT (Figure 3). Total FAS scores significantly decreased in both responders and nonresponders at 3 months after FMT. Meanwhile, the physical and mental fatigue of responders decreased, while only the mental fatigue of nonresponders decreased (Figure 3).

The total IBS-QoL and SF-NDI scores did not differ between responders and nonresponders at baseline (Figure 4). The scores for both IBS-QoL and SF-NDI changed in responders but not in nonresponders after FMT (Figure 4).

3.3 | Bacteria analysis

DIs did not differ between responders and nonresponders at baseline and at 3 months after FMT (Figure 5).

At baseline, the fluorescence signals of six bacteria differed between responders and nonresponders (Figure 6). These bacteria were *Alistipes*, *Actinobacteria*, *Bacteroides fragilis*, *Bacteroides pectinophilus*, *Streptococcus salivarius* ssp. *thermophilus*, *and sanguinis*, and *Akkermansia muciniphila*. The fluorescence signals

TABLE 1 Characteristics of irritable bowel syndrome (IBS) patient who respond and do not respond to fecal microbiota transplantation

	Overall	Responders	Nonresponders
Number	109	90	19
Age, years (mean ± SD)	38.6 ± 12.5	38.5 ± 12.6	39.1 ± 12.3
Sex (female/male)	133/32	75/15	10/9
IBS-D	41	35	6
IBS-C	40	32	8
IBS-M	28	23	5
IBS duration, years (mean ± SD)	18.9 ± 9.7	19.5 ± 10.0	16.2 ± 8.1

Note: IBS-D, diarrhea-predominant IBS; IBS-C, constipation-predominant IBS; IBS-M, mixed-diarrhea-and-constipation IBS.

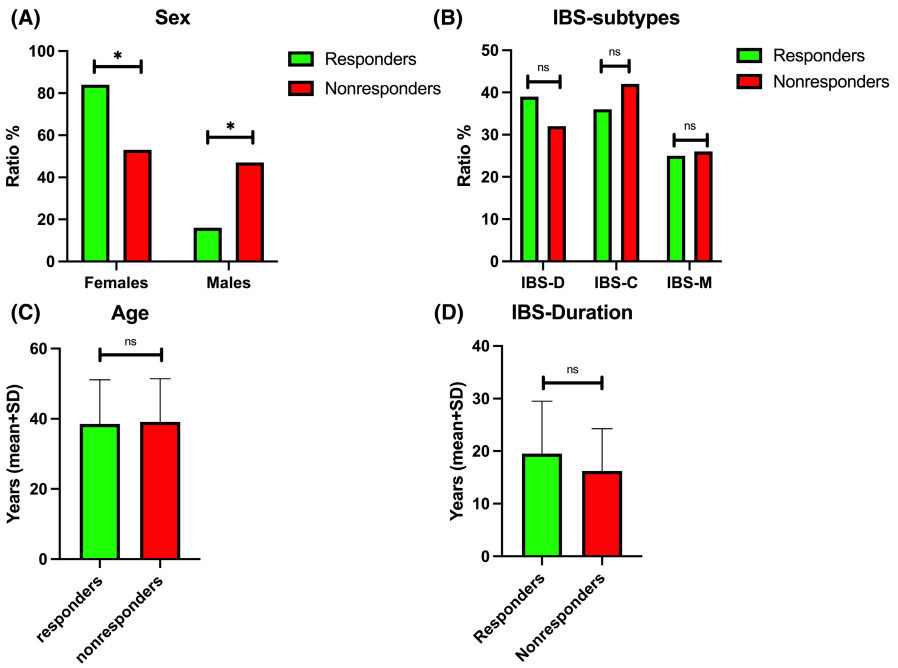

FIGURE 1 Differences between responders and nonresponders in sex (A), proportions of IBS subtypes (B), age (C), and irritable bowel syndrome (IBS) duration (D). ns, not significant; *, $p < 0.05$

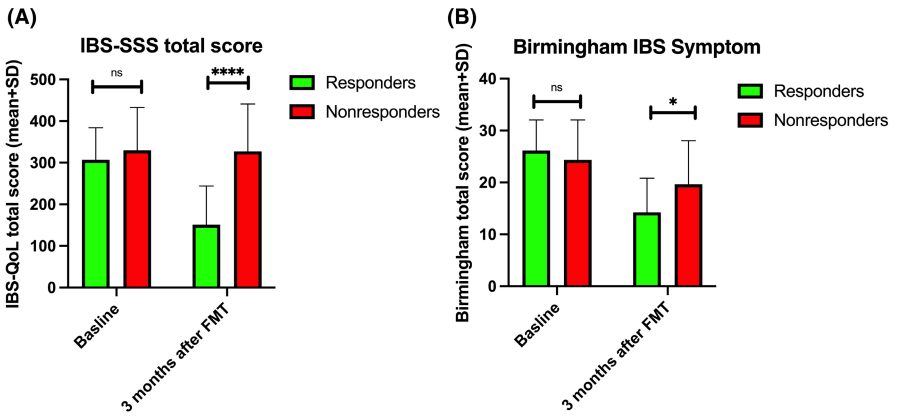

FIGURE 2 Total IBS Severity Scoring System "IBS-SSS" (A), and Birmingham IBS Symptom (B) scores in responders and nonresponders at baseline and at 3 months after fecal microbiota transplantation (FMT). ns, not significant; $^{*}p < 0.05$; $^{****}p < 0.0001$

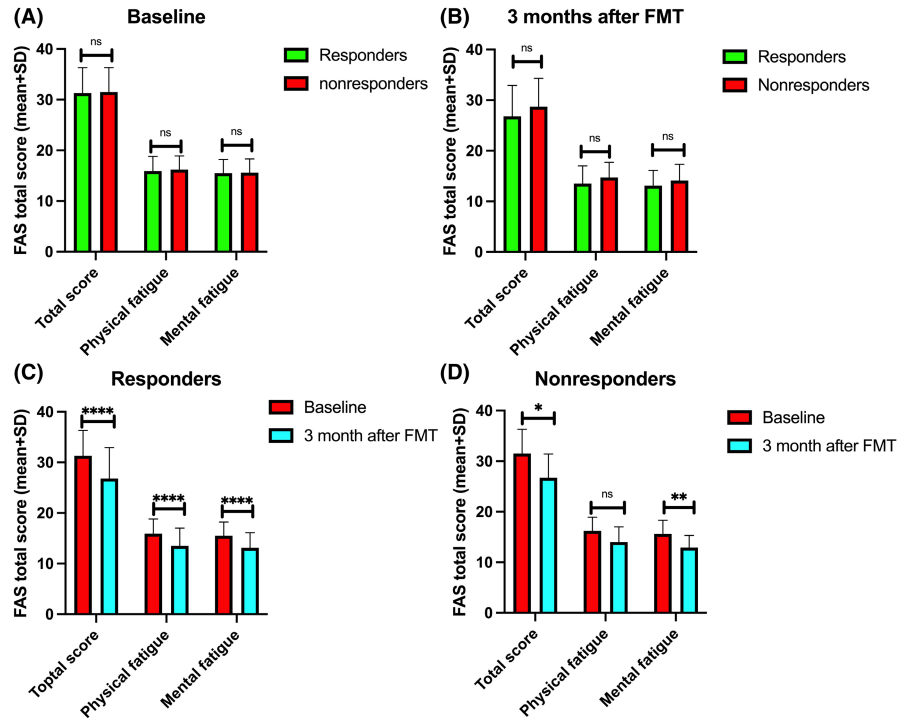

FIGURE 3 Total Fatigue Assessment Scale (FAS) scores and its two subitems in responders and nonresponders at baseline (A) and at 3 months after FMT (B). Changes in total FAS scores and scores for its subitems in responders (C) and nonresponders (D) at the baseline and 3 months after FMT. ns, not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$

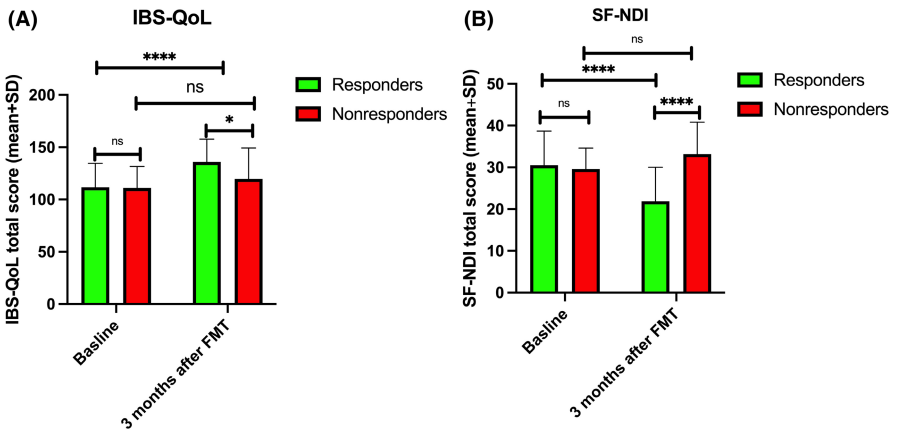

FIGURE 4 Total Quality of Life Scale (A) and Short-Form Nepean Dyspepsia Index (B) scores of responders and nonresponders at baseline and at 3 months after FMT. ns, not significant; $^{*}p < 0.05$; $^{****}p < 0.0001$

of *Alistipes*, *Bacteroides fragilis*, *Streptococcus salivarius* ssp. *thermophilus*, and *Streptococcus sanguinis* were higher in responders than in nonresponders, and the fluorescence signals of *Actinobacteria*, *Bacteroides pectinophilus*, and *Akkermansia muciniphila* were lower in responders than in nonresponders (Figure 6). There were no differences in the fluorescence signals of all these bacteria except for *Alistipes* after FMT. Although the fluorescence signals of *Alistipes* increased significantly after FMT in nonresponders, it never reached the levels of the responders (Figure 6). The fluorescence signals of *Proteobacteria*, *Shigella* spp., and *Escherichia* spp. decreased in nonresponders at 1 month after FMT (Figure 7).

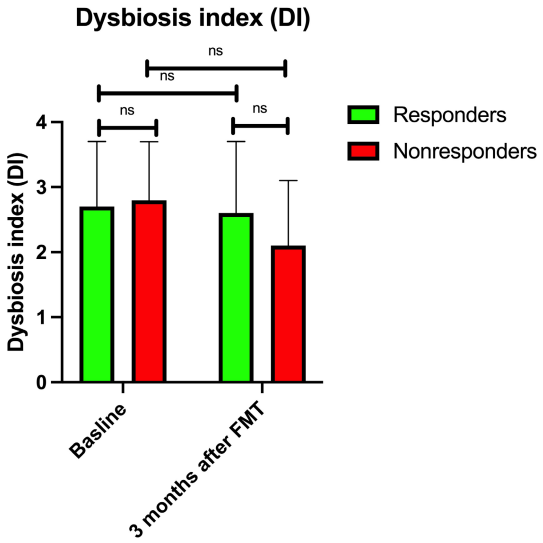

FIGURE 5 Dysbiosis index (DI) of responders and nonresponders at baseline and at 1 month after FMT. ns, not significant

4 | DISCUSSION

There is currently no effective treatment for IBS, and so the available clinical treatments focus on symptom relief. Applying FMT with a carefully selected donor and with a proper protocol is a promising intervention.[12] A single FMT improves abdominal symptoms, fatigue, and increases the quality of life in most treated patients for at least 1 year.[25] Moreover, when this intervention is done properly, it is safe despite the possibility of mild self-limiting adverse events.[12,16,25] Restricting this effective treatment to specific IBS subsets has been discussed.[13]

The present study found no differences between responders and nonresponders regarding age, IBS duration, IBS subtype, IBS symptoms, fatigue, quality of life, or DI. However, most nonresponders were males. Recently published studies have indicated that males respond to FMT less than females.[13,26] Speculating about the cause of this sex-related difference in FMT responses is difficult, but it could be due to differences in social, psychological, and/or biological factors between females and males. It should also be remembered that the prevalence of IBS in females is double that in males.[27]

The fluorescence signals of six different bacteria differed between responders and nonresponders at baseline. These bacterial differences disappeared after FMT, except for those of *Alistipes*. The fluorescence signals of *Alistipes* were lower in nonresponders than in responders at baseline, and they increased after FMT in nonresponders, but never reached the levels of the responders. *Alistipes* levels have been reported to be lower at baseline in those who responded to FMT after 3 months but relapsed after 1 year than in those who maintained a response 1 year after FMT.[25] Furthermore, *Alistipes* levels were negatively correlated with total IBS-SSS and FAS scores.[25] *Alistipes* is a genus of bacteria that was described in 2003. It is anaerobic, gram-negative, rod-shaped, and does not form spores.[28] *Alistipes* levels are associated with several diseases such as depression, anxiety, chronic fatigue syndrome, autism, cirrhosis, and aging.[28] Several immuno-inflammatory and metabolic mechanisms have been proposed for its role in human health and its association with diseases.[28] *Alistipes* hydrolyze tryptophan to indole. As tryptophan is a precursor for serotonin, an increase in level of *Alistipes* would decrease serotonin availability.[28] *Alistipes* has also been shown to express glutamate decarboxylase, an enzyme that metabolizes glutamate into γ-aminobutyric acid (GABA) in chickens.[28] Moreover, *Alistipes* produces the short chain fatty acids (SCFAs) acetic, succinic and propionic acids.[28,29] These SCFAs have anti-inflammatory effects.[28] Propionic acid has neurobiological effects in rats.[30] Furthermore, *Alistipes* expresses methylmalonyl-CoA epimerase, in which the gene for this enzyme is located on an operon with the acetyl-CoA carboxylase gene.[28]

While no improvements were found for nonresponders regarding abdominal symptoms or quality of life, fatigue did improve, in terms of mental fatigue. The fluorescence signals of *Alistipes* increased after FMT in nonresponders, and *Alistipes* is known to affect mental health.[28] It is yet to be determined to what extent this change in *Alistipes* levels contributes to this improvement.

In conclusion, this study has revealed that male IBS patients and IBS patients with low fecal *Alistipes* levels are not likely to respond to FMT treatment. Further studies are needed to establish whether *Alistipes* levels can be used as indicator to the FMT outcome.

The strengths of this study are that it included a relatively large IBS patient cohort (delet), comprising three IBS subtypes, males and females, and used a single well-defined donor. These allowed a subgroup analysis. Limitations of this study are that it did not include the fourth IBS subtype, IBS-U, and it only investigated predetermined targets of the intestinal bacterial contents.

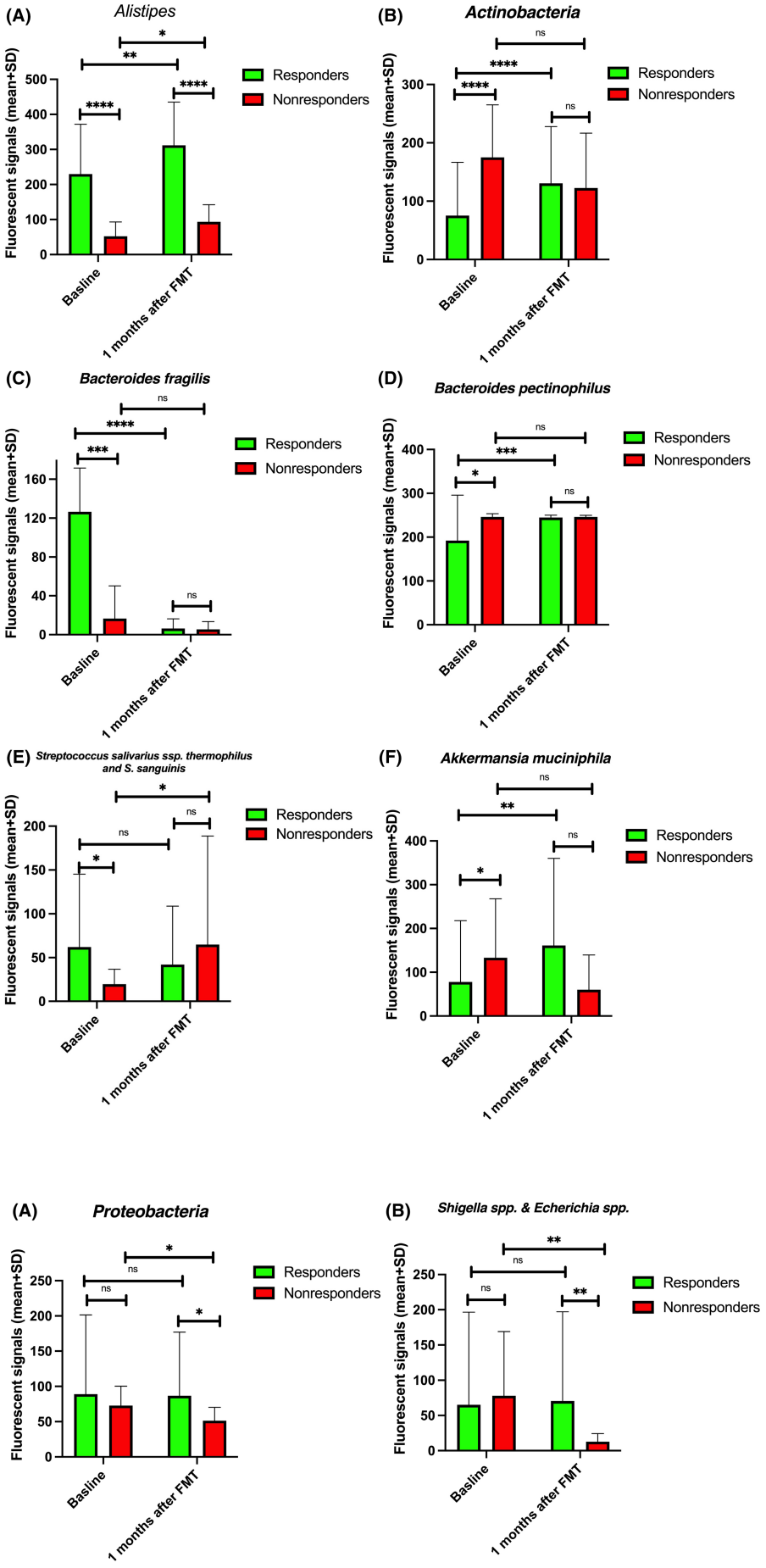

FIGURE 6 Fluorescence signals of *Alistipes* (A), *Actinobacteria* (B), *Bacteroides fragilis* (C), *Bacteroides pectinophilus* (D), *Streptococcus salivarius* ssp. *thermophilus* and *Streptococcus sanguinis* (E), and *Akkermansia muciniphila* (F) of responders and nonresponders at baseline and at 1 month after FMT. ns, not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$

FIGURE 7 Fluorescence signals of *Proteobacteria* (A) and *Shigella* spp. and *Escherichia* spp. (B) at baseline and at 1 month after FMT. ns, not significant; $^{*}p < 0.05$; $^{**}p < 0.01$

CONFLICT OF INTEREST

The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

M.E.S. designed the study, obtained the funding, administered the study, recruited the patients, performed FMT, collected, analyzed, and interpreted the data, and drafted the manuscript. T.M., T.H., and J.G.H. contributed to the design of the study, to data analysis, and critically revised the manuscript for important intellectual content.

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Clinical Gastroenterology and Hepatology 2022;20:2876–2887

FUNCTIONAL DISORDERS

Efficacy and Acceptability of Dietary Therapies in Non-Constipated Irritable Bowel Syndrome: A Randomized Trial of Traditional Dietary Advice, the Low FODMAP Diet, and the Gluten-Free Diet

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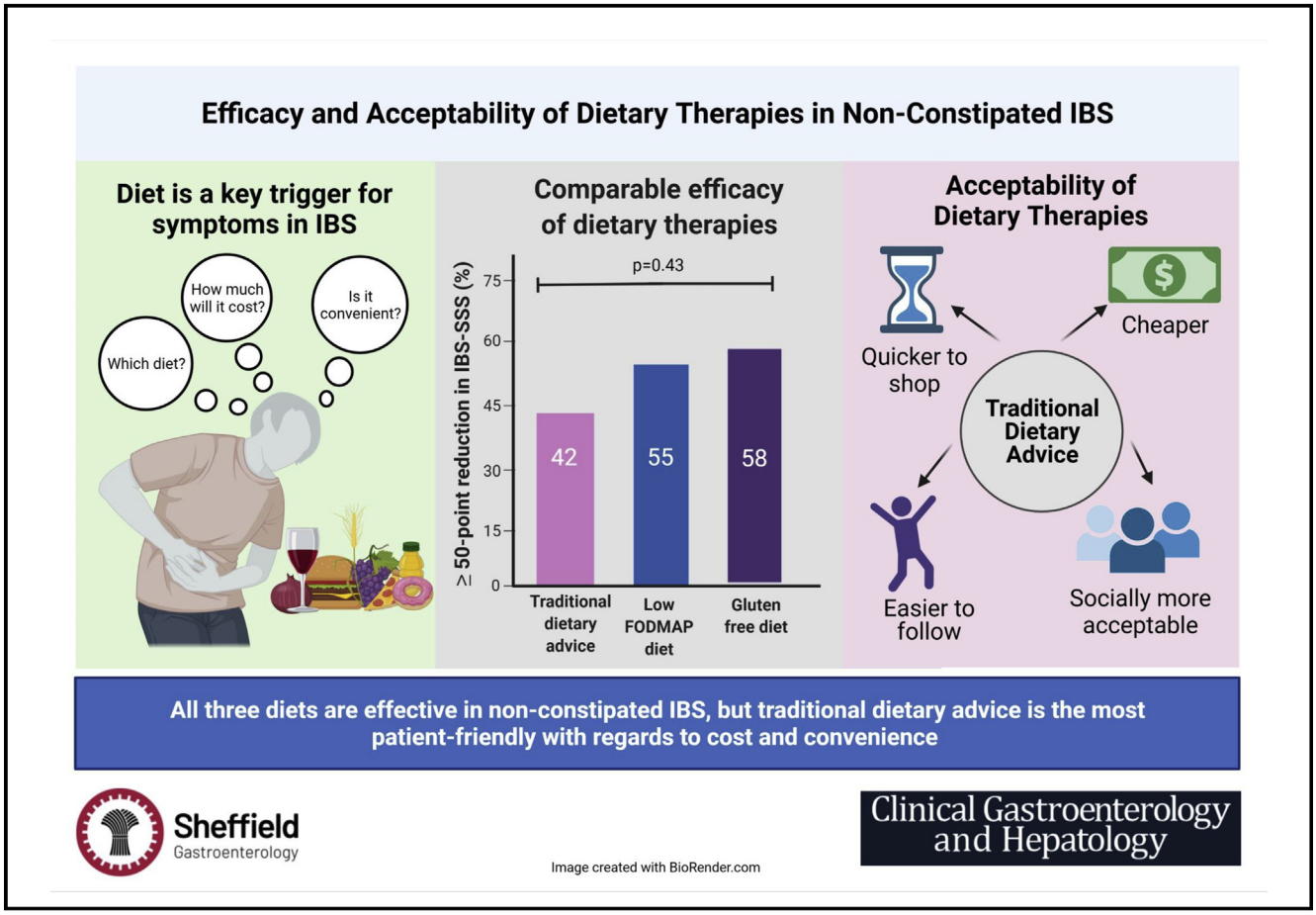

Abbreviations used in this paper: COVID-19, coronavirus disease 2019; DI, dybiosis index; DRV, dietary reference value; FODMAP, fermentable oligosaccharides; disaccharides, monosaccharides; and polyols, GFD; gluten-free diet, GI; gastrointestinal, IBS; irritable bowel syndrome, IBS-D; IBS-diarrhea, IBS-M; IBS-mixed type, IBS-SSS; IBS symptom severity score, LFD; diet low in fermentable oligosaccharides, disaccharides; monosaccharides, and polyols; QOL, quality of life; TDA, traditional dietary advice.

Most current article

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BACKGROUND & AIMS: **Various diets are proposed as first-line therapies for non-constipated irritable bowel syndrome (IBS) despite insufficient or low-quality evidence. We performed a randomized trial comparing traditional dietary advice (TDA) against the low FODMAP diet (LFD) and gluten-free diet (GFD).**

METHODS: **Patients with Rome IV–defined non-constipated IBS were randomized to TDA, LFD, or GFD (the latter allowing for minute gluten cross-contamination). The primary end point was clinical response after 4 weeks of dietary intervention, as defined by ≥50-point reduction in IBS symptom severity score (IBS-SSS). Secondary end points included (1) changes in individual IBS-SSS items within clinical responders, (2) acceptability and food-related quality of life with dietary therapy, (3) changes in nutritional intake, (4) alterations in stool dysbiosis index, and (5) baseline factors associated with clinical response.**

RESULTS: **The primary end point of ≥50-point reduction in IBS-SSS was met by 42% (n = 14/33) undertaking TDA, 55% (n = 18/33) for LFD, and 58% (n = 19/33) for GFD ($P = .43$). Responders had similar improvements in IBS-SSS items regardless of their allocated diet. Individuals found TDA cheaper ($P < .01$), less time-consuming to shop ($P < .01$), and easier to follow when eating out ($P = .03$) than the GFD and LFD. TDA was also easier to incorporate into daily life than the LFD ($P = .02$). Overall reductions in micronutrient and macronutrient intake did not significantly differ across the diets. However, the LFD group had the greatest reduction in total FODMAP content (27.7 g/day before intervention to 7.6 g/day at week 4) compared with the GFD (27.4 g/day to 22.4 g/day) and TDA (24.9 g/day to 15.2 g/day) ($P < .01$). Alterations in stool dysbiosis index were similar across the diets, with 22%–29% showing reduced dysbiosis, 35%–39% no change, and 35%–40% increased dysbiosis ($P = .99$). Baseline clinical characteristics and stool dysbiosis index did not predict response to dietary therapy.**

CONCLUSIONS: **TDA, LFD, and GFD are effective approaches in non-constipated IBS, but TDA is the most patient-friendly in terms of cost and convenience. We recommend TDA as the first-choice dietary therapy in non-constipated IBS, with LFD and GFD reserved according to specific patient preferences and specialist dietetic input. Clinicaltrials.gov: NCT04072991.**

Keywords: Irritable Bowel Syndrome; Diet; Acceptability; Nutrition; Microbiome.

Irritable bowel syndrome (IBS) is a common functional bowel disorder characterized by chronic abdominal pain, bloating, and altered bowel habit.[1] Dietary therapies are frequently recommended in IBS, because more than 80% of individuals report food-related symptoms,[2] with almost 63% wanting to know which food(s) they should avoid.[3]

The last decade has seen 3 diets popularized for non-constipated IBS, which are (1) traditional dietary advice (TDA), (2) a diet low in fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAP) (LFD), and (3) a gluten-free diet (GFD).[4] Of these, TDA is the first-line dietary therapy within the United Kingdom and is based on guidance provided by the National Institute for Health and Care Excellence and the British Dietetic Association.[5–7] Its principles include adopting healthy, sensible eating patterns such as having regular meals, never eating too little/too much, maintaining adequate hydration, and reducing the intake of (1) alcohol/caffeine/fizzy drinks, (2) fatty/spicy/processed foods, (3) fresh fruit to a maximum of 3 per day, (4) fiber and other commonly consumed gas-producing foods (eg, beans, bread, sweeteners, etc), and (5) addressing any perceived food intolerances (eg, dairy). The LFD is the second-line dietary therapy for IBS within the United Kingdom,[5–7] although in North America it is first-line.[8,9] FODMAPs are short-chain fermentable carbohydrates found in a variety of fruits, vegetables, dairy products, artificial sweeteners, and wheat. They increase small intestinal water volume and colonic gas production that, in those with visceral hypersensitivity, induce gastrointestinal (GI) symptoms.[7] The LFD initially eliminates all FODMAPs for 4–6 weeks, followed by their gradual reintroduction and personalization. Finally, taking a GFD without celiac disease has become a global phenomenon, with ∼10% of the population reporting gluten-based products to provoke intestinal symptoms compatible with IBS.[10] The mechanism for symptom improvement on a GFD are extensively debated but appear, in the main, not to be via the removal of gluten per se but rather through reducing fructan content (a FODMAP) due to wheat exclusion.[11]

Although heavily promoted, these diets are limited in evidence.[7,12] Recommendations for TDA are mainly based on clinical experience and the potential mechanisms by which these foods may induce symptoms, as opposed to randomized controlled trials.[6] With regard to a LFD, historical and contemporary reviews report an efficacy approaching ∼75%,[13,14] although a 2018 systematic review and meta-analysis of randomized

controlled trials concluded there to be low-quality evidence, mainly because of small sample sizes and significant heterogeneity between studies.[12] Interestingly, the few studies that compared the LFD with TDA demonstrated the least magnitude of effect,[12] with a response rate of 40%–50%, although some debated whether the LFD was (sub)optimally delivered and its efficacy underestimated.[15–17] Additional studies have since been performed,[18–20] with a 2021 network meta-analysis ranking the LFD first among the dietary therapies for IBS, deeming it superior to TDA.[21] Yet, trials of TDA were limited to 5 studies, had far fewer participants compared with a LFD, and some modified its recommended instructions (Supplementary Table 1).[16–21] For example, 4 of 5 studies did not advise patients to reduce commonly consumed gas-producing foods,[17–20] which contradicts the TDA concept and conceivably underestimates its efficacy. Although it may be argued that TDA overlaps with the LFD, they are appreciable differences in that the former advises reducing commonly consumed gas-producing foods, whereas the latter initially eliminates them all. A GFD in IBS has also come under scrutiny because despite reports of ~70% efficacy, a systematic review and meta-analysis identified only 2 randomized trials and concluded insufficient evidence.[4,12]

In addition, some previous IBS dietary trials have been feeding studies that, despite being a powerful proof-of-concept tool,[12] do not address the challenges placed on patients to incorporate the diets into their everyday personal and social life. This may be of relevance with the conceivably more complex LFD and GFD, which also require specialist dietetic input before implementation and incur substantial pressures on publicly funded healthcare services.[7] Concerns have also been raised that restrictive diets may induce potentially detrimental nutritional and stool microbial changes.[4]

In summary, there is no pragmatic head-to-head trial comparing the efficacy and acceptability of the LFD and GFD against TDA. We hypothesized that the LFD and GFD will be superior to TDA in improving IBS symptoms and performed a randomized trial to address this. We also investigated the acceptability, nutritional and stool microbial changes associated with these diets. Finally, we evaluated whether baseline factors predict a response to dietary intervention, because this could lead to future provision of personalized care.

What You Need to Know

Background

Dietary therapies are popular for the management of irritable bowel syndrome (IBS), yet data on their comparative efficacy and acceptability are limited.

Findings

Traditional dietary advice is effective like the low FODMAP and gluten-free diet but is more patient-friendly with regard to cost, time to shop, and ease of implementation.

Implications for patient care

Traditional dietary advice should be considered the first-choice dietary therapy in IBS, with the low FODMAP and gluten-free diet reserved according to specific patient preferences and with specialist dietetic counseling.

Methods

Participants and Setting

All authors had access to the study data and reviewed and approved the final manuscript. Patients were recruited via 2 secondary care centers in the United Kingdom. The inclusion criteria were adults aged ≥18 years with Rome IV IBS-diarrhea (IBS-D) or mixed-type (IBS-M), and an IBS-symptom severity score (IBS-SSS) of >75. The exclusion criteria are in the Supplementary Methods.

Randomization

Patients were allocated TDA, the LFD, or GFD (the latter not being strict as in celiac disease, because gluten cross-contamination was allowed, eg, sharing the same household toaster). Individuals were block-randomized into groups of up to 5, with diets given in a 1:1:1 ratio. The randomization was computer-generated and performed by an individual not involved in recruitment or treatment. Participants were seen face-to-face by specialist dietitians where they were educated on their allocated diet via a standardized 45- to 60-minute presentation, including time for questions, followed by appropriate dietary information sheets. However, after the onset of coronavirus disease 2019 (COVID-19), delivery of dietetic advice was transferred to a web-based live virtual consult, with the same information provided as with face-to-face. Participants commenced their allocated diet for 4 weeks, with outcomes at week 4 compared with baseline.

Questionnaires

Participants provided baseline demographic data. Their socioeconomic status was also determined by using the Index of Multiple Deprivation 2019 scale, because this may contribute toward an individual's biopsychosocial model and their response to dietary therapy.

The following questionnaires were completed before and after dietary intervention, with further information provided in Supplementary Material.

(1) IBS-SSS,

(2) Hospital Anxiety and Depression Scale,

(3) Patient health questionnaire-12 non-GI somatic symptoms scale,

(4) IBS quality of life (QOL) questionnaire,

(5) Acceptability of dietary restriction questionnaire,

(6) Food-related QOL questionnaire, and

(7) Comprehensive Nutrition Assessment Questionnaire (CNAQ).

Stool Samples

Participants provided stool samples before and after dietary intervention. However, this process was temporarily suspended at the start of COVID-19 and resumed once allowed. Hence, stool samples were collected in around half of cases. Data were analyzed using the GA-map Dysbiosis Test, with bacterial profiles assigned a dysbiosis index (DI) on a scale from 0 to 5, with >2 indicating a bacteria composition differing from a healthy normobiotic reference range and, as such, considered dysbiotic. Further information regarding stool sample analysis is in Supplementary Material.

End Points

The primary end point was % clinical responders after 4 weeks of dietary intervention, as defined by ≥50-point reduction in IBS-SSS, which has been shown to represent a clinically significant improvement. Secondary end points included (1) changes in individual IBS-SSS items in those with a clinical response, (2) changes in anxiety, depression, somatization, QOL, nutritional intake, gut microbiota, and (3) acceptability and food-related QOL associated with dietary therapy. An assessment was also made on whether baseline factors (age, gender, Index of Multiple Deprivation, mood, somatization, stool DI) might be associated with clinical response to dietary therapy.

Sample Size and Statistical Analysis

The sample size calculation considered the aforementioned ambiguities regarding the true efficacy of a LFD or GFD, with some groups reporting ~75% response,[13,14,22,23] and questioning the lower response rates from randomized trials.[15] Furthermore, detecting a large effect size might be desirable if demanding diets (ie, LFD/GFD) were to be considered first choice over the relatively straightforward TDA. Assuming a response rate of 75% with LFD or GFD and 40% with TDA, 31 subjects per arm were required to detect a 35% difference with 80% power at $\alpha = 0.05$. To accommodate 10% dropout rate, we aimed for 33 individuals per arm. Of note, the effect size is comparable with previous studies[16,17] and those published recently.[18–20]

Full details on statistical analyses are in Supplementary Material. The P value was significant at <.05, with post hoc Bonferroni corrections performed as required.

Results

Of 114 participants recruited, 101 commenced dietary intervention (TDA = 35, LFD = 33, GFD = 33), with 2 excluded as lost to follow-up. A total of 99 participants, 33 per arm, completed the study (Supplementary Figure 1). There was no difference in baseline variables across groups (Supplementary Table 2). The mean age was 37 years, with 71% female, 88% white, 75% IBS-D, and 25% IBS-M. The mean baseline IBS-SSS was 301, with 9% having mild IBS, 47% moderate IBS, and 45% severe IBS ($P = .5$ across groups).

Clinical Response

The primary end point of ≥50-point reduction in IBS-SSS was met by 42% (n = 14/33) taking TDA, 55% with LFD (n = 18/33), and 58% with GFD (n = 19/33), with no significant difference across groups ($P = .43$) (Figure 1).

Of those who experienced ≥50-point reduction in IBS-SSS, there were significant within-group improvements in individual IBS-SSS items. This was seen with each dietary therapy but with no significant difference across groups (Table 1).

A ≥50-point reduction in IBS-SSS was seen in 52% (n = 15/29) receiving face-to-face consult vs 51% (n = 36/70) receiving live virtual consult ($P = .98$). This was seen to a similar extent irrespective of the allocated dietary therapy (data not shown).

A ≥50-point reduction in IBS-SSS was seen in 54% (n = 40/74) with IBS-D vs 44% (n = 11/25) with IBS-M, with no difference between groups ($P = .38$). There was no statistical difference in response rates between IBS-D vs IBS-M on the basis of a particular dietary therapy (data not shown).

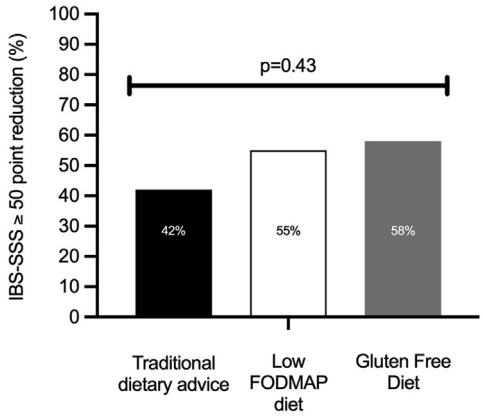

Figure 1. Response rate to dietary therapies.

Table 1. Change in IBS-SSS and Its Individual Items in Patients Responding to Dietary Therapy (n = 51 of 99)

IBS-SSS and its individual items, mean (standard deviation)	Intervention									Difference in reduction across groups, *P* value
	TDA (n = 14)			LFD (n=18)			GFD (n=19)			
	Baseline	Week 4	Within-group change[a]	Baseline	Week 4	Within-group change[a]	Baseline	Week 4	Within-group change[a]	
IBS-SSS	330 (74)	199 (93)	131	311 (80)	148 (87)	163	299 (73)	180 (89)	119	.13
Abdominal pain severity	51 (22)	28 (30)	23	55 (23)	18 (17)	37	52 (22)	25 (21)	27	.11
No. of days in pain every 10 days	6.1 (2.5)	3.5 (3.2)	2.6	6.0 (2.9)	3.1 (2.3)	2.9	6.4 (1.8)	3.7 (2.6)	2.7	.93
Abdominal distention severity	63 (24)	28 (26)	35	54 (32)	22 (23)	32	49 (24)	21 (22)	28	.61
Satisfaction with bowel habits	78 (19)	58 (30)	20	75 (25)	44 (32)	31	71 (24)	52 (28)	19	.50
Interference with life in general	76 (15)	50 (26)	26	67 (22)	33 (28)	34	63 (22)	44 (32)	19	.17

NOTE. Values presented as mean (standard deviation).
[a]Significant within-group reductions for all IBS-SSS items.

Impact on Mood, Somatization, and IBS-QOL

Individuals allocated a LFD had a significant improvement in depression compared with TDA. Changes in anxiety, somatization, and IBS QOL did not differ across groups, except for the LFD having a significant improvement in dysphoria compared with TDA and GFD (Supplementary Table 3).

Acceptability of Dietary Restriction and Food-Related QOL

Individuals found TDA cheaper ($P < .01$), less time-consuming to shop ($P < .01$), and easier to follow when eating out at family and friends ($P = .03$) compared with a GFD and LFD. Individuals found TDA and GFD easier to incorporate into their life than the LFD ($P = .02$) (Table 2).

The proportions of individuals who would consider continuing the diets were 70% (n = 23) for TDA, 67% (n = 22) for LFD, and 61% (n = 20) for GFD, with no difference across groups ($P = .73$).

Nutritional Intake and FODMAP Composition

Although macronutrients and micronutrients reduced within each dietary group, there was no significant difference across groups besides a trend toward more fiber reduction on the LFD compared with the GFD and TDA ($P = .06$) (Table 3).

The proportion of individuals meeting recommended dietary reference values (DRVs) for macronutrients did not change before to after intervention for any of the diets. However, DRVs for the micronutrients of potassium and iron were significantly reduced with TDA, whereas thiamine and magnesium were significantly reduced with the LFD and GFD. The majority of individuals across all 3 diets failed to meet DRVs for total energy intake both before and after intervention (Supplementary Table 4).

Significant within-group reduction in total FODMAP intake occurred with all 3 diets (Table 4). However, the greatest reduction was with a LFD (27.7 g/day before intervention to 7.6 g/day at week 4) compared with TDA (24.9 g/day to 15.2 g/day) and GFD (27.4 g/day to 22.4 g/day) ($P < .01$).

As expected, the LFD led to significant reductions in each individual FODMAP component, whereas with TDA it was for fructo-oligosaccharides, lactose, and mannitol, and with the GFD it was for fructo- and galacto- oligosaccharides. The LFD led to a significantly greater reduction in fructo-oligosaccharides, galacto-oligosaccharides, and mannitol compared with TDA and a significantly greater reduction in lactose, excess fructose, and mannitol compared with the GFD (Table 4).

Table 2. Acceptability of Dietary Restriction and Food-Related QOL

	Agree, n *(%)*			Neutral, n *(%)*			Disagree, n *(%)*			Comparison across groups *p-value*
	TDA	LFD	GFD	TDA	LFD	GFD	TDA	LFD	GFD	
Acceptability of dietary restriction										
I find it easy to buy suitable foods for my current diet at my normal supermarkets or shops.	19 (58)	12 (36)	18 (55)	13 (39)	13 (39)	11 (33)	1 (3)	8 (24)	4 (12)	.1
I am able to buy foods suitable for my current diet at my normal supermarkets or shops.	23 (70)	18 (55)	26 (79)	7 (21)	11 (33)	6 (18)	3 (9)	4 (12)	1 (3)	.3
I use high street/online specialty shops (eg, health food shops) to buy food for my current diets.	8 (24)	3 (9)	9 (27)	7 (21)	7 (21)	7 (21)	18 (55)	23 (70)	17 (52)	.4
It takes extra time to shop for my current diet.	13 (39)	26 (79)	23 (70)	7 (21)	4 (12)	8 (24)	13 (39)	3 (9)	2 (6)	<.01[a]
I find food labelling is adequate to allow me to confidently choose suitable foods.	18 (55)	20 (61)	28 (85)	13 (39)	9 (27)	3 (9)	2 (6)	4 (12)	2 (6)	.04
The cost of my current diet is more expensive.	16 (49)	27 (82)	27 (82)	8 (24)	3 (9)	6 (18)	9 (27)	3 (9)	0 (0)	<.01[a]
Does eating out at restaurants make it more difficult for you to follow your current diet?	19 (58)	20 (61)	19 (58)	9 (27)	12 (36)	11 (33)	5 (15)	1 (3)	3 (9)	.5
Does eating out at friends/families make it more difficult for you to follow your current diet?	17 (52)	22 (67)	22 (67)	7 (21)	10 (30)	9 (27)	9 (27)	1 (3)	2 (6)	.03[a]
Does travel (overseas/United Kingdom) make it more difficult for you to follow your current diet?	18 (55)	15 (46)	13 (39)	11 (33)	16 (49)	18 (55)	4 (12)	2 (6)	2 (6)	.5
Overall, I find my current diet tasty and enjoyable.	17 (52)	14 (42)	13 (39)	11 (33)	9 (27)	18 (55)	5 (15)	10 (30)	2 (6)	.04
I can incorporate my current diet easily into my life.	18 (55)	11 (33)	15 (46)	13 (39)	11 (33)	15 (46)	2 (6)	11 (33)	3 (9)	.02[b]
My current dietary needs have created stress with my family/friends.	3 (9)	8 (24)	8 (24)	13 (39)	11 (33)	10 (30)	17 (52)	14 (42)	15 (46)	.5
Food-related QOL										
Food and meals are positive elements of my life.	17 (52)	17 (52)	22 (67)	13 (39)	6 (18)	8 (24)	3 (9)	10 (30)	3 (9)	.05
I am generally pleased with my food.	22 (67)	15 (46)	17 (52)	8 (24)	9 (27)	14 (42)	3 (9)	9 (27)	2 (6)	.05
My life in relation to food and meals is close to my ideal.	6 (18)	7 (21)	7 (21)	14 (42)	11 (33)	19 (58)	13 (39)	15 (46)	7 (21)	.3
With regard to food, the conditions of my life are excellent.	4 (12)	6 (18)	9 (27)	18 (55)	12 (36)	17 (52)	11 (33)	15 (46)	7 (21)	.2
Food and meals give me satisfaction in daily life.	20 (61)	15 (46)	18 (55)	9 (27)	6 (18)	10 (30)	4 (12)	12 (36)	5 (15)	.1
I wish my meals were much more pleasant part of my life.	11 (33)	20 (61)	13 (39)	11 (33)	7 (21)	13 (39)	11 (33)	6 (18)	7 (21)	.2
When I think of my next meal, I only see problems, obstacles, and disappointments.	4 (12)	11 (33)	7 (21)	11 (33)	13 (39)	13 (39)	18 (55)	9 (27)	13 (39)	.2

[a]Statistically significant difference between TDA vs LFD and GFD on post hoc analysis.
[b]Statistically significant difference between LFD vs TDA and GFD on post hoc analysis.

Table 3. Nutritional Intake at Baseline and Week 4 of Dietary Therapy

	Intervention						
	TDA		LFD		GFD		Difference in reduction across groups, *P* value
Nutritional parameter	Baseline	Week 4	Baseline	Week 4	Baseline	Week 4	
Energy, *kcal/d*	2373 (1774–2923)	1861 (1579–2411)	2338 (1574–2764)	1738 (1210–2231)	2366 (2030–2928)	1958 (1406–2770)	.63
Protein, *g/d*	104.2 (81.6–160.4)	90.9 (65.6–108.6)	97.1 (74.1–118.6)	80.4 (51.6–95.7)	99.7 (74.4–132.6)	79.1 (61.5–105.3)	.52
Carbohydrate, *g/d*	268 (224–342)	222 (203–320)	277 (211–357)	223 (141–277)	307 (231–375)	227 (174–306)	.55
Fat, *g/d*	86.8 (57.9–112.2)	65.3 (44.5–87.8)	82.3 (56.9–114.9)	64.6 (43.5–95.2)	86.1 (71.0–115.8)	77.9 (49.3–113.0)	.66
Dietary fiber, *g/d*	32.6 (27.4–40.7)	28.5 (21.4–35.4)	23.5 (16.8–44.1)	18.7 (14.3–31.7)	32.7 (23.4–39.3)	25.9 (21.1–35.0)	.06
Folate, *μg/d*	449 (351–583)	353 (273–496)	362 (219–592)	291 (175–407)	392 (311–524)	335 (254–496)	.22
Thiamine, *mg/d*	1.70 (1.38=2.73)	1.40 (1.10–1.73)	1.40 (1.00–2.40)	1.00 (0.60–1.45)	1.50 (1.30–2.55)	1.10 (0.80–1.70)	.13
Riboflavin, *mg/d*	2.50 (1.70–4.25)	2.00 (1.20–2.63)	1.90 (1.43–3.28)	1.65 (1.13–2.53)	2.10 (1.80–3.70)	2.00 (1.30–2.90)	.12
Niacin, *mg/d*	24.0 (16.1–30.3)	19.6 (16.6–23.9)	19.1 (13.3–25.3)	14.8 (11.5–20.9)	20.2 (18.6–27.7)	17.7 (15.1–23.2)	.72
Vitamin C, *mg/d*	185 (143–280)	172 (115–250)	111 (78–251)	94 (73–213)	163 (124–244)	150 (117–204)	.16
Sodium, *mg/d*	2772 (1695–3204)	1947 (1516–2485)	2220 (1641–2915)	1761 (1372–2642)	2424 (1980–3217)	1910 (1446–2902)	.97
Potassium, *mg/d*	4394 (3739–5620)	3704 (2967–4807)	4042 (2819–5077)	3119 (2097–3813)	4039 (3469–5370)	3518 (2582–4577)	.50
Magnesium, *mg/d*	377 (288–517)	315 (252–423)	324 (238–440)	247 (169–333)	347 (292–426)	298 (234–379)	.36
Calcium, *mg/d*	1122 (917–2030)	896 (625–1357)	991 (714–2069)	888 (520–1330)	1057 (792–1699)	1049 (605–1510)	.14
Phosphorus, *mg/d*	1771 (1424–2569)	1476 (1202–1750)	1472 (1146–2111)	1365 (853–1793)	1606 (1200–2382)	1435 (1067–1956)	.41
Iron, *mg/d*	13.4 (9.4–14.3)	11.5 (9.4–14.3)	11.7 (8.5–15.6)	10.4 (6.5–14.1)	12.7 (10.0–16.0)	10.6 (8.6–13.9)	.70
Zinc, *mg/d*	11.8 (8.6–14.9)	10.7 (8.3–13.2)	11.0 (8.7–13.3)	11.1 (6.3–12.9)	11.2 (8.7–15.0)	10.9 (7.5–15.0)	.70

Footnote; Values presented as median (IQR).
Significant within-group reductions seen with most macro- and micro- nutrients following dietary intervention, except for zinc (all diets), vitamin C (TDA and GFD), and fibre/folate/riboflavin (GFD)
NOTE. Values presented as median (interquartile range). Significant within-group reductions seen with most macronutrients and micro nutrients after dietary intervention, except for zinc (all diets), vitamin C (TDA and GFD), and fiber/folate/riboflavin (GFD).

Table 4. FODMAP Intake at Baseline and Week 4 of Dietary Therapy

	Intervention									
	TDA			LFD			GFD			
FODMAP	Baseline	Week 4	Baseline vs Week 4, *P* value	Baseline	Week 4	Baseline vs. Week 4, *P* value	Baseline	Week 4	Baseline vs Week 4, *P* value	Difference in change across groups, *P* value
Oligosaccharides										
Fructo-oligosaccharides, *g/d*	3.8 (2.7–4.7)	2.9 (2.2–3.7)	<.01	3.3 (1.8–6.2)	1.6 (0.8–2.5)	<.01	3.9 (3.0–4.5)	2.4 (1.6–4.0)	<.01	<.01[a]
Galacto-oligosaccharides, *g/d*	1.1 (0.8–1.5)	1.1 (0.7–1.3)	.05	1.2 (0.6–2.2)	0.6 (0.3–1.1)	<.01	1.2 (0.9–2.2)	0.9 (0.7–1.6)	.02	<.01[a]
Disaccharides										
Lactose, *g/d*	11.7 (4.3–26.4)	4.9 (1.0–15.0)	<.01	12.5 (3.3–24.0)	1.9 (0.5–6.5)	<.01	14.3 (7.0–26.0)	13.0 (4.6–22.0)	.22	.02[b]
Monosaccharides										
Excess fructose, *g/d*	5.2 (2.6–7.0)	2.8 (1.7–6.8)	.31	3.5 (2.0–10.4)	1.5 (0.8–3.5)	<.01	4.0 (2.3–6.6)	4.0 (2.2–6.4)	.95	<.01[b]
Polyols										
Sorbitol, *g/d*	1.9 (0.7–3.0)	1.4 (0.4–2.8)	.18	1.3 (0.6–2.2)	0.3 (0.1–1.0)	<.01	2.1 (1.1–3.2)	1.9 (0.9–3.4)	.84	.05
Mannitol, *g/d*	0.8 (0.5–1.1)	0.6 (0.4–1.0)	<.01	0.6 (0.3–0.8)	0.1 (0.0–0.3)	<.01	0.7 (0.4–1.1	0.6 (0.3–1.1)	.70	<.01[a,b]
Total FODMAPs, *g/d*	24.9 (13.8–53.4)	15.2 (9.1–28.0)	<.01	27.7 (13.9–46.3)	7.6 (2.8–13.7)	<.01	27.4	22.4	.03	<.01[a,b]

NOTE. Values presented as medians (interquartile range).
[a]Statistically significant difference between LFD and TDA on post hoc analysis.
[b]Statistically significant difference between LFD and GFD on post hoc analysis.

Stool Analysis

A total of 55 paired stool samples were analyzed (TDA = 18, LFD = 17, GFD = 20). Changes in DI did not differ across groups ($P = .99$), with 22%–29% having an improvement, 35%–39% having no change, and 35%–40% having worsening DI (Figure 2). Changes in DI did not differ between responders and non-responders (Supplementary Table 5).

No significant changes in functional bacterial profiles were noted (Supplementary Table 6), with specific alterations in bacterial abundance reported in Supplementary Tables 7–9.

Factors Associated With Clinical Response

Age, gender, IBS subtype, Index of Multiple Deprivation, somatization, and mood did not predict response to dietary therapies (Supplementary Table 10), and baseline stool DI did not (Supplementary Figure 2).

Discussion

This is a randomized trial comparing the efficacy and convenience of TDA, LFD, and GFD in non-constipated IBS. The pragmatic study design, whereby the responsibility was left on patients to undertake the diets following appropriate education, means our findings can be generalized. The main results are that the diets did not significantly differ in clinical efficacy, with 42%–58% experiencing a ≥50-point reduction in IBS-SSS. Responders had similar improvements in IBS-SSS items regardless of their allocated diet. Individuals found TDA cheaper, less time-consuming to shop, and easier to follow when eating out than the GFD and LFD. It was also easier to implement into everyday life than the LFD. Neither clinical characteristics nor stool DI predicted response to dietary therapy. Finally, the modes of dietary education, either face-to-face or virtual, were equally effective.

Our study has notable strengths. First, it is among the largest studies assessing dietary therapies in IBS.[12] Second, we provided dietary education as per recommended instructions,[5,6] whereas 4 of 5 previous randomized trials of TDA have been limited to providing a modified or incomplete version.[17–20] This could partly explain TDA being ranked inferior to a LFD in a recent network meta-analysis.[21] Our findings shed further clarity on the efficacy of TDA and are in line with a Swedish randomized trial that provided TDA instructions as per guidance and noted no difference versus the LFD.[16] The added value of our study is its assessment of dietary acceptability, as well as evaluating a GFD, which has become increasingly popular in modern times. Although a recent Italian study demonstrated similar clinical efficacy between the LFD, GFD, and a balanced Mediterranean diet, with 86% of patients subsequently expressing a preference for the latter, it was limited to being a small non-randomized trial of 42 patients, and the Mediterranean diet did not resemble TDA.[24]

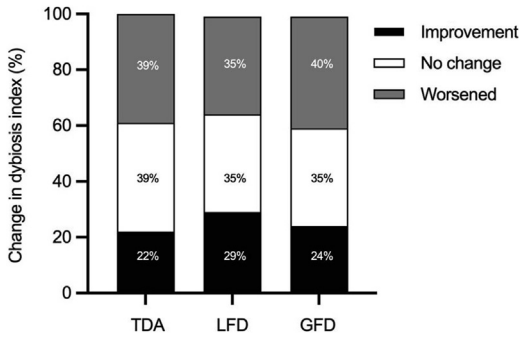

Figure 2. Change in stool dysbiosis index.

Third, objective evidence to support dietary adherence can be inferred from the reductions seen in specific FODMAPs. For example, there was a marked reduction in all FODMAPs within the LFD group (27.7 g/day to 7.6 g/day, with <12 g per day being the desired cutoff),[25] appropriate reductions with the GFD (ie, fructo- and galacto- oligosaccharides), and for TDA a decrease in fructo-oligosaccharides, lactose, and mannitol (which is to be expected when reducing some gas-producing foods, eg, bread, fruits, dairy, and sweeteners).

Fourth, because of COVID-19, dietary education moved away from face-to-face to virtual consults and was also provided in group settings. We found similar clinical efficacy to dietary therapy irrespective of the mode of educational delivery, with response rates in the group setting being comparable with studies where patients have been seen individually.[16,17] Moving forward, this delivery of care model will have cost-saving implications for healthcare services and alleviate concerns patients may have attending health centers in the current climate.

Current national guidelines demonstrate some differences regarding dietary therapies in IBS.[7–9] Whereas British guidelines recommend TDA as the first choice followed by a LFD, the North American guidelines only mention a LFD.[7–9] Because of insufficient evidence, neither recommends a GFD, although our study (among other recent publications) suggests it deserves future reevaluation.[4] On balancing the efficacy and acceptability of dietary therapies plus the demands they place on healthcare services, we suggest TDA be considered first. Although the LFD and GFD are beneficial, they are costlier, harder to follow, and more inconvenient. Furthermore, their implementation requires specialized and extensive dietetic input, which incurs a substantial burden on healthcare services. Indeed, even within countries with highly established healthcare systems (eg, United Kingdom and United States), there is inequity of GI dietetic services available across regions[26] and a failure to correctly implement a LFD despite it frequently being recommended and prescribed.[27] Because IBS is a global condition, then arguably countries with less

established healthcare systems may be falling even shorter of optimally delivering a LFD. Hence, we suggest a GFD or LFD be reserved according to specific patient preferences and with specialist dietetic input. It would also be of interest to evaluate their efficacy in patients not responding to TDA. However, because costs are critical determinants of IBS treatment value to patients and providers, alternate cheaper options should also be considered (eg, antispasmodics, neuromodulators).[28]

The diets reduced total FODMAP intake, mostly in the LFD group compared with TDA and GFD. This suggests a degree of overlap and that moderate FODMAP restriction, as seen with TDA and a GFD, may be similarly effective as a strict LFD. To reintroduce FODMAPs to tolerance and avoid over-restriction, it is important to emphasize that the LFD is altogether a 3-stage process, and that after its strict 4- to 6-week elimination phase comes reintroduction and personalization, all done under dietetic supervision.[6,7] However, real-world Canadian healthcare experience suggests that only 40% satisfactorily complete all 3 phases of the LFD program, implying that a proportion might remain within the strict elimination phase and are at risk of developing overly restrictive eating patterns and nutritional inadequacies.[25] There are suggestions that a "bottom-up" or "FODMAP-gentle" approach to the LFD may overcome its extensive 3-phase program.[4] For example, in the long-term, many patients on a personalized LFD reduce fructan intake to manage their symptoms and facilitate this through purchasing gluten-free or wheat-free products.[29] This raises the hypothesis that a GFD might be an option before considering the complete LFD program. Other reasons for a GFD in IBS are in anti-gliadin antibody positive patients, as well as those with non-celiac gluten/wheat sensitivity.[10,30] Our study, among another recent publications, suggests that a GFD in IBS does not need to be strict as that seen in celiac disease.[30] However, because the GFD generally comes in one form, future studies should determine the level of gluten restriction required to derive symptom benefit, regardless of whether they start with this diet or reach it via a personalized LFD.

The study limitations are similar to previous randomized trials in that dietary intervention was of 4-week duration, and long-term outcomes are relatively unknown.[12] A few studies have demonstrated ongoing efficacy with a GFD and personalized LFD,[23,29] although as mentioned there is currently no guidance regarding gluten reintroduction. The study was also powered to detect a large 35% difference in clinical benefit between the LFD and GFD compared with TDA; thus it was underpowered to detect smaller yet significant differences, potentially leading to a type II error. Interestingly, when combining our results with that of a similarly designed and powered Swedish study,[16] essentially doubling the sample size to ~70 patients per arm, the proportion of responders with a ≥50-point reduction in IBS-SSS is ~44% (range 42%–46%) with TDA and ~53% with LFD (range 50%–55%), suggesting a difference of 9%. In our study, a GFD showed 16% gain over TDA. To ascertain whether a 9% to 16% therapeutic gain with a LFD and GFD is significant over TDA, then on the basis of our primary end point, studies with a sample size of more than 950 and almost 300 patients, respectively, would be needed. However, whether this would lead to TDA being displaced from pole position is debatable because of its relative simplicity and minimal healthcare service requirements, and that the LFD and GFD are still viable options that can be considered afterwards. In addition, although our study was geared toward comparing different diets head-to-head, their true benefit (if any) over placebo is unknown in the absence of a control group. Pharmacologic trials in IBS suggest a pooled placebo response rate of approximately 30%,[31] but this is yet to be adequately explored with dietary interventions. We also excluded patients with IBS-constipation on the presumption that reducing FODMAP intake might aggravate constipation and worsen overall symptoms; however, there are emerging data to suggest a LFD might benefit this patient group and, alongside the other dietary interventions, merits further independent study.[16] Other limitations relating to the tools used to assess nutritional intake and nutritional considerations when prescribing dietary therapies are detailed in Supplementary Discussion. Here we also discuss issues regarding the stool normobiotic reference range, and that only 50% of stool samples were collected, which precludes firm conclusions on the stool DI being made.

In conclusion, TDA, GFD, and a LFD are effective approaches in non-constipated IBS. We recommend TDA as the first-choice dietary option because of its widespread availability and patient friendliness. The LFD or GFD are alternative options based on specific patient preferences and with specialist dietetic counseling.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Clinical Gastroenterology and Hepatology* at www.cghjournal.org, and at https://doi.org/10.1016/j.cgh.2022.02.045.

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Conflicts of interest

This author discloses the following: DSS receives an educational grant from Schaer (a gluten-free food manufacturer). The remaining authors disclose no conflicts.

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Supplementary Methods

Inclusion and Exclusion Criteria

Patients with IBS were recruited across 2 secondary care centers in the region of South Yorkshire, United Kingdom (Sheffield Teaching Hospitals and Doncaster Royal Infirmary). The inclusion criteria were adults aged 18 years and older, meeting the Rome IV criteria for IBS-D or IBS-M, and with an IBS-SSS >75. Additional inclusion criteria included being English literate, able to travel to hospital, and having telephone or internet access.

Exclusion criteria were those not meeting the inclusion criteria listed above or anyone with a history of inflammatory bowel disease, celiac disease (as per positive tissue transglutaminase), GI cancer, previous abdominal surgery, scleroderma, poorly controlled diabetes, severe liver/respiratory/cardiac/psychiatric disease (with "severe" defined as repeated flares, recurrent hospital or general practitioner attendances, numerous medications, clinically appearing unwell because of that disease process), memory impairment, pregnant, current dietary interventions, recurrent or current use of probiotics/antibiotics/narcotics, or currently titrated antidepressants (ie, not on a stable dose).

Questionnaires

The following questionnaires were completed before and after dietary intervention:

(1) IBS-SSS[1]: this is a frequently used assessment in clinical studies where responders rate, over the preceding 10 days, abdominal pain severity, pain frequency, bloating, bowel habit dissatisfaction, and life interferences related to bowel symptoms. The maximum cumulative score available is 500, and subjects can be classified as having no symptoms (<75) to mild (75–175), moderate (175–300), and severe IBS (>300). A reduction of 50 points is considered to confer a clinical improvement and was the primary end point of this study.

(2) Hospital Anxiety and Depression Scale (HADS)[2]: a psychological screening tool that has in total 14 items, 7 each for depression and anxiety. Each item is rated from 0 (not present) to 3 (maximum), giving a cumulative score for each subscale to range from 0 to 21.[2] A subscale score ≥11 is used to indicate a clinically significant level of anxiety or depression.

(3) The patient health questionnaire (PHQ)-12 non-GI somatic symptoms scale[3]: The PHQ-12 records bothersome non-GI symptoms over the past month. The 12 symptoms assessed are back pain, limb pain, headaches, chest pain, dizziness, fainting spells, palpitations, breathlessness, menstrual cramps, dyspareunia, insomnia, and lethargy. Subjects were asked to rate how much they had been troubled by these 12 symptoms over the last 4 weeks as 0 ("not bothered at all"), 1 ("bothered a little"), or 2 ("bothered a lot"). Responses were used to calculate the number of sites reporting somatic symptoms (ranging from 0 to 12) and the somatization severity score (ranging from 0 to 24), which was categorized as minimal (≤3), low (4–7), medium (8–12), and high (≥13).

(4) The IBS quality of life (IBS-QOL) questionnaire[4]: this consists of 34 questions that are summed and averaged for a total score, in addition to 8 subscale scores (Dysphoria, Interference with Activity, Body Image, Health Worries, Food Avoidance, Social Reaction, Sexuality, Social Relationship). Total and subscale scores are transformed to a 0–100 scale. Higher scores indicate better IBS-specific QOL.

(5) The acceptability of dietary restriction questionnaire is based on the adapted nutrition-related QOL questionnaire.[5] Responses are recorded using a Likert scale, with the responses of agree, neutral, and disagree.

(6) The food-related QOL questionnaire is a 7-item questionnaire based on the food-related QOL tool (Satisfaction with Food-related Life).[6] Responses are recorded on Likert scale as agree, neutral, or disagree.

(7) Comprehensive Nutrition Assessment Questionnaire (CNAQ): this is a semiquantitative food frequency questionnaire, consisting of 297 questions, assessing macronutrient and micronutrient intake, as well as FODMAPs, fiber, starch, glycemic index, and glycemic load.[7] The questionnaire asks about food intake over the last 6 months, although the answers range from over the last month to daily. Importantly, we asked individuals not to provide their food intake over the last 6 months (as written on the CNAQ) but rather pre-specified that it has to be over the last 4 weeks only.

Stool Samples

Stool samples were collected both before and after dietary intervention. Sample storage experiments performed by GA-map have shown that fecal samples are stable for GA analysis up to 5 days at room temperature (data not shown), allowing enough time for sending of samples through the post. In our study, the samples were collected from the patient's address within a day and then batch stored in a −80° freezer until completion of the study. On study completion, samples were shipped on dry ice to Norway for analysis. The GA-map Dysbiosis test was used to analyze samples, which is a gut microbiota DNA analysis tool that can identify and characterize

dysbiosis from a fecal sample.[8] The test allows for mapping of select bacteria and is based on DNA profiling using probes to target variable regions (V3 to V7 regions) of bacteria 16S ribosomal RNA (rRNA) gene to characterize whether bacteria are present.[8] Each probe was designed to target a bacterial species or group on the basis of their 16S rRNA sequence.[8] Probes were selected on ability to differentiate between healthy individuals, IBS, and inflammatory bowel disease.[8] The probe set consisted of 48 probes detecting bacteria within the 6 phyla: Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria. Tenericutes, and Verrucomicrobia. On analysis, bacterial profiles were assigned an overall DI index on a scale set from 0 to 5.[8] A DI score of 2 or lower was classified as being within the non-dysbiotic region compared with the normobiotic reference cohort. A DI of greater than 2 was considered to be dysbiotic, with a higher DI number indicating greater dysbiosis from the reference range.[8] Relative bacterial abundance was also supplied for each probe in comparison with the normobiotic reference range, with values ranging from −3 to +3 (dependent upon probe), with −3 being strongly reduced levels of bacteria compared with the reference range and +3 being strongly elevated levels of bacteria. In addition, functional bacterial profiles were given, with functional properties deduced from specific bacteria profiles; the bacterial markers of *Anaerobutyricumhallii, [Eubacterium] rectale,* and *Faecalibacterium prausnitzii* were used to assess butyrate producing bacteria, *Faecalibacterium prausnitzii* and *Akkermansiamuciniphila* were used to assess gut mucosa protective bacteria, *Faecalibacterium prausnitzii* was used to assess gut intestinal health, *Faecalibacterium prausnitzii, Ruminococcusgnavus, Proteobacteria, Shigella spp.,* and *Escherichia spp.* were used to assess intestinal epithelial barrier, and *Proteobacteria, Shigella spp.,* and *Escherichia spp.* were used to assess proinflammatory bacteria.

Statistical Analysis

Analysis was performed per protocol. All data were analyzed using SPSS version 26 (International Business Machines, Armonk, NY) and GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA). Statistical significance was considered when $P < .05$.

Categorical variables were summarized by descriptive statistics, including total numbers and percentages, with comparisons between groups performed using χ^2 testing, with post hoc tests (if required). Normality was assessed by using the Shapiro-Wilk test. Parametric data were summarized by mean and standard deviation, with the difference across multiple groups performed using one-way analysis of variance, with post hoc tests (if required) using the Bonferroni correction. Within-group comparisons for parametric data were analyzed by using paired *t* tests. Non-parametric data were summarized by median and range, with the difference across multiple groups performed using the Kruskal-Wallis test, with post hoc tests performed if required. Within-group comparisons for non-parametric data were performed by using the Wilcoxon test. Missing data were replaced by using the last observation carried forward method. Where no baseline outcome data were available, data were excluded from analysis.

Binary logistic regression was used to assess predictors for response to dietary therapies, with univariate analysis used initially and with multivariate analysis if significance was noted.

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Supplementary Discussion

Nutritional Implications and Limitations

Nutritional inadequacy can arise after dietary therapies in IBS.[1] In fact, before seeking professional dietary advice, patients with IBS might have restricted eating patents and fail to meet DRVs for multiple nutrients.[2] In our study, most individuals failed to meet DRVs for total energy intake before intervention. After dietary interventions, reductions in absolute macronutrient and micronutrient counts were observed, with a significant proportion subsequently failing to meet DRVs for certain micronutrients (potassium and iron on TDA, thiamine and magnesium on both LFD and GFD) but not macronutrients. Another noteworthy observation is that more patients were willing to continue with the diet they were allocated than had a symptomatic response. Reasons for this are unclear, but it might be that patients are in pursuit of obsessive healthy eating, which puts them at risk of developing avoidant restrictive food intake disorders and needs to be carefully monitored.[1] In all, this emphasizes the importance of carefully screening patients at high risk of eating disorders before recommending dietary interventions. This can be achieved through the use of simple eating disorder questionnaires (eg, SCOFF) and identifying those with high levels of psychological distress.[3–5]

A limitation of our nutritional analysis was that the food frequency questionnaire tool used (CNAQ) was based on the Australian diet. Although this tool is the most objective tool currently available in the literature and has been used in previous United Kingdom studies,[6] the nutritional and FODMAP assessments may have been underestimated or overestimated. However, in this study, the CNAQ tool was used both before and after intervention to assess change between all 3 groups to ensure consistency.

Stool Microbial Implications and Limitations

Diet is thought to be a key environmental component in the composition of the gut microbiome,[7] with the LFD having previously been shown in short-term studies to reduce the proportion of luminal *Bifidobacterium*, as well as total bacterial abundance.[8–10] On the GFD, reduction in *Bififobacterium* has also been noted, as well as *Faecalibacterium prausnitzii* proportions.[11] Although changes in bacterial abundance after all 3 interventions were noted, the clinical significance of this is unclear, with no significant difference in functional bacterial profiles noted. A previous study using the same method of stool analysis demonstrated that response to a LFD, but not TDA, could be determined from baseline fecal bacterial profiles.[9] However, we were unable to replicate these findings, highlighting the uncertainty in this area, although admittedly our stool sample size was only 50% and underpowered because of the impact of COVID-19. Finally, the stool DI was based on a normobiotic reference range derived from a predominantly Scandinavian cohort,[12] although the purpose of our study was to compare within- and across-group changes after dietary intervention.

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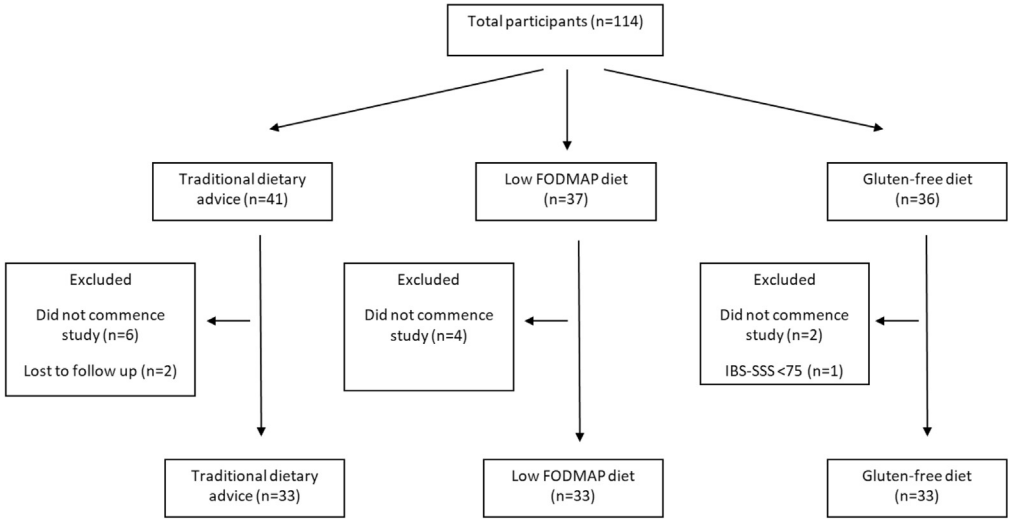
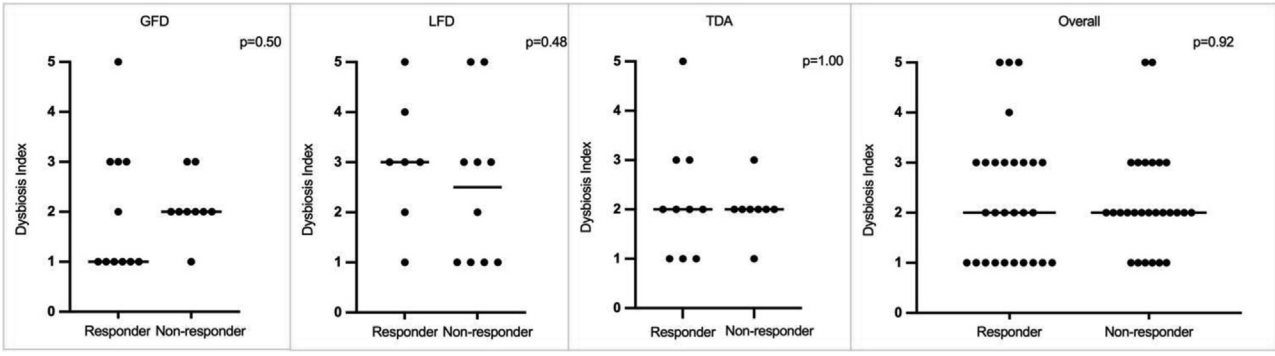


Supplementary Figure 1. Consort diagram of patient flow throughout study.



Supplementary Figure 2. Baseline stool DI between responders and non-responders to dietary therapy.

Supplementary Table 1. Randomized Trials of TDA (vs a low FODMAP Diet) Assessing Whether Patients Allocated TDA Were Advised to Reduce Commonly Consumed Gas-Producing Foods as per Recommendations

Author	Country	Advised to reduce common gas-producing foods as per TDA recommendations?	Detectable reduction in consumption of gas-producing foods with TDA?
Bohn et al[1]	Sweden	Yes	Yes
Eswaran et al[2]	United States	No, instead termed as "modified" diet	No
Zahedi et al[3]	Iran	Partial, only advised avoidance of chewing gums and sweeteners containing polyols	No
Goyal et al[4]	India	No	No
Zhang et al[5]	China	No	No

[1]Böhn L, Störsrud S, Liljebo T, et al. Diet low in FODMAPs reduces symptoms of irritable bowel syndrome as well as traditional dietary advice: a randomized controlled trial. Gastroenterology 2015;149:1399–1407.e1392.
[2]Eswaran SL, Chey WD, Han-Markey T, et al. A randomized controlled trial comparing the low FODMAP diet vs modified NICE guidelines in US adults with IBS-D. Am J Gastroenterol 2016;111:1824–1832.
[3]Zahedi MJ, Behrouz V, Azimi M. Low fermentable oligo-di-mono-saccharides and polyols diet versus general dietary advice in patients with diarrhea-predominant irritable bowel syndrome: a randomized controlled trial. J Gastroenterol Hepatol 2018;33:1192–1199.
[4]Goyal O, Batta S, Nohria S, et al. Low fermentable oligosaccharide, disaccharide, monosaccharide, and polyol diet in patients with diarrhea-predominant irritable bowel syndrome: a prospective, randomized trial. J Gastroenterol Hepatol 2021;36:2107–2115.
[5]Zhang Y, Feng L, Wang X, et al. Low fermentable oligosaccharides, disaccharides, monosaccharides, and polyols diet compared with traditional dietary advice for diarrhea-predominant irritable bowel syndrome: a parallel-group, randomized controlled trial with analysis of clinical and microbiological factors associated with patient outcomes. Am J Clin Nutr 2021;113:1531–1545.

Supplementary Table 2. Baseline Demographics

Demographics	TDA (n = 33)	LFD (n = 33)	GFD (n = 33)	Overall (n = 99)	Comparison across groups, *P* value
Female, n (%)	24 (73)	25 (76)	21 (64)	79 (70)	.57
Mean age (SD), *y*	40 (15)	35 (12)	36 (13)	37 (13)	.22
Mean BMI (SD), kg/m^2	27 (10)	26 (11)	24 (8)	25 (10)	.33
Mean IBS-SSS (SD)	291 (89)	296 (87)	316 (92)	301 (89)	.47
Mean HADS (SD)	16 (7)	18 (9)	16.5 (9)	17 (8)	.58
Mean PHQ-12 (SD)	9.6 (5)	8.5 (4)	8.4 (4)	8.8 (4)	.41
Mean IBS-QOL (SD)	52 (18)	51 (21)	60 (26)	54 (22)	.15
Index of Multiple Deprivation quintiles					
Q1, n *(%)* – most deprived	7 (21)	9 (27)	12 (36)	28 (29)	.1
Q2, n *(%)*	3 (9)	9 (27)	1 (3)	13 (13)	
Q3, n *(%)*	10 (30)	6 (18)	8 (24)	24 (25)	
Q4, n *(%)*	7 (21)	7 (21)	4 (12)	18 (18)	
Q5, n *(%)* – least deprived	6 (18)	2 (6)	7 (21)	15 (15)	

BMI, body mass index; HADS, Hospital Anxiety and Depression scale; PHQ-12, Patient Health Questionnaire-12; SD, standard deviation.

Supplementary Table 3. HADS, PHQ-12, and IBS-QOL

Symptom score	Intervention						Comparison of change across groups, *P* value
	TDA		LFD		GFD		
	Baseline	Week 4	Baseline	Week 4	Baseline	Week 4	
HADS anxiety, mean (SD)	9.5 (4.4)	9.5 (4.4)	10.6 (5.4)	8.9 (5.1)	9.8 (5.4)	8.4 (4.9)	.08
HADS depression, mean (SD)	6.8 (3.3)	7.6 (3.5)	7.6 (4.5)	6.5 (5.2)	6.7 (4.6)	6.4 (5.0)	.03
PHQ-12 score, mean (SD)	9.6 (4.7)	8.7 (3.7)	8.5 (4.0)	7.7 (3.7)	8.4 (3.6)	7.9 (4.2)	.8
IBS-QOL							
IBS-QOL score, mean (SD)	52 (18)	55 (21)	51 (21)	61 (24)	60 (26)	65 (26)	.10
Dysphoria	51 (22)	55 (24)	48 (26)	65 (27)	60 (32)	65 (32)	<.01
Interference with activity	49 (20)	54 (25)	45 (27)	56 (27)	57 (29)	62 (28)	.21
Body image	52 (23)	55 (21)	45 (25)	54 (25)	63 (25)	67 (30)	.43
Health worries	56 (20)	54 (25)	62 (25)	69 (25)	62 (27)	69 (24)	.09
Food avoidance	39 (26)	38 (29)	45 (32)	48 (27)	45 (33)	47 (30)	.88
Social reaction	51 (22)	57 (26)	51 (28)	62 (29)	62 (26)	66 (28)	.37
Sexuality	62 (30)	65 (30)	70 (30)	70 (28)	75 (27)	75 (31)	.83
Social relationship	62 (25)	67 (24)	64 (29)	68 (29)	70 (25)	75 (26)	1.00

HADS, Hospital Anxiety and Depression scale; PHQ-12, Patient Health Questionnaire-12; SD, standard deviation.

Supplementary Table 4. Proportion of Individuals Meeting DRVs

	Intervention								
	TDA			LFD			GFD		
Nutritional parameter	Baseline DRV met (%)	Week 4 DRV met (%)	Baseline vs Week 4, *P* value	Baseline DRV met (%)	Week 4 DRV met (%)	Baseline vs Week 4, *P* value	Baseline DRV met (%)	Week 4 DRV met (%)	Baseline vs Week 4, *P* value
Energy, *kcal/d*	41	29	.31	45	26	.11	55	42	.32
Protein, *g/d*	91	74	.06	81	72	.38	88	82	.49
Carbohydrate, *g/d*	44	41	.81	53	38	.21	42	42	1.00
Fat, g/d	74	76	.78	53	50	.80	61	61	1.00
Dietary fiber, *g/d*	62	38	.05	38	28	.42	55	39	.22
Folate, *μg/d*	97	85	.09	88	72	.12	100	94	.55
Thiamine, *mg/d*	94	88	.39	94	63	<.01	97	79	.02
Riboflavin, *mg/d*	85	88	.72	84	78	.52	91	91	1.00
Niacin, *mg/d*	88	85	.72	81	58	.05	91	85	.45
Vitamin C, *mg/d*	100	100	1.00	97	91	.30	100	100	1.00
Sodium, *mg/d*	79	71	.40	78	69	.40	67	52	.21
Potassium, *mg/d*	79	53	.02	56	34	.08	67	52	.21
Magnesium, *mg/d*	79	62	.11	63	38	<.05	79	55	.04
Calcium, *mg/d*	82	68	.16	75	59	.18	85	70	.14
Phosphorus, *mg/d*	100	97	.31	100	97	.31	100	100	1.00
Iron, *mg/d*	71	47	<.05	61	52	.44	61	52	.46
Zinc, *mg/d*	79	79	1.00	81	69	.37	91	79	.17

Supplementary Table 5. Change in DI by Intervention and Responders

	Change in DI			Comparison of responders vs non-responders, *P* value
	Improved, n *(%)*	No change, n *(%)*	Worsened, n *(%)*	
TDA				
Responders	3 (30)	2 (20)	5 (50)	.18
Non-responders	1 (13)	5 (63)	2 (25)	
LFD				
Responders	2 (29)	2 (29)	3 (43)	.84
Non-responders	3 (30)	4 (40)	3 (30)	
GFD				
Responders	2 (18)	4 (36)	5(46)	.72
Non-responders	3 (33)	3 (33)	3 (33)	
Overall				
Responders	7 (25)	8 (29)	13 (46)	.37
Non-responders	7 (26)	12 (44)	8 (30)	

Supplementary Table 6. Functional Bacterial Profiles With Dietary Interventions

	TDA			LFD			GFD		
	Baseline, n *(%)*	Follow-up, n *(%)*	*P* value	Baseline, n *(%)*	Follow-up, n *(%)*	*P* value	Baseline, n *(%)*	Follow-up, n *(%)*	*P* value
Low levels of butyrate producing bacteria	5 (28)	2 (22)	.70	4 (24)	2 (12)	.37	3 (15)	5 (25)	.43
Low levels of gut mucosa protective bacteria	3 (17)	3 (17)	1.00	3 (18)	4 (24)	.67	4 (20)	6 (30)	.47
Low levels of F prausnitzii	0 (0)	3 (17)	.07	3 (18)	2 (12)	.63	2 (10)	4 (20)	.38
Imbalance between selected gut barrier protective and potentially harmful bacteria	2 (11)	2 (11)	1.00	4 (24)	5 (29)	.70	6 (30)	3 (15)	.26
High levels of proinflammatory bacteria	1 (6)	1 (6)	1.00	4 (24)	4 (18)	.67	0 (0)	1 (5)	.31

Supplementary Table 7. Bacterial Abundance Following TDA

Genus/species	Class	Phylum	DI at follow-up vs baseline	*P value*
Actinobacteria	Actinobacteria	Actinobacteria	No difference	.67
Actinomycetales	Actinobacteria	Actinobacteria	No difference	.18
Bifidobacterium spp.	Actinobacteria	Actinobacteria	No difference	1.00
Alistipes	Alistipes	Bacteroidetes	No difference	.32
Alistipes onderdonkii	Alistipes	Bacteroidetes	No difference	.66
Bacteroides fragilis	Bacteroides	Bacteroidetes	No difference	.56
Bacteroide spectinophilus	Bacteroides	Bacteroidetes	No difference	.08
Bacteroides spp.	Bacteroides	Bacteroidetes	No difference	.26
Bacteroides spp., Prevotella spp.	Bacteroides	Bacteroidetes	No difference	.33
Bacteroides stercoris	Bacteroides	Bacteroidetes	No difference	.71
Bacteroides zoogleoformans	Bacteroides	Bacteroidetes	No difference	.26
Parabacteroides johnsonii	Parabacterioides	Bacteroidetes	No difference	1.00
Parabacteroides spp.	Parabacterioides	Bacteroidetes	No difference	.32
Firmicutes	Negativicutes/Clostridia	Firmicutes	No difference	.19
Bacilli	Bacilli	Firmicutes	No difference	.60
Catenibacterium	Erysipelotrichia	Firmicutes	No difference	1.00
Clostridia	Clostridia	Firmicutes	No difference	.72
Clostridium methylpentosum	Ruminiclostridium	Firmicutes	No difference	1.00
Clostridium sp.	Clostridia	Firmicutes	No difference	1.00
Coprobacillus cateniformis	Erysipelotrichia	Firmicutes	No difference	1.00
Dialister invisus	Negativicutes	Firmicutes	No difference	1.00
Dialister invisus, Megasphaera micronuciformis	Negativicutes	Firmicutes	No difference	.32
Dorea spp.	Clostridia	Firmicutes	Decreased	$<$.05
Eubacterium biforme	Clostridia	Firmicutes	No difference	.26
Eubacterium hallii	Clostridia	Firmicutes	No difference	.56
Eubacterium rectale	Clostridia	Firmicutes	No difference	1.00
Eubacterium siraeum	Clostridia	Firmicutes	No difference	.41
Faecalibacterium prausnitzii	Clostridia	Firmicutes	No difference	.53
Lachnospiraceae	Clostridia	Firmicutes	No difference	.71
Lactobacillus ruminis, Pediococcus acidilactici	Bacilli	Firmicutes	No difference	.16
Lactobacillus spp.	Bacilli	Firmicutes	No difference	.76
Lactobacillus spp. 2	Bacilli	Firmicutes	No difference	.71
Phascolarctobacterium sp.	Negativicutes	Firmicutes	No difference	.32
Ruminococcus albus, R bromii	Clostridia	Firmicutes	No difference	.78
Ruminococcus gnavus	Clostridia	Firmicutes	No difference	.56
Streptococcus agalactiae, Eubacterium rectale	Bacilli	Firmicutes	No difference	.56
Streptococcus salivarius ssp. Thermophiles, S sanguinis	Bacilli	Firmicutes	No difference	.71

Supplementary Table 7. Continued

Genus/species	Class	Phylum	DI at follow-up vs baseline	*P value*
Streptococcus salivarius ssp. Thermophilus	Bacilli	Firmicutes	No difference	1.00
Streptococcus spp.	Bacilli	Firmicutes	No difference	.78
Streptococcus spp. 2	Bacilli	Firmicutes	No difference	.85
Veillonella spp.	Negativicutes	Firmicutes	No difference	.60
Firmicutes (various)	—	Firmicutes/Tenericutes/ Bacteroidetes species	No difference	1.00
Proteobacteria	—	Proteobacteria	No difference	.41
Acinetobacter junii	Gammaproteobacteria	Proteobacteria	No difference	1.00
Enterobacteriaceae	Gammaproteobacteria	Proteobacteria	No difference	.16
Shigella spp., Escherichia spp	Gammaproteobacteria	Proteobacteria	No difference	1.00
Mycoplasma hominis	Mollicutes	Tenericutes	No difference	1.00
Akkermansia muciniphila	Verrumicrobiae	Verrucomicrobia	No difference	.41

Supplementary Table 8. Bacterial Abundance Following a Low FODMAP Diet

Genus/species	Class	Phylum	Abundance at follow-up vs baseline	*P* value
Actinobacteria	Actinobacteria	Actinobacteria	Decreased	<.05
Actinomycetales	Actinobacteria	Actinobacteria	No difference	.56
Bifidobacterium spp.	Actinobacteria	Actinobacteria	No difference	.16
Alistipes	Alistipes	Bacteroidetes	Increased	.02
Alistipes onderdonkii	Alistipes	Bacteroidetes	No difference	.18
Bacteroides fragilis	Bacteroides	Bacteroidetes	Decreased	.02
Bacteroides pectinophilus	Bacteroides	Bacteroidetes	No difference	.71
Bacteroides spp.	Bacteroides	Bacteroidetes	No difference	.38
Bacteroides spp., Prevotella spp.	Bacteroides	Bacteroidetes	No difference	.18
Bacteroides stercoris	Bacteroides	Bacteroidetes	No difference	1.00
Bacteroides zoogleoformans	Bacteroides	Bacteroidetes	No difference	.53
Parabacteroides johnsonii	Parabacterioides	Bacteroidetes	Increased	<.05
Parabacteroides spp.	Parabacterioides	Bacteroidetes	No difference	.13
Firmicutes	Negativicutes/Clostridia	Firmicutes	No difference	.18
Bacilli	Bacilli	Firmicutes	No difference	.16
Catenibacterium	Erysipelotrichia	Firmicutes	No difference	.32
Clostridia	Clostridia	Firmicutes	No difference	.38
Clostridium methylpentosum	Ruminiclostridium	Firmicutes	Increased	.03
Clostridium sp.	Clostridia	Firmicutes	No difference	.32
Coprobacillus cateniformis	Erysipelotrichia	Firmicutes	No difference	.32
Dialister invisus	Negativicutes	Firmicutes	No difference	.32
Dialister invisus, Megasphaera micronuciformis	Negativicutes	Firmicutes	No difference	.66
Dorea spp.	Clostridia	Firmicutes	No difference	.48
Eubacterium biforme	Clostridia	Firmicutes	No difference	.16
Eubacterium hallii	Clostridia	Firmicutes	No difference	.48
Eubacterium rectale	Clostridia	Firmicutes	No difference	.76
Eubacterium siraeum	Clostridia	Firmicutes	No difference	1.00
Faecalibacterium prausnitzii	Clostridia	Firmicutes	No difference	.74
Lachnospiraceae	Clostridia	Firmicutes	Increased	.01
Lactobacillus ruminis, Pediococcus acidilactici	Bacilli	Firmicutes	No difference	.32
Lactobacillus spp.	Bacilli	Firmicutes	No difference	.32
Lactobacillus spp. 2	Bacilli	Firmicutes	No difference	.26
Phascolarctobacterium sp.	Negativicutes	Firmicutes	No difference	.16
Ruminococcus albus, R bromii	Clostridia	Firmicutes	No difference	.71
Ruminococcus gnavus	Clostridia	Firmicutes	No difference	.89
Streptococcus agalactiae, Eubacterium rectale	Bacilli	Firmicutes	No difference	.48
Streptococcus salivarius ssp. Thermophiles, S sanguinis	Bacilli	Firmicutes	No difference	.48

Supplementary Table 8. Continued

Genus/species	Class	Phylum	Abundance at follow-up vs baseline	*P* value
Streptococcus salivarius ssp. Thermophilus	Bacilli	Firmicutes	No difference	.32
Streptococcus spp.	Bacilli	Firmicutes	No difference	.06
Streptococcus spp. 2	Bacilli	Firmicutes	No difference	.19
Veillonella spp.	Negativicutes	Firmicutes	No difference	.21
Firmicutes (various)	—	Firmicutes/Tenericutes/ Bacteroidetes species	No difference	.66
Proteobacteria	—	Proteobacteria	No difference	.23
Acinetobacter junii	Gammaproteobacteria	Proteobacteria	No difference	1.00
Enterobacteriaceae	Gammaproteobacteria	Proteobacteria	No difference	.66
Shigella spp., Escherichia spp	Gammaproteobacteria	Proteobacteria	No difference	.16
Mycoplasma hominis	Mollicutes	Tenericutes	No difference	1.00
Akkermansia muciniphila	Verrumicrobiae	Verrucomicrobia	No difference	.20

Supplementary Table 9. Bacterial Abundance Following a GFD

Genus/species	Class	Phylum	Abundance at follow-up vs baseline	*P* value
Actinobacteria	Actinobacteria	Actinobacteria	Reduced	.03
Actinomycetales	Actinobacteria	Actinobacteria	No difference	.41
Bifidobacterium spp.	Actinobacteria	Actinobacteria	No difference	.08
Alistipes	Alistipes	Bacteroidetes	No difference	.37
Alistipes onderdonkii	Alistipes	Bacteroidetes	No difference	1.00
Bacteroides fragilis	Bacteroides	Bacteroidetes	No difference	.32
Bacteroides pectinophilus	Bacteroides	Bacteroidetes	No difference	.48
Bacteroides spp.	Bacteroides	Bacteroidetes	No difference	.74
Bacteroides spp., Prevotella spp.	Bacteroides	Bacteroidetes	No difference	1.00
Bacteroides stercoris	Bacteroides	Bacteroidetes	No difference	.16
Bacteroides zoogleoformans	Bacteroides	Bacteroidetes	No difference	1.00
Parabacteroides johnsonii	Parabacterioides	Bacteroidetes	Reduced	.01
Parabacteroides spp.	Parabacterioides	Bacteroidetes	No difference	.26
Firmicutes	Negativicutes/Clostridia	Firmicutes	No difference	1.00
Bacilli	Bacilli	Firmicutes	No difference	.21
Catenibacterium mitsuokai	Erysipelotrichia	Firmicutes	No difference	1.00
Clostridia	Clostridia	Firmicutes	No difference	.78
Clostridium methylpentosum	Ruminiclostridium	Firmicutes	No difference	.26
Clostridium sp.	Clostridia	Firmicutes	No difference	1.00
Coprobacillus cateniformis	Erysipelotrichia	Firmicutes	No difference	1.00
Dialister invisus	Negativicutes	Firmicutes	No difference	.32
Dialister invisus, Megasphaera micronuciformis	Negativicutes	Firmicutes	No difference	.05
Dorea spp.	Clostridia	Firmicutes	No difference	1.00
Eubacterium biforme	Clostridia	Firmicutes	No difference	.08
Eubacterium hallii	Clostridia	Firmicutes	No difference	.27
Eubacterium rectale	Clostridia	Firmicutes	Reduced	.02
Eubacterium siraeum	Clostridia	Firmicutes	No difference	.52
Faecalibacterium prausnitzii	Clostridia	Firmicutes	No difference	.21
Lachnospiraceae	Clostridia	Firmicutes	No difference	.32
Lactobacillus ruminis, Pediococcus acidilactici	Bacilli	Firmicutes	No difference	.56
Lactobacillus spp.	Bacilli	Firmicutes	No difference	.56
Lactobacillus spp. 2	Bacilli	Firmicutes	No difference	.33
Phascolarctobacterium sp.	Negativicutes	Firmicutes	No difference	.08
Ruminococcus albus, R bromii	Clostridia	Firmicutes	Reduced	.04
Ruminococcus gnavus	Clostridia	Firmicutes	No difference	.17
Streptococcus agalactiae, Eubacterium rectale	Bacilli	Firmicutes	No difference	.16
Streptococcus salivarius ssp. Thermophiles, S sanguinis	Bacilli	Firmicutes	No difference	.06

Supplementary Table 9. Continued

Genus/species	Class	Phylum	Abundance at follow-up vs baseline	*P* value
Streptococcus salivarius ssp. Thermophilus	Bacilli	Firmicutes	No difference	.10
Streptococcus spp.	Bacilli	Firmicutes	No difference	.59
Streptococcus spp. 2	Bacilli	Firmicutes	No difference	.42
Veillonella spp.	Negativicutes	Firmicutes	No difference	.77
Firmicutes (various)	—	Firmicutes/Tenericutes/ Bacteroidetes species	No difference	.10
Proteobacteria	—	Proteobacteria	No difference	.41
Acinetobacter junii	Gammaproteobacteria	Proteobacteria	No difference	1.00
Enterobacteriaceae	Gammaproteobacteria	Proteobacteria	No difference	1.00
Shigella spp., Escherichia spp	Gammaproteobacteria	Proteobacteria	No difference	.32
Mycoplasma hominis	Mollicutes	Tenericutes	No difference	1.00
Akkermansia muciniphila	Verrumicrobiae	Verrucomicrobia	No difference	.10

Supplementary Table 10. Binary Logistic Regression Analysis of Clinical Factors Associated With a Response to Dietary Therapies

	Intervention			
	TDA	LFD	GFD	Overall
Variable	OR (CI)	OR (CI)	OR (CI)	OR (CI)
Age (*y*)	1.04 (1.00 –1.10)	0.99 (0.94 –1.05)	0.99 (0.94 –1.05)	1.00 (0.98 –1.04)
Gender				
Male	1	1	1	1
Female	0.50 (0.10 –2.44)	1.67 (0.32 –8.59)	0.62 (0.15 –2.58)	0.81 (0.34 –1.93)
IBS subtype				
IBS-D	1	1	1	1
IBS-M	0.40 (0.07 –2.42)	0.32 (0.06 –1.62)	1.69 (0.34 –8.40)	0.65 (0.26 –1.62)
IMD quintiles				
Q1–3	1	1	1	1
Q4–5	1.69 (0.42 –6.72)	4.14 (0.71 –24.16)	0.29 (0.06 –1.32)	1.17 (0.51 –2.69)
Somatization severity				
Minimal	1	1	1	1
Low	1.43 (0.10 –20.44)	1.50 (0.10 –23.07)	4.00 (0.27 –58.56)	1.69 (0.41 –6.98)
Medium	1.67 (0.12 –24.26)	0.11 (0.01 –1.52)	4.50 (0.31 –65.23)	0.90 (0.22 –3.63)
High	2.50 (0.16 –38.60)	0.33 (0.02 –5.33)	0.50 (0.02 –12.90)	0.82 (0.18 –3.74)
HADS anxiety levels (clinical)				
Normal	1	1	1	1
Abnormal	0.46 (0.11 –1.96)	0.53 (0.13 –2.14)	0.40 (0.10 –1.68)	0.51 (0.23 –1.14)
HADS depression levels (clinical)				
Normal	1	1	1	1
Abnormal	1.21 (0.15 –9.76)	0.25 (0.04 –1.54)	0.21 (0.03 –1.32)	0.38 (0.13 –1.12)

HADS, Hospital Depression and Anxiety scale; IMD, Index of Multiple Deprivation.

Arthritis Research & Therapy

RESEARCH ARTICLE Open Access

Gut dysbiosis associated with worse disease activity and physical function in axial spondyloarthritis

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Abstract

Background: Based on clinical and genetic associations, axial spondyloarthritis (axSpA) and inflammatory bowel disease (IBD) are suspected to have a linked pathogenesis. Gut dysbiosis, intrinsic to IBD, has also been observed in axSpA. It is, however, not established to what degree gut dysbiosis is associated with axSpA disease severity.

The objective of this study was to compare gut dysbiosis frequency between controls, non-radiographic axial spondyloarthritis (nr-axSpA), and ankylosing spondylitis (AS) patients and investigate whether gut dysbiosis is cross-sectionally associated with axSpA disease activity, physical function, mobility, or pain.

Methods: Gut dysbiosis was assessed by 16SrRNA analysis of feces from 44/88 nr-axSpA/AS patients (ASAS/mNY criteria) without inflammatory bowel disease (IBD) and 46 controls without IBD or rheumatic disease. The GA-map™ Dysbiosis Test was used, grading gut microbiota aberrations on a 1-5 scale, where ≥3 denotes dysbiosis. Proportions with dysbiosis were compared between the groups. Furthermore, standard axSpA measures of disease activity, function, mobility, and pain were compared between patients (nr-axSpA and AS combined) with and without dysbiosis, univariately, and adjusted for relevant confounders (ANCOVA).

Results: Gut dysbiosis was more frequent in AS than controls (36% versus 17%, p=0.023), while nr-axSpA (25% dysbiosis) did not differ significantly from either AS or controls. Univariately, most axSpA measures were significantly worse in patients with dysbiosis versus those without: ASDAS-CRP between-group difference 0.6 (95% CI 0.2–0.9); BASDAI 1.6 (0.8–2.4); evaluator's global disease activity assessment (Likert scale 0–4) 0.3 (0.1–0.5), BASFI 1.5 (0.6–2.4), and VAS pain (cm) 1.3 (0.4–2.2). Differences remained significant after adjustment for demographics, lifestyle factors, treatments, gut inflammation (fecal calprotectin ≥50 mg/kg), and gut symptoms, except for VAS pain. BASMI and CRP were not associated with dysbiosis.

Conclusion: Gut dysbiosis, more frequent in AS patients than controls, is associated with worse axSpA disease activity and physical function, seemingly irrespective of both gut inflammation and treatments. This provides further evidence for an important link between disturbances in gastrointestinal homeostasis and axSpA.

Keywords: Spondyloarthritis, Ankylosing spondylitis (AS), Non-radiographic axial spondyloarthritis (nr-axSpA), Physical function, Microbiota, Disease activity, Dysbiosis, Gut inflammation, Inflammatory bowel disease

Background

The link between spondyloarthritis (SpA) and inflammatory bowel disease (IBD) is well recognized. IBD patients are at increased risk of developing SpA, and the reverse

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is likewise true, with prevalence estimates of diagnosed IBD in axial SpA (axSpA) cohorts between 5 and 10% [1–3]. Several shared genetic risk loci are known [4, 5], and a common pathobiological pathway suggested [6, 7]. Additionally, approximately 50–60% of SpA patients display microscopic intestinal inflammation in biopsies of the ileum or colon, often reminiscent of Crohn's disease [8–10].

IBD is associated with an altered intestinal bacteria composition [11], a condition known as intestinal or gut dysbiosis [12]. The intestinal inflammation characterizing IBD is thought to arise through a complex interplay between genetic, environmental, immunological, and gut microbial factors [13]. A variation of microbial aberrations has been reported in IBD, but a recurrent finding is a reduced species richness, particularly within the Firmicutes phylum [14–16]. Moreover, several studies have shown a decreased presence of *Fecalibacterium prausnitzii*, a species with protective, immune regulatory features, and increased abundance of *Escherichia coli*, possessing pathogenic properties [11, 17].

Recently, several studies have reported gut dysbiosis also in ankylosing spondylitis (AS) [18–23], whereas little is known about gut microbiota in non-radiographic axSpA (nr-axSpA) patients [24]. Furthermore, previous studies have linked intestinal inflammation, assessed either histologically [10, 25, 26], or estimated via elevated fecal calprotectin (F-calprotectin) levels [27, 28], to more active axSpA disease and worse prognosis. Yet, it remains largely unknown whether gut microbiota aberrations alone, independent of intestinal inflammation, is associated with more severe axSpA.

Methods

The objectives of this study were to compare the frequency and degree of gut dysbiosis between well-characterized nr-axSpA and AS patients without known IBD and controls and to investigate whether gut dysbiosis is cross-sectionally associated with worse axSpA disease activity, physical function, mobility, or pain.

Study population

Nr-axSpA and AS patients for the present study were enrolled from the population-based SPARTAKUS cohort of validated axSpA cases from southern Sweden. Comprehensive information about the SPARTAKUS cohort and its inclusion and diagnosis validation process has been published earlier [27]. Briefly, all patients from a defined geographical area of Skåne county, Sweden, with at least one outpatient visit to the Department of Rheumatology, Skåne University Hospital, with an ICD-10 diagnosis consistent with axial SpA (M45.9/M46.0/M46.1/M46.8/M46.9) during 2011–2014, were invited to enroll in the cohort. Among patients with a clinical diagnosis of undifferentiated SpA (M46.8/M46.9), only those reporting back pain ≥3 months with an onset before age 45 were eligible. Enrolled patients were extensively characterized through questionnaires, clinical examinations, blood/feces/urine sampling and imaging (X-ray and, if needed for the diagnosis validation/classification, also magnetic resonance imaging [MRI] of the sacroiliac [SI] joints), enabling classification into nr-axSpA or AS according to the Assessment of SpondyloArthritis international Society (ASAS) axSpA and modified New York criteria, respectively [29, 30]. For the present gut dysbiosis study, patients were consecutively enrolled from the SPARTAKUS cohort, whereby the first 150 subjects classified as nr-axSpA (n=50) and AS (n=100) without comorbid IBD, and who had provided a fecal sample (available for 88% of SPARTAKUS patients), were included. The study visits for these patients occurred 2015–2018.

A control group of 50 persons without rheumatic disease or IBD, and frequency-matched to the overall SPARTAKUS cohort by age and sex, was gathered among friends/colleagues/relatives of the authors. The controls followed a shortened protocol, including questionnaires and provision of blood and fecal samples (study visits during 2018).

Patients (n=18) and controls (n=4) reporting treatment with antibiotics within the last 3 months before they provided the fecal sample were excluded from the present study, with the final study population thus comprising 44 patients with nr-axSpA, 88 with AS and 46 controls.

Gut microbiota analysis

Gut microbiota was analyzed using the GA-map™ Dysbiosis Test (Genetic Analysis, Oslo, Norway), a validated method designed to identify and grade gut dysbiosis in fecal samples by means of a pre-determined target approach, assessing the presence and abundance of a selected panel of 48 bacteria markers at different taxonomic levels and translating this into a dysbiosis index [31]. In brief, the test employs DNA probes, based on the 16SrRNA sequence in seven variable regions (V3–V9), to measure the abundance of bacteria according to the strength of fluorescent signal detection (probe signal intensity). The DNA probes included in the GA-map™ Dysbiosis Test were selected based on published observations regarding gut microbiota aberrations in IBD and IBS (irritable bowel syndrome), and in all target ≥300 bacteria in the six phyla Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Tenericutes, and Verrucomicrobia.

Based on bacterial composition and abundance by this sampling technique and applying a healthy Scandinavian control group as reference of normobiosis (normal microbiota composition), an algorithm was then developed to grade microbial aberrations from normobiosis, with a resulting Dysbiosis Index (DI) score of 1–5 [31]. A state of gut dysbiosis is considered present at DI ≥3 and severe dysbiosis at DI 5. A validation study of the GA-map™ Dysbiosis Test found dysbiosis (DI ≥3) in 16% of healthy volunteers and 74% of IBD patients [31]. Regarding the determination of gut dysbiosis, good agreement has also been demonstrated between the GA-map™ Dysbiosis Test and deep-sequencing by MiSeq Illumina sequencing technology [31].

The participants of the present study were instructed to collect their fecal sample as close in time to the study visit as possible and to store it in a −18°C freezer before delivery. After the visit, samples were stored frozen at −80°C until analysis (all samples were analyzed at the same time).

Outcomes

Mean DI score 1–5, as well as the frequency of dysbiosis (DI ≥3), were compared between axSpA patients (AS and nr-axSpA combined) and controls. Similar comparisons were also performed separately for nr-axSpA versus controls, AS versus controls, and for nr-axSpA versus AS.

Distributions of the following standard axSpA measures were assessed in relation to DI score 1–5 and presence of gut dysbiosis (DI ≥3), respectively: measures of disease activity (Ankylosing Spondylitis Disease Activity Score using CRP [ASDAS-CRP]; Bath Ankylosing Spondylitis Disease Activity Index [BASDAI]; Evaluator's Global disease activity assessment [EvalGlobal, Likert scale 0–4, signifying remission/low/medium/high/maximal]; CRP), physical function (Bath Ankylosing Spondylitis Functional Index [BASFI]), mobility (Bath Ankylosing Spondylitis Metrology Index [BASMI]), and pain (patient's visual analog scale for pain [VAS pain]).

While, as outlined above, the focus of the present study was on the DI as a global measure of gut microbiota aberrations, in order to also allow for some comparisons with prior findings, exploratory analyses comparing the probe signal intensities for the 48 bacterial markers included in the GA-map™ Dysbiosis Test between the axSpA patients (nr-axSpA and AS combined) and controls are presented in Additional file 1.

Statistics

Demographic, disease, and treatment characteristics were compared between groups by chi^2-test, Fisher's exact test or Mann-Whitney *U* test, as appropriate. Between-group comparisons of DI score 1–5 were performed univariately, as well as adjusted for age, sex, body mass index (BMI), and smoking (categorized as never smoker/quit ≥6 months ago/intermittent smoker or quit <6 months ago/everyday smoker) by analysis of covariance (ANCOVA). Due to the ordinal nature of the DI score 1-5, non-parametric bootstrapping with 10,000 iterations was used to calculate 95% confidence intervals (CI). BMI and smoking were adjusted for based on evidence that both are associated with intestinal microbiota alterations [32, 33]. The frequency of dysbiosis (DI ≥3) was compared between groups using chi^2 analysis.

Comparisons of overall between-group differences in the various axSpA measures (regarding disease activity, function, mobility, and pain) between patients with different levels of DI score 1–5 were performed using one-way analysis of variance (ANOVA) or Kruskal-Wallis test, as appropriate. DI levels 4 and 5 were combined into one group, as there were too few cases with each score separately (n=3/n=5). Bootstrapped 95% CI were used for non-normally distributed measures (with normality assessed by Shapiro-Wilk test).

Comparisons of the axSpA measures (regarding disease activity, function, mobility, and pain) between patients with and without dysbiosis (DI ≥3) were performed univariately, as well as multivariately by ANCOVA, again applying bootstrapped 95% CI for non-normally distributed measures. The multivariate analyses were adjusted for age, sex, BMI, smoking (categorized as above), axSpA subtype (nr-axSpA/AS), HLA-B27 status, ongoing anti-TNF (tumor necrosis factor) therapy (yes/no), ASAS NSAID score for the preceding 3 months [34], gut inflammation as measured by F-calprotectin ≥50 mg/kg (yes/no; Calpro AS, Lysaker, Norway), and symptoms meeting the ROME III criteria for IBS (yes/no) [35]. Adjustment for axSpA subtype was considered relevant based on the numerically higher frequency of gut dysbiosis in AS, as compared to nr-axSpA, observed in the present study (see the "Results" section below) and the well-known differences in measures such as CRP and BASMI between the groups. Regarding treatments, anti-TNF agents are used to treat IBD and have been shown to affect gut microbiota in axSpA [20, 36], while NSAIDs can contribute to intestinal inflammation and also have potential to alter the gut microbiome [37]. Gut inflammation, as measured by F-calprotectin elevation, has been previously associated with both worse dysbiosis [19] and axSpA disease activity and function [27, 28] and was thus adjusted for to try to separate the effect of dysbiosis from that of inflammation. Finally, since a previously published study on the SPARTAKUS cohort found a high prevalence (30%) of IBS symptoms and that such symptoms were linked to comorbid fibromyalgia and worse patient-reported axSpA measures of disease activity, function,

and pain [38], it was also deemed relevant to adjust for this, as gut dysbiosis is central to IBS [39].

To further ascertain that any observed associations between gut dysbiosis (DI ≥3) and axSpA measures (regarding disease activity, function, mobility, and pain) were linked to dysbiosis, and not to simultaneous intestinal inflammation or IBS, sensitivity analyses were performed excluding patients with elevated F-calprotectin (≥50 mg/kg) and IBS symptoms, respectively.

Welch's *t* test with bootstrapped 95%CI (due to non-normal distributions; 10,000 iterations) was used for the exploratory comparisons of probe signal intensities for the 48 bacteria markers of the GA-map™ Dysbiosis Test between axSpA patients and controls.

Logarithmic values of ASAS NSAID score were used due to its skewed distribution. No imputations of missing data were performed. $p<0.05$ was considered statistically significant. SPSS, version 27 (IBM Corporation, NY, USA) was used for statistical analyses.

Results

Characteristics of the study population

Characteristics of the axSpA patients and controls are displayed in Table 1. Controls were well matched to the total patient group for age and gender. Compared to the AS group, nr-axSpA patients were on average younger and had shorter symptom duration, and a larger proportion were females. Ongoing or prior smoking was more common among AS patients than in the nr-axSpA or control groups. A numerically larger proportion of AS than nr-axSpA patients displayed gut inflammation (F-calprotectin ≥50 mg/kg), while presence of IBS symptoms was evenly distributed between the two axSpA groups. Similar proportions of nr-axSpA and AS patients received conventional synthetic and/or biologic DMARDs, with 41% anti-TNF use in both groups.

Regarding axSpA measures, BASMI values were significantly higher and CRP values numerically higher in the AS group, while other measures of disease activity, function, and pain were evenly distributed between the axSpA subtypes.

Gut dysbiosis in axSpA and controls

DI score 1–5 was significantly higher among axSpA patients than controls (unadjusted between-group difference 0.45 [95% CI 0.16 to 0.74], $p=0.003$; adjusted between-group difference 0.34 [0.04 to 0.64], $p=0.028$); Fig. 1A). Yet, the frequency of gut dysbiosis (DI ≥3) was only numerically more common in axSpA versus controls (33% versus 17%; $p=0.050$). Of the 8 controls with dysbiosis, none displayed more pronounced aberrations from normobiosis, as measured by a DI of 4 or 5, whereas in the axSpA group this was observed in 19% of the dysbiotic patients ($n=8$ out of 43 patients with dysbiosis).

In our predefined subgroup analysis, both DI score 1–5 (results in Fig. 1B) and presence of gut dysbiosis were significantly higher in the AS group compared to controls (DI ≥3: 36% versus 17%; $p=0.023$). Conversely, for nr-axSpA, neither DI score 1–5 (results in Fig. 1B), nor the frequency of gut dysbiosis differed significantly from controls (DI ≥3: 25% versus 17%; $p=0.377$), although in both cases the nr-axSpA point-estimates fell between those of the control and AS groups. Analogously, no significant between-group differences in DI score 1–5 (results in Fig. 1B) or presence of dysbiosis ($p=0.189$) were detected between the nr-axSpA and AS groups.

Results of the exploratory analyses comparing probe signal intensity for the 48 bacterial markers of the GA-map™ Dysbiosis Test between axSpA patients and controls are presented in Additional file 1.

AxSpA measures in relation to dysbiosis index levels

Among the axSpA patients (nr-axSpA and AS combined), higher DI score 1–5 (combining DI 4 and 5 into one group, due to too few cases) was associated with worse scores of most assessed axSpA measures (Fig. 2). Significant overall between-group differences across the DI score 1–5 categories were found for ASDAS-CRP, BASDAI, BASFI, and VAS pain, but not for EvalGlobal, CRP, or BASMI (Figs. 2 and **3**; $p=0.055$ for EvalGlobal).

Differences in axSpA measures in patients with versus without gut dysbiosis

When instead splitting the axSpA group (nr-axSpA and AS combined) according to presence of gut dysbiosis (DI <3 [$n=89$] versus ≥3 [$n=43$]), patients displaying dysbiosis had higher BMI and were more often ongoing or prior smokers (Table 2). HLA-B27 positivity was less frequent in the dysbiotic group, while gut inflammation (F-calprotectin ≥50 mg/kg) was numerically and IBS symptoms significantly more common in subjects with dysbiosis (Table 2).

Comparing the axSpA measures between patients with versus without gut dysbiosis, univariate analyses showed significantly higher ASDAS-CRP, BASDAI, BASFI, VAS pain, and EvalGlobal scores for patients with versus without dysbiosis (Fig. 4; Table 2). These between-group differences remained significant also after adjustment, except for VAS pain ($p=0.066$) (Fig. 4). Regarding CRP and BASMI, no differences were detected between patients with versus without dysbiosis, neither univariately nor in the adjusted analyses (Fig. 4).

In the sensitivity analyses, the comparisons displayed in Fig. 4 were repeated after having respectively limited the study population to patients without gut

Table 1 Characteristics of the study population

	All axSpA (Nr-axSpA+AS)	Nr-axSpA	AS	Controls
	***n*=132**	***n*=44**	***n*=88**	***n*=46**
Male sex, *n* (%)	72 (55%)	17 (39%)	55 (63%) ‡	23 (50%)
Age, years	53 (13)	48 (12)	55 (13) ‡	51 (14)
BMI, kg/m^2	27 (4.3)	27 (4.2)	27 (4.3)‡	25 (3.3) ‡‡
Smoking status				
Never smoker, *n* (%)	89 (67%)	35 (80%)	54 (61%)	32 (71%)
Quit smoking >6 months ago, *n* (%)	31 (24%)	7 (16%)	24 (27%)	11 (24%)
Intermittent smoker or quit <6 months ago, *n* (%)	5 (3.8%)	1 (2.3%)	4 (4.5%)	0
Every day smoker, *n* (%)	7 (5.3%)	1 (2.3%)	6 (6.8%)	2 (4.4%)
Family history of SpA, *n* (%)	58 (44%)	17 (39%)	41 (47%)	
Symptom duration, years	26 (14)	21 (11)	28 (14) ‡	
HLA-B27 positive, *n* (%)	114 (87%)	41 (93%)	73 (84%)	
Back pain ≥3 months:				
With onset <45 years, *n* (%)	127 (96%)	44 (100%)	83 (94%)	
Improved by exercise and not relieved by rest, *n* (%)	103 (79%)	33 (75%)	70 (81%)	
Inflammatory back pain (ASAS definition), *n* (%)	112 (85%)	37 (84%)	75 (85%)	
Sagittal lumbar flexion (Modified Schober's test), cm	4.2 (1.6)	4.2 (1.2)	4.2 (1.8)	
Lateral lumbar flexion, cm[a]	14 (4.9)	15 (4.9)	13 (4.8) ‡	
Chest expansion, cm	4.8 (1.8)	5.0 (1.9)	4.7 (1.7)	
Sacroiliitis on plain X-ray, *n* (%)	88 (67%)	0 (0%)	88 (100%) ‡	
SI joint MRI available, *n* (%)	65 (49%)	25 (57%)	40 (45%)	
SI joint bone marrow oedema on MRI, *n* (%) [b]	32 (49%)	9 (36%)	23 (58%)	
Good response of back pain to NSAID, *n* (%)	103 (78%)	33 (75%)	70 (80%)	
Elevated CRP in the presence of back pain, *n* (%)	82 (62%)	25 (57%)	57 (65%)	
Peripheral arthritis, *n* (%)	68 (52%)	26 (59%)	42 (48%)	
Dactylitis, *n* (%)	15 (11%)	9 (21%)	6 (6.8%) ‡	
Heel enthesitis, *n* (%)	57 (43%)	22 (50%)	35 (40%)	
History of uveitis, *n* (%)	46 (35%)	11 (25%)	35 (40%)	
Skin and/or nail psoriasis, *n* (%)	8 (6.1%)	3 (6.8%)	5 (5.7%)	
IBD, *n* (%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
F-calprotectin, mg/kg				
Mean (SD)	74 (131)	48 (55)	87 (155)	
Median (IQR)	33 (60)	29 (39)	39 (65)	
Elevated F-calprotectin ≥50 mg/kg, *n* (%)	46 (35%)	12 (28%)	34 (39%)	
IBS symptoms, *n* (%)[c]	43 (33%)	15 (34%)	28 (32%)	
ASDAS-CRP	1.8 (0.9)	1.9 (0.9)	1.8 (0.9)	
BASDAI	3.1 (2.2)	3.3 (1.9)	3.0 (2.4)	
EvalGlobal, 0–4, median (IQR)[d]	1 (0-1)	1 (0-1)	1 (0–1)	
CRP, mg/l	3.7 (5.3)	2.3 (2.4)	4.3 (6.1)	
BASFI	2.0 (2.1)	2.0 (1.7)	2.1 (2.2)	
BASMI	3.0 (1.4)	2.5 (1.1)	3.2 (1.5) ‡	
VAS pain, cm	3.3 (2.5)	3.4 (2.2)	3.2 (2.7)	
ASAS 3-month NSAID score	37 (44)	36 (44)	37 (44)	
Use of proton-pump inhibitors last 3 months, *n* (%)	53 (40%)	19 (43%)	34 (39%)	
Ongoing csDMARD, *n* (%)	24 (18%)	9 (20%)	15 (17%)	
Methotrexate, *n* (%)	14 (10.6%)	4 (9.1%)	10 (11.4%)	
Sulfasalazine, *n* (%)	8 (6.1%)	3 (6.8%)	5 (5.7%)	
Other csDMARD, *n* (%)	2 (1.5%)	2 (4.5%)	0 (0%)	
Ongoing bDMARD, *n* (%)	56 (42%)	19 (43%)	37 (42%)	

Table 1 (continued)

	All axSpA (Nr-axSpA+AS)	Nr-axSpA	AS	Controls
	n=132	*n*=44	*n*=88	*n*=46
Adalimumab, *n* (%)	10 (7.6%)	2 (4.5%)	8 (9.1%)	
Certolizumab pegol, *n* (%)	11 (8.3%)	7 (15.9%)	4 (4.5%)‡	
Etanercept, *n* (%)	19 (14.4%)	5 (11.4%)	14 (15.9%)	
Golimumab, *n* (%)	5 (3.8%)	2 (4.5%)	3 (3.4%)	
Infliximab, *n* (%)	9 (6.8%)	2 (4.5%)	7 (8.0%)	
Secukinumab, *n* (%)	2 (1.5%)	1 (2.3%)	1 (1.1%)	

Mean (SD) if not otherwise stated. [a]Mean of right and left lateral lumbar flexion. [b]Previous or current SI joint bone marrow oedema according to the ASAS definition. [c]Meeting the ROME III criteria for IBS. [d]Likert scale 0–4, corresponding to Remission/Low/Medium/High/Maximal. ‡$p<0.05$ for between-group difference between nr-axSpA and AS by chi^2-test/Fisher´s Exact test or Mann-Whitney *U* test, as appropriate. ‡‡$p<0.05$ for between-group difference between axSpA (nr-axSpA and AS combined) and controls by Mann-Whitney *U* test. Missing data, *n* (%): Symptom duration 1 (0.8%); HLA-B27 1 (0.8%); back pain improved by exercise but not by rest 1 (0.8%); chest expansion 1 (0.8%); F-calprotectin 2 (1.5%); IBS symptoms 1 (0.8%); ASDAS-CRP 16 (12%); BASDAI 7 (5.3%), EvalGlobal 5 (3.8%); CRP 15 (11%); BASFI 9 (6.8%); BASMI 2 (1.5%); VAS pain 2 (1.5%); Smoking, controls 1 (2.2%). *AS* ankylosing spondylitis, *ASDAS-CRP* ankylosing spondylitis disease activity score using CRP, *ASAS* Assessment of SpondyloArthritis international Society, *AxSpA* axial spondyloarthritis, *BASDAI* Bath ankylosing spondylitis disease activity index, *BASFI* Bath ankylosing spondylitis functional index, *BASMI* Bath ankylosing spondylitis metrology index, *bDMARD* biologic disease-modifying anti-rheumatic drug, *CRP* C-reactive protein, *csDMARD* conventional synthetic disease-modifying anti-rheumatic drug, *EvalGlobal* evaluator's global assessment of disease activity, *HLA* human leukocyte antigen, *IBD* inflammatory bowel disease, *IBS* irritable bowel syndrome, *IQR* interquartile range, *MRI* magnetic resonance imaging, *nr-axSpA* non-radiographic axial spondyloarthritis, *NSAID* non-steroidal anti-inflammatory drug, *SD* standard deviation, *SI* sacroiliac, *SpA* spondyloarthritis, *VAS* visual analog scale

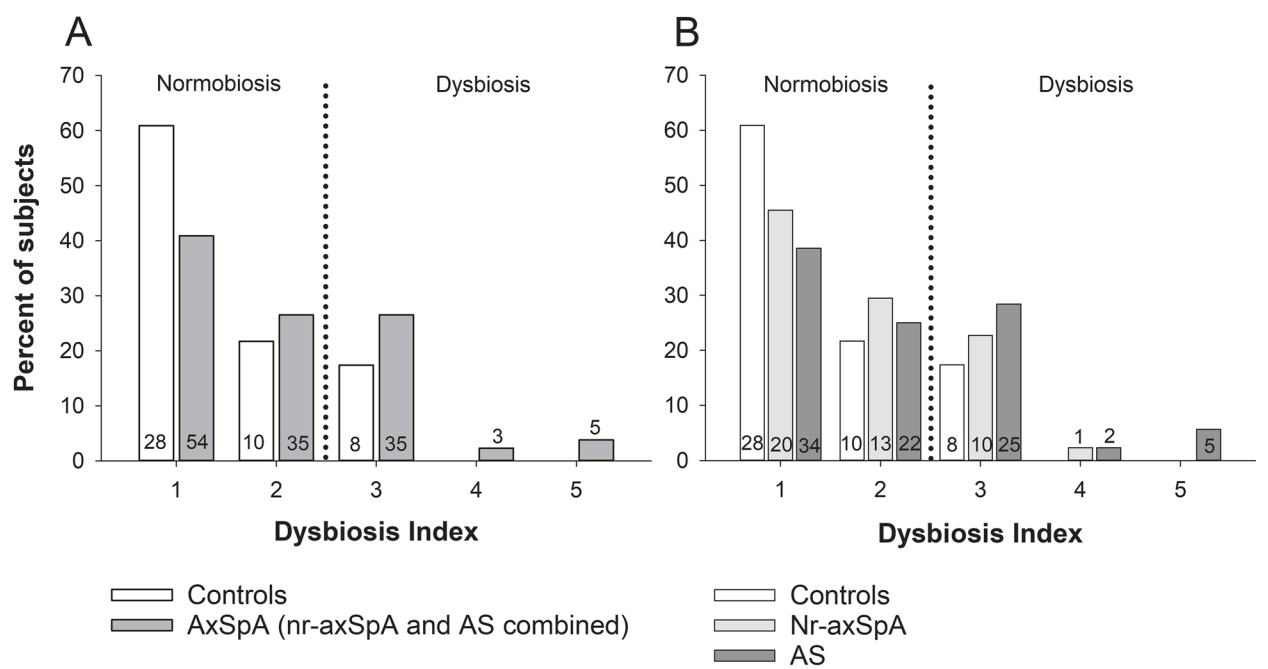

Fig. 1 Dysbiosis index distributions among axSpA patients and controls. **A** Distributions of dysbiosis index (DI) score 1–5 among axSpA patients (nr-axSpA and AS combined) and controls. Unadjusted between-group difference in DI score 1–5: 0.45 (95% CI 0.16 to 0.74), p=0.003. Adjusted (for age, sex, BMI, and smoking) between-group difference in DI score 1–5: 0.34 (0.04 to 0.64), p=0.028. **B** Distributions of DI score 1–5 among nr-axSpA patients, AS patients, and controls. Nr-axSpA versus controls: unadjusted between-group difference in DI score 1–5: 0.25 (−0.09 to 0.60), p=0.154; adjusted (as above): 0.17 (−0.19 to 0.54), p=0.409. AS versus controls: unadjusted: 0.55 (0.22 to 0.88), $p<0.001$; adjusted: 0.43 (0.07 to 0.78), p=0.019. AS versus nr-axSpA: unadjusted: 0.30 (−0.06 to 0.64), p=0.098; adjusted: 0.20 (−0.18 to 0.56), p=0.286. Number of subjects per group presented at the bottom of (or above) the bars. AS ankylosing spondylitis, axSpA axial spondyloarthritis, BMI body mass index, CI confidence interval, DI dysbiosis index, nr-axSpA non-radiographic axial spondyloarthritis

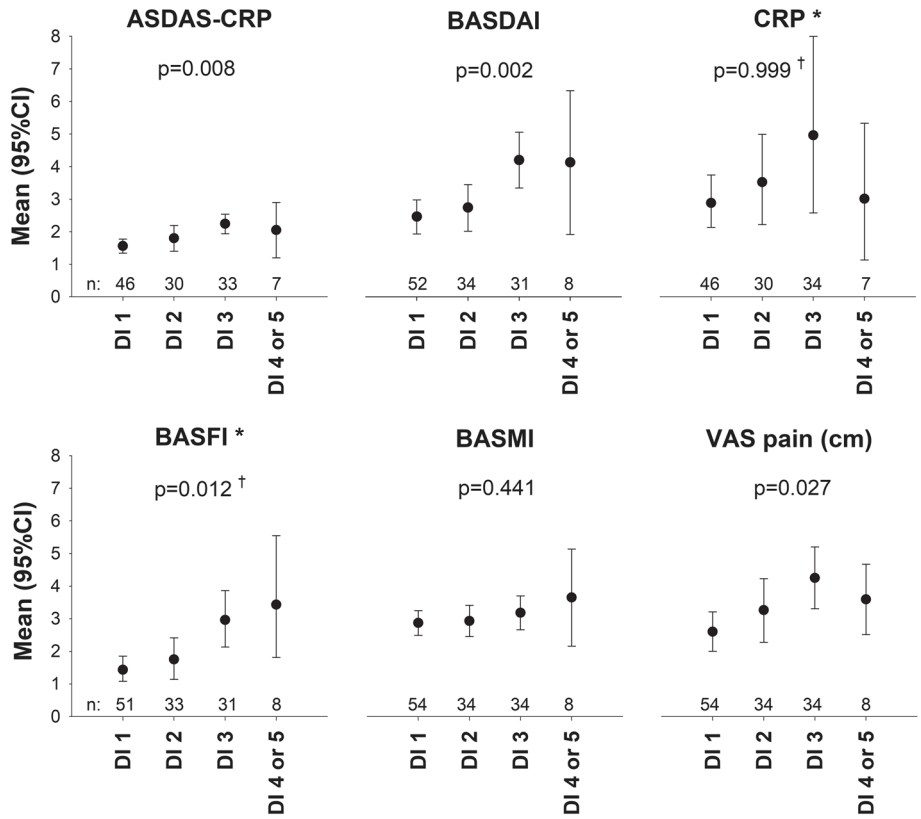

Fig. 2 AxSpA measures in relation to dysbiosis index levels. Distributions of measures of disease activity, physical function, mobility, and pain among axSpA patients (nr-axSpA and AS combined) with different levels of dysbiosis index (DI) score 1–5. Number of analyzed subjects per group presented above the *x*-axes (DI 4 and 5 combined into one group due to too few cases). *P* values for overall between-group differences by one-way ANOVA (or Kruskal-Wallis test †) displayed in the graphs. *Bootstrapped 95% CI. ANOVA analysis of variance, AS ankylosing spondylitis, axSpA axial spondyloarthritis, ASDAS-CRP ankylosing spondylitis disease-activity score using C-reactive protein, BASDAI Bath ankylosing spondylitis disease activity index, BASFI Bath ankylosing spondylitis functional index, BASMI Bath ankylosing spondylitis metrology index, CI confidence interval, CRP C-reactive protein, DI dysbiosis index, nr-axSpA non-radiographic axial spondyloarthritis, VAS visual analog scale

inflammation (F-calprotectin <50 mg/kg; n=84) or to those not reporting IBS symptoms (n=88). In both cases, between-group differences for patients with versus without dysbiosis seen in the main analysis were confirmed or found even more distinct regarding BASDAI and VAS pain, and in relation to gut inflammation also for ASDAS-CRP and BASFI (see Additional file 1, Figures S2 and S3). However, regarding Eval-Global, no significant difference remained between the groups after adjustment in the patient sample without gut inflammation (p=0.061; see Additional file 1, Figure S2). Also in the sample without IBS symptoms, no between-group differences were observed for Eval-Global in either the univariate or adjusted analysis, nor for ASDAS-CRP or BASFI in the adjusted analysis (see Additional file 1, Figure S3).

Discussion

Main findings

In this population-based, cross-sectional study of well-characterized axSpA patients without IBD, we found gut dysbiosis to be significantly more frequent in AS (36%) than controls (17%). Likewise, the degree of gut microbiota aberration (as measured by DI score 1–5) was significantly higher in AS than controls. Conversely, neither dysbiosis frequency nor DI score 1–5 were significantly increased in nr-axSpA compared to controls, although numerically the nr-axSpA point-estimates fell between those of the AS patients and controls.

More importantly (and to the best of our knowledge not this clearly demonstrated before), in the overall axSpA group (nr-axSpA and AS combined), a state of gut dysbiosis was associated with significantly worse

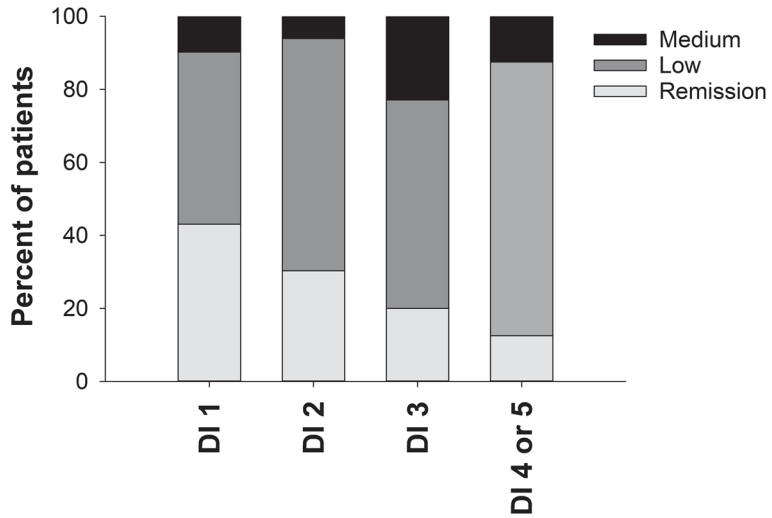

Fig. 3 Evaluator's global assessment of disease activity in relation to dysbiosis index levels. Distribution of Evaluator's global assessment of disease activity (EvalGlobal; Likert scale 0–4, corresponding to Remission/Low/Medium/High/Maximal) among axSpA patients (nr-axSpA and AS combined) with different levels of dysbiosis index (DI) score 1–5. Number of patients in each group: DI 1 *n*=51, DI 2 *n*=33, DI 3 *n*=35, DI 4, or 5 *n*=8 (DI 4 and 5 combined into one group due to too few cases). No patients in any DI group were assessed as having high or maximal disease activity. *P*=0.055 for overall between-group difference by Kruskal-Wallis test. AS ankylosing spondylitis, axSpA axial spondyloarthritis, DI dysbiosis index, nr-axSpA non-radiographic axial spondyloarthritis

disease activity, physical function, and pain as measured by ASDAS-CRP, BASDAI, EvalGlobal, BASFI, and VAS pain. The associations remained also after adjustment for relevant confounders, including gut inflammation, anti-TNF, and NSAID treatment, except regarding VAS pain (where statistical significance was just barely lost). Restricting the analyses to include only patients without gut inflammation (as measured by elevated F-calprotectin ≥50 mg/kg) further demonstrated the associations to be present regardless of such inflammation. Thus, similar to prior observations regarding gut inflammation in axSpA [10, 25–28], but seemingly independent of such inflammation, gut dysbiosis may be a marker of more severe axSpA.

Previous research

Gastrointestinal pathology, including dysbiosis, and the pathogenesis of SpA

Gut dysbiosis is believed to play an important role in IBD pathogenesis through complex and incompletely understood interactions with genetic and environmental risk factors, gut barrier defects, and abnormal immune responses [13]. SpA is closely associated with IBD—in particular Crohn's disease—both genetically and in terms of clinical comorbidity, and many of their shared genetic risk loci govern mucosal immune defenses [7]. Furthermore, several pathological, gastrointestinal processes and aberrations reminiscent of those in IBD have been observed also in SpA, including gut dysbiosis [18–24, 40], gut inflammation (present in 50–60% of SpA patients, although subclinical in most cases) [8–10], and signs of gut barrier defects [41]. Based on this, one central hypothesis for SpA pathogenesis implicates such IBD-like, gastrointestinal processes, including dysbiosis, as an upstream event, subsequently inducing an IL-23/IL-17-pathway driven inflammation of the spine and joints [6, 7, 42, 43]. The present results, linking gut dysbiosis to worse axSpA phenotype and more active disease appear to fit well with such a theory. This hypothesis remains unproven, however, and the close links between IBD/IBD-like pathology and SpA may also be due to their shared genetic predisposition, without a causal relationship [44]. Regardless of which, ongoing gut inflammation has been repeatedly associated with more severe SpA [10, 25–28], demonstrating an important interaction between the gut and the musculoskeletal apparatus in this disease.

Gut dysbiosis in axSpA

The current results are congruent with previous findings which have repeatedly associated AS with altered gut microbiota [18–23], whereas little prior information is available regarding nr-axSpA [24]. While compositional analyses of the gut microbiome have consistently been able to distinguish AS from healthy controls [18–21, 23], the specific microbiota alterations observed have been heterogeneous, and no specific changes at any level of the bacterial taxonomic hierarchy have so far been identified as typical for axSpA. Furthermore, several

Table 2 Characteristics of the axSpA patients (nr-axSpA and AS combined), stratified by the presence of gut dysbiosis

	AxSpA without dysbiosis (DI <3) *n*=89	**AxSpA with dysbiosis (DI ≥3) *n*=43**
Male sex, *n* (%)	49 (55%)	23 (54%)
Age, years	51 (13)	55 (13)
BMI, kg/m^2	26 (3.9)	28 (4.7) ‡‡
Smoking status		
Never smoker, *n* (%)	67 (75%)	2 (51%)
Quit smoking >6 months ago, *n* (%)	17 (19%)	14 (33%)
Intermittent smoker or quit <6 months ago, *n* (%)	1 (1.1%)	4 (9.3%)
Every day smoker, *n* (%)	4 (4.5%)	3 (7.0%)
Symptom duration, years	25 (13)	28 (15)
HLA-B27 positive, *n* (%)	83 (94%)	31 (72%) ‡
Nr-axSpA, *n* (%)	33 (37%)	11 (26%)
F-calprotectin, mg/kg		
Mean (SD)	56 (71)	112 (206)
Median (IQR)	30 (55)	39 (69)
Elevated F-calprotectin ≥50 mg/kg, *n* (%)	29 (33%)	17 (41%)
IBS symptoms, *n* (%)[a]	22 (25%)	21 (49%) ‡
ASDAS-CRP	1.7 (0.9)	2.2 (0.9) ‡
BASDAI	2.6 (2.0)	4.2 (2.4) ‡
EvalGlobal, 0–4, median (IQR)[b]	1 (0-1)	1 (1-1) ‡
CRP, mg/l	3.1 (3.3)	4.6 (7.6)
BASFI	1.6 (1.6)	3.1 (2.5) ‡
BASMI	2.9 (1.4)	3.3 (1.5)
VAS pain, cm	2.9 (2.5)	4.1 (2.5) ‡
ASAS 3-month NSAID score	35 (41)	39 (50)
Use of proton-pump inhibitors last 3 months, *n* (%)	33 (37%)	20 (47%)
Ongoing csDMARD, *n* (%)	17 (19%)	7 (16%)
Methotrexate, *n* (%)	10 (11.2%)	4 (9.3%)
Sulfasalazine, *n* (%)	5 (5.6%)	3 (7.0%)
Other csDMARD, *n* (%)	2 (2.2%)	0 (0%)
Ongoing bDMARD, *n* (%)	37 (42%)	19 (44%)
Adalimumab, *n* (%)	8 (9.0%)	2 (4.7%)
Certolizumab pegol, *n* (%)	7 (7.9%)	4 (9.3%)
Etanercept, *n* (%)	14 (15.7%)	5 (11.6%)
Golimumab, *n* (%)	3 (3.4%)	2 (4.7%)
Infliximab, *n* (%)	4 (4.5%)	5 (11.6%)
Secukinumab, *n* (%)	1 (1.1%)	1 (2.3%)

Mean (SD) if not otherwise stated. [a]Meeting the ROME III criteria for IBS. [b]Likert scale 0–4, corresponding to Remission/Low/Medium/High/Maximal. ‡$p<0.05$ for between-group difference between axSpA patients (nr-axSpA and AS combined) with versus without gut dysbiosis by chi^2-test/Fisher's exact test or Mann-Whitney *U* test, as appropriate. Missing data, *n* (%): Symptom duration 1 (0.8%); HLA-B27 1 (0.8%); F-calprotectin 2 (1.5%); IBS symptoms 1 (0.8%); ASDAS-CRP 16 (12%); BASDAI 7 (5.3%), EvalGlobal 5 (3.8%); CRP 15 (11%); BASFI 9 (6.8%); BASMI 2 (1.5%); VAS pain 2 (1.5%). *AS* ankylosing spondylitis, *ASDAS-CRP* ankylosing spondylitis disease activity score using CRP, *ASAS* Assessment of SpondyloArthritis international Society, *AxSpA* axial spondyloarthritis, *BASDAI* Bath ankylosing spondylitis disease activity index, *BASFI* Bath ankylosing spondylitis functional index, *BASMI* Bath ankylosing spondylitis metrology index, *bDMARD* biologic disease-modifying anti-rheumatic drug, *CRP* C-reactive protein, *csDMARD* conventional synthetic disease-modifying anti-rheumatic drug, *DI* dysbiosis index, EvalGlobal Evaluator's global assessment of disease activity, *IBS* irritable bowel syndrome, *IQR* interquartile range, *nr-axSpA* non-radiographic axial spondyloarthritis, *NSAID* non-steroidal anti-inflammatory drug, *SD* standard deviation, *VAS* visual analog scale

features reminiscent of the gut dysbiosis of IBD have been reported in AS [18–21, 40]. Compositional analyses, however, clearly distinguish the gut microbiome of AS from that of IBD [19, 23], showing that despite certain similarities, the type of dysbiosis in the two diseases is not the same.

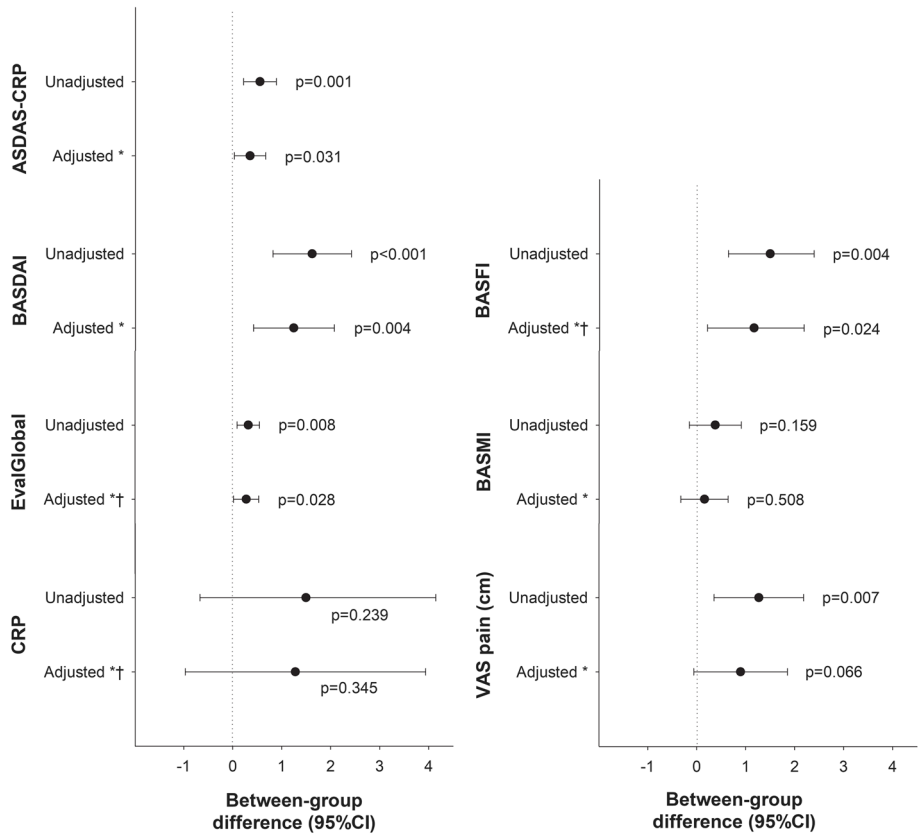

Fig. 4 Differences in axSpA measures in patients with versus without gut dysbiosis. Differences in measures of disease activity, physical function, mobility, and pain between axSpA patients (nr-axSpA and AS combined) with gut dysbiosis (DI ≥3) versus those without dysbiosis (DI <3). The data shown represent point-estimate differences (dots) with 95% CI (whiskers) and corresponding *p* values from unadjusted analyses, and after adjustment (*) for age, sex, BMI, smoking, axSpA subtype (nr-axSpA/AS), HLA-B27 status (positive/negative), ongoing anti-TNF therapy (yes/no), ASAS 3-month NSAID score, gut inflammation (F-calprotectin ≥50 mg/kg, yes/no), and IBS-symptoms (yes/no) (ANCOVA). † Bootstrapped 95% CI. ANCOVA analysis of covariance, AS ankylosing spondylitis, ASAS Assessment of SpondyloArthritis international Society, axSpA axial spondyloarthritis, ASDAS-CRP ankylosing spondylitis disease-activity score using C-reactive protein, BASDAI Bath ankylosing spondylitis disease activity index, BASFI Bath ankylosing spondylitis functional index, BASMI Bath ankylosing spondylitis metrology index, BMI body mass index, CI confidence interval, CRP C-reactive protein, DI dysbiosis index, EvalGlobal Evaluator's global assessment of disease activity, F fecal, HLA human leukocyte antigen, IBS irritable bowel syndrome, nr-axSpA non-radiographic axial spondyloarthritis, NSAID non-steroidal anti-inflammatory drug, TNF tumor necrosis factor, VAS visual analog scale

As outlined above, 50–60% of SpA patients display microscopic gut inflammation [8–10], but whether there is a causal relationship between this and gut dysbiosis—and if so, in which direction—remains incompletely understood. The two processes are, however, intertwined, since the presence of inflammation has been associated with a significantly different gut microbiota composition compared to SpA patients without histological gut inflammation [24]. Furthermore, another study showed the gut microbiota composition to differ between AS patients with elevated versus normal F-calprotectin [19].

In the present study, gut microbiota aberrations were assessed by the validated, semi-quantitative DI of the GA-map™ Dysbiosis Test. Klingberg et al. previously applied the same method in a cohort of 150 AS patients, finding a substantially higher gut dysbiosis (DI ≥3) prevalence of 87% [19], as compared to the 36% in our AS sample. Of interest in light of our results, however, their cohort also displayed higher disease activity and worse physical function, with median BASDAI/BASFI scores of 3.2/2.3 versus 2.2/1.2 in our AS group, and thus represented a patient group with worse disease.

AxSpA disease severity in relation to gut dysbiosis

To our knowledge, only a few studies have previously investigated gut microbiota aberrations in SpA in relation to disease severity, most seeming to indicate an association with disease activity in line with our results.

Tito et al. studied the intestinal microbiota in biopsies from 27 axSpA patients (AS and nr-axSpA) and found a significant positive correlation between the abundance of the genus *Dialister* and disease activity as measured by ASDAS (r_s=0.62), and a similar trend for BASDAI (r_s=0.53) [24]. In another study, Breban et al. observed an enrichment in *Ruminococcus gnavus* in SpA, which was significant in relation to controls only for cases with active disease (BASDAI ≥3) [40]. This enrichment in association with higher SpA disease activity was observed regardless of concomitant IBD, but the elevation was more pronounced in those with comorbid IBD, for whom there was also a significant positive correlation between *Ruminucoccus gnavus* abundance and BASDAI (r_s=0.77). Finally, a recent abstract from a multinational study on gut microbiota in AS showed that the bacterial composition differed significantly between patients with different BASDAI levels (categorized as <2.5/2.5-5/5-7.5/>7.5), congruent with our findings [23].

Conversely, Klingberg et al. reported no associations between gut microbiota composition, assessed by the GA-map™ Dysbiosis Test, and ASDAS-CRP, BASDAI, BASFI, BASMI, CRP, or ESR (erythrocyte sedimentation rate) [19]. However, in contrast to our analyses, their study did not relate these measures to the DI, but performed compositional analysis, comparing the gut microbiota composition between patient groups with better or worse (dichotomized) levels of the different indices. Notably, as stated above, Klingberg et al. found dysbiosis (DI ≥3) in as many as 87% of their AS patients (n=130 versus n=20 without dysbiosis), a distribution which may have limited their ability to detect differences in disease measures based on microbiota composition.

Apart from excluding [19, 24] or separately analyzing [40] patients with comorbid IBD, none of the above-cited studies [19, 23, 24, 40] adjusted their analyses regarding the link between gut microbiota aberrations and disease severity for intestinal inflammation. Thus, some reported observations may have been driven by the known link between gut inflammation and more severe disease [10, 25–28], rather than by the dysbiosis per se. Here, we both adjusted for F-calprotectin and performed a sensitivity analysis excluding all patients with elevated F-calprotectin (≥50 mg/kg), aiming to more specifically assess a potential association with gut dysbiosis. In this context, it should be acknowledged that the ability of a F-calprotectin <50 mg/kg to rule out asymptomatic, microscopic (histological) gut inflammation, which is much more common in SpA than overt IBD [8, 27], has not been widely studied, and F-calprotectin may also be less sensitive for small-bowel than colonic inflammation [45]. Cypers et al., however, did report significantly elevated F-calprotectin in SpA patients displaying microscopic gut inflammation in the terminal ileum and/or colon, with an optimal F-calprotectin cut-off of 85 mg/kg for the detection of such cases [46]. Furthermore, the presence of macroscopic inflammation at capsule endoscopy and/or ileocolonoscopy in SpA patients with previously undiagnosed IBD has also been shown to be significantly associated with elevated F-calprotectin levels (>100 mg/kg) [47, 48]. Taken together, the current results seem to indicate that gut dysbiosis may be associated with worse axSpA disease activity and physical function, independently of intestinal inflammation.

Similarly, since IBS symptoms have previously been associated with worse patient-reported axSpA measures in the SPARTAKUS cohort [38] and that gut dysbiosis is intrinsic to IBS [39], the same adjustment and sensitivity analysis approach was also applied for IBS symptoms, again without relevant changes to our results. Anti-TNF therapy, used by 41% of our patients, has previously shown a potential to ameliorate gut dysbiosis in axSpA [20, 36] and was therefore adjusted for, without any considerable impact on the findings. Moreover, proton-pump inhibitor (PPI) use was considered as an additional adjustment factor due to its described association with gut dysbiosis [49], but was omitted since PPI-use during the last 3 months did not differ significantly between patients with versus without dysbiosis (DI ≥3) in our material (p=0.300 by chi^2-test) and since no causal relationship between PPI use and the axSpA measures was suspected.

Strengths and limitations

This study provides new knowledge regarding how gut dysbiosis relates to axSpA phenotype and disease measures (regarding disease activity, function, mobility, and pain)—associations previously investigated by only a limited number of studies. Patients were consecutively enrolled from a population-based cohort study and were well characterized and classified according to the ASAS axSpA and modified New York criteria. The detailed protocol allowed for extensive adjustment for possible confounders, including BMI, smoking, axSpA subtype, HLA-B27 status, anti-TNF and NSAID therapy, intestinal inflammation, and IBS symptoms. By including F-calprotectin, we could—to a certain degree—disentangle a link between gut dysbiosis and worse axSpA disease activity and function from the impact of simultaneous intestinal inflammation.

Another strength is that, in addition to AS, our study also encompasses nr-axSpA, a disease subtype for which data regarding gut microbiota are scant. The relatively low number of nr-axSpA patients (n=44) means that we cannot rule out type 2 error from contributing to the lack of significant differences between this group and both

AS and controls. Yet, the pattern by which the nr-axSpA point-estimates fall in between those of AS and controls is similar to prior observations for F-calprotectin [27]. The relatively long mean symptom duration of our nr-axSpA group should also be remembered when assessing the results, since it likely entails a lower average risk of future progression to AS than in a cohort of newly diagnosed nr-axSpA.

Regarding generalizability, only comorbid IBD and recent antibiotic use were exclusion criteria. Without restrictions regarding disease subtype (nr-axSpA or AS) or severity, treatment, or other comorbidities, we believe our results to be fairly generalizable to the wider axSpA population. However, in the absence of any concurrent ICD-10 codes for SpA (usually added in case of axial involvement), patients clinically diagnosed with psoriatic, IBD-associated, or reactive arthritis (by ICD-10 codes) were not included, although these conditions may sometimes have axial disease.

Regarding limitations, endoscopic examinations and biopsies were not part of the SPARTAKUS protocol, but could have provided valuable information regarding histological inflammation and mucosa-associated microbiota (fecal and mucosa-associated microbiota have been shown to differ [50, 51]). The use of deep-sequencing or shotgun metagenomics sequencing methods, rather than the pre-determined target approach of the GA-map™ Dysbiosis Test, would have provided a much more detailed characterization of the gut microbiota. Assessment of associations between specific bacteria and axSpA disease measures were, however, beyond the scope of the present study. For the determination of gut dysbiosis, the validated GA-map™ Dysbiosis Test has shown good agreement with deep-sequencing methodology [31], and by the DI, it provides a readily comprehensible, semiquantitative score of global dysbiosis, calibrated to healthy individuals as reference of normobiosis. Finally, information regarding probiotics use is missing, and the cross-sectional design did not allow for longitudinal evaluation of microbiota shifts or disease development.

Conclusions

The presence of gut dysbiosis is linked to worse axSpA phenotype and more active disease. While dysbiosis was more prevalent in AS than controls, no significant difference was observed between nr-axSpA and controls. In the overall axSpA group (nr-axSpA and AS combined), the presence of gut dysbiosis was independently associated with worse disease activity and physical function, seemingly irrespective of both gut inflammation and ongoing treatments. Further research is needed to assess the validity of these findings also in other axSpA cohorts, as well as to gain a deeper understanding of the connection between gut microbiota and axSpA, and examine whether a causal relationship exists. This would offer guidance as to whether targeting the intestine therapeutically, with regard to the luminal microbiota composition and/or mucosal inflammation, could be a relevant approach to try to interfere with axSpA development or mitigate the disease course. Nonetheless, the current results add to the steadily growing body of evidence for an important, potentially pathobiological, link between disturbances in gastrointestinal homeostasis and axSpA.

Abbreviations

ANCOVA: Analysis of covariance; AS: Ankylosing spondylitis; ASAS: Assessment of SpondyloArthritis international Society; ASDAS-CRP: Ankylosing spondylitis disease activity score using CRP; axSpA: Axial spondyloarthritis; BASDAI: Bath ankylosing spondylitis disease activity index; BASFI: Bath ankylosing spondylitis functional index; BASMI: Bath ankylosing spondylitis metrology index; bDMARD: Biologic disease-modifying anti-rheumatic drug; BMI: Body mass index; CI: Confidence interval; CRP: C-reactive protein; csDMARD: Conventional synthetic disease-modifying anti-rheumatic drug; DI: Dysbiosis index; DMARD: Disease-modifying anti-rheumatic drug; ESR: Erythrocyte sedimentation rate; EvalGlobal: Evaluator's global assessment of disease activity; HLA-B27: Human leukocyte antigen B27; IBD: Inflammatory bowel disease; IBS: Irritable bowel syndrome; IL: Interleukin; IQR: Interquartile range; mNY: Modified New York criteria; Nr-axSpA: Non-radiographic axial spondyloarthritis; NSAID: Non-steroidal anti-inflammatory drug; PPI: Proton-pump inhibitor; SD: Standard deviation; TNF: Tumor necrosis factor; VAS pain: Visual analog scale for pain.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13075-022-02733-w.

Additional file 1. Exploratory analyses of bacterial abundance in axial spondyloarthritis patients versus controls, and sensitivity analyses regarding disease activity, physical function, mobility and pain in relation to gut dysbiosis status in the patient group. Exploratory analyses comparing probe signal intensity for the 48 bacterial markers of the GA-map™ Dysbiosis Test between the axial spondyloarthritis patients and controls (including **Figure S1**). **Figure S2.** Differences in measures of disease activity, physical function, mobility and pain between axial spondyloarthritis patients with gut dysbiosis versus those without dysbiosis, when only including patients without gut inflammation. **Figure S3.** Differences in measures of disease activity, physical function, mobility and pain between axial spondyloarthritis patients with gut dysbiosis versus those without dysbiosis, when only including patients without irritable bowel syndrome symptoms.

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Authors' contributions

JS participated in the study design, analysis, and interpretation of the data and draft and revision of the manuscript. JKW participated in the study design, acquisition of data, analysis and interpretation of data, and draft and revision of the manuscript. TO, EM, and EL participated in the study design, acquisition of data, interpretation of data, and revision of the manuscript (i.e., revising it critically for important intellectual content). KA, JM, MG, and LEK participated in the study design, interpretation of data, and revision of the manuscript (i.e., revising it critically for important intellectual content). The authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and analyzed for the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Ethical approval for the SPARTAKUS study was granted by the Regional Ethics Committee in Lund, Sweden (Dnr. 2015/436 with amendment Dnr. 2018/238). Oral and written informed consent was acquired from all patients and controls before entry into the study, which was performed in accordance with the Helsinki Declaration.

Consent for publication
Not applicable.

Competing interests
JS: None declared. TO: Consultant of Eli Lilly, Merck Sharp & Dohme. EM: Consultant of Novartis. JM: Speakers bureau of AbbVie, Bristol-Myers Squibb, Ferring, Janssen Pharmaceuticals, Pfizer, Takeda, Tillotts; Consultant of AbbVie, Janssen Pharmaceuticals, Pfizer, Takeda; Grant/research support from AbbVie, Ferring, Pfizer, Takeda. KA: None declared. MG: Speakers bureau of AbbVie, Novartis, Pfizer, UCB Pharma. LEK: Speakers bureau of AbbVie, Amgen, Bristol-Myers Squibb, Celgene, Eli Lilly, Janssen Pharmaceuticals, Merck Sharp & Dohme, Novartis, Pfizer, UCB Pharma; Consultant of AbbVie, Amgen, Bristol-Myers Squibb, Celgene, Eli Lilly, Janssen Pharmaceuticals, Merck Sharp & Dohme, Novartis, Pfizer, UCB Pharma; EL: None declared. JKW: Consultant of AbbVie, Amgen, Celgene, Eli Lilly, Novartis.

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Predictors of Symptom-Specific Treatment Response to Dietary Interventions in Irritable Bowel Syndrome

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Abstract: (1) Background: Predictors of dietary treatment response in irritable bowel syndrome (IBS) remain understudied. We aimed to investigate predictors of symptom improvement during the low FODMAP and the traditional IBS diet for four weeks. (2) Methods: Baseline measures included faecal Dysbiosis Index, food diaries with daily energy and FODMAP intake, non-gastrointestinal (GI) somatic symptoms, GI-specific anxiety, and psychological distress. Outcomes were bloating, constipation, diarrhea, and pain symptom scores treated as continuous variables in linear mixed models. (3) Results: We included 33 and 34 patients on the low FODMAP and traditional IBS diet, respectively. Less severe dysbiosis and higher energy intake predicted better pain response to both diets. Less severe dysbiosis also predicted better constipation response to both diets. More severe psychological distress predicted worse bloating response to both diets. For the different outcomes, several differential predictors were identified, indicating that baseline factors could predict better improvement in one treatment arm, but worse improvement in the other treatment arm. (4) Conclusions: Psychological, nutritional, and microbial factors predict symptom improvement when following the low FODMAP and traditional IBS diet. Findings may help individualize dietary treatment in IBS.

Keywords: prediction; treatment response; gastrointestinal symptoms; irritable bowel syndrome; disorders of the gut brain interaction; functional gastrointestinal disorders; diet; low FODMAP diet; NICE guidelines; dysbiosis

1. Introduction

Irritable bowel syndrome (IBS) is a disorder of gut-brain interaction (DGBI), formerly known as a functional gastrointestinal (GI) disorder, which affects 4% of the adult global population [1]. According to the diagnostic Rome IV criteria, IBS is characterized by weekly abdominal pain associated with altered bowel habits [2]. Apart from abdominal pain, altered stool frequency, diarrhea, and constipation, a large proportion of patients with IBS also experience other GI symptoms such as abdominal distention, bloating and flatulence [3].

The pathophysiology of IBS is only partly understood and multifactorial. Pathophysiological factors that are described up until today include altered gut-brain interactions, visceral hypersensitivity [4], abnormal GI motility [5,6], psychosocial factors, altered mucosal immune function and permeability [7–9], altered gut microbial environment with potential dysbiosis (imbalance in the microbiota homeostasis) [10,11], and food hypersensitivity [12].

The majority of patients with IBS report an association between food intake and symptoms [13,14]. A subset of patients with meal-related symptoms therefore severely self-restricts and avoids certain foods [15]. This distinct subgroup of patients may have a more severe symptom pattern, poorer quality of life, lower caloric intake, and reduced intake of several nutrients.

Due to the renewed focus on this meal-related factor in IBS, the interest in dietary treatment has increased. Many dietary interventions, such as a gluten-free, a low lactose, and a high fiber diet, as well as many more, have been studied in IBS [16]. However, the clinical guidelines currently focus primarily on two dietary treatment approaches. A first-line diet approach in IBS is to follow the traditional IBS diet described in the guidelines of the British Dietetic Association and National Institute for Health and Care Excellence (NICE) [17,18]. These guidelines include general lifestyle and dietary advice. They encourage physical activity, regular meal intake, a reduction of fat intake, and to avoid large meals, excessive insoluble fiber intake, and a few dietary triggers. Alternatively, or when patients do not respond to the traditional IBS diet or an initial pharmacological treatment approach, patients can be referred to a specialized dietician. They can then recommend a diet that limits the intake of poorly absorbed short-chain carbohydrates, i.e., fermentable oligo-, di-, monosaccharides, and polyols (FODMAPs)—the low FODMAP diet. In patients with IBS, symptoms can be triggered by the ingestion of these short-chain carbohydrates that are incompletely absorbed in the small intestine. FODMAPs are osmotically active, interfere with water absorption, and when entering the colon are precursors to gas production through fermentation by intestinal bacteria. This could cause luminal distention and altered motility, which in turn may lead to symptoms [19]. Randomized controlled trials show that approximately 50–75% of patients with IBS experience adequate symptom relief when following a low FODMAP diet [20–22]. Unfortunately, this leaves 25–50% of the patients with IBS not responding. Therefore, determining potential predictors of who would potentially benefit from dietary treatment and who would not, could be of major help to individualize the treatment approach.

Previous research has identified several potential predictors of response to the low FODMAP diet. A lower FODMAP intake before starting diet, a higher age, being female, and having fructose malabsorption was associated with responding better to the intervention [22,23]. Higher hydrogen levels after a nutrient challenge test were also predictive of a better response [24]. Specific volatile organic compound patterns found in the faeces of patients with IBS can separate responders from non-responders [25]. High baseline colonic methane and short-chain fatty acid production, high baseline symptom severity, and a high saccharolytic fermentation activity have also been identified as predictors of response [26,27]. Furthermore, since gut microbiota are involved in fermentation of FODMAPs, studies also focused on the predictive capacity of baseline faecal microbiota. Low FODMAP diet responders were characterized by a microbiota composition more closely related to the composition of a healthy reference group [28]. Specifically, responders presented a lower abundance of *Clostridia*, *Actinomycetales*, *Anaerotruncus*, and *Escherichia*, and a greater abundance of *Bacteroides fragilis* and *acidifiens, Acinetobacter, Ruminiclostridium, Streptococcus, Eubacterium, Streptococcus, Dorea,* and *Ruminococcus gnavus* [27–30]. One study even identified a gut microbial signature in IBS patients with diarrhea and mixed bowel habits that shows enhanced clinical responsiveness to the low FODMAP diet. The microbiomes in these patients were depleted in *Bacteroidetes* species and enriched in *Firmicutes* and genes for amino acid and carbohydrate metabolism [30]. However, there are also studies indicating that microbial factors do not predict response to the low FODMAP diet [21,31]. Studies that investigate the prediction of response to the traditional IBS diet

are lacking. Apart from IBS-C patients and patients with a high saccharolytic fermentation activity being less likely to respond, no predictors of response to the traditional IBS diet have been described [23,27].

Most studies investigating predictors of treatment response dichotomized their outcome into responder vs. non-responder. However, treating the outcome as a continuous variable maximizes statistical power. Furthermore, response can also occur for one symptom, but not for the other. Therefore, our aim for this study was to investigate predictors of treatment response to the low FODMAP and the traditional IBS diet while focusing on the four core IBS symptoms, i.e., bloating, constipation, diarrhea, and pain as a post-hoc analysis of a previously published randomized controlled trial [23]. Hereby, treatment response was defined as the improvement of these symptoms during the intervention of four weeks. As potential predictors, we aimed to focus on different baseline factors that are of importance in the pathophysiology of IBS. We selected a psychological factor (i.e., psychological distress), non-GI somatic symptom severity, nutritional factors (i.e., energy, FODMAP, oligo-, di-, monosaccharide, and polyol intake), and microbial characteristics (i.e., an index that represents how severe the microbiota composition differs from the compositions of a healthy control sample). We studied psychological distress and non-GI somatic symptom severity, because the severity of both factors is closely related to more severe GI symptoms and may therefore influence the severity of symptom scores during the interventions [32]. Dietary variables were included because eating habits before the intervention might influence the dietary changes that the patients need to make during the intervention. Moreover, if patients are eating FODMAP restrictive prior to the intervention, their response could be dampened. Lastly, the microbial factor was added due to existing evidence that indicates that baseline feacal microbial composition together with fermentation activity influences the response to the dietary intervention [27–29].

2. Materials and Methods

2.1. Study Cohort

The study participants and general study methods that are not described here have been previously described in detail by Böhn et al. [23]. Briefly, we included patients fulfilling the Rome III criteria for IBS from the gastroenterology outpatient clinics in Gothenburg and Stockholm, Sweden. Patients with other GI diseases, such as coeliac disease or inflammatory bowel disease, severe liver, neurological, cardiac or psychiatric disease were excluded. Patients on excessively nutrient restrictive diets prior to the study were also not included. All patients provided written and verbal informed consent before the study start.

2.2. Study Design

During a 10-day screening period, patients collected a faecal sample, completed a 4-day food diary, and a daily stool diary based on the Bristol Stool Form (BSF) scale [33] that was used to subgroup patients according to bowel habits. After these ten days, patients completed several GI and non-GI symptom questionnaires including the IBS severity scoring system (IBS-SSS) [34]. If the patients scored $\geq$ 175 corresponding to moderate or severe IBS, they were randomized to the low FODMAP diet or the traditional IBS diet by an online software. The allocated diet was then thoroughly explained by trained dieticians and followed for four weeks. During the intervention, patients weekly completed the symptom severity measure, the Gastrointestinal Symptom Rating Scale-IBS (GSRS-IBS) [35].

2.3. Dietary Interventions

2.3.1. The Traditional IBS Diet

Patients randomized to the traditional IBS diet received instructions that were focused on 'how' and 'when' to eat. Patients needed to eat regularly, i.e., three meals and three snacks per day of adequate portion size. They needed to avoid feeling hungry or too full. Chewing thoroughly, eating in peace and quiet, and eating fibers (evenly distributed throughout the day) was encouraged. A few dietary products needed to be avoided or

reduced (fatty and spicy foods, coffee, alcohol, onions, cabbage, beans, soft drinks and carbonated beverages, chewing gums, and sweeteners that ends with "-ol").

2.3.2. The Low FODMAP Diet

Patients randomized to the low FODMAP diet were instructed to limit the intake of all FODMAP containing foods [36]. Patients received a study specific brochure with detailed information about foods to avoid. The intake of foods rich in fructan (e.g., barley, rye, wheat, onion, leak, and garlic), galacto-oligosaccharides (GOS) (e.g., legumes and nuts), lactose (e.g., milk, yoghurt, and ice cream), fructose in excess of glucose (e.g., apples, honey, and orange juice), and polyols (e.g., avocado, celery, and artificial sweeteners) needed to be restricted. The brochure also contained lists of alternative foods that were allowed (e.g., lactose-free milk, gluten-free pasta, carrots, kiwi, and strawberries).

2.4. Dietary Intake

Food intake was recorded in 4-day food diaries in the week before the randomization and in the last week of the intervention period (Wednesday to Saturday). All food products and drinks were entered in the nutrient calculation software DIETIST XP version 3.1 (Kostdata.se, Stockholm, Sweden) added with an extensive FODMAP database [37]. For this analysis, we used the average daily intake of energy, FODMAP, oligosaccharide (i.e., GOS and fructan), disaccharide (i.e., lactose), monosaccharide (i.e., excess fructose), and polyols from the 4-day food diaries before the randomization. FODMAP intake was calculated by adding up the content of GOS, fructan, lactose, polyol, and excess fructose in every meal for four days. An average intake per day for all the dietary data was computed, which was then used to obtain the average daily intakes for all four days.

2.5. Questionnaires

2.5.1. GI Symptoms

The primary outcome in this analysis was the improvement of four core IBS symptoms as measured by the GSRS-IBS, recorded retrospectively to reflect the last 7 days [35]. GSRS-IBS is a self-administered 13-items questionnaire with a 7-day recall period. Questions are scored on a 7-point Likert scale where one means 'no symptoms' and seven 'severe symptoms'. There are 5 GSRS-IBS subscales with their respective sum score ranges: pain (2–14), bloating (3–21), constipation (2–14), diarrhea (4–28), and satiety (2–14). In order have an identical score range for all subscales, the average scores were considered for all subscales in this analysis (score ranges all 1–7). The satiety subscale, which is not considered as a key IBS symptom, was not included in this analysis. The other validated subscales thus assess the severity of four symptoms pivotal in the clinical picture of IBS. Due to our specific interest in symptom-based responses to the dietary interventions, we choose the GSRS-IBS subscales as our primary outcome instead of the validated response definition of >50 points reduction on the IBS-SSS, as information on individual key IBS symptoms cannot be assessed with the same level of detail with IBS-SSS.

2.5.2. Non-GI Symptoms

Non-GI somatic symptoms were assessed with the Patient Health Questionnaire (PHQ)-12, which is a modified version of the PHQ-15 without three GI-related items [38]. The 12 items are scores on a scale from zero, 'not bothered at all', to two, 'bothered a lot'. Summing up all symptom scores created a total score (women: 0–24, men: 0–22) with a high score representing a high non-GI somatic symptom burden during the last two weeks. One item assesses menstrual cramps/other period problems, leading to potentially higher PHQ-12 scores in women.

Psychological distress, i.e., anxiety and depression, was measured using the 14-item Hospital Anxiety and Depression scale (HADS) that has a one-week recall period [39]. Response scores range from zero to three that are used to create a sum score (0–42) with high scores representing more severe psychological distress.

GI-specific anxiety was assessed with the Visceral Sensitivity Index (VSI) [40], a 15-item questionnaire that contains statements related to fear of GI symptoms. Each item can be scored from one, 'strongly agree', to six, 'strongly disagree'. High sum scores represent more severe GI-specific anxiety.

2.6. Gut Bacterial Analysis

Gut bacterial analysis was performed with the GA-map Dysbiosis Test (Genetic Analysis AS, Oslo, Norway) [41], which is a commercially available test that is described in more detail elsewhere [28]. In summary, the GA-map Dysbiosis Test created a bacterial profile and a Dysbiosis Index score ranging from one to five. A Dysbiosis Index score >2 indicates a microbiota composition that substantially differs from the composition of a healthy reference group and can thus be described as dysbiosis. In this analysis, we only used the Dysbiosis Index score as a potential predictor.

2.7. Data and Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 26 software (SPSS Inc., Chicago, IL, USA) and SAS 9.4 software (SAS Institute, Cary, NC) for the univariate and multivariate analyses, respectively. Summary descriptive statistics are presented as mean ± standard deviation (SD) for continuous variables and *n* (%) for categorical variables. Significance was set at $p < 0.05$. Before performing the multivariate analysis, the distribution of all variables was tested for normality using the Shapiro–Wilk test. Non-normal distributions were transformed using Box Cox or log transformations [42]. The GSRS-IBS subscales used in the linear mixed (i.e., random-effect) models were BoxCox transformed. For the transformation of the GSRS-IBS subscales, the following BoxCox formula were used for all GSRS-IBS subscales: $y(\lambda) = (y^{\lambda} - 1)/\lambda$. The following specific formulas were used for pain, constipation, diarrhea and bloating subscale: $y(1) = (y^{1} - 1)/1$, $y(0.75) = (y^{0.75} - 1)/0.75$, $y(0.25) = (y^{0.25} - 1)/0.25$, and $y(1) = (y^{1} - 1)/1$, respectively. This led to changes in the original score range score of 1–7 to 0–6, 0–3.29, 0–2.51, and 0–6, respectively. Variables unable to achieve normal distributions were ordinalized into tertiles (i.e., Dysbiosis index score).

To test our hypothesis that nutritional, microbial, and psychological factors could potentially predict symptom improvement when following a low FODMAP or traditional IBS diet, we used linear mixed (i.e., random-effect) models. Hereby, treatment response was not dichotomized, but GSRS-IBS subscale scores before, and during the intervention were used as continuous outcome variables. The models included the main effect of baseline predictors (testing the association between the predictor and GSRS-IBS score at the baseline timepoint) and the main effect of time (as a linear slope, one baseline timepoint and four during the intervention). Our main interest in this analysis was the included interaction effect between baseline predictor × time (testing the hypothesis that the baseline variable predicts the response slope). To test whether the baseline variables differentially predicted the response to the low FODMAP and traditional IBS diets, diet was added as a dichotomous variable to the models, including its main effect and all interactions, with the three-way baseline predictor × diet × time interaction effect testing this hypothesis.

3. Results

3.1. Clinical Characteristics

In total, 33 patients on low FODMAP diet and 34 patients on the traditional IBS diet were included in the analysis. Figure 1 provides further information about the included patients and which study measurements they completed. Demographic factors, distributions of IBS subtypes based on a BSF stool diary, baseline symptom severity, and baseline psychological, nutritional, and microbial factors of the two treatment arms are shown in Table 1.

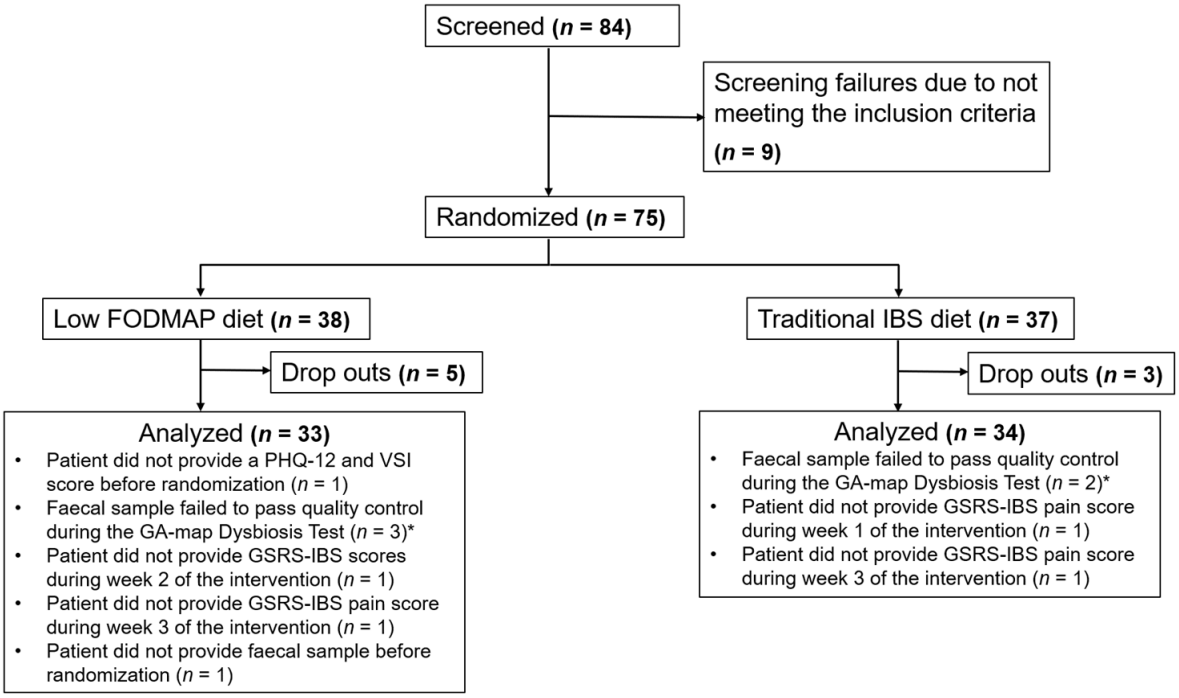

Figure 1. Flow chart indicating the number of patients included in each study phase, for details see text. Fermentable oligo-, di-, monosaccharides, and polyols, FODMAP; Patient Health Questionnaire-12, PHQ-12; Visceral Sensitivity Index, VSI; Gastrointestinal Symptom Rating Scale-Irritable Bowel Syndrome, GSRS-IBS.

3.2. Symptom Improvement during Dietary Interventions

In models with only the main effect of time as independent variable, both diets combined were shown to be effective at the group level for most symptoms. The dietary interventions significantly reduced the severity of pain, diarrhea, bloating (all $p < 0.0001$), but not of constipation ($p = 0.15$). Estimates can be found in Supplementary Material, Table S1. When diet was added to the models, the results indicated absence of significant differences in response between both dietary interventions for any of the symptoms (pain: $p = 0.80$, constipation: $p = 0.63$, diarrhea: $p = 0.96$, bloating: $p = 0.35$). The models including diet and its interaction with time are displayed in Supplementary Material, Table S2. This confirmed an earlier analysis on total IBS symptom severity assessed with the IBS-SSS by Böhn et al. [23].

Table 1. Clinical characteristics of the two treatment arms at baseline *.

Clinical Characteristic	Dietary Intervention	
	Low FODMAP Diet	**Traditional IBS Diet**
Gender (%) (*n* = 67)		
Female	85.8	83.6
Male	15.2	16.4
Age (years) (*n* = 67)	45 ± 15	40 ± 17
BMI (kg/m^2) (*n* = 65)	24.39 ± 3.93	24.07 ± 3.75
IBS subtypes (%) (*n* = 67)		
IBS-C	27.3	31.3
IBS-D	30.3	28.4
IBS-nonCnonD	42.4	40.3
IBS-SSS (score) (*n* = 67)	317 ± 70	306 ± 66
GSRS-IBS total (score) (*n* = 67)	3.91 ±0.95	3.64 ± 0.75
GSRS-IBS pain	4.05 ± 1.00	4.13 ± 0.93
GSRS-IBS bloating	4.98 ± 1.08	5.13 ± 1.38
GSRS-IBS constipation	3.03 ± 1.57	3.29 ± 1.65
GSRS-IBS diarrhea	3.41 ± 1.01	3.29 ± 1.12
PHQ-12 (score) (*n* = 66)	8.16 ± 3.89	7.47 ± 3.40
VSI (score) (*n* = 66)	49.13 ± 12.83	47.59 ±16.85
HADS total (score) (*n* = 67)	13.55 ± 7.39	10.74 ± 6.74
FODMAP intake (gram/day) (*n* = 67)	18.49 ± 9.90	18.49 ± 7.92
Oligosaccharide intake (gram/day)	2.72 ± 1.21	2.87 ± 1.42
Disaccharide intake (gram/day)	8.80 ± 8.33	8.47 ± 5.69
Monosaccharide intake (gram/day)	5.91 ± 5.42	6.03 ± 5.49
Polyols intake (gram/day)	1.07 ±1.56	1.12 ± 1.44
Energy intake (Kcal/day) (*n* = 67)	2097 ± 430	2100 ± 451
Dysbiosis index score (% 1/2/3/4/5) (*n* = 60)	10.3/20.7/48.3/10.3/10.3	6.7/16.7/53.3/13.3/10.0

* NOTE: Irritable bowel syndrome (IBS) with predominant constipation, IBS-C; IBS with predominant diarrhea, IBS-D; IBS-non-constipation, non-diarrhea, IBS-nonCnonD; IBS severity scoring system, IBS-SSS; gastrointestinal symptom rating scale for IBS, GSRS-IBS; patient health questionnaire-12, PHQ-12; visceral sensitivity index, VSI; hospital anxiety and depression scale, HADS; fermentable oligo-, di-, monosaccharides, and polyols, FODMAP.

3.3. Predictors of Pain Improvement

A higher energy intake significantly predicted better response to the low FODMAP and the traditional IBS diet when focusing on pain (time × energy intake interaction effect: $p = 0.03$), (Table 2). Figure 2a indicates that patients with a higher energy intake at baseline presented a significantly better pain improvement during the low FODMAP and the traditional IBS diet compared to patients with a lower energy intake. A lower Dysbiosis Index score (less severe dysbiosis) was associated with a better response to the diets (time × Dysbiosis Index (lowest vs. highest tertile) interaction effect: $p = 0.05$) (Table 2). Figure 2b shows the mean estimates over time of Dysbiosis Index scores grouped in tertiles. Patients with the lowest scores at baseline improved significantly more during the interventions compared to the tertile with the highest scores. When diet was included in the models, the intake of oligosaccharides was differentially associated with treatment response between both diets (time × oligosaccharide intake × diet interaction effect: $p = 0.05$) (Supplementary Material Table S3). This was driven by a significant association between higher baseline oligosaccharide intake and a better response to the traditional IBS diet, which was absent in the low FODMAP diet (time × oligosaccharide intake interaction effect in the low FODMAP diet and the traditional IBS diet: $p = 0.49$ and $p = 0.02$, respectively) (Figure 2c,d). All linear mixed models that contain the diet factor, including the models that did not identify significant differential predictors, are shown in Supplementary Material, Table S3.

Table 2. Mixed linear random-effect models without diet effects and GSRS-IBS subscales as outcome *.

	Pain			**Constipation**			**Diarrhea**			**Bloating**		
Independent variables: PHQ-12 and time	β	SE	*p*-value	β	SE	*p*-value	β	SE	*p*-value	β	SE	*p*-value
(intercept)	3.02	0.11	**<0.0001**	1.48	0.11	**<0.0001**	1.32	0.05	**<0.0001**	3.72	0.15	**<0.0001**
Time	−0.18	0.03	**<0.0001**	−0.03	0.02	0.18	−0.06	0.01	**<0.0001**	−0.30	0.04	**<0.0001**
PHQ-12	0.37	0.11	**0.001**	0.01	0.11	0.91	0.13	0.05	**0.01**	0.24	0.15	0.11
Time × PHQ-12	−0.01	0.03	0.67	0.01	0.02	0.60	−0.008	0.01	0.54	0.01	0.04	0.70
Independent variables: HADS and time	β	SE	*p*-value	β	SE	*p*-value	β	SE	*p*-value	β	SE	*p*-value
(intercept)	3.01	0.12	**<0.0001**	1.48	0.11	**<0.0001**	1.33	0.05	**<0.0001**	3.73	0.15	**<0.0001**
Time	−0.17	0.03	**<0.0001**	−0.03	0.02	0.15	−0.06	0.01	**<0.0001**	−0.30	0.04	**<0.0001**
HADS	0.03	0.12	0.80	−0.10	0.11	0.37	0.03	0.05	0.55	−0.16	0.15	0.28
Time × HADS	0.01	0.03	0.70	0.01	0.02	0.75	0.004	0.01	0.72	0.08	0.04	**0.03**
Independent variables: VSI and time	β	SE	*p*-value	β	SE	*p*-value	β	SE	*p*-value	β	SE	*p*-value
(intercept)	3.02	0.11	**<0.0001**	1.48	0.11	**<0.0001**	1.32	0.05	**<0.0001**	3.72	0.15	**<0.0001**
Time	−0.18	0.03	**<0.0001**	−0.03	0.02	0.18	−0.06	0.01	**<0.0001**	−0.30	0.04	**<0.0001**
VSI	0.29	0.11	**0.01**	0.17	0.11	0.11	0.14	0.05	**0.01**	0.11	0.15	0.47
Time × VSI	0.01	0.03	0.68	−0.001	0.02	0.97	−0.01	0.01	0.60	0.06	0.04	0.11
Independent variables: FODMAP intake and time	β	SE	*p*-value	β	SE	*p*-value	β	SE	*p*-value	β	SE	*p*-value
(intercept)	3.01	0.12	**<0.0001**	1.48	0.10	**<0.0001**	1.33	0.05	**<0.0001**	3.73	0.15	**<0.0001**
Time	−0.17	0.03	**<0.0001**	−0.03	0.02	0.15	−0.06	0.01	**<0.0001**	−0.30	0.04	**<0.0001**
FODMAP intake	0.02	0.12	0.83	0.18	0.10	0.09	0.05	0.05	0.33	−0.12	0.15	0.41
Time × FODMAP intake	0.01	0.03	0.80	0.01	0.02	0.71	−0.01	0.01	0.69	0.001	0.04	0.97

Table 2. *Cont.*

	Pain			**Constipation**			**Diarrhea**			**Bloating**		
Independent variables: Dysbiosis Index (DI) score (in tertiles) and time	β	SE	*p*-value	β	SE	*p*-value	β	SE	*p*-value	β	SE	*p*-value
Independent variables: Energy intake and time	β	SE	*p*-value	β	SE	*p*-value	β	SE	*p*-value	β	SE	*p*-value
(intercept)	3.01	0.12	**<0.0001**	1.48	0.11	**<0.0001**	1.33	0.05	**<0.0001**	3.73	0.14	**<0.0001**
Time	−0.17	0.03	**<0.0001**	−0.03	0.02	0.15	−0.06	0.01	**<0.0001**	−0.30	0.04	**<0.0001**
Energy intake	−0.10	0.12	0.39	0.01	0.11	0.94	−0.06	0.05	0.24	−0.34	0.14	**0.03**
Time × Energy intake	−0.07	0.03	**0.03**	−0.01	0.02	0.72	−0.02	0.01	0.20	0.01	0.04	0.81
(intercept)	2.80	0.25	**<0.0001**	1.35	0.23	**<0.0001**	1.42	0.11	**<0.0001**	3.72	0.32	**<0.0001**
Time	−0.08	0.07	0.24	0.01	0.05	**0.05**	−0.07	0.03	**0.01**	−0.25	0.08	**0.003**
DI score (lowest vs. highest ertile)	−0.03	0.32	0.92	−0.08	0.30	0.79	−0.25	0.15	0.09	−0.34	0.41	0.41
DI score (mid vs. highest tertile)	0.45	0.30	0.13	0.31	0.28	0.26	−0.02	0.13	0.87	0.25	0.39	0.51
DI score (lowest vs. mid tertile)	−0.49	0.26	0.06	−0.39	0.24	0.11	−0.22	0.12	0.06	−0.59	0.34	0.08
Time × DI score (lowest vs. highest tertile)	−0.17	0.09	**0.05**	−0.18	0.06	**0.01**	0.03	0.04	0.36	−0.06	0.10	0.59
Time × DI score (mid vs. highest tertile)	−0.08	0.08	0.31	−0.17	0.06	**0.01**	0.01	0.03	0.81	−0.05	0.10	0.62
Time × DI score (lowest vs. mid tertile)	−0.09	0.07	0.20	−0.01	0.05	0.81	0.03	0.03	0.40	−0.008	0.09	0.92
Independent variables: Oligosaccharide intake and time	β	SE	*p*-value	β	SE	*p*-value	β	SE	*p*-value	β	SE	*p*-value
(intercept)	3.34	0.22	**<0.0001**	1.50	0.20	**<0.0001**	1.29	0.10	**<0.0001**	4.00	0.28	**<0.0001**
Time	−0.12	0.06	0.06	−0.04	0.05	0.33	−0.04	0.02	0.11	−0.33	0.07	**<0.0001**
Oligosaccharide intake	−0.27	0.15	0.07	−0.02	0.14	0.91	0.04	0.07	0.60	−0.22	0.19	0.25
Time × Oligosaccharide intake	−0.05	0.04	0.25	−0.01	0.03	0.80	−0.01	0.02	0.42	0.03	0.05	0.53

Table 2. *Cont.*

	Pain			**Constipation**			**Diarrhea**			**Bloating**		
Independent variables: Polyols intake and time	β	SE	*p*-value	β	SE	*p*-value	β	SE	*p*-value	β	SE	*p*-value
(intercept)	3.07	0.14	**<0.0001**	1.50	0.13	**<0.0001**	1.39	0.06	**<0.0001**	3.67	0.19	**<0.0001**
Time	−0.17	0.04	**<0.0001**	−0.04	0.03	0.15	−0.06	0.02	**0.001**	−0.26	0.05	**<0.0001**
Polyols intake	0.05	0.08	0.47	0.02	0.07	0.79	0.05	0.04	0.16	−0.06	0.11	0.56
Time × Polyols intake	0.0001	0.02	>0.99	−0.01	0.02	0.61	0.001	0.01	0.93	0.03	0.02	0.21
Independent variables: Disaccharide intake and time	β	SE	*p*-value	β	SE	*p*-value	β	SE	*p*-value	β	SE	*p*-value
(intercept)	2.62	0.22	**<0.0001**	0.89	0.19	**<0.0001**	1.27	0.10	**<0.0001**	3.70	0.30	**<0.0001**
Time	−0.13	0.06	**0.04**	0.01	0.05	0.82	−0.05	0.03	0.07	−0.30	0.07	**0.0003**
Disaccharide intake	0.16	0.08	**0.05**	0.24	0.07	**0.001**	0.03	0.04	0.48	0.01	0.10	0.92
Time × Disaccharide intake	−0.02	0.02	0.45	−0.02	0.02	0.26	−0.004	0.001	0.68	−0.01	0.03	0.80
Independent variables: Monosaccharide intake and time	**β**	SE	*p*-value	β	SE	*p*-value	β	SE	*p*-value	β	SE	*p*-value
(intercept)	3.05	0.19	**<0.0001**	1.59	0.18	**<0.0001**	1.32	0.09	**<0.0001**	3.81	0.25	**<0.0001**
Time	−0.18	0.05	**0.001**	−0.07	0.04	0.08	−0.04	0.02	**0.03**	−0.25	0.06	**0.0001**
Monosaccharide intake	−0.04	0.11	0.75	−0.07	0.10	0.49	0.003	0.05	0.96	−0.06	0.14	0.67
Time × Monosaccharide intake	0.004	0.03	0.89	0.03	0.02	0.22	−0.01	0.01	0.64	−0.03	0.04	0.35

* NOTE: Gastrointestinal symptom rating scale for IBS, GSRS-IBS; patient health questionnaire-12, PHQ-12; visceral sensitivity index, VSI; hospital anxiety and depression scale, HADS; fermentable oligo-, di-, monosaccharides, and polyols, FODMAP; dysbiosis index, DI; effect size, β; standard error, SE; the GSRS-IBS subscales used in the linear mixed (i.e., random-effect) models were BoxCox transformed. The bold indicates the significant *p*-values in this large table.

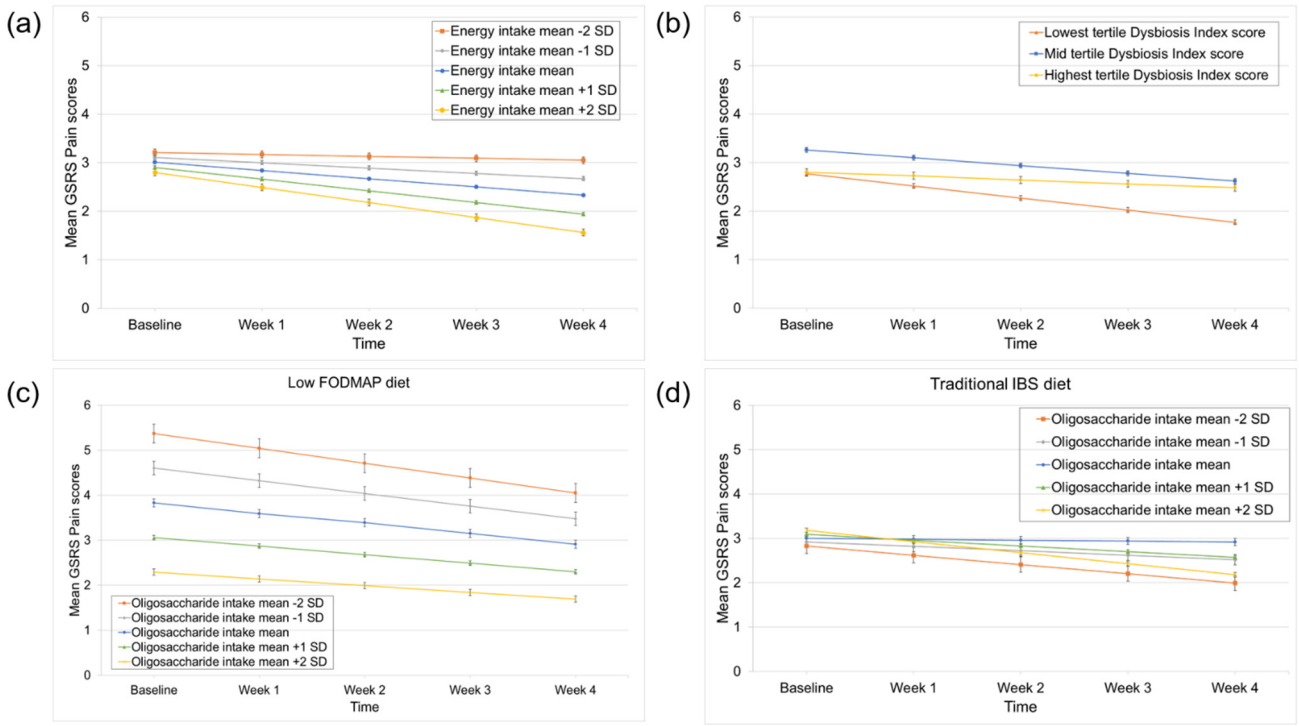

Figure 2. Predictors of pain improvement during the low FODMAP and traditional IBS diet. (**a**) Higher energy intake predicted better response to both diets. (**b**) Lower Dysbiosis Index scores predicted better response to both diets. (**c**) Oligosaccharide intake was a differential predictor with a non-significant association between higher baseline oligosaccharide intake and a worse response in low FODMAP treatment arm and (**d**) a significant association between higher baseline oligosaccharide intake and a better response in traditional IBS treatment arm. The GSRS-IBS subscales used in the linear mixed (i.e., random-effect) models were BoxCox transformed. For the transformation of the pain subscale, the following BoxCox formula was used: $y(\lambda) = \frac{y^{\lambda}-1}{\lambda}$. This led to changes in the original score range score of 1–7 to 0–6, using the formula: $y(1) = \frac{y^{1}-1}{1}$.

3.4. Predictors of Constipation and Diarrhea Improvement

When focusing on the improvement of constipation, a lower Dysbiosis Index score significantly predicted a better response to the dietary interventions (time × Dysbiosis Index (lowest vs. highest tertile) interaction effect: $p = 0.01$) (Table 2 and Figure 3a). When diet was added to the models, disaccharide (i.e., lactose) intake was identified as a differential predictor (time × disaccharide intake × diet interaction effect: $p = 0.02$), (Supplementary Material, Table S3). This was again driven by a higher baseline disaccharide intake associated with a better response to the traditional diet, which was absent in the low FODMAP diet (time × disaccharide intake interaction effect in the low FODMAP diet and the traditional IBS diet: $p = 0.46$ and $p = 0.01$, respectively), (Figure 3b,c). For diarrhea, no predictors of treatment response were identified (Table 2). All linear mixed models that contain the diet factor, including the models that did not identify significant differential predictors, are shown in Supplementary Material, Table S3.

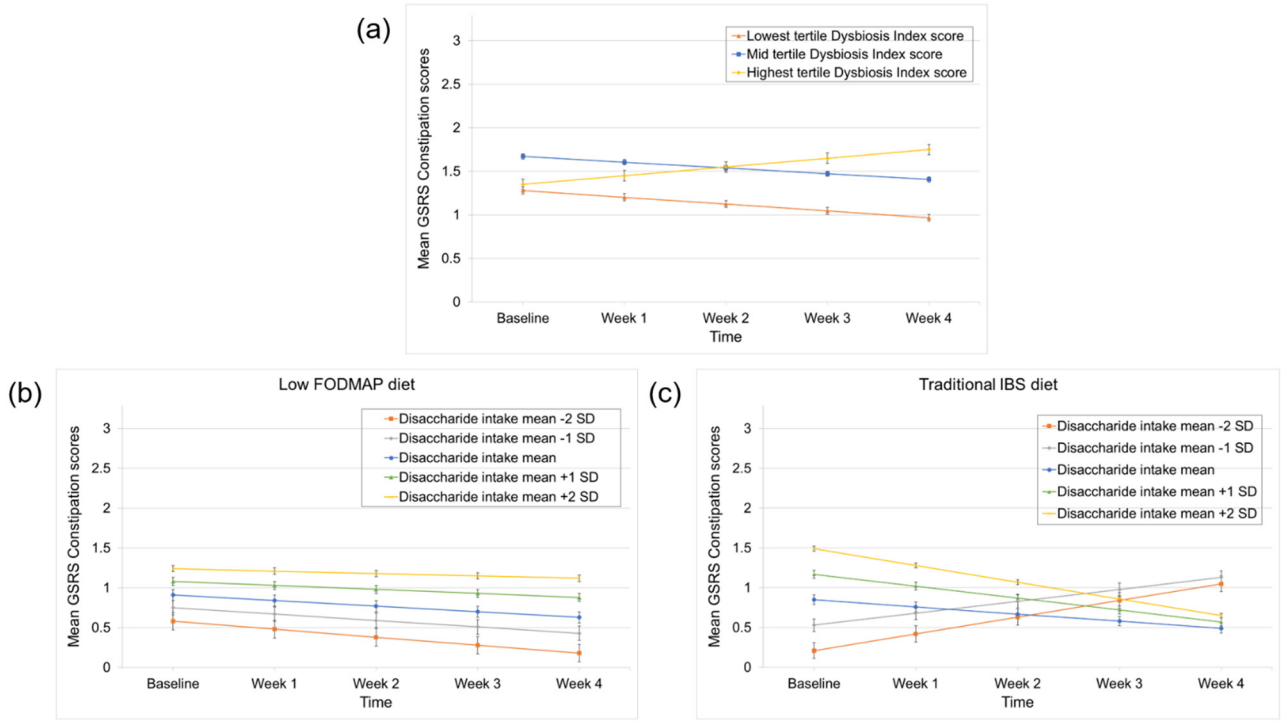

Figure 3. Predictors of constipation improvement during the low FODMAP and traditional IBS diet. (**a**) A lower Dysbiosis Index score predicted better response to both diets. (**b**) Disaccharide intake was a differential predictor with a non-significant association between higher baseline disaccharide intake and a worse response in low FODMAP treatment arm and (**c**) a significant association between higher baseline disaccharide intake and a better response in traditional IBS treatment arm. The GSRS-IBS subscales used in the linear mixed (i.e., random-effect) models were BoxCox transformed. For the transformation of the constipation subscale, the following BoxCox formula was used: $y(\lambda) = \frac{y^{\lambda}-1}{\lambda}$. This led to changes in the original score range score of 1–7 to 0–3.29, using the formula: $y(0.5) = \frac{y^{0.5}-1}{0.5}$.

3.5. Predictors of Bloating Improvement

More severe psychological distress significantly predicted worse response to the interventions (time × HADS interaction effect: $p = 0.03$) (Table 2 and Figure 4a). When diet was included in the models, oligosaccharide intake emerged as a differential predictor (time × oligosaccharide intake × diet interaction effect: $p = 0.005$), (Supplementary Material, Table S3). Higher baseline oligosaccharide intake predicted worse response to the low FODMAP diet, but not to the traditional IBS diet (time × oligosaccharide intake interaction effect in the low FODMAP diet and the traditional IBS diet: $p = 0.01$ and $p = 0.16$, respectively), (Figure 4b,c). All linear mixed models that contain the diet factor, including the models that did not identify significant differential predictors, are shown in Supplementary Material, Table S3.

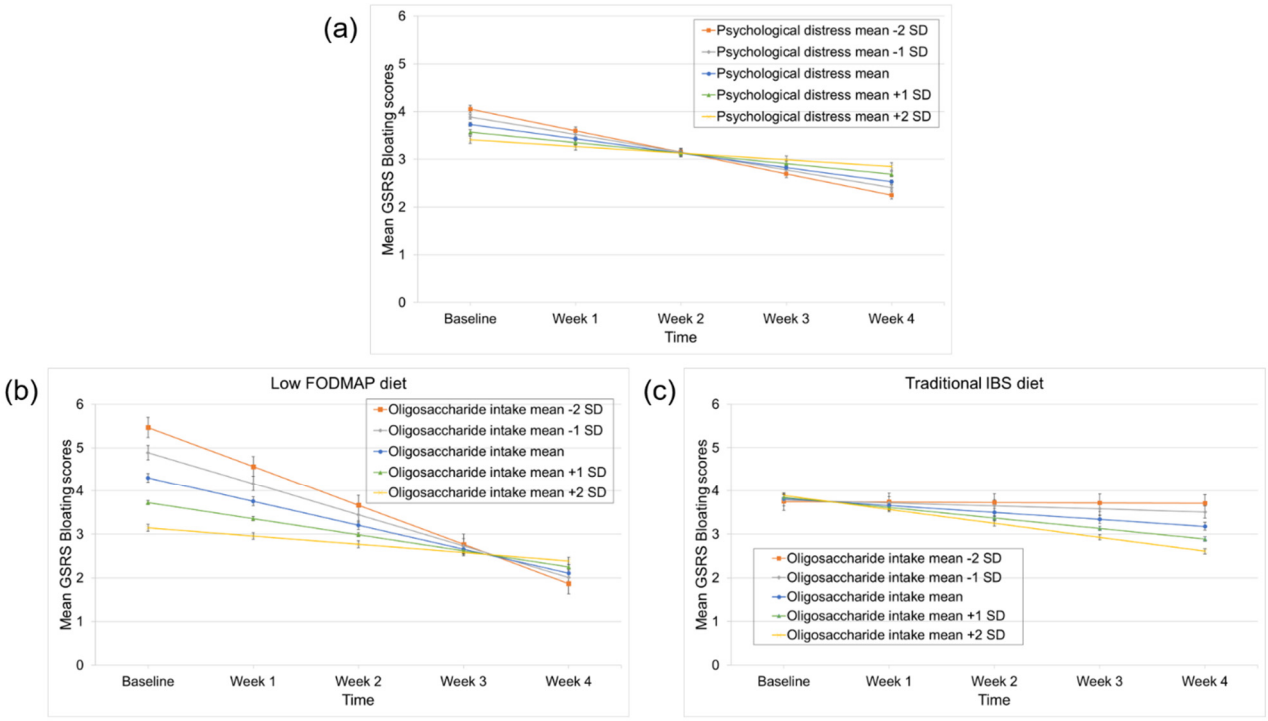

Figure 4. Predictors of bloating improvement during the low FODMAP and traditional IBS diet. (**a**) Higher psychological distress predicted worse response to both diets. (**b**) Oligosaccharide intake was a differential predictor with a significant association between higher baseline oligosaccharide intake and a worse response in low FODMAP treatment arm and (**c**) a non-significant association between higher baseline oligosaccharide intake and a better response in traditional IBS treatment arm. The GSRS-IBS subscales used in the linear mixed (i.e., random-effect) models were BoxCox transformed. For the transformation of the bloating subscale, the following BoxCox formula was used: $y(\lambda) = \frac{y^{\lambda}-1}{\lambda}$. This led to changes in the original score range score of 1–7 to 0–6, using the formula: $y(1) = \frac{y^{1}-1}{1}$.

4. Discussion

This study evaluated potential predictors of symptom response to the traditional IBS and the low FODMAP diet in patients with IBS. Instead of grouping participants in responders and non-responders, we used the improvement of four core IBS symptoms during the intervention weeks as our outcome. We showed that a microbial and dietary factor predicted better pain improvement due to the interventions. The same microbial factor predicted better constipation improvement when following both dietary regimes. We also identified a psychological factor that was associated with worse bloating improvement during the dietary interventions. Multiple dietary factors were also found to be differential predictors, meaning that they predicted either better or worse symptom improvement during one treatment, but not to the other.

When studies investigate treatment response predictors, a straightforward predictor that can be identified is the level of baseline symptom severity. A previous study found that patients who respond to the low FODMAP diet indicate higher baseline symptom severity scores when completing the IBS-SSS questionnaire [27]. However, in our sample, there were indications that patients with less severe clinical characteristics responded better to the treatments. Patients with a higher energy intake at baseline and a lower Dysbiosis Index score, i.e., a score more closely related to that of healthy controls, improved more than patients with a lower energy intake and a higher Dysbiosis Index score at baseline. A recent study showed that patients with a more severe symptom pattern, but still a normal BMI, have a lower energy intake possibly due to food avoidance and restriction [15]. Our findings suggest that patients who have a lower energy intake and who have a

microbial composition that substantially differs from that of healthy controls might need different treatment approaches. When Vervier et al. described a gut microbial signature that predicted a better response to a low FODMAP intervention, they indicated the opposite [30]. Microbiota profiles that resembled a more pathogenic phenotype had a greater reduction in IBS-SSS after the low FODMAP diet compared to patients with a profile more closely related to healthy controls. However, it is not clear whether the differences between our findings could be attributable to methodological differences. Our patient population (i.e., specific IBS subtypes vs. all subtypes), but also our primary outcome, and the way microbial factors were studied differed. Based on our results, the patients with a more severe disease might not have adequate symptom relief when they follow the traditional IBS or low FODMAP diet alone. They may potentially benefit from more elaborate treatment approaches potentially using different combinations of diet, peripherally and centrally acting pharmaceutical interventions and behavioral therapies.

Furthermore, patients with more severe psychological distress appear to respond worse to both diets. It has been shown that patients who demonstrate more severe psychological distress also report more severe GI symptoms in general [32]. Patients with more severe psychological distress, that could also partly be food-related, might be hypersensitive and potentially more skeptical towards modifying their diets. However, a survey including 256 patients with IBS indicated that most IBS patients (around 80%) prefer receiving pharmacological as well as dietary therapy [43]. Another possible explanation for the worse response in patients with more severe psychological distress could therefore again be that these patients might need combinations of pharmacological, behavioral, and other treatment options. Physicians should consider the potential food-related psychological distress that these patients might experience. When Peters et al. compared the effect of gut-directed hypnotherapy, the low FODMAP diet or a combination therapy, no significant differences in efficacy were found between the treatments [44]. However, in this study, there were no differences in the psychological measurements at baseline between the treatment arms. What they did report is that hypnotherapy resulted in superior improvements of the psychological measures. When patients thus report severe psychological symptoms, it can be hypothesized that the food-related psychological distress may need to be targeted first before making dietary changes in order to alleviate symptoms.

Our study also indicated that it is possible to identify baseline characteristics that are of importance for the response to one diet, but not for the other diet. These differential predictors included the intake of different FODMAP groups at baseline. Our findings might indicate that when it comes to dietary advice, we need to stick to the originally proposed treatment algorithm by recommending the traditional IBS diet first and only proceeding with the low FODMAP diet when this does not adequately relieve symptoms. In this study, patients who have higher baseline intakes of different FODMAP groups responded well to the traditional IBS diet. This observation was not present in the treatment arm that followed the low FODMAP diet. In fact, patients with higher oligosaccharide intake at baseline had a worse bloating response to the low FODMAP diet. Patients who have not identified FODMAPs as triggers and do not avoid and restrict them in daily life might not benefit from a diet as restrictive as the low FODMAP diet. For these patients following the traditional diet and applying lifestyle changes proposed by the British Dietetic Association and the NICE guidelines could already adequately relieve symptoms. Even if the low FODMAP diet shows efficacy in IBS, there are also negative consequences of this diet, such as deterioration of the Dysbiosis Index score, and reduction of potentially beneficial bacteria, such as *Bifidobacterium* [28,45,46]. Therefore, it is recommended to start with the less restrictive first-line therapy, i.e., the traditional IBS diet, and if this does not adequately relieve symptoms, only then proceed with the second-line treatment, i.e., the low FODMAP diet.

The key strength of our study is the novel approach to not categorize the patients in two groups, i.e., responders and non-responders, but to focus on individual core IBS symptoms. In order to develop suitable treatment algorithms, we do believe that considering the

heterogeneity of IBS symptom patterns is of importance. One patient might be considered a non-responder based on an inadequate reduction of the IBS-SSS total score, but the patient could have experienced substantial improvement of one specific bothersome IBS symptom. Furthermore, we preselected potential predictors from three different domains that are highly important in the pathophysiology of IBS, i.e., psychological, microbial and nutritional factors. Nevertheless, this study also has limitations including the use of a convenience sample. When designing the initial study, the calculation of the sample size was not based on this analysis. Additionally, we conducted this study with a limited number of patients appointed to two treatment groups. Moreover, preselecting potential predictors of importance was based on the limited number of studies that have already looked into predictors of treatment response together with the study data that were available.

Since the current treatment options in IBS remain symptom-based with efficacy levels that are often limited, developing predictive evidence-based treatment algorithms remains a priority for future research. Repeating this analysis in larger samples, powered for this approach, should be encouraged. Future studies should consider including potential predictors that could be easily measured in clinical practice and assess more treatment options than the ones we addressed. Investigators should preferably select a practical and effective treatment response measure that considers the heterogeneity of IBS symptom patterns.

In conclusion, this study shows that patterns of psychological, dietary, and microbial factors can predict IBS symptom response to two dietary advice systems—the traditional IBS and the low FODMAP diet. A clinical profile with less severe IBS features appears to predict a better symptom-specific response to both dietary interventions. These findings should be confirmed in larger prospective randomized controlled trials, preferably including different treatment approaches that have been shown to be efficacious in IBS. Results may help to optimize personalized treatment algorithms in IBS.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nu14020397/s1, Table S1: mixed linear random-effect models with time as an independent variable without covariates (diet) and GSRS-IBS subscales as outcome, Table S2: mixed linear random-effect models with time as an independent variable with covariates (diet) and GSRS-IBS subscales as outcome, Table S3: mixed linear random-effect models with all independent variables, covariates (diet) and GSRS-IBS subscales as outcome.

Author Contributions: Conceptualization, E.C., L.V.O. and M.S.; methodology, L.V.O. and E.C.; software, L.V.O.; validation, E.C., L.V.O., M.S. and S.N.; L.V.O. and E.C.; investigation, L.B., S.S. and S.B.; resources, M.S.; data curation, E.C. and S.N.; writing—original draft preparation, E.C.; writing—review and editing, all authors; visualization, E.C.; supervision, M.S., L.V.O., H.T., J.T., L.Ö.; project administration, M.S.; funding acquisition, M.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was registered at ClinicalTrials.gov with ID NCT02107625 (assessed on 8 April 2014). The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Regional Ethical Review Boards in Gothenburg and Stockholm, Sweden, Dnr: 619-13.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

Conflicts of Interest: S.S. has served as advisory board member for Takeda and speaker for Viatris. H.T. has served as advisory board member/consultant for Almirall and Shire. L.V.O. is assistant research professor of the K.U. Leuven Special Research Fund (Bijzonder Onderzoeksfonds, BOF). J.T. has given scientific advice to Adare, AlfaWassermann, Allergan, Arena, Bayer, Christian Hansen, Clasado, Danone, Devintec, Falk, Grünenthal, Ironwood, Janssen, Kiowa Kirin, Menarini, Mylan, Neurogastrx, Neutec, Novartis, Noventure, Nutricia, Shionogi, Shire, Takeda, Theravance, Tramedico, Truvion, Tsumura, Zealand, and Zeria Pharmaceutical; received research support from Shire, Sofar, and Tsumura; and served on the Speakers Bureau for Abbott, Allergan, AstraZeneca, Janssen, Kyowa Kirin, Menarini, Mylan, Novartis, Shire, Takeda, Truvion, and Zeria Pharmaceutical, all outside the submitted work. L.Ö. has received a financial support for research by Genetic Analysis AS, Biocodex, Danone Research and AstraZeneca and served as a consultant/advisory board member for Genetic Analysis AS, and as a speaker for Biocodex, Ferring Pharmaceuticals, Takeda, AbbVie, and Meda. M.S. has received grants and personal fees from Glycom and Danone Nutricia Research; personal fees from Ironwood, Menarini, Biocodex, Adnovate, Arena, Tillotts, Kyowa Kirin, Takeda, Alimentary Health, AlfaSigma, and the Falk Foundation; and grants from Genetic Analysis AS, outside the submitted work. The remaining authors disclose no conflicts. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. The authors declare no conflict of interest.

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ORIGINAL ARTICLE

WILEY

Gut microbiota profiles and the role of anti-CdtB and anti-vinculin antibodies in patients with functional gastrointestinal disorders (FGID)

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Abstract

Background: Distinct faecal microbiota profiles are reported to be associated with various subtypes of IBS. Circulating antibodies to cytolethal distending toxin B (CdtB) and vinculin are proposed as biomarkers to identify post-infectious IBS. The aim of our study was to analyse serum levels of anti-CdtB and anti-vinculin antibodies in patients with different functional gastrointestinal disorders (FGID) and their correlation with the composition of faecal microbiome.

Methods: The study cohort comprised 65 prospectively recruited individuals: 15 with diarrhoea-type-IBS (IBS-D), 13 with constipation-type-IBS (IBS-C), 15 with functional dyspepsia (FD) and 22 healthy controls. FGID subgroups were defined according to Rome III criteria. Serum levels of anti-CdtB and anti-vinculin antibodies were measured by ELISA. Faecal microbiome composition analysis and assessment of dysbiosis were performed by GA-map® Dysbiosis Test.

Results: Positivity rate either for anti-CdtB or anti-vinculin antibodies was higher in the IBS-C group (76.9%) compared to IBS-D (40.0%), FD (60%) and healthy (63.6%) groups.

Dysbiosis was more frequent in subjects positive for anti-CdtB antibodies and in IBS-C patients, who showed an increased amount of opportunistic/pro-inflammatory bacteria and reduced gut protective bacteria. IBS-C patients showed a high inter-individual variation of bacterial communities compared to other FGID subgroups and healthy individuals, whereas microbial profiles of patients with IBS-D and FD were overlapping with those of healthy controls. No bacteria markers showed significant differences between FGID subgroups and healthy controls.

Conclusion: Neither anti-CdtB/anti-vinculin antibodies nor faecal microbial profiles allowed to discriminate between specific FGID subgroups. Dysbiosis was more frequent in patients presenting with anti-CdtB antibodies and in IBS-C patients.

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KEYWORDS
cytolethal distending toxin/ vinculin, dysbiosis, faecal microbiota, functional dyspepsia, irritable bowel syndrome

1 | INTRODUCTION

Irritable bowel syndrome (IBS) and functional dyspepsia (FD) are two distinct but often overlapping gastrointestinal disorders (FGID), that affect up to 20% of the general population.[1] FGID lead to frequent medical consultations and diagnostic examinations with a significant burden on the healthcare system. Diagnosis of IBS and FD is based on the presence of characteristic symptoms, currently defined by the Rome criteria with exclusion of organic disease. Also, the selection of therapy is prioritized according to the leading symptom in FD and IBS as proposed in the Rome consensus.[2]

Many aspects in the aetiology and pathophysiology of IBS are still unclear and symptoms are only partially helpful in guiding the selection of the most appropriate therapy. Therefore, the availability of accurate biomarkers that allow to diagnose and categorize patients with FGID would be very helpful in clinical management. In this context, gut microbial signatures are a promising tool and have been reported to be altered (i.e., dysbiosis) in IBS patients when compared to healthy individuals.[3] Factors that trigger faecal microbial dysbiosis that need to be taken into account include dietary changes, stress, intake of antibiotics and previous infections.[4,5] Dysbiotic changes of microbial communities were shown in various subtypes of IBS. Past episodes of acute gastroenteritis leave persisting alterations of the gut microbiota and promote the development of long-lasting predominantly diarrhoea-type IBS (IBS-D).[6] Based on this notion, a serological tool, measuring circulating anti-CdtB and anti-vinculin antibodies, has been proposed for the discrimination of patients with IBS-D from patients with other conditions of diarrhoea, including inflammatory bowel disease (IBD) and celiac disease, as well as from healthy individuals.[7,8] The diagnostic contribution of cytolethal distending toxin (Cdt), a bacterial toxin produced by most common bacteria causing gastroenteritis, including *Clostridioides difficile*, *Campylobacter jejuni*, *Salmonella*, *Escherichia coli*, and *Shigella*, and vinculin, a host cell adhesion protein that cross-react with anti-CdtB antibodies, had originally been studied in animal models of post-infectious IBS (PI-IBS).[9,10] Also, dyspepsia originating as sequelae from previous gastrointestinal infections has been reported.[11] *Helicobacter pylori* may be considered as a model condition for post-infectious dyspepsia. Successful *H. pylori* eradication leads to relief of dyspeptic symptoms in a subset of patients,[12] while in some patients dyspeptic symptoms recur or persist even after cure of the infectious gastritis. This condition, referred as post-infectious functional dyspepsia, may be considered analogous to post-infectious IBS.

Aims of this explorative study were (a) to determine the prevalence in serum of anti-CdtB and anti-vinculin antibodies, (b) to determine the faecal microbiota structure and a previously validated Dysbiosis Index (DI), and (c) to correlate the serum antibodies with specific microbial signatures and the degree of abnormalities reflected in the DI in different patient subsets with Functional Gastrointestinal Disorders (FGID) including IBS subtypes and FD.

2 | MATERIAL AND METHODS

2.1 | Subjects and specimen collection

Patients with different FGID and healthy controls were prospectively recruited at Magdeburg University Hospital from October 2015 to March 2017. The study cohort comprised a total of 65 individuals including 15 patients with IBS-D, 13 patients with IBS-C, 15 patients with FD and 22 healthy controls. Subjects were recruited within the EMGASTA project (DRKS-ID: DRKS00009737), a large-scale prospective study focussed on research into gut microbiota profiles in healthy individuals and in patients with functional GI diseases. The study was approved by the local ethics committee and government authorities and was conducted in accordance with the current Good Clinical Practice guidelines and the Declaration of Helsinki.[13] All recruited subjects provided their written informed consent to participate in the study.

Patients with IBS were diagnosed according to Rome III criteria after an accurate anamnesis and clinical evaluation.[14] Healthy controls did not report any GI disease, tumour disease, severe metabolic or cardiovascular disease requiring therapy, or neurodegenerative disease. Study participants must not have any antibiotic intake in the 8 weeks prior to inclusion. From each study, participant blood samples for routine laboratory tests and serological analysis were collected. A subset of participants provided also faecal samples for microbiome analysis. This subgroup included 13 patients with IBS-D, 11 with IBS-C, 14 with FD and 15 healthy controls. Faecal samples were collected with a sterile stool sample collection kit (PT26.1; Carl Roth, Karlsruhe, Germany) and kept in the refrigerator at 4°C (for a maximum of 8 h) until transferred to the laboratory. After aliquoting, faecal samples were stored at −80°C until further analysis. All patients with dyspepsia underwent esophagogastroduodenoscopy (EGD) for further

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clinical evaluation and diagnosis of structural disorders. In those patients, a standard histopathologic assessment was performed according to the updated Sydney System. Testing of dyspeptic patients for *H. pylori* infection was performed by histology, serology and stool antigen test, as previously described.[13] Concordant negative results in all tests except for *H. pylori* serology were required to exclude *H. pylori* infection.

2.2 | ELISA analysis

Blood samples were obtained from all study participants by venipuncture into a blood collection tube (BD Vacutainer RST, BD, Franklin Lakes, NJ, USA) and then centrifuged at 3050 rpm for 10 min and stored after aliquoting at −80°C. Anti-CdtB and anti-vinculin antibody levels in plasma were measured by a second-generation Enzyme-linked immunosorbent assays (ELISA) as previously described (see also Supplementary Material). Based on previous literature, the cut-offs for positivity were set at OD values of ≥ 1.68 for anti-vinculin and ≥ 2.80 for anti-CdtB antibodies, respectively.[7]

2.3 | Microbiome analysis

To assess gut microbiota composition, we used GA-map® Dysbiosis Test Lx (Genetic Analysis), which is a targeted approach to evaluate gut microbiota profiles.[15] In brief, the test utilizes faecal homogenization, mechanical and enzymatic bacterial cell disruption to isolate and bind total bacterial genomic DNA to magnetic beads. Then, 16S rRNA hypervariable regions V3-V9 are amplified by polymerase chain reaction (PCR). Bacterial DNA labelling is done by single nucleotide extension and hybridized to a complementary DNA strand coupled to beads. Finally, the abundance of bacteria is assessed by the strength of fluorescent signal (probe intensity), detected and measured by a Luminex® L×200™ instrument (Luminex Corporation). In total, the relative abundance of 48 bacteria markers targeting more than 300 bacteria species is measured. The test also algorithmically assesses faecal bacterial abundance and profile in comparison with a reference healthy population. A deviation in the microbiome from normobiosis is summarized in a Dysbiosis Index (DI) 1 to 5. DI of 1–2 indicates normobiosis, 3 indicates mild dysbiosis, and 4–5 indicates severe dysbiosis. In addition, abundance scores within the range of −3 to +3 (where 0 indicates within the normal range) for all 48 bacteria markers are assigned for each sample analysed (see Table S1). Principal component analysis (PCA) was used to explore the variation in microbiota between the groups. The analysis was performed on scaled and log-transformed GA-map® signal strength data in R using the Stats package. Additional information on the microbiome data analysis is provided in Supplementary Material.

2.4 | Statistical analyses

Descriptive statistical analysis was performed using IBM SPSS Statistics 21.0.0 (IBM Corporation) and GraphPad Prism 7. Numerical variables were expressed by mean values ±standard deviation. Chi-square test was used for comparison of categorical data. Shapiro-Wilk test was used to assess the normality of the data. To evaluate statistical differences between two or multiple groups, Mann-Whitney U tests or Kruskal-Wallis tests were performed for non-normally distributed data, respectively, while normally distributed variables were compared across more than two groups by one-way ANOVA and Dunnett's post hoc tests. All tests were carried out two-sided with a level of significance set to 0.05.

Reporting of the study conforms to broad EQUATOR guidelines (Simera et al. January 2010 issue of EJCI).[16]

3 | RESULTS

3.1 | Characteristics of the study cohort

Four patients with dyspepsia were tested *H. pylori*-positive and showed in EGD mild to moderate signs of chronic gastritis without severe structural alterations (i.e., ulcer). No statistically significant differences were found in age distribution and gender proportions between groups. The characteristics of the study population are shown in Table 1.

TABLE 1 Characteristics of the study population. Mean and standard deviation (in brackets) are shown. Anti-CdtB and anti-vinculin antibodies titres are indicated by optical densities (OD)

	Healthy controls ($n = 22$)	IBS-D ($n = 15$)	IBS-C ($n = 13$)	FD ($n = 15$)	p
Age	54.8 (11.2)	43.5 (20.6)	50.6 (18.2)	48.7 (17.6)	ns
Male/Female	10 / 12	5 / 10	3 / 10	7 / 8	ns
Anti-CdtB antibodies (OD)	2.53 (0.74)	2.35 (0.81)	2.72 (0.71)	2.62 (0.66)	ns
Anti-vinculin antibodies (OD)	1.60 (1.01)	0.86 (0.85)	1.26 (0.70)	1.30 (0.84)	ns

Note: p values were calculated with the Kruskal-Wallis test. 'ns' denotes no significant difference. Abbreviations are defined in Materials and Methods.

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3.2 | Comparison of circulating antibody titres among groups

The highest mean values of circulating anti-CdtB antibodies titres indicated by optical density (OD) were found in patients with IBS-C (2.72 ± 0.71), followed by patients with FD (2.62 ± 0.66), healthy subjects (2.53 ± 0.74) and patients with IBS-D (2.35 ± 0.81). Anti-vinculin antibodies were higher in healthy individuals (1.60 ± 1.01) and slightly reduced in patients with IBS-D (0.86 ± 0.85), while patients with FD and IBS-C showed similar levels (1.30 ± 0.84 and 1.26 ± 0.70, respectively, Table 1). No significant changes in the mean levels of measured biomarker were found between FGID groups and healthy controls, both for anti-CdtB and for anti-vinculin antibodies ($p = .598$ and $p = .076$, respectively, Figure 1).

According to the cut-off values proposed by Pimentel et al.,[7] anti-CdtB positivity rates were higher in the IBS-C group (53.8%) compared to IBS-D group (33.3%), FD group (40.0%) and healthy controls (40.9%), Figure 2. Individuals positive for anti-vinculin antibodies were found less frequently among patients with IBS-D (13.3%) compared to patients with IBS-C (30.8%), patients with FD (33.3%) and healthy subjects (36.4%). However, no significant differences were observed comparing the positivity rates between the four groups neither for anti-CdtB ($p = .742$) nor for anti-vinculin antibodies ($p = .473$). The IBS-C group showed the highest positivity rate for at least one biomarker with 76.9% of patients being positive either for anti-CdtB or anti-vinculin antibodies (or both), followed by healthy subjects (63.6%), patients with FD (60.0%) and patients with IBS-D (40.0%). Also, this parameter was not statistically different between groups ($p = .243$), Figure 2.

3.3 | Microbiota composition and Dysbiosis Index in patients with different subtypes of FGID and healthy controls

According to the GA-map® Dysbiosis Test Lx, the majority of patients with IBS-C showed a tendentially higher degree of dysbiosis (81.1% of patients with DI >2 vs. 18.9% of patients with DI = 1–2: 18.9%, $p = .068$), whereas the IBS-D, FD and healthy controls groups had lower rates of subjects with dysbiosis (46.2%, 57.1% and 46.7% with DI >2, respectively), Figure 3 & Table S1.

Principal component analysis (PCA) was used to visualize variation in signal data from GA-map® test. In general, we observed a marked inter-individual variation of bacterial communities' composition. Microbial structures from patients with IBS-D and FD were similar to those from healthy controls and covered a mostly overlapping area (Figure 4). No significant differences were observed between dyspeptic patients positive for *H. pylori* and negative ones, neither in faecal microbiota composition nor with regards to DI.

Faecal samples from patients with IBS-C showed a different profile with a larger internal variation and a distinct distribution compared to the other groups. No bacterial markers were found to display significant differences (padj <0.05) between healthy individuals and FGID subgroups. However, Proteobacteria, *Ruminococcus albus & Ruminococcus bromii* and *Phascolarctobacterium sp*. were found to be more abundant individuals with high DI score and in those with antibody positivity.

The OPLS-DA model was used to define differences in faecal bacterial communities between the groups. According to multivariate factor discriminant analysis, the microbiota composition could not discriminate between healthy subjects and IBS or FD patients, as demonstrated by poor fitness ($R^2Y < 0.5$) and limited discriminant predictive ability ($Q^2 < 0.5$) of the model. Notably, whilst the limited fitness of the OPLS-DA, the IBS-C group showed the highest Q^2 value (−0.375) compared to the other groups (Figure S1).

Functional bacterial profile analysis revealed that the majority of IBS-C patients showed a higher degree of imbalance in all the five profiles compared to other FGID groups. In IBS-C patients and in subjects with severe dysbiosis, the abundance of essential butyrate-producing, and gut mucosa protective bacteria were reduced, while pro-inflammatory and opportunistic bacteria abundance were found in increased abundance.

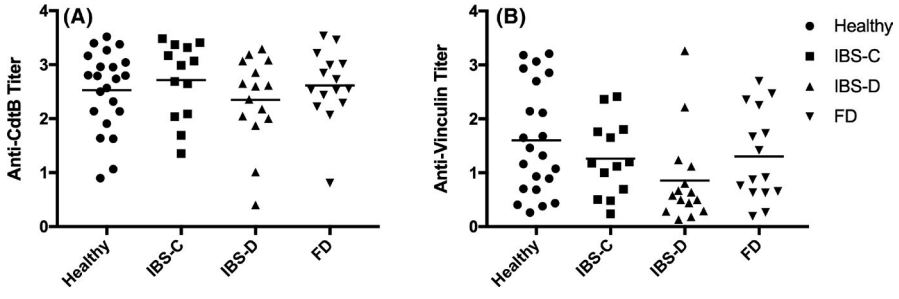

FIGURE 1 Anti-CdtB (A) and anti-vinculin (B) antibodies titres in patients with IBS-D ($n = 15$), patients with IBS-C ($n = 13$), patients with FD ($n = 15$) and in healthy controls ($n = 22$). Data are shown as optical densities (OD) in scatter plots where the horizontal line marks the mean value

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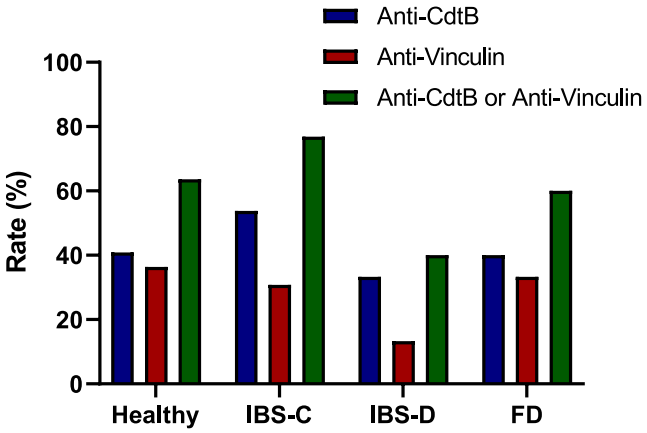

FIGURE 2 Comparison of antibody positivity rates (anti-CdtB, anti-vinculin, anti-CdtB OR anti-vinculin) among the study groups. Comparisons of categorical variables between groups were performed by Pearson's chi-square test. No significant differences were observed in inter-groups comparisons

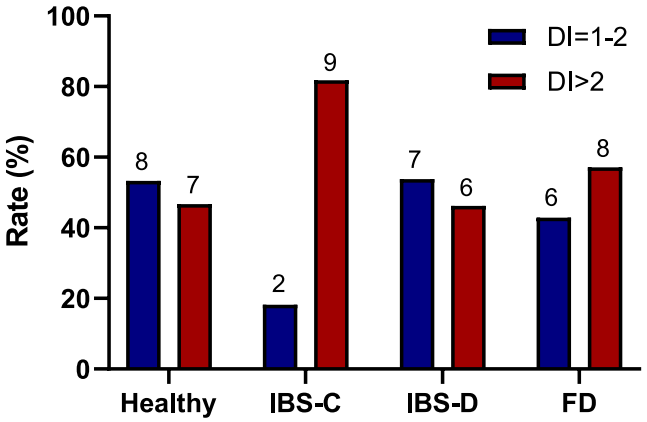

FIGURE 3 Distribution of the Dysbiosis Index (DI) among patients with IBS-C ($n = 11$), patients with IBS-D ($n = 13$), patients with FD ($n = 14$) and in healthy controls ($n = 15$). Dysbiosis was evaluated in faecal samples by the GA-map® Dysbiosis Test. DI is scored between 1 and 5, where a score of 1 and 2 signifies normobiosis (blue) and 3–5 dysbiosis of increasing severity (red). Digits above the columns represent the number of subjects within each column. Comparisons of categorical variables between groups were performed by Pearson's chi-square test. IBS-C patients showed more frequently higher DI ($p = .068$) compared to controls. No other significant differences were observed in inter-groups comparisons

3.4 | Microbiota changes in dysbiotic patients with FIGD positive for anti-CdtB or anti-vinculin antibodies

Dysbiosis was tendentially more common in individuals who were positive for at least one biomarker between anti-CdtB or anti-vinculin antibodies (63% showed DI >2) or for both biomarkers (100% of these subjects presented with DI >2) compared to subjects negative for both antibodies (48% with DI >2, $p = .053$, Figure S2). In the subgroup analysis, a significantly higher proportion of individuals with moderate/severe dysbiosis was observed in patients with high levels of anti-CdtB antibodies compared to subjects negative for anti-CdtB (72.7% vs. 45.1%, $p = .046$), suggesting that the presence of anti-CdtB antibodies may be associated with a more dysbiotic microbiota profile. No significant association was detected between presence of dysbiosis and positivity to anti-vinculin antibodies (Figure 5).

The faecal microbiota composition of individuals positive for anti-CdtB or anti-vinculin antibodies showed a larger variation compared to individuals negative for both antibodies. There was a substantial grade of overlap in the PCA between microbiota profiles of antibody-positive and those of antibody-negative subjects (Figure 6).

The OPLS-DA model could not discriminate the group of antibody positives from that of antibody-negative subject ($R^2Y = .199$, $Q^2 = -0.146$).

However, Proteobacteria, *Ruminococcus albus & R. bromii* and *Phascolarctobacterium* sp. appeared to have a higher effect on the DI score for antibody-positive subjects with high DI (DI >4) than for the remaining subjects. This effect is explained by the DI algorithm, in which the bacteria markers influence differently, in combination with measured signal strength.

4 | DISCUSSION

Gut microbiota is suggested to be a contributing factor to the pathophysiology of FGID and presents with distinct profiles in different subforms of IBS.[17] One such subform is post-infectious IBS which is well documented in a subset of patients following gastrointestinal infections.[18] The assessment and characterization of FGID relies on the patient's history, exclusion of organic pathologies and definition of symptom clusters.[19] No specific biomarkers and specific faecal microbiota profiles to objectively confirm the diagnosis of FGID are available for clinical use at present.

For the purpose of progressing in this field, we assessed the composition of faecal microbiota in patients with FGID and in healthy controls combined with the determination of plasma levels of 2 selected biomarkers. The most notable finding in our study was that severe dysbiosis was most prevalent in patients with IBS-C. Severe dysbiosis was also more frequently detected in individuals positive for circulating anti-CdtB antibodies compared to other groups. However, a discrimination between different subgroups of FGID based on the positivity for anti-CdtB/anti-vinculin antibodies or on the Dysbiosis Index (DI) was not possible.

In our cohort, we were not able to discriminate patients with IBS and different IBS subtypes from healthy controls by the determination of circulating anti-CdtB and anti-vinculin

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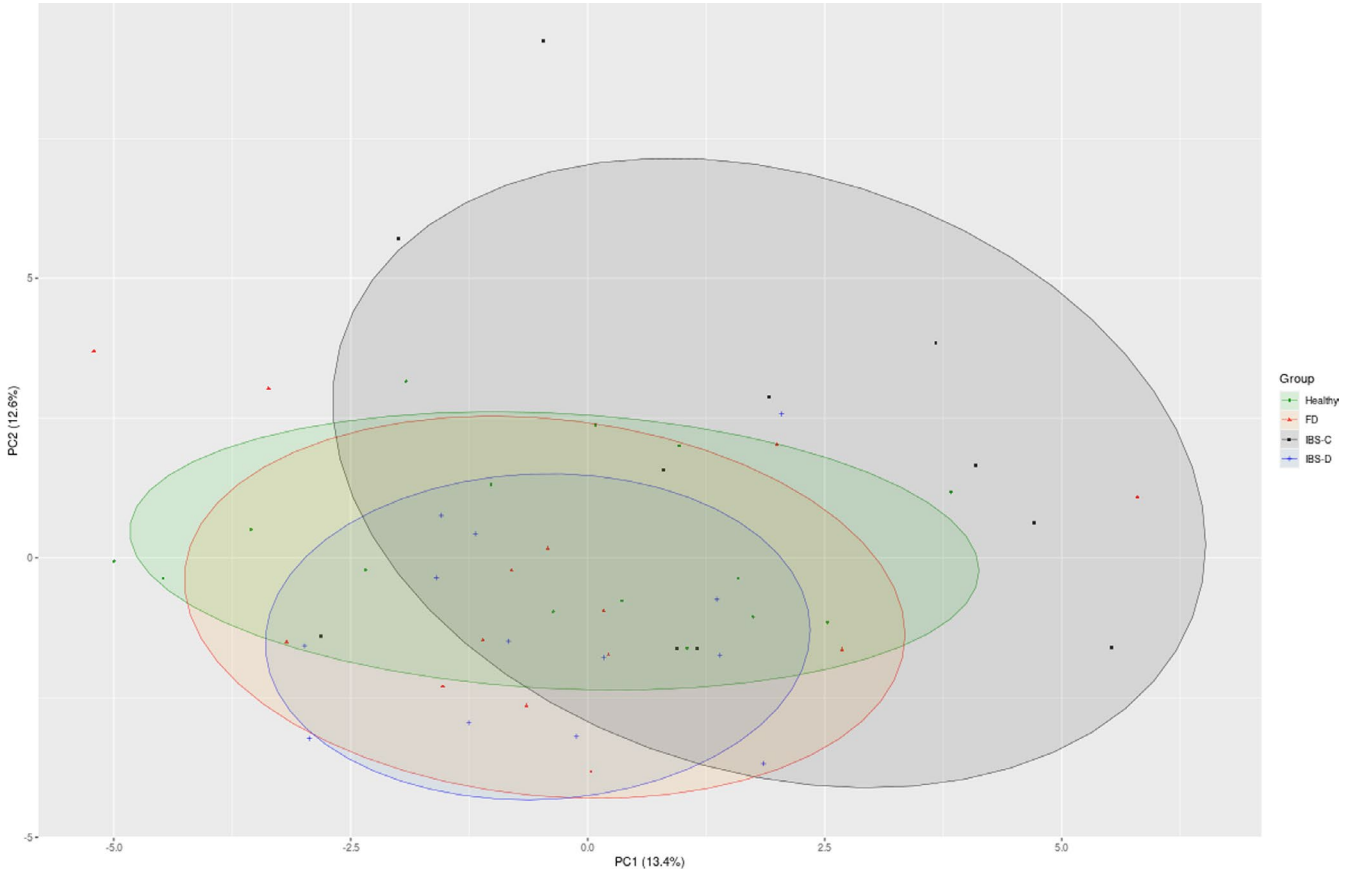

FIGURE 4 PCA score plot showing the differences in global bacterial community structures between the groups (IBS-C ($n = 11$), IBS-D ($n = 13$), FD ($n = 14$) and healthy controls ($n = 15$)). The ellipses cover approximately 80% of the scores for each group

antibodies. This is in contrast with a previous study of Pimentel et al.,[7] that reported increased plasma levels of these antibodies in IBS-D patients compared to IBD patients and healthy subjects. A further study by Rezaie et al.[8] described a gradual increase in the prevalence of anti-CdtB and anti-vinculin positivity rates according to IBS subtypes, being highest in patients with diarrhoea-predominant IBS (IBS-D and IBS-M) and lowest in subjects with constipation (IBS-C) and healthy controls. The most likely explanation for this discrepancy is the selection of our patient cohort in Germany, differing in environment, lifestyle, nutritional habits and 'microbial world' compared to the previously studied patient populations. Gut microbiota structures vary depending on geographic location of the host and on the related differences of environmental factors.[20] In some of previous studies with a positive association of the serological antibacterial antibodies, the group of patients with IBS-D was overrepresented compared to other IBS groups and healthy controls (i.e., 2375 patients in the IBS-D group vs. 25 patients with IBS-M, 30 patients with IBS-C and 43 healthy controls[8]).

A study from Australia reported findings similar to ours.[21] Using two cohorts, one community population-based ($n = 331$) and one outpatient clinic-based ($n = 460$) Talley et al. found no significant differences in levels of both anti-CdtB and anti-vinculin biomarkers between IBS patients and healthy controls, between IBS-D and IBS-C individuals and between IBS and FD groups. Interestingly, authors of that study found that patients with FD and IBS/FD overlap presented with higher levels of anti-CdtB compared to healthy subjects. To further investigate the possible association of anti-CdtB with post-infectious, FD we extended our analysis to a group of patients presenting with dyspepsia. However, we did not detect increased anti-CdtB antibodies levels nor did we find bacterial profiles of patients with FD that were distinct from those of healthy controls.

Analysis of changes in the composition and function of the gut microbiota in patients with IBS compared to normal healthy subjects leave us with inconsistent results. A meta-analysis on 24 studies published up till 2018 highlighted the lack of consistency between findings and the relevant differences in methodologies used for microbiota analysis.[22] Most frequent findings considered being of relevance in IBS were pointing out dysbiotic changes consisting in decreased biodiversity of microbial communities,[23–27] reduction in relative abundancies of uncultured Clostridiales,[28-30] *Faecalibacterium*[23,30] and *Bifidobacterium*[24,29,31] and increase in relative abundancies of potentially harmful bacteria, such as Proteobacteria,[23,30] Enterobacteriaceae (e.g.,

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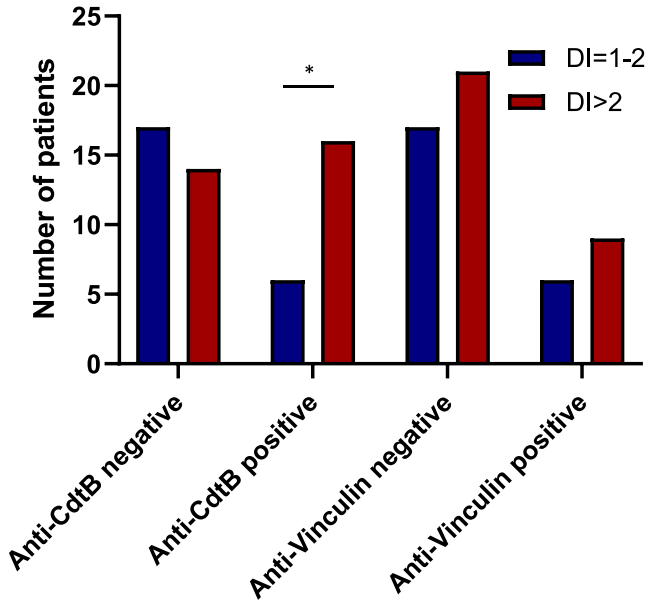

FIGURE 5 Distribution of the Dysbiosis Index (DI) among individuals according to serological status of anti-CdtB and anti-vinculin antibodies. Antibody positivity was defined according to the cut-off values proposed by Pimentel et al.[6] * $p < .05$

Escherichia, *Shigella*, *Campylobacter* and *Salmonella*),[23,32] *Streptococcus*[23,33] and *Bacteroides*[24,31,34] in patients with IBS compared to healthy individuals. None of these previous studies could, however, distinguish different IBS subtypes from healthy controls on the ground of specific microbial signatures both in mucosal and in luminal samples.[22,35] In our study, there was great overlap among IBS groups and healthy controls. Even moderate/severe dysbiosis (DI >2) were found with similar prevalence in both patients and healthy subjects. Only patients with IBS-C showed a trend for higher scores of DI compared to controls, even though this difference did not reach statistical significance, likely due to the small size of the cohort. These findings are in contrast with previous reports, where dysbiosis frequency in IBS cases was around 73% and 16% in healthy subjects,[15] and might be explained by the different inclusion criteria adopted and by the different geographical locations. Dysbiosis Index as general tool for the diagnosis of FGID cannot find clinical application unless regional, cultural, dietetic and lifestyle factors are taken into consideration. This suggests the need for local validation of DI for defined populations.

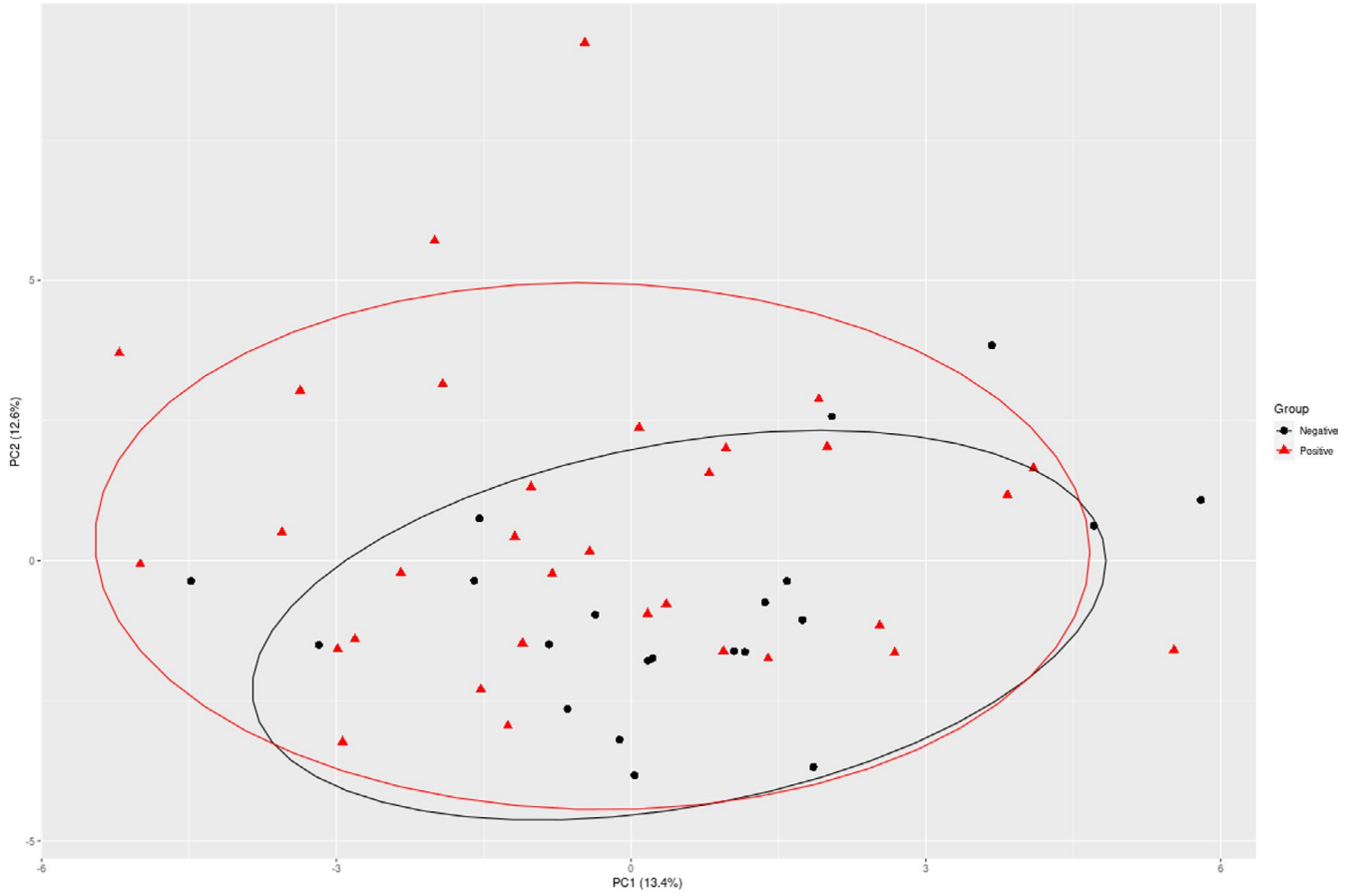

FIGURE 6 PCA score plot showing the differences in global bacterial community structures between individuals positive for anti-CdtB or anti-vinculin antibodies (Ab-positive, $n = 32$) and individuals negative for both antibodies (Ab-negative, $n = 21$)

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This is the first study that analysed the microbial profiles of patients with FD and subforms of IBS in their correlation with specific microbial antibodies serum markers. In patients positive for anti-CdtB antibodies, severe dysbiosis was more frequent and bacterial profiles were characterized by impaired functions including essential butyrate production (*Eubacterium* spp., *Faecalibacterium prausnitzii*) and gut mucosa protection (*Akkermansia muciniphila*, *Faecalibacterium prausnitzii*). Similar alterations were reported from other authors.[22] This also goes along with a recent meta-analysis including 15 studies that reports reduction in butyrate and propionate levels in patients with IBS-C.[36] The role of SCFAs in gut barrier protection and gastrointestinal motility is well documented and has important implications with the pathogenesis of IBS.[37] Our results indicate that functional bacterial profiles leading to severe DI were associated with anti-CdtB antibodies and characterized by an increase in relative abundancies of pro-inflammatory and opportunistic bacteria (e.g.,. *Salmonella*, *Escherichia coli*, and *Shigella*). Several of these bacteria are able to produce CdtB and considered to possess a possible trigger function in post-infectious-IBS.[6,38]

There is an important limitation of our study due to the small number of patients in each cohort, which, with the further reduction of numbers for stratification of patients, leads to an underpowered statistical analysis. The small number of IBS cases in our exploratory study, compared with the large cohorts in two previous studies,[7,8] carries the risk of a potential sampling bias. This, independently of the geographical location where our study was carried out, and of similar findings to ours reported from another world region,[21] does not allow to draw definitive conclusions on the role of anti-CdtB and anti-vinculin as biomarkers in IBS. Nevertheless, even though patient numbers were low, our findings indicate high inter-individual variations in each subgroup with IBS. This indication suggests that patterns of microbial signatures, combined with specific antibacterial antibodies in plasma as used in our approach, are not significantly contributing to the allocation of the individual patient to a subgroup of FGID.

Large-scale prospective studies will help to better define the role of dysbiosis following episodes of gastroenteritis in the pathogenesis of FGID. Future studies may also benefit from extending the analysis to other members of the gut microbiota, such as fungi and bacteriophages, that may also play an important role in FGID pathogenesis, as recent evidences have shown.[39,40]

In conclusion, we found a high inter-individual variation of faecal microbiota profiles and selected serum antibodies. This precludes the identification of clear distinctive microbial signatures and the discrimination of FGID subgroups on the basis of bacterial markers. Analysis of faecal microbiota profiles and functions should be therefore at present cautiously interpreted for FGID subgroup definitions. On the horizon, their role appears a promising tool within the personalized medicine approach and deserves to be intensively studied whether it can provide useful information for tailored treatments to modulate the gut microbiota in the individual patient with FGID.

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CONFLICT OF INTEREST

The authors have nothing to disclose. CC, GTK, KHK are employees of Genetic Analysis AS. CC and KHK own stocks and shares in Genetic Analysis AS.

AUTHOR CONTRIBUTIONS

KS, CS and PM performed the study design. KS and PM obtained funding and supervised the study procedures. RV, MS, CS and PM drafted the manuscript. RV, CS and KS recruited suitable subjects, collected samples and registered data. CC, GTK, KHK, MS performed laboratory workup, NGS analysis, bioinformatic and statistical analyses. RV, CS, MS, LM, CC, GTK, KHK, KS and PM interpreted the data. PM is the guarantor of the study. All authors red and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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ORIGINAL ARTICLE

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Long-term effects of fecal microbiota transplantation (FMT) in patients with irritable bowel syndrome

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Abstract

Background: We recently found fecal microbiota transplantation (FMT) in irritable bowel syndrome (IBS) patients to be an effective and safe treatment after 3 months. The present follow-up study investigated the efficacy and safety of FMT at 1 year after treatment.

Methods: This study included 77 of the 91 IBS patients who had responded to FMT in our previous study. Patients provided a fecal sample and completed five questionnaires to assess their symptoms and quality of life at 1 year after FMT. The dysbiosis index (DI) and fecal bacterial profile were analyzed using a 16S rRNA gene-based DNA probe hybridization. The levels of fecal short-chain fatty acids (SCFAs) were determined by gas chromatography.

Results: There was a persistent response to FMT at 1 year after treatment in 32 (86.5%) and 35 (87.5%) patients who received 30-g and 60-g FMT, respectively. In the 30-g FMT group, 12 (32.4%) and 8 (21.6%) patients showed complete remission at 1 year and 3 months, respectively; the corresponding numbers in the 60-g FMT group were 18 (45%) and 11 (27.5%), respectively. Abdominal symptoms and the quality of life were improved at 1 year compared with after 3 months. These findings were accompanied by comprehensive changes in the fecal bacterial profile and SCFAs.

Conclusions: Most of the IBS patients maintained a response at 1 year after FMT. Moreover, the improvements in symptoms and quality of life increased over time. Changes in DI, fecal bacterial profile and SCFAs were more comprehensive at 1 year than after 3 months. www.clinicaltrials.gov (NCT03822299).

KEYWORDS
fatigue, microbiome, short-chain fatty acids, superdonor, therapy

This study was previously presented at the UEG week virtual congress in October 2020.

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1 | INTRODUCTION

Irritable bowel syndrome (IBS) is a common chronic gastrointestinal disorder, with IBS patients representing 12–14% of primary-care patient visits and 28% of referrals to gastroenterologists.[1-5] The current treatments used in the clinic focus on symptom relief. The intestinal bacterial profile of IBS patients deviates from that of healthy subjects, with a low diversity and or abnormal bacteria profiles (dysbiosis). However, there is disagreement regarding the distinct microbial signature of IBS patients.[6] The intestinal microbiota is considered to be one of the factors that plays crucial roles in the etiology of IBS.[1]

A recent randomized, double-blind, placebo-controlled study performed by our group found that fecal microbiota transplantation (FMT) was effective in improving the abdominal symptoms, fatigue, and quality of life in IBS patients.[7] These changes were accompanied by changes in the fecal bacterial profile.[7] However, these results were observed only at 3 months after FMT, and several questions remained to be answered, such as whether the clinical effect of FMT is sustained over the long term, whether the bacterial profile changes over time and whether there are any long-term adverse events. The present study therefore performed a 1-year follow-up of the responders to FMT in our previous study in order to answer these questions.

Key Points

- A recently published randomize controlled trial (RCT) from our group showed that fecal microbiota transplantation (FMT) improves IBS symptoms and the quality of life at 3 months after treatment. Mild self-limited adverse events were observed.
- The improvement in symptoms and quality of life in this RCT was accompanied marked changes in the fecal profiles of active treated patients.
- Most responders to FMT after 3 months maintain their response after 1 year. The effects of FMT on IBS symptoms, the quality of life, and the changes in the fecal bacteria increase over time. Furthermore, the microbial metabolism changed from a saccharolytic to a proteolytic fermentation pattern in IBS patients at 1 year after FMT.

2 | MATERIALS AND METHODS

2.1 | Study design

The design of this has been described in detail previously.[7] In brief, patients provided a fecal sample and completed five questionnaires to assess their symptoms and quality of life at baseline, and then provided another fecal sample and completed a new set of questionnaires at 1 month after FMT. They also completed an additional set of questionnaires at 3 months after FMT.[7] At 1 year after FMT, the patients completed another set of the questionnaires and provided a new fecal sample. The patients were asked to keep a diary to record bowel habits and register any adverse events. Polyethylene glycol and loperamide were allowed during the previous randomized, double-blind, placebo-controlled study as rescue medication. The patients were asked to continue using this rescue medication and to record their consumption. They were asked also to record the use of other IBS medication. The patients continued with their medication listed in Table 1 including proton-pump inhibitor (PPI).

2.2 | Patients

This study included 77 of the 91 IBS patients who responded to FMT in our previous study, defined as a decrease of ≥50 points in the IBS Severity Scoring System (IBS-SSS) total score.[7] The original 91 patients comprised 42 and 49 who received 30-g and 60-g FMT, respectively,[7] and 14 patients were either excluded or dropped out. Three patients were excluded because of pregnancy, one because of breast cancer diagnosis, three because of treatment with antibiotics for pharyngitis, pyelonephritis, and diverticulitis, one because of a bout of gastroenteritis caught abroad, and one because of abdominal surgery, while five patients dropped out. The remaining 77 patients comprised 37 and 40 who received 30-g and 60-g FMT. The characteristics of these patients at baseline are given in Table 1.

The inclusion and exclusion criteria of the present study are also provided in our previous report.[7] In summary, the inclusion criteria were being aged 18 to 85 years and having moderate-to-severe IBS symptoms, which were defined as an IBS-SSS total score of ≥175. The exclusion criteria were the presence of systemic disease, immune deficiency, or being treated by immune-modulating medication, pregnant, planning pregnancy, lactating, having a severe psychiatric disorder, having alcohol or drug abuse, or taking probiotics, antibiotics or IBS medications within 8 weeks prior to study inclusion.

2.3 | Donor characteristics and fecal sample collection, preparation, and administration

The single donor was a superdonor who has been described in detail previously.[7] Fecal samples were frozen immediately and kept at −20°C until they were delivered frozen to the laboratory, where they were kept at −80°C. The fecal bacterial and short-chain fatty acids analysis were performed on 63 fecal samples: 32 patients belonging to the 30-g group (28 responders and 4 non-responders) and 31 patients belonging to the 60-g group (27 responders and 4 non-responders). Fourteen fecal samples were discarded due to thawing during transport. The process of FMT has also been described in detail previously.[7] In brief, the patients who were randomized to receive FMT were divided into two groups: the first group received 30 g of donor feces and the second group received 60 g. The fecal

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TABLE 1 Baseline characteristics of the included patients

	Overall	30-g FMT	60-g FMT
Number	77	37	40
Age, years	39.6 ± 11.6	39.6 ± 11.6	38.1 ± 14.4
Sex, female/male	62/15	29/8	33/7
IBS-D	32	17	15
IBS-C	25	11	14
IBS-M	20	9	11
IBS duration, years	15.5 ± 7.9	17.2 ± 9.3	14.7 ± 5.7
IBS-SSS score	311.3 ± 72.5	322.8 ± 64.1	315.3 ± 72.5
FAS score	31.3 ± 4.9	31.4 ± 5.1	31.2 ± 4.8
PPI medication	35 (45.5)	18 (48.6)	17 (42.5)
Birth-control medication	48 (62.3)	22(59.5)	26 (65.0)
Antimigraine medication	7 (9.1)	4 (10.8)	3 (7,5)
Medication against asthma/allergies	11 (14.3)	4 (10.8)	7(17.5)
Medication with levothyroxine	1 (1.3)	0 (0)	1 (2.5)
Medication with heart/vascular drugs	3 (3.9)	2 (5.4)	1 (2.5)

Note: Data are mean ± SD, *n*, or *n* (%) values.

Abbreviations: IBS-C, constipation-predominant IBS-C; IBS-D, diarrhea-predominant IBS; IBS-M, mixed-diarrhea-and-constipation IBS; PPI, proton-pump inhibitor.

material was thawed for 2 days at 4°C, mixed with 40 mL of sterile saline, filtered, and administered to the distal duodenum via the working channel of a gastroscope.

2.4 | Symptoms and quality of life assessment

Symptoms were assessed using the IBS-SSS and the Birmingham IBS Symptom Questionnaire, while fatigue was measured using the Fatigue Assessment Scale (FAS).[8,9] Patients who exhibited a decrease of ≥50 points in the IBS-SSS total score after FMT were considered responders, while those who had an IBS-SSS total score of ≤75 were considered to be in complete remission.[8] Quality of life was measured using the IBS Quality of Life (IBS-QoL) questionnaire and the Short-Form Nepean Dyspepsia Index (SF-NDI) questionnaire.[10-12]

2.5 | Fecal bacterial analysis

The fecal bacteria were analyzed using the GA-map Dysbiosis Test® method as described in detail previously.[13,14] In brief, the test uses the 16S rRNA gene to measure the fluorescence signals for 48 bacterial markers. This method was used for calculating both the bacterial profile and dysbiosis index (DI). The 48 bacterial markers detected bacteria within five phyla (Firmicutes, Proteobacteria, Bacteroidetes, Tenericutes, and Verrucomicrobia) that cover 10 bacterial classes, 36 genera, and 32 species.[13] This test assesses >300 bacteria at different taxonomic levels.[14] The DI was measured on a 5-point scale from 1 (normal) to five (severe dysbiosis), where DI values >2 indicate the presence of dysbiosis.[13]

2.6 | Determination of fecal levels of short-chain fatty acids

About 0.5 g of the fecal samples was homogenized with a solution containing 3 mmol/L 2-ethylbutyric acid (as internal standard) and 0.5 mmol/L H_2SO_4. A 2.5-mL aliquot of the homogenate was vacuum distilled, and the levels of short-chain fatty acids (SCFAs) were determined by gas chromatography (Agilent 7890 A, Agilent) using a capillary column (serial no. USE400345H, Agilent J&W GC columns, Agilent) and flame ionization detection.[15,16] The levels of total SCFAs and acetic, propionic, isobutyric, n-butyric, isovaleric, n-valeric acid, isocaproic, and n-caproic acids, with the results expressed in units of mmol kg^{-1} wet weight.

2.7 | Statistical analysis

The difference between the patients exhibiting complete remission at 3 months and 1 year after FMT was calculated using the chi-square test. Differences between patients at baseline, 3 months, and 1 year in the scores on the IBS-SSS, the Birmingham IBS Symptom, FAS, IBS-QoL, and SF-NDI questionnaires, and in the levels of SCFAs were analyzed using the Kruskal–Wallis test, with Dunn's multiple-comparisons test as a post-test. The Kruskal–Wallis test was also used to detect difference in the bacterial markers at baseline between responders, patients in remission, and patients who relapsed 1 year after FMT. The correlations between the changes in bacterial profiles and the IBS-SSS and FAS scores were analyzed using the non-parametric Spearman test and reported as correlation coefficient (r), *p* value and Bonferroni adjusted *p* values. Differences in

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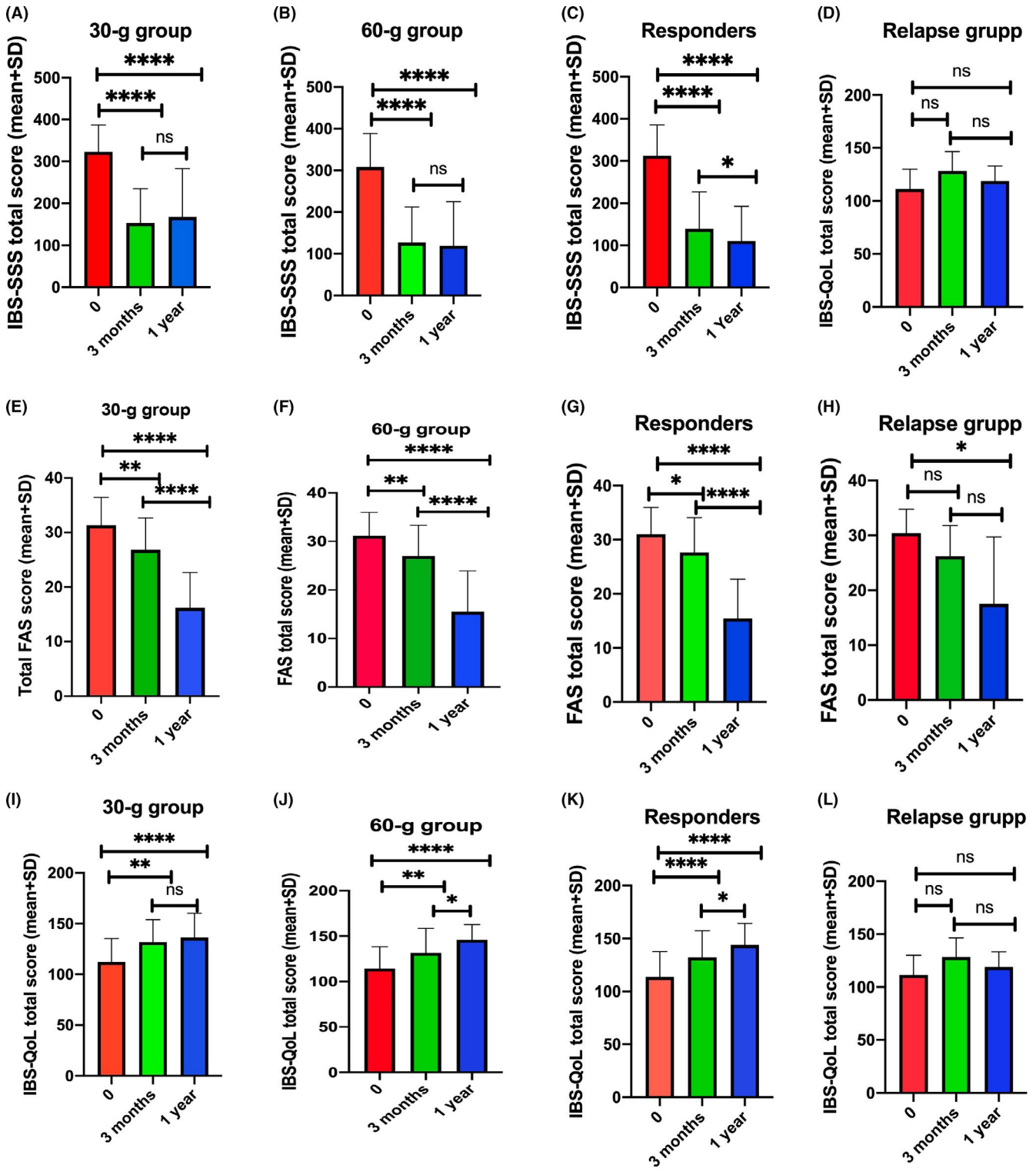

FIGURE 1 Total scores of IBS-SSS (A-D), of FAS (E-H) and of IBS-QoL (I-L) in the IBS patients who received 30 g transplant (30-g group), 60 g transplant (60-g group), responder (patients who exhibited a decrease of ≥50 points in the IBS-SSS total score 1 year after FMT) and relapse (patients who responded at 3 months, but did not respond 1 year after FMT) groups at baseline, at 3 months and 1 year after FMT. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$

DI score between baseline, 1 month, and 1 year were tested using the paired Wilcoxon test. The difference in fluorescence signals between baseline and 1 year after FMT was also tested using the paired Wilcoxon test. Adjusted *p* values were calculated using Bonferroni correction, with $p < 0.05$ considered significant. Scaled principal components analysis (PCA) of log-transformed fluorescence signals was performed, with the results visualized using different colors for different groups, together with ellipses that covered 80% of the

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TABLE 2 IBS-SSS total score and the scores for its four subitems after FMTH

Time	Group	Total score	Subitem 1	Subitem 2	Subitem 3	Subitem 4
0	30-g FMT	322.8 ± 64.1	113.6 ± 39.2	55.0 ± 25.4	76.4 ± 19.2	75.6 ± 17.8
	60-g FMT	308.2 ± 79.8	106.5 ± 45.8	56.8 ± 27.2	80.0 ± 20.3	75.6 ± 20.8
3 months	30-g FMT	153.1 ± 82.1[a]	53.7 ± 38.9[a]	30.5 ± 21.7[a]	44.6 ± 29.7[a]	45.3 ± 30.9[a]
	60-g FMT	126.9 ± 95.5[a]	56.5 ± 47.5[a]	30.4 ± 26.4[a]	35.3 ± 27.5[a]	40.3 ± 27.5[a]
1 year	30-g FMT	167.0 ± 115.9[a]	59.0 ± 54.9[a]	27.3 ± 22.6[a]	45.1 ± 30.0[a]	41.5 ± 31.8[a]
	60-g FMT	119.4 ± 105.3[a]	41.7 ± 50.5[a]	19.4 ± 20.2[a]	30.0 ± 27.9[a]	30.9 ± 28.2[a]

Note: Data are mean ± SD values.

Subitems: 1, abdominal pain; 2, abdominal distension; 3, dissatisfaction with bowel habits; 4, interference with quality of life.

[a]$p < 0.0001$ compared with baseline.

TABLE 3 FAS scores in FMT-treated patients.

Time	Group	Total score	Physical fatigue	Mental health
0	30-g FMT	31.4 ± 5.1	16.0 ± 3.0	15.6 ± 2.8
	60-g FMT	31.2 ± 4.8	15.9 ± 2.8	15.5 ± 2.7
3 months	30-g FMT	26.8 ± 5.8[b]	13.3 ± 3.5	13.5 ± 3.0
	60-g FMT	27.0 ± 6.3[b]	14.0 ± 3.5[b]	13.0 ± 3.1[a]
1 year	30-g FMT	16.2 ± 6.5[c,d]	8.9 ± 3.9[c,d]	7.6 ± 3.3[c,d]
	60-g FMT	15.5 ± 8.4[c,d]	8.0 ± 4.5[c,d]	7.6 ± 4.0[c,d]

Note:: Data are mean±SD values.

[a]$p < 0.05$.

[b]$p < 0.01$.

[c]$p < 0.0001$ compared with baseline.

[d]$p < 0.0001$ compared with 3 months.

samples within each group. These analyses were performed using GraphPad Prism software (version 8.4.1, La Jolla) and R (version 3.6.3, R Foundation for Statistical Computing).

2.8 | Ethics

The study was approved by the Regional Committee for Medical and Health Research Ethics West, Bergen, Norway (approval no. 2017/1197/REK vest). All subjects provided both oral and written consents to participate. The study was registered at www.clinicaltrials.gov (NCT03822299).

All authors had access to the study data and reviewed and approved the final version of the manuscript.

3 | RESULTS

3.1 | Patient responses and use of medications

In the 30-g FMT group, 5 (13.5%) patients relapsed and 32 (86.5%) maintained their response to FMT. Twelve (32.4%) patients showed complete remission at 1 year, compared with 8 (21.6%) after 3 months ($p = 0.4$). In the 60-g FMT group, 5 (12.5%) patients relapsed and 35 (87.5%) continued to respond for FMT. Eighteen (45%) patients were in complete remission, compared with 11 (27.5%) after 3 months ($p = 0.1$).

All the relapsed patients (n = 10) used regularly medications. Four patients used loperamide, two patients used polyethylene glycol, and two patients used both polyethylene glycol and loperamide. The remaining two patients used linaclotide and prucalopride. In the patients that maintained response, two patients used loperamide only once during the following period and one patient used polyethylene glycol twice.

3.2 | Abdominal symptoms, fatigue, and quality of life

The maintained response in females was 90.3% and in males was 86.7% and the complete remission rate was 40.3% in females and 33.3% in males. The response rate and the complete remission rates did not differ significantly between females and males ($p = 0.6$ and 0.8, respectively). The maintained response rates were 90.6%, 88.0%, and 90.0% in IBS-D, IBS-C, and IBS-M patients, respectively. The complete remission rates were 41%, 40%, and 30% in IBS-D, IBS-C, and IBS-M patients, respectively. There was no significant difference between the IBS sub-types regarding either response or complete remission ($p = 0.9$ and 0.7, respectively).

The IBS-SSS total score and the scores for its four subitems were significantly lower than at baseline in both 30-g and 60-g groups. There were no significant differences in the IBS-SSS total scores or subitem scores between 3 months and 1 year after FMT (Figure 1 and Table 2). The IBS-SSS total score in responders was significantly lower at 1 year than at 3 months after FMT (Figure 1). In the patients who relapsed, while the IBS-SSS total score at the baseline was significantly higher than that at 3 months after FMT, it did not differ from that at 1 year after FMT (Figure 1). The IBS-SSS total scores were 135.9 ± 109.9, 141.2 ± 126.2, and 162.0 ± 102.5 in IBS-D, IBS-C, and IBS-M, respectively. The IBS-SSS total score did not differ significantly between the IBs sub-types ($p = 0.5$).

The total score for the Birmingham IBS Symptom Questionnaire and the scores in its three domains were significantly lower than those at baseline (Table S1). There was no difference in the

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TABLE 4 IBS-QoL total scores and scores in its eight domains in FMT-treated patients

Time	Group	Total score	1	2	3	4	5	6	7	8
0	30-g FMT	12.2 ± 23.1	26.2 ± 6.9	20.0 ± 6.0	11.2 ± 3.4	10.1 ± 2.8	5.9 ± 2.8	13.6 ± 3.4	7.5 ± 2.1	10.8 ± 2.6
	60-g FMT	114.2 ± 24.0	27.4 ± 6.7	21.4 ± 5.5	11.5 ± 2.7	10.5 ± 2.7	6.7 ± 2.8	14.1 ± 3.4	7.7 ± 1.6	10.8 ± 2.8
3 months	30-g FMT	131.5 ± 22[b]	32.1 ± 6.9[d]	24.2 ± 5.2[c]	14.0 ± 3.0[c]	12.6 ± 2.9[d]	8.8 ± 2.8[d]	16.1 ± 2.6[c]	8.2 ± 1.6	12.6 ± 1.8[c]
	60-g FMT	131.5 ± 27.1[b]	32.2 ± 6.7[d]	24.0 ± 4.7[b]	14.3 ± 3.7[d]	12.7 ± 2.4	9.6 ± 3.6[d]	15.7 ± 3.4[a]	8.3 ± 1.6	12.1 ± 2.6[a]
1 year	30-g FMT	136.0 ± 24.0[d]	33.8 ± 5.9[d]	24.0 ± 5.0[b]	14.7 ± 3.2[d]	16.7 ± 3.0[d, g]	9.2 ± 3.7[d]	16.5 ± 3.3[c]	8.5 ± 1.7[a]	12.7 ± 2.1[c]
	60-g FMT	145.71 ± 16.9[d, e],	35.0 ± 4.9[d]	26.3 ± 3.4[d, e],	15.9 ± 2.8[d]	17.4 ± 2.3[d, h],	11.3 ± 2.8[d, g],	17.6 ± 1.9[d, e],	9.3 ± 1.1[d, f],	13.2 ± 1.7[d]

Note: Data are mean ± SD values. IBS-QoL domains: 1, dysphoria; 2, interference with daily activities; 3, body image; 4, health worries; 5,food avoidance; 6, social reaction; 7, sexual function; 8, impact on relationships.

[a] $p < 0.05$,
[b] $p < 0.01$,
[c] $p < 0.001$,
[d] $p < 0.0001$ compared with placebo.
[e] $p < 0.05$,
[f] $p < 0.01$,
[g] $p < 0.001$,
[h] $p < 0.0001$ compared with 30-g FMT.

Birmingham IBS Symptom Questionnaire total score or its three domain scores between 3 months and 1 year after FMT in both the 30-g and 60-g groups (Table S1).

In both the 30-g and 60-g FMT groups, the FAS score was significantly lower at 1 year after FMT than at baseline (Figure 1 and Table 3). The FAS scores in both the 30-g and 60-g FMT groups were significantly lower at 1 year than at 3 months after FMT. Similar results were observed in the responders. In the group exhibiting clinical relapse, the FAS score was significantly lower at 1 year after FMT than at baseline.

The IBS-QoL total scores increased significantly at 1 year after FMT in the 30-g FMT group, 60-g FMT group, and responders compared with those at baseline (Figure 1 and Table 4). In addition, the IBS-QoL total score was significantly higher at 1 year than at 3 months after FMT. In the patients who relapsed at 1 year after FMT, there was no significant improvement in the IBS-QoL at either at 3 months or 1 year after FMT. The deterioration in the quality of life as measured by the SF-NDI was significantly reduced at 1 year after FMT in both the 30-g and 60-g FMT groups (Table S2).

3.3 | Bacterial analysis

The median DI for all patients who received FMT was 3 (range: 1–5) at baseline and 2 (range: 1–4) at 1 year after FMT ($p < 0.0001$). In the 30-g FMT group, the median DI was 3 (range: 1–5) at baseline and 1 (range: 1–4) at 1 year after FMT ($p = 0.004$). In the 60-g FMT group, the median DI was 3 (range: 1–5) at baseline and 2 (range: 1–4) at 1 year after FMT ($p = 0.003$). The median DIs in the patients in complete remission were 3 (range: 1–5) and 2 (range: 1–3) at baseline and 1 year after FMT, respectively ($p = 0.01$); the corresponding values were 2 (range: 1–5) and 2 (range: 1–4), respectively ($p = 0.004$), in responders, and 3 (range: 1–3) and 2 (range: 1–3) ($p = 0.12$) in patients who relapsed.

There was a marked change in the bacterial profiles at 1 year after FMT compared to baseline in all patients who received FMT as well as in the 30-g and 60-g FMT groups, as detected by PCA and by analyzing differences in fluorescence signals (Figure 2).

The levels of 10 bacteria groups differed significantly between baseline and 1 year after FMT in patients who received FMT (Table 5). In the 30-g FMT group, the levels of three bacteria were significantly increased at 1 year after FMT: *Bacteroides stercoris*, *Alistipes* spp., and *Bacteroides* spp. & *Prevotella* spp. The levels of these bacteria were correlated inversely with both the IBS-SSS and FAS total scores (Table S3). The levels of *Eubacterium biforme* and *Parabacteroides* spp. also increased significantly at 1 year after FMT, but they were not correlated with either the IBS-SSS or FAS total score (Table S3).

In the 60-g FMT group ($N = 31$), the levels of *Bacteroides Spp. & Prevotella spp*, *Alistipes* spp., *Bacteroides stercoris*, *Parabacteroides* spp., and *Bacteroides zoogleoformans* increased at 1 year after FMT compared to the baseline sample and were inversely correlated correlate with both the IBS-SSS and FAS total scores, with the exception

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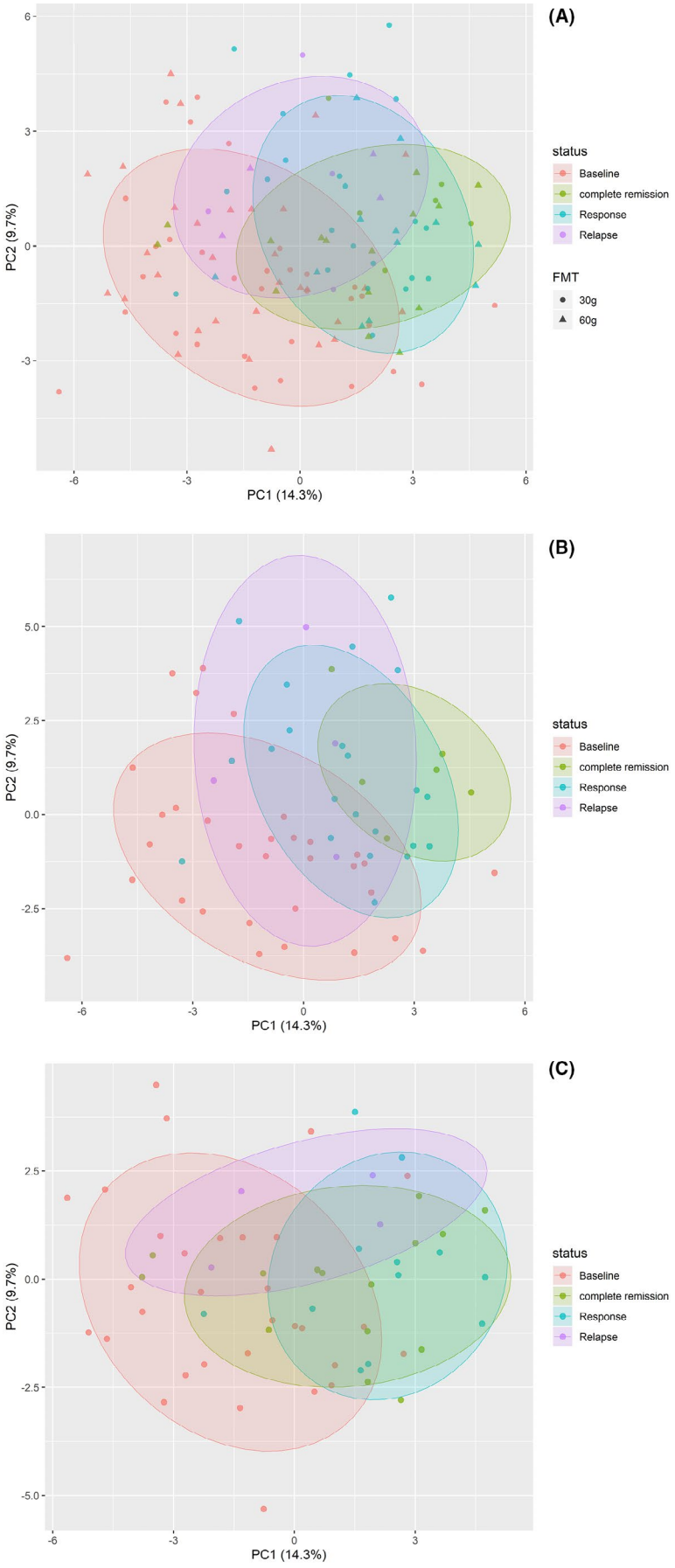

FIGURE 2 PCA plots of all FMT-treated patients (A), 30-g FMT group (B) and 60-g FMT group (C) at 1 year after FMT. The bacterial profile is indicated in orange for baseline, green for complete remission, purple for responders, and blue for relapses. Bacteria in the patients who received 30-g and 60-g FMT are indicated using circles and triangles, respectively. Ellipses of different colors enclose approximately 80% of the samples within the different groups. The bacterial profiles of the FMT-treated patients are grouped more to the right along the first PCA axis

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TABLE 5 Bacteria whose levels differed significantly between baseline and 1 year after FMT in all patients who received FMT

Prob bacteria markers	Baseline	1 year after FMT	*p*	Adjusted *p*
206 Bacteroides spp. & *Prevotella* spp.	334 (9–1349)	944 (106–1330)	<0.001	<0.001
Alistipes spp.	43 (2–512)	228 (2–512)	<0.001	<0.001
207 Bacteroides stercoris	9 (2–69)	26 (2–212)	<0.001	<0.001
327 Streptococcus salivarius ssp. *thermophilus*	84 (5–907)	22 (2–732)	<0.001	<0.001
210 Parabacteroides spp.	6 (2–137)	38 (2–248)	<0.001	<0.001
208 Bacteroides zoogleoformans	20 (2–137)	45 (2–248)	<0.001	0.001
404 Shigella spp. & *Echerichia* spp.	20 (2–1167)	2 (2–725)	<0.001	0.002
314 Eubacterium hallii	151 (8–466)	102 (41–777)	<0.001	0.006
300 Firmicutes spp.	468 (68–1080)	677 (143–1304)	<0.001	0.016
500 Proteobacteria spp.	45 (16–1306)	29 (11–376)	<0.001	0.038

Note: Data are median (range) values.

of *Bacteroides zoogleoformans* (Table S4). On the other hand, the levels of *Streptococcus salivarius* ssp. *thermophilus* and *Eubacterium hallii* were decreased, and correlated significantly with the IBS-SSS and FAS total scores (Table S4).

In responders, the levels of nine bacteria were changed at 1 year after FMT (Table S5). The levels of these bacteria were correlated significantly with the IBS-SSS and FAS total scores, with the exceptions of *Parabacteroides* spp., *Shigella* spp. & *Escherichia* spp., and *Dorea* spp.

In patients exhibiting complete remission at 1 year after FMT, the levels of the following bacteria were significantly increased: *Bacteroides* spp. & *Prevotella* spp., *Alistipes* spp., and *Bacteroides stercoris*. In contrast, those of *Streptococcus salivarius* ssp. *thermophilus* and *Eubacterium hallii* were significantly decreased (Table S6).

No bacterial markers were significantly changed in the group of patients who had clinically relapsed at 1 year after FMT.

The only bacteria that differed at baseline between relapsed patients, patients in remission and responders were *Alistipes* spp. The level of *Alistipes* spp. was significantly lower in the relapsed patients than in the patients with remission and responders ($p = 0.008$, adjusted $p = 0.15$) (Figure S1).

3.4 | Fecal SCFA levels

In both the 30-g and 60-g FMT groups, the fecal levels of isobutyric and isovaleric acids increased at 1 year after FMT (Table S7). In the 60-g FMT group, the fecal level of total SCFAs increased at 1 year after FMT, whereas that of acetic acid decreased (Table S7). In the patients exhibiting complete remission and responders, the fecal levels of isobutyric and isovaleric acids increased at 1 year after FMT, whereas those of acetic and propionic acids decreased (Table 6). Moreover, in the patients exhibiting complete remission, the fecal levels of total SCFAs and butyric acid increased (Table 6). In the relapsed patients, the levels of fecal isobutyric and isovaleric acids increased at 1 year after FMT.

3.5 | Adverse events

Apart from the mild intermittent abdominal pain, diarrhea and constipation occurring at the first 2 days after FMT,[7] no adverse events were reported under the follow-up period. The 2 patients developed diverticulitis at 2 and 3 months after FMT reported previously did not have new diverticulitis attacks.[7]

4 | DISCUSSION

The present findings indicate that most of the patients who responded 3 months after FMT remained responders after 1 year.[7] Furthermore, there were more patients exhibiting clinically complete remission at 1 year after FMT than after 3 months.[7] Moreover, the abdominal symptoms and fatigue were significantly less severe and the quality of life was significantly better at 1 year than at 3 months after FMT.[7] Thus, it seems that the effect of FMT intensified in those who remained responding after 1 year. The finding that FMT induced remission in about half of the patients with IBS emphasizes the importance of the intestinal microbiota as an etiological factor of IBS.

Similar to our previous observation 3 months after FMT,[7] there was no difference between IBS sub-types regarding the response rate or IBS symptoms 1 year following FMT. In contrast to the recently published observation,[17] the response rate and IBS symptoms did not differ between females and males in the present study.

The DI did not improve at 1 month after FMT in our previous study,[7] whereas it had improved at 1 year after FMT in the present study. This indicates that the intestinal bacterial diversity had

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TABLE 6 The SCFAs concentration in the feces of the remission, responders, and relapse groups at the baseline and 1 year after FMT

Acid	Remission group			Responders			Relapse group		
	Baseline	1 year After FMT	*p* values	Baseline	1 year After FMT	*p* values	Baseline	1 year After FMT	*p* values
Total SCFA	74.5 ± 49.3	111-0 ± 23.0	0.008	71.5 ± 37.1	78.0 ± 42.1	0.5	69.6 ± 18.2	77.3 ± 26.6	0.6
Acetic acid	44.1 ± 21.4	31.7 ± 11.4	0.04	44.4 ± 21.3	37.5 ± 15.4	0.04	39.8 ± 10.4	40.3 ± 16.2	0.9
Propionic acid	14.8 ± 12.1	8.7 ± 4.5	0.02	13.2 ± 6.7	10.4 ± 5.9	0.03	11.9 ± 6.0	14.2 ± 7.1	0.6
Iso-butyric acid	1.2 ± 0.5	1.7 ± 0.7	0.03	1.3 ± 0.8	1.7 ± 1.8	0.002	1.0 ± 0.5	1.7 ± 0.6	0.03
Butyric acid	10.3 ± 7.6	13.6 ± 7.5	0.03	12.0 ± 9.1	10.3 ± 6.8	0.7	7.4 ± 3.1	11.3 ± 5.0	0.04
Iso-valeric acid	2.1 ± 1.5	2.9 ± 1.6	0.04	2.1 ± 1.4	2.4 ± 1.3	0.02	1.5 ± 0.9	2.3 ± 1.0	0.1
Valeric acid	1.4 ± 0.5	1.6 ± 0.6	0.6	1.8 ± 1.6	1.8 ± 0.9	0.3	1.1 ± 0.7	2.1 ± 0.6	0.01
Iso-capronic acid	0.018 ± 0.078	0.004 ± 0.021	0.5	0.010 ± 0.058	0.004 ± 0.21	0.9	0.000 ± 0.000	0.125 ± 0.315	0.5
Capronic acid	0.8 ± 1.0	0.6 ± 0.5	0.9	0.6 ± 0.5	0.6 ± 0.7	0.2	0.1 ± 0.3	0.4 ± 0.6	0.2

Note: The values were expressed as mmol kg^{-1} wet weight.

improved at 1 year after FMT. The changes in the bacterial profile in patients at 1 year after receiving FMT were more comprehensive than those we observed previously at 1 month after FMT, with 10 bacteria taxa changed after 1 year compared with 4 after 1 month.[7] In responders, the levels of nine bacteria taxa were significantly changed at 1 year after FMT compared with those at baseline. The levels of six of these bacteria taxa were correlated with the IBS-SSS and FAS scores. *Alistipes* are strictly anaerobic gram-negative rods that are resistant to bile and have limited ability to ferment carbohydrates.[18] *Alistipes* spp. levels in the relapsed patients were significantly lower at baseline than in responders and patients in remission at 1 year after FMT. This suggests that the levels of *Alistipes* spp. can be used to predict the outcome of FMT. Furthermore, *Alistipes* spp. levels increased as early as 1 month after FMT and remain high in responders at 1 year after FMT.[7] Moreover, they were strongly correlated with the total scores for both the IBS-SSS and FAS. Together these findings suggest that *Alistipes* spp.— which belong to the phylum Bacteroidetes—play a central role in the improvements seen after FMT. Further investigations are needed to clarify such a role.

The fecal SCFAs in IBS patients differ from those in healthy subjects, and it has been suggested that this difference plays a role in the pathophysiology of IBS.[19] It has been reported recently that FMT increases the fecal SCFA levels in IBS patients at 1 month after FMT.[20] Moreover, fecal butyric acid levels increased at 1 month after FMT and were inversely correlated to IBS-SSS and FAS total score.[20] The present study found a dose-dependent effect of FMT on the fecal SCFAs profile, with increased levels of the branched SCFAs isobutyric and isovaleric acids, and a decreased level of the straight SCFA acetic acid. These changes were more pronounced in patients with a sustained symptomatic effect at 1 year of FMT than in patients who relapsed. These results suggest an overall shift of the microbial metabolism, from a saccharolytic to a proteolytic fermentation pattern, which is conceivably of pathophysiological relevance.[21] The reduction in acetic acid levels in responders and patients in the 60-g FMT group may be particularly relevant since acetic acid has been found to induce visceral hypersensitivity in rodents.[22] It is noteworthy that similar changes of the SCFAs profile have been demonstrated previously in patients with IBS following adherence to a low-FODMAP (fermentable oligo-, di-, monosaccharides, and polyols) diet, an intervention that is also associated with relief of IBS symptoms.[23] The changes observed here in the fecal SCFAs may be caused by either changes in diet or changes in the intestinal bacterial profile. However, since the patients did not change their diet and that comprehensive change in the intestinal bacterial profile occurred after FMT, it is more likely that the changes in SCFAs are caused by the changes in the bacterial profile following FMT.

There were no reported adverse events at 1 year after FMT, which means that this treatment can be considered to be safe. However, one should keep in mind that the patients included in this study did not have systemic disease, immune deficiency (or were receiving an immune-modulating medication), a severe psychiatric disorder, or alcohol or drug abuse.

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The major limitation of this study is that it did not follow-up the placebo group in our previous RCT study. In an earlier study, about 33.3% of IBS patients become asymptomatic in the long term.[24] Assuming that this would be the same in the placebo group this would be much lower than that observed in the active-treated groups. However, this limitation makes it difficult to assess the response using FDA/EMA endpoints or the difference medications use between the placebo and active treated groups in the follow-up period. Other limitations of this study were that it investigated only a rather small proportion of the total fecal bacterial content and that the levels of SCFAs were measured in feces, which does not accurately reflect the colonic production of SCFAs, since that mainly occurs in the caecum. Moreover, the findings for SCFAs reported here should be interpreted with caution, since luminal SCFA levels result from a complex interplay between diet (i.e., input of substrates for microbial fermentation), host (i.e., intestinal absorption capacity and transit of both substrates and SCFAs) and microbes (i.e., fermentation capacity).

In conclusion, the present follow-up study found that most patients who had responded to FMT after 3 months maintained their response after 1 year. Furthermore, their abdominal symptoms, fatigue, and quality of life were improved at 1 year compared with at 3 months after FMT. Finally, the changes in the intestinal bacterial profile and fecal SCFAs were more comprehensive at 1 year compared with at 3 months after FMT.

CONFLICT OF INTEREST

The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

M.E.S. designed the study, obtained the funding, administered the study, recruited the patients, performed FMT, collected, analyzed and interpreted the data, and drafted the manuscript. A.B.K. and C.C. contributed to the design of the study, analyzed and interpreted the results of the microbiome analysis, and critically revised the manuscript for important intellectual content. J.V. contributed to the design of the study, interpreted the results for SCFAs and critically revised the manuscript for important intellectual content. J.G.H., O.H.G., and T.H. contributed to the design of the study and to the analysis and interpretation of the data, and critically revised the manuscript for important intellectual content.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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A Distinct Faecal Microbiota and Metabolite Profile Linked to Bowel Habits in Patients with Irritable Bowel Syndrome

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Abstract: Patients with irritable bowel syndrome (IBS) are suggested to have an altered intestinal microenvironment. We therefore aimed to determine the intestinal microenvironment profile, based on faecal microbiota and metabolites, and the potential link to symptoms in IBS patients. The faecal microbiota was evaluated by the GA-map^{TM} dysbiosis test, and tandem mass spectrometry (GC-MS/MS) was used for faecal metabolomic profiling in patients with IBS and healthy subjects. Symptom severity was assessed using the IBS Severity Scoring System and anxiety and depression were assessed using the Hospital Anxiety and Depression Scale. A principal component analysis based on faecal microbiota (n = 54) and metabolites (n = 155) showed a clear separation between IBS patients (n = 40) and healthy subjects (n = 18). Metabolites were the main driver of this separation. Additionally, the intestinal microenvironment profile differed between IBS patients with constipation (n = 15) and diarrhoea (n = 11), while no clustering was detected in subgroups of patients according to symptom severity or anxiety. Furthermore, ingenuity pathway analysis predicted amino acid metabolism and several cellular and molecular functions to be altered in IBS patients. Patients with IBS have a distinct faecal microbiota and metabolite profile linked to bowel habits. Intestinal microenvironment profiling, based on faecal microbiota and metabolites, may be considered as a future non-invasive diagnostic tool, alongside providing valuable insights into the pathophysiology of IBS.

Keywords: irritable bowel syndrome; pathophysiology; intestinal microenvironment; microbiota; microbial metabolites

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1. Introduction

Irritable bowel syndrome (IBS) is a multifactorial disease involving a perturbed gut–brain interaction [1], visceral hypersensitivity [2], altered gastrointestinal (GI) motility [3], increased permeability, immune activation [4] and an altered gut microenvironment [5]. Currently, the diagnosis of IBS is based on symptom-based criteria, and in most cases, a

limited number of tests are carried out to exclude other organic GI diseases [6]. However, reliable disease-specific biomarkers for IBS are still lacking [6].

The microbiota inhabiting the human GI tract coexist under a mutualistic relationship and benefit the host by participating in immunity, resistance to pathogen colonization and intestinal development [7,8]. The gut microbiota produces a diverse range of metabolites from the anaerobic fermentation of exogenous undigested dietary compounds. These small and diverse molecules reach the colon and are used as energy sources and substrates in metabolic or signalling pathways, as well as influencing host immune response [9,10], reflecting the hosts' physiological status [11]. Metabolites may be regarded as a "fingerprint" of the functional interactions taking place between the host and microbiota [9]. Recent studies have described an altered composition of gut microbiota and metabolites in several diseases of the GI tract such as colorectal cancer and inflammatory bowel disease [11,12]. In addition, IBS has been linked to an unbalanced gut microbiota profile [13–16] and possibly also to an altered metabolite profile [17,18], with links to GI [17,19] and psychological symptoms [20], at least in subsets of patients. There are also contradictory reports of lower, as well as higher, concentrations of faecal short-chain fatty acids in IBS with diarrhoea (IBS-D) as compared to healthy subjects [21]. However, no clear differences have been depicted in the microbiome or metabolome profiles between IBS subtypes [22]. The published data within the field are still relatively limited and studies combining analyses of faecal microbiota and metabolites are rare and inconclusive [22–26]. Despite inconsistencies, the overall picture indicates that subsets of IBS patients have an altered intestinal microenvironment [27].

We hypothesized that the intestinal microenvironment differs between IBS patients and healthy subjects. Analyses of the joined faecal microbiota and metabolite composition may provide valuable insights into host–microbiota interactions and how these interactions can play a role in the pathophysiology of IBS. In this study, we aimed to determine the intestinal microenvironment, based on both faecal microbiota and metabolites, and its link to bowel habits, symptom severity and psychological symptoms in patients with IBS.

2. Materials and Methods

2.1. Study Cohort

This is a retrospective study analysing faecal samples collected from IBS patients and healthy subjects recruited as described previously [16,28]. Briefly, IBS patients (18–70 years old) meeting the Rome III criteria [29] were recruited from patients referred to the gastroenterology outpatient clinic at the Sahlgrenska University Hospital, Gothenburg, as well as through advertisement in local newspapers. Exclusion criteria were the presence of severe diseases such as malignancy, heart disease, kidney disease; neurological or psychiatric diseases; or a GI disease other than IBS that could explain their current symptoms (e.g., inflammatory bowel disease, coeliac disease). Patients on special diets such as the low FODMAP diet and low fibre diet were not included in the study. IBS patients were characterized as having IBS with constipation (IBS-C), diarrhoea (IBS-D) [29], mixed IBS (mixed loose and hard stools) (IBS-M) or unclassified IBS (IBS-U) by evaluation of bowel movements (number of stools per day) and stool consistency according to Bristol Stool Form (BSF) scale in a two-week stool diary. For analyses, the last two subtypes, IBS-M and IBS-U, were grouped together as IBS-nonCnonD [29].

All IBS patients completed validated self-report questionnaires assessing the severity of their IBS symptoms (IBS Severity Scoring System; IBS-SSS) [30], and anxiety and depression (Hospital Anxiety and Depression Scale; HADS) [31]. These questionnaires have validated cut-off levels used to categorize patients according to symptom severity and the presence or absence of anxiety or depression, respectively [30,32] (see Supplementary Material for more details). Healthy subjects (18–70 years old) with no current or prior history of GI diseases were included as controls. Chronic disorders, use of any immunosuppressive agents, antibiotics or any other medication during the 3 months prior to sample collection, and being on restrictive diets (i.e., vegan, glute-free and lactose-free diet), were

reasons for exclusion from the healthy cohort. The use of other special diets was recorded as previously shown [16]. Pregnant or lactating women were not included. Additionally, all study subjects were requested to abstain from the intake of pre- and probiotics. All IBS patients and healthy subjects were given verbal and written information before giving their written consent to participate in the study. The study protocol was approved by the Regional Ethical Review Board in Gothenburg. All authors had access to the study data and have reviewed and approved the final manuscript.

2.2. Faecal Sample Collection

Faecal samples were collected by all study subjects at home. Study subjects were provided with faecal sample collection kits, including an empty tube and a sampling spatula. Samples were kept in a freezer at −20 °C until being transferred to the laboratory in a mini cooler bag with an ice pack. Samples were stored in the laboratory at −80 °C until analysis. All samples were analysed for microbiota and metabolomics profiles, respectively.

2.3. Faecal Microbial Analysis

Bacterial populations were detected at Genetic Analysis AS (Oslo, Norway) using the commercially available analysis, the GA-map™ dysbiosis test. The GA-map™ test targets ≥ 300 bacteria belonging to different taxonomic levels by using a set of 54 highly specific DNA probes previously defined to discriminate between healthy subjects and patients with IBS in faecal samples. The protocol is described in detail elsewhere [33]. Briefly, this test includes faecal sample homogenization, mechanical bacterial cell disruption and automated total bacterial genomic DNA extraction with magnetic beads. Amplification of 16S rRNA (V3–V9 regions) is performed by polymerase chain reaction (PCR), followed by a single-nucleotide extension reaction. In this step, the complementary PCR amplicon hybridizes with a labelling probe, which is then extended with a labelled nucleotide. Subsequent addition of complementary probes coupled with magnetic beads hybridize to the labelled probes. This last hybridisation provides the signal from the nucleotide labelled-labelling probes that is acquired by the BioCode 1000A 128-Plex Analyzer (Applied BioCode, Santa Fe, CA, USA). This signal corresponds to the bacteria present in the sample, which are identified with the help of magnetic beads [33]. As a result, the GA-map™ test generates a bacterial profile based on absolute faecal bacterial abundance, which is denoted as probe signal intensity [33].

2.4. Faecal Supernatant Preparation and Metabolite Analysis

Faecal supernatants extracted from faecal samples were used for metabolomics analysis. Faeces were mixed with 2 weight volumes of ice-cold PBS, followed by centrifugation for 10 min at 1600× g. The resulting supernatant was ultra-centrifuged at 35,000× g for 2 h, at 4 °C. The faecal supernatants were collected and stored at −80 °C until analysis. The metabolomic profile of faecal supernatant samples was analysed using gas chromatography coupled to a tandem mass spectrometer (GC-MS/MS) using the method of Savolainen et al. [34]. Briefly, samples were extracted with water:methanol (1:9 v/v) containing ten stable isotope labelled internal standards [34], followed by drying and derivatization by using oxymation and silylation. The derivatised extracts were injected into a GC-MS/MS system (Shimadzu GCMS TQ-8030 system, Shimadzu Europa GmbH, Duisberg, Germany) and GC-MS scan data (between m/z 50–750) were analysed for targeted peak detection. Metabolites present in the Swedish Metabolomics Centre library were screened against the scan data using a Matlab script (Mathworks, Natick, MA, USA) and database developed at the Swedish Metabolomics Centre (Umeå, Sweden). This uses the retention index, diagnostic ion and spectral matching to score and identify likely metabolites. Peaks were visually inspected to ensure they were correct. Data were normalized based on the internal standard peak intensities [35].

2.5. Statistical Analyses

Univariate statistical analysis was performed using the SPSS Statistical Package (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Alrmonk, NY, USA: IBM Corp.). Categorical variables were compared with the χ^2 test, whereas continuous variables were compared using either the independent samples and paired samples *t*-test or the corresponding non-parametric Mann–Whitney U test and Wilcoxon signed rank test, based on the normality of distribution determined by histograms and Kolmogorov–Smirnov statistic tests. The data in text, figures and tables are shown, corresponding to the distribution, as mean ±SD or median (interquartile range), respectively. p values < 0.05 were considered statistically significant.

Multivariate analyses were applied to the combined dataset containing faecal microbiota and metabolites data. Principal component analysis (PCA) was conducted using the *prcomp*-function with z-score scaling, and visualized using the *pca3d*-package in R (version 3.6.2, Vienna, Austria) [36]. The multivariate group means were represented as centroids. To test the statistical significance of comparisons between the two groups (e.g., IBS vs. healthy), i.e., whether the groups differed in their mean scores on the two principal components, we iteratively randomized the group labels to simulate a null-distribution of centroid differences, and ranked the actual value within this null-distribution. p values < 0.05 were interpreted as significantly different multivariate group means. Orthogonal partial least squares-discriminant analysis (OPLS-DA) was implemented to investigate if the combined dataset, containing faecal microbiota and metabolites profiles (X variables), could discriminate IBS patients from healthy subjects (Y variables). All discriminant analyses were performed using SIMCA® software (version 15.0.2, MKS Umetrics AB, Umeå, Sweden). The quality of OPLS-DA was determined based on the parameters R^2, i.e., the goodness of fit of the model (values of ≥0.5 define good discrimination, where $R^2 = 1$ is the best possible fit), and Q^2, i.e., the goodness of prediction of the model (values of ≥0.4 are considered good) [37]. The reliability of the models was confirmed using analysis of variance testing of cross-validated predictive residuals (CV-ANOVA), where a p value < 0.05 indicated significantly different residuals of the compared groups. Student's *t*-test was used to verify the robustness of the selected variables from the simplified OPLS-DA model.

Volcano scatter plots were used for the identification of differentiating faecal microbiota and metabolites for which the fold change for each metabolite and bacterial taxon between IBS patients and healthy subjects was calculated, i.e., (metabolite A in IBS/metabolite A in healthy) and (bacterial taxon A in IBS/bacterial taxon A in healthy). Significance ($-\log_{10}$ (p value < 0.05), Student's *t*-test) versus $\log_2$ (mean fold change) [38] were plotted, and only most relevant variables were labelled. Visualization was performed in R using the *ggplot2* and *ggrepel* packages. Further, Cleveland plot analysis was performed in R using the *ggplot2*, *dplyr* and *tidyr* packages.

In addition, the least absolute selection and shrinkage operator (LASSO) method with regularization was used to extract a set of most relevant discriminative variables amongst microbial taxa and metabolites to help distinguish IBS patients from healthy subjects and further facilitate the interpretability of our findings by simplifying our model. LASSO was performed using the *caret* [39] and *glmnet* [40] packages in R and is described in detail in the Supplementary material.

The ingenuity pathway analysis (IPA, version 2.3) (Qiagen Inc., https://www.qiagenbio-informatics.com/products/ingenuitypathway-analysis, trial version accessed on 18 March 2020) [41] was used to identify biological networks associated with mean fold change values (IBS patients/healthy subject) for each of the analysed faecal metabolites. A p value < 0.05 was set as the cut-off and activation z-scores were calculated and used to predict increased (z-score > 2.0) or decreased (z-score < −2.0) biological functions that could explain the differences on the metabolite dataset.

3. Results

3.1. Characteristics of the Study Cohort

The study cohort consisted of 40 IBS patients and 18 healthy subjects. The demographics and clinical characteristics of the study subjects are shown in Table 1. Females and males were unequally distributed between the study groups, and healthy subjects were younger than IBS patients ($p < 0.001$). All IBS subtypes (IBS with diarrhoea or IBS-D, IBS with constipation or IBS-C; and IBS-nonCnonD or combined mixed-type IBS and IBS unclassified) were represented in the study population. The majority of patients presented with moderate to severe IBS symptoms (IBS-Severity Scoring System; IBS-SSS) and had higher Hospital Anxiety and Depression Scale (HADS) total scores than healthy subjects. Likewise, a higher proportion of individuals with anxiety was found among the IBS patients compared to healthy subjects, whereas the proportion of individuals with depression did not differ between the two groups.

Table 1. Demographics and clinical characteristics of the study cohort.

Baseline Characteristics	IBS (n = 40)	Healthy (n = 18)	p Value
Sex (F:M)	31:9	9:9	0.04
Age, years †	52 (24–70)	26 (19–54)	<0.001
IBS subtypes IBS-C:IBS-D:IBS-nonCnonD	15:11:14	N/A	
IBS-SSS ‡	236 (174–327)	11 (0–20)	<0.001
Mild:moderate:severe §	10:17:13	N/A	
HADS total score ¶	9.9 ± 5.9	6.8 ± 3.9	<0.05
Anxiety:no anxiety ᵠ	14:17	3:15	0.04
Depression:no depression ᵠ	3:28	0:18	0.17

Note: Abbreviations: N/A, not applicable; IBS-C, IBS with constipation; IBS-D, IBS with diarrhoea; IBS-nonCnonD, mixed-type IBS and unclassified IBS; IBS-SSS, IBS Severity Scoring System; HADS, Hospital Anxiety and Depression Scale. † Data shown as median (min–max). ‡ Data shown as median (interquartile range). § Based on IBS-SSS. ¶ Data shown as mean ±SD. ᵠ Based on HADS.

3.2. The Intestinal Microenvironment Differs between IBS Patients and Healthy Subjects

First, we investigated whether the intestinal microenvironment differed between IBS patients and healthy subjects. A principal component analysis (PCA) based on the total number of identified faecal microbiota (n = 54 variables) and metabolites (n = 155 variables) showed a clear separation between IBS patients and healthy subjects, with a large distance between the centroids (scores averages) of the two groups ($p < 0.001$) (Figure 1A). This separation was not seen to be influenced by age and gender differences (Figure S1A,B). An orthogonal partial least squares-discriminant analysis (OPLS-DA), based on the total number of identified variables, further supported the differentiation seen between IBS patients and healthy subjects (Table 2). As visualized by Cleveland plots, the majority of the differentiating variables were found in higher abundance in IBS patients (bacterial taxa; n = 3 and metabolites; n = 67) compared to healthy subjects (bacterial taxa; n = 5 and metabolites; n = 2) (Figure 1B, Figure S2). Furthermore, a volcano plot showed that a large number of metabolites such as citric acid, alanine, glycine, tryptophan, xanthine, hypoxanthine and pyruvic acid along with *Acinetobacter junii* were higher in IBS patients as compared to healthy subjects, whereas only a few variables such as *Clostridium* sp., glucose-6-phosphate and nicotinic acid were found in lower abundance (Figure 1C). In addition, LASSO variable selection identified a total of 21 variables (bacterial taxa; n = 5 and metabolites; n = 16), providing an even more robust model (accuracy of 90.9%) for distinction of IBS patients from healthy subjects. The variables identified by the LASSO model as being most important for the separation between IBS patients and healthy subjects were depicted by an OPLS-DA (Figure 1D).

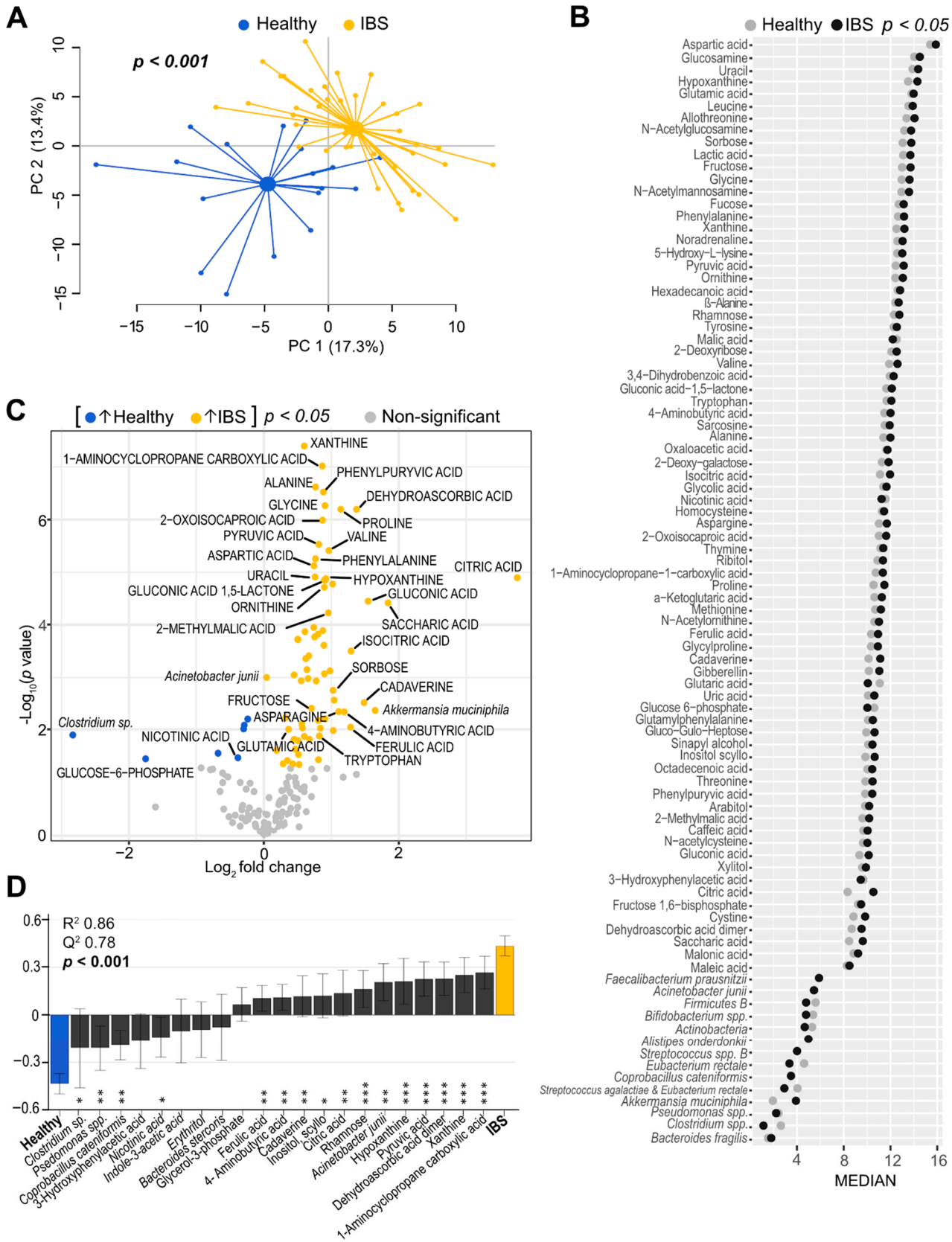

Figure 1. Faecal microbiota and metabolite profiles of IBS patients and healthy subjects. (**A**) Principal component analysis (PCA) score scatter plot based on faecal bacteria and metabolites showing IBS patients (n = 40, yellow dots) and healthy

subjects (n = 18, blue dots). The p value depicts statistical significance of the distance between centroids (scores average) of the groups. (**B**) Cleveland dot plot depicting the differentiating microbiota and metabolite variables ($p < 0.05$) between IBS patients and healthy subjects. The horizontal axis represents bacterial probe intensity (n = 14) and metabolite signal intensity (n = 76) values and the vertical axis shows the variable IDs. Log-transformed median values for each X-variable are depicted for the IBS group (black dots) and healthy group (grey dots), respectively. Mann–Whitney U test was performed to evaluate differences between the groups. (**C**) Volcano plot illustrating mean fold change vs. statistical significance based on 54 bacteria (probe intensity) and 155 metabolites (signal intensity) in IBS patients vs. healthy subjects (IBS/H). Each dot corresponds to a bacterial probe or metabolite signal. Variables increased in IBS are coloured in yellow (n = 70), while decreased variables compared to healthy subjects are coloured in blue (n = 7). Mean fold change is shown as $\log_2$. p values are shown as $-\log_{10}$; p value < 0.05 are coloured; non-significant p values are shown in grey. Student's t-test was used to identify differences between the groups. For visual clarity, only some bacteria and metabolites with $\log_2$ (IBS/H) < −1 and >+1 and p values < 0.05 are labelled. (**D**) Orthogonal partial least squares-discriminant analysis (OPLS-DA) loading column plot based on the discriminatory bacterial taxa (n = 5) and metabolites (n = 16), selected after the least absolute selection and shrinkage operator (LASSO) method with regularization, between IBS patients and healthy subjects. The R^2 value indicates goodness of the fit; the Q^2 value expresses prediction ability. The p value depicts statistical significance based on the cross-validated residuals (CV-ANOVA) test. Error bars indicate 95% confidence intervals. Asterisks represent significant p-values: * < 0.05; ** < 0.01; *** < 0.001.

Table 2. Parameter values for orthogonal partial least squares-discriminant analysis (OPLS-DA) models.

OPLS-DA Model	R^2	Q^2	p Value
IBS vs. Healthy	0.96	0.79	<0.001
IBS vs. Healthy (LASSO) †	0.86	0.78	<0.001
IBS-C vs. IBS-D	0.99	0.56	0.04
IBS-C vs. IBS-nonCnonD	0.46	0.14	0.13
IBS-D vs. IBS-nonCnonD ‡	0.75	−0.26	>0.99
IBS mild vs. IBS moderate §	0.38	−0.005	>0.99
IBS mild vs. IBS severe ‡,§	0.84	−0.02	>0.99
IBS moderate vs. severe §	0.42	0.05	0.52
IBS with anxiety vs. IBS without anxiety ¶	0.72	0.12	0.51

Note: All OPLS-DA models are based on the intestinal microenvironment profile, including 54 microbial taxa and 155 metabolites analysed, unless specified. R^2 value indicates goodness of the fit of the model; Q^2 value describes the prediction ability of the model. The p value depicts statistical significance based on ANOVA of the cross-validated residuals (CV-ANOVA). Abbreviations: IBS-C, IBS with constipation; IBS-D, IBS with diarrhoea; IBS-nonCnonD, mixed-type IBS and unclassified IBS. † IBS vs. healthy (LASSO) model based on 21 selected variables (n = 5 bacterial taxa and n = 16 metabolites). ‡ OPLS-DA model calculated by adding two first forced components. § Based on IBS-SSS, IBS Severity Scoring System. ¶ Based on Hospital Anxiety and Depression Scale.

3.3. The Intestinal Microenvironment Differs between IBS Subgroups

We further explored whether the intestinal microenvironment composition differed between subgroups of IBS patients classified according to the predominant bowel habit, symptom severity and anxiety. A PCA based on the total number of identified faecal microbiota (n = 54) and metabolites (n = 155) showed that IBS-nonCnonD (n = 14) overlapped with IBS-C (n = 15) and IBS-D (n = 11) (Figure 2A), whereas patients with IBS-D clustered separately from IBS-C with significant distance between their centroids ($p < 0.001$) (Figure 2B). The separation between the predominant bowel habit subtypes was supported by an OPLS-DA based on the total number of measured variables (Table 2). Furthermore, a volcano plot identified the variables, mostly metabolites, which contributed to the separation between IBS-D (bacterial taxa; n = 2 and metabolites; n = 27) and IBS-C patients (bacterial taxa; n = 4 and metabolites; n = 5) (Figure 2C). IBS-D patients were defined by higher levels of numerous metabolites, whereas IBS-C patients were associated with higher levels of δ-tocopherol, *Mycoplasma hominins*, Firmicutes A, actinomycetales and *Shigella* sp. and *Escherichia* spp. (Figure 2C).

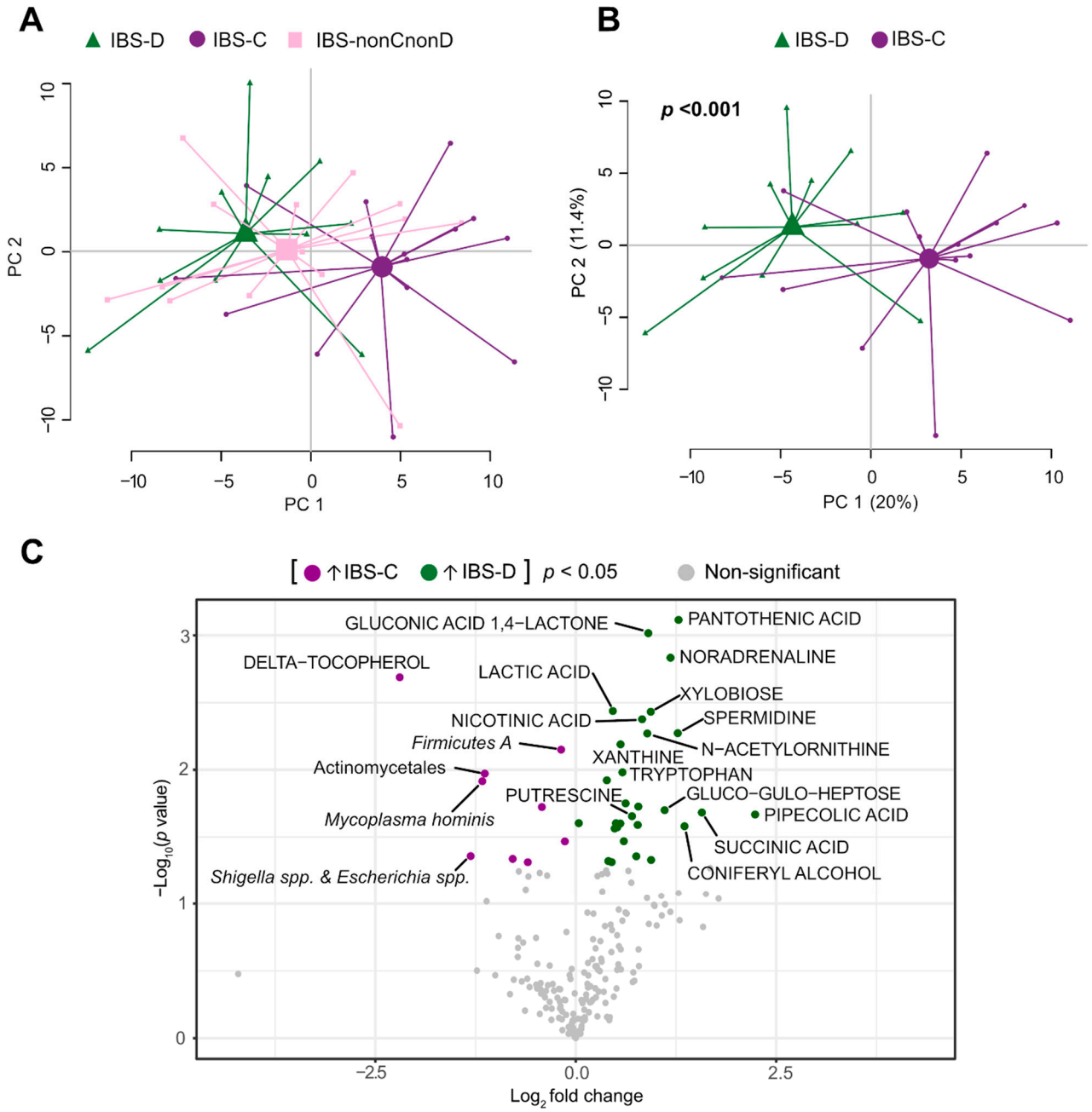

Figure 2. Faecal microbiota and metabolite profiles of IBS patients based on the predominant bowel habit. Principal component analysis (PCA) score scatter plot based on faecal bacteria and metabolites showing (**A**) IBS-D (n = 11, green triangles), IBS-C (n = 15, purple circles) and IBS-nonCnonD (n = 14, pink squares) patients and (**B**) patients with the predominant bowel habit, IBS-D and IBS-C. The p value depicts the statistical significance of the distance between the centroids (scores averages) of the groups. (**C**) Volcano plot illustrating statistical significance versus mean fold change of 54 bacteria (probe intensity) and 155 metabolites (signal intensity) between IBS-D patients respect to IBS-C patients (IBS-D/IBS-C). Each dot corresponds to a bacteria probe or metabolite signal. Variables increased in IBS-D are coloured in green (n = 29), while decreased variables compared to IBS-C are coloured in purple (n = 9). Mean fold change is shown as $\log_2$. p values are shown as $-\log_{10}$; p values < 0.05 are coloured; non-significant p values are shown in grey. Student's t-test was used to identify differences between the groups. Not all variables were labelled, only some bacteria and metabolites with $\log_2$ (IBS/H) < −1 and >+1 and p values < 0.05.

In contrast, the intestinal microenvironment profile did not differ between patients with mild (n = 10), moderate (n = 17) and severe IBS symptoms (n = 13) (Figure 3A, Table 2). Additionally, no clustering, based on the faecal microbiota and metabolites, was seen

between IBS patients with and without anxiety (cut-off HADS anxiety score ≥ 8) (Figure 3B, Table 2). Too few patients met the criteria for borderline or clinically significant depression to allow exploration of the relationship with intestinal microenvironment profile.

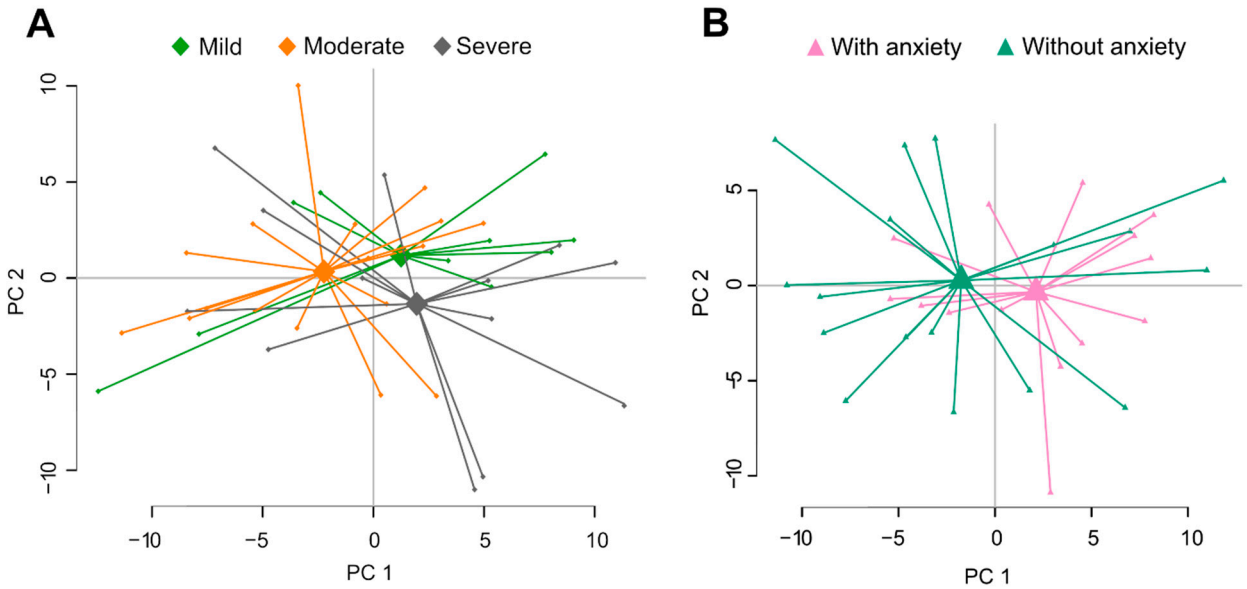

Figure 3. Faecal microbiota and metabolite profiles of IBS patients classified according to symptoms. (**A**) Principal component analysis (PCA) score scatter plot based on faecal bacteria and metabolites showing patients with mild (n = 15, light green), moderate (n = 18, orange) and severe (n = 7, grey) IBS symptoms (IBS-SSS). (**B**) PCA score scatter plot based on faecal bacteria and metabolites showing IBS patients with (n = 17, pink) and without anxiety (n = 14, teal) (based on validated cut-off levels on HAD-A, ≥8).

3.4. Altered Amino Acid Metabolism and Cell Signaling-Related Pathways in IBS Patients

Finally, IPA "core analysis" was performed to explore if the distinct metabolite profile seen in IBS patients could be predicted to have an effect on biological functions. In all, from 500 total biochemical pathways identified with various biological function annotations, 22 biological functions were predicted to be differently regulated in IBS patients as compared to healthy subjects (Figure 4, Table S1). As identified by IPA, several functions related to metabolism, in particular amino acid metabolism, were predicted to be altered in IBS patients, based on upregulation in efflux of L-alanine along with downregulation in the uptake of L-proline and L-alanine, among others. Furthermore, functions such as the growth of bacteria and apoptosis of epithelial cells, included in the categories of proliferation of bacteria and cell death and survival, were predicted to be upregulated in IBS patients. Moreover, the biological functions of cell cycle, cellular growth and molecular transport were predicted to be upregulated in IBS, as seen in Figure 4 and Table S1.

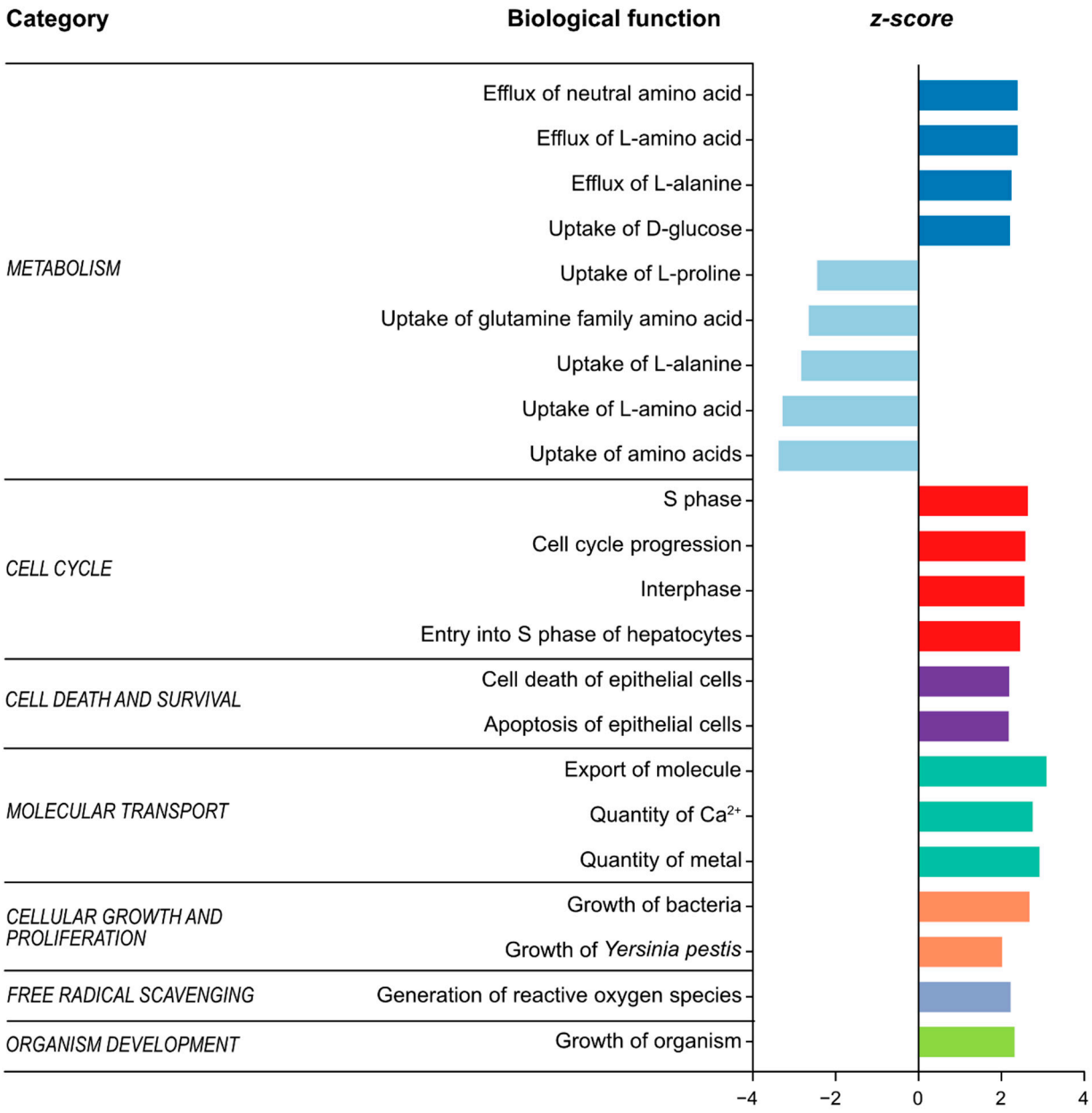

Figure 4. Significantly enriched biological functions predicted to be associated with changes in metabolites in patients with IBS compared to healthy subjects. The bar plot represents 22 biological functions predicted to be altered in IBS patients with significant activation Z-score > 2 or < −2 identified by IPA core analysis. Z-scores > 2 or < −2 indicate predicted decrease or increase, respectively. The analysis used a total of 35 metabolites (p value < 0.05). Each bar denotes a different biological function where each colour corresponds to a simplified category.

4. Discussion

In this study, we demonstrated that IBS patients can be differentiated from healthy subjects based on the intestinal microenvironment, comprising the combined faecal microbiota and metabolite profiles. Additionally, we showed that IBS patients classified according to their predominant bowel habits, but not severity of IBS symptoms or presence/absence of anxiety, can be distinguished on the basis of the intestinal microenvironment. Further analysis suggested that altered amino acid metabolism and certain cell signalling pathways may be involved in the pathophysiology of IBS.

During the last decade, numerous studies have addressed the role of gut microbiota in IBS pathophysiology [13–16]. More recently, reports focusing on intestinal metabolites [17,18],

alongside studies integrating the gut microbiome and metabolome [22,24,25], as well as the host epigenome and transcriptome [26], have explored host–microbiota interactions and the link to GI symptoms in IBS patients. Our current study demonstrates that a combined analysis of faecal microbiota and metabolite profiles separates a mixed group of IBS patients from healthy subjects. This is in agreement with a report by Shankar et al., who previously described this joint classifier to facilitate diagnosis of IBS and differentiate paediatric IBS-D patients from age-matched controls [24]. Furthermore, our study demonstrated that the combined microbiota and metabolite profiling model differentiated between IBS-D and IBS-C patients. This finding is in line with the work by Mars et al., reporting IBS subtype-specific and symptom-related variation in microbial composition and function [26]. Nevertheless, to emphasize the complexity of these analyses and their interpretation, there is also a report demonstrating a lack of differences between IBS subtypes based on the individual microbiota or metabolite profiles [22].

In this study, however, we did not detect differences in the intestinal microenvironment profile between IBS subgroups based on IBS symptom severity. Interestingly, our group previously demonstrated faecal bacteria to be associated with bowel habit-based IBS subtyping, whereas mucosa-associated bacteria were linked to IBS symptom severity [16]. It may therefore be suggested that the link between the intestinal microenvironment profile and bowel habits but not symptom severity in this study was due to the sampling site. Faecal samples, although not fully representative of the entire GI tract, are widely used in studies to reflect the intestinal microenvironment since they are non-invasive and easy to collect, compared to mucosal biopsies. Moreover, the analysis techniques used in this study to determine the microenvironment profile (GA-map^{TM} test and GC-MS/MS) are both relatively easier and quick to perform. This facilitates their use in a clinical setup in comparison to other explorative techniques, e.g., 16S rRNA gene sequencing and LC-MS/MS. The bacterial profiles generated by the commercially available GA-map^{TM} test based on faecal bacterial absolute abundance have the additional advantage of being closely associated with gastrointestinal disorders. Thus, the combination of the accessibility of faecal samples, and these two quick and reliable analyses may constitute an asset for development of non-invasive diagnostic tools.

Altered production or availability of metabolites such as amino acids, short-chain fatty acids, vitamins, bile salts, lipids and organic acids, associated with microbial metabolism or host-microbial co-metabolism, could result in alteration of pathways involved in host physiology [9]. Our study depicts a distinct IBS gut microenvironment characterized by increased amino acid metabolism seen as increase in alanine, glycine and proline, and amino acid metabolism intermediates such as phenylpyruvic acid and pyruvic acid. This may suggest higher epithelial cell turnover processes related to low-grade inflammation such as collagen degradation [42], potential malabsorption of amino acids [23], an increase in protein catabolism by the gut microbiota [25] or even increased efflux of amino acids in IBS patients. Additionally, increased levels of molecules involved in the Krebs cycle such as α-ketoglutaric acid, citric acid and pyruvic acid, but also hypoxanthine and xanthine, which are metabolic forms of purines, known to promote intestinal barrier development [43], were recorded in patients with IBS. This may indicate an altered energy metabolism and proliferation or degradation of bacterial and/or epithelial cells [23,44] in patients with IBS. Hypoxanthine is reported to promote intestinal barrier function and may, as a substrate for oxidase activity, play a role in IBS pathophysiology [43,45]. Indeed, IBS patients have been reported to display altered hypoxanthine levels, although results are inconclusive [18,26]. Still, the as yet scarce and somewhat inconsistent data of hypoxanthine in IBS highlight the complexity of this metabolic pathway, but also suggest that it may be of importance for underlying disease mechanisms. Moreover, our LASSO-extracted variables provided a refined discriminatory model, further implicating the importance of certain metabolic pathways such as the amino acid and purine metabolism. Altogether, our findings suggest that IBS patients may present alterations in their intestinal metabolic function and activity that could play an important role in the pathophysiology of IBS.

Growing evidence further indicates that IBS pathogenesis involves a dysregulated and bidirectional gut-brain interaction where microbiota and microbial metabolites influence neuro-immune crosstalk [1,20,46], and in return, the central nervous system influences the intestinal microenvironment [1]. A recent study demonstrated altered profiles of urine metabolites and faecal bacteria in IBS, and with specific correlations with anxiety and depression [20]. Even though the intestinal microenvironment profile defined in this study could not discriminate patients based on IBS severity or psychological symptoms, the detection of increased abundance of molecules related to neuro-immune signalling, such as tryptophan, ornithine, glycine and glutamic acid, may support an underlying perturbed gut-brain interaction in patients with IBS. Tryptophan, an intermediate metabolite in serotonin metabolism, has been suggested to be essential for the proliferation and effector functions of T cells [47] and the enhancement of serotonin production. Serotonin may regulate the activity of innate immune cells through interaction with receptors in mast cells, dendritic cells and macrophages/monocytes. Furthermore, microbial tryptophan catabolites are known to activate gut immune response via the aryl hydrocarbon receptor [48], potentially strengthening the intestinal barrier integrity [49]. Thus, an altered tryptophan metabolism may contribute to the aberrant host–microbial crosstalk associated with IBS patients. In addition, we also recorded increased levels of ornithine and its precursor glutamic acid in IBS patients. Both molecules are known to participate in the synthesis of polyamines as putrescine, spermidine and spermine, with a suggested importance in lymphocyte activation and development [50]. Moreover, tryptophan, glycine and glutamate which are metabolites involved in amino acid metabolism, have also been associated with IBS-specific brain changes in a recent study integrating functional neuroimaging and faecal metabolites [51]. All in all, these findings support the importance of metabolites in the aberrant brain–gut interaction implicated in the pathophysiology of IBS, warranting further investigations.

The IPA core analysis was carried out to evaluate the direction of the change in the metabolomic profile and predict effects on biological functions in IBS patients compared to healthy subjects. Interestingly, IPA further predicted the uptake of various amino acids to be altered in IBS patients, similarly to studies proposing differences in the ability of IBS patients to metabolize dietary substrates [23]. Additionally, the altered amino acid uptake may be associated with IBS-specific dietary habits suggested to be characterized by an increased protein and dietary fibre intake [52], although studies are inconclusive [53]. Additionally, apoptosis and cell death of epithelial cells were predicted to be increased in patients with IBS, supporting the notion of an impaired epithelial barrier in IBS pathophysiology [6]. It is also important to point out that the interactions between the intestinal microenvironment and the host are complex and multidimensional. Even though our results predict changes in several biological functions potentially associated with IBS disease pathophysiology, further metaproteomic analyses would provide supplementary functional information which would enhance the biological meaning of such distinct intestinal microenvironment. In line with this, a recent study identified peptides from potentially pathogenic *Brachyspira* in a subset of patients with IBS using metaproteomics, as well as described the immune response associated with this infection [54].

Additionally, our results suggest a relationship between bowel habits and the intestinal microenvironment, where a potential shift in microbial function rather than the microbial composition may be of importance and reflect an altered faecal metabolite profile in IBS subtypes. The separation between IBS-D and IBS-C patients was primarily attributed to higher levels of succinic acid, nicotinic acid and xanthine, which are involved in energy metabolism-related pathways. Similar to findings from Lee et al. [18], higher levels of tryptophan were also depicted in IBS-D patients. Tryptophan, as described above, is an intermediate metabolite in serotonin metabolism. Serotonin is known to promote motility, secretion and visceral hypersensitivity in IBS patients with diarrhoea [18,42,55] and, hence, drug development has to a large extent focused on 5-hydroxytryptamine (5-HT) receptor antagonist for symptom management [55]. Moreover, altered levels of trypto-

phan and putrescine observed in IBS-D could be implicated in symptom generation of abdominal pain and discomfort [56]. Overall, the distinct intestinal microenvironment associated with IBS-D patients described in our study is consistent with the current available literature [21,25,26].

While metabolites had greater influence when discriminating between IBS patients and healthy subjects, a few bacterial taxa were identified to be linked to health whereas others were associated with the IBS-specific profile. For instance, IBS patients had lower abundance of *Clostridium* sp. levels, suggested to be relevant in gut health [57]. A previous study has also associated members of Clostridiales including *Clostridium* sp., with GI sensorimotor function in healthy controls, but not in IBS patients [58]. These differences have been suggested to contribute to the altered brain-gut-microbe interaction and pain perception in patients with IBS [58]. Moreover, a higher abundance of Firmicutes, *Escherichia* spp. and *Shigella* sp. and *Mycoplasma homini* was a distinct feature of IBS-C patients in our study. These bacterial taxa have previously been associated with IBS, however not to any specific IBS subtype [14,59,60]. Altogether these results suggest that these different taxa may be associated with the predominant bowel habits in IBS patients.

Previous reports evaluating the effects of faecal microbiota transplantation (FMT) from healthy subjects, described that FMT normalized either microbiota [61] or microbiota together with short-chain fatty acids [62] in IBS-D patients and improved IBS symptoms. Altogether, these studies highlight differences in the intestinal microenvironment of IBS and further support the necessity to account for the role of both microbiota and metabolites when investigating new microbiota-based treatments for IBS management.

Although presenting promising results, this study has its limitations and weaknesses. First of all, human microbiome studies have raised concerns regarding the contribution of age and gender in the human gut microbiota [63]. Nevertheless, in spite of this retrospective study with limited study size was not matched for age and gender, this did not influence our findings. Moreover, dietary habits which can affect both the gut microbiota and metabolite composition [64] were not taken into account. Although this study collected only single time-point samples, longitudinal sampling has been shown to overcome heterogeneity and improve accuracy of results [26], and it would be a beneficial add on for future validation studies. Further, correction for stool water content or even the addition of a second healthy control group with an accelerated transit triggered by laxatives would have been valuable to support the effect of bowel habit on the intestinal microenvironment, as depicted in our study. Despite the limitations of the selected heterogeneous IBS cohort, we could demonstrate that a mixed IBS cohort could be differentiated from healthy subjects based on their intestinal microenvironment. Altogether, our study highlights the importance of the combined microbiota and metabolite profile in improving the ability to separate IBS patients and healthy subjects, and our refined model based on the LASSO selected variables provides possibilities for future explorative studies.

In conclusion, IBS appears to be associated with a shift in microbial function rather than microbial composition, with bowel habits also being reflected in the intestinal microenvironment profile of IBS patients. Accordingly, intestinal microenvironment profiling through measurement of specific faecal microbes and metabolites may be considered as a future non-invasive diagnostic tool alongside providing valuable insights into the pathophysiology of IBS.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cells10061459/s1, Supplementary Methods: Symptom Assessment Questionnaires; and Selection of variables from the intestinal microenvironment profile, Figure S1: Faecal microbiota and metabolite profiles of IBS patients and healthy subjects, Figure S2: Cleveland dot plot representing the distinct combined microbiota and metabolite profile in IBS patients and healthy subjects, Table S1: Significantly enriched functions predicted by Ingenuity Pathway Analysis (IPA) "Core analysis".

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ORIGINAL ARTICLE

Clinical Trials Study

Responses to faecal microbiota transplantation in female and male patients with irritable bowel syndrome

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Author contributions: El-Salhy M designed the study, obtained the funding, administered the study, recruited the patients, performed faecal microbiota transplantation, collected, analyzed and interpreted the data, and drafted the manuscript; Casen C contributed to the design of the study, analyzed the faecal bacteria and critically revised the manuscript for important intellectual content; Valeur J contributed to the design of the study, analyzed the short-chain fatty acids and critically revised the manuscript for important intellectual content; Hausken T and Hatlebakk JG contributed to the design of the study and to the analysis and interpretation of the data, and critically revised the manuscript for important intellectual content.

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Abstract

BACKGROUND

Faecal microbiota transplantation (FMT) seems to be a promising treatment for irritable bowel syndrome (IBS) patients. In Western countries (United States and Europe), there is a female predominance in IBS. A sex difference in the response to FMT has been reported recently in IBS patients.

AIM

To investigate whether there was a sex difference in the response to FMT in the IBS patients who were included in our previous randomized controlled trial of the efficacy of FMT.

METHODS

The study included 164 IBS patients who participated in our previous randomized controlled trial. These patients had moderate-to-severe IBS symptoms belonging to the IBS-D (diarrhoea-predominant), IBS-C (constipation-predominant) and IBS-M (mixed) subtypes, and had not responded to the National Institute for Health and Care Excellence (NICE)-modified diet. They belonged in three groups: placebo (own faeces), and active treated group (30-g or 60-g superdonor faeces). The patients completed the IBS severity scoring system (IBS-SSS), Fatigue Assessment Scale (FAS) and the IBS quality of life scale (IBS-QoL) questionnaires

This study is registered at www.clinicaltrials.gov (NCT03822299) and www.cristin.no (ID657402).

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrolment.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

Data sharing statement: No additional data are available.

CONSORT 2010 statement: The authors have read the CONSORT 2010 statement, and the manuscript was prepared and revised according to the CONSORT 2010 statement.

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at the baseline and 2 wk, 1 mo and 3 mo after FMT. They also provided faecal samples at the baseline and 1 mo after FMT. The faecal bacteria profile and dysbiosis were determined using the 16S rRNA gene polymerase chain reaction DNA amplification covering V3-V9; probe labelling by single nucleotide extension and signal detection. The levels of short-chain fatty acids (SCFAs) were determined by gas chromatography and flame ionization.

RESULTS

There was no sex difference in the response to FMT either in the placebo group or active treated group. There was no difference between females and males in either the placebo group or actively treated groups in the total score on the IBS-SSS, FAS or IBS-QoL, in dysbiosis, or in the faecal bacteria or SCFA level. However, the response rate was significantly higher in females with diarrhoea-predominant (IBS-D) than that of males at 1 mo, and 3 mo after FMT. Moreover, IBS-SSS total score was significantly lower in female patients with IBS-D than that of male patients both 1 mo and 3 mo after FMT.

CONCLUSION

There was no sex difference in the response to FMT among IBS patients with moderate-to-severe symptoms who had previously not responded to NICE-modified diet. However, female patients with IBS-D respond better and have higher reduction of symptoms than males after FMT.

Key Words: Dysbiosis; Fatigue; Microbiome; Quality of life; Short-chain fatty acids; Superdonor

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Core Tip: A sex difference in the response to faecal microbiota transplantation (FMT) was previous reported for a subgroup of refractory irritable bowel syndrome (IBS) patients with severe bloating who had not responded to at least three conventional therapies for IBS. This subgroup only contained patients with diarrhoea-predominant (IBS-D) or mixed (IBS-M) IBS. The present study found no sex difference in the response to FMT among IBS patients with moderate-to-severe symptoms of IBS-D, constipation-predominant (IBS-C) and IBS-M. However, female patients with IBS-D respond better and have higher reduction of symptoms than males after FMT.

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INTRODUCTION

The gut microbiota plays an important role in the pathophysiology of irritable bowel syndrome (IBS)[1,2]. The composition of the gut bacteria in IBS patients differs from that of healthy subjects[2-6]. IBS patients have lower abundances of the butyrate-producing bacteria, *Erysipelotrichaceae* and *Ruminococcaceae* compared with healthy controls[7,8]. Methane-producing bacteria, *Methanobacteriales* were found to be more abundant in IBS patients with constipation as a predominant symptom (IBS-C) and less abundant in IBS patients with diarrhoea as a predominant symptom (IBS-D) compared with healthy individuals[7,8]. Moreover, IBS patients have been found to have increased abundances of *Veillonella*, *Lactobacillus* and *Ruminococcus* bacteria and decreased abundances of *Bifidobacterium*, *Faecalibacterium* and *Erysipelotrichaceae* methanogens[7,8]. IBS patients also have a lower diversity of gut bacteria (dysbiosis) than healthy subjects[4-6,9].

Faecal microbiota transplantation (FMT) has previously been performed in IBS patients in seven randomized controlled trials (RCTs)[10-16]. Four of these RCTs

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showed that FMT had good effects on symptoms and the quality of life[10,12,15,16]. while the other three RCTs found no effects[11,13,14]. It soon became clear that carefully selecting the donor based on clinical and microbial criteria as well as the dose of the transplant are important for a successful outcome of FMT[17].

In Western countries (United States and Europe) there is a sex difference in IBS, with a female:male ratio of 2:1[18-20]. However, in Asia there is no such female predominance[21-24]. A recently published RCT on FMT in IBS found that females responded better to FMT than did males[16]. A recent RCT of IBS patients performed by our group found that FMT led to marked reductions in IBS symptoms and fatigue and an improvement in the quality of life[12]. These improvements were accompanied by marked changes in the faecal bacteria profile and the profile of short-chain fatty acids (SCFAs) of the patients[12,25].

The present study investigated whether there is a sex difference in the response to FMT in terms of symptoms, dysbiosis, and bacteria and SCFA profiles in the same cohort of patients that we had investigated in our previous study[12].

MATERIALS AND METHODS

Study design and randomization of patients

The design of this study has been described in detail previously[12]. In brief, patients completed three questionnaires to assess their symptoms and quality of life at the baseline and 2 wk, 1 mo and 3 mo after FMT. They also provided faecal samples at the baseline and 1 mo after FMT. Polyethylene glycol and loperamide were allowed as rescue medication during the study. The patients were randomized 1:1:1 to placebo (own faeces), 30-g (superdonor faeces) or 60-g (superdonor faeces) FMT[12]. The 30- and 60-g superdonor-faeces groups were pooled together and called the active treated group in order to increase the sample size and reduce the probability of type-II statistical errors.

Patients

This study included 164 patients who had participated in our previous study[12]. The characteristics of these patients are given in Table 1. The patients enrolled in this study have been described in detail previously[12]. In brief, patients attending the outpatient clinic at Stord Hospital who fulfilled the Rome IV criteria for a diagnosis of IBS were recruited. All of the recruited patients had previously not responded to consuming the National Institute for Health and Care Excellence (NICE)-modified diet for at least 3 mo[12]. They also received a course of IBS treatment that slightly improved their symptoms.

The inclusion criteria were being aged between 18 and 75 years and having moderate-to-severe IBS symptoms, as indicated by a score of 175 on the IBS severity scoring system (IBS-SSS). The exclusion criteria were being pregnant or planning pregnancy, lactating, the presence of systemic disease, having immune deficiency or being treated by immune-modulating medication, or having a psychiatric illness, excessive alcohol consumption or drug abuse. Patients who took probiotics, antibiotics or IBS medications within 8 wk prior to study inclusion were also excluded[12].

Donor

The single superdonor used in this study has been described in detail previously[12]. Briefly, he was screened according to the European guidelines for FMT donors[26]. He was a healthy 36-year-old male, non-smoker, not taking any medication regularly and had a normal body mass index. He had been born *via* a vaginal delivery, breastfed and had taken only a few courses of antibiotics during his life. He exercised regularly and took sport-specific dietary supplements, which made his diet richer than average in protein, fibre, minerals and vitamins. He was normobiotic, but his faecal bacteria profile deviated from the healthy subjects abundance in 14 of the 39 bacteria markers[12].

Collection, preparation and administration of faecal samples

Faecal samples were frozen immediately and kept at -20 °C until they were delivered frozen to the laboratory, where they were kept at -80 °C. The process of FMT has been described in detail previously[12]. In brief, the patients randomized to the placebo FMT group received 30 g of their own faeces (autologous), while those in the 30-g and 60-g FMT groups received 30 g and 60 g of the superdonor's faeces (allogenic),

Table 1 Characteristics of the patients in the placebo and active treated groups

	Placebo				Active treated			
	Total	Females	Males	*P* value	Total	Females	Males	*P* value
n	**55**	**47**	**8**		**109**	**85**	**24**	
Age, yr (median, range)	38.5 (18-75)	38.0 (18-73)	47.0 (20-75)	0.3	39.0 (18-73)	40.0 (18-73)	32.0 (21-65)	0.07
IBS-D	21	19	2	0.7	42	30	12	0.4
IBS-C	22	18	4		40	32	8	
IBS-M	12	10	2		27	23	4	
IBS duration, yr	15.5 ± 7.9	16.2 ± 8.0	15.0 ± 9.0	0.9	17.3 ± 8.9	16.8 ± 8.2	18.0 ± 9.2	0.9
Age at IBS onset, yr (median, range)	20.0 (15-35)	20.5 (16-35)	19.0 (15-30)	0.4	20.0 (15-36)	20.0 (16-35)	20 (15-33)	0.6
IBS-SSS total score	315.2 ± 77.1	320.1 ± 77.8	286.9 ± 69.3	0.5	312.9 ± 82.0	319.1 ± 77.3	297.7 ± 82.0	0.4
Moderate symptoms[1] (%)	23 (42)	17 (36)	6 (75)	0.06	45 (41)	30 (35)	13 (54)	0.1
Severe symptoms[2] (%)	32 (58)	30 (64)	2 (25)		64 (59)	55 (65)	11 (46)	

Data are *n*, *n* (%) or mean ± SD values.

[1]Irritable bowel syndrome severity scoring system total score between 175 and 300.

[2]Irritable bowel syndrome severity scoring system total score of ≥ 300. IBS: Irritable bowel syndrome; IBS-D: Irritable bowel syndrome with diarrhoea-predominant; IBS-C: Irritable bowel syndrome with constipation-predominant; IBS-M: Irritable bowel syndrome with mixed diarrhoea and constipation; IBS-SSS: Irritable bowel syndrome severity scoring system.

respectively. The transplant was administered to the distal duodenum *via* a gastroscope[12].

Symptom and quality-of-life assessments

Symptoms were assessed using the IBS-SSS and the Fatigue Assessment Scale (FAS)[27-31]. Quality of life was measured using the IBS quality of life scale (IBS-QoL)[32-34]. Response was defined as a decrease of ≥ 50 points in the IBS-SSS total score after FMT.

Microbiome analysis and dysbiosis index

The faecal bacteria profile and dysbiosis were determined by the GA-map Dysbiosis Test (Genetic Analysis, Oslo, Norway) using the 16S rRNA gene polymerase chain reaction DNA amplification covering V3-V9; probe labelling by single nucleotide extension and signal detection by BioCode 1000A 128-Plex Analyzer (Applied BioCode, Santa Fe Springs, CA, United States)[6]. The bacterial markers used detected bacteria within 5 phyla (Firmicutes, Proteobacteria, Bacteroidetes, Tenericutes and Verrucomicrobia) that cover 10 bacterial classes, 36 genera and 32 species[6]. This test assesses > 300 bacteria at different taxonomic levels[9]. The dysbiosis index (DI) was measured on a 5-point scale from 1 to 5, where DI values 1-2 indicates normobiosis, 3-5 indicates dysbiosis[6].

Determination of faecal SCFA levels

The method used to determine faecal SCFA levels has been described in detail previously[25]. Briefly, the faecal samples were homogenized with a solution containing 3 mmol/L 2-ethylbutyric acid and 0.5 mmol/L H_2SO_4. The homogenate was vacuum distilled, and the SCFA levels were determined by gas chromatography (Agilent 7890 A, Agilent, CA, United States) using a capillary column (serial no. USE400345H, Agilent J&W GC columns, Agilent) and flame ionization[35,36] levels of total SCFAs, acetic, propionic, iso-butyric, n-butyric, iso-valeric, n-valeric acid, isocapronic and n-capronic acids, were determined and were expressed in units of mmol/kg wet weight.

Statistical analysis

The sample size required in each arm of the previously published trial was calculated by assuming that a placebo effect was 40% and an effect response was 80%. The total

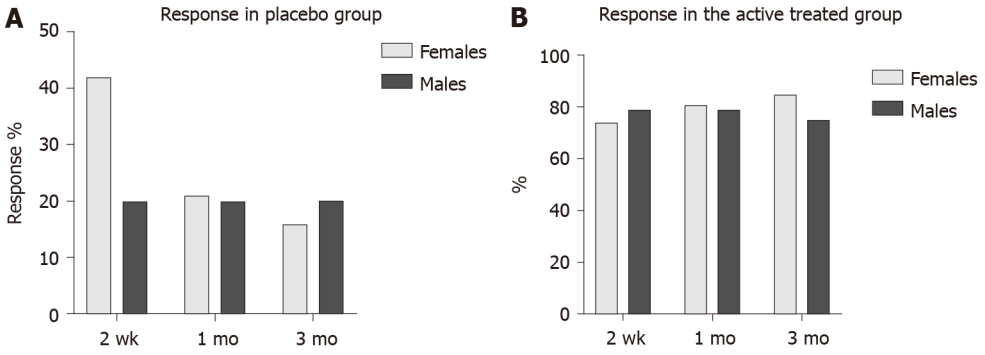

Figure 1 Response to faecal microbiota transplantation in females and males at different intervals after faecal microbiota transplantation in the two groups. A: Placebo group; B: Active treated group.

sample size was estimated to be 60 patients, with 20 in each arm ($\alpha = 0.05$, $1\text{-}\beta = 0.80$)[12]. In the present study a new calculation for the sample size was done based on the response rates obtained from our previous RCT[12]. Thus, assuming that the females' response is 90% and males' response is 60%, The total sample size was estimated to be 22 with 11 females and 11 males ($\alpha = 0.05$, $1\text{-}\beta = 0.80$). The 30- and 60-g superdonor-faeces groups were pooled together and called the active treated group in order to increase the sample size and reduce the probability of type-II statistical errors. Differences in response and dysbiosis between females and males in the placebo and the active treated group were analyzed using the χ^2 test. Differences between females and males in the total scores on the IBS-SSS, FAS and IBS-QoL, and in faecal bacteria and SCFA levels were analyzed using the Mann-Whitney test. These analyses were performed using GraphPad Prism (version 8, La Jolla, CA, United States).

Ethics

The Regional Committee for Medical and Health Research Ethics West, Bergen, Norway approved the study (approval No. 2017/1197/REK vest). All subjects provided both oral and written consents to participate. The study was registered at www.clinicaltrials.gov (NCT03822299) and www.cristin.no (ID657402).

RESULTS

Symptom and quality-of-life assessments

In the placebo group, the response did not differ between females and males at 2 wk, 1 mo and 3 mo after FMT ($P = 0.4$, 0.9 and 0.8, respectively). The responses in the active treated group did not differ between females and males after 2 wk, 1 mo and 3 mo ($P = 0.6$, 0.8 and 0.3, respectively) (Figure 1). The response rate was significantly higher in females with IBS-D than that of males at 1 mo, and 3 mo after FMT (Table 2 and Figure 2). There was no significant difference of response rates between female and male patients with either moderate or severe IBS symptoms (Table 3 and Figure 3).

The IBS-SSS total score did not differ significantly between female and male IBS patients in either the placebo or the active treated group (Table 4 and Figure 4). However, IBS-SSS total score was significantly lower in female patients with IBS-D than that of male patients both 1 mo and 3 mo after FMT (Table 5 and Figure 5). The IBS-SSS total score did not differ significantly between females and males in patients with moderate or severe IBS symptoms (Table 6 and Figure 3).

The FAS total score also did not differ significantly between female and male IBS patients in the active treated group (Table 7 and Figure 6), but it was lower in males than females in the placebo group at 3 mo after FMT. This could have been due to a type-I statistical error. There was no significant difference between female and male IBS patients belonging to different IBS-subtypes IBS symptoms (Table 8 and Figure 7). However, the FAS total score was lower in males IBS patients with IBS-D than that of females 2 wk after FMT.

The IBS-QoL total score did differ between females and males in both the placebo and active treated groups (Table 9 and Figure 8), being higher in males than in females at the baseline. IBS-QoL total scores did not differ significantly between female and male patients belonging to different IBS-subtypes (Table 10 and Figure 9).

Table 2 The response rates of females and males in different irritable bowel syndrome-subtypes at different intervals after faecal microbiota transplantation

Time after FMT	IBS-D			IBS-C			IBS-M		
	Females	Males	*P* value	Females	Males	*P* value	Females	Males	*P* value
2 wk (%)	73	58	0.3	65	50	0.7	72	55	0.3
1 mo (%)	90	42	**0.0003**	69	75	0.7	65	60	0.9
3 mo (%)	90	42	**0.0003**	70	75	0.3	63	80	0.6

IBS-D: Irritable bowel syndrome with diarrhoea-predominant symptom; IBS-C: Irritable bowel syndrome with constipation-predominant symptom; IBS-M: Irritable bowel syndrome with mixed diarrhoea and constipation; FMT: Faecal microbiota transplantation.

Table 3 The response rates in females and males with either moderate or severe irritable bowel syndrome symptoms

Time after FMT	Moderate symptoms[1]			Severe symptoms[2]		
	Females	Males	*P* value	Females	Males	*P* value
2 wk (%)	58	61	0.999	78	91	0.4
1 mo (%)	61	61	0.999	78	91	0.4
3 mo (%)	63	56	0.778	77	82	0.999

[1]Irritable bowel syndrome severity scoring system total score between 175 and 300.
[2]Irritable bowel syndrome severity scoring system total score of ≥ 300. FMT: Faecal microbiota transplantation.

Table 4 Irritable bowel syndrome severity scoring system total scores of females and males in the two study groups at different times after faecal microbiota transplantation

Time	Placebo		*P* value	Active treated		*P* value
	Females	Males		Females	Males	
0	320 ± 78	287 ± 69	0.2	319 ± 77	297 ± 82	0.3
2 wk	254 ± 106	256 ± 90	0.9	199 ± 102	205 ± 95	0.6
1 mo	277 ± 98	272 ± 89	0.8	196 ± 108	193 ± 94	0.9
3 mo	288 ± 90	266 ± 100	0.6	173 ± 116	183 ± 105	0.5

Data are mean ± SD values.

Microbiome analysis

The faecal bacteria levels in the placebo group did not differ between female and male IBS patients at the baseline and 1 mo after FMT (Table 11 and Figure 10). Similarly, there were no differences in the faecal bacteria levels between female and male IBS patients in the active treated group (Table 12 and Figure 11).

In the placebo group, 26 females (55%) and 4 males (50%) had dysbiosis ($P = 0.8$) at the baseline, while 25 females (53%) and 4 males (50%) had dysbiosis ($P = 0.9$) at 1 mo after FMT. In the active treated group, 52 females (61%) and 13 males (54%) had dysbiosis ($P = 0.3$) at the baseline, while 41 females (48%) and 9 males (38%) had dysbiosis ($P = 0.2$) at 1 mo after FMT.

Faecal SCFA levels

The faecal levels of total SCFAs and acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocapronic and capronic acids did not differ between female and males IBS patients in both the placebo and active treated groups at the baseline and 1 mo after FMT (Table 13 and Figure 12).

Table 5 The irritable bowel syndrome severity scoring system total scores in females and males belonging to different irritable bowel syndrome-sub-types

Time after FMT	IBS-D			IBS-C			IBS-M		
	Females	Males	*P* value	Females	Males	*P* value	Females	Males	*P* value
2 wk	190.5 ± 191.4	204.0 ± 92.2	0.6	228.1 ± 116.2	239.2 ± 113.8	0.5	202.8 ± 121.3	225.0 ± 65.8	0.5
1 mo	177.8 ± 94.9	226.9 ± 73.3	**0.02**	228.8 ± 118.1	215.8 ± 115.7	0.6	219.9 ± 136.6	197.0 ± 65.2	0.8
3 mo	157.8 ± 102.9	212.3 ± 96.9	**0.03**	212.8 ± 124.0	234.6 ± 131.8	0.5	219.2 ± 146.3	149.0 ± 36.0	0.5

Data are mean ± SD values. IBS-D: Irritable bowel syndrome with diarrhoea-predominant symptom; IBS-C: Irritable bowel syndrome with constipation-predominant symptom; IBS-M: Irritable bowel syndrome with mixed diarrhoea and constipation; FMT: Faecal microbiota transplantation.

Table 6 Irritable bowel syndrome severity scoring system total scores in females and males with moderate or severe irritable bowel syndrome symptoms

Time after FMT	Moderate symptoms[1]			Severe symptoms[2]		
	Females	Males	*P* value	Females	Males	*P* value
2 wk	166.5 ± 70.0	167.2 ± 55.8	0.8	225.1 ± 114.0	259.5 ± 102.3	0.3
1 mo	162.1 ± 73.1	179.4 ± 63.8	0.5	229.2 ± 120.2	214.1 ± 118.1	0.7
3 mo	155.3 ± 76.9	175.3 ± 100.2	0.5	214.6 ± 131.8	202.3 ± 120.6	0.8

Data are mean ± SD values.
[1]Irritable bowel syndrome severity scoring system total score between 175 and 300.
[2]Irritable bowel syndrome severity scoring system total score of ≥ 300. FMT: Faecal microbiota transplantation.

Table 7 Fatigue Assessment Scale total scores of females and males in the two study groups at different times after faecal microbiota transplantation

Time	Placebo			Active treated		
	Females	Males	*P* value	Females	Males	*P* value
0	31 ± 5	29 ± 4	0.3	32 ± 5	30 ± 5	0.09
2 wk	31 ± 6	29 ± 6	0.2	28 ± 6	28 ± 5	0.5
1 mo	31 ± 6	27 ± 7	0.1	27 ± 7	29 ± 5	0.5
3 mo	30 ± 4	26 ± 4	**0.01**	29 ± 6	27 ± 5	0.7

Data mean ± SD values.

DISCUSSION

The present study found that the response to FMT did not differ between females and males. Furthermore, the total scores on the IBS-SSS, FAS and IBS-QoL did not differ between females and males in the active treated groups before FMT and at different times after FMT. In the placebo group, the total score of IBS-QoL was higher in males than males and the FAS total score was lower in males than females 3 mo after FMT. This indicates that the effects of active treated FMT did not differ between males and females regarding IBS symptoms, fatigue and quality of life. Moreover, there was no difference between females and males regarding dysbiosis or the faecal bacteria.

SCFA profiles following FMT did not differ between females and males in both the placebo and the active treated groups. SCFAs regulate intestinal motility and the secretion and absorption of water and electrolytes[37,38]. Moreover, SCFAs increase also the secretion and up-regulate the gene expression of peptide YY (PYY)[39,40]. PYY is a mediator of the ileal brake and stimulates the absorption of water and electrolytes in the colon[37]. The faecal level of total SCFAs increased significantly in IBS patients after 1 mo and remained elevated at 1 year after FMT (unpublished

Table 8 Fatigue Assessment Scale total scores of females and males irritable bowel syndrome patients belonging to different irritable bowel syndrome-subtypes

Time after FMT	IBS-D			IBS-C			IBS-M		
	Females	Males	*P* value	Females	Males	*P* value	Females	Males	*P* value
2 wk	30.1 ± 3.6	27.0 ± 3.6	**0.04**	28.0 ± 6.3	29.3 ± 7.4	0.5	26.7 ± 5.4	26.3 ± 2.1	0.9
1 mo	27.1 ± 5.2	26.6 ± 4.6	0.6	27.1 ± 6.7	31.3 ± 6.1	0.1	28.3 ± 8.3	28.3 ± 4.0	0.9
3 mo	27.5 ± 5.7	27.8 ± 5.0	0.4	26.0 ± 6.2	29.2 ± 5.0	0.2	26.8 ± 7.6	24.8 ± 2.2	0.7

Data are mean ± SD values. IBS-D: Irritable bowel syndrome with diarrhoea-predominant symptom; IBS-C: Irritable bowel syndrome with constipation-predominant symptom; IBS-M: Irritable bowel syndrome with mixed diarrhoea and constipation; FMT: Faecal microbiota transplantation.

Table 9 Irritable bowel syndrome quality of life scale total scores of females and males in the two study groups at different times after faecal microbiota transplantation

Time	Placebo			Active treated		
	Females	Males	*P* value	Females	Males	*P* value
0	116 ± 20	130 ± 11	**0.03**	111 ± 23	114 ± 21	0.9
2 wk	123 ± 29	120 ± 23	0.7	122 ± 24	118 ± 27	0.6
1 mo	123 ± 26	121 ± 26	0.8	126 ± 24	119 ± 29	0.4
3 mo	112 ± 24	118 ± 26	0.2	132 ± 23	131 ± 25	0.9

Data are mean ± SD values.

Table 10 Irritable bowel syndrome quality of life scale total scores of females and males irritable bowel syndrome patients belonging to different irritable bowel syndrome-subtypes

Time after FMT	IBS-D			IBS-C			IBS-M		
	Females	Males	*P* value	Females	Males	*P* value	Females	Males	*P* value
2 wk	123.3 ± 98	123.7 ± 25.5	0.8	120.5 ± 23.1	102.4 ± 28.0	0.06	123.1 ± 24.6	131.5 ± 13.0	0.4
1 mo	131.3 ± 20.8	129.6 ± 28.2	0.9	121.9 ± 24.4	111.4 ± 29.1	01	125.4 ± 25.2	130.5 ± 11.3	0.8
3 mo	136.4 ± 16.6	134.5 ± 22.7	0.9	128.7 ± 24.4	129.1 ± 31.3	0.6	129.5 ± 28.6	124.8 ± 22.0	0.5

Data are mean ± SD values. IBS-D: Irritable bowel syndrome with diarrhoea-predominant symptom; IBS-C: Irritable bowel syndrome with constipation-predominant symptom; IBS-M: Irritable bowel syndrome with mixed diarrhoea and constipation; FMT: Faecal microbiota transplantation.

data)[25]. Similarly, the faecal level of butyric acid increased in IBS patients after 1 mo and remained elevated at 1 year after FMT (unpublished data)[25]. Butyrate is a major energy source for colonic epithelial cells, interacts with the immune response, modulates the oxidative stress, and decreases both intestinal-cell permeability and intestinal motility[41]. Butyrate modulates also colonic hypersensitivity[42-44]. At 1 year after FMT levels of isobutyric and isovaleric acids were increased in IBS patients, indicating a shift in microbial fermentation from a saccharolytic to a proteolytic pattern[45]. It is worthy of note that in IBS patients, who adhered to a low-FODMAPs (fermentable oligosaccharides, disaccharides, monosaccharides, and polyols) diet an increase in the levels of isobutyric and isovaleric acids have been found[46]. Moreover, the level of acetic acid which induces visceral hypersensitivity decreased significantly at 1 year after FMT[47].These changes in SCFAs after FMT appear to be one of the mechanisms underlying the effects seen in IBS patients after FMT. That is why the difference between females and males regarding the changes in SCFAs was assessed.

Holvoet *et al*[16] reported that females responded better to FMT than did males. That RCT differed from the present study in terms of the characteristics of the included patients, the size of the patient cohort and the dose of the faecal transplants[16]. The trial of Holvoet *et al*[16] included a subgroup of refractory IBS

Table 11 Faecal bacteria levels in the female and male irritable bowel syndrome patients in the placebo group at the baseline and 1 mo after faecal microbiota transplantation

Bacteria	Baseline		1 mo after FMT	
	Females	Males	Females	Males
Actinobacteria	-0.235 ± 0.763	-0.365 ± 0.768	-0.250 ± 0.954	-0.375 ± 0.838
Actinomycetales	0.118 ± 0.382	-0.212 ± 0.536	0.175 ± 0.594	0.100 ± 0.496
Bifidobacterium spp.	-0.020 ± 0.607	-0.154 ± 0.539	0.025 ± 0.660	-0.075 ± 0.572
Alistipes	-0.863 ± 0.895	-0.885 ± 0.900	-0.875 ± 0.853	-0.800 ± 0.709
Alistipes onderdonkii	-0.667 ± 0.792	-0.615 ± 0.718	-0.650 ± 0.834	-0.550 ± 0.783
Bacteroides fragilis	-0.255 ± 0.689	-0.212 ± 0.637	0.175 ± 0.501	0.050 ± 0.221
Bacteroides spp. and *Prevotella spp.*	-0.980 ± 1.157	-0.885 ± 1.182	-0.750 ± 1.032	-0.900 ± 0.687
Bacteroides stercoris	-0.137 ± 0.448	-0.154 ± 0.415	0.025 ± 0.158	0.100 ± 0.304
Bacteroides zoogleoformans	0.078 ± 0.272	0.038 ± 0.194	0.025 ± 0.158	0 ± 0
Parabacteroides johnsonii	0.039 ± 0.196	0.077 ± 0.269	0.050 ± 0.221	0.100 ± 0.304
Parabacteroides spp.	-0.451 ± 0.642	-0.327 ± 0.706	-0.425 ± 0.747	-0.225 ± 0.480
Firmicutes	-0.431 ± 0.575	-0.385 ± 0.566	-0.325 ± 0.526	-0.400 ± 0.591
Bacilli	0.235 ± 1.124	0.192 ± 0.991	0.271 ± 1.132	0.150 ± 1.001
Catenibacterium mitsuokai	0.000 ± 0.400	0.135 ± 0.525	0.050 ± 0.289	0.100 ± 0.441
Clostridia	-0.020 ± 0.244	-0.077 ± 0.269	-0.025 ± 0.276	0.0 ± 0.036
Clostridium spp.	0.039 ± 0.196	0.038 ± 0.194	0.0 ± 0.0	0.050 ± 0.316
Dialister invisus	0.118 ± 0.381	-0.173 ± 0.474	0.200 ± 0.405	0.225 ± 0.158
Dialister invisus and *Megasphaera micronuciformis*	0.059 ± 0.238	0.173 ± 0.474	0.125 ± 0.335	0.025 ± 0.158
Dorea spp.	0.569 ± 0.700	0.500 ± 0.700	0.625 ± 0.628	0.667 ± 0.806
Eubacterium biforme	0.412 ± 0.753	0.269 ± 0.598	0.275 ± 0.640	0.400 ± 0.633
Eubacterium hallii	0.804 ± 0.939	0.673 ± 0.879	0.655 ± 0.730	0.650 ± 0.597
Eubacterium rectale	0.078 ± 0.337	0.058 ± 0.235	0.050 ± 0.221	0.025 ± 0.158
Eubacterium siraeum	-1.412 ± 0.963	-1.288 ± 0.161	-1.475 ± 1.086	-1.200 ± 1.265
Faecalibacterium prausnitzii	-0.431 ± 0.671	-0.500 ± 0.804	-0.550 ± 0.745	-0.500 ± 0.599
Lachnospiraceae	0.196 ± 0.566	0.269 ± 0.630	0.325 ± 0.730	0.275 ± 0.640
Lactobacillus ruminis and *Pediococcus acidilactici*	0.059 ± 0.311	0.077 ± 0.334	0.0 ± 0.0	0.025 ± 0.158
Lactobacillus spp.	0.353 ± 0.594	0.269 ± 0.528	0.325 ± 0.616	0.475 ± 0.680
Phascolarctobacterium spp.	0.078 ± 0337	0.077 ± 0.337	0.125 ± 0.404	0.075 ± 0.350
Ruminococcus albus and *Ruminococcus bromii*	0.353 ± 0.658	0.404 ± 0.721	0.325 ± 0.616	0.450 ± 0.749
Ruminococcus gnavus	0.431 ± 0.878	0.577 ± 0.878	0.450 ± 0.815	0.325 ± 0.764
Streptococcus agalactiae & Eubacterium rectale	0.157 ± 0.367	0.250 ± 0.480	0.110 ± 0.304	0.125 ± 0.345
Streptococcus salivarius ssp. Thermophilus and *Streptococcus sanguinis*	0.412 ± 0.606	0.346 ± 0.556	0.675 ± 0.888	0.475 ± 0.751
Streptococcus salivarius ssp. thermophilus	0.628 ± 0.871	0.577 ± 0.915	0.500 ± 0.934	0.600 ± 0.928
Streptococcus spp.	0.471 ± 0.833	0.423 ± 0.696	0.400 ± 0.709	0.450 ± 0.815
Veillonella spp.	-0.177 ± 0.518	-0.173 ± 0.648	-0.175 ± 0.385	-0.150 ± 0.534
Proteobacteria	0.294 ± 0.576	0.289 ± 0.499	0.275 ± 0.599	0.325 ± 0.616
Shigella spp. and *Escherichia spp.*	-0.275 ± 0.940	-0.212 ± 0.893	-0.200 ± 0.853	-0.335 ± 0.920
Mycoplasma hominis	-0.451 ± 0.503	-0.404 ± 0.496	-0.450 ± 0.504	-0.450 ± 0.503

Akkermansia muciniphila	0.471 ± 0.644	0.365 ± 0.627	0.450 ± 0.714	0.650 ± 0.802

The bacterial levels are mean ± SD relative values to a normobiotic microbiota profile of 165 healthy subjects. FMT: Faecal microbiota transplantation.

patients with severe bloating who had not responded to at least three conventional therapies for IBS. This subgroup contained only patients with the IBS-D or mixed (IBS-M) subtypes. The patients included in the present study had moderate-to-severe IBS symptoms belonging to the IBS-D, IBS-C and IBS-M subtypes, and had not responded to the NICE-modified diet. The patient cohort investigated by Holvoet *et al*[16] included 62 patients: 19 in the placebo group and 43 in the active treated group. The present study investigated a cohort of 164 patients: 55 in the placebo group and 109 in the active treated group. It is worthy of note that in the trial of Holvoet *et al*[16] included 30 females and 13 males in the active treated group and 8 females and 11 males in the placebo group. The present study included 85 females and 24 males in the active treated group and included 47 females and 8 males in the placebo group. Thus, this makes the present study less constrained than the RCT of Holvoet *et al*[16] regarding power and sample sizes. Moreover, the dose of the faecal transplant from the donor was not reported for the RCT by Holvoet *et al*[16], while in the present study the active treated group received either 30 g or 60 g of a superdonor transplant. In our previously published RCT we showed that the response rates increased with increased dose[12]. These differences make it difficult to compare the outcomes of the present study with those of Holvoet *et al*[16]. However, in the present study, female patients with IBS-D had a significant higher response rate to FMT and lower IBS-SSS score after FMT than males. These observations could explain the discrepancy between the findings of Holvoet *et al*[16] and the present study as in Holvoet *et al*[16] study the cohort of patients included were only IBS-D and IBS-M IBS-subtypes.

CONCLUSION

In conclusion, there is no sex difference in the response to FMT in IBS patients with moderate-to-severe IBS symptoms belonging to the three of IBS subtypes of IBS-C and IBS-M in patients who did not responded to NICE-modified diet. Female patients with IBS-D had a significant higher response rate to FMT and lower IBS-SSS score after FMT than males.

Table 12 Gut bacteria levels in female and male irritable bowel syndrome patients in the active treated group at the baseline and 1 mo after faecal microbiota transplantation

Bacteria	Baseline		1 mo after FMT	
	Females	Males	Females	Males
Actinobacteria	-0.250 ± 0.954	-0.375 ± 0.838	-0.250 ± 0.719	-0.2350 ± 0.636
Actinomycetales	0.175 ± 0.594	0.100 ± 0.496	0.068 ± 0255	0.145 ± 0.412
Bifidobacterium spp.	0.025 ± 0.660	-0.075 ± 0.572	-0.045 ± 0.526	-0.063 ± 0.433
Alistipes	-0.875 ± 0.853	-0.800 ± 0.709	-0.886 ± 0.869	-0.783 ± 0.821
Alistipes onderdonkii	-0.650 ± 0.834	-0.550 ± 0.783	-0.523 ± 0.699	-0.354 ± 0.565
Bacteroides fragilis	0.175 ± 0.501	0.050 ± 0.221	0.159 ± 0.480	0.104 ± 0.371
Bacteroides spp. and *Prevotella spp.*	-0.750 ± 1.032	-0.800 ± 0.687	-1.091 ± 1.996	-0.708 ± 0.967
Bacteroides stercoris	0.025 ± 0.158	0.100 ± 0.304	0.023 ± 0.151	0.146 ± 0.357
Bacteroides zoogleoformans	0.025 ± 0.158	0 ± 0	0.091 ± 0.291	0.083 ± 0.347
Parabacteroides johnsonii	0.050 ± 0.221	0.100 ± 0.304	0.045 ± 0.302	0.021 ± 0.144
Parabacteroides spp.	-0.425 ± 0.747	-0.225 ± 0.480	-0.455 ± 0.504	-0.313 ± 0.468
Firmicutes	-0.325 ± 0.526	-0.400 ± 0.591	-0.546 ± 0.627	-0.454 ± 0.483
Bacilli	0.271 ± 1.132	0.150 ± 1.001	0.205 ± 1.047	0.042 ± 0.824
Catenibacterium mitsuokai	0.050 ± 0.289	0.100 ± 0.441	0.023 ± 0.151	0.104 ± 0.515
Clostridia	-0.025 ± 0.276	0.0 ± 0.036	0.068 ± 0.255	0.021 ± 252
Clostridium spp.	0.0 ± 0.0	0.050 ± 0.316	0.223 ± 0.151	0.063 ± 0.245
Dialister invisus	0.200 ± 0.405	0.225 ± 0.158	0.091 ± 0.362	0.146 ± 0.505
Dialister invisus and *Megasphaera micronuciformis*	0.125 ± 0.335	0.025 ± 0.158	0.068 ± 0.034	0.104 ± 0.308
Dorea spp.	0.625 ± 0.628	0.667 ± 0.806	0.727 ± 0.758	0.663 ± 0.796
Eubacterium biforme	0.275 ± 0.640	0.400 ± 0.633	0.477 ± 0.791	0.563 ± 0.769
Eubacterium hallii	0.655 ± 0.730	0.550 ± 0.597	0.886 ± 0.993	0.979 ± 1.021
Eubacterium rectale	0.050 ± 0.221	0.025 ± 0.158	0-068 ± 0.255	0.042 ± 0.202
Eubacterium siraeum	-1.475 ± 1.086	-1.200 ± 1.265	-1.295 ± 0.930	-1.208 ± 0.988
Faecalibacterium prausnitzii	-0.550 ± 0.745	-0.500 ± 0.599	-0.568 ± 0.759	-0.521 ± 0.825
Lachnospiraceae	0.325 ± 0.730	0.275 ± 0.640	0.205 ± 0.553	0.125 ± 0.489
Lactobacillus ruminis and *Pediococcus acidilactici*	0.0 ± 0.0	0.025 ± 0.158	0.021 ± 0.146	0.188 ± 0.571
Lactobacillus spp.	0.325 ± 0.616	0.475 ± 0.680	0.500 ± 0.731	0.583 ± 0.679
Phascolarctobacterium spp.	0.125 ± 0.404	0.075 ± 0.350	0.091 ± 0.362	0.083 ± 0.347
Ruminococcus albus and *Ruminococcus bromii*	0.325 ± 0.616	0.450 ± 0.749	0.205 ± 0.553	0.271 ± 0.574
Ruminococcus gnavus	0.450 ± 0.815	0.325 ± 0.764	0.364 ± 0.810	0.250 ± 0.636
Streptococcus agalactiae & Eubacterium rectale	0.110 ± 0.304	0.125 ± 0.345	0.267 ± 0.495	0.083 ± 0.279
Streptococcus salivarius ssp. thermophilus and *Streptococcus sanguinis*	0.675 ± 0.888	0.475 ± 0.751	0.455 ± 0.504	0.292 ± 0.459
Streptococcus salivarius ssp. thermophilus	0.500 ± 0.934	0.600 ± 0.928	0.523 ± 0.821	0.604 ± 0.844
Streptococcus spp.	0.400 ± 709	0.450 ± 0.815	0.444 ± 0.841	0.396 ± 0.610
Veillonella spp.	-0.175 ± 0.385	-0.150 ± 0.534	-0.273 ± 0.544	-0.208 ± 0.504
Proteobacteria	0.275 ± 0.599	0.325 ± 0.616	0.717 ± 0.750	0.583 ± 0.498
Shigella spp. and *Escherichia spp.*	-0.200 ± 0.853	-0.335 ± 0.920	-0.151 ± 1.077	-0.188 ± 0.790
Mycoplasma hominis	-0.450 ± 0.504	-0.450 ± 0.503	-0.500 ± 0.506	-0.479 ± 0.505

Akkermansia muciniphila	0.450 ± 0.714	0.650 ± 0.802	0.741 ± 0.713	0.813 ± 0.915

The bacterial levels are relative values to a normobiotic microbiota profile of 165 healthy subjects (mean ± SD). FMT: Faecal microbiota transplantation.

Table 13 The short-chain fatty acids concentration in the faeces of the placebo group and the patients that received donor's faecs (faecal microbiota transplantation) at the baseline and 1 mo after faecal microbiota transplantation

Acids	Placebo				Active treated			
	Baseline		1 mo after FMT		Baseline		1 mo after FMT	
	Females	Males	Females	Males	Females	Males	Females	Males
Total SCFAs	72 ± 37	69 ± 23.	73 ± 37	69 ± 23	77 ± 40	72 ± 40	87 ± 42	89 ± 26
Acetic acid	42 ± 18	40 ± 15	41 ± 17	40 ± 14	44 ± 21	44 ± 20	46 ± 13	40.2 ± 15.0
Propionic acid	12 ± 8	11 ± 5	12 ± 8	11 ± 5	13 ± 10	13 ± 8	14 ± 4	11 ± 7
Iso-butyric acid	2 ± 2	1 ± 1	1 ± 2	1 ± 1	1 ± 1	1 ± 1	2 ± 2	1 ± 1
Butyric acid	14 ± 9	12 ± 6	13 ± 8	12 ± 6	11 ± 8	12 ± 9	18 ± 14	16 ± 10
Iso-valeric acid	2 ± 2	2 ± 1	2 ± 2	2 ± 1	2 ± 1	2 ± 2	2 ± 2	2 ± 1
Valeric acid	2 ± 2	1 ± 1	2 ± 2	1 ± 1	2 ± 2	2 ± 2	2 ± 1	2 ± 1
Iso-capronic acid	0.1 ± 0.04	0.01 ± 0.07	0.5 ± 0.7	0.4 ± 0.7	0.0 ± 0.0	0.02 ± 0.08	0.01 ± 0.04	0.0 ± 0.0
Capronic acid	0.5 ± 0.7	0.5 ± 0.7	0.01 ± 0.04	0.1 ± 0.08	0.7 ± 1.3	0.6 ± 0.8	0.6 ± 0.9	0.5 ± 0.9

The values were expressed as mmol/kg wet weight (mean ± SD). FMT: Faecal microbiota transplantation; SCFAs: Short-chain fatty acids.

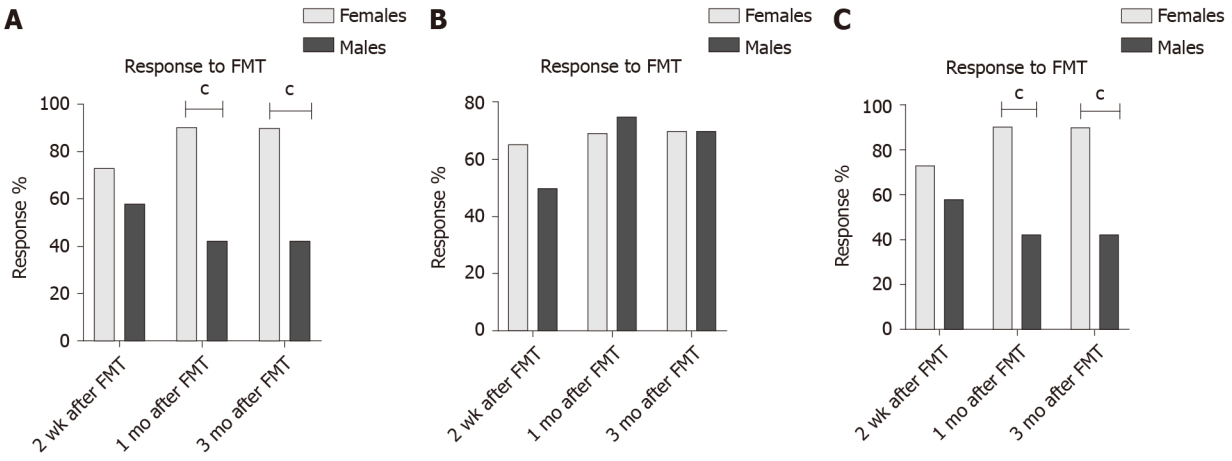

Figure 2 Response rates to faecal microbiota transplantation of female and male irritable bowel syndrome patients. A: Irritable bowel syndrome with diarrhoea-predominant; B: Irritable bowel syndrome with constipation-predominant; C: Irritable bowel syndrome with mixed diarrhoea and constipation. $^{c}P < 0.001$. FMT: Faecal microbiota transplantation.

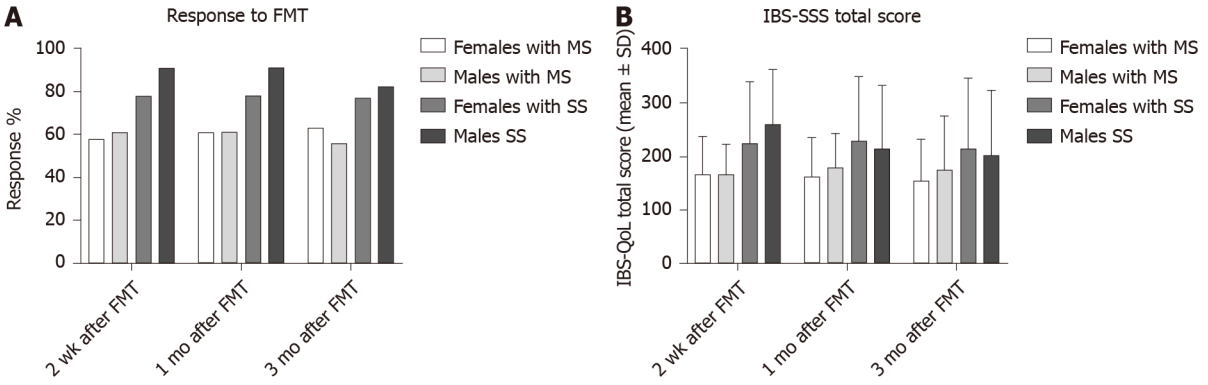

Figure 3 Response rates to faecal microbiota transplantation and the total irritable bowel syndrome-severity scoring system scores of irritable bowel syndrome patients with moderate irritable bowel syndrome symptoms (irritable bowel syndrome-severity scoring system total score between 175 and 300) and with severe irritable bowel syndrome symptoms (irritable bowel syndrome-severity scoring system total score of ≥ 300). A: Faecal microbiota transplantation; B: Irritable bowel syndrome severity scoring system total score. MS: Moderate irritable bowel syndrome symptoms; SS: Severe irritable bowel syndrome symptoms; FMT: Faecal microbiota transplantation.

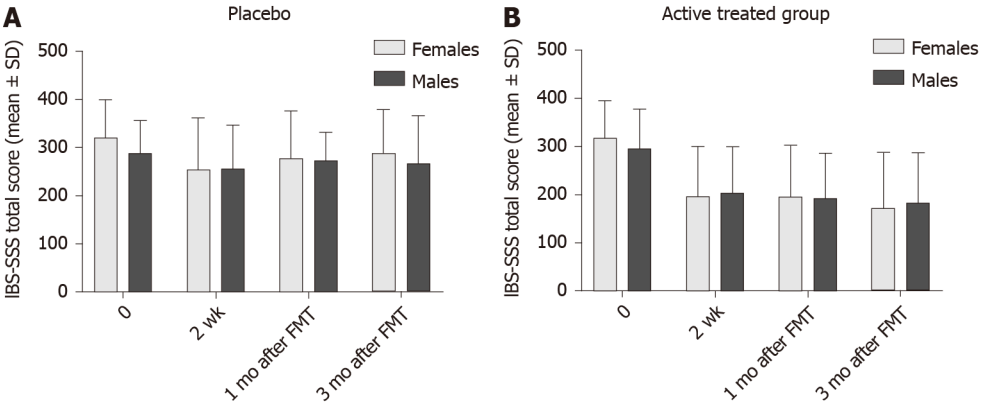

Figure 4 The total irritable bowel syndrome-severity scoring system scores in females and males. A: Placebo group; B: Active treated group. FMT: Faecal microbiota transplantation.

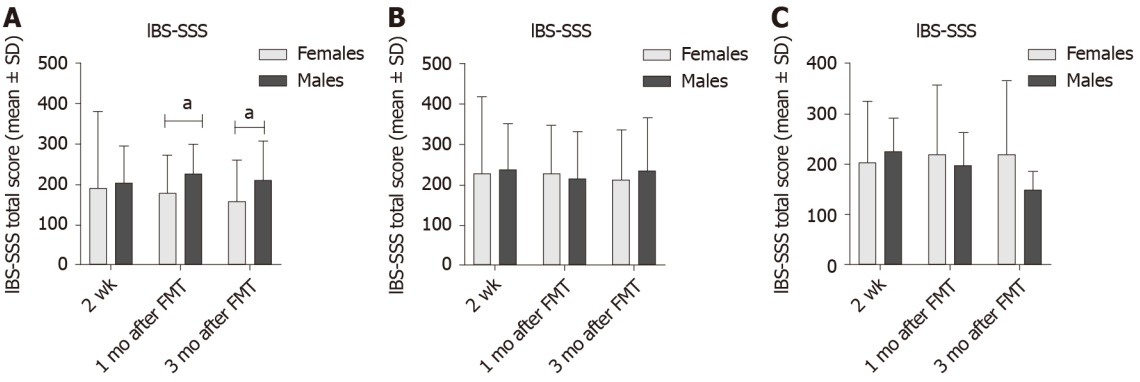

Figure 5 The total irritable bowel syndrome-severity scoring system scores in females and males. A: Irritable bowel syndrome with diarrhoea-predominant; B: Irritable bowel syndrome with constipation-predominant; C: Irritable bowel syndrome with mixed diarrhoea and constipation. $^{a}P < 0.05$. IBD-SSS: Irritable bowel syndrome-severity scoring system; FMT: Faecal microbiota transplantation.

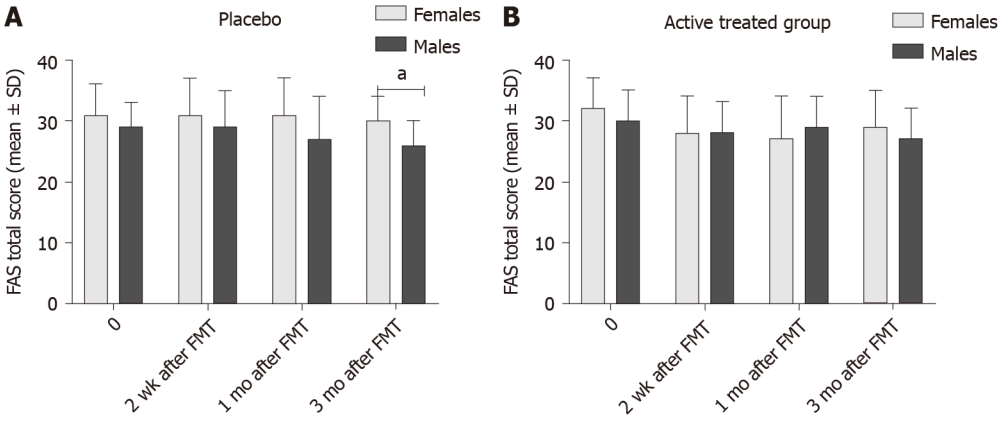

Figure 6 The total Fatigue Assessment Scale scores in female and male irritable bowel syndrome patients. A: Placebo group; B: Active treated group. [a]*P* < 0.05. FAS: Fatigue Assessment Scale; FMT: Faecal microbiota transplantation.

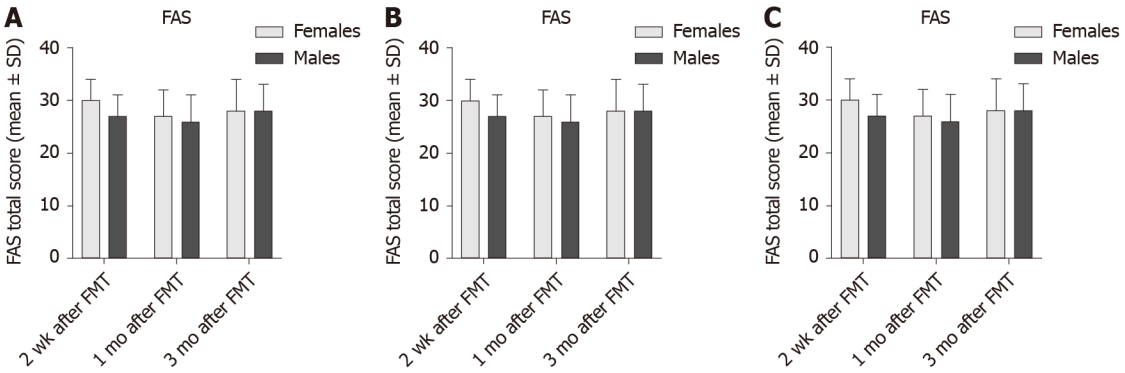

Figure 7 Total Fatigue Assessment Scale scores in female and male patients. A: Irritable bowel syndrome with diarrhoea-predominant; B: Irritable bowel syndrome with constipation-predominant; C: Irritable bowel syndrome with mixed diarrhoea and constipation. FAS: Fatigue Assessment Scale; FMT: Faecal microbiota transplantation.

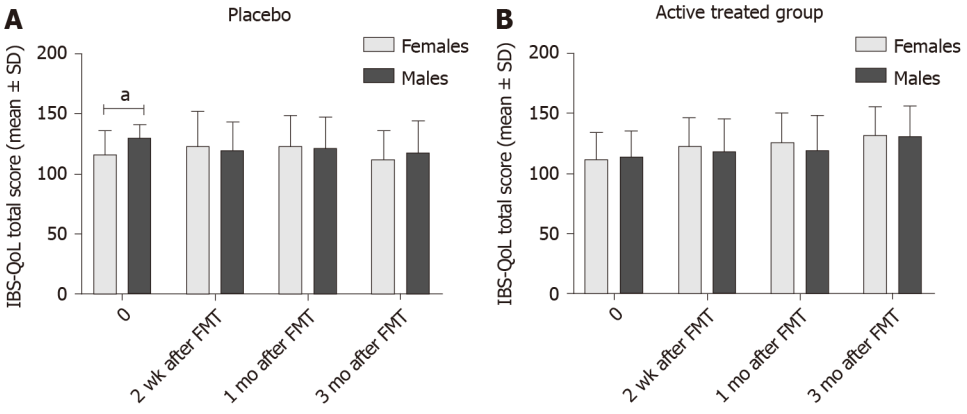

Figure 8 Total irritable bowel syndrome quality of life scale scores in female and male patients. A: Placebo group; B: Active treated group. [a]*P* < 0.05. IBS-QoL: Irritable bowel syndrome quality of life scale; FMT: Faecal microbiota transplantation.

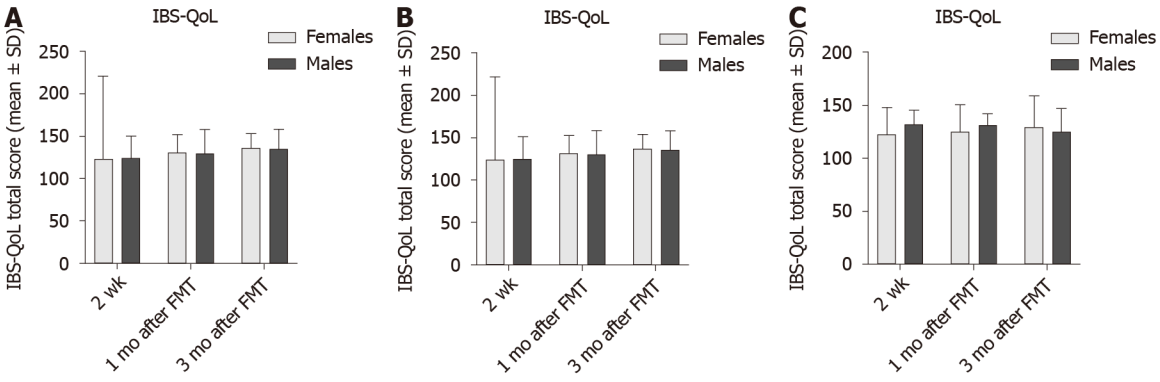

Figure 9 Total irritable bowel syndrome quality of life scale scores in females and males. A: Irritable bowel syndrome with diarrhoea-predominant; B: Irritable bowel syndrome with constipation-predominant; C: Irritable bowel syndrome with mixed diarrhoea and constipation. IBS-QoL: Irritable bowel syndrome quality of life scale; FMT: Faecal microbiota transplantation.

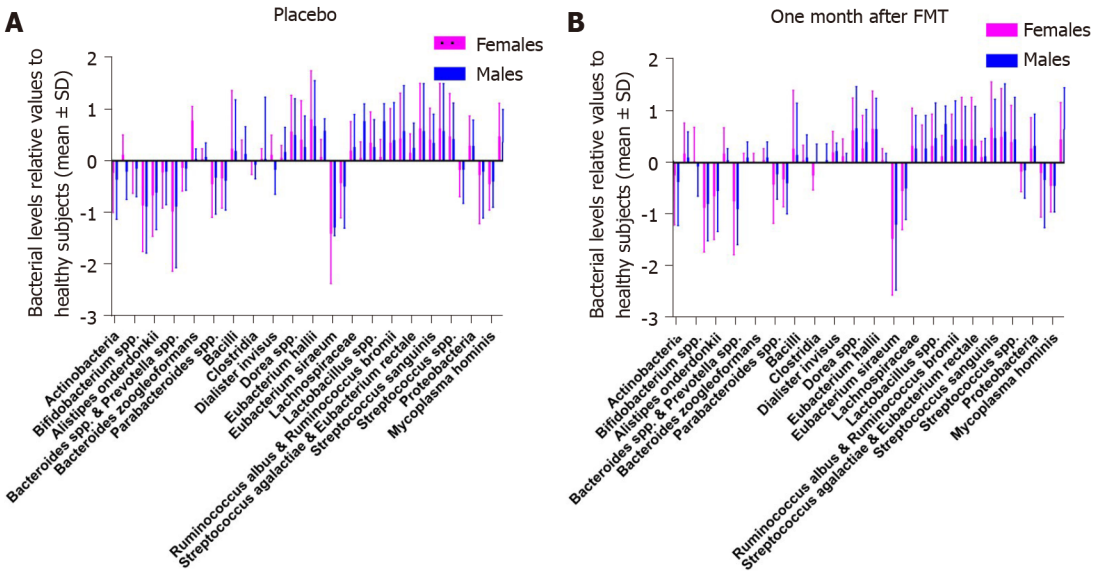

Figure 10 Bacteria levels in the faeces of female and male irritable bowel syndrome patients in the placebo group at the baseline and 1 mo after faecal microbiota transplantation. A: Baseline; B: 1 mo after faecal microbiota transplantation. The bacterial levels are relative values to a normobiotic microbiota profile of 165 healthy subjects. FMT: Faecal microbiota transplantation.

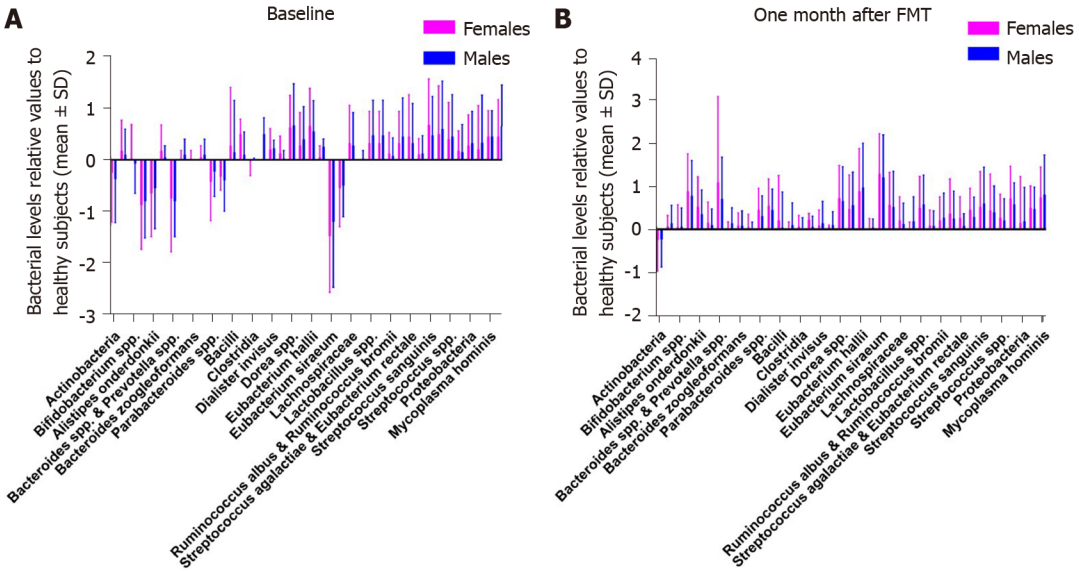

Figure 11 Faecal bacteria levels in the female and male irritable bowel syndrome patients in the active treated group at the baseline and 1 mo after faecal microbiota transplantation. A: Baseline; B: 1 mo after faecal microbiota transplantation. The bacterial levels are expressed as in Figure 10. FMT: Faecal microbiota transplantation.

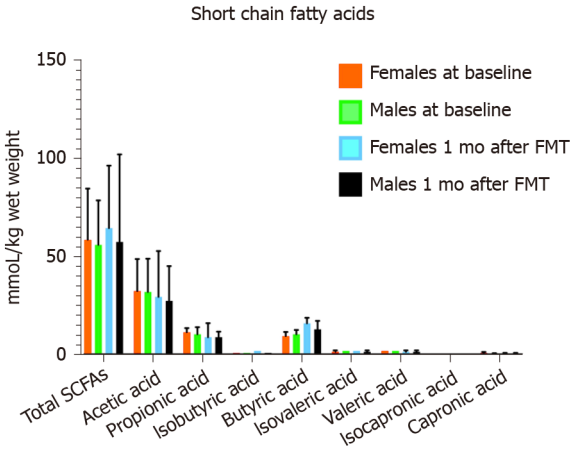

Figure 12 The faecal short chain fatty acids concentration of female and male patients in both the placebo and active treated groups at the baseline and 1 mo after faecal microbiota transplantation. The values were expressed as mmol/kg wet weight (mean ± SD).

ARTICLE HIGHLIGHTS

Research background

Irritable bowel syndrome (IBS) is a common chronic disorder, where intestinal microbiota plays a pivotal role in its pathophysiology. Faecal microbiota transplantation for IBS appears to be a promising treatment of IBS.

Research motivation

In Western countries, there is a female predominance in IBS with female:male ratio of 2:1. In a recent randomized double-blind placebo-controlled trial on faecal microbiota transplantation (FMT) in IBS females responded better to FMT than did males.

Research objectives

We aimed to investigate whether there is a sex difference in the response to FMT in terms of symptoms, dysbiosis, and bacteria and short-chain fatty acids (SCFAs) profiles in the same cohort of patients that we had investigated in our previous randomized controlled trial.

Research methods

This study included 164 patients who fulfilled the Rome IV criteria for the diagnosis of IBS. These patient's cohort included IBS diarrhoea-predominant (IBS-D), IBS-constipation predominant (IBS-C) and mixed diarrhoea and constipation (IBS-M) subtypes. They were randomized to placebo (own faeces), 30 g or 60 g donor's faeces at a ratio of 1:1:1. The faecal transplant was administered *via* gastroscope to the duodenum. Patients completed IBS severity scoring system (IBS-SSS), the Fatigue Assessment Scale (FAS) and the IBS quality of life scale (IBS-QoL) questionnaires at the baseline and 2 wk, 1 mo and 3 mo after FMT. They also provided faecal samples at the baseline and 1 mo after FMT. Response was defined as a decrease of ≥ 50 points in the IBS-SSS total score after FMT. The faecal bacteria profile and dysbiosis were determined by the GA-map Dysbiosis Test (Genetic Analysis, Oslo, Norway) using the 16S rRNA gene. The levels of faecal SCFAs were determined by gas chromatography.

Research results

There was no sex difference in the response to FMT either in the placebo group or active treated group. There was no difference between females and males in either the placebo group or actively treated groups in the total score on the IBS-SSS, FAS or IBS-QoL, in dysbiosis, or in the faecal bacteria or SCFA level. However, the response rate was significantly higher in females with IBS-D than that of males at 1 mo, and 3 mo after FMT. Moreover, IBS-SSS total score was significantly lower in female patients with IBS-D than that of male patients both 1 mo and 3 mo after FMT.

Research conclusions

There is no sex difference in the response to FMT in IBS patients with moderate-to-severe IBS symptoms belonging to the three of IBS subtypes of IBS-C and IBS-M in patients who did not responded to National Institute for Health and Care Excellence-modified diet. However, female patients with IBS-D had a significant higher response rate to FMT and lower IBS-SSS score after FMT than males.

Research perspectives

The present observation that female patients with IBS-D respond better to FMT than males raise several questions as to the cause of this difference. Further studies are needed to explore the difference in diet and life style between females and males as possible causes for this difference.

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The Effects of Fecal Microbiota Transplantation on the Symptoms and the Duodenal Neurogenin 3, Musashi 1, and Enteroendocrine Cells in Patients With Diarrhea-Predominant Irritable Bowel Syndrome

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Introduction: Interactions between the gut microbiota and enteroendocrine cells play important role in irritable bowel syndrome (IBS). Reduced stem cell densities and their differentiation into enteroendocrine cells may cause abnormal densities of the duodenal enteroendocrine cells in IBS patients.

Materials and Methods: We aimed to investigate the effects of fecal microbiota transplantation (FMT) on stem cell differentiation into enteroendocrine cells as detected by neurogenin 3, stem cells as detected by Musashi 1, and the enteroendocrine cells in the duodenum of IBS patients. The study included 16 IBS patients according to Rome III criteria. Four patients were excluded. The remaining patients (n = 12, four females and eight males) were divided according to the cause of IBS into post-infectious (n = 6) and idiopathic (n = 6) IBS. They completed the following questionnaires before and 3 weeks after FMT: IBS-Symptom Severity Scoring system (IBS-SSS) and IBS-Symptom Questionnaire (IBS-SQ). Feces donated by healthy relatives of the patients were transplanted *via* gastroscope. Biopsies were taken from the descending part of the duodenum at baseline and 3 weeks after FMT. They were immunostained for neurogenin 3, Musashi 1, and all types of duodenal enteroendocrine cells and quantified by computerized image analysis. Microbiota analyses of feces collected just before and 3 weeks after FMT were performed using GA-map™ Dysbiosis test (Genetic Analysis AS, Oslo, Norway).

Results: The total scores for IBS-SSS and IBS-SQ were significantly improved 3 weeks after receiving FMT, P = 0.0009 and <0.0001, respectively. The stem cell densities of

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neurogenin 3 increased significantly following FMT ($P = 0.0006$) but not for Musashi 1 ($P = 0.42$). The cell densities of chromogranin A, cholecystokinin, gastric inhibitory peptide, serotonin, and somatostatin, but not for secretin, have significantly changed in both IBS groups after 3 weeks from receiving FMT.

Conclusion: More than two-thirds of IBS patients experienced improvement in their symptoms parallel to changes in the enteroendocrine cells densities 3 weeks after FMT. The changes in the enteroendocrine cell densities do not appear to be caused by changes in the stem cells or their early progenitors rather by changes in the differentiation progeny as detected by neurogenin 3. The study was retrospectively registered at ClinicalTrials.gov (ID: NCT03333291).

Clinical Trial Registration: ClinicalTrials.gov, identifier NCT03333291.

Keywords: cell densities, duodenum, neuroendocrine, fecal microbiota transplantation, gut hormones, microbiota, irritable bowel syndrome, stem cells

INTRODUCTION

The neuroendocrine peptides/amines produced by the enteroendocrine cells regulate several functions of the gastrointestinal (GI) tract such as motility, secretion, absorption, visceral sensitivity, local immune-defense, cell proliferation, and appetite (El-Salhy et al., 2014; El-Salhy et al., 2017). The enteroendocrine cells possess sensory tentacle protruding into the intestinal lumen that sense the changes in the intestinal lumen and respond by releasing their neuroendocrine peptides/amines (Sternini et al., 2008; El-Salhy et al., 2016). Enteroendocrine cells, stem cells, and their progenitor cells are abnormal in the proximal and distal parts of the small intestine of patients with irritable bowel syndrome (IBS) (Dizdar et al., 2010; El-Salhy et al., 2010; El-Salhy et al., 2014a; El-Salhy, 2015; El-Salhy and Gilja, 2017). The abnormalities in enteroendocrine cells seem to explain the dysmotility, visceral hypersensitivity, and abnormal intestinal secretion seen in IBS patients (El-Salhy et al., 2014).

Patients with IBS also have an altered gut microbiota profile (Carroccio et al., 2010; Ohman and Simren, 2013; Hong and Rhee, 2014) *i.e.*; fewer *Lactobacillus* and *Bifidobacterium* spp. and more *Clostridium* spp. that ferment fibers and fermentable oligosaccharides, disaccharides and monosaccharides, and polyols (FODMAPs), and produce gas leading to luminal distension with symptoms of the abdominal pain and bloating (Si et al., 2004; Kassinen et al., 2007; El-Salhy et al., 2014b). Probiotics (live bacterial cultures) have been used to alleviate the symptoms of IBS (Sisson et al., 2014), but unfortunately, they have shown limited effectiveness (Malikowski et al., 2017). Human feces from a healthy subject are used to treat patients with recurrent *Clostridium difficile* colitis through re-establishing the balance in the gut microbiota (van Nood et al., 2013). A new concept is emerging where FMT is used as a treatment option for IBS symptoms (Pinn et al., 2014; Halkjaer et al., 2018; Johnsen et al., 2018; Mazzawi et al., 2018; El-Salhy et al., 2019).

The gut microbiota affect both the enteroendocrine cells and the enteric nervous system (Reigstad et al., 2015; Greiner and Backhed, 2016). A published study by our group (Mazzawi et al., 2018) has shown that using FMT in IBS patients reduces their symptoms, improves their quality of life within the first week, and restores the imbalance in the gut microbiota within 3 weeks, which lasts for 28 weeks after receiving FMT. The mechanisms behind this improvement are still unknown. The aim of this study was to investigate whether the improvement brought about by FMT in these IBS patients was caused by the interaction of the microbiota with the stem or/and enteroendocrine cells. Thus, the possible changes in the densities of Musashi 1 (Msi-1, a marker for stem cells and their early progenitors) (Bjerknes and Cheng, 2006), neurogenin 3 (NEUROG3, a marker for enteroendocrine cells progenitor) (Montgomery and Breault, 2008), and enteroendocrine cells in IBS patients following FMT were studied.

MATERIALS AND METHODS

Patients, Donors, and Controls

A recipient group of 16 IBS patients who met the Rome III criteria for IBS and were referred to the gastroenterology outpatient clinic, Haukeland University Hospital, Bergen, Norway during the year of 2015 were included in the study. The patients had moderate to severe abdominal symptoms as defined by IBS-Symptom Severity Scoring system (IBS-SSS) score >175. The exclusion criteria included history of inflammatory bowel diseases, GI malignancy, blood in stool, immunocompromised patients who are taking immunosuppressive medications, history of opportunistic infections within 1 year prior to FMT, oral thrush, or disseminated lymphadenopathy. Patients who were scheduled for

Abbreviations: CgA, Chromogranin A; CCK, Cholecystokinin; FMT, Fecal microbiota transplantation; FODMAP, fermentable oligosaccharides, disaccharides, monosaccharides, and polyols; GI, Gastrointestinal; GIP, Gastric inhibitory peptide; IBS, Irritable Bowel Syndrome; PI, post-infectious; IBS-SQ, Irritable Bowel Syndrome Symptom Questionnaire; IBS-SSS, Irritable Bowel Syndrome Symptom Severity Scale; Msi-1, Musashi 1; NEUROG3, neurogenin 3; SEM, Standard error of mean.

abdominal surgery, pregnant or lactating women, and patients taking probiotics or antibiotics within 1 month prior to FMT were also excluded.

A donor group of healthy family members, seven males and nine females with an age range 20–55 (mean age 35) years were also included. The exclusion criteria of the donors were pregnancy, history of inflammatory bowel diseases, IBS, chronic abdominal pain, GI malignancy, diarrhea, blood in stool, antibiotic and probiotic use within 1 month prior to FMT, immunocompromised patients who were consuming immunosuppressive medications, history of opportunistic infections within 1 year prior to FMT, oral thrush, and disseminated lymphadenopathy.

Biopsy samples from a group of 12 healthy volunteers, 10 females and two males with an age range 20–42 (mean age 39) years, recruited at Stord Hospital, Stord, Norway by advertising in the local newspapers, were used as controls to study duodenal stem cells and enteroendocrine cells.

The study was performed in accordance with the Declaration of Helsinki (World Medical Association Declaration of Helsinki, 2013), adhered to CONSORT guidelines and was approved by the Regional Committee for Medical and Health Research Ethics in Western Norway (reference no.: 2013/1497). The current study was retrospectively registered on 03.Nov.2017 at ClinicalTrials.gov (ID: NCT03333291). All of the participants provided written informed consent.

Study Design

The donors and patients were screened one week before FMT according to the reviews and guidelines that were published prior to the start of the study in the year 2015 (Aroniadis and Brandt, 2013; Cammarota et al., 2014). They were physically examined, and blood tests were taken to investigate previous exposure to contagious infectious agents including serologic testing for the donors (hepatitis A, B and C, HIV, Epstein–Barr virus and cytomegalovirus) and for the patients [hemoglobin, leucocytes, platelets, creatinine, aspartate aminotransferase, alanine aminotransferase, International normalized ratio (INR), chromogranin A (CgA), and electrolytes]. Stool samples from both donors and patients were examined for fecal calprotectin, cultured for enteric bacterial pathogens, and screened for viruses, parasites, and eggs. The patients were told not to change their lifestyle or diet before the start or during the study and to report any changes in their lifestyle or use of new medications during the study.

FMT Procedure

On transplantation day, 30 g of fresh donors' feces (<2 h from production to donation) was mixed with 60 ml normal saline for preparing the fecal suspension using manual effort and sieved in order to avoid the clogging of infusion syringes and tubes (Aroniadis and Brandt, 2013; Cammarota et al., 2014). All of the patients fasted overnight and received only once a 60 ml of fecal suspension followed by 60 ml normal saline, using a 60 ml syringe, infused through the gastroscope into the descending part of the duodenum past the duodenal papilla at a steady rate within 7–10 seconds to avoid a fast flush and regurgitation of fecal material into the stomach. Tissue biopsies were taken from the descending part of the duodenum before installation of feces and again 3 weeks after FMT. Fecal samples were collected on the same day of FMT (before the procedure) and 3 weeks after FMT and sent for microbiota analysis using GA-map™ Dysbiosis test (Genetic Analysis AS, Oslo, Norway) as previously explained in details (Mazzawi et al., 2018).

Questionnaires

The patients completed the following questionnaires before and 3 weeks after FMT for IBS symptoms assessment: a) IBS-Symptom Severity Scoring system (IBS-SSS), in which higher scores indicated worse symptoms (scores <175 represented mild IBS symptoms, 175–300 moderate severity, and >300 severe IBS) and a decrease of 50 points correlated with improvement in clinical symptoms (Francis et al., 1997), and b) IBS symptom questionnaire (IBS-SQ) reported IBS symptoms (nausea, bloating, abdominal pain, constipation, diarrhea, and anorexia) using a severity scale from 0 to 10, where 0 = no symptoms and 10 = severe symptoms (Mathias et al., 1994; Kane et al., 2003).

Gastroscopy and Immunohistochemistry

The patients fasted overnight prior to undergoing a gastroscopy. During the gastroscopy, four tissue biopsies were collected from the descending part of the duodenum, distal to the papilla. The biopsy samples were fixed in 4% buffered paraformaldehyde, paraffin-embedded biopsies were cut into 5 μm thick sections. The sections were then stained with hematoxylin–eosin and immunostained with an ultraView Universal DAB Detection Kit (cat.no. 760-500, Ventana Medical Systems, Basal, Switzerland) and the BenchMark Ultra IHC/ISH staining module (Ventana Medical Systems). The sections were incubated with the primary antibodies for 32 min at 37°C. The primary antibodies that were diluted according to the suppliers' recommendations were as follows: polyclonal rabbit anti-synthetic peptide conjugated to keyhole limpet hemocyanin derived from within residues 1–100 of human Msi-1 (code ab21628, Abcam, Cambridge, United Kingdom), monoclonal mouse-anti-protein expressed in 293T cells transfected with human NEUROG3 expression vector (code ab87108, Abcam), monoclonal mouse antibody raised against the N-terminal of purified CgA (code no. M869, Dako, Glostrup, Denmark), polyclonal rabbit anti-human secretin for the detection of secretin (code no. sc-20938, Santa Cruz Biotechnology, Santa Cruz, CA, United States), rabbit antibodies for the detection of cholecystokinin (CCK; code no. A0568, Dako, Glostrup, Denmark), mouse antibodies against human synthetic gastric inhibitory peptide (GIP; code no. sc-57162, Santa Cruz Biotechnology), mouse antibodies against serotonin (code no. R87104 B56-1, Dako), and rabbit antibodies against synthetic cyclic somatostatin (code no. A0566, Dako). Labeled secondary antibodies locate the specific antibody. The complex is then visualized with hydrogen peroxide substrate and 3, 3′-diaminobenzidine tetrahydrochloride (DAB) chromogen, which produces a brown precipitate, and counterstained with hematoxylin.

Computerized Image Analysis

1) The densities of Msi-1 and NEUROG3 were quantified using ×40 objective on the light microscope equipped with a digital

camera (DP 26, Olympus, Tokyo, Japan) connected to a computer equipped with a software cellSens imaging program (version 1.7, Olympus, Tokyo, Japan). The number of immunoreactive cells and crypts was quantified in 10 randomly chosen fields. Each field (frame) represented a tissue area of 0.035 mm^2. Msi-1 cell density is expressed as the number of cells/10 crypts and the density of NEUROG3 as number of cells/field.

2) Enteroendocrine cell densities were quantified using a light microscope with ×40 objective and a computer software Cell^B imaging program (Olympus, Tokyo, Japan). The number of immunoreactive cells was quantified in 10 randomly chosen fields. Each field (frame) of epithelial cells represents a tissue area measured at 0.09 mm^2. The density of each enteroendocrine cell type was expressed as the number of cells/mm^2 of epithelium.

By keeping the identity of the slides concealed, the quantification of Msi-1 and NEUROG3 was performed by M.E.S., and the quantification of the enteroendocrine cells was performed by T.M.

Gut Microbiota Analysis

The gut microbiota analysis was previously described in details (Mazzawi et al., 2018). Briefly, GA-map™ Dysbiosis test is based on fecal homogenization, mechanical bacterial cell disruption, and automated total bacterial genomic DNA extraction using magnetic beads. Fifty-four DNA probes were used targeting more than 300 bacterial strains based on their 16S rRNA sequence in seven variable regions (V3–V9). Twenty-six bacteria probes are species specific, 19 detect bacteria on the genus level, and nine probes detect bacteria at higher taxonomic levels. Probe labeling is by single nucleotide extension and hybridization to complementary probes coupled to magnetic beads and signal detection by using BioCode 1000A 128-Plex Analyzer (Applied BioCode, Santa Fe Springs, CA, USA) (Casen et al., 2015).

Statistical Analysis

Kruskal–Wallis with Dunn's *post hoc* test was used to compare the cell densities between the controls and the patients before and after receiving FMT. Paired *t*-test is used to compare between the cell densities of the patients before and after receiving FMT. Mann–Whitney U test was used to compare the microbiota profiles of the patients before and after FMT to their respective donors. Correlations were performed using Spearman non-parametric test. The data are presented as mean ± SEM values. $P < 0.05$ is considered to be statistically significant.

RESULTS

Patients

Four out of the 16 originally included patients were excluded after withdrawing their consents to participate for practical reasons ($n = 1$), failed gastroscope intubation after FMT ($n = 1$), being diagnosed with functional dyspepsia ($n = 1$), and positive stool culture for *Clostridium difficile* ($n = 1$). The remaining patients ($n = 12$) were four females and eight males, with age range 20–44 years. Six patients suffered from post-infectious IBS (PI-IBS), and six had idiopathic IBS. They completed the whole study by filling out the questionnaires and delivered stools for microbiota analysis at the same day of undergoing gastroscopy with duodenal biopsies *i.e.* the day of FMT and after 3 weeks. No change in lifestyle, diet, or use of any/new medications has been registered during the study.

Questionnaires

Three weeks following FMT, the symptoms of IBS as assessed by IBS-SSS improved in 9/12 (75%) of total IBS patients, 5/6 (83.3%) of PI-IBS and 4/6 (66.6%) of idiopathic IBS patients. The total scores of the questionnaires showed a significant reduction from before FMT to 3 weeks after FMT as follows: IBS-SSS (326.6 ± 22.3 and 240.2 ± 33.6, respectively, $P = 0.0009$) and IBS-SQ (30.8 ± 3.3 and 11.6 ± 2.1, respectively, $P < 0.0001$). The scores of the different domains of IBS-SQ are shown in **Table 1**, which show a significant improvement in all of its domains except for anorexia.

Gastroscopy and Immunohistochemistry

The duodenum of the patients was normal both endoscopically and microscopically. Msi-1 immunoreactivity was observed in both the cytoplasm and nucleus, and immunoreactive cells were found in the crypts of the duodenum. NEUROG3 immunoreactivity was found exclusively in the nuclei of cells that were observed in both the crypts and along side the villi. The immunoreactive enteroendocrine cells; CgA, secretin, CCK, GIP, somatostatin, and serotonin, were localized mostly in the crypts.

Computerized Image Analysis

The densities of the stem cells and the enteroendocrine cells in the duodenum in controls and IBS patients are shown in **Tables 2** and **3**, respectively, before and after receiving FMT. The densities of the immunoreactive cells before and after FMT are shown in **Figures 1** and **2** for NEUROG3 and **Figure 3** for Msi-1. The cell densities of NEUROG3 were significantly lower ($P = 0.04$) for the total group of IBS patients before FMT than controls, but no significant difference was shown after FMT ($P = 0.47$). The cell densities of Msi-1 for the total group of IBS patients before and after FMT were significantly lower than the controls ($P = 0.046$ and 0.004, respectively). No significant changes were shown in the NEUROG3 and Msi-1 cells densities for both subgroups before and after FMT compared to controls (except for Msi-1 cell densities in idiopathic IBS after FMT, $P = 0.02$). When we compared cell densities for the patients

TABLE 1 | The scores of IBS-symptom questionnaire (IBS-SQ) domains in patients with irritable bowel syndrome.

Questionnaire	Before FMT	3 weeks after FMT	P-value
Nausea	3.7 ± 0.9	1.4 ± 0.6	**0.0025**
Bloating	7.9 ± 0.5	3.4 ± 0.8	**<0.0001**
Abdominal pain	6.3 ± 0.9	3.3 ± 0.8	**0.0012**
Constipation	4.3 ± 1.1	1.5 ± 0.7	**0.046**
Diarrhea	6.4 ± 0.9	1.2 ± 0.4	**<0.0001**
Anorexia/loss of appetite	2.3 ± 0.7	0.9 ± 0.4	0.087

Data are presented as the mean ± SEM. Comparison: Paired t test. FMT, fecal microbiota transplantation.

Significant differences are seen in the different domains (except for anorexia/loss of appetite) when comparing the symptoms of the patients before to after FMT.

The bolded numbers are significant values <0.05.

TABLE 2 | Densities of stem cells in the duodenum of total IBS group, PI-IBS, and idiopathic IBS patients before and after receiving FMT.

Marker	Enteroendocrine cell densities (cells/mm^2)							^{a}P-value	^{b}P-value	^{c}P-value	^{d}P-value	^{e}P-value	^{f}P-value	^{g}P-value	^{h}P-value	^{i}P-value
	Control, *n* = 12	Total IBS, Before, *n* = 12	Total IBS, After, *n* = 12	PI-IBS, before, *n* = 6	PI-IBS, after, *n* = 6	Idiopathic IBS, before, *n* = 6	Idiopathic IBS, after, *n* = 6									
Neurogenin 3	340 ± 40	222.3 ± 13.8	394.3 ± 30.7	214.2 ± 18.5	430.5 ± 28.9	230.5 ± 21.5	358.2 ± 52.9	**0.004**	0.47	0.1	0.2	0.1	0.99	**0.0006**	**0.0007**	0.1
Musashi 1	8.75 ± 0.95	5.7 ± 0.4	5 ± 0.5	5.3 ± 0.7	5.2 ± 0.8	6 ± 0.4	4.8 ± 0.7	**0.046**	**0.004**	0.07	0.05	0.2	**0.02**	0.42	0.9	0.2

Data are presented as the mean ± SEM. Comparison: Kruskal–Wallis with Dunn's post test, [a]controls vs. total IBS patients before FMT, [b]controls vs. total IBS patients after FMT, [c]controls vs. PI IBS patients before FMT, [d]controls vs. PI IBS patients after FMT, [e]controls vs. idiopathic IBS patients before FMT, [f]controls vs. idiopathic IBS patients after FMT; and Paired t test, [g]total IBS patients before vs. after FMT, [h]PI-IBS patients before vs. after FMT, [I]Idiopathic IBS patients before vs. after FMT. FMT, fecal microbiota transplantation; IBS, irritable bowel syndrome. PI, post-infectious.
The bolded numbers are significant values <0.05.

TABLE 3 | Densities of enteroendocrine cells in the duodenum of controls, total IBS group, PI-IBS, and idiopathic IBS patients before and after receiving FMT.

Hormone	Enteroendocrine cell densities (cells/mm^2)							P^a-value	P^b-value	P^c-value	P^d-value	P^e-value	P^f-value	P^g-value	P^h-value	P^i-value
	Control, *n* = 12	Total IBS, Before, *n* = 12	Total IBS, After, *n* = 12	PI-IBS, before, *n* = 6	PI-IBS, after, *n* = 6	Idiopathic IBS, before, *n* = 6	Idiopathic IBS, after, *n* = 6									
Chromogranin A	228 ± 33.6	370.3 ± 21	269.8 ± 22	340.8 ± 34	422.7 ± 31	399.8 ± 20.9	316.8 ± 10.2	**0.0099**	**0.0133**	0.2	**0.006**	**0.005**	0.4	0.98	**0.0006**	**0.0065**
Secretin	74 ± 3.8	83.8 ± 4.9	86.7 ± 5.9	80.5 ± 8.8	89.7 ± 10.7	87.2 ± 4.8	83.7 ± 5.9	0.32	0.21	0.99	0.5	0.1	0.3	0.5	0.099	0.6
Cholecystokinin	80 ± 4.7	122.8 ± 6.7	110.7 ± 8.1	113 ± 10.4	126.5 ± 0.5	132.5 ± 7.2	94.8 ± 9	**0.0007**	**0.023**	0.055	**0.005**	**0.0008**	0.5	0.2	0.052	**0.0006**
Gastric inhibitory peptide	53.3 ± 5.7	65.1 ± 3.8	70.3 ± 6.2	60 ± 3 ± 3.7	84 ± 7.1	69.8 ± 6.3	57.2 ± 7	0.33	0.19	0.96	**0.01**	0.2	0.99	0.5	**0.014**	0.2
Serotonin	76.5 ± 13.6	135.1 ± 14.7	142 ± 12.8	100.5 ± 7.1	160.7 ± 16.6	169.7 ± 20.6	123.3 ± 17.3	**0.04**	**0.0088**	0.7	**0.005**	**0.01**	0.2	0.7	**0.012**	**0.034**
Somatostatin	42.3 ± 5.6	58.6 ± 4	66.2 ± 6.3	52.3 ± 2.7	78.5 ± 8.1	64.8 ± 7	53.8 ± 6.8	**0.033**	**0.012**	0.3	**0.006**	**0.03**	0.3	0.3	**0.011**	**0.017**

Data are presented as the mean ± SEM. Comparison: Kruskal–Wallis with Dunn's post test, [a]controls vs. total IBS patients before FMT, [b]controls vs. total IBS patients after FMT, [c]controls vs. PI IBS patients before FMT, [d]controls vs. PI IBS patients after FMT, [e]controls vs. idiopathic IBS patients before FMT, [f]controls vs. idiopathic IBS patients after FMT; and Paired t test, [g]total IBS patients before vs. after FMT, [h]PI-IBS patients before vs. after FMT, [I]Idiopathic IBS patients before vs. after FMT. FMT, fecal microbiota transplantation; IBS, irritable bowel syndrome; PI, post-infectious.
The bolded numbers are significant values <0.05.

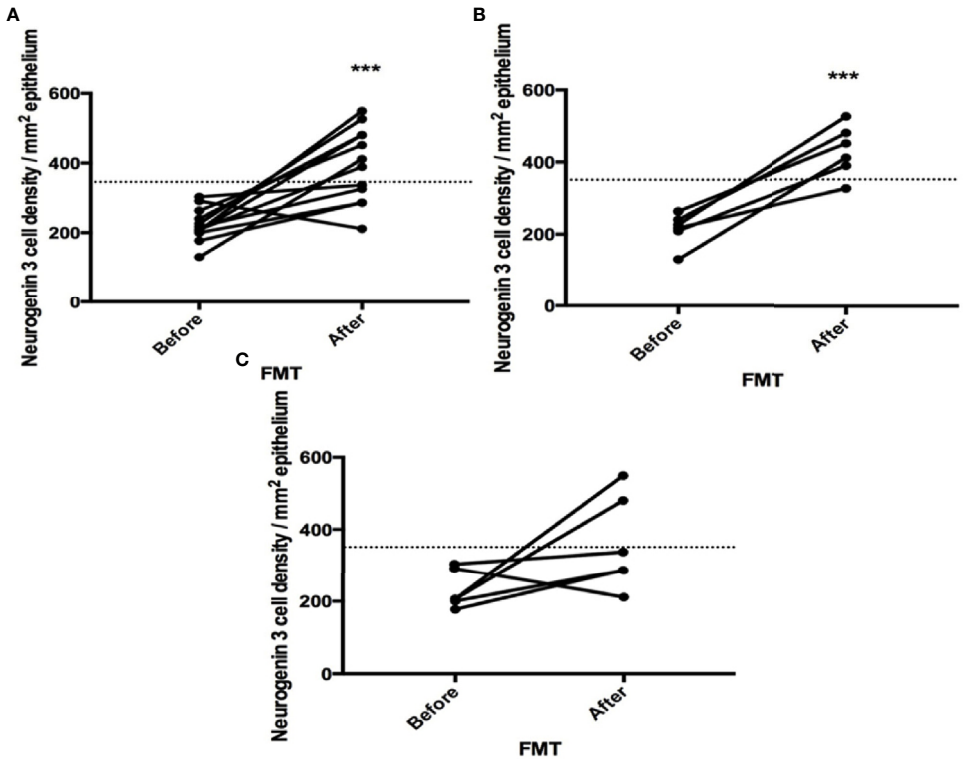

FIGURE 1 | Neurogenin 3 cell densities before and after fecal microbiota transplantation (FMT) in **(A)** total, **(B)** post-infectious and **(C)** idiopathic irritable bowel syndrome patients. The dotted line represents the mean cell densities for the healthy control group. *** indicates $P < 0.001$.

before to after FMT, significant changes were seen in the cell densities of NEUROG3 densities for the total group of IBS and PI-IBS patients ($P = 0.0006$ and 0.007, respectively) but not for idiopathic IBS subgroup. No changes were seen in the Msi-1 cell densities when comparing IBS patients before and after FMT in all groups, **Table 2**. Several enteroendocrine cells showed significant differences in their cells densities compared to controls except for secretin (**Figure 4**) and GIP. The densities of the enteroendocrine cells increased in PI-IBS and decreased in the idiopathic IBS subgroup following FMT compared to before FMT. The directions of these changes were either towards or away from the values measured for controls, **Table 3**.

Gut Microbiota

The analysis of the gut microbiota for the total group of IBS patients has been previously described (Mazzawi et al., 2018). In the current study, patients with PI-IBS before FMT had a significant increase in the bacterial signals for *Streptococcus*

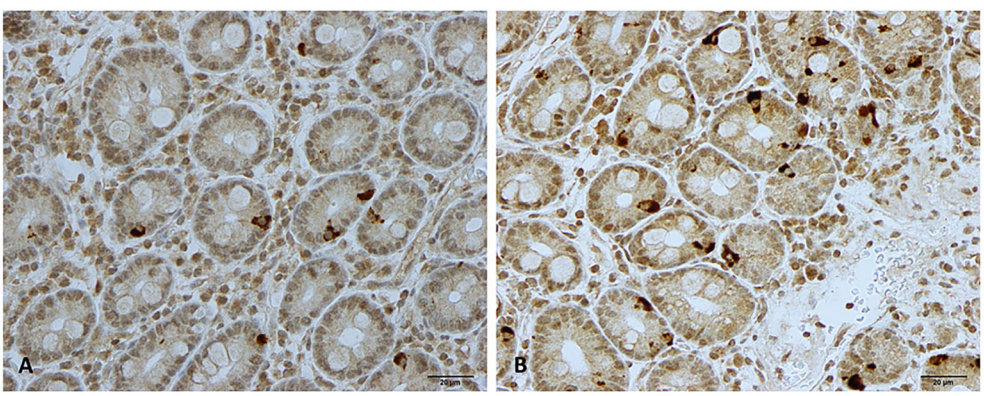

FIGURE 2 | Neurogenin 3 immunoreactive cells in patients with irritable bowel syndrome **(A)** before and **(B)** after fecal microbiota transplantation.

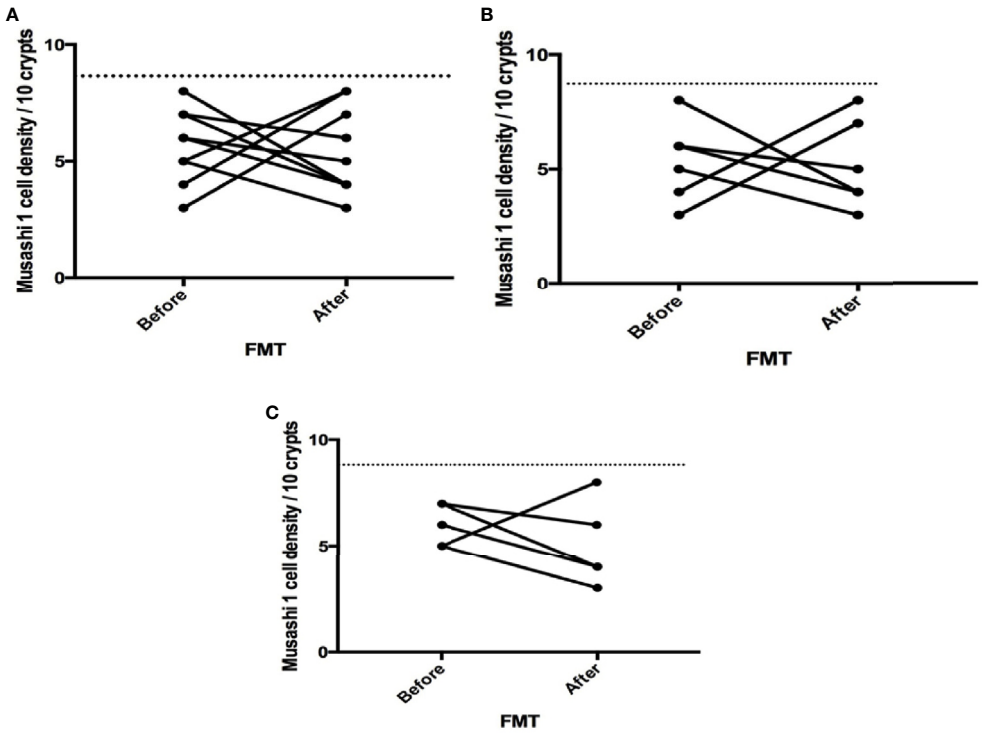

FIGURE 3 | Musashi 1 cell densities before and after fecal microbiota transplantation (FMT) in **(A)** total, **(B)** post-infectious and **(C)** idiopathic irritable bowel syndrome patients. The dotted line represents the mean cell densities for the healthy control group.

compared to donors at the beginning of the study and significantly reduced bacterial signals for *Megasphaera/Dialister, Actinobacteria*, and *Bifidobacterium* compared to donors at the beginning of the study, **Table 4**. Patients with idiopathic IBS before FMT had a significant decrease in the bacterial signals for *Firmicutes/Tenericutes/Bacteroidetes*, *Megasphaera/Dialister, Actinobacteria, Bifidobacterium*, and *Actinomycetales* compared to donors at the beginning of the study, **Table 4**.

Correlations

The correlation coefficient (r) and value of significance (*P*) for NEUROG3 cells with secretin were r = −0.89, $P = 0.033$ after FMT in the PI-IBS subgroup, and for NEUROG3 cells with serotonin were r = 0.89, $P = 0.03$ before FMT, and also with CCK were r = 0.89, $P = 0.03$ after FMT in the idiopathic IBS subgroup.

Certain strains of the gut microbiota correlated with stem and enteroendocrine cells. Before FMT, *Parabacteriodes* correlated with NEUROG3 cells in the total group (r = 0.76, $P = 0.037$) and with

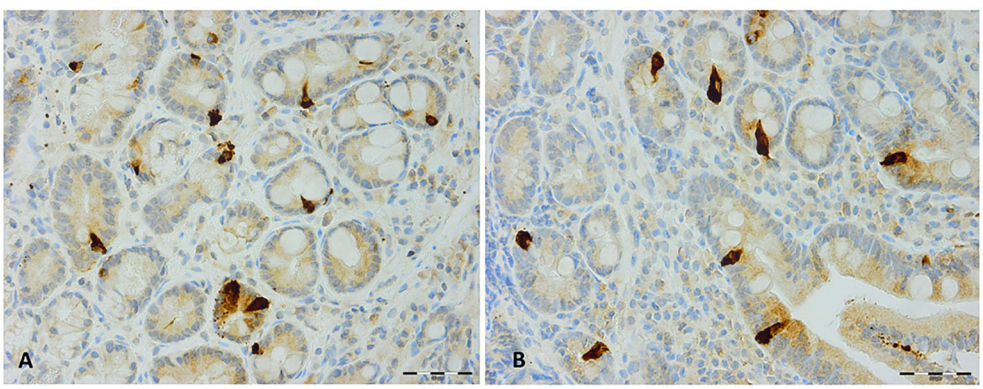

FIGURE 4 | Secretin immunoreactive cells in patients with irritable bowel syndrome **(A)** before and **(B)** after fecal microbiota transplantation.

TABLE 4 | Fecal bacterial strains for donors at the beginning of the study and post-infectious IBS and idiopathic IBS patients before and after FMT.

Bacteria strain	Donors	Patients		*P*-values*	*P*-values**
		Before FMT	Week 3 after FMT		
Post-infectious IBS					
Streptococcus	47 (41, 59)	88 (59, 114)	79 (44, 210)	**0.038**	0.26
Megasphaera/Dialister	162 (58, 286)	19 (3, 57)	10 (5, 143)	**0.038**	0.11
Actinobacteria	160 (99, 321)	61 (21, 73)	219 (107, 435)	**0.0095**	0.9
Bifidobacterium	160 (96, 370)	62 (19, 86)	212 (92, 447)	**0.019**	0.9
Idiopathic IBS					
Firmicutes/Tenericutes/Bacteroidetes	260 (103, 475)	51 (24, 134)	133 (14, 279)	**0.048**	0.18
Megasphaera/Dialister	4 (3.8, 247)	3.3 (3, 3.6)	3.1 (2.7, 98)	**0.048**	0.37
Actinobacteria	296 (251, 499)	89 (36, 115)	89 (44, 268)	**0.0025**	**0.02**
Bifidobacterium	358 (256, 645)	70 (38, 110)	100 (45, 282)	**0.0025**	**0.02**
Actinomycetales	15 (11, 18)	8.4 (8.1, 13)	13 (10,24)	**0.03**	0.95

*Data are presented as the median (first, third quartiles). Comparison: Mann-Whitney U test. * donors compared to patients before FMT and ** donors compared to patients after FMT. FMT, fecal microbiota transplantation; IBS, irritable bowel syndrome.*
The bolded numbers are significant values <0.05.

GIP cells in the PI-IBS group (r = 0.89, $P = 0.03$). In the PI-IBS subgroup, inverse correlations were found between *Streptococcus sanguinis and thermophiles* and the following: CgA (r = −0.94, $P = 0.017$), CCK (r = −0.89, $P = 0.03$), and serotonin (r = −0.89, $P = 0.03$) cells and between *Actinobacteria* and CgA (r = −0.89, $P = 0.03$), CCK (r = −0.94, $P = 0.017$) and serotonin (r = −0.94, $P = 0.02$) cells. After FMT, a correlation was found between NEUROG3 cells and *Actinobacteria* (r = 0.79, $P = 0.025$) also *Bifidobacterium* (r = 0.79, $P = 0.025$) in the total group of IBS patients and between GIP cells and *Actinobacteria* (r = −1, $P = 0.017$) also *Bifidobacterium* (r = −1, $P = 0.017$) in the idiopathic-IBS subgroup. In addition, correlations were found between *Lactobacillus* and NEUROG3 cells (r = 0.75, $P = 0.038$) in the total group of IBS patients and also somatostatin cells (r = 1, $P = 0.017$) in the idiopathic-IBS group. No correlations were found between Msi-1 and gut microbiota before or after FMT in any group of IBS patients.

The correlations between IBS symptoms using IBS-SQ questionnaire and the different enteroendocine cells were as follows: before FMT, pain correlated with the cell densities of each of CgA (r = 0.87, $P = 0.02$), serotonin (r = 0.87, $P = 0.02$) and CCK (r = 0.92, $P = 0.01$) and anorexia correlated with GIP (r = 0.91, $P = 0.01$) in the PI-IBS subgroup. In addition, bloating correlated with CgA (r = 0.85, $P = 0.03$), anorexia correlated with each of secretin (r = −0.91, $P = 0.01$) and GIP (r = −0.95, P=0.003), and nausea correlated with each of CCK (r= −0.89, P=0.01) and GIP (r= −0.95, P=0.003) in the idiopathic IBS subgroup. After FMT, diarrhea correlated with secretin (r = 0.83, $P = 0.04$) in PI-IBS and nausea with CCK cell density (r = −0.89, $P = 0.02$) in the idiopathic IBS subgroup.

Adverse Events

No adverse events were reported during or following FMT.

DISCUSSION

In this study, the changes in IBS symptoms are associated with changes in the densities of the enteroendocrine cells and their progenitors NEUROG3 in the duodenum and in the gut microbiota of these patients 3 weeks after receiving FMT.

As previously reported (Mazzawi et al., 2018), approximately 75% of the total IBS patients showed improvement in their IBS symptoms following FMT. The densities of stem cells and enteroendocrine cells in the duodenum are abnormal in IBS patients before FMT, which is consistent with previous publications (El-Salhy et al., 2010; El-Salhy et al., 2014c; El-Salhy et al., 2015). Significant changes were mainly seen in the densities of NEUROG3 but not Msi-1 cells and all of the enteroendocrine (except for secretin) cells in IBS patients 3 weeks following FMT compared to before FMT. This indicates that the changes in the enteroendocrine cell densities are caused by a change in the differentiation of stem cells into enteroendocrine cells (as presented by NEUROG3). The observation that gut microbiota influence the differentiation of stem cells into enteroendocrine cells has been previously reported in the jejunum and colon of mice (Cani et al., 2007; Everard et al., 2011; Wichmann et al., 2013; Cani and Knauf, 2016). However, the exact mechanism explaining how the gut microbiota shape the fate of stem cells into enteroendocrine cells remains unknown.

A previous study showed that the CgA cell density, a common marker for endocrine cells (Deftos, 1991; Taupenot et al., 2003), correlated with NEUROG3 cell density in the small intestine of IBS patients (El-Salhy and Gilja, 2017). The correlations between the stem cells and enteroendocrine cells may be explained by that all of the enteroendocrine cells types arise from endocrine progenitors expressing NEUROG3 then the opposing activities of Pax4- and Arx-homeodomain transcription factors (Beucher et al., 2012). The inactivation of Pax4 leads to the up-regulation of Arx and the differentiation of these progenitors into different types of enteroendocrine cells and *vice versa*. Serotonin-producing enterochromaffin cells can differentiate in a NEUROG3 dependent and independent pathway. Similar to serotonin, somatostatin cells are generated from progenitors expressing NEUROG3 then Pax4. GIP-, secretin-, and CCK-cells arise from endocrine progenitors expressing NEUROG3 then Pax4 and Arx. The inactivation of Arx can cause these progenitors to be reallocated into the expression of somatostatin cells while GIP-, secretin-, and CCK-cells' expression becomes impaired. However, an inactivation of Pax4 leads to an up-

regulation of Arx; thus the differentiation of these progenitors into L cells (producing glucagon-like peptide-1 and peptide YY), while the differentiation of somatostatin-, serotonin-, and GIP-cells becomes impaired (Beucher et al., 2012). Therefore, the positive correlations between NEUROG3 cell densities and the different enteroendocrine cells along with the negative correlation between NEUROG3 cell density with secretin cell density, before and after FMT in the different IBS subgroups, may be attributed to the progenitor differentiation to other enteroendocrine cell types as previously explained. The densities of the enteroendocrine cells in the duodenum have changed 3 weeks following the FMT procedure. On one hand, the changes in the idiopathic-IBS patients are towards the healthy controls, which is similar to a previous study by our group using diet in managing IBS symptoms (Mazzawi and El-Salhy, 2017a; Mazzawi and El-Salhy, 2017b). On the other hand, the cell densities in PI-IBS patients increase away from the values measured for healthy controls, which may be due to the infectious origin of IBS in these patients (Dizdar et al., 2010).

The alterations in the strain signals of the gut microbiota have become normal after 3 weeks following FMT except for *Actinobacteria* and *Bifidobacterium*, which showed some changes towards normal but remained significantly different in the idiopathic IBS subgroup compared to the donors. The bacteria that correlated with the changes in the differentiation of stem cells into enteroendocrine cells as detected by NEUROG3 namely, *Parabacteroides*, *Actinobacteria*, *Bifidobacterium*, and *Lactobacillus*, also correlated with different enteroendocrine cells before and after FMT in the different groups. The changes in the bacterial strain signals occurred parallel to the changes in the differentiation progeny of stem cells and enteroendocrine cells densities, which indicated that gut microbiota might influence other enteroendocrine cell types than the previously reported enterochromaffin and L cells (Everard et al., 2011). This may also have shed some light on the mechanisms lying behind FMT and the positive effects of using these bacterial strains in the management of IBS symptoms (Whorwell et al., 2006; Drouault-Holowacz et al., 2008; Bixquert Jimenez, 2009; Sisson et al., 2014). The gut microbiota interact with the enteroendocrine cells through their microbial metabolites, short-chain fatty acids (especially butyrates) (Nohr et al., 2013; Cani and Knauf, 2016). Searching for a mechanism of action, we reasoned that because the vast majority of the gut microbiota are located in the ileum and in the colon and due to the paracrine and endocrine functions of the enteroendocrine cells throughout the gastrointestinal tract, the beneficial effects of FMT on the gut microbiota and their products (short-chain fatty acids mainly butyrate) (Mazzawi et al., 2019) might be related but not exclusive to this area of the gastrointestinal tract.

Visceral hypersensitivity plays an important role in the pathogenesis of upper gastrointestinal symptoms especially nausea, abdominal bloating and pain (Salet et al., 1998). Correlations of pain with serotonin and CCK cells can be explained since serotonin and CCK are involved in sensory and motor responses to distension in the intestinal tract and so perception of pain in IBS (Varga et al., 2004; Wendelbo et al., 2014). Although negatively correlated in this study, CCK is a satiety hormone and is involved in anorexia and nausea (Chua and Keeling, 2006; El-Salhy et al., 2014). GIP correlates negatively with anorexia (Stock et al., 2005) and does not induce nausea (Mentis et al., 2011). Secretin contributes to the rapid gastric emptying and pancreatic bicarbonate and fluid secretions therefore diarrhea in IBS (El-Salhy et al., 2014).

Two recently published meta-analyses have shown that fresh or frozen donor stool delivered *via* colonoscopy or nasojejunal tube may be beneficial in IBS (Xu et al., 2019; Ianiro et al., 2019). A newly published randomized, double-blinded, placebo-controlled study using a large cohort of IBS patients show that transplanting frozen stool *via* a gastroscope is an effective treatment for IBS patients (El-Salhy et al., 2019). In the current study, we have chosen to use gastroscopy because it is an easy and fast procedure and because patients with IBS often have small intestinal bacterial overgrowth, which would have escaped the suspension, had we chosen to use colonoscopy for FMT. The amount of transplanted fecal material, 30 g, used in this study is consistent with the published recommendations (Aroniadis and Brandt, 2013; Cammarota et al., 2014) before the starting point of the study in 2015 and with the European consensus on FMT (Cammarota et al., 2017). According to the newly published study (El-Salhy et al., 2019), both 30 g and 60 g FMT have shown significant responses compared to placebo in IBS patients.

No adverse events were noted following FMT in the current study despite previous data of minor adverse events that resolved spontaneously (El-Salhy and Mazzawi, 2018). We have previously published about the daily changes that occur to these symptoms during the first 3 days and 7 days following FMT, which showed a dramatic improvement in IBS symptoms shortly after FMT (Mazzawi et al., 2018). One can hypothetically assume that due to the severity of IBS symptoms before FMT, either that the patients did not relate to any worsening of their originally experienced bothersome symptoms or that they actually have benefited from the FMT with rapid improvement of the aforementioned symptoms.

The strength of the current study is the usage of validated methods to study the changes in the gut microbiota and immunoreactive cells' densities and validated questionnaires to assess the changes in IBS symptoms. However, there are several limitations, such as the small number of the participants and the lack of a placebo-control group from which tissue biopsies could have been taken for the study of the stem and enteroendocrine cells. In spite of the small sample size of the different IBS groups, we found significant differences in the immunoreactive cells' densities before and after FMT. However, further studies with larger groups of participants are required to be able to draw firm conclusions regarding the effect of FMT on the stem and enteroendocrine cells' densities.

CONCLUSIONS

Fecal microbiota transplantation improves the symptoms after 3 weeks in of IBS patients, both PI and idiopathic IBS. This improvement is associated with a change in the enteroendocrine cell density and the gut microbiota. The changes in the

enteroendocrine cell density do not appear to be caused by changes in the stem cells or their early progenitors but rather by changes in the differentiation progeny as detected by changes in neurogenin 3.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Regional Committee for Medical and Health Research Ethics in Western Norway (reference no.: 2013/1497). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TM: included the patients, performed gastroscopies and FMT, quantified the enteroendocrine cells, drafted the manuscript, analyzed data, and planned the study. TM is a postdoctoral fellow, Helse-Vest (project number: 912309) www.helse-vest.no. ME-S: bought the necessary antibodies and covered all the expenses related to immunohistochemistry, quantified the stem cells, commented on the manuscript, analyzed data, and planned the study. GL: included the patients, performed gastroscopies and FMT, commented on the manuscript, and planned the study. TH: included the patients, performed gastroscopies and FMT, commented on the manuscript, and planned the study. All authors contributed to the article and approved the submitted version.

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Fecal microbiota dynamics during disease activity and remission in newly diagnosed and established ulcerative colitis

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Patients with ulcerative colitis (UC) have an altered gut microbiota composition, but the microbial relationship to disease activity needs to be further elucidated. Therefore, temporal dynamics of the fecal microbial community during remission and flare was determined. Fecal samples were collected at 2–6 time-points from UC patients during established disease (cohort EST) and at diagnosis (cohort NEW). Sampling range for cohort EST was 3–10 months and for cohort NEW 36 months. Relapses were monitored for an additional three years for cohort EST. Microbial composition was assessed by Genetic Analysis GA-map Dysbiosis Test, targeting ≥ 300 bacteria. Eighteen patients in cohort EST (8 with maintained remission and 10 experiencing a flare), provided 71 fecal samples. In cohort NEW, 13 patients provided 49 fecal samples. The microbial composition showed no clustering related to disease activity in any cohort. Microbial dissimilarity was higher between than within patients for both cohorts, irrespective of presence of a flare. Microbial stability within patients was constant over time with no major shift in overall composition nor modification in the abundance of any specific species. Microbial composition was not affected by intensified medical treatment or linked to future disease course. Thus in UC, the gut microbiota is highly stable irrespective of disease stage, disease activity or treatment escalation. This suggests that prolonged dietary interventions or repeated fecal transplantations are needed to be able to induce permanent alterations of the gut microbiota.

Ulcerative colitis (UC) is a chronic inflammatory disease of the colon with a disease course characterized by periods of active disease with flares of abdominal pain, diarrhea and hematochezia followed by periods of remission[1]. Multiple cross-sectional studies have demonstrated that patients with inflammatory bowel disease (IBD), comprising UC and Crohn's disease (CD), have an altered gut microbiota composition as compared to healthy individuals, with lower microbial richness and diversity as compared to healthy individuals, reviewed in[2,3]. These differences emphasize the potential role for the gut microbiota for development and/or progression of IBD.

Longitudinal profiling studies demonstrate that the intra-individual stability of the microbial composition over time is high in both healthy individuals and IBD patients[4,5], although with a higher degree of fluctuation in CD patients[2,6,7]. However, few studies have explored gut microbiota dynamics in relationship to disease activity over time. Studies that prospectively monitor gut microbiota in IBD before and during a flare of the disease report no evidence for overall changes in microbiota composition linked to the flare[6,8,9], even though some patient-specific taxonomic shifts have been detected[8]. Recent studies further demonstrate that the fecal microbiota composition of CD patients is subject to little intra-individual variation over time and is not influenced by disease activity, neither in the short- nor long-term perspective[9,10]. However, the relationship between gut microbiota dynamics and the disease activity during different phases of the disease in UC still needs to be explored. Also, multiple sampling times spanning before, during and after a flare are warranted. Therefore, in this longitudinal

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	Cohort EST			
	Remission group (n = 8)	Flare group (n = 10)	p-value	Cohort NEW (n = 13)
Age (median, range)	40 (31–60)	36 (20–50)	0.32	34 (22–55)
Gender (female)	4	6	0.67	7
Disease extent (E1/E2/E3[a])	0/3/5	0/6/4	0.34	2/2/9
Disease duration, years (median, range)	3 (1–13)	5 (1–16)	0.36	NA
Smoking (no/yes)	7/1	9/1	0.87	12/1
Fecal calprotectin, µg/g (median, IQR)	40 (20–80)	50 (30–90)	0.59	650 (380–1600)
Months since previous flare, median (range)	17 (11–24)	9 (5–18)	0.006	NA
Maintenance treatment			0.36	NA
5ASA	8	9		
5ASA/AZA	0	1		

Table 1. Patient demographics at inclusion for patients during established disease (cohort EST) and at diagnosis (cohort NEW). *NA* not applicable, *5ASA* 5-aminosalicylic acid, *AZA* azathioprine. [a]E1, proctitis; E2, left-sided colitis; E3, extensive colitis.

study we determined the temporal dynamics of fecal microbiota composition during remission and flare in UC patients with either established disease or at diagnosis.

Results

Study populations. For cohort EST, 39 patients were included to the initial study and 17 experienced a flare. Of these 17, 10 patients fulfilled the inclusion criteria for this study. In addition, the 8 first included patients fulfilling the criteria for maintained remission were selected. For patient demographics see Table 1, time intervals for fecal sampling and relation to flares before and after sampling are shown in Fig. 1A,B. Together the patients provided 71 fecal samples. At inclusion, when all patients were in remission, 17 of the patients had an endoscopic mayo score of 0 while one patient had an endoscopic mayo score of 1 (belonging to the flare group). All patients were on stable 5ASA treatment and one patient was on concomitant treatment with azathioprine since 9 months. During remission, medicines were kept stable. Presence of a flare was determined by sigmoidoscopy (n = 5) or by an increase in symptoms and calprotectin levels > 300 µg/g (n = 5). During a flare, treatment included standardized 5-ASA dose escalation without (N = 7) or with oral corticosteroids (N = 3). During follow-up, three years after the last study sample, median flare numbers were 0 (range 0–3) and 2 (0–4), for remission and flare group, respectively (p = 0.01).

For cohort NEW, 100 patients were included to the initial study but only 13 patients had enough quantity of stool sample available for analysis from the visit at diagnosis and could enter this study (Table 1), resulting in 49 fecal samples. Time intervals for fecal sampling and flares during the 36 months sampling time are shown in Fig. 1C. Treatment of the flare at diagnosis included 5-ASA without (N = 3) or with corticosteroids (N = 10, 3 topical, 7 oral). After inducing remission from the initial flare, two patients maintained in remission, four experienced 1 flare and seven experienced ≥ 2 flares the coming 3 years.

Fecal microbiota dynamics over time in UC. Temporal dynamics in microbial composition was first evaluated for cohort EST. Principal component analysis (PCA) of all samples, coded for remission group and flare group during either remission or flare showed no clustering for any of the groups (Fig. 2A). Linking of intra-individual samples to their centroid in the PCA revealed that samples from the same individual tended to localize close to one another (Fig. 2B). This was confirmed by assessing Bray–Curtis dissimilarities, which quantify the compositional dissimilarity between different samples based on the microbial probe intensities. Results showed that the dissimilarity was higher between patients than within patients, both for the remission and flare group (Fig. 2C). Next, we analyzed temporal microbial dissimilarities within patients for the remission and flare groups, which showed constant stability over time with no alterations before, during or after a flare (Fig. 2D).

Then, we examined alterations in microbial composition for cohort NEW. PCA analysis showed no specific clustering linked to active disease at diagnosis (Fig. 3A) and samples from the same individual tended to localize close to one another (Fig. 3B). Again dissimilarities were higher between than within patients (Fig. 3C) and temporal microbial dissimilarities were stable over time when compared to the time for diagnosis (Fig. 3D).

Despite a change in fecal calprotectin levels at the flare for cohort EST (visit -1 *vs.* flare; 80 µg/g (60–190) *vs.* 310 µg/g (250–1020), p < 0.0001) and at diagnosis for cohort NEW (inclusion *vs.* month 3; 650 µg/g (380–1600) *vs.* 70 µg/g (40–570), p = 0.03) there were no significant changes in probe signal intensity over time for the four major phyla, Firmicutes, Bacteroides spp., Proteobacteria or Actinobacteria, in any of the cohorts (Fig. 4A,B), or for any of the other 50 probes detecting bacteria on different taxonomic levels (data not shown). No effect related to treatment escalation could be detected for the flare group of cohort EST between Flare and Visit + 1 (Fig. 4A, left) or for initiation of treatment in cohort NEW comparing diagnosis and month 3 (Fig. 4B). Finally, there was no link between the microbiota composition and manifestations of flares the coming 3 years in any of the patient cohorts (Fig. 5).

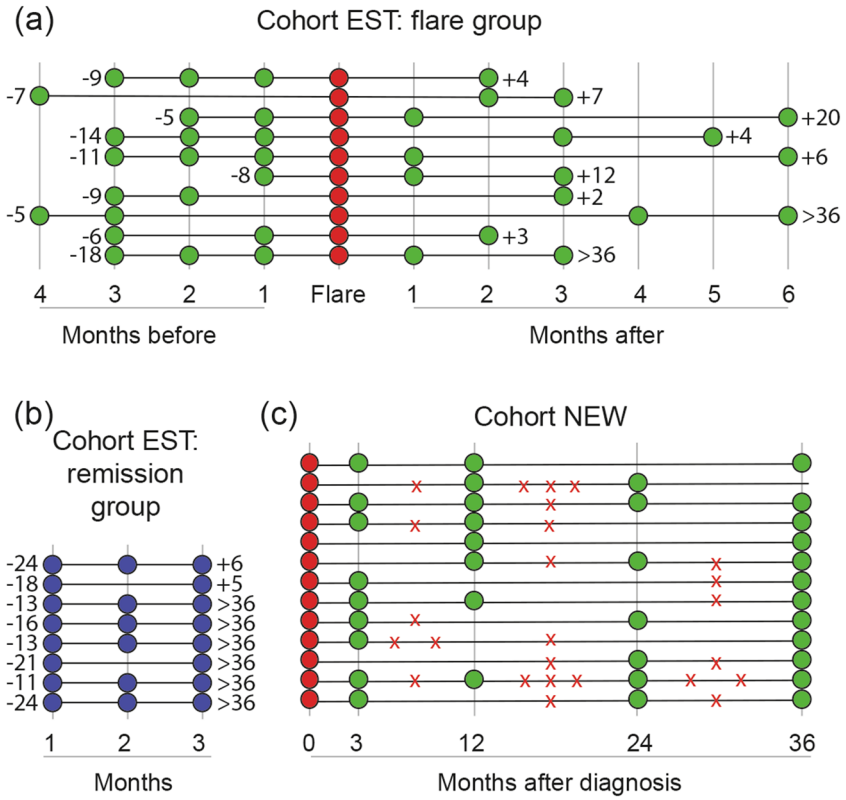

Figure 1. Time intervals for fecal sampling and time indication for flares for the study cohorts. Fecal samples were obtained at time-points indicated by the circles, each row represents one patient. Sampling for patients with established UC (cohort EST) are divided into **(A)** flare group and **(B)** remission group. Numbers to the left indicate months to previous flare and numbers to the right indicate months to the next flare. **(C)** Sampling time-points for patients with newly diagnosed UC (cohort NEW). The symbol X indicates the time period when the patient experienced a flare. Note that the X does not indicate an exact time-point, the flare occurred any time during the specific time interval.

Discussion

In this longitudinal study of UC patients, we have demonstrated that the microbiota composition is constant over time with no alterations before, during, or after a flare of the disease or during remission, both at the time of diagnosis and in established UC. Our results further show that the microbial dissimilarity is higher between than within patients, irrespective of disease activity or time since diagnosis, supporting the notion of the long-term stability of an individual's gut microbiota.

During the last decade several studies have reported differences in fecal microbial composition between active and inactive IBD[6,11–13]. However, most previous studies have had a cross-sectional design comparing fecal microbiota from patients with active disease and patients in remission. Our results clearly show that the inter-individual differences in fecal microbiota composition are larger than the intra-individual differences over time, confirming previous reports on the matter[8,9]. Thus, differences in fecal microbiota composition related to disease activity demonstrated using a cross sectional set-up most likely reflect individual differences in microbiota composition rather than disease activity.

More recently, data from long-term investigations have suggested that gut microbiota composition within an individual is relatively constant over time, although microbiota of IBD patients has been proposed to fluctuate more than those of healthy subjects[6,8–10]. Potential instability of gut microbiota of IBD patients may be linked to inflammation and our approach was therefore to follow patients before, during and after flare of disease. Our study demonstrates a high microbial stability over time in UC, both at the time of diagnosis and with established disease, with no changes in the overall microbial composition related to disease activity. Moreover, the high stability of the microbial profile during the three years after time of diagnosis indicates that the deviation from a healthy microbiota was firmly established before or during early development of disease. Thus, by including several consecutive samples obtained before, during, and after a flare of the disease linked to thorough clinical follow-up we confirm the stability of the microbiota community both at the time of diagnosis and in established UC.

Similar to the lack of influence by disease activity, increased dose of 5ASA or addition of corticosteroids did not affect the microbial community in the present study, neither when being introduced to patients with newly diagnosed disease, nor when being intensified in patients with established disease. Comparable results have

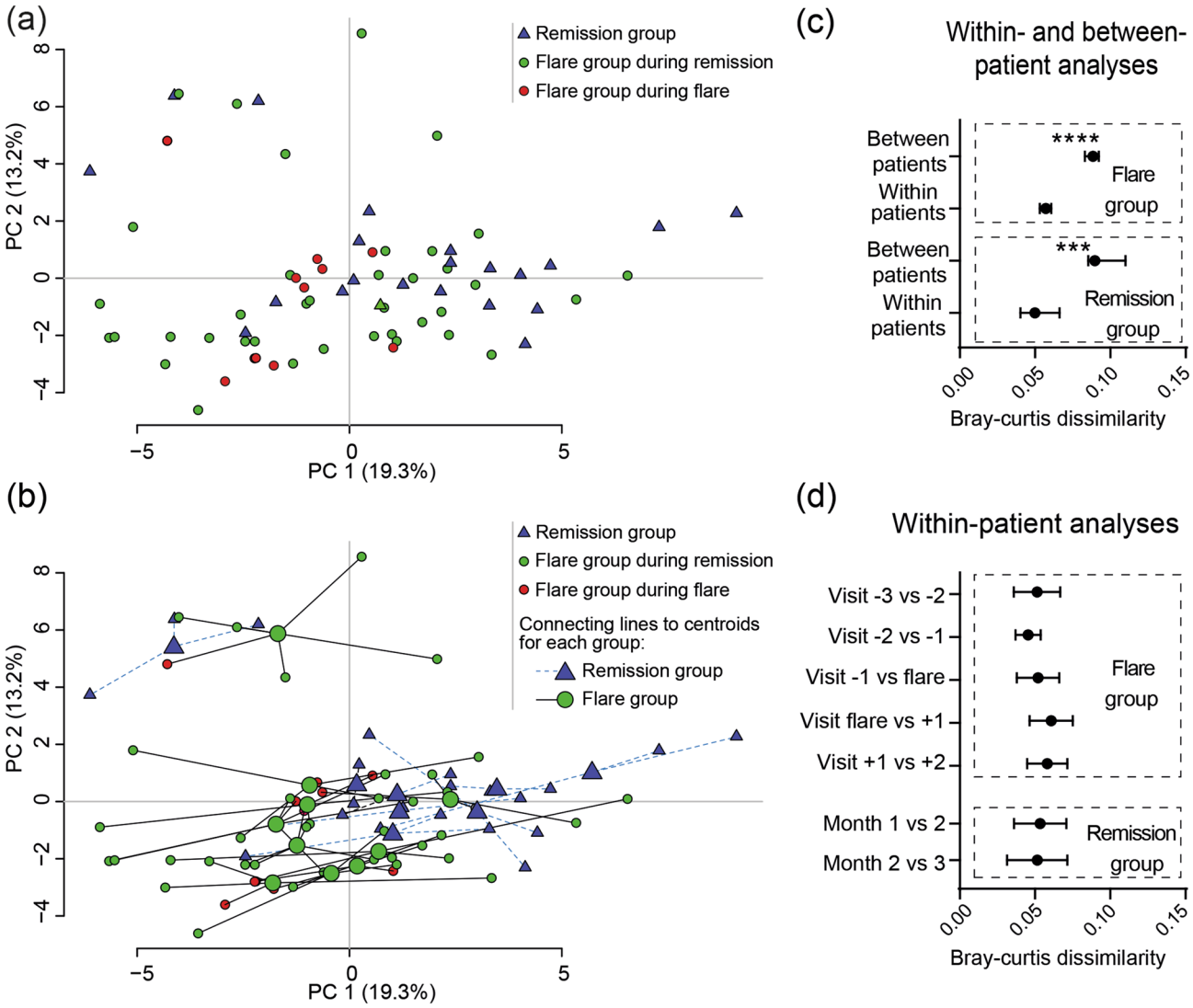

Figure 2. Fecal microbiota dynamics during remission and active flare in patients with established UC. Fecal samples were obtained from patients with established UC (cohort EST) and analysed by the GA-map Dysbiosis Test (remission N = 8, flare N = 10, total number of samples N = 71). The flare group include samples from visit − 3, − 2, − 1, flare, + 1 and + 2 where samples − 3 to − 1 were obtained 1–4 months before the flare and samples + 1 to + 2 were obtained 1–6 months after the flare. The remission group samples were obtained at two to three months in a row. **(a,b)** Principal component analysis showing remission group (blue triangles), flare group during remission (green circles) and flare group during active flare (red circles). In **(b)** samples originating from individual patients are linked to their centroids by dotted lines (remission group) or solid lines (flare group). **(c)** Within- and between-patient and **(d)** within-patient microbial dissimilarities for flare and remission group. The dissimilarities were analyzed by Bray–Curtis dissimilarity index. Data in **(c,d)** are shown as median (IQR), ***$p < 0.001$, ****$p < 0.0001$.

recently been reported for both CD and UC with no impact of medication on microbial stability[9,14]. Nevertheless, microbial diversity was improved in pediatric IBD patients responding to anti-TNF therapy[15]. The inconsistent reports of potential impact of medical regimens on gut microbiota raises the question whether the different outcomes is related to the type of drug or that gut microbiota of adolescent patients are more volatile than that of adult patients. It might also be considered if the stability of the microbiota profile in adult IBD patients may be a potential explanation for fecal transplantation only partly meeting the high expectations as a therapeutic option for IBD patients[16,17]. Furthermore, the usefulness of autologous fecal transplantation, i.e. one's own microbiota, for treatment in IBD patients could also be disputed[18]. Concerning diets and the microbiota, UC patients on vegetarian or gluten free diets show altered gut microbiota profiles in comparison to omnivores but without any clinical benefits[19]. For diet interventions, a study employing a low fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAP) diet for 4 weeks in IBD patients reported higher relief of gut symptoms *vs.* normal diet and showed lower abundance of *Bifidobacterium adolescentis*, *Bifidobacterium longum* and *Feacalibacterium prausnitzii* but no alterations in phyla distribution or α- and β-diversity[20]. A 4-week diet of low fat, high fiber was evaluated in UC patients in comparison to an improved standard American diet

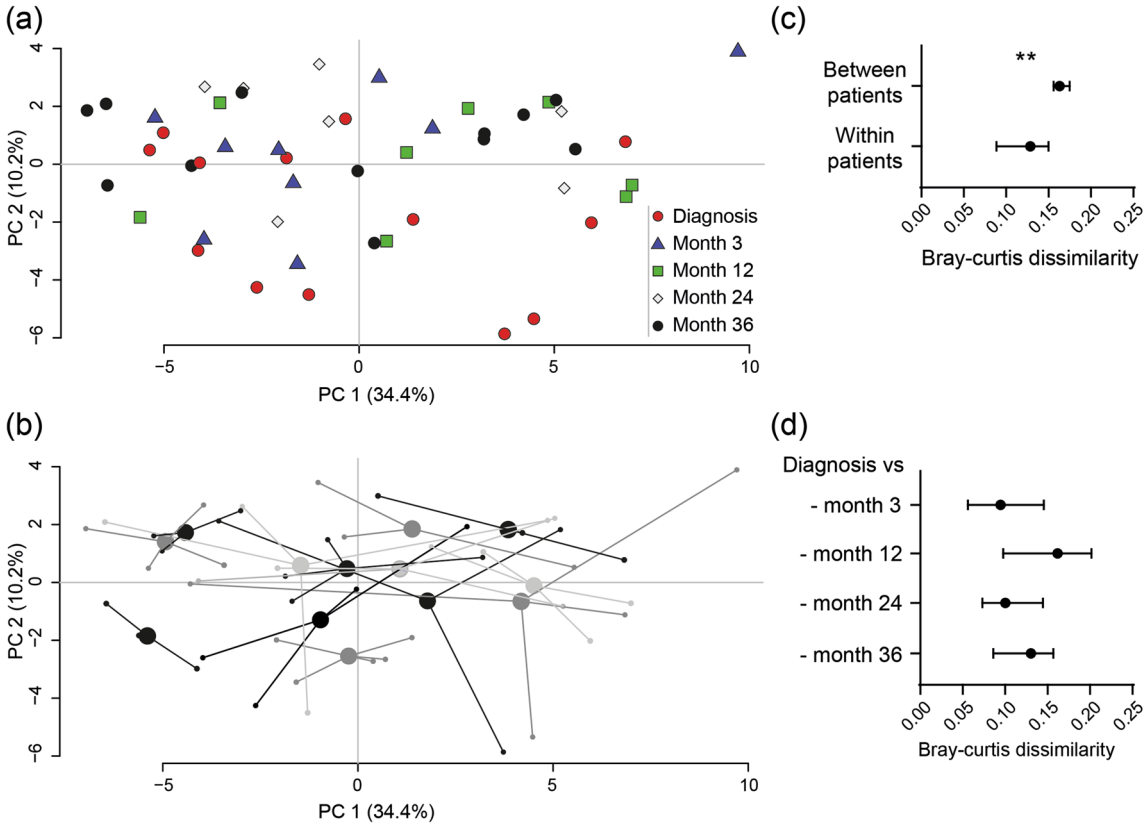

Figure 3. Fecal microbiota dissimilarity dynamics in patients with newly diagnosed UC. Fecal samples were obtained from patients with newly diagnosed UC (cohort NEW) and analysed by the GA-map Dysbiosis Test (N = 13, total number of samples N = 49). Samples were obtained at diagnosis and at month 3, 12, 24 and 36. Principal component analyses with **(a)** the sampling time indicated by color/shape of the dots and **(b)** samples originating from individual patients linked to their centroids. **(c)** within- and between-patient and **(d)** within-patient microbial dissimilarities analyzed by Bray–Curtis dissimilarity index. Data are shown as median (IQR), $^{**}p < 0.01$.

and resulted in decreased markers of inflammation and reduced dysbiosis[21]. However, long term persistence of the altered microbiota is still unknown and considering the high stability of the microbiota such studies are warranted. Altogether this further emphasizes the need of longitudinal studies evaluating the dynamics of gut microbiota related to different treatment regimens including medical therapies, diets and fecal transplantation.

The main strength of our study is the longitudinal study design, which allows for assessment of associations between microbiota composition and disease activity within patients over time; this is particularly important considering the inter-individual differences in microbiota composition and medication use. Compared to previous studies with similar research focus[8,9], we have included more samples per individual, for the established phase encompassing time-points both before and after the flare to evaluate possible changes preceding or following a flare, and for the newly diagnosed encompassing both short- and long-term sampling. We also used a different mode of analyzing the microbiota, as the GA-map technology is based on a pre-selected set of bacteria known to discriminate IBD patients from healthy subjects. The use of the GA-map Dysbiosis Test has limitations since it only determines defined bacterial sequences, ruling out the possibility to find new strains or to deliver full taxonomic details and α and β diversity values. On the other hand, strengths of the method include the generation of absolute values instead of relative abundance, enabling direct comparisons between samples, and the ease of data handling as compared to deep or shotgun sequencing.

A limitation of our study is that the limited number of patients, however, the standardized treatment regimens, the large number of samples per individual and the use of two different cohorts strengthen the findings of the study. Another limitation is that we have not ruled out the presence of an infectious agent, such as Clostridium difficile, as a cause for the flares in the established cohort. However, symptoms were consistent with UC exacerbation and the patients responded well to an increased dose of 5-ASA with or without corticosteroids. Also, no conclusions concerning thiopurines and microbiota could be drawn for this study since only one patient was on thiopurines, but a previous study has reported decreased alpha-diversity in patients treated with thiopurines[8].

In summary, we have shown that the gut microbiota in UC is highly stable irrespective of stage of the disease, disease activity or treatment escalation. This suggests that previous studies showing differences in microbiota composition between patients in remission and patients experiencing a flare reflect individual differences, rather

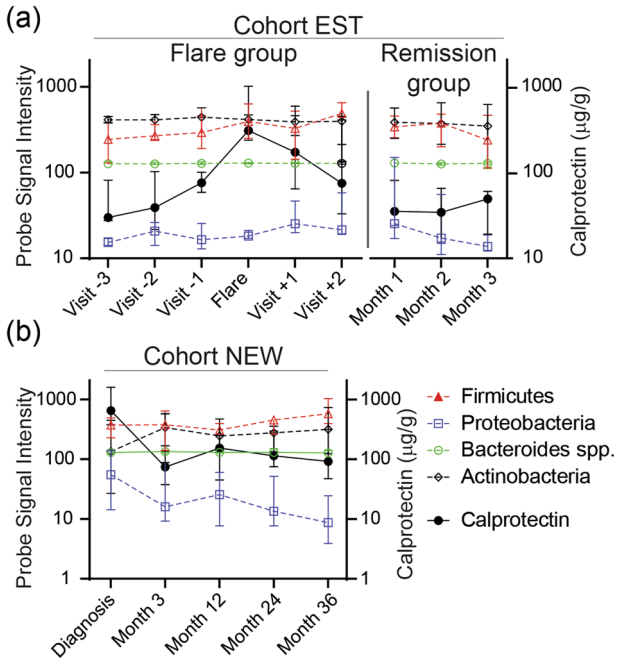

Figure 4. Bacterial probe intensity for the four major phyla in relation to levels of calprotectin in fecal samples during remission and active flare in patients with established and newly diagnosed UC. Fecal samples were analysed by the GA-map Dysbiosis Test and for levels of calprotectin by ELISA. **(a)** Patients with established disease (cohort EST, remission N = 8, flare N = 10, total number of samples N = 71). The flare group include 1–3 samples before, 1 during and 1–2 after the flare and the remission group includes samples obtained two to three months in a row. **(b)** Patients with newly diagnosed UC (cohort NEW, N = 13, total number of samples N = 49). Probe signal intensity for the bacteria is indicated on the left y-axis and calprotectin concentration on the right y-axis. Lines linking bacteria are dotted and symbols are open, lines linking calprotectin are solid and symbols are filled. The Probe Signal Intensity shows absolute values and can be compared between different samples, however a high Probe Signal Intensity for one bacterial species does not imply higher presence when compared to a low signal of a different bacterial species. Data are shown as median (interquartile range).

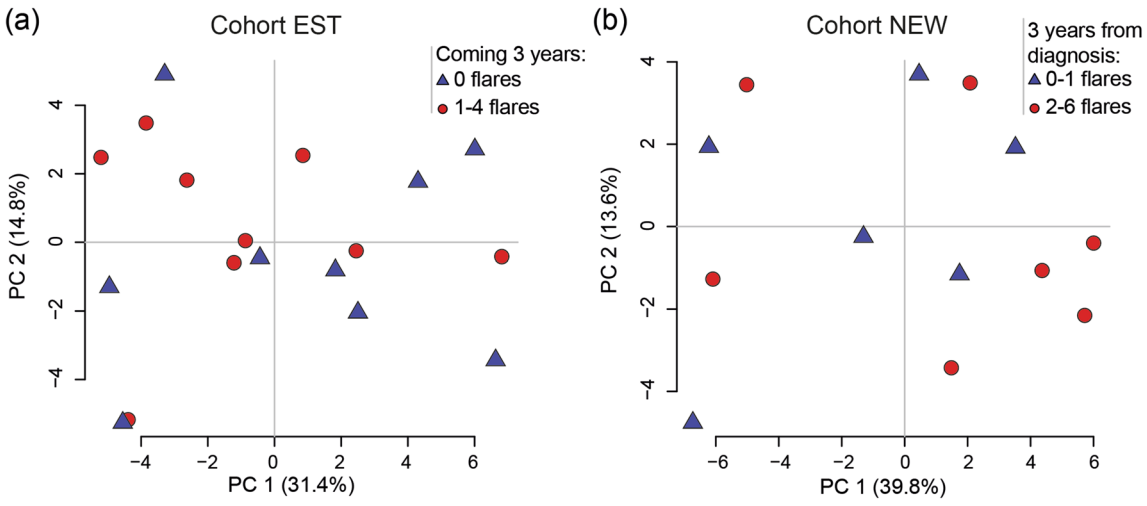

Figure 5. Fecal microbiota and disease outcome the coming 3 years. Fecal samples were obtained from patients with UC and analysed by the GA-map Dysbiosis Test. The microbial compositions are shown by principal component analyses. **(a)** For patients with established disease (cohort EST), results for the last sample obtained is shown in relation to disease outcome after 3 years grouped into maintained remission (N = 9) and 1–4 flares (N = 9). **(b)** For patients at diagnosis (cohort NEW), results for the first sample obtained is shown in relation to disease outcome after 3 years grouped into 0–1 flares (N = 6) and 2–6 flares (N = 7).

than disease activity. It also suggests that nutritional modulation of the gut microbiota as a strategy to control the disease requires long-term commitment.

Methods

Study population. This study encompassed two cohorts of UC patients recruited from five gastroenterology units in Western Sweden; cohort EST comprised patients with established disease being in remission at inclusion, whereas cohort NEW comprised patients included at the time for diagnosis.

Cohort EST was initially recruited from the control group of a 5-ASA intervention study[22]. Patients were > 18 years old, on standardized maintenance treatment with oral 5-ASA. Exclusion criteria were ongoing anti-TNF, corticosteroid or non-steroidal anti-inflammatory drug treatment, pregnancy or prior colon resection. At inclusion, all patients were in remission defined as a Mayo score ≤ 2, with no single variable > 1, confirmed by a flexible sigmoidoscopy. Patients were asked to provide stool samples by regular mail every month during 1.5 years, but the compliance of sending samples differed from patient to patient. A relapse was defined by colonoscopy or by an increase in symptoms, consistent with UC, together with a calprotectin level > 300 µg/g. For the present study, selection criteria for patients with a flare included clinically diagnosed relapse with 1–3 stool samples before the flare (1–4 months prior), one stool sample at the flare and 1–2 stool samples after the flare (1–6 months post). Selection criteria for patients without a relapse were 2–3 stool samples during consecutive months with no flares the previous six months. The remission group was chosen to be of similar size as the relapse group and patients were included by date order (from inclusion start). None of the patients had been taking antibiotics the last month prior to sample collection or did so during the sampling time. After the last stool sample, numbers of relapses were monitored for an additional three years.

Cohort NEW was included at the time for diagnosis and consisted of patients with new onset of UC[23]. Inclusion criteria were newly diagnosed UC, age 18–75 years without medical treatment for IBD. Exclusion criteria were other severe diseases such as heart, lung, or neurological disease, and active malignancies, as well as antibiotic use during the month before inclusion. The extent of the disease and the endoscopic disease activity were established with colonoscopy or sigmoidoscopy. Stool samples were sent by regular mail at the time of the diagnosis and at month 3, 12, 24 and 36. Numbers of relapses were monitored until month 36.

Stool samples from both cohorts arrived within 24 h from sampling to the clinic and were immediately frozen at − 20 °C. Within one week from sampling the fecal calprotectin was determined by ELISA according to the manufacturer's instructions (Bühlmann Laboratories AG, Basel, Switzerland). Detection range was 10–600 µg/g and samples above the higher threshold were diluted and re-run.

Sample storage experiments at room temperature have shown stability up to 3 days for calprotectin[24] and 5 days for the GA-map Dysbiosis Test[25].

Microbiota analysis, data analyses and statistics. Targeted microbiota analysis of 1 g of fecal samples was performed using the commercially available GA-map Dysbiosis Test (Genetic Analysis AS, Oslo, Norway). The GA-map Dysbiosis Test consists of 54 DNA probes targeting ≥ 300 bacteria on different taxonomic levels assessed as Probe Signal Intensity[25]. Probes are listed in Supplementary Table S1.

Statistical and data analyses were performed in IBM SPSS 25 and R Studio 1.1.456 (R 3.6.1), respectively. Principal component analyses were performed using the pca3d package in R. Bray–Curtis dissimilarity was used in the R vegan package to investigate differences within and between study subjects. Within-patient analyses over time show dissimilarities between one sample to the next for the same patient (established disease) or diagnosis sample to each of the following samples for the same patient (newly diagnosed). Within- vs. between-patient analyses show mean of all dissimilarities between samples for a unique patient (= within) vs. mean of all dissimilarities for a unique patient to all non-related samples (= between). Lower numbers infer higher similarity between samples. For *post-hoc* analysis, Mann–Whitney U test or Kruskal–Wallis test followed by Dunn's multiple comparisons test were used. For demographic patient data, Mann–Whitney U and chi-squared tests were used. Data are shown as median (interquartile range, IQR) unless otherwise stated.

Ethical considerations. All subjects provided their verbal and written informed consent before participation according to the declaration of Helsinki. Approval was obtained by the Regional Ethical Review Board in Gothenburg prior to start of the studies (Dnr 403-12 and 154-09).

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Author contributions

The study protocol was conceived and designed by L.Ö., A.L., H.S., M.S. and M.K.M. Analysis of data was performed by S.I., M.H., A.S. and M.K.M. A.L. and H.S. enrolled the patients and collected patient data. L.Ö. obtained funding. The draft manuscript was written by L.Ö., A.S. and M.K.M. All the authors reviewed the final manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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MINIREVIEW

Determining Gut Microbial Dysbiosis: a Review of Applied Indexes for Assessment of Intestinal Microbiota Imbalances

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ABSTRACT Assessing "dysbiosis" in intestinal microbial communities is increasingly considered a routine analysis in microbiota studies, and it has added relevant information to the prediction and characterization of diseases and other adverse conditions. However, dysbiosis is not a well-defined condition. A variety of different dysbiosis indexes have been suggested and applied, but their underlying methodologies, as well as the cohorts and conditions for which they have been developed, differ considerably. To date, no comprehensive overview and comparison of all the different methodologies and applications of such indexes is available. Here, we list all types of dysbiosis indexes identified in the literature, introduce their methodology, group them into categories, and discuss their potential descriptive and clinical applications as well as their limitations. Thus, our focus is not on the implications of dysbiosis for disease but on the methodological approaches available to determine and quantify this condition.

KEYWORDS dysbiosis, dysbiosis index, gut, imbalance, intestine, microbiome, microbiota

ASSESSMENT OF DYSBIOSIS

Imbalance, dysfunction, or disturbance of the gut microbiota is increasingly recognized as an indicator of a given disease or a poor health status. Due to the complexity and huge interindividual variation in the microbial communities, no gold standard exists to determine the presence or extent of a given imbalance or disturbance, although a multitude of studies refer to it broadly with the term "dysbiosis." An important part of the challenge with defining dysbiosis arises from the fact that due to the huge interindividual variation existing in the healthy population, no clear definition of a healthy gut microbiota has been established to date.

Dysbiosis of the gut microbiota has been associated with numerous adverse conditions, such as *Clostridioides difficile* (formerly designated *Clostridium difficile*) infection (CDI) (1), metabolic syndrome (2, 3), inflammatory bowel disease (IBD) (4), colorectal cancer (5), chronic hepatitis (6), common variable immunodeficiency (7), and even schizophrenia (8). Dysbiosis has also been observed in nonintestinal microbial communities, such as those of the gums (9), oral mucosa and saliva (10), and scalp and forehead (11). The application of the term dysbiosis is quite broad, ranging from a change of a single species to the perturbation of entire microbial communities (12).

To qualify the term dysbiosis, several indexes have been defined and applied. Such indexes may help to characterize diseases and adverse conditions, predict treatment outcomes, and provide information other than the commonly used alpha and beta diversity assessments. It is worth noting that some researchers have applied the term dysbiosis even to conditions associated with improved health status, e.g., as a result of metformin treatment against diabetes mellitus type 2 (13). However, since the prefix "dys" (Greek for "bad," "difficult," or "disordered") implies an adverse condition, in the current context, we will refer to dysbiosis as a condition differing from the normal or

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healthy state. It should, however, be highlighted that due to a significant interindividual variation, defining the normal or healthy gut microbiota remains an inherent challenge (14); consequently, most dysbiosis indexes are based on comparison to a set of individuals or samples used as references. Even so, it is important to emphasize that dysbiosis is not a well-defined condition, and that dysbiosis indexes differ with respect to methodology and clinical context and were developed in different cohorts of individuals to describe a variety of different conditions.

Basic methods and principles related to microbiota assessment in a clinical context were recently reviewed (15). In the present review, we provide a summary of currently applied dysbiosis indexes and explain their calculation and performance in the context of specific diseases and conditions. We consider this very relevant for the choice and use of dysbiosis indexes in future studies. However, the potential causal links between intestinal dysbiosis and human health are not captured by any of the dysbiosis indexes and, thus, are outside the scope of this review.

TYPES OF DYSBIOSIS INDEXES

In May 2020, we searched the scientific literature indexed in the PubMed database for the various combinations of the search terms "dysbiosis" or "disruption" and "score" or "index," together with "gut" or "intestine," in all search fields. We assessed all previous studies regarding the definition and application of gut dysbiosis indexes appearing in these searches.

We grouped the identified dysbiosis indexes into five categories based on the methodology, including large-scale bacterial marker profiling, relevant taxon-based methods, neighborhood classification, random forest prediction, and combined alpha-beta diversity (Fig. 1). The generalizations and extensions of a given method were described when possible. A detailed overview of the different indexes was compiled (Table 1). Below, we introduce the five categories of indexes, starting from the most prevalently used.

Category 1: large-scale bacterial marker profiling. Large-scale profiling of bacterial markers determines the dysbiosis of the gut microbiota by simultaneously identifying a large number of carefully selected marker species or taxa. This type of dysbiosis determination is exemplified by the GA-map dysbiosis test (Genetic Analysis AS, Oslo, Norway), which is one of a few commercial products designed to determine and characterize the dysbiosis of gut microbiota (16). The technology of this product is based on a set of 54 probes targeting the 16S rRNA gene (V3 to V7) at different bacterial taxonomic levels, thereby covering six phyla, *Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Tenericutes,* and *Verrucomicrobia*, which includes 10 bacterial classes, 36 genera, and more than 300 species (Fig. 1, index 1.1). The GA-map dysbiosis test assigns each sample a score from 1 to 5, where a score greater than 2 indicates that the microbial profile is different from a selected reference population and, thus, designated "dysbiosis," while a score of 2 or lower is defined as "normobiosis," indicating a healthy state. Additionally, the species targeted by the test are scored from −3 to 3, where a negative value refers to reduced abundance and a positive value indicates increased abundance compared to the reference population. The details in score calculation for samples and taxa are proprietary and not available in the public domain. The GA-map dysbiosis test was developed based on samples from 668 adults, including healthy controls ($n = 297$), patients with irritable bowel syndrome (IBS) ($n = 236$), and patients with inflammatory bowel disease (IBD) ($n = 135$). The test has been validated in independent cohorts and showed proportions of dysbiosis of 73% in IBS, 70% in treatment-naive IBD, 80% in IBD in remission, and 16% in healthy individuals (16).

Generalizations and extensions. The GA-map dysbiosis test, outlined above, was originally developed and validated for diagnosis and prediction of IBD and IBS; however, studies have also applied the test to evaluate its correlation with the effect of various interventions, including fecal microbiota transplantation, or FMT (17–19), dietary interventions (20–22), and anti-tumor necrosis factor (anti-TNF) therapy against ulcerative colitis, or UC (23). The GA-map index score was reportedly reduced following FMT

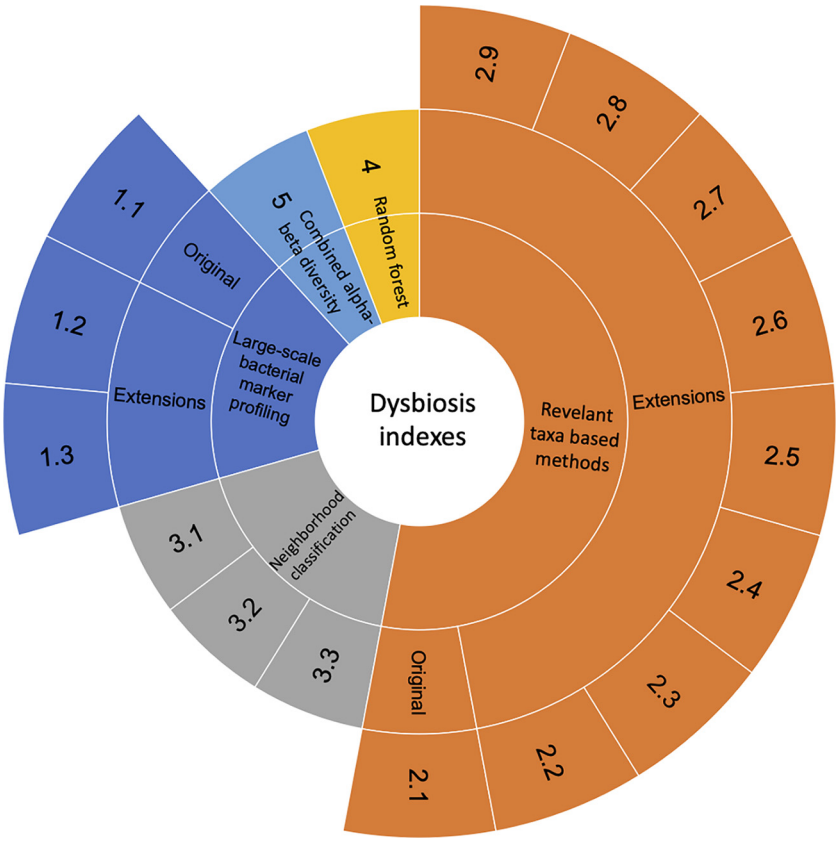

FIG 1 Hierarchical chart to show different indexes used to characterize the dysbiosis of microbial communities. Numbers showing in the outmost layer refer to different indexes. Original corresponds to indexes that are defined early in time and used prevalently. Extension indicates the indexes that are either derived or generalized from the original indexes.

(18, 19), and this was further lowered by repetitive FMT (17). The index was additionally reported to capture the response of the gut microbiota to a diet low in fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAP), which, notably, led to an increase of the dysbiosis index (17, 21). However, other reports find that a low FODMAP diet did not have any effect (22). Magnusson et al. (23) showed that the effect of anti-TNF therapy for patients with UC was partially determined by the gut microbiota composition before treatment and that nonresponders had a higher GA-map index than responders. Another study assessed the performance of the GA-map dysbiosis index for detection of IBS when morbid obesity was considered (24). Four groups of subjects with or without IBS and with or without morbid obesity were compared. Dysbiosis was more frequently detected in morbidly obese subjects, regardless of IBS conditions, than in healthy volunteers. This underlines that confounding factors should be considered when using the GA-map dysbiosis test.

The GA-map dysbiosis test has also been applied to assess the effect of weight loss interventions followed by bariatric surgery (25), nonnutritive sweeteners, or NNSs (26), and primary Sjögren's syndrome, or pSS (27). The dysbiosis index increased after weight loss interventions followed by bariatric surgery and was positively associated with the intake of NNSs. Dysbiosis was additionally found to be more prevalent in pSS patients than in healthy controls.

Some indexes were adapted for specific applications from the GA-map dysbiosis test (13, 22). Farup et al. (13) adjusted the GA-map dysbiosis test and thereby created a new index, named alternative index (ADI) (index 1.2), which allowed differentiation between microbiota disturbances caused by metformin, which has antihyperglycemic and weight-reducing effects (28, 29), and microbiota disturbances caused by NNSs,

TABLE 1 Different indexes to characterize the dysbiosis of microbial communities

Dysbiosis index	Method type	Description	Disease/condition	Method	Reference(s)
1.1	Large-scale bacterial marker profiling	A set of 54 probes targeting 16S RNA gene (V3–V7) and covering more than 300 bacterial markers; dysbiosis index score calculation is a commercial secret, and scores range from 1 to 5 (2 indicates dysbiosis); scores of each taxon range from −3 to 3, where a negative value refers to reduced abundance compared to the reference population	IBS, IBD	Microarray	16
1.2	Modified large-scale bacterial marker profiling	Summed GA map score of 7 taxa selected to differ between metformin and NNS treatment (*Alistipes*, *Proteobacteria*, *Shigella* spp., *Escherichia* spp., *Bacteroides fragilis*, *Ruminococcus gnavus*, *Bacteroides* spp., *Prevotella* spp., and *Dialister invisus*); the index is scaled from −14 to 14	NNSs versus metformin	Microarray	13
1.3	Modified large-scale bacterial marker profiling	Median bacterial scores for 10 taxa differing between responders and the nonresponders (higher abundance of *Bacteroides fragilis*, *Acinetobacter*, *Ruminiclostridium*, *Streptococcus*, and *Eubacterium* in responders; higher abundance of *Clostridia*/*Negativicutes*/*Bacilli*, *Actinomycetales*, *Anaerotruncus*, *Clostridiales*, and *Shigella*/*Escherichia* in nonresponders) are defined as the cutoff; each sample is given a point for each differential taxon; the points for each sample are then summed up, resulting in an index between 0 and 10	FODMAPs diet for patients with IBS	Microarray	22
2.1	Relevant taxon-based methods	Dysbiosis was calculated as $\log_e$ of (summed abundance of taxa increased in patients with CD/summed abundance of taxa decreased in patients with CD)	CD	16S ribosomal amplicon sequencing and shotgun metagenomics	32
2.2	Relevant taxon-based methods	Dysbiosis was calculated as (summed abundance of taxa increased in patients with cirrhosis/summed abundance of taxa decreased in patients with cirrhosis)	Cirrhosis	Multitag pyrosequencing of 16S genes	34
2.3	Relevant taxon-based methods	Dysbiosis was calculated as (summed abundance of taxa increased in patients with CHB/no. of CHB-increased taxa) − (summed abundance of taxa increased in subjects without CHB/no. of taxa increased in the absence of CHB)	CHB	16S ribosomal amplicon sequencing	6
2.4	Relevant taxon-based methods	Dysbiosis was calculated as [(summed abundance of taxa increased in patients with stroke/no. of stroke-increased taxa) − (summed abundance of taxa increased in subjects without stroke/no. of taxa increased in the absence of stroke)]×100	Stroke	16S ribosomal amplicon sequencing	35
2.5	Relevant taxon-based methods	Dysbiosis was calculated as [(summed abundance of taxa increased in patents with gout/no. of gout-increased taxa) − (summed abundance of taxa increased in subjects without gout/no. of taxa increased in the absence of gout)] × 1,000,000	Gout	16S ribosomal amplicon sequencing	36
2.6	Relevant taxon-based methods	Dysbiosis was calculated as $\log_{10}$[(summed abundance of taxa increased in case 1/summed abundance of taxa decreased in case 1) × (summed abundance of taxa	Colorectal cancer (tumorigenesis)	16S ribosomal amplicon sequencing	5

(Continued on next page)

TABLE 1 (Continued)

Dysbiosis index	Method type	Description	Disease/condition	Method	Reference(s)
		increased in case 2/summed abundance of taxa decreased in case 2) × (summed abundance of taxa increased in case 3/summed abundance of taxa decreased in case 3) × (summed abundance of taxa increased in case 4/summed abundance taxa decreased in case 4) + 1]; case 1, 2, 3, and 4 refer to carcinoma, carcinoma adjacent, adenoma, and adenoma adjacent			
2.7	Relevant taxon-based methods	*Acinetobacter johnsonii* and *Streptococcus salivarius* were either positively or negatively associated with RAS; dysbiosis was calculated as 5.35 × (abundance of *A. johnsonii*) − 0.309 × (abundance of *S. salivarius*)	RAS	16S ribosomal amplicon sequencing	10
2.8	Relevant taxon-based methods	Dysbiosis was calculated as (abundance of *Firmicutes*)/(abundance of *Bacteroidetes*)	LC, HF, IBS	16S ribosomal amplicon sequencing	37–39
2.9	Relevant taxon-based methods	Dysbiosis was calculated as (abundance of *Basidiomycota*)/(abundance of *Ascomycota*)	IBD	16S ribosomal amplicon sequencing and ITS2	42
3.1	Neighborhood classification	Dysbiosis index was calculated as the median Bray-Curtis distance between the test sample and the reference	UC, CD	Shotgun metagenomics	43
3.2	Neighborhood classification	Seven selected taxa were reported to be associated with CE in dogs; their abundances were determined by qPCR; dysbiosis was calculated as the difference between (Euclidean distance between the test sample and the healthy class centroid) and (Euclidean distance between the test sample and the diseased class centroid)	CE	qPCR	46
3.3	Neighborhood classification	Dysbiosis was calculated as $\log_2$-transformed CLOUD statistic	SIBO	16S ribosomal amplicon sequencing	54, 55
4	Random forest prediction	Dysbiosis was calculated as out-of-bag probability of random forest to differentiate patients with SIBO from healthy patients	SIBO	16S ribosomal amplicon sequencing	55
5	Combined alpha-beta diversity	Dysbiosis was calculated as (mean difference of Shannon diversity between the test sample and each of donors) × (mean Jensen-Shannon divergence of the test sample and each donor)	CDI	16S ribosomal amplicon sequencing	1

which may induce glucose intolerance (30). While the ADI showed opposite responses to these two interventions, the unmodified GA-map dysbiosis test yielded increased scores in both cases.

For quantification and prediction of the response of IBS patients to a 4-week FODMAP-restricted diet (22), a specific response index (RI) based on the GA-map dysbiosis test was created (index 1.3). Response was defined as a reduction in IBS severity scores of greater than 50%. First, the responders' median bacterial scores (derived from the GA-map dysbiosis test) of 10 selected taxa, which differed between responders and nonresponders, were determined and defined as the cutoff. Each sample was then given a point for each taxon if the less abundant taxa (responders

versus nonresponders) had lower scores than the cutoff or if the more abundant taxa (responders versus nonresponders) had higher scores than the cutoff. The summed points for each sample resulted in an RI range between 1 and 10. Thus, RI was more sensitive than the GA-map dysbiosis test to distinguish and predict treatment outcomes, since it was designed to consider only taxa differing between responders and nonresponders.

Category 2: relevant taxon-based methods. A large number of studies have utilized relevant taxa to create dysbiosis indexes. Such indexes only require the abundances of specific taxa and have been widely used in studies due to their simplicity, especially when sequencing data are available. They are easy to interpret and visualize and are typically calculated based on ratios between abundances, differences between abundances, or abundance-based linear regressions. The dysbiosis indexes introduced here are all based on relative abundances; however, other types of normalizations are also possible (31).

Among these approaches, the method of Gevers et al. was the first introduced and is currently the most widely used (32) (index 2.1). It is known that an altered gut microbial community composition is associated with the pathogenesis of IBD (33). To quantify and define the associations between gut microbiota and Crohn's disease (CD), Gevers et al. compared ileum, rectum, and stool microbiotas from a large number of subjects with CD (n = 447) with those from healthy controls (n = 221). An overall decrease in richness as well as an altered gut microbiota composition was observed in CD subjects. One set of genera was positively correlated with CD, while another set of genera was negatively correlated. Based on this, the authors developed a dysbiosis index (index 2.1), defined as

$$\log_e \frac{\sum_{i=1}^{n} \text{abundance}(\text{CD-enriched taxon})_i}{\sum_{i=1}^{n} \text{abundance}(\text{CD-depleted taxon})_i}$$

This index was found to be negatively associated with species richness and positively associated with CD severity.

Generalizations, extensions, and variants. Gevers' dysbiosis index was later modified for detection of other diseases and conditions. In a study of cirrhosis, Bajaj et al. (34) used the ratio between summed relative abundance of taxa, which in previous studies had been found to be reduced in cirrhosis patients (*Lachnospiraceae*, *Ruminococcaceae*, and *Clostridiales* XIV), and summed relative abundances of previously identified cirrhosis-associated taxa (*Enterobacteriaceae* and *Bacteroidaceae*) (index 2.2):

$$\frac{\sum_{i=1}^{n} \text{abundance}(\text{health-related taxon})_i}{\sum_{i=1}^{n} \text{abundance}(\text{patient-related taxon})_i}$$

Low index values indicated dysbiosis. The index was observed to be higher in healthy controls than in cirrhosis patients and was negatively correlated with endotoxin, death, and organ failures.

In a study of chronic hepatitis B (CHB), Wang et al. (6) defined the dysbiosis index (index 2.3) as

$$\frac{\sum_{i=1}^{n} \text{abundance}(\text{patient enriched taxon})_i}{n} - \frac{\sum_{i=1}^{n} \text{abundance}(\text{health enriched taxon})_i}{n}$$

Thus, this type of index uses the normalized abundance difference instead of the ratio between abundances to quantify dysbiosis. n refers to the number of taxa enriched in either the diseased or healthy state. The larger the index value, the more severe the dysbiosis. Thereafter, the authors applied Youden's J statistic to look for the optimal index value as a diagnostic threshold to differentiate healthy from CHB patients. The

cutoff was set at −25.36, thereby achieving 0.77, 0.75, and 0.81 for accuracy, sensitivity, and specificity, respectively.

Similar indexes include a multiplication by 100 or even by 10^6. For example, Xia et al. (35) defined a dysbiosis index for acute ischemic stroke (index 2.4) as

$$\left[\frac{\sum_{i=1}^{n}\text{abundance(patient enriched taxon)}_i}{n} - \frac{\sum_{i=1}^{n}\text{abundance(health enriched taxon)}_i}{n}\right] \times 100$$

The index was calculated based on 18 discriminant taxa, and it achieved AUCs (area under the receiver operating characteristic curves) of 0.749 in the training cohort and 0.843 in the validation cohort, which indicates a good power for differentiating stroke patients from controls. Patients with stroke showed significantly higher index values than healthy controls.

Along the same lines, a study of patients with gout (36) defined a microbial index of gout (index 2.5) as

$$\left(\frac{\sum_{i=1}^{n}\text{abundance(patient-enriched taxon)}_i}{n} - \frac{\sum_{i=1}^{n}\text{abundance(health-enriched taxon)}_i}{n}\right) \times 10^6$$

The index threshold was set at −2.157 by use of Youden's J statistic. An index above the threshold indicated increased risk of suffering from gout. This index achieved an AUC of 0.817 for the identification of individuals diagnosed with gout.

Some reports define more complicated dysbiosis indexes. To have an index to describe the tumor burden in colorectal cancer development, Nakatsu et al. (5) devised a composite index including the microbiota at multiple sample sites, such as carcinoma (case 1), carcinoma-adjacent (case 2), adenoma (case 3), and adenoma-adjacent (case 4). The dysbiosis index (index 2.6) was then formulated to incorporate the differences in all cases:

$$\log_{10}\left[\left(\frac{\sum_{i=1}^{n}\text{abundance (enriched taxon in case 1)}_i}{\sum_{i=1}^{n}\text{abundance (depleted taxon in case 1)}_i} \times \frac{\sum_{i=1}^{n}\text{abundance (enriched taxon in case 2)}_i}{\sum_{i=1}^{n}\text{abundance (depleted taxon in case 2)}_i} \times \frac{\sum_{i=1}^{n}\text{abundance (enriched taxon in case 3)}_i}{\sum_{i=1}^{n}\text{abundance (depleted taxon in case 3)}_i} \times \frac{\sum_{i=1}^{n}\text{abundance (enriched taxon in case 4)}_i}{\sum_{i=1}^{n}\text{abundance (depleted taxon in case 4)}_i}\right) + 1\right]$$

In contrast to these complex approaches, some studies defined a dysbiosis index based on only a very few taxa. For example, a study included only two relevant taxa in a linear regression to create a dysbiosis index (10). Two relevant taxa found to be significantly associated with recurrent aphthous stomatitis (RAS) (*Acinetobacter johnsonii*) and absence of RAS (*Streptococcus salivarius*), respectively, were identified based on logistic regression. The coefficients of these two species were then incorporated in a linear regression, and the dysbiosis index was defined as 5.35 × [*A. johnsonii*] − 0.309 × [*S. salivarius*], using the relative abundance of *A. johnsonii*

and *S. salivarius* in the mucosa (index 2.7). This dysbiosis index correctly predicted 83% of the total cases for the presence or absence of RAS in the investigated cohort.

Even simpler, some studies used the ratio of only two phyla, such as *Firmicutes* and *Bacteroidetes*, to calculate an index for the description of the gut microbiota (index 2.8). Together, these two phyla constitute the majority of the human gut bacteria, and they represent the Gram-positive and the Gram-negative populations, respectively. Jeffery et al. (37) applied the *Firmicutes*-to-*Bacteroidetes* (F/B) ratio and successfully differentiated two subgroups (F/B high and F/B low) of patients with IBS. Stratification into high and low F/B ratios allowed the identification of different gut microbiotas in IBS versus healthy controls, which was only found in the high F/B group. Liu et al. (38) applied the F/B ratio for studies of patients with liver cirrhosis and observed higher ratio values in patients than in healthy controls. In patients with heart failure (HF), the microbiota was characterized by a decreased F/B ratio and a reduced bacterial diversity, which was associated with clinical outcome (39). In addition, F/B ratio has been linked with obesity; however, this is still controversial (40, 41).

While most gut dysbiosis indexes are based on the bacterial community, Sokol et al. (42) defined the index as the abundance ratio between the two fungal phyla *Basidiomycota* and *Ascomycota*, since these two phyla showed differential abundances across phenotypes classified as IBD, IBD with flare (IBDf), IBD in remission (IBDr), and healthy controls. These two fungal phyla additionally exhibited a strong inverse correlation to each other (index 2.9). The index distinctly separated samples originating from different phenotypes, as healthy subjects scored significantly lower values than IBD, IBDf, and IBDr subjects and IBDf subjects scored significantly higher values than IBDr subjects.

Category 3: neighborhood classification. Neighborhood classification is a way to quantify the deviation of samples from a reference sample set based on the microbial composition assessed with the distance or dissimilarity matrices. Hence, it has been used as a measurement of microbial dysbiosis in given individuals compared to a population of healthy controls. By its application, Lloyd-Price et al. (43) defined a dysbiosis score as the median value of Bray-Curtis dissimilarity between the test sample and a healthy reference population (non-IBD metagenomes) (index 3.1). This index was developed from either the taxonomic or the metabolomic composition of a sample with the computational tools MetaPhlAn2 and HUMAnN2, respectively (44, 45). The tools include internal normalization to the total pool of taxa or metabolites. To determine whether a sample was dysbiotic, a threshold was defined as the 90th percentile of the dysbiosis score in the non-IBD samples. Hence, the median distance value of a test sample above the threshold indicates dysbiosis. This dysbiosis index was observed to distribute differently across disease phenotypes (non-IBD, UC, and CD).

In a more complicated way, AlShawaqfeh et al. (46) introduced a dysbiosis index for dogs with chronic inflammatory enteropathy (CE) (index 3.2), defined as

$$\begin{aligned}&(\text{Euclidean distance between the test sample and the healthy class centroid}) -\\&(\text{Euclidean distance between the test sample and the diseased class centroid})\end{aligned}$$

Thus, the index expresses the difference between the distances to the average healthy and diseased populations for a given microbiota sample. To be disease specific, the Euclidean distance was calculated based on quantification by quantitative PCR (qPCR) of seven carefully selected taxa that were shown to be significantly correlated with CE. An index of zero indicated that the sample had an equal distance to the center of both (healthy and diseased) populations. An index above zero designated deviation from the normal healthy state. This index achieved 74% sensitivity and 95% specificity for separation of healthy and CE dogs. Due to this good performance, numerous

following studies utilized this index to quantify microbial dysbiosis for dogs or cats with CE (47, 48), response to food (49, 50), anthelmintic treatment (51), FMT (52), and alterations after intensive physical activity (53).

Montassier et al. (54) introduced a test named "CLOUD" to look for outliers within a given set of microbiota samples. Although the test was not directly intended to be a measure of dysbiosis, the $\log_2$-transformed CLOUD statistic was used as a dysbiosis score (55) (index 3.3). The normalization of taxon abundances depends on the distance matrix used to determine the CLOUD statistic. Here, normalization based on geometric means of pairwise ratios (GMPR) was used (56). A sample was considered dysbiotic if the CLOUD distance between the test sample and the healthy reference set was more than two standard deviations (SD) larger than the CLOUD distance mean within the healthy reference set. The CLOUD is a nonparametric test and makes no assumptions about the distribution of the reference sample set; thus, it may prove useful to identify less healthy microbial signatures under different conditions, diseases compared to healthy controls, or assessment of the restoration of microbiota following FMT.

Category 4: random forest prediction. The output from the machine learning algorithm random forest (out-of-bag probability, an internal estimation of prediction performance for samples left out of the bootstrap), fed with microbiota data from patients suffering from small intestinal bacterial overgrowth (SIBO) and healthy controls, has been suggested as a dysbiosis index (designated "symptom index") (55) (index 4). This index was based on the operational taxonomic unit (OTU) abundances normalized by GMPR (56). This index ranges from 0 to 1, where values approaching 1 indicate high likelihood of the gut microbiota coming from symptomatic patients. The index successfully differentiated patients with SIBO from healthy controls (AUC, 0.896). Additionally, the index was observed to be associated with specific patient characteristics, such as age and antibiotics use.

Category 5: combined alpha and beta diversity. Alpha and beta diversity have routinely been used in sequencing-based microbiota studies and provide a general description of microbial communities. Alpha diversity, applied for describing the amount of unique taxa (richness) and their distribution (evenness) within a microbial community, is often considered a biomarker of health, since a low gut bacterial alpha diversity in adults is known to be associated with risk markers related to metabolic health (57, 58). Beta diversity, used for assessing differences in microbial community composition between individuals, is also commonly applied to assess differences between patients and healthy controls. Recently, a study combined alpha (Shannon index) and beta diversity (Jensen Shannon divergence, or JSD) and created a dysbiosis index for patients with *Clostridioides difficile* treated with FMT (1). The dysbiosis index of a sample (index 5) was defined as

$$(\text{average difference in Shannon index between the test sample and each of the donors}) \times (\text{average JSD between the test sample and each of the donors})$$

This index generally ranged from 0 to 5. Healthy controls usually have an index between 0 and 1, and patients with dysbiosis have index values larger than 1. This index achieved an AUC of 0.922 at differentiating pre-FMT samples from post-FMT samples.

APPLICATIONS OF DYSBIOSIS INDEXES

Here, we reviewed existing methods to determine and quantify dysbiosis, including large-scale bacterial marker profiling, relevant taxon-based methods, neighborhood classification, random forest prediction, and combined alpha and beta diversity. These approaches all successfully captured the differences between microbiota related to specific conditions of disease or intervention and those present in healthy patients or at baseline (before an intervention).

The large-scale bacterial marker profiling measures a large group of bacterial markers to assess the divergence of a sample from the healthy controls. The GA-map dysbiosis test was specifically designed to diagnose IBD and IBS and has been widely used. However, confounding factors, such as obesity, need to be considered when using this test to avoid misleading conclusions (24). The indexes 1.2 and 1.3 represent modifications of the GA-map dysbiosis test and show the potential to reformulate the basic GA-map dysbiosis test scores for specific purposes.

The relevant taxon-based methods were also developed to be disease specific. Because the relevant taxa can be readily identified under any deviating conditions, they can easily be adapted to different diseases or conditions. Relevant taxon-based methods are convenient dysbiosis index alternatives when next-generation sequencing data are available, such as 16S rRNA gene amplicon sequences or shotgun metagenomic sequences. While the relevant taxon-based dysbiosis index 2.1 was developed in a large sample set, subsequently validated in a different cohort of patients with CD, and prevalently used in other studies, the remaining indexes were much less validated and often developed and utilized to explain the same data set upon which they were based. Thus, the specifically chosen relevant taxa may not be valid in other studies due to the differences in sequencing techniques, statistical analysis, individual differences, and confounding factors.

The neighborhood classification methods utilize distance or dissimilarity matrices to quantify if a test sample is significantly different from a set of healthy controls. Index 3.1 is a simple way of using the distance matrices by subjectively choosing a threshold of distance to distinguish between dysbiotic and nondysbiotic samples. Index 3.2 assesses the closeness of a test sample to the groups of diseased and healthy samples, respectively, in a more complicated way. Because the distance matrix is based on seven carefully selected taxa, index 3.2 is restricted to the disease CE, but generalizing this approach to other conditions remains a possibility. Index 3.3 is a robust, nonparametric outlier test, which makes it an appropriate index for many different conditions.

Random forest is a popular choice for researchers that use machine learning techniques for large and complex biological data sets (59), such as gut microbiota sequence data. Index 4 uses the original out-of-bag probability from random forest as the dysbiosis index to quantify the similarity between a test sample and the dysbiotic samples. This index is not restricted to a specific disease and can be used to differentiate dysbiotic samples from healthy controls. However, index 4 basically is the same as a binary classification of samples by random forest and does not provide much additional information, although the continuous index value potentially can be correlated with clinical characteristics (55).

The combined alpha and beta diversity takes advantage of the commonly used alpha and beta diversity and quantifies the difference between samples originating from patients and from donors of FMT. More validations are needed, because the definitions of dysbiosis and nondysbiosis are not clearly defined in the study.

CONCLUSIONS

Several dysbiosis indexes have been successfully applied to characterize the gut microbiota in patients with different diseases or conditions. They may have important applications in the context of given diseases and treatments.

However, it is important to emphasize that the existence of dysbiosis as measured by a specific index does not imply that the dysbiosis is in any way causal to the given disease. In fact, the altered microbiota characterizing a given disease or intervention often results from alterations in factors such as diet, medication, oxygen availability, or immune reactions (14, 60–63), in which case a dysbiosis index is applicable as a diagnostic marker but not necessarily as a predictor. Difficulties in inferring general principles for the assessment of dysbiosis are, to a large extent, attributed to the huge variation between healthy individuals, leading to a lack of a clear definition of a "normal" gut microbiota. In fact, the existence of a balance of the microbiota that can suddenly

tip over remains to be proven and is highly debated (12). It is important to note that dysbiosis indexes are not standalone measurements and have to be interpreted in the context of the clinical findings. Nevertheless, the value of dysbiosis indexes as simple tools to describe complex differences between intestinal microbial communities remains.

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ORIGINAL CONTRIBUTION

Effects of high intake of cod or salmon on gut microbiota profile, faecal output and serum concentrations of lipids and bile acids in overweight adults: a randomised clinical trial

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Abstract

Purpose To explore whether high intake of cod or salmon would affect gut microbiota profile, faecal output and serum concentrations of lipids and bile acids.

Methods Seventy-six adults with overweight/obesity with no reported gastrointestinal disease were randomly assigned to consume 750 g/week of either cod or salmon, or to avoid fish intake (Control group) for 8 weeks. Fifteen participants from each group were randomly selected for 72 h faeces collection at baseline and end point for gut microbiota profile analyses using 54 bacterial DNA probes. Food intake was registered, and fasting serum and morning urine were collected at baseline and end point.

Results Sixty-five participants were included in serum and urine analyses, and gut microbiota profile was analysed for 33 participants. Principal component analysis of gut microbiota showed an almost complete separation of the Salmon group from the Control group, with lower counts for bacteria in the *Bacteroidetes* phylum and the *Clostridiales* order of the *Firmicutes* phyla, and higher counts for bacteria in the *Selenomonadales* order of the *Firmicutes* phylum. The Cod group showed greater similarity to the Salmon group than to the Control group. Intake of fibres, proteins, fats and carbohydrates, faecal daily mass and output of fat, cholesterol and total bile acids, and serum concentrations of cholesterol, triacylglycerols, non-esterified fatty acids and total bile acids were not altered in the experimental groups.

Conclusion A high intake of cod or salmon fillet modulated gut microbiota but did not affect faecal output or serum concentrations of lipids and total bile acids.

Clinical trial registration This trial was registered at clinicaltrials.gov as NCT02350595.

Keywords Cod · Salmon · Gut microbiota · Faeces · Lipids

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Introduction

Gut microbiota may affect human nutrition and health by actively contributing to food digestion, energy harvesting and production of metabolic active compounds including short chain fatty acids and bile acids, but a definition of healthy human gut microbiome is still lacking [1]. The breakdown of proteins from the diet, followed by absorption of small peptides or free amino acids in the small intestine, is an efficient process in healthy adults; however even easily digestible proteins such as egg proteins escape digestion and absorption in the small intestine and may serve as substrate for microbial energy metabolism in the colon to produce mainly short chain fatty acids [2, 3]. Also dietary fibres, some starch and very small quantities of simple sugars enter the colon for fermentation to short chain fatty acids by bacteria [2].

The short chain fatty acids acetate, propionate and butyrate are generally accepted to be beneficial for the gut function and morphology [4], and improve glycaemic control and reduce appetite in addition to serve as an energy source for the host [5]. Gut microbiota also plays important roles in the microbial deconjugation and dehydroxylation of bile acids and in the enterohepatic circulation of bile acids, and may directly regulate circulating cholesterol concentration [6, 7]. Glycine or taurine conjugated bile acids produced from cholesterol in the liver is deconjugated to secondary bile salts by bile salt hydrolases, found in all major bacterial divisions, before reabsorption as a part of the enterohepatic cycle [6, 7]. Thus, changes in diets can be expected to affect the gut microbiota and through changes in physiological processes this may affect health and development of diseases.

Clinical studies have shown that fish intake or fish protein supplementation is associated with increased HDL-cholesterol and/or lower LDL-cholesterol concentrations [8–13]. Lower circulating cholesterol concentration is also found in rats fed fish or fish proteins [14–20], and it has been suggested that this may in part be regulated through a higher faecal bile acid excretion [14, 19–21]. A reduction in circulating cholesterol concentration through increased faecal bile acid output may therefore be associated with changes in gut microbiota composition.

The information on the effect of diets on gut microbiota composition and function in humans is scarce. Short-term effects on the microbial community structure have been reported in healthy adults after intake of plant- or animal-based diets, or high-fat/low-fibre or low-fat/high-fibre diets [22, 23], and non-digestible carbohydrates (dietary fibres) beneficially affect gut function and affects gut microbiota [24]. The effects of intake of protein-rich foods such as fish on gut microbiota composition and function are still little investigated. A 4-week crossover study with 2235 g of lean seafood per week showed only marginal effects on gut microbiome composition [25], and also supplementation with an n-3 PUFA preparation with high EPA content (2–10 g/day) for 12 weeks in healthy humans had little effect on intestinal bacteria [26]. When fed to rats, proteins from fish affected the composition of gut bacteria in the caecum differently from proteins from beef, pork, casein or soy, resulting in higher abundance of *Firmicutes* and less *Bacteroidetes* [27]. Fish may also affect gut microbiota due to its content of trimethylamine N-oxide (TMAO), found in various fish species including Atlantic salmon and Atlantic cod [28]. TMAO may reduce bile acid production from cholesterol in liver [29], and evidence suggests that changes in bile acid pool size can affect the gut microbiome community structure [30].

In a previous paper, we showed that a high intake of Atlantic salmon (750 g/week) for 8 weeks improved postprandial glucose regulation in study participants with overweight/obesity, whereas high intake of Atlantic cod (750 g/week) did not affect glucose regulation in this study setting [31]. The TMAO content is higher in cod compared to salmon [28], and we recently demonstrated that high cod intake, but not high salmon intake, markedly increased the TMAO concentration in serum and urine of this study population as a result of high TMAO intake from cod fillet [28]. In the present study, we aimed to further explore the biological materials from this randomised clinical trial to investigate if a high intake of cod or salmon would affect gut microbiota composition, faecal output and serum concentrations of lipids and bile acids. Little is known about the effects of high intake of cod or salmon on gut microbiota composition, and whether this could be linked to lipid homeostasis. Our hypothesis was that high intake of cod or salmon would affect gut microbiota and faecal output of lipids and bile acids, and thus improve circulating lipid status in a group of adults with overweight/obesity.

Methods

Participants, study setting and ethics

The study design, the description of study participants, the study setting and the protocol for study visits have been published in detail previously [31]. In brief, the study population consisted of adults with overweight or obesity, and all participants were of Norwegian ethnic origin (Caucasian) living in the Bergen area in South-Western Norway. Inclusion criteria were: BMI ≥ 27 kg/m^2, fasting blood glucose ≤ 7.0 mmol/l and age 18–69 years. Exclusion criteria were high habitual fish/seafood intake (> 500 g/week), pregnancy, incompatibility with fish

consumption (allergies, intolerance and/or dislike), diagnosed diabetes mellitus, heart disease or gastrointestinal diseases, use of medications affecting lipid metabolism or glucose homoeostasis, use of anti-inflammatory medications, use of supplements containing n-3 PUFAs, intentional weight loss and large fluctuation in body weight (> 3 kg) over the previous 2 months. Participants were interviewed about their fish/seafood intake before they were included in the study, and those with a regular fish intake > 1 fish dinner per week were instructed to avoid eating fish for 4 weeks before the baseline visit. Very few participants (< 10%) had an intake above 1 fish dinner per week before enrolment to the study and went through a 4-week fish-free period, and those with a habitual fish/seafood intake > 500 g/week were not included in the study.

The study was designed as a randomised, controlled intervention study with a parallel group design, with three intervention arms: Atlantic cod (wild-caught *Gadus morhua*) in weekly doses of 750 g, Atlantic salmon (farmed *Salmo salar*) in weekly doses of 750 g, and no-fish intake as the Control group. The intervention period was 8 weeks. In total, 76 participants were included in the study and were randomly assigned to the Cod group ($N=27$), the Salmon group ($N=27$) or the Control group ($N=22$). The participants were randomised into the different groups by the project manager by drawing lots, and the participants were informed about their group allocation during the baseline visit. All examinations were conducted at the Clinical Research Unit at the Haukeland University Hospital, Bergen, Norway. To enhance compliance, the participants were contacted by phone approximately 1 week prior to baseline and end point visits, during which they were informed of the schedule and procedures for the following visit. Also, a text message was sent 1–3 days before the 8-week visit, as a reminder of how to prepare for the upcoming visit. For any inquires during the trial period, members of the research group could be reached by email or telephone. Compliance was monitored through interviews; after 1, 4 and 8 weeks intervention, the participants in the fish-eating groups were asked how many dinners with cod/salmon they had not eaten since the last contact, instead of asking how well they had complied, to lower the bar for reporting missing intake. Participants in the Control group were interviewed about their intake of fish/seafood after 1, 4 and 8 weeks. Noncompliance was defined as not following the protocol with regard to fish intake (omitting more than 3 fish dinners in the fish-eating groups), other dietary changes or use of prescription medicine not compatible with the inclusion criteria, or changes in physical activity. As reward for completing the study, participants were offered a dietary consultation with a student dietician and all results from blood analyses.

The study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures were approved by the Regional Committee for Medical and Health Research Ethics of Western Norway (REC no.: 2011/572). Written informed consent was obtained from all subjects.

Health professionals performing blood sampling, and personnel conducting the laboratory analyses, were all blinded to participants' identity and group allocation. All data were analysed anonymously. This trial was registered at clinicaltrials.gov as NCT02350595.

Interventions

Cod and salmon fillets were provided to the participants as frozen skin- and boneless fillet portions (mean weight with standard deviation; 150 (SD 10) g; Lerøy Seafood Group ASA), and pallets of fish were chosen at random from Lerøy's warehouse in Bergen, Norway. The cod and salmon fillets were supplied free of charge to the participants, and were distributed at the baseline visit or at any time during the study period, if preferred. Participants in the Cod group were instructed to eat five dinners per week containing 150 g of cod fillet, and participants in the Salmon group were instructed to eat five dinners per week containing 150 g of salmon fillet. The participants in the fish-eating groups were instructed not to exceed a total amount of 750 g of fish/week, not to consume any other types of fish or seafood during the study period, and to otherwise maintain their normal eating habits throughout the study period apart from eating the mandatory amount of 750 g fish/week. The Control group was also instructed to continue their normal eating habits, except to avoid fish and seafood intake. Participants in both fish intervention groups received a booklet with recipes for inspiration and to help them to increase the variation of their meals, as previously described [13]. The participants in the Control group did not receive any recipes, to avoid them being inspired to change their dietary habits during the study period.

Subjects in all groups were instructed not to change their physical activity level during the intervention period. The participants' dietary intake and habitual lifestyle were recorded at the baseline and the end point visits, using food record charts and a questionnaire for reporting physical activity. The participants were asked about the types of physical activity they engaged in, such as whether they worked out in a gym, were members of sports clubs or whether they worked out individually, the type of physical activity (e.g. hiking, running, biking) and the number of hours of light physical activity (not sweaty/not breathless) or hard physical activity (with sweat/breathless). The participants completed the questionnaire at the baseline and end point visits. The weekly number of hours and the intensities of the physical activities were coded as continuous variables. Reported energy and macronutrient intake and physical activity were

similar between the groups at baseline and did not change within the groups during the study period [31].

Protocol for study visits

The total study period was 8 weeks, with baseline visits between August 22, 2011 and September 19, 2011. Examinations were conducted in the morning after an overnight fast. The participants were instructed not to eat or drink anything except water, and not to use substances containing nicotine after 22.00 h the previous day, and to avoid physical exercise and alcohol for 24 h before each visit.

Body height was measured at the baseline, using a wall-mounted stadiometer (Seca 222; Seca). Body weight and body composition were measured in a fasted state using a bioelectrical impedance analysis device (InBody 720; Biospace Co. Ltd) at the baseline and the end point visits.

Fasting blood samples and morning urine were collected at baseline and end point. Blood was drawn into BD Vacutainer SST II Advance gel tubes (Becton, Dickinson and Company) for isolation of serum and in Vacuette K2EDTA tubes (Greiner Bio-one, Austria) for collection of whole blood. The staff complied with a strict protocol for pre-analytical sample handling to ensure high sample quality. Serum, whole blood and urine were aliquoted and frozen at − 80 °C until analyses.

Estimation of dietary intakes

Participants completed dietary records of the five preceding days before the baseline visit and the 8-week visit, including at least 1 weekend day. The intakes of non-digestible dietary fibre, starch, total fat, cholesterol and protein were calculated from the participants' dietary records using the 'Mat på Data 5.1' software [32], which contains information on the nutrient contents in food items sold in Norway. In this database, dietary fibres are defined as non-digestible carbohydrates, and the collective term carbohydrate is used for available carbohydrates, i.e. including sugars and starch but not dietary fibres. Food records were checked for completeness at both study visits.

Faeces collection and analysis

Fifteen subjects from each experimental group were selected by the project manager (by drawing lots) to do a 72-h faeces collection at baseline and end point, and the participants were informed about this during the baseline visit. Participants were interviewed about their opportunity to store faeces in their home freezer before receiving equipment for collection of faeces. The participants received detailed written and verbal instructions on how to conduct a 72-h faeces collection and were provided with appropriate plastic containers with tight lids (1 l) and a holder to support the containers in the toilet. Faeces were collected on three subsequent days, and participants in the fish groups were instructed to complete the baseline faeces collection before they started consuming cod or salmon.

The participants were instructed to store the samples immediately after each bowel movement in a new container in their own freezer, and they could deliver the faeces samples to our laboratory at any time throughout the intervention period. The faecal samples were stored at − 20 °C until further processing. The faeces were weighed in day by day and the total faeces from 72 h collection for each participant at each time point were pooled, mixed with twice the amount of water, homogenised with a T 50 basic ULTRA-TURRAX® (IKA® Werke, Staufen, Germany) and stored at − 20 °C. Aliquots of faecal samples were weighed before and after freeze drying and the dry mass content of the stool samples was calculated.

Gut microbiota profile analysis was performed using the GA-map® Dysbiosis Test (Genetic Analysis AS, Oslo, Norway) by algorithmically assessing faecal bacterial abundance [33]. Briefly, the test is based on mechanical and chemical bacterial cell disruption and automated total bacterial genomic DNA extraction using magnetic beads, followed by PCR amplification of the 16S rRNA gene. 54 DNA bacterial markers targeting more than 300 bacteria based on their 16S rRNA sequence in seven variable regions (V3–V9) were analysed; 28 bacteria probes are species specific, 18 detect bacteria at genus level and 8 probes detect bacteria at higher taxonomic levels. Probes are listed in Supplemental Table 1. Probe labelling is by single nucleotide extension and hybridisation to complementary probes coupled to magnetic beads, and signal detection by using BioCode 1000A 128-Plex Analyzer (Applied Bio-Code, Santa Fe Springs, CA, USA).

Cholesterol was measured in lipid extracts from faeces as previously described [34, 35] using the Cholesterol Gen.2 kit for Cobas c111 from Roche Diagnostics GmbH (Mannheim, Germany). Total bile acids (3α-hydroxy bile acids) concentration was measured in freeze-dried faeces after solid phase extraction with Chromabond C18 ec (3 ml/200 mg, Macherey–Nagel, Düren, Germany) as described by Suckling et al. [36] and quantification using the enzymatic bile acid assay from Diazyme Laboratories, Inc. on the Cobas c111 system (Roche).

Faecal fatty acids were quantified using a modified van de Kamer method, described in detail elsewhere [37, 38]. In brief, acylglycerols and cholesterol esters were hydrolysed by boiling homogenised faecal samples with KOH and ethanol, before acidifying with HCl. Free fatty acids were extracted from the mixture using petroleum ether, and the molar amount of fatty acids was determined by

titration against 0.1 N NaOH using thymol blue as an indicator.

Analyses of serum, whole blood and urine samples

Analyses of total cholesterol, HDL-cholesterol, LDL-cholesterol, triacylglycerol, total bile acids, glucose, C-reactive protein (CRP) and alanine transaminase (ALT) in blood serum were performed by routine methods at the Laboratory of Clinical Biochemistry at Haukeland University Hospital. Serum NEFA was analysed with the Cobas c111 system (Roche Diagnostics GmbH, Marburg, Germany) using the NEFA FS kit (DiaSys; Diagnostic Systems GmbH). Urine creatinine and HbA1c in whole blood were analysed on the Cobas c 111 system using the CREP2 (Creatinine plus ver.2) kit and the A1C-3 kit with A1CD2 haemolysing reagent (Roche Diagnostics GmbH) for Cobas c111. Trimethylamine N-oxide (TMAO) and 1-methylhistidine (1-MeHis, π-methylhistidine) were measured in serum and urine at Bevital AS (Bergen, Norway, https://www.bevit al.no) using liquid chromatography combined with tandem mass spectrometry, as previously described [39] by adding ion pairs for the analytes and isotope-labelled internal standards to the existing assay.

Outcome measurements

The primary outcome of the present study was the gut microbiota profile after a weekly intake of 750 g fillet from either cod or salmon, or a fish-free diet for 8 weeks. Secondary outcomes were changes in faecal output and serum concentrations of lipids and bile acids after cod or salmon intake, and possible correlations between markers of cod and salmon intake and gut microbiota.

Sample size estimation

The sample size calculation for this trial was originally conducted with the aim of investigating the effects of high intake of cod or salmon on postprandial glucose regulation after a standardised breakfast in participants with overweight or obesity [31]. We estimated that it was necessary to include 76 participants divided into three groups to ensure that 20 participants in each group completed the trial with satisfactory compliance, with a power of 80% and α of 0.05, and of these, 65 participants were included in statistical analyses [31]. In the present study, we wanted to further explore the biological materials from this randomised clinical trial to investigate the effects of a high intake of cod or salmon on gut microbiota composition, faecal output of lipids and bile acids, and circulating lipids. The primary aim of the present study was to investigate the effects of high intake of cod or salmon on gut microbiota. Forty-five participants (15 participants in each experimental group) were selected for collection of all faeces produced in 72 h at baseline and end point, and of these three participants withdrew from the study, four participants were not able to complete their collection of faeces, and one participant was excluded from analysis due to poor compliance; thus, a total of 37 sets of faeces samples were collected for gravimetric and biochemical analyses. We were able to obtain complete microbiota profiles from faeces sampled at both baseline and end point from 33 participants.

This is the first study to investigate the effects of 8 weeks of high intake (750 g/week) of cod or salmon on gut microbiota and faecal output in adults with overweight/obesity; therefore, no data were available for sample size calculation for the present study.

Statistical analyses

Subjects who did not complete the study were excluded from the statistical analyses. For lipids and bile acids in faeces and serum, and for estimated intake of nutrients from dietary records, most data were not normally distributed according to the Shapiro–Wilk test, and nonparametric tests were used to investigate changes within groups (the Wilcoxon-signed ranks test). For these nonparametric data, the Kruskal–Wallis test was used to compare values between the three groups at baseline. Changes within the groups were compared using univariate analysis of covariance (ANCOVA) with adjustment for baseline values after log transformation. Data are expressed as medians and 25th, 75th percentiles. Categorical data were compared using the Pearson's χ^2 test. All comparisons were two-sided. Correlations between parameters were tested using two-tailed Spearman's correlations test. Statistical analyses were conducted using SPSS Statistics 25 (SPSS, Inc., IBM Company) and $P < 0.05$ was considered statistically significant. Patterns of microbiota profiles were investigated by performing principal component analysis (PCA) on matrixes containing centred and standardised concentrations using SigmaPlot 14.0 (Systat Software, Inc.).

Results

Participant characteristics

Seventy-six participants were included in the study and completed the first study visit, and 68 participants completed the trial. Two participants (one woman in the Cod group and one man in the Salmon group) were excluded from analysis because they did not comply with the protocol, and one woman in the Salmon group was excluded from statistical analysis after analyses of postprandial blood glucose revealed that she had prediabetes. In total, 65 subjects (29

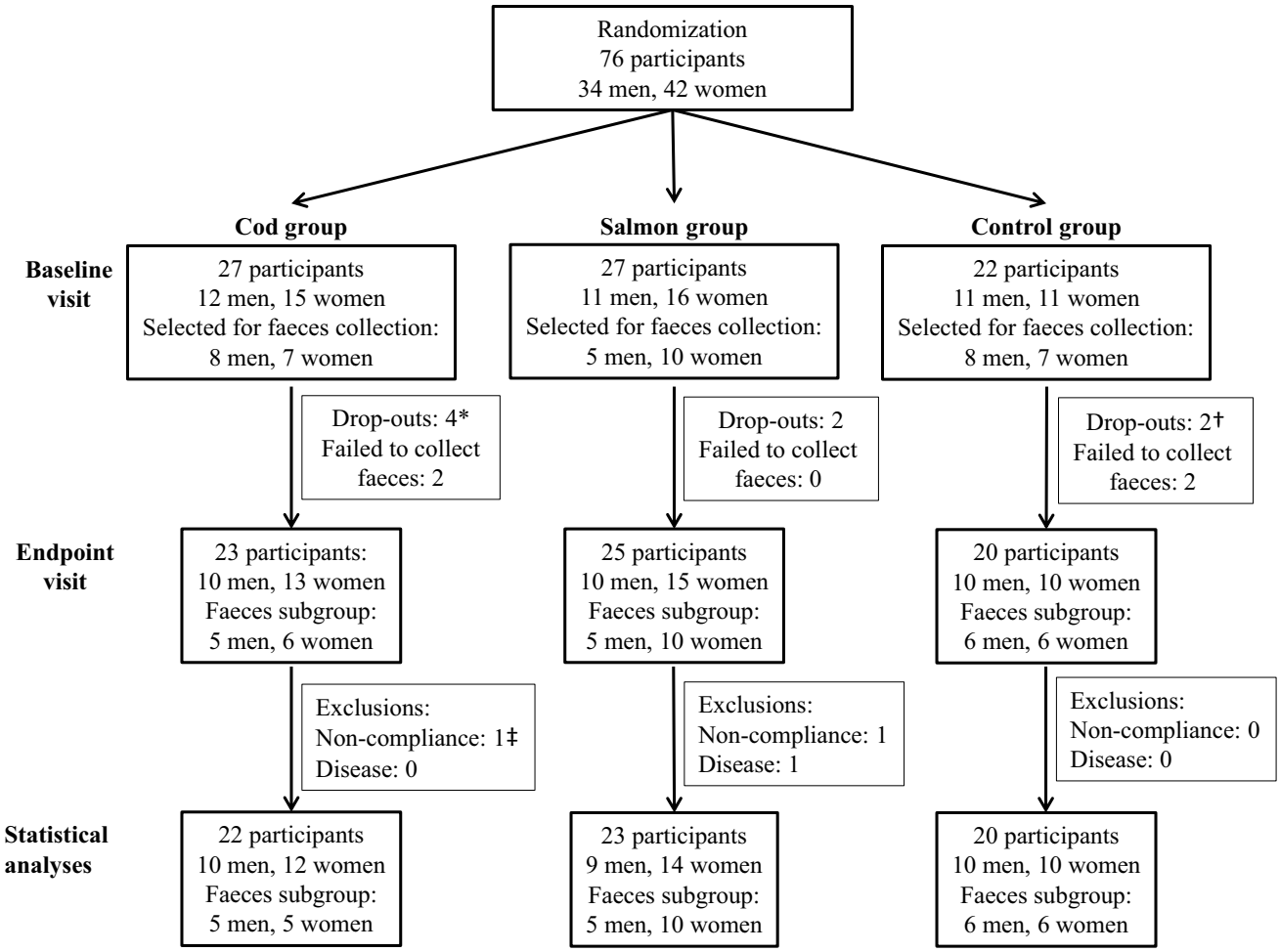

Fig. 1 Study overview of participants. Participants not complying with the protocol were not included in the statistical analyses. Non-compliance was defined as not following the protocol in regard to fish intake (omitting more than 3 fish dinners in the fish-eating groups), other dietary changes or use of prescription medicine not compatible with the inclusion criteria, or changes in physical activity. *Of these, two participants (one man and one woman) in the Cod group were selected for faeces collection. †Of these, one participant (a woman) in the Control group was selected for faeces collection. ‡Of these, one participant (a woman) selected for faeces collection in the Cod group was excluded from analysis

men and 36 women) were included in the statistical analyses. The flow of participants in the study is presented in Fig. 1. The groups were similar at baseline in regard to sex distribution, age, BMI, percentage body fat, percentage muscle mass, reported light (not sweaty/not breathless) and hard (with sweat/breathless) physical activity, and fasting serum concentrations of glucose, CRP and ALT (Table 1), with median age 45.5 (25th, 75th percentiles 36.2, 53.2) years and median BMI 32.3 (25th, 75th percentiles 29.6, 35.7) kg/m^2. After 8 weeks, no changes were seen in any of the groups for BMI, percentage body fat or muscle mass (data not presented). None of the participants reported using antibiotics, probiotics or prebiotics at baseline or end point, or at any time during the intervention period.

Fifteen participants from each of the experimental groups were chosen for collection of two periods of faeces for 72 h at baseline and at the end of the intervention period. Of these 45 participants, 3 withdrew from the study (1 man and 1 woman in the Cod group, 1 woman in the Control group) and 4 men (2 in the Cod group and 2 in the Control group) were not able to complete their collection of faeces. One woman in the Cod group was excluded from analysis due to poor compliance. Table 2 presents the baseline characteristics for the 37 participants collecting faeces.

Estimated dietary intakes and calculated faecal output

The food intake was registered by participants for 5 days before the baseline and end point visits. We have previously shown that there was no difference in estimated energy intake from baseline to end point within any of the groups

Table 1 Participant characteristics at baseline: overview of all participants that completed the study and were included in the statistical analysis

	Cod group ($N=22$)		Salmon group ($N=23$)		Control group ($N=20$)		P^*
Men/women	10/12		9/14		10/10		0.77
Age, years	47.2	38.0, 54.2	45.9	43.1, 52.2	39.8	31.1, 52.7	0.39
BMI, kg/m^2	31.0	29.2, 35.9	31.9	29.9, 34.6	34.4	29.3, 36.5	0.64
Body fat, %	39.3	28.3, 42.9	39.9	30.0, 42.9	39.1	32.5, 41.0	0.99
Muscle mass, %	34.4	32.5, 41.4	33.3	31.5, 40.0	34.0	32.4, 38.5	0.85
Light physical activity, h/week	1	0, 4	2	2, 3	2	1, 3	0.35
Hard physical activity, h/week	2	0, 3	2	1, 2	2	0, 3	0.97
Glucose, mmol/l	5.2	4.9, 5.6	5.2	4.9, 5.5	5.3	4.8, 5.6	0.96
CRP, mg/l	2.0	0.78, 3.25	2.0	0.9, 3.0	2.5	2.0, 5.8	0.084
ALT, U/l	27	20, 43	26	17, 39	28	16, 36	0.56

Medians and 25th and 75th percentiles

CRP C-reactive protein, *ALT* alanine transaminase

*Groups were compared at the baseline using the Pearson's χ^2 (categorical data) or the Kruskal–Wallis test (continuous data). Glucose, CRP and ALT were measured in serum collected in a fasting state

Table 2 Participant characteristics at baseline: overview of participants that collected faeces

	Cod group ($N=10$)		Salmon group ($N=15$)		Control group ($N=12$)		P^*
Men/women	5/5		10/5		6/6		0.58
Age, years	49.4	36.6, 54.5	46.4	43.2, 53.9	39.8	31.1, 52.7	0.28
BMI, kg/m^2	30.4	28.8, 36.7	31.5	29.5, 34.6	33.3	29.1, 36.2	0.90
Body fat, %	37.9	28.2, 40.8	39.9	30.0, 42.2	39.1	33.0, 40.1	0.84
Muscle mass, %	34.6	32.6, 40.7	33.3	31.8, 40.0	34.0	33.2, 37.9	0.81
Light physical activity, h/week	1	1, 3	2	1, 3	2	1, 3	0.81
Hard physical activity, h/week	2	0, 4	2	1, 2	2	0, 3	0.80
Glucose, mmol/l	5.2	4.9, 5.6	5.3	5.1, 5.6	5.2	4.7, 5.8	0.75
CRP, mg/l	2.0	0.9, 4.3	2.0	0.9, 3.0	4.0	2.0, 9.8	0.23
ALT, U/l	25	20, 44	25	17, 32	23	15, 34	0.56

Medians and 25th and 75th percentiles

CRP C-reactive protein, *ALT* alanine transaminase

*Groups were compared at the baseline using the Pearson's χ^2 (categorical data) or the Kruskal–Wallis test (continuous data). Glucose, CRP and ALT were measured in serum collected in a fasting state

[31]. Here, we show that the estimated median intakes of non-digestible dietary fibre, starch, total fat, protein, cholesterol and carbohydrates were not different between the experimental groups at baseline (Table 3). The estimated daily intake of non-digestible dietary fibres, starch, protein, cholesterol and carbohydrates were not changed from baseline to end point. The estimated daily total fat intake was significantly increased in the Control group (15 of the 20 participants showed increased fat intake), but this change was not significantly different when compared to the Cod group and the Salmon group. The combined intake of the macronutrients fat, protein and carbohydrates was not changed within any of the group or between the groups (p ANCOVA for group comparisons was 0.29, data not presented).

In the subgroups of participants that collected all faeces produced for 72 h before the baseline and end point visits, the median daily total faeces wet weight, dry weight, cholesterol, total fat (measured as fatty acids) or total bile acids outputs were not different between the groups at baseline and did not change from baseline to end point within any of the groups (Table 4).

Gut microbiota

The signals for 54 gut microbiota DNA probes in total 72 h faeces collected at baseline and at end point were compared for 33 participants ($N=9$ in Cod group, $N=13$ in Salmon group and $N=11$ in Control group) using PCA. For the baseline analyses, the PCA score plot revealed no

Table 3 Estimated daily dietary intake of dietary fibre, starch, fat, protein, cholesterol and carbohydrates based on 5 days dietary records at baseline and after 8 weeks*

	Baseline		8 weeks		$P^{\dagger}$	$P^{\ddagger}$
	Median	25th, 75th percentile	Median	25th, 75th percentile		
Non-digestible dietary fibre (g/day)						
Cod group	20.0	16.2,24.5	20.6	17.4,27.3	0.43	0.67
Salmon group	18.7	15.5,21.8	18.8	16.3,23.5	0.78	
Control group	17.9	13.8,20.9	17.7	13.8,25.2	0.31	
Starch (g/day)						
Cod group	115	82,141	107	88,136	0.78	0.81
Salmon group	114	93,143	96	80,133	0.41	
Control group	133	99,168	102	95,143	0.21	
Fat (g/day)						
Cod group	90	68,115	83	66,108	0.45	0.096
Salmon group	94	64,120	93	84,120	0.14	
Control group	91	74,113	91	81,126	0.036	
Protein (g/day)						
Cod group	94	84,122	100	74,114	0.86	0.88
Salmon group	86	77,104	92	83,98	0.85	
Control group	92	80,106	91	72,122	0.83	
Cholesterol (mg/day)						
Cod group	411	260,491	400	305,516	0.88	0.79
Salmon group	372	287,602	367	324,474	0.47	
Control group	308	207,438	363	223,502	0.30	
Carbohydrates (g/day)						
Cod group	203	168,248	181	162,225	0.18	0.49
Salmon group	197	167,231	188	144,233	0.74	
Control group	209	164,272	195	141,281	0.90	

Medians and 25th and 75th percentiles

*No differences were seen between the groups at the baseline (Kruskal–Wallis test). Results are presented for 22 participants in the Cod group, 23 participants in the Salmon group and 20 participants in the Control group

†Within-group changes are tested using the Wilcoxon's signed-ranks test

‡Changes within Cod group, Salmon group and Control group are compared using analysis of covariance (ANCOVA) with adjustment for baseline values after log transformation

separation between the dietary groups by the two first principal components (PC1 explained 26.22% and PC2 explained 9.82% of the variation in the dataset, Fig. 2a). The loading plot showed no clear grouping of the bacteria at baseline (Fig. 2b). The PCA showed no relation of the microbiota profile with participants' age, sex, BMI, percent body fat, percent muscle mass, dietary intake (fibre, starch, fat, protein, carbohydrates, cholesterol), serum lipids or NEFA, serum CRP, whole blood HbA1c, stool content of dry mass and moisture, and daily faecal output of fat, cholesterol and total bile acids at baseline (data not presented).

In the PCA for end point data, the participants showed a propensity to cluster according to their experimental group affiliation (PC1 and PC2 explained 25.67 and 10.11%, respectively, of the variation in the dataset, Fig. 3a). The Salmon group was almost completely separated from the Control group, whereas the Cod group showed greater similarity to the Salmon group than to the Control group. The loading plot revealed that the separation was in part due to a trend of lower counts for bacteria that belong to the *Bacteroidales* order in the *Bacteroidetes* phylum and the *Clostridiales* order of the *Firmicutes* phyla, and higher counts for a few bacteria in the *Firmicutes* phylum including the *Selenomonadales* order in the Cod and Salmon groups when compared to the Control group (Fig. 3b).

Serum lipids

Serum concentrations of total cholesterol, LDL-cholesterol, triacylglycerol, NEFA and total bile acids were not changed from baseline to end point within any of the experimental groups, and no differences were seen

Table 4 Faecal output at baseline and after 8 weeks*

	Baseline		8 weeks		*P*†	*P*‡
	Median	25th, 75th percentile	Median	25th, 75th percentile		
Total wet weight of faeces, g/day						
Cod group	189	107,238	183	129,227	0.65	0.53
Salmon group	133	80,228	131	68,190	0.61	
Control group	145	81,198	130	77,197	0.53	
Total dry weight of faces, g/day						
Cod group	42	27,63	39	29,58	0.65	0.61
Salmon group	34	16,51	27	23,30	0.12	
Control group	29	22,39	20	16,37	0.73	
Cholesterol, µmol/day						
Cod group	829	532,1139	938	703,1255	0.65	0.56
Salmon group	699	276,1592	524	258,941	0.14	
Control group	760	347,2201	538	253,1460	0.24	
Total fatty acids, g/day						
Cod group	6.47	3.94,12.52	7.21	4.18,9.33	0.80	0.61
Salmon group	5.63	3.40,8.22	5.88	3.49,9.48	0.31	
Control group	4.24	3.16,7.79	4.62	2.23,8.33	0.53	
Total bile acids, µmol/day						
Cod group	199	183,208	205	188,209	0.14	0.63
Salmon group	198	182,204	203	192,210	0.67	
Control group	193	171,210	197	163,211	0.72	

Medians and 25th and 75th percentiles

*No differences were seen between the groups at the baseline (Kruskal–Wallis test). Results are presented for 10 participants in the Cod group, 15 participants in the Salmon group and 12 participants in the control group

†Within-group changes are tested using the Wilcoxon's signed-ranks test

‡Changes within the Cod group, Salmon group and Control group are compared using the analysis of covariance (ANCOVA) with adjustment for baseline values after log transformation

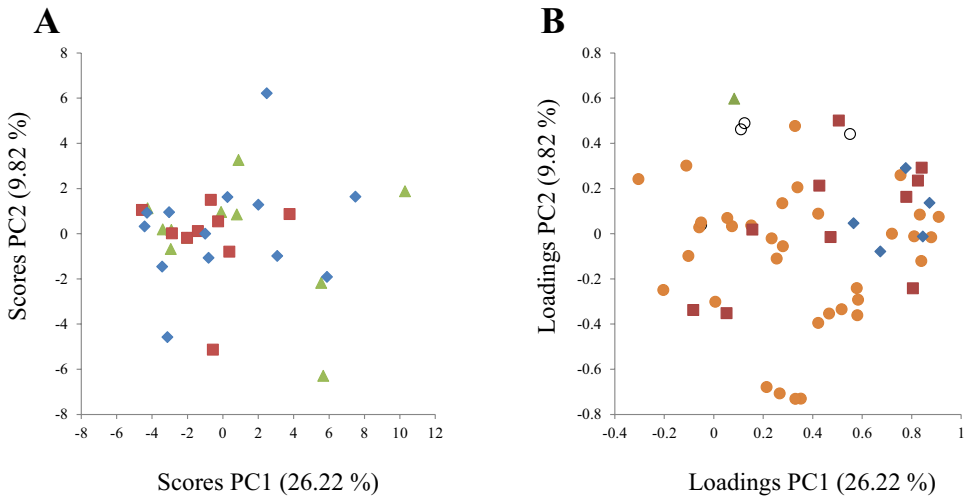

Fig. 2 Scores (**a**) and loadings (**b**) from the first two principal components (PC1 and PC2) obtained from principal component analysis using centred and standardised gut bacteria counts in faeces at baseline for $N=9$ subjects in the Cod group, $N=13$ subjects in the Salmon group, and $N=11$ subjects in the Control group. The score plot (**a**) shows the experimental groups (blue diamond; Cod group, red squares; Salmon group, green triangles; Control group). The loading plot (**b**) show bacterial signals by phylum (open black circles; *Actinobacteria*, red squares, *Bacteroidetes*, orange circles; *Firmicutes*, blue diamond; *Proteobacteria*, green triangle; *Verrucomicrobia*)

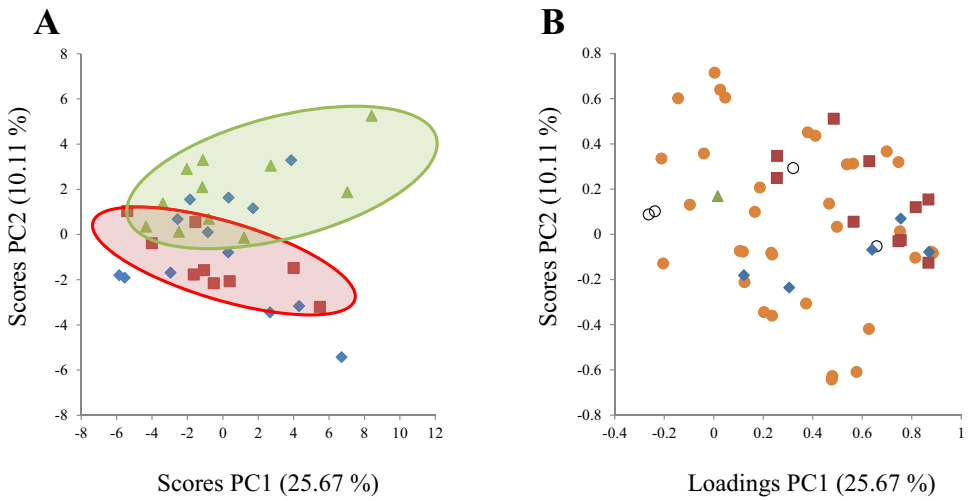

Fig. 3 Scores (**a**) and loadings (**b**) from the first two principal components (PC1 and PC2) obtained from principal component analysis using centred and standardised gut bacteria counts in faeces at end point for $N=9$ subjects in the Cod group, $N=13$ subjects in the Salmon group, and $N=11$ subjects in the Control group. The score plot (**a**) shows the experimental groups (blue diamond; Cod group, red squares; Salmon group encircled by a red ellipse, green triangles; Control group encircled by a green ellipse). The loading plot (**b**) show bacterial signals by phylum (open black circles; *Actinobacteria*, red squares, *Bacteroidetes*, orange circles; *Firmicutes*, blue diamond; *Proteobacteria*, green triangle; *Verrucomicrobia*)

between the groups for changes over time (Table 5). Serum HDL-cholesterol was reduced after 8 weeks in the Control group, but this change was not significantly different when compared to the Cod group and the Salmon group.

Correlations between estimated dietary intakes, faecal outputs and serum lipid concentrations

The combined baseline and end point data sets were analysed using Spearman's two-tailed correlation analysis (Table 6). The dietary intakes of fibre, starch, fat, proteins, cholesterol and carbohydrates were correlated with wet and dry faecal weight, with the strongest correlation between fibre intake and faecal dry weight (Spearman's correlation 0.418, $p=2.4\times10^{-4}$). Faecal cholesterol output correlated with intakes of starch and carbohydrates, and faecal fat output correlated with intakes of fibre, fat, proteins and cholesterol, whereas total bile acid faecal output was not significantly correlated to dietary intakes. Serum cholesterol, HDL-cholesterol, LDL-cholesterol, triacylglycerols and total bile acids concentrations were not correlated to faecal outputs, but serum NEFA concentration was inversely correlated to faecal wet and dry weights and faecal outputs of fat and total bile acids. The strongest associations for faecal outputs of total cholesterol and fat were seen for faecal wet and dry weights, with $p<10^{-8}$, however correlations were not statistically significant between faecal weights and faecal total bile acids output. The correlation between wet and dry daily faecal weights were 0.850 ($p=1.1\times10^{-21}$).

Correlations between biomarkers of Cod and Salmon intake and gut microbiota

We recently found that serum and urine concentrations of TMAO and 1-MeHis were promising biomarkers of fish intake, as both serum and urine concentrations of TMAO and 1-MeHis were significantly increased after cod and salmon intake, respectively, in samples from participants in the present study [28]. Here, we explored the correlations between these biomarkers and gut microbiota signals, and bacteria showing at least one statistically significant correlation are presented in Table 7. Serum and urine TMAO concentrations were positively correlated with the *Akkermansia muciniphila* signal at baseline, whereas these correlations were not siginficant at end point. Serum TMAO concentration was inversely correlated with *Clostridium methylpentosum* at baseline, but not at end point. Urine TMAO concentration was positively correlated with *Bacteroides* sp. at baseline and negatively correlated with *Bacteroides* sp. at end point, but only baseline urine TMAO concentration correlated with *Proteobacteria*.

Baseline serum concentration of 1-MeHis was positively correlated with *Akkermansia muciniphila* and *Lachnosp* Incertae Sedis, *Phascolarctobacterium faecium*, whereas no correlations with gut microbiota were seen for urine 1-MeHis at baseline. At end point, serum 1-MeHis was negatively correlated with *Eubacterium rectale* and urine 1-MeHis was negatively correlated with *Coprobacillus cateniformis*, *Eubacterium rectale*, *Staphylococcus epidermidis* and *Streptococcus agalactiae*, and positively correlated with *Mycoplasma hominis*.

Table 5 Serum fasting concentrations of lipids, NEFA and total bile acids at baseline and after 8 weeks*

	Baseline		8 weeks		*P*†	*P*‡
	Median	25th, 75th percentile	Median	25th, 75th percentile		
Serum total cholesterol, mmol/l						
Cod group	5.7	4.5,6.1	5.5	4.3,6.4	0.84	0.26
Salmon group	5.0	4.4,5.5	4.8	4.2,5.4	0.10	
Control group	5.2	4.4,5.5	5.0	4.3,5.4	0.19	
Serum HDL-cholesterol, mmol/l						
Cod group	1.3	1.0,1.5	1.3	0.9,1.5	0.59	0.21
Salmon group	1.3	1.2,1.4	1.3	1.0,1.4	0.48	
Control group	1.3	1.1,1.6	1.2	1.0,1.5	0.0075	
Serum LDL-cholesterol, mmol/l						
Cod group	3.5	2,6,4.5	3.6	2.6,4.4	0.30	0.39
Salmon group	3.2	2.6,3.6	3.1	2.4,3.5	0.30	
Control group	3.4	2.5,3.6	3.2	2.5,3.5	0.97	
Serum triacylglycerols, mmol/l						
Cod group	1.26	1.08,1.93	1.20	0.91,1.64	0.78	0.33
Salmon group	0.98	0.76,1.45	1.00	0.73,1.53	0.76	
Control group	1.35	0.90,1.69	1.41	0.91,1.83	0.32	
Serum NEFA, mmol/l						
Cod group	0.50	0.39,0.70	0.52	0.37,0.64	0.88	0.92
Salmon group	0.51	0.43,0.71	0.48	0.37,0.56	0.38	
Control group	0.56	0.35,0.66	0.52	0.45,0.63	0.89	
Serum total bile acids, µmol/l						
Cod group	1.0	0.7,2.3	1.5	0.5,2.0	0.81	0.41
Salmon group	1.0	0.6,3.0	1.0	0.8,2.0	0.70	
Control group	1.0	0.8,3.0	2.5	1.0,3.0	0.42	

Medians and 25th and 75th percentiles

*No differences were seen between the groups at the baseline (Kruskal –Wallis test). Results are presented for 22 participants in the Cod group, 22 participants in the Salmon group and 19 participants in the control group. NEFA, non-esterified fatty acids

†Within-group changes are tested using the Wilcoxon's signed-ranks test

‡Changes within Cod group, Salmon group and Control group are compared using the analysis of covariance (ANCOVA) with adjustment for baseline values after log transformation

Discussion

In the present study, we investigated the effects of high intake of cod or salmon on the gut microbiota profile. We observed that a weekly intake of 750 g of cod or salmon changed the microbiota composition, but did not change faecal output and serum concentrations of lipids and total bile acids.

Dietary proteins are substrates for microbial energy metabolism in the colon [2, 3] and may affect gut microbiota profile. Here, we show that high intake of cod or salmon resulted in a separation of bacteria by the PCA, with lower counts for bacteria in the *Bacteroidetes* phylum and the *Clostridiales* order of the *Firmicutes* phyla, and higher counts for bacteria in the *Selenomonadales* order in the *Firmicutes* phylum when compared to the Control group. The separation from Control group was almost complete for participants in the Salmon group, and less clear for those in the Cod group. Although the first two components from the PCA explained merely 35.78% of the variation in the dataset, this observed separation between the experimental groups are of potentially great interest. A higher abundance of *Bacteroidetes* has been found in patients with type 2 diabetes [40], and the present finding of lower counts of *Bacteroidetes* especially in the Salmon group fits nicely with our previous report of improved postprandial glucose regulation after high salmon intake [31]. Lower numbers of *Bacteroides* spp. have also been observed in fish fermentations compared to chicken in vitro for human faeces [41]. Similar findings have been reported for gut bacteria in the caecum in Sprague–Dawley rats fed fish as the sole protein source, with lower abundance of *Bacteroidetes* however with higher abundance of *Firmicutes* [27], whereas intake of lean seafood or n-3 PUFA supplementation did

Table 6 Spearman's correlations of estimated daily intakes, faecal daily outputs and serum concentrations of lipids and total bile acids, combined for baseline and end point data sets

	Estimated daily intake						Faecal daily output					Serum concentrations				
	Fibre	Starch	Fat	Protein	TC	CH	Wet weight	Dry weight	TC	Fat	TBA	TC	HDL	LDL	TAG	NEFA
Estimated daily intake ($N=130$)																
Starch	0.375***															
Total fat	0.201*	0.302***														
Protein	0.269**	0.356***	0.699***													
TC	−0.010	−0.061	0.479***	0.612***												
CH	0.439***	0.753***	0.304***	0.233**	−0.140											
Faecal daily output ($N=74$)																
Wet weight	0.335**	0.322**	0.307**	0.393***	0.267*	0.336**										
Dry weight	0.418***	0.368**	0.326**	0.368**	0.214	0.353**	0.850***									
TC	0.042	0.317**	0.014	0.078	−0.086	0.368**	0.739***	0.604***								
Fat	0.431***	0.136	0.294*	0.358**	0.325**	0.150	0.713***	0.778***	0.368**							
TBA	−0.034	0.176	0.070	0.087	−0.034	0.204	−0.175	0.001	−0.187	−0.224						
Serum fasting concentrations ($N=130$)																
TC	−0.152	−0.231**	−−0.009	0.088	0.310***	−0.210*	0.024	0.053	−0.056	−0.003	0.119					
HDL	−0.054	−0.130	−0.070	−0.166	0.011	−0.155	0.006	−0.057	−0.084	0.073	−0.203	0.005				
LDL	−0.115	−0.209*	−0.006	0.085	0.281**	−0.182*	−0.004	0.044	−0.099	−0.011	0.148	0.955***	−0.150			
TAG	−0.104	0.072	0.021	0.109	0.143	0.085	0.069	0.073	0.137	−0.056	0.159	0.453***	−0.381***	0.373***		
NEFA	0.017	−0.073	−0.238**	−0.181*	−0.202*	−0.103	−0.233*	−0.252*	−0.105	−0.262*	0.245*	0.043	0.018	0.006	0.092	
TBA	−0.015	0.070	−0.004	−0.097	−0.109	−0.030	−0.071	0.058	0.065	−0.009	0.101	−0.121	−0.073	−0.156	0.042	−0.015

Fibre non-digestible dietary fibre, *TC* total cholesterol, *CH* carbohydrates, *TBA* total bile acids, *HDL* HDL-cholesterol, *LDL* LDL-cholesterol, *TAG* triacylglycerols, *NEFA* Non-esterified fatty acids

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

Table 7 Spearman's correlations of serum and urine concentrations of trimethylamine N-oxide (TMAO) and 1-methylhistidine (1-MeHis) with gut microbiota combined for Cod group, Salmon group and Control group, showing only correlations where at least one bacterial probe is significantly correlated with TMAO or 1-MeHis

Counts	Serum (μmol/l)		Urine (μmol/mmol creatinine)	
	TMAO	1-MeHis	TMAO	1-MeHis
Baseline				
Akkermansia muciniphila	0.416*	0.366*	0.375*	0.084
Bacteroides sp.	0.200	−0.068	0.372*	−0.066
Clostridium methylpentosum	−0.350*	0.096	−0.073	0.218
Coprobacillus_cateniformis	0.229	−0.085	0.047	−0.011
Eubacterium rectale	−0.115	0.086	−0.071	0.141
Lachnosp Incertae Sedis	−0.186	0.382*	−0.127	0.331
Mycoplasma hominis	0.253	0.100	0.298	0.232
Phascolarctobacterium faecium	0.129	0.437*	0.212	0.222
Proteobacteria	0.201	0.166	0.358*	0.064
Staphylococcus epidermidis	−0.213	0.125	−0.294	0.136
Streptococcus agalactiae	−0.297	−0.117	−0.148	0.085
Endpoint				
Akkermansia muciniphila	0.306	−0.022	−0.142	−0.106
Bacteroides sp.	−0.128	0.103	−0.417*	−0.087
Clostridium methylpentosum	0.135	−0.077	0.106	−0.097
Coprobacillus cateniformis	−0.025	−0.079	−0.163	−0.415*
Eubacterium rectale	−0.130	−0.384*	−0.286	−0.482**
Lachnosp Incertae Sedis	−0.059	−0.214	−0.265	−0.338
Mycoplasma hominis	0.080	0.151	−0.040	0.371*
Phascolarctobacterium faecium	0.201	0.272	−0.223	−0.216
Proteobacteria	0.089	−0.062	0.126	0.034
Staphylococcus epidermidis	−0.121	−0.321	−0.307	−0.364*
Streptococcus agalactiae	−0.233	−0.310	−0.282	−0.513**

*Correlation is significant at the 0.05 level (two-tailed)

**Correlation is significant at the 0.01 level (two-tailed)

not affect gut microbiome composition [25, 26]. The differences in gut microbiota profile between the experimental groups in the present study could be a consequence of the high intake of cod or salmon in the fish groups, but it may also be due to the higher intake of meat in the Control group where nearly 100% of dinners contained red or white meat as opposed to around only 29% of the dinners in the fish-eating groups [28]. Since there is no consensus regarding what can be considered a healthy human gut microbiome [1], an interpretation of the present findings cannot be made beyond stating that high intake of cod or salmon has the capacity to alter the gut microbiome profile compared to a Control group consuming red or white meat.

The faecal microbiota richness and variance of bacteria is strongly influenced by stool moisture, which in turn is determined by transit time and residence time in the colon and rectum [1]. Fibre intake, especially non-digestible fibre, and physical activity are associated with shorter intestinal transit time and increased gastrointestinal motility, leading to a higher water content in the stool [42, 43]. Also total food intake, body weight and diets are important determinants for total stool mass [42]. The medians for measured daily faecal wet weight and outputs of dry mass, fat, cholesterol and total bile acids were within the normal ranges and indicated healthy gastrointestinal function in our participants [42]. Non-digestible fibres undergo minimal changes in the digestive tract and have a high water holding capacity and thus increase faecal mass, but also digestible fibres can cause an increase in faecal weights since the presence of fermentable substrates will stimulate proliferation of bacteria and results in soft, bulky and water-retaining stools [42]. In line with this, the unchanged faecal wet and dry weights (from two 72 h faecal collection periods) during the course of the present trial corresponds well with the unaltered intake of dietary fibre and the unchanged levels of physical activity [31]. The significant correlation between estimated dietary fibre intake and measured faecal dry mass output (Spearman's correlation 0.418, $p = 2.4 \times 10^{-4}$) suggests that both the registration of food intake and the collection and analysis of faeces were successful. Since no changes were seen in wet and dry faecal mass within any of the experimental groups, we conclude that the differences in gut microbiota

profile between the groups were due to genuine differences in composition and were not a result of a 'dilution effect'.

The role of faecal output in overweight and obesity is unclear, but the removal of excess energy by increased faecal excretion of lipids represents an attractive target and is the principle of weight loss drugs such as Orlistat. The faecal fat consists of undigested fat from dietary intake, bacteria and shredded epithelial cells [42], and the faecal fat excretion in healthy persons is ≤ 7 g per day when the daily fat intake from the diet is between 50 and 150 g [44]. In the present study, the estimated median fat intake from food diaries at baseline and end point was 90.5 (interquartile range 72–114) g/day and the measured median faecal fat excretion for these time points was 5.5 (interquartile range 3.5–8.9) g/day, and we found a weak but significant correlation between fat intake and faecal fat excretion (Spearman's correlation 0.294, $p = 0.012$). Diets that are rich in dietary fibres will increase the faecal output of fat by reducing the absorption capacity in the gut [42], and in line with this we found a strong correlation between dietary fibre intake and faecal fat output (Spearman's correlation 0.431, $p = 1.4 \times 10^{-4}$). Fat contributed to a median 19% (interquartile range 14–23) of the faecal dry weight in the present study and these parameters were strongly correlated (Spearman's correlation 0.778, $p = 3.6 \times 10^{-16}$). Also exercise may affect gastrointestinal and gallbladder motility [43]; therefore, since the participants in this study were instructed to not change their eating habits except for the mandatory fish intake in the Cod group and the Salmon group and no-fish intake in the Control group, together with no change in the level of physical activity during the intervention period [31], it is not surprising that we did not observe any changes in faecal fat excretion.

The circulating concentration of cholesterol is controlled by the rate of cholesterol biosynthesis regulated by HMG-CoA reductase, by the uptake of LDL-particles facilitated by LDL-receptors and through faecal excretion of cholesterol and bile acids. Bile formation is essential for total body cholesterol balance, as biliary excretion of cholesterol and conversion of cholesterol to bile acids are the principal routes of cholesterol excretion and catabolism, and the gut microbiota plays a key role in the enterohepatic circulation of bile acids [6, 7]. An increase in faecal bile acid excretion would enhance the biosynthesis of cholesterol and trigger an increased production of bile acids and up-regulate LDL-receptors leading to lower circulating LDL-cholesterol concentrations. We recently investigated the effects of replacing 25 wt% of casein in the regular AIN-93G diet [45] with corresponding amounts of proteins from cod or salmon fillets on cholesterol metabolism in obese Zucker fa/fa rats. Cod fillet feeding resulted in lower serum total, HDL- and LDL-cholesterol concentrations and lower hepatic mRNA expressions of HMG-CoA reductase and LDL-receptor, without affecting serum and faecal total bile acid concentration, faecal and liver cholesterol amounts in faeces, or liver cholesterol 7 alpha-hydroxylase mRNA concentration in these rats [16]. These findings indicate that the lower circulating cholesterol concentration was not regulated through increased faecal output of cholesterol and/or bile acids, but was a result of lower endogenous production of cholesterol in rats fed cod fillet. Similar results were seen when obese Zucker fa/fa rats were fed salmon fillet as 25 wt% of the diet; serum concentrations of total, HDL- and LDL-cholesterol were lower in these rats compared to those fed the regular AIN-93G diet, with no effect on faecal excretion of cholesterol and total bile acids [17]. In contrast to our findings in rats, we observed no effects on serum cholesterol after high intake of cod or salmon in the present clinical trial. However, the observation that faecal outputs of cholesterol and total bile acids were not affected by high cod or salmon intake in our participants with obesity/overweight corresponded well with previous studies in rats fed cod or salmon fillet [16, 17].

TMAO concentrations in serum and urine are affected by gut microbiota production of trimethylamine from dietary precursors, dietary intake of TMAO and/or its precursors, and kidney function [46, 47]. Participants in the present study appeared to have normal kidney function since serum creatinine and urine albumin concentrations were within normal range both at start and the end of the intervention period, and TMAO seems to be a good biomarker for cod intake in this study population with a significant increase in TMAO concentrations in serum and urine in this dietary group after 8 weeks intervention [28]. Trimethylamine, the bacterial precursor for TMAO, is generated by gut microbiota through three pathways, either from choline, trimethylglycine (betaine), ergothioneine, dimethylglycine or carnitine, which are found in common foodstuffs such as seafood, terrestrial meat, egg, dairy products, vegetables, mushrooms, kidneys and liver; from dietary L-carnitine that may be converted to gamma-butyrobetaine [46]; or by reduction of TMAO by the gut microbiota (predominantly *Enterobacteriaceae* in the phyla *Proteobacteria*) [48]. Trimethylamine is transported to the liver for oxidation to TMAO by flavin-containing monooxygenases [46]. Various bacterial phyla including *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* are associated with the first pathway (mentioned above) in the human colon [46], whereas especially *Akkermansia muciniphila* has been demonstrated to be associated with the carnitine–gamma-butyrobetaine–TMAO pathway [49]. In the present study, we found positive correlations between serum and urine TMAO concentrations and the *Akkermansia muciniphila* signal (phylum: *Verrucomicrobia*) at baseline, and correlations were also found between serum TMAO concentration and *Clostridium methylpentosum* (phylum: *Firmicutes*), and between urine TMAO concentration and *Bacteroides* sp. (phylum: *Bacteroidetes*) and unspecified bacteria of the phylum *Proteobacteria*.

Thus, TMAO may have been produced by either of the microbiota pathways for trimethylamine at baseline. The hepatic production of TMAO from gut microbiota-originating trimethylamine is small compared to the TMAO contribution from fish intake [46]. Therefore, it is not surprising that the correlations between TMAO and gut bacteria for our study population were fewer (the only correlation was for urine TMAO concentration with *Bacteroides* sp.) after interventions with cod or salmon in two of the three experimental groups, since TMAO predominately originated from fish intake and not microbial production.

1-MeHis, a potential biomarker for salmon intake [28], has not been described as a metabolite of or substrate for the gut microbiota. We have recently shown that serum and urine concentrations of 1-MeHis were significantly increased in the Salmon group in the present study [28]. 1-MeHis from diet is not reutilised for protein synthesis or metabolised, and it is estimated that 90% of 1-MeHis from the diet is excreted in urine as 1-MeHis in humans [50]. At end point, urine concentration of 1-MeHis was negatively correlated with a few bacterial species, mainly in the *Firmicutes* phylum, with the strongest negative correlations with *Eubacterium rectale* (class: *Clostridia*) and *Streptococcus agalactiae* (class: *Bacilli*), and with positive correlation with *Mycoplasma hominis*. Since little information is available regarding the consumption and production of 1-MeHis by gut microbiota, it is difficult to conclude if these correlations are a result of changes in consumption or production of 1-MeHis by gut microbiota or simply non-causal associations. The described correlations between urine 1-MeHis concentration and gut microbiota correspond well with the results from PCA using end point data, thus urine 1-MeHis concentration may be a useful biomarker for salmon intake in future studies when interpreting the effects of diets on gut microbiota in humans, as an alternative or an addition to food diaries or food-frequency questionnaires.

We have recently shown that a high intake of salmon, but not of cod, for 4 weeks reduced triacylglycerol and increased HDL-cholesterol serum concentrations in healthy, normal weight adults [13]. This is in sharp contrast to findings in adults with overweight or obesity in the present study, where we found no effect of high cod or salmon intake on any of the measured serum lipid concentrations. This may be a consequence of larger heterogeneity in regard to factors such as age and percentage of body fat and muscle mass in the latter study.

The present study has some strengths and limitations. A strength is the use of 72 h faeces collection at baseline and end point, rather than using spot samples since the day to day variation in the faecal mass can vary widely for the same individual [1]. Also, faecal mass is affected by diet and physical activity [42], and gut microbiota is affected by medications including antibiotics [51] and metformin [52]. Our study participants did not change their diet except the mandatory intake of cod or salmon in the fish-eating groups or their physical activity, and none of the participants reported using antibiotics, probiotics, prebiotics or antidiabetics at baseline or end point, or at any time during the intervention period. Limitations for this study include shortcomings associated with the participants' faeces collection, such as failing to collect all produced stool over the 72 h periods and immediately depositing the filled containers in the freezer as instructed. The choice of analysing only 54 bacterial DNA probes using a method originally developed for identifying dysbiosis [33] can be criticised; still, the findings are of interest since the Salmon group and the Control group were partially separated by PCA. Complete gut bacteria characterisation should be conducted in future studies with similar design. The intakes of prebiotics such as polyphenols and inulin found in fruits, vegetables and berries may also affect gut microbiota [1, 53], but were not estimated in the present study. The sample size was relatively small, and a type II error may mask significant effects of the intervention in both gut microbiota and faecal output and serum concentrations of lipids and total bile acids due to low statistical power.

To conclude, despite the small sample size and the limited number of bacterial probes used, this study is unique since it demonstrates that a high intake of cod or salmon fillet may modulate gut microbiota in adults with overweight/obesity without known gastrointestinal disease. The high intake of cod or salmon did not affect faecal output or serum lipids and bile acids.

Authors contribution GR, HS, GM and OAG formulated the research question and designed the study. MB, IVH, AH and OAG conducted the clinical study. MB, IVH, AH, FE, ØM, PMU, TH and OAG analysed the data and performed statistical analyses. OAG drafted the paper and had primary responsibility for the final content. All authors have contributed to the writing and approved the final manuscript. We thank all participants who have contributed to the current study.

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Compliance with ethical standards

Conflict of interest GR and HS are employed in Skretting Aquaculture Research Centre AS and Lerøy Seafood Group ASA, respectively. Skretting Aquaculture Research Centre AS is a global leader in providing innovative and sustainable nutritional solutions for the aquaculture industry. Lerøy Seafood Group ASA is the leading exporter of sea-

food from Norway and the world's second largest producer of Atlantic salmon. Skretting Aquaculture Research Centre AS and Lerøy Seafood Group ASA were not involved in on-site data collection. The other authors declare no conflicts of interest.

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Mycophenolate mofetil for systemic sclerosis: drug exposure exhibits considerable inter-individual variation—a prospective, observational study

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Abstract

Objective: Mycophenolate mofetil (MMF) is an established therapy for systemic sclerosis (SSc), but its pharmacokinetics in this disease remains unexplored. We have investigated drug exposure in MMF-treated patients with SSc in relation to clinical features of the disease and common concomitant drugs.

Methods: This study was predefined to include 35 MMF-treated SSc patients who were using MMF at a fixed dose of 0.5, 1.0 or 1.5 g twice daily since at least 3 months. The 12-h drug exposure of the active MMF metabolite mycophenolic acid (MPA) was estimated by repeated analysis of plasma MPA over a 6-h period. This 12-h drug exposure was dose normalised to a daily intake of 3 g MMF (MPA_AUC_{3g}) in order to compare subjects using MMF at different doses. Drug exposure was analysed in reference to the clinical characteristics including body weight, renal function, autoantibodies, intestinal dysbiosis, intestinal inflammation assessed by faecal (F)-calprotectin, intestinal symptoms assessed by the University of California Los Angeles Scleroderma Trial Consortium Gastrointestinal Tract Instrument 2.0 and concomitant drug usage including proton-pump inhibitors (PPI).

Results: Thirty-four out of 35 study participants completed the study. The mean daily MMF dose was 2.1 g. Drug exposure expressed as MPA_AUC_{3g} varied up to 8-fold between patients (median 115, range 27–226 mg h/L). MPA_AUC_{3g} was inversely related to body weight ($r_s = -0.58$, $p < 0.001$) and renal function ($r_s = -0.34$, $p = 0.054$). Anti-topoisomerase-1 antibodies and male sex were associated with lower MPA_AUC_{3g} (87 vs 123 and 71 vs 141; $p = 0.008$ and $p = 0.015$, respectively). MPA_AUC_{3g} was inversely related to the intestinal abundance of lactobacilli and to F-calprotectin ($r_s = -0.54$, $p = 0.004$; $r_s = -0.36$, $p = 0.034$), but not to gastrointestinal symptoms. MPA_AUC_{3g} was inversely related to PPI usage ($r_s = -0.45$, $p = 0.007$). We found no association between MPA_AUC_{3g} and disease subtype, disease duration or disease activity.

Conclusion: MMF-treated SSc patients exhibit considerable inter-individual variation in drug exposure, and lower MPA levels were primarily found in PPI users with poor prognostic factors. Body weight, renal function, sex, serology, gastrointestinal manifestations and/or measuring individual MPA exposure should be considered when using MMF for SSc.

Keywords: Systemic sclerosis, Scleroderma, Mycophenolate mofetil, Dysbiosis

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Introduction

Mycophenolate mofetil (MMF) was introduced in 1995 as an immunosuppressant to prevent graft rejection in recipients of solid organ transplants [1]. Today, MMF is also an established therapy in systemic sclerosis (SSc) [2–5].

The pharmacokinetics of MMF is complex [6, 7]. Briefly, ingested MMF is hydrolised to the active metabolite mycophenolic acid (MPA) in the stomach and then rapidly absorbed. MPA is converted to the inactive metabolite MPA-7-*O*-glucoronide (MPAG) in the liver and kidney and excreted in the bile and urine. MPAG that reaches the intestine is deconjugated to MPA by anaerobe bacteria and readily reabsorbed [7].

Individual MPA exposure is difficult to estimate based on a single analysis of plasma MPA. A good estimation of MPA exposure can be made by measuring plasma MPA multiple times during a 3–6-h period and calculating MPA area under the concentration-time curve 0–12 h (MPA_AUC_{0-12}) [6–8].

The treatment effect of MMF in recipients of solid organ transplants has been studied in relation to MPA_AUC_{0-12}. Levels below 30 mg h/L have been associated with increased risk of irreversible transplant failure while exposure above 60 mg h/L did not improve outcomes, but caused more frequent side effects. Consequently, monitoring of MPA exposure has been advocated for recipients of solid organ transplants [6].

The pharmacokinetics of MMF has also been investigated in systemic lupus erythematosus (SLE) and chronic graft versus host disease (GvHD), where data suggests that drug efficacy is dependent on plasma concentration and not on dose [9, 10]. In SLE, the treatment effect levelled out when MPA exposure exceeded 45 mg h/L and a target MPA_AUC_{0-12} level of 35 mg h/L has been suggested for this disease [9]. In GvHD, a drug exposure of at least 30 mg h/L has been suggested [10].

Gastrointestinal involvement in SSc is multifaceted and has been associated with dysbiosis and malabsorption [11, 12]. Dysbiosis in SSc has been characterised by a relative abundance of lactobacilli in three independent studies [12–14]. Malnutrition and malabsorption have been associated with poor outcome in new-onset SSc and altered uptake of the drug penicillamine [15, 16].

In SSc, MMF at a target dose of 3 g daily has shown equivalent efficacy as cyclophosphamide against lung fibrosis [2]. Later studies have confirmed a role for MMF in the treatment of skin and lung fibrosis in SSc, albeit our knowledge on optimal dosing remains limited [3–5, 17]. Both the immunosuppressive and the antifibrotic effects of MPA are concentration-dependent [6, 18].

The purpose of this study was to investigate MPA exposure in SSc subjects treated with MMF. Our secondary objective was to investigate how the pharmacokinetics of MMF relates to clinical characteristics including gastrointestinal factors and to medications commonly used in SSc.

Patients and methods

Patients

This prospective open-label study encompassed patients with SSc fulfilling the 2013 ACR/EULAR classification criteria who were using MMF tablets in a fixed dose twice daily since ≥ 3 months [19]. Participants were recruited from all over Sweden with the help of the national patient organisation for SSc. Based on our current knowledge on the pharmacokinetics of MMF, patients with a history of gastrointestinal surgery including resection of any part of the gastrointestinal tract, renal failure or pulmonary arterial hypertension were excluded [7, 20]. Because of the necessity of multiple venepunctures, we excluded patients with a history of complicated venepunctures or anaemia. Further exclusion criteria included pregnancy, ongoing infection and previous solid organ transplantation.

Patient and public involvement

The study design was developed in collaboration with the national patient organisation for SSc.

Mycophenolate mofetil

Participants spent the night at the hospital or in direct adjunction to the hospital. They were instructed to follow their usual morning routine regarding concomitant intake of food and other medications together with MMF. Blood samples were drawn right before ingestion of the morning dose (which was administered under supervision) and 60, 180, and 360 min later. Plasma MPA levels were measured using high-performance liquid chromatography. MPA exposure was estimated based on a model previously developed for autoimmune diseases: $MPA_AUC_{0-12} = 12.3 + 4.7 \times C_0 + 1.2 \times C_1 + 2.7 \times C_3 + 1.8 \times C_6$ mg h/L [8].

Patient characteristics

Disease subtype, antibody status, disease duration since first non-Raynaud's manifestation, disease activity and skin involvement were defined in accordance with the EUSTAR recommendations [21]. Renal function was estimated based on a combination of measurement of plasma creatinine and plasma cystatin C [22]. Gastrointestinal microbiota dysbiosis was analysed with the GA-map™ Dysbiosis Test (Genetic Analysis, Oslo, Norway) and the relative abundance of lactobacilli analysed [12]. Gastrointestinal inflammation was assessed by faecal (F)-calprotectin (Calpro AS, Norway), and levels above 50 μg/g were considered pathological [11]. Gastrointestinal symptoms were evaluated by the University of California Clinical Trial Consortium Gastrointestinal Tract Instrument (UCLA SCTC GIT) 2.0 [23].

Disease activity was assessed by the EULAR revised activity index [24] and malnutrition risk by the Malnutrition Universal Screening Tool (MUST) [25]. Participants were asked to report recent intake of proton-pump inhibitors (PPI), calcium channel blockers and NSAID by filling out designated forms.

Statistical analyses

The study was predefined to include thirty-five patients, a number that was chosen together with a senior statistician to be able to do subgroup analyses [6]. In order to compare the relative drug exposure in patients prescribed different doses of MMF, we constructed the variable MPA_AUC_{3g} corresponding to a daily intake of 3 g MMF. This estimate was made by multiplying MPA_AUC_{0-12} by 3 or 1.5, respectively, in patients using MMF at a dose of 1 or 2 g daily [26, 27]. Descriptive data were presented using mean ± (SD) or median (IQR or range), and comparative analyses were done using non-parametric statistics including Spearman's rank correlation (r_s) coefficient, Kruskal-Wallis test and the Mann-Whitney *U* test.

Based on our current knowledge on the pharmacokinetics of MMF, we studied MPA exposure in relation to weight, renal function and concomitant medications [6, 28].

Based on previous reports on malabsorption in SSc, we also set out to explore MPA exposure in relation to gastrointestinal symptoms and inflammation, the MUST and intestinal microbiota [11–16, 23, 25].

Based on current knowledge on SSc prognosis for specific SSc subset, we also set out to explore MPA exposure in relation to skin involvement and serological profile [29].

Ethics

This study was approved by the Swedish Ethical Review Authority (Dnr 2018/490) and the Swedish Medical Products Agency (EudraCT 2018-002105-54) and prospectively registered at ClinicalTrials.gov (NCT03678987, posted September 20, 2018). All participants gave their written informed consent prior to entering the study. The study was conducted in accordance with the Declaration of Helsinki.

Results

Thirty-four out of predefined 35 study participants completed the study. Patient characteristics are presented in Table 1 and show some heterogeneity with regard to the MMF dose. Most notably, renal function that was lower in patients using MMF at a lower dose.

The mean daily MMF dose was 2.1 g. MPA exposure exhibited considerable variation between patients (Table 2). Two subjects exhibited an estimated MPA_AUC_{0-12} < 30 mg h/L while 25 subjects exhibited an estimated MPA_AUC_{0-12} > 60 mg h/L. The MPA_AUC_{0-12} displayed a linear dose-dependent relationship with MMF intake (Table 2), and MPA_AUC_{3g} was therefore used for further analyses.

MPA_AUC_{3g} varied inversely with body weight (r_s = − 0.58, $p < 0.001$) and the estimated glomerular filtration rate (r_s = − 0.34, $p = 0.054$). Male subjects had lower MPA_AUC_{3g} levels than their female counterparts (74 vs 121 mg h/L; $p = 0.015$; Supplemental Figure 1). Of note, the correlation between MPA_AUC_{3g} and renal function and sex could not be corroborated when relating these variables to MPA exposure expressed as MPA_AUC0–12 (Table 3).

MPA exposure exhibited significant heterogeneity in relation to four serological groups (Table 3, p value for between-group differences = 0.005). Patients with anti-topoisomerase-1 autoantibodies had lower MPA_AUC_{3g} compared to other participants (median 87 vs 123 mg h/L; $p = 0.008$; Table 3, Fig. 1).

Dysbiosis was present in 14 of the 27 patients tested and was not associated with altered MPA exposure (Table 3). However, there was a negative association between the relative prevalence of lactobacilli and MPA_AUC_{3g} (r_s = 0.54, $p = 0.004$; Fig. 2). Patients with normal F-calprotectin had higher MPA_AUC_{3g} compared to patients with pathological F-calprotectin levels (127 vs 99 mg h/L, p = 0.040), and F-calprotectin levels correlated inversely with MPA_AUC_{3g} (r_s = − 0.36, $p = 0.025$). We were unable to find an association between MPA exposure and gastrointestinal symptoms or malnutrition assessed by the UCLA SCTC GIT 2.0 and MUST (Table 3). However, MPA_AUC_{3g} was negatively associated with PPI usage (r_s = − 0.45, $p = 0.007$; Supplemental Figure 1).

We were not able to find any association between MPA exposure and calcium channel blockers or NSAID usage, nor disease duration, diffuse cutaneous disease subtype or disease activity (Table 3).

All five patients prescribed MMF at a dose of 1 g daily had previously used the drug at a higher dose. Of these, three had experienced suspect adverse events resulting in lowering of the dose while two patients were prescribed the lower dose of 1 g twice daily when their disease was considered stable. In total, five patients reported adverse events associated with MMF. A history of adverse events was not associated with MPA_ AUC_{3g} ($p = 0.508$).

Discussion

MMF is an immunosuppressive and anti-fibrotic drug that shows a plasma concentration-dependent efficacy when used for solid organ transplantation, SLE and GvHD [1, 9, 10]. It is an established therapy in SSc where available data support its use at a target dose of 3 g daily [2]. We report considerable inter-individual variation in MPA exposure following oral MMF treatment

Table 1 Patient characteristics. Data are shown as numbers and per cent (%) or means ± standard deviation (SD) or median interquartile range (IQR)/range and in relation to daily dose MMF

	***N* (%)**	**Daily dose MMF (*n*)**		
		1 g (5)	**2 g (21)**	**3 g (8)**
Disease subtype (dcSSc/lcSSc)	12/22 (35/65)	0/5	9/12	3/5
Sex (females/males)	30/4 (88/12)	5/0	19/2	6/2
ANA positivity, of which	33 (97)	5	20	8
ATA	8 (24)	1	5	2
ARA	4 (12)	0	4	0
PM-SCL (75 or 100)	10 (29)	3	4	3
MMF producer (Roche/Actavis/Sandoz)	8/24/2 (24/71/6)	1/4/0	4/16/1	3/4/1
MMF indication				
Lung fibrosis	21 (62)	4	10	7
Skin fibrosis	8 (24)		8	0
Combination of disease manifestations	5 (15)	1	3	1
Dysbiosis Index (1–5) ($n = 27$)	13/14 (48/52)	1/2	9/7	3/5
PPI (daily, sporadic, none)	26/2/6 (76/6/18)	5/0/0	16/1/4	5/1/2
CCB (daily, sporadic, none)	26/0/8 (76/0/24)	3/0/2	17/0/4	6/0/2
NSAID (daily, sporadic, none)	2/1/31 (6/3/91)	1/0/4	1/0/20	0/1/7
MUST (0, ≥ 1)	30/4 (88/12)	5/0	3/18	1/8
F-calprotectin pathological (yes/no)	14/20 (41/59)	3/2	9/12	2/6
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
Age (years)	58 (15)	62 (14)	59 (15)	51 (16)
Weight (kg)	73 (16)	70 (15)	71 (14)	80 (23)
EUSTAR Revised Activity Index	2.30 (1.48)	3.0 (1.8)	2.2 (1.6)	2.1 (1.1)
Leukocyte count (10^9/L)	7.3 (2.6)	8.4 (4.2)	7.2 (2.3)	6.6 (2.3)
Lymphocyte count(10^9/L)	1.4 (0.6)	1.7 (1.0)	1.4 (0.47)	1.7 (0.83)
Albumin (g/L)	42 (3.5)	40 (3.7)	42 (3.6)	44 (2.9)
eGFR (ml/min/1.73 m^2)	76 (12)	66 (6.1)	76 (12)	83 (9.0)
	Median (IQR)	**Median (range)**	**Median (range)**	**Median (range)**
Disease duration (years)*	4.9 (2.7–8.1)	4.8 (1.4–25)	5.8 (1.0–17)	3.7 (0.3–6.1)
Duration since SSc diagnosis (years)	3.5 (1.2–7.1)	3.6 (1.0–9.8)	4.3 (0.0–18)	1.2 (0.7–10)
Duration of MMF treatment (years)	2.1 (1.1–4.3)	2.5 (0.8–9.5)	2.6 (0.3–6.9)	0.9 (0.5–9.3)
Faecal calprotectin	39 (12–102)	74 (32–194)	41 (4–443)	17 (1–201)
UCLA SCTC GIT 2.0 Total score ($n = 31$)	0.24 (0.04–0.66)	0.125 (0.0–1.2)	0.31 (0.0–1.5)	0.25 (0.0–1.2)

dcSSc diffuse cutaneous systemic sclerosis, *lcSSc* limited cutaneous systemic sclerosis, *ATA* anti-topoisomerase-1 antibodies, *ARA* anti-RNA-polymerase III antibodies, *PM-SCL* anti-polymyositis-scleroderma, *MMF* mycophenolate mofetil, *PPI* proton-pump inhibitor, *SD* standard deviation, *UCLA SCTC GIT 2.0* University of California Los Angeles Scleroderma Clinical Trials Consortium Gastrointestinal Tract Instrument 2.0, *eGFR* estimated glomerular filtration rate
*Since first non-Raynaud's manifestation

in SSc. We also report that 25 out of 34 of the study participants exhibited MPA_AUC_{0-12} above the recommended interval for recipients of solid organ transplantation (30–60 mg h/L) [6], even though the average daily dose was only 2.1 g. These results need to be validated but indicate that it is common that SSc patients treated according to the current guidelines are exposed to higher levels of MPA than has been recommended for other diseases. One should be careful not to extrapolate safety data from previous MMF studies, to SSc patients prescribed MMF at a daily dose of 2 or 3 g.

In this study, we found that anti-topoisomerase-1 positivity and male sex predicted lower level of MPA exposure. We also identified that both an SSc-specific alteration in the microbiota and gastrointestinal inflammation were associated with lower levels of MPA exposure, suggesting that gastrointestinal manifestations of

Table 2 MPA exposure in MMF-treated systemic sclerosis. The mean and median MPA exposure correlated to MMF intake in a dose-dependent manner. There was a considerable inter-individual variation in MPA exposure in all three subgroups

Daily dose MMF	MPA_AUC_{0-12} (mg h/L)				MPA_AUC_{3g} (mg h/L) ($n = 34$)
	0.5 g × 2 ($n = 5$)	1 g × 2 ($n = 21$)	1.5 g × 2 ($n = 8$)	All subjects ($n = 34$)	
Mean	50	75	102	78	115
Median	48	72	119	72	114
Interquartile range	33–69	60–86	72–135	58–102	87–139
Range	25–75	43–120	27–139	25–139	27–226

MPA_AUC_{0-12} mycophenolate acid area under the concentration-time curve 0–12 h (mg h/L), *MMF* mycophenolate mofetil
MPA_AUC_{3g} MPA_AUC_{0-12} adjusted to a daily intake of 1.5 g MMF twice daily (mg h/L)

SSc may alter the bioavailability of MPA. We do not know what drug exposure is optimal for SSc. Our findings could be of clinical importance for newly diagnosed anti-topoisomerase-1 positive male patients with malabsorption. These patients have a poor prognosis, partly because of progressive lung fibrosis, and are in need of the full therapeutic potential of MMF [15, 29].

We found that PPI intake was associated with lower MPA exposure which is in line with previous reports. The mechanism behind this finding has been suggested to be explained the pH-dependent hydrolisation of MMF to MPA in the stomach [30]. This finding is of clinical significance considering the frequency by which PPIs are used for SSc [5].

Patients with the highest drug exposure had poor renal function and/or low body weight. This finding is in line with previous studies in other diseases and questions the rationale of a fixed target dose for all SSc patients.

The limitations of this study include the fact that we estimated MPA exposure using a 4-point model that has been designed for autoimmune diseases but not validated for SSc or for MPA measurement using high-performance liquid chromatography [8]. This study is limited to 34 patients which must be taken into consideration when extrapolating data from our subgroup analyses. Also, this study includes patients using MMF at three different daily doses with some heterogeneity between the groups, e.g. renal function, age, weight, disease duration and years on MMF (Table 1). Furthermore, we have not investigated the impact of MPA exposure on drug toxicity and drug efficacy. This issue needs further exploration before we can recommend therapeutic drug monitoring in MMF-treated SSc on a routine basis. Finally, because some of the patients that were excluded from the study were likely to suffer from a more severe disease (e.g. patients with a history of complicated venepunctures, renal failure or anaemia), the results may not be representative for the whole SSc population. There may also exist a selection bias towards participants who were able to tolerate this medication.

The optimal therapeutic interval for MPA in SSc remains to be defined. Considering the multiple modes of action by which this drug may modulate autoimmune fibrotic disease [31], it is possible that higher plasma concentrations compared to other diseases are needed in order for the drug to exhibit its full effect in SSc. In vitro studies have shown that not only the immunosuppressive function but also the anti-fibrotic effect of MPA is concentration-dependent [18]. Future studies are needed to investigate the clinical outcome of MMF therapy in SSc in relation to MPA_AUC_{0-12}, as has been done in SLE [9]. We therefore advocate MPA exposure to be analysed in clinical SSc trials as was alluded to already by the authors of the SLS-II trial: "while serum levels of MMF [sic] might have provided some insight, they were not obtained" [2].

Individual therapeutic monitoring of MMF together with an appropriate dose adjustment has successfully been used for recipients of solid organ transplants because of the concentration-dependent efficacy of this drug [6]. Based on these experiences, measurement of plasma MPA and estimation of MPA_AUC_{0-12} are currently available in many clinics worldwide.

In the era of personalised medicine, we suggest that individual MPA exposure and its relation to body weight, renal function, prognostic markers and PPI usage should be considered when using MMF in SSc.

Conclusions

MMF-treated SSc patients exhibit considerable inter-individual variation in drug exposure. Higher levels are found in female patients with poor renal function and low body weight. Relatively lower levels are found in overweight PPI users with poor prognostic factors. Body weight, renal function, sex, serology, gastrointestinal manifestations and the possibility of individual analysis of MPA levels should be considered when using and evaluating MMF for SSc.

Table 3 Associations between MPA exposure and clinical characteristics

MPA exposure estimate, daily dose MMF	All patients ($N = 34$)		MPA_AUC_{0-12}, mg h/L, median (range)		
	MPA_AUC_{3g}, mg h/L, median (IQR)	MPA_AUC_{0-12}, mg h/L, median (IQR)	1 g × 2 ($n = 21$)	1 g × 2 ($n = 21$)	1.5 g × 2 ($n = 8$)
Disease subtype (lcSSc vs dcSSc)	119 (90–132) vs 98 (85–150), $p = 0.929$	72 (48–85) vs 75 (59–116), $p = 0.383$	N/A	73 (43–101) vs 65 (56–121)	118 (27–139) vs 125 (83–139)
Sex (male vs female)	74 (36–103) vs 121 (94–141), $p = 0.015$	58 (31–81) vs 72 (60–103), $p = 0.262$	N/A	58 (43–73) vs 71 (47–121)	55 (27–83) vs 123 (68–139)
Topoisomerase (positive vs rest)	87 (67–108) vs 123 (97–146), $p = 0.008$	58 (31–71) vs 78 (63–103), $p = 0.039$	25 (25) vs 56 (41–75)	61 (43–72) vs 77 (47–121)	72 (27–118) vs 123 (68–139)
F-calprotectin (pathological vs normal)	99 (74–119) vs 127 (100–144), $p = 0.025$	57 (42–71) vs 83 (69–116), $p = 0.001$	41 (25–63) vs 62 (48–75)	58 (43–101) vs 82 (61–121)	72 (27–118) vs 123 (68–139)
Dysbiosis index ≥ 3	114 (74–139) vs 120 (86–141), $p = 0.756$	71 (57–119) vs 81 (61–94), $p = 0.943$	44 (25–63) vs 75	65 (43–104) vs 81 (56–121)	125 (68–139) vs 83 (27–120)
MUST pathological	108 (87–134) vs 114 (87–141), $p = 0.817$	72 (58–124) vs 72 (58–102), $p = 0.857$	N/A	N/A	N/A
PPI (user vs non-user)	108 (84–137) vs 121 (108–143), $p = 0.341$	67 (56–86) vs 92 (72–125), $p = 0.060$	N/A	66 (43–120) vs 76 (31–103)	100 (27–139) vs 130 (120–139)
CCB (user vs non-user)	109 (87–141) vs 122 (87–135), $p = 0.765$	73 (60–102) vs 63 (49–115) $p = 0.591$	48 (25–75) vs 52 (41–63)	72 (43–121) vs 59 (47–83)	100 (27–139) vs 132 (125–139)
NSAID (user vs non-user)	120 vs 108 (84–139) $p = 0.524$	73 vs 72 (58–101) $p = 0.909$	N/A	N/A	N/A
Spearman's rank correlation coefficient					
Body weight	$r_s = -0.58$, $p < 0.001$	$r_s = -0.38$, $p = 0.029$	$r_s = -0.50$	$r_s = -0.55$	$r_s = -0.48$
Age	$r_s = 0.33$, $p = 0.053$	$r_s = 0.10$, $p = 0.574$	$r_s = 0.90$	$r_s = 0.32$	$r_s = -0.24$
Disease duration	$r_s = -0.10$, $p = 0.566$	$r_s = -0.244$, $p = 0.164$	$r_s = -0.30$	$r_s = -0.21$	$r_s = 0.29$
Years on MMF	$r_s = 0.29$, $p = 0.102$	$r_s = 0.52$, $p = 0.002$	$r_s = 0.50$	$r_s = 0.18$	$r_s = 0.53$
Disease activity	$r_s = -0.06$, $p = 0.728$	$r_s = -0.17$, $p = 0.349$	$r_s = -0.50$	$r_s = 0.08$	$r_s = -0.667$
eGFR	$r_s = -0.34$, $p = 0.054$	$r_s = -0.024$, $p = 0.894$	$rs = -0.30$	$rs = -0.21$	$rs = -0.56$
Albumin	$r_s = -0.17$, $p = 0.351$	$r_s = 0.06$, $p = 0.762$	$r_s = 0.95$	$r_s = -0.34$	$r_s = -0.22$
PPI dose	$r_s = -0.45$, $p = 0.007$	$r_s = -0.43$, $p = 0.012$	$r_s = -0.71$	$r_s = -0.52$	$r_s = -0.31$
UCLA GIT 2.0	$r_s = 0.16$, $p = 0.358$	$r_s = 0.15$, $p = 0.385$	$r_s = 0.90$	$r_s = 0.159$	$r_s = -0.048$
F-calprotectin	$r_s = -0.36$, $p = 0.034$	$r_s = -0.60$, $p = 0.010$	$r_s = -0.31$	$r_s = -0.53$	$r_s = -0.50$
Lactobacilli	$r_s = -0.54$, $p = 0.004$	$r_s = -0.49$, $p = 0.010$	N/A	$r_s = -0.60$	$r_s = -0.14$
Kruskal-Wallis one-way analysis of variance					
MMF indication	$p = 0.699$	$p = 0.513$	N/A	N/A	N/A
Serology	$p = 0.005$	$p = 0.297$	N/A	N/A	N/A
MMF brand	$p = 0.897$	$p = 0.746$	N/A	$p = 0.512$	$p = 0.547$

MPA_AUC_{0-12} mycophenolate acid area under the concentration-time curve 0–12 h (mg h/L), *MPA_AUC_{3g}* MPA_AUC_{0-12} adjusted to a daily intake of 1.5 g MMF twice daily (mg h/L), *MMF* mycophenolate mofetil, *dcSSc* diffuse cutaneous systemic sclerosis, *lcSSc* limited cutaneous systemic sclerosis, *MUST* Malnutrition Universal Screening Tool [25], *NSAID* non-steroidal anti-inflammatory drug, *CCB* calcium channel blocker, *PPI* proton-pump inhibitor, *eGFR* estimated glomerular filtration rate, *UCLA GIT 2.0* University of California Los Angeles Scleroderma Trial Consortium Gastrointestinal Tract Instrument 2.0 [23]

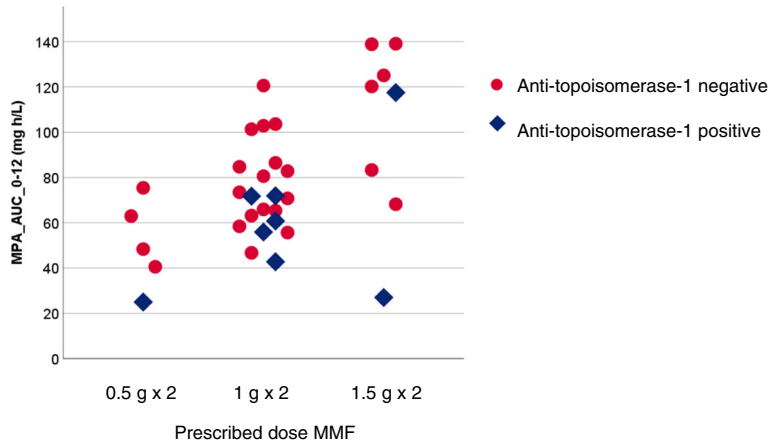

Fig. 1 MPA exposure in relation to daily MMF intake. MPA exposure, defined by the variable MPA_AUC_{0-12} varied considerably between patients. Patients with anti-topoisomerase-1 antibodies had significantly lower drug exposure compared to the other subjects

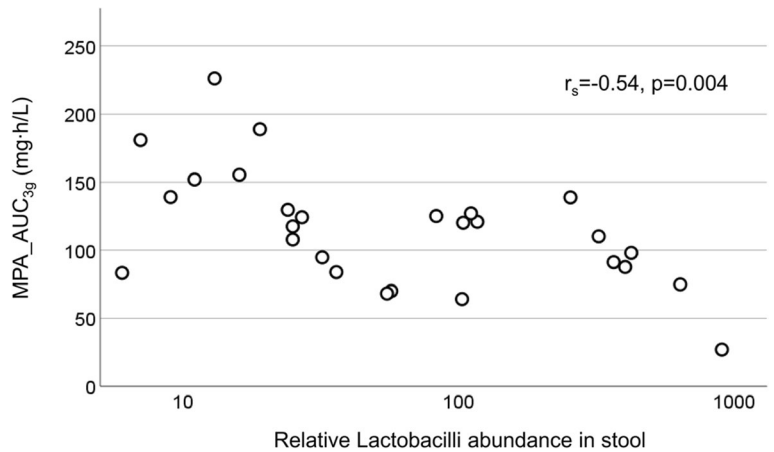

Fig. 2 MPA exposure in relation to intestinal lactobacilli levels. Patients with increased levels of lactobacilli in their stool had lower MPA exposure

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13075-020-02323-8.

Additional file 1: Supplemental Figure 1A-E. Scatter plot of MPA_AUC_{3g} in relation to sex, body weight, renal function, relative omeprazole dose and F-calprotectin.

Abbreviations

ACR: American College of Rheumatology; EudraCT: European Union Drug Regulating Authorities Clinical Trials Database; EULAR: European League Against Rheumatism; EUSTAR: The European Scleroderma Trials and Research group; GvHD: Graft versus host disease; MMF: Mycophenolate mofetil; MPA: mycophenolic acid; MPA_AUC_{0-12}: MPA area under the concentration-time curve 0–12 h; MPA_AUC_{3g}: MPA_AUC_{0-12} adjusted to a daily intake of 1.5 g MMF twice daily; MPAG: MPA-7-*O*-glucoronide; MUST: Malnutrition Universal Screening Tool; PPI: Proton-pump inhibitor; SLE: Systemic lupus erythematosus; SSc: Systemic sclerosis; UCLA SCTC GIT: University of California Clinical Trial Consortium Gastrointestinal Tract Instrument

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Authors' contributions

KA and RH planned the study and wrote the manuscript. KA and KN conducted the study and interpreted the results together with DW, DH, JM and RH. KN, DH and DW participated in the planning of the study. KN, DW, DH and JM reviewed the manuscript for intellectual content. The authors read and approved the final manuscript.

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Availability of data and materials

The dataset supporting the conclusions of this article is available upon reasonable request.

Ethics approval and consent to participate

This study was approved by the Swedish Ethical Review Authority (Dnr 2018/490) and the Swedish Medical Products Agency (EudraCT 2018-002105-54) and prospectively registered at ClinicalTrials.gov (NCT03678987, posted September 20, 2018). All participants gave their written informed consent prior to entering the study. The study was conducted in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable.

Competing interests

All authors declare no competing interests.

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ORIGINAL ARTICLE

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Faecal microbiota signatures of IBD and their relation to diagnosis, disease phenotype, inflammation, treatment escalation and anti-TNF response in a European Multicentre Study (IBD-Character)

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ABSTRACT

Method: We examined faecal samples, using the GA-map™ Dysbiosis Test, to associate gut microbiota composition with Crohn's disease (CD) and ulcerative colitis (UC) and to identify markers for future biomarker identification. We conducted a prospective case-control study (EU-ref. no. 305676) in an inception cohort of 324 individuals (64 CD, 84 UC, 116 symptomatic non-IBD controls and 44 healthy controls) across five European centres and examined 54 predetermined bacterial markers. We categorized patients according to the Montreal Classification and calculated the dysbiosis index (DI). Non-parametric tests were used to compare groups and the Bonferroni correction to adjust for multiple comparisons.

Results: The fluorescent signals (FSSs) for *Firmicutes* and *Eubacterium hallii* were lower in inflammatory bowel disease (IBD) vs. symptomatic controls ($p<.05$). FSS for Firmicutes, *Lachnospiraceae, Eubacterium hallii* and *Ruminococcus albus/bromii* were lower, whereas the signal for *Bacteroides Fragilis* was higher in UC vs. symptomatic controls ($p<.05$). FSS was higher for *Bifidobacterium spp.*, *Eubacterium hallii*, Actinobacteria and Firmicutes among patients with ulcerative proctitis, compared to extensive colitis ($p<.05$). In CD, we observed no association with disease location. The DI correlated with faecal-calprotectin in both CD and in UC ($p<.001$). In terms of treatment escalation and anti-TNF response, differences were observed for some bacterial markers, but none of these associations were statistically significant.

Conclusion: Our data reveal that the GA-map™ Dysbiosis Test holds the potential to characterize the faecal microbiota composition and to assess the degree of dysbiosis in new-onset IBD. On the other hand, our results cannot demonstrate any proven diagnostic or predictive value of this method to support clinical decision making.

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Introduction

Inflammatory bowel disease (IBD), comprising the predominant forms Crohn's disease (CD) and ulcerative colitis (UC), is a complex disease, characterized by a chronic inflammation in the gastrointestinal tract. The inflammation seems to arise due to a dysregulated immune response to gut microbiota in genetically susceptible individuals [1,2]. However, the complex interactions between gut microbiota and the host are still poorly understood and the precise pathophysiology of IBD is yet to be determined.

Clinically, IBD represents a heterogeneous disorder with a wide variety of clinical presentations and manifestations at diagnosis. The subsequent disease course may vary from an indolent disease to a severe course, refractory to medical treatment and with need of repeated surgery. Patients with an aggressive course are at increased risk of disease complications and morbidity [3]. As a result of the clinical

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heterogeneity, efforts have been made to identity prognostic biomarkers of future disease course. Most previous studies have defined the severity of the disease course, based on the need of surgery or advanced therapies, such as biological agents during follow-up [4]. Both in CD and in UC clinical factors as age, disease location and behavior, have been demonstrated to predict clinical outcome [3,5–7]. More recently, studies utilising -omic technologies have identified molecular markers of prognostic potential [8–10].

To tailor treatment in IBD, there has been a great focus on the identification of predictors of response to specific treatments such as anti-TNF. A number of clinical variables, including age, body mass index, smoking and concurrent use of immunomodulators, have been identified as potential predictive biomarkers of anti-TNF response [11–13]. However, previous data are inconsistent, and few clinical variables are used as predictors of individual therapies in clinical practice. To guide clinical decision-making, objective tools to categorize patients, predict disease course and treatment response, are needed [14]. The gut microbiota is of great interest in this respect, since its composition is believed to play a central role in the initiation and propagation of the gastrointestinal inflammation [15,16]. Driven by technology improvements and the establishment of next-generation sequencing platforms, significant progress has also been achieved with respect to our possibility to characterize the microbiota. Features of gut microbiota composition, including the degree of dysbiosis, have been associated with various subtypes of IBD, including phenotypes of CD [17]. However, the extent to which the clinical heterogeneity of IBD is explained by differences in gut microbiota composition is largely unknown, since most previous studies have examined prevalent patients with long-standing disease, where results may be explained by differences in previous treatments and disease history. Attempts to identify predictive biomarkers of disease course and response to individual therapies based on microbiota signatures have shown diverging results [18–20]. In the present study, we assessed the faecal microbiota composition and dysbiosis by the GA-map™ Dysbiosis Test, a kit designed to separate patient groups and healthy individuals based on their bacterial profile of the faecal microbiota [21].

We examined newly diagnosed IBD patients and healthy controls, and the primary aim was to identify faecal microbiota signatures associated with IBD and their phenotypes. The secondary aim was to identify faecal microbiota signatures associated with future disease course and treatment response.

Materials and methods

Study design

This is a prospective, multi-centre case-control study, where patients with newly confirmed IBD, symptomatic controls without IBD (non-IBD) and healthy controls, without any history of chronic gastrointestinal disease, were recruited across five European gastrointestinal clinics, as part of the 'Inflammatory Bowel Disease CHARACTERization by a multimodal integrated biomarker study' (IBD-Character, reference no. 305676) [22].

Study population

Patients, 18–70 years of age, referred to the gastroenterological unit at five European hospitals, between May 2012 and September 2015, for suspected IBD, were invited to take part in the study. Presence of gastrointestinal symptoms, such as diarrhoea, abdominal pain and blood or mucus in stool for >2 weeks, indicative of IBD, was an inclusion criterion. Exclusion criteria were antibiotic use within 30 days prior to baseline, gastrointestinal infections and other gastrointestinal inflammatory diseases. The diagnosis of IBD was established according to internationally accepted criteria, following thorough clinical, microbiological, endoscopic, histological and radiological evaluation [23]. We used the Montreal classification to categorize the phenotypes of CD, UC and IBD-U [24,25]. To assess clinical activity, the Harvey-Bradshaw Index (HBI) was used for CD and the simple clinical colitis activity index (SCCAI), as well as the partial Mayo score for UC. Individuals with gastrointestinal symptoms, with no endoscopic or histological signs of inflammation at inclusion, and no discernible evidence of IBD during follow-up, were classified as symptomatic non-IBD controls. To exclude other inflammatory or infectious disease in the symptomatic non-IBD patients, further investigations were done on clinical indication. In non-IBD cases with persisting symptoms 1–2 months after inclusion, a follow-up f-calprotectin was assessed. MRI and capsule-endoscopy were performed on selected cases with high f-calprotectin. Upper endoscopy was performed on clinical indication to exclude IBD and other organic disease.

Treatment-naivety within the IBD cohort was defined as no exposure to any IBD related medical therapies such as 5-ASA, corticosteroids, immunomodulators and biologics. In addition, we included healthy controls, without any history of chronic gastrointestinal symptoms (Table 1). Healthy controls were not matched for age or sex, and they were recruited from the two Swedish centers.

Clinical course of IBD

Clinical outcome data were collected from the medical records for patients with IBD. We classified the disease course as mild or severe based on the need of treatment escalation (Table 2). A severe disease course was defined as treatment escalation to a biologic agent, cyclosporine or surgery, introduced for a disease flare or disease refractory to primary treatment.

Predicting remission from anti-TNF therapy

For descriptive purposes, we also evaluated response to anti-TNF treatment, among patients with IBD who were treated with an anti-TNF agent during follow-up. Remission was assessed at 14 weeks after initiation of anti-TNF treatment (Supplementary Figure 1). For CD, remission was defined as

Table 1. Demographic data, smoking status, clinical indices, biochemical and faecal markers, and phenotypes according to the Montreal classification.

	CD	UC	IBDU	Non-IBD	HC
n	68	84	12	116	44
Demographic data					
Median age at diagnosis (range)	29 (18–78)	33 (18–77)	35 (25–56)	29 (18–65)	26 (21–32)
Male, *n* (%)	39 (57)	41 (49)	5 (42)	45 (39)	21 (48)
Smoking current/former/non (% current)	21/18/46[c] (31.3)	7/32/77 (8.3)	0/8/12 (0)	30/21/85[c] (26.1)	3/9/41 (6.8)
Clinical indices					
HBI (median, range)	6 (0–19)				
SCCAI (median, range)		7 (0–15)			
Biochemical parameters					
CRP, mg/l (median, range)	4.3 (0.3–263)	3.7 (0.3–300)	1.1 (0.3–130)	1.8 (0.3–80)	1.5 (0.3–130)
Faecal calprotectin mg/kg (median, range)	888 (32–>6000)	1617 (32–>6000)	1465 (41–>6000)	70 (7–2647)	43 (7–312)
Montreal classification					
Age at diagnosis CD					
A1 < 17 years	0 (0)				
A2 17–40 years	53 (77.9)				
A3 > 40 years	15 (22.1)				
Location/extension					
L1 ileal[a]/E1 proctitis[b]	25 (36.8)	25 (29.8)	1 (8.3)		
L2 colonic[a]/E2 left-sided colitis[b]	20 (29.4)	23 (27.4)	4 (33.3)		
L3 ileocolonic[a]/E3 extensive colitis[b]	23 (33.8)	36 (42.9)	7 (58.3)		
Behaviour CD[c]					
B1 non-stricturing, non-penetrating	56 (83.6)				
B2 stricturing	2 (3.0)				
B3 penetrating	5 (7.5)				
B1p perianal modifier	2 (3.0)				
B3p perianal modifier	2 (3.0)				

All data for Montreal classification presented with number of cases (*n*) and percentage (%).
[a]CD.
[b]UC.
[c]Missing data for one patient.

Table 2. Escalation to anti TNF or cyA and/or surgery.

CD	Escalation (*n* = 21)	Non-escalation (*n* = 47)
Median (range) age at diagnosis, years	27 (18–66)	30 (20–78)
Location		
L1 ileal, *n* (%)	5 (24)	15 (32)
L2 colonic, *n* (%)	6 (29)	19 (41)
L3 ileocolonic, *n* (%)	10 (47)	13 (27)
Median (range) CRP, mg/L	12.0 (0.34–263)	3.1 (0.3–78)
Median (range) f-calprotectin, µg/g	1524.5 (160 to >6000)	600.0 (32 to >6000)
UC	Escalation (*n* = 20)	Non-escalation (*n* = 64)
Median (range) age at diagnosis, years	29 (18–60)	36 (18–77)
Extension UC		
Proctitis (E1), *n* (%)	0 (0)	25 (40)
Left-sided colitis (E2), *n* (%)	5 (25)	17 (27)
Extensive colitis (E3), *n* (%)	15 (75)	21 (33)
Median (range) CRP, mg/L	12.0 (0.43–300)	2.3 (0.30–204.0)
Median (range) f-calprotectin, µg/g	4425 (58 to >6000)	1384 (32 to >6000)

Baseline characteristics at inclusion in CD and UC.

HBI ≤3 points, CRP ≤3 mg/l and no concomitant corticosteroid use [12,26,27]. For UC, remission was defined as partial Mayo score ≤1, no concomitant steroid use and at least one of the following criteria: (I) faecal calprotectin (f-calprotectin) <250 mg/kg, (II) CRP ≤3 mg/l and (III) an endoscopic Mayo subscore ≤1 [19,27].

Faecal calprotectin

f-Calprotectin was extracted and analysed with ELISA in a single batch at the end of recruitment, according to the manufacturer's protocol (BÜHLMANN fCAL™, Schönenbuch, Switzerland) [28].

GA-map™ Dysbiosis Test

Faecal samples were collected in screw-capped plastic containers within two days prior to inclusion, kept at room temperature for ≤2 days before being brought to the clinical centre and stored in –20 °C, until shipment on dry ice to Genetic Analysis. Bacterial DNA was extracted and processed through 16S PCR [29]. The test measures relative bacterial abundance based on the fluorescent signal (FSS) strength of 54 predetermined bacterial DNA markers targeting variable regions of V3 to V9 of the bacterial 16S rRNA gene (Supplementary Figure 2), giving a dysbiosis index (DI); range 1–5. The index gives information on the degree of deviation of the gut microbiota composition in the test sample compared to a healthy population [21,29]. The bacterial markers

were selected based on previous literature, and 165 healthy individuals were used to develop a dysbiosis model and DI score output. The representation of pathobionts is correlated to increasing DI, whereas bacteria affiliated with anti-inflammatory properties decrease the DI. The method was further validated, when 330 samples were analysed and demonstrated dysbiosis in 70% of treatment naïve IBD, 80% of IBD in remission, 73% in IBS and only 16% among healthy individuals [21]. All samples were analysed in a single batch and lab-technicians were blinded for clinical data.

Statistical analyses

Continuous variables, representing clinical characteristics, are presented as median and range, and categorical data are presented as frequencies. Only markers with a detectable level in $\geq$10% of the samples were included in the analyses. The DI was calculated based on the GA-map® algorithm [21]. Normalized signal levels of each bacterial marker were used for univariable comparisons. To account for multiple comparisons, *p* values were adjusted using Bonferroni's correction approach with adjusted *p* value reported. Principal component analyses (PCAs) plots of log-transformed data were performed and score-plots were visually inspected to identify possible differences between groups. Analysis of similarities was calculated based on the two first PCA axis with 1000 permutations and *p* value describing the differences between groups reported. To compare differences between groups univariably, we used the Kruskal–Wallis rank sum test and the Wilcoxon rank sum test. In the comparison between single bacterial markers, all results with a Bonferroni adjusted *p* value were reported together with a non-adjusted *p* value $<$.05. A heatmap was visualising hierarchical cluster analysis based on Euclidian distance, here each bacterial marker was normalized by extracting the median of the normal population, scaling the variance to 1 and truncating extreme values to -2 and 2. In the heatmap, blue indicates lower signal than the median of the normal population while red indicates higher signal. Statistical analyses and data processing were performed in R version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria) [30].

Ethics

The study was conducted with informed consent and ethical approval, in accordance with the Helsinki declaration. The study has approval by Committees for Medical Research Ethics in all countries.

Results

Altogether, 432 individuals provided a faecal sample at inclusion. We excluded unfit samples (Supplementary Figure 3). Clinical demographics of the final study cohort ($N = 324$) are shown in Table 1. Among the 164 IBD patients, 117 were treatment-naive and 47 were exposed to IBD-related medical therapies (topical or systemic 5-ASA, $N = 20$; topical or systemic corticosteroids, $N = 37$; azathioprine (AZA), $N = 3$; anti-TNF $N = 1$) during the last six months before inclusion. The mean duration from diagnosis to inclusion in the study was 16 days.

Microbial composition among IBD patients, symptomatic controls and healthy controls

We compared the microbial composition of samples from IBD patients, symptomatic non-IBD controls and healthy controls. Samples from IBD patients grouped closer to samples from symptomatic non-IBD controls than to samples from healthy controls. Further, samples from the healthy controls were more similar to each other, grouped closer together, than samples from the IBD and non-IBD samples, respectively. Anosim *p* value was non-significant (Figure 1(A)). The gut microbiota composition of treatment-naive IBD patients was compared to the composition of IBD patients who had been exposed to IBD-related drugs. No significant difference was observed between the two groups (Supplementary Figure 4) and the samples from treated and treatment-naive IBD patients were, therefore, analysed together as one group.

When comparing the five diagnostic groups, the FSS for the markers detecting *Lachnospiraceae*, *Mycoplasma hominis*, *Eubacterium hallii*, *Clostridia*, Firmicutes and *Ruminococcus albus/bromii* significantly differed between CD, UC, IBD-U, symptomatic controls and healthy controls (adjusted *p* value $<$.05). The FSSs for Firmicutes and *Eubacterium hallii* were significantly lower (adjusted *p* value $<$.05) in samples from patients with IBD compared to symptomatic non-IBD controls. Further, there were significantly lower FSSs (adjusted *p* value $<$.05) for *Lachnospiraceae* and for *Clostridia* in UC compared to CD (Figure 1(B)).

When comparing samples from patients with UC and healthy controls, the FSSs for *Mycoplasma hominis*, *Eubacterium hallii*, Firmicutes and *Ruminococcus albus/R. bromii* were lower (adjusted *p* value $<$.05) in UC samples, while the signals for *Shigella spp.*, *Escherichia spp.* and *Streptococcus spp. 2* were higher (adjusted *p* value $<$.05) (Supplementary Figure 5(A)). Correspondingly, the FSSs for *Mycoplasma hominis*, *Eubacterium hallii* and *Faecalibacterium prausnitzii* were lower (adjusted *p* value $<$.05) and the signal for *Ruminococcus gnavus* was higher (adjusted *p* value $<$.05) when samples from CD patients were compared to those from healthy controls (Supplementary Figure 5(B)).

When comparing samples from patients with UC and symptomatic non-IBD controls, the FSSs for the markers detecting Firmicutes, *Lachnospiraceae*, *Eubacterium hallii* and *Ruminococcus albus/bromii* were lower (adjusted *p* value $<$.05) in UC, whereas the signal for *Bacteroides fragilis* was higher (adjusted *p* value=.04) (Supplementary Figure 6(A)). No significant differences were observed between the patients with CD and symptomatic non-IBD controls with respect to FSSs (Supplementary Figure 6(B)).

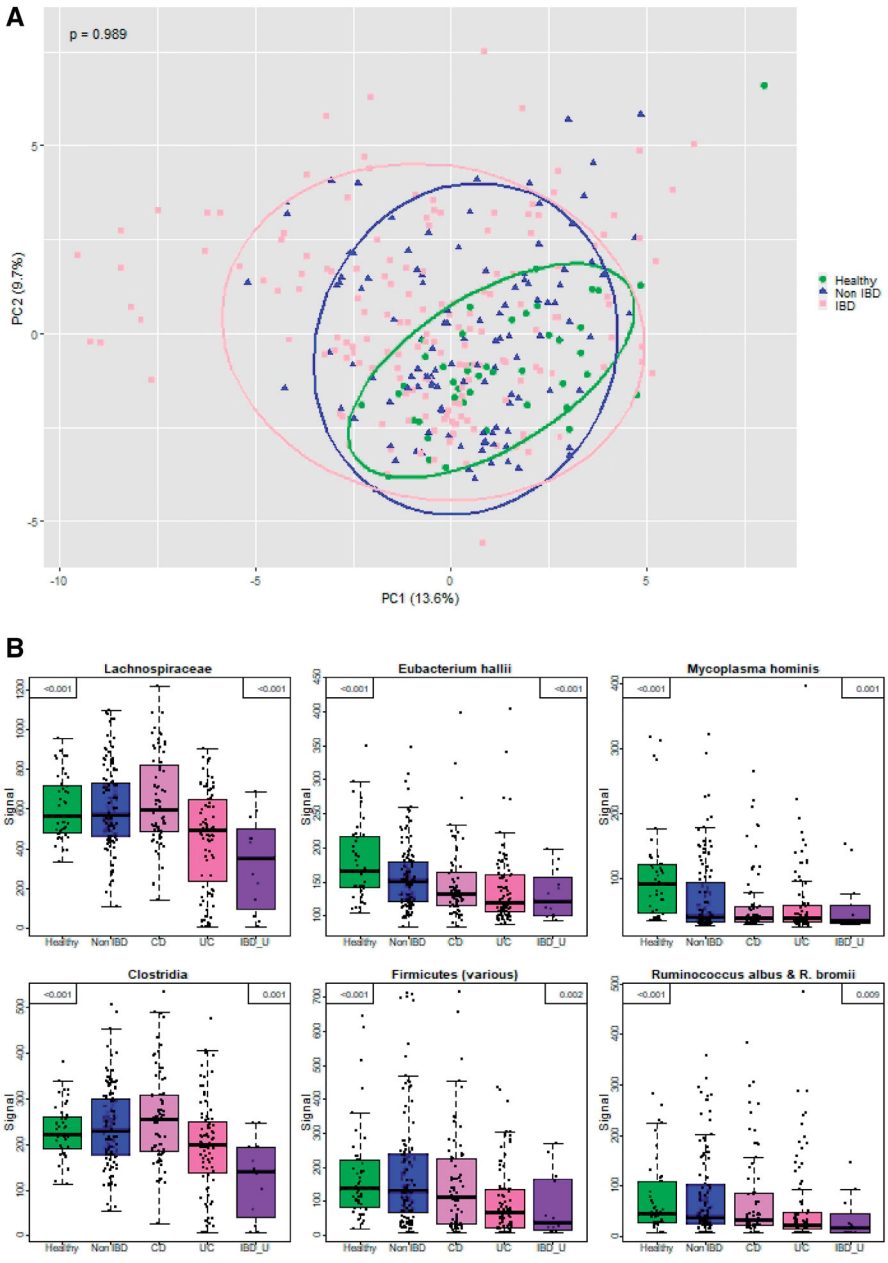

Figure 1. (A) PCA of log transformed and scaled fluorescent signal strength, microbiota composition separating diagnostic groups (IBD, non-IBD and healthy controls). The variation of all samples explained by each axis is given in breaks in the axis label. Ellipsoids are made to include 90% of the data in one group. Anosim *p* value (the largest difference between two groups) in upper left corner. (B) Bacterial markers separating the diagnostic groups. Median is plotted as a thick line, 50% of the data visualized as a box from the 25% to the 75% quantile, whiskers indicate min and max, or min and max assuming that the data are normally distributed, while outliers are marked with circles. Non-adjusted *p* value is given in the upper left corner. Bonferroni's adjusted *p* value is given in upper right corner.

Microbial composition in different phenotypes of UC and CD

In order to examine if there is an association between microbial composition and phenotypes of UC, samples from patients with extensive colitis (E3), left-sided colitis (E2), proctitis (E1) and healthy controls were compared. The microbial compositions of patients with extensive and left-sided colitis were more similar, and differed from the composition of patients with proctitis and healthy controls (p=.001) (Figure 2(A)). The FSS for *Bifidobacterium spp.*, *Eubacterium hallii*, Actinobacteria and Firmicutes was significantly lower in patients with extensive/left-sided colitis compared to patients with proctitis (adjusted p value <.05) (Figure 2(B)).

In contrast to the difference in microbial composition across different phenotypes of UC, we did not observe any difference when samples from patients with ileal (L1), colonic (L2), ileocolonic (L3) CD and healthy controls were compared. In fact, the microbial composition of CD patients with various disease locations largely overlapped (anosim p=.287). On the other hand, they significantly differed from the healthy controls (p=.001) (Figure 2(C)). Numerically higher FSS strength was observed for some bacterial markers, but none of these

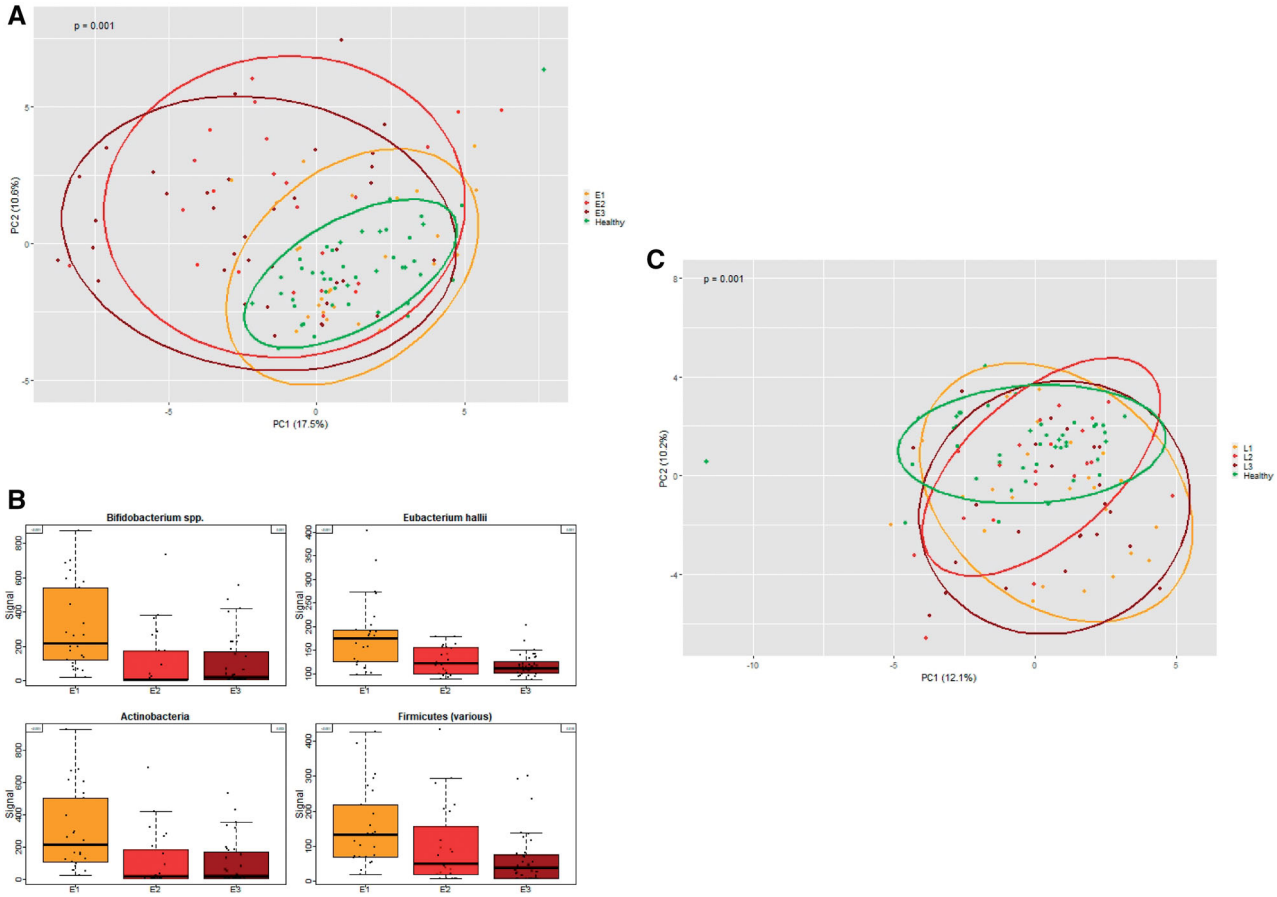

Figure 2. (A) PCA of log transformed and scaled fluorescent signal strength for microbiota composition separating UC phenotypes. Anosim *p* value (the largest difference between two groups) in upper left corner. (B) Bacterial markers separating UC phenotypes [24]. (C) PCA of log transformed and scaled fluorescent signal strength for microbiota composition separating CD phenotypes. Anosim *p* value (the largest difference between 2 groups) in upper left corner. Anosim *p* value=.287 for CD phenotypes.

associations were statistically significant (Supplementary Figure 7).

Unsupervised cluster analyses

A heatmap with all the 54 bacterial markers demonstrates the complexity of the gut microbiota without recognizing distinct clusters regarding diagnosis, phenotype or f-calprotectin (Supplementary Figure 8).

Correlation between dysbiosis index and inflammatory activity

To examine the correlation between degree of dysbiosis and inflammatory activity, we correlated the DI with the concentration of f-calprotectin. When all samples from patients (IBD and symptomatic non-IBD) were analysed, the concentration of f-calprotectin increased with the degree of DI independent of diagnosis (Figure 3(A)). The correlation remained significant, when stratifying by diagnosis and analysing patients with UC (Figure 3(B)), CD (Figure 3(C)) and symptomatic non-IBD (Supplementary Figure 9) separately (log linear regression, $p<.001$).

The FSSs for the 54 bacterial markers are all influencing the DI. Supplementary Figure 10 demonstrates the association between every single bacterial marker and f-calprotectin. When focusing on some dominating phylae in the figure, a rise in Proteobacteria and a fall in Firmicutes associate to increasing f-calprotectin, even though no strong correlation between single bacterial markers and f-calprotectin can be found.

Microbial composition as predictor of disease course

Information on medical therapy and surgery during follow-up was available for 158/164 (96%) patients with IBD. In total, 41 patients (26%) required treatment escalation (anti-TNF treatment, $N=29$; cyclosporine A treatment, $N=1$ and surgery, $N=11$).

Among patients who escalated therapy, high symptom scores were accompanied by endoscopic findings, increased f-calprotectin levels and/or increased CRP concentrations, whereas non-escalators had normal levels of clinical and biochemical markers.

We aimed for a minimum of 12 months follow-up after diagnosis, since most patient who experience treatment escalation, escalate within 12 months [31]. In our cohort, 95% of the patients had a follow-up period of >12 months. Of those who escalated, 90% of the patients with UC and 86% of the patients with CD escalated within 1 year (Supplementary Figure 11). Among patients with UC, 20/84

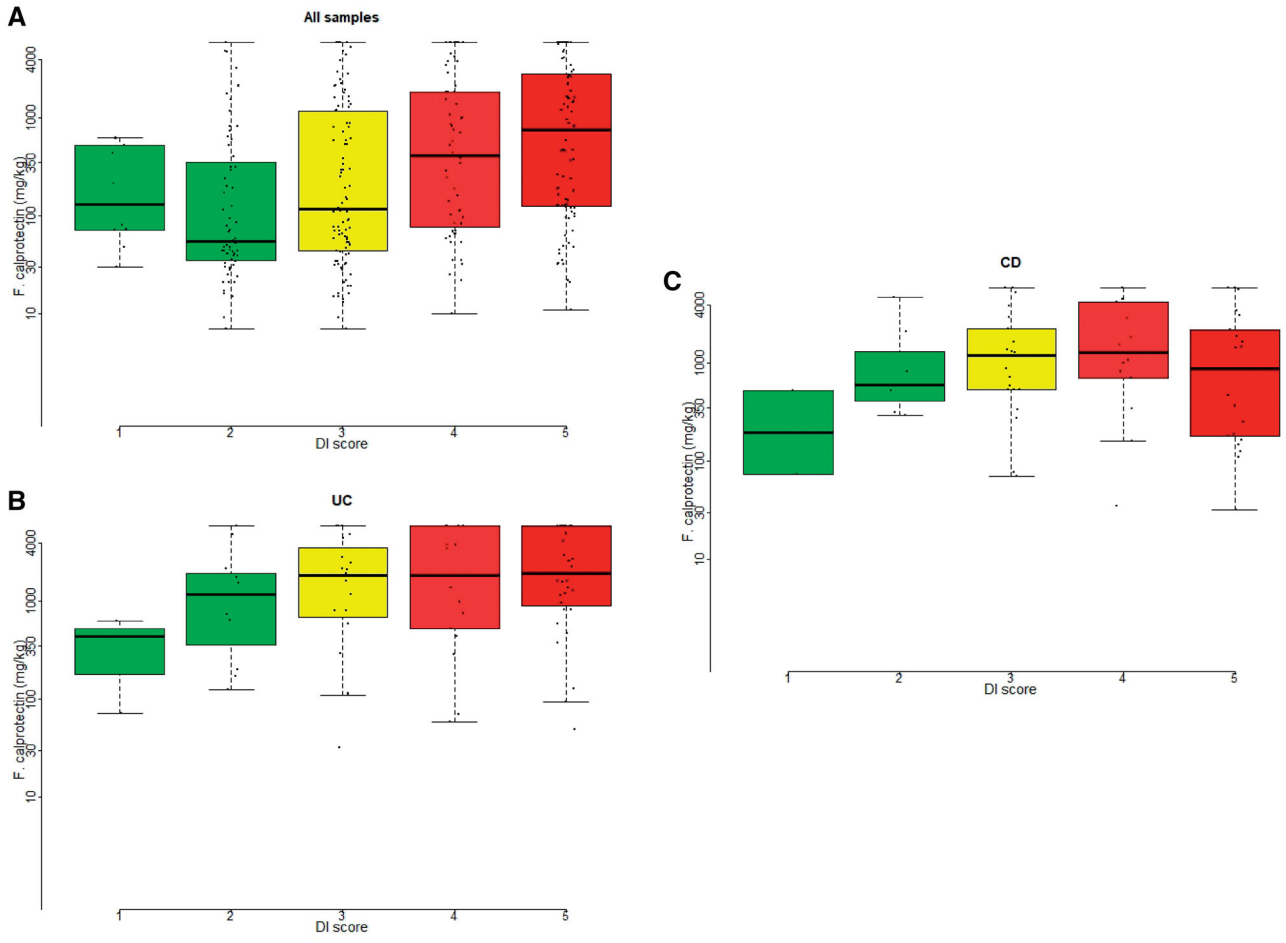

Figure 3. Log10 f-calprotectin (mg/kg) on vertical axis. Dysbiosis index score (DI) on horizontal axis. Dots are individual samples. (A) All samples. (B) UC samples and (C) CD samples. Linear regression between log10 f-calprotectin (mg/kg) as outcome and dysbiosis index (DI) score as dependent variable explained 75.6% (A), 87.3% (B) and 83.6% (C) of the variation in the observations ($p<.001$ for all three regressions).

(24%) experienced treatment escalation, whereas 21/68 (31%) patients with CD experienced treatment escalation. In UC, concentrations of CRP and f-calprotectin at inclusion were significantly higher among patients who escalated therapy compared to patients who did not escalate therapy during follow-up ($p=.02$ and $p=.004$, respectively). Correspondingly, the concentration of CRP at inclusion was significantly higher among patients with CD who eventually escalated ($p=.005$). The concentration of f-calprotectin at inclusion was only numerically higher among patients with CD who escalated ($p=.11$). However, the microbial composition at inclusion largely overlapped between escalators and non-escalators in both UC (Supplementary Figure 12) and CD (Supplementary Figure 14). No significant difference was observed between escalating patients and non-escalating patients when the FSSs for individual bacterial markers were compared, even though numerically different signals were observed for some bacterial markers in both UC and CD (Supplementary Figures 13 and 15).

Microbial composition as predictor of response to future anti-TNF treatment

Data on response to anti-TNF therapy 14 weeks after initiation of treatment, were available for 24/29 (83%) patients who were treated with an anti-TNF agent at some time during follow-up. Anti-TNF responders experienced a large drop in CRP, f-calprotectin and symptoms, in compliance with the definition of anti-TNF-induced remission (Supplementary Figure 16). Altogether, 3/9 (33%) patients with UC and 5/15 (33%) patients with CD achieved remission 14 weeks after the initiation of anti-TNF treatment. No significant difference was found in the overall gut microbiota composition or in specific bacterial markers between patients who achieved remission and those who did not achieve remission, even though numerically different FSS strengths were observed for some bacterial markers when comparing samples from remitters and non-remitters (Supplementary Figures 17–20).

Discussion

Using the GA-map™ Dysbiosis Test and faecal samples mostly collected prior to initiation of treatment in new-onset patients, we studied the gut microbiota composition in one of the largest adult IBD inception cohorts to date.

Numerous studies have reported alterations in the faecal microbiota of patients with established IBD [17,32–34]. Bacterial taxa as *Eubacterium hallii* [35,36] and *Faecalibacterium prausnitzii* [18,31,37] have demonstrated properties promoting a healthy gut environment. On the

other hand, taxa as *Ruminococcus gnavus* [38] and *Shigella spp*. and *Escherichia spp.* [39,40], have been shown to act as pathobionts with pro-inflammatory and mucin-degrading capabilities. In accordance with previous literature [19,21,41], we observed lower FSSs for *Mycoplasma hominis*, *Eubacterium hallii*, Firmicutes and *Ruminococcus albus/R. bromii* and higher FSSs for *Shigella spp*. and *Escherichia spp*., as well as *Streptococcus spp. 2* in UC compared to healthy controls (Supplementary Figure 5(A)). When comparing CD and healthy controls, our findings also were in accordance with previous literature [18,42–44], with lower signals of markers detecting *Mycoplasma hominis*, *Eubacterium hallii* and *Faecalibacterium prausnitzii*, and higher signal for *Ruminococcus gnavus* in CD (Supplementary Figure 5(B)) already in this early disease state.

To examine the potential diagnostic capacity of the faecal microbiota, we compared IBD patients with symptomatic controls. Fluorescent signals for Firmicutes and *Eubacterium hallii* were significantly lower in samples from patients with new-onset IBD compared to symptomatic non-IBD controls (Figure 1(B)). However, it is doubtful if the assessment of dysbiosis with this method has diagnostic value applicable for clinical use, since established methods as f-calprotectin measurement have shown sovereign capabilities in IBD diagnostics [45–47].

In UC, the FSSs for Firmicutes, *Lachnospiraceae*, *Eubacterium hallii* and *Ruminococcus albus/bromii* were lower, whereas the signal for *Bacteroides fragilis* was higher among patients with UC compared to symptomatic controls (Supplementary Figure 6(A)). Interestingly, *Bacteroides fragilis* holds anti-inflammatory properties [48,49], and an upregulation of *Bacteroides fragilis* observed in parallel with a downregulation of *Ruminococcus* in UC, could possibly demonstrate a response of the host microbiota cross-talk promoting intestinal homeostasis.

Between patients with CD and symptomatic controls, however, the microbiota composition largely overlapped, with non-significant differences among the bacterial markers (top hits in Supplementary Figure 6(B)). These findings elucidate the diagnostic potential of the faecal microbiota, and this potential to separate IBD from non-IBD in symptomatic patients seems to be especially strong in UC, and rather weak in CD. In contrast, earlier studies have found a diagnostic potential also in CD. It has been proposed that a group of eight microorganisms, including a decreased abundance of *Faecalibacterium*, an unknown Peptostreptococcaceae, *Anaerostipes*, *Methanobrevibacter*, an unknown *Christensenellaceae*, *Collinsella* and an increased abundance of *Fusobacterium* and *Escherichia*, could be used to differentiate stool samples of patients with CD from non-CD, i.e., healthy controls, patients with anorexia, IBS or UC(42). The extent to which these findings are causative or a consequence of disease history, various types of treatments and inflammation is largely unknown, since samples were obtained from patients with established disease. On the other hand, another study of treatment-naïve paediatric IBD has also demonstrated a diagnostic potential related to the gut microbiota, in CD [50]. Gevers et al. examined faecal samples and mucosal biopsies obtained from a large inception cohort of treatment-naïve children with paediatric onset CD and children with non-inflammatory conditions. The authors identified a microbiota signature in mucosal biopsies that correlated strongly with disease status. In contrast to the discriminative capacity of rectal and ileal biopsies, the microbiome composition of faecal samples was less shifted towards a dysbiotic state and, thereby, far less discriminative [50].

Notably, the faecal microbiota composition of symptomatic non-IBD patients shared similarities with the composition of patients with IBD (Figure 1(A)). Additionally, we found an association between DI and f-calprotectin in this group (Supplementary Figure 9). This could possibly indicate that the gut microbiota plays a role in several gastrointestinal diseases, since symptomatic non-IBD patients were represented by different gastrointestinal diagnoses, including IBS. This finding is to some extent supported by previous literature, where alterations in gut microbiota composition have been reported among other gastrointestinal diseases such as IBS [21].

We compared the faecal microbiota of different phenotypes of UC and CD. In UC, the microbiota was more dysbiotic in extensive and left-sided disease compared to disease limited to the rectum (Figure 2(A)). This difference was mainly driven by lower FSSs for *Bifidobacterium spp*., *Eubacterium hallii*, Actinobacteria and Firmicutes in samples from patients with extensive disease, compared to samples from patients with proctitis (Figure 2(B)). The extent to which these findings reflect causative differences or are explained by possible differences in stool consistency [51] and inflammatory activity [19,52,53], can only be speculated on.

Previous studies have reported differences in gut microbiota between CD patients with different phenotypes. In general, a more pronounced dysbiosis has been observed in patients with ileal CD compared with patients with colonic disease [54]. Our data indicate that these findings do not seem to be evident at diagnosis (Figure 2(C) and Supplementary Figure 7) and suggest that these differences could be secondary to inflammation or treatment. Notably, we were not able to examine all dysbiosis-associated taxa, since our analyses were restricted to 54 pre-defined bacterial markers.

We observed a strong correlation between the degree of dysbiosis and the inflammatory activity, defined by f-calprotectin levels. This correlation between DI and f-calprotectin was independent of diagnosis, when samples from symptomatic individuals, both IBD and non-IBD, were analysed (Figure 3(A)). The correlation remained significant, when stratifying by diagnosis and analysing patients with UC (Figure 3(B)) and CD (Figure 3(C)) separately. The FSSs for the 54 bacterial markers are all influencing the DI. Supplementary figure 10 demonstrates the relation of every single bacterial marker to f-calprotectin level. When focusing on two well-known phylae among the 54 bacterial markers, Proteobacteria and Firmicutes, we demonstrate a rise in Proteobacteria and a fall in Firmicutes as f-calprotectin levels increase. These findings are consistent with the shown association between DI and f-

calprotectin, as a rise in Proteobacteria and a fall in Firmicutes, enhances the DI. However, the correlation between single markers and f-calprotectin is weaker than the correlation of all signals together, elucidating the significance of calculation the DI when applying this method for faecal microbiota assessment.

Our findings indicate that alterations in the gut microbiota composition are present already at diagnosis, suggesting that changes in the gut microbiota occurred at an early disease phase, potentially even before diagnosis of adult-onset IBD and independent of medical therapy. Our data support previous studies which have examined the inflammatory activity in the context of specific taxa, rather than overall degree of dysbiosis. Even though a recent meta-analysis indicates that the decrease in *Faecalibacterium prausnitzii* seems to be most pronounced in patients with clinically active disease, few cross-sectional studies have demonstrated a correlation between abundance of specific bacterial taxa and inflammatory activity [19,53]. We have recently reported an inverse correlation between temporal changes in the relative abundance of *Faecalibacterium prausnitzii* and concentrations of f-calprotectin [52]. Importantly, inflammation may translate into higher oxygen concentrations which may induce an environment that negatively affects obligate anaerobes and results in a diminished mucus layer [55].

Finally, we explored the prognostic and predictive potential of the GA-map™ Dysbiosis Test to foresee treatment escalation and response to future anti-TNF treatment. The identification of prognostic biomarkers has been the aim of many studies, including our own parallel studies of glycomics, methylation and protein profiling [9,56,57]. Lee et al. identified expression profiles of T cell exhaustion in CD8 T cells that predicted treatment escalation in IBD [4], defining escalation as the need for $\geq$2 immunosuppressants and/or surgery after initial disease remission. We recognise that clinical decisions and timing on treatment escalations may vary across centres. We used more stringent criteria to define treatment escalation than previous studies, and included biological agents, cyclosporin A or surgery in the definition, rather than introduction of immunosuppression per se. Immunosuppressant as methotrexate and AZA was mainly used as co-medication to biologics in this cohort, but there is a substantial number of patients treated with AZA without experiencing further treatment escalation. Avoiding immunomodulators in the definition, could potentially add limitations to the study, as the AZA-treated patients also experience a more aggressive disease course than those treated with 5-ASA alone (UC), or a short period of GC, with further de-escalation of therapy. However, the fact that the proportion of patients experiencing treatment escalation to biologics, cyA and/or surgery was quite similar between the centres (22–29%), indicates that patients were treated according to rather similar principles across the five European centres.

Several bacterial markers showed numerically lower FSSs among UC patients who escalated therapy compared to those who remained on their initial treatment during follow-up (Supplementary Figure 13). This might reflect a less rich and diverse gut microbiota already at diagnosis of UC among patients with a poor prognosis. Prognostic clinical variables such as extensive disease and possibly inflammatory activity [7] may have influenced our results. An explanation for this might be that patients with a severe disease course more often were diagnosed with extensive colitis, and had higher concentrations of CRP and f-calprotectin compared to those who experienced an indolent disease course, without need of treatment escalation (Table 2).

Alterations in microbiota composition have previously been associated with response to biological agents, including anti-TNF treatment [19,58,59]. In contrast to these previous studies, we could not identify any difference in microbial composition or DI between responders and non-responders. However, the low number of anti-TNF treated patients limited our analyses, and the absence of significant differences may reflect low statistical power.

A major strength of this study is the inclusion of newly diagnosed patients with a high proportion being treatment-naïve (69%). Thus, results are less influenced by previous treatments and inflammation compared to most foregoing studies. The main benefit of using the GA-map® technology over other applications comes from the standardisation and consistency of results given, which in turn are indexed against a normal control population. The lack of clustering demonstrated in the heatmap (Supplementary Figure 8) underscores the potential of the DI to characterize the faecal microbiota and to separate diagnostic groups.

Nevertheless, there are also limitations of a predetermined primer-based methodology compared to 16S rRNA sequencing technology. Metagenomics provide a broader characterization of the gut microbiota and may unravel the functional potential of the gut microbiota [60,61]. In contrast to faecal samples, analyses of mucosal biopsies may also shed light on the host-microbiota crosstalk. The absence of information on dietary intake is another limitation of the study, since dietary factors differ between geographic areas and impact on the gut microbiota [62,63].

Conclusions

By using the GA-map™ Dysbiosis Test, we demonstrate that the microbiota composition of patients with new-onset UC and CD differed from healthy individuals. UC samples also differed from symptomatic non-IBD patients. Differences in microbiota composition were also seen between different phenotypes of UC, but not in CD. Using the DI, we observed a correlation between degrees of inflammation and dysbiosis, irrespectively of disease status. Our data reveal that the GA-map™ Dysbiosis Test holds the potential to characterize the faecal microbiota composition and to assess the degree of dysbiosis in new-onset IBD. On the other hand, our results cannot demonstrate any proven diagnostic or predictive value of this method to support clinical decision making.

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Disclosure statement

ABK and CC are employed by Genetic Analysis (GA). MHV is member of the expert panel in GA. The other authors have no conflicts of interest to declare.

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Study protocol of the Bergen brain-gut-microbiota-axis study

A prospective case-report characterization and dietary intervention study to evaluate the effects of microbiota alterations on cognition and anatomical and functional brain connectivity in patients with irritable bowel syndrome

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Abstract

Introduction: Irritable bowel syndrome (IBS) is a common clinical label for medically unexplained gastrointestinal (GI) symptoms, recently described as a disturbance of the brain-gut-microbiota (BGM) axis. To gain a better understanding of the mechanisms underlying the poorly understood etiology of IBS, we have designed a multifaceted study that aim to stratify the complex interaction and dysfunction between the brain, the gut, and the microbiota in patients with IBS.

Methods: Deep phenotyping data from patients with IBS (n = 100) and healthy age- (between 18 and 65) and gender-matched controls (n = 40) will be collected between May 2019 and December 2021. Psychometric tests, questionnaires, human biological tissue/samples (blood, faeces, saliva, and GI biopsies from antrum, duodenum, and sigmoid colon), assessment of gastric accommodation and emptying using transabdominal ultrasound, vagal activity, and functional and structural magnetic resonance imaging (MRI) of the brain, are included in the investigation of each participant. A subgroup of 60 patients with IBS-D will be further included in a 12-week low FODMAP dietary intervention-study to determine short and long-term effects of diet on GI symptoms,

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All patient data will be published anonymously and no individual participant will be possible to identify. Patients have been informed and signed a consent form.

Future datasets used and/or analysed during the current study may be available from the corresponding author on reasonable request and ethical approval.

The authors report no conflicts of interest.

Trial results will be published in peer-reviewed journals. Professional writers will not be used.

The datasets generated during and/or analyzed during the current study are not publicly available, but are available from the corresponding author on reasonable request.

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microbiota composition and functions, molecular GI signatures, cognitive, emotional and social functions, and structural and functional brain signatures. Deep machine learning, prediction tools, and big data analyses will be used for multivariate analyses allowing disease stratification and diagnostic biomarker detection.

Discussion: To our knowledge, this is the first study to employ unsupervised machine learning techniques and incorporate systems-based interactions between the central and the peripheral components of the brain-gut-microbiota axis at the levels of the multiomics, microbiota profiles, and brain connectome of a cohort of 100 patients with IBS and matched controls; study long-term safety and efficacy of the low-FODMAP diet on changes in nutritional status, gut microbiota composition, and metabolites; and to investigate changes in the brain and gut connectome after 12 weeks strict low-FODMAP-diet in patients with IBS. However, there are also limitations to the study. As a restrictive diet, the low-FODMAP diet carries risks of nutritional inadequacy and may foster disordered eating patterns. Strict FODMAP restriction induces a potentially unfavourable gut microbiota, although the health effects are unknown.

Trial registration number: NCT04296552 (ClinicalTrials.gov)
Protocol version 1 May 2019

Abbreviations: AFNI = analysis of functional neuroimages, AQ = autism-spectrum quotient, ASRS = adult attention deficit hyperactivity disorder self-report scale, BGM = brain-gut-microbiota, BIS = bergen insomnia scale, BIS = bristol stool scale, BRIEF-A = the behavior rating inventory of executive – adult, CFQ-11 = chalder fatigue scale, CPT-3 = the conner's continuous performance test, dMRI = diffusion magnetic resonance imaging, DNA = deoxyribonucleic acid, dsDNA = double stranded deoxyribonucleic acid, EDA = exploratory data analysis, EF = executive functions, ENS = enteric nervous system, fMRI = functional magnetic resonance imaging, FODMAP = fermentable oligo-, di-, mono-saccharides and, FSL = FMRIB software library, GA-map = gentic analysis map, GI = gastrointestinal, GSRS-IBS = gastrointestinal symptom rating scale for irritable bowel syndrome, HADS = hospital anxiety and depression scale, HC = healthy controls, HRV = heart rate variability, HUH = haukeland university hospital, IBI = interbeat intervals, IBS = irritable bowel syndrome, IBS-D = Irritable bowel syndrome with diorraeha, IBS-QOL = irritable bowel syndrome quality of life measure, IBS-SQ = irrritable bowel syndrome symptom questionnaire, IBS-SSS = irritable bowel syndrome symptom severity score, MRI = magnetic resonance imaging, NEO-FFI-3 = neuroticism-extraversion-openness five-factor inventory, PCR = polymerase chain reaction, PET = positron emission tomography, RBANS = the repeatable battery for the assessment of neuropsychological status, RMSSD = root-mean-square of successive R-R-interval differences, ROI = region of interest, SCFA = short chain fatty acids, UMAT = ultrasound and the meal accommodation tes, VSI = visceral sensitivity index, VSI = visceral sensitivity index, WURS = wender utah rating scale for attention deficit hyperactivity disorder in adults.

Keywords: brain-gut-microbiota axis, functional magnetic resonance imaging, irritable bowel syndrome, machine learning, neurogastroenterology, psychometric tests, transabdominal ultrasound

1. Introduction

Irritable bowel syndrome (IBS) is a symptom-based diagnosis characterized by chronic abdominal pain associated with altered bowel habits, in the form of diarrhea, constipation, or a mix between the two.[1] IBS is the most common condition encountered by gastroenterologists, with a global pooled prevalence of 11.2%[2] and a prevalence around 20% in the western world.[3] In the absence of a structural or organic cause, growing evidence suggests that there is a plethora of factors that may elicit abnormal responses in patients with IBS. Collectively, these factors are described as dysregulation of the brain-gut-microbiota (BGM) interaction. As a bidirectional link between the brain and the gut, the axis reciprocally interacts with key sensorimotor functions, sympathetic and parasympathetic branches of the autonomic nervous system, the endocrine and immune system, the hypothalamic-pituitary-adrenal-axis, the enteric nervous system (ENS), and gut microbiota and metabolites. Alterations in gut microbiota composition or functions is known to impact human behaviour and brain physiology, and dysbiosis is commonly reported in patients with IBS.[4,5] However, whether IBS-symptoms are caused by changes in microbiota still remains elusive and multiple pathways and mechanisms have been suggested to be involved. These include immune, endocrine, and neural signalling pathways, which the gut microbiota and metabolites may modulate. For instance, structural components of intestinal bacteria and bacterially produced metabolites [e.g., short chain fatty acids (SCFAs), tryptophan metabolites] may regulate immune function and cytokine production, with upstream effects on the blood–brain barrier or brain function through modulation of neuroinflammation. The gut microbiota, directly or through interactions with the host or diet, produce neuroactive compounds (gamma-aminobutyric acid, 5-hydroxytryptamine receptor, norepinephrine, etc) that may affect neuroendocrine functions locally or through humoral or neural routes such as signaling along the vagal nerve.[6]

Indeed, there is a substantial knowledge gap in regards to understanding pathophysiology in both the brain and the gut. The current view on disorders of the BGM axis has replaced the conventional focus on individual brain regions and cell types of the gut, integrating brain networks (brain connectome) and networks of gut cells and microbiota (gut connectome).[7] In this study, we will employ cutting-edge omics and advanced computational methods to unravel gastrointestinal (GI) pathophysiology of IBS, link this information to results from multimodal brain imaging examination, gastric accommodation test, and information from psychometric tests and questionnaires, and translate these into clinical biomarkers. By this, we aim to stratify the diagnosis and improve treatment for patients with IBS.

2. Methods/Design

2.1. Objectives

The primary objective of this study is to increase knowledge and understanding of BGM axis dysfunction and identify covariance across multiple phenotypes leading to identification of biomarkers.

The secondary objectives are addressing the multifaceted manifestations of BGM axis dysfunction, specifically

- To identify and quantify structural and functional brain connectivity signatures, including visceral pain provocation and psychophysiological measures, using advanced neuroimaging techniques and statistical approaches.
- To investigate clinical biochemistry in blood (plasma, serum).
- To evaluate upper GI motility patterns using transabdominal ultrasound.
- To investigate GI biopsies for multi-omics analysis and complex molecular subclassification.
- To identify microbiota signatures and metabolites associated with gut-brain equilibrium or dysfunction in IBS patients and healthy controls.
- To investigate clinical effects of short and long-term strict low-FODMAP diet.
- To examine and compare the compositional and functional response of gut microbiota at baseline and after the low-FODMAP-dietary intervention, and the relationship with clinical and psychophysiological outcomes.
- To evaluate if the GA-map technology can be used to predict the response to treatment (to distinguish responder and non-responders prior to low-FODMAP diet).
- To investigate cognitive and emotional function associated with brain structures and functions known to be affected in adults with IBS, and personality traits and behaviour expected to be of importance to the patient's everyday functioning and effects of a strict low-FODMAP diet.
- To define phenotypes and biomarkers from all our above-mentioned data using high dimensional data-driven and machine learning techniques.

2.2. Study population, recruitment, and consent

Eligible patients from the Bergen area are recruited from the IBS outpatient clinic at Haukeland University Hospital and through social media. Patients are informed about the study online (www.braingut.no.) and are individually informed by telephone by a study nurse, before inclusion. If a patient decides to participate in the study, (s)he signs an informed consent. Patient recruitment will take place between May 2019 and December 2021. Inclusion criteria for the studyincluded age between 18 and 65 years; fulfillment of the Rome-IV criteria for IBS: Reports of recurrent abdominal pain on average at least 1 day per week during the previous 3 months that is associated with alterations in bowel habits; duration at least 6 months and an IBS-symptom severity score (SSS) >175.[8] Exclusion criteria for the study included pharmacological treatment affecting the GI-tract, including treatment for depression; presence of an organic disease such as coeliac disease, inflammatory bowel disease, diabetes, active *Helicobacter pylori* infection, polycystic ovary syndrome, and neurological diseases such as multiple sclerosis, Parkinson's disease, and amyotrophic lateral sclerosis; treated with systemic antibiotics within the last 3 months; probiotics or low-FODMAP-diet within the last 3 weeks or being vegan or vegetarian; regular use of analgesics; pregnancy; previous intestinal surgery except appendectomy; claustrophobia or having metallic implants that are not MRI compatible; been travelling outside Europe within the last 3 weeks (or plan to travel in the nearest future); participation in any other simultaneous clinical study; inability to comprehend and respond to questionnaires or follow dietary guidance.

Three patients and one healthy volunteer will be included in the study every 4 weeks until the necessary number of participants have been enrolled (IBS n = 100 patients, HC n = 40) at baseline. Sixty candidates with IBS-D will enroll in the 12-week strict low-FODMAP dietary intervention. Additional patients will be enrolled to replace patients who drop out during baseline until the end of the inclusion period. Patients who unexpectedly get claustrophobic during brain scanning may be included in the dietary intervention without functional magnetic resonance imaging (fMRI) examination.

2.3. Study design

The study is an open, single-center, case–control characterization study, followed by open label dietary intervention for a subgroup of subjects with IBS-D. Table 1 gives an overview of the study procedures and events during case–control characterization (baseline) and the following 12-week strict low-FODMAP diet intervention.

3. Outcomes

3.1. Primary outcome

Primary outcomes included proportion of patients with treatment success in the lowFODMAP dietary intervention. Treatment success is defined as an improvement of ≥50 points on the IBS symptom severity score (IBS-SSS) at 12 weeks after treatment start compared with the score at baseline.

3.2. Secondary outcomes

- ≥50 points decrease in the IBS symptom severity score (IBS-SSS) from the scores 4 weeks after treatment compared to baseline.
- ≥13 points increase in the IBS-Qualiy of Life (IBS-QoL) week 4 and/or week 12 after treatment when compared to the scores at baseline
- Twenty percent reduction on visual analog scale on nausea, pain, and bloating measured post lactulose-test at week 12, compared with baseline scores.
- Proportion of patients with change dysbiosis index at week 4 and week 12 according GA-map technology, compared with baseline and/or healthy controls.

3.3. Explorative outcomes

- Changes in structural and functional brain connectivity signatures, including visceral pain provocation and psychophysiological measures at 12 weeks compared with baseline, and healthy controls.
- Changes in resting state regional brain interactions at 12 weeks compared with baseline and healthy controls, as measured with fMRI.
- Changes in cortical thickness (FA values) as measured with DTI (MRI)
- Differences in taxonomy and function of the microbiome, the immune system, metabolome and gut epithelial barrier in IBS

Table 1

Brain-gut study procedures and events during baseline and 12-week strict low-FODMAP diet intervention.

	Diet Intervention						
Baseline	**Wk 1**	**Wk 2**	**Wk 4**	**Wk 6**	**Wk 8**	**Wk 10**	**Wk 12**
	Cons. w/dietitian		Cons. w/dietitian		Cons. w/dietitian		
UMAT							UMAT
Endoscopy							endoscopy
fMRI with pain provocation test							fMRI with pain provocation test
Measures of vagal function							Measures of vagal function
Psychometric testing							Psychometric testing
RBANS							RBANS
BRIEF-A							BRIEF-A
NEO-FFI-3							CPT3
CPT3							
Sampling biol. material			Sampling biol. material				Sampling biol. material
Blood			Faeces				Blood
GI biopsies			Saliva				GI biopsies
Faeces							Faeces
Saliva							Saliva
3-d dietary record			3-d dietary record		3-d dietary record		3-d dietary record
Questionnaires	Questionnaires			Questionnaires		Questionnaires	Questionnaires
ROME-III	IBS-SSS			IBS-SSS		IBS-SSS	ROME-III
IBS-SSS	HADS			HADS		HADS	IBS-SSS
HADS	IBS-SQ			IBS-SQ		IBS-SQ	HADS
IBS-QOL	BIS			BIS		BIS	IBS-QOL
BIS	BSS			BSS		BSS	BIS
BSS							BSS
VSI							VSI
GSRS-IBS							GSRS-IBS
IBS-QS							IBS-QS
CFQ-11							CFQ-11
WURS							WURS
AQ							AQ

AQ = Autism-spectrum quotient, ASRS = Adult Attention Deficit Hyperactivity Disorder self-report scale, BIS = Bergen Insomnia Scale, BIS = Bristol Stool Scale, BRIEF-A = The Behavior Rating Inventory of Executive – Adult, CFQ-11 = Chalder fatigue scale, CPT-3 = The Conner's Continuous Performance Test, GSRS-IBS = Gastrointestinal Symptom Rating Scale for Irritable Bowel Syndrome, HADS = Hospital Anxiety and Depression scale, IBS-QOL = Irritable Bowel Syndrome Quality of Life Measure, IBS-SQ = irritable bowel syndrome symptom questionnaire, IBS-SSS = irritable bowel syndrome symptom severity scale, NEO-FFI-3 = Neuroticism-Extraversion-Openness Five-Factor Inventory, RBANS = The Repeatable Battery for the Assessment of Neuropsychological Status, VSI = Visceral sensitivity index, VSI = Visceral Sensitivity Index, WURS = Wender Utah Rating Scale for Attention Deficit Hyperactivity Disorder in adults.

patients at 12 weeks compared with baseline measures, and healthy controls.
- Changes in levels of SCFA composition, compared with baseline, and heathy controls.
- Changes in cognitive and emotional function, personality traits, and behavior at 12 weeks compared with baseline, and healthy controls.
- Improvements in The Conner's Continuous Performance Test (CPT-3) at 12 weeks in IBS patients, compared with baseline.

3.4. Sample size estimation

Generally, for samples size calculation and statistical power, we would make the following assumptions: Anticipated effect size (f^2) is medium (0.20), desired statistical power level is 0.8 (80%) with type-I error of 5% ($\alpha = 0.05$). On the basis of these assumptions, the minimum required samples size is 59 in each group for multiple regression analyses. Previous experience with this patient group has shown that we may expect a high level of missing data. To allow diagnostic subclassification, we therefore have increased the baseline sample size to n = 100, and the dietary intervention samples size n = 60 in patients with IBS-D and n = 40 for healthy controls. However, to identify biomarkers and patient subpopulations, comprehensive analysis methods will be employed to combine neuroimaging and neuromics data with clinical patient data. Although IBS is a highly heterogeneous patient group, we believe machine learning techniques and possibly pre-trained models will enable us to make inferences about an IBS population from the given sample, considering the limitation of cost, time, and convenience of data collection.

3.5. Clinical assessment

3.5.1. Structural and functional brain MRI. Participants will be scanned using a Siemens Biograph mMR PET/MRI, incorporating a 3 T MRI Verio scanner with MQ gradients (45 mT/m @200 T/m/s), a 12-channel RF head coil and paralell imaging. The following MRI sequences will be applied: Scout (136 images, voxelsize $1.6 \times 1.6 \times 1.6\,mm^3$; iPAT = 3; TA 0:14); T1-w 3D MPRAGE (192 slices, voxelsize $1.0 \times 1.0 \times 1.0\,mm^3$; TR/TE/TI = 2400/2.26/900 ms, FA = 8 deg; iPAT = 2; TA_5:35), T2-w 3D TSE (192 slices; voxelsize $1.0 \times 1.0 \times 1.0\,mm^3$; TR/TE = 3200/407 ms, FA = 120 deg; iPAT = 2; TA 5:12), GRE field map (36 slices,

voxelsize 3.0 x 3.0 x 3.75 mm^3; TR/TE1/TE2 = 400/4.92/7.38 ms, FA = 60 deg; iPAT = 1; TA 0:54); rs-fMRI epi2d moco (47 slices, voxelsize 2.4 x 2.4 x 3.12 mm^3, 200 vols; TR/TE = 2890/30 ms, FA = 90 deg; iPAT = 2; TA 9:48), rs-fMRI epi2d moco (32 slices, voxelsize 2.4 x 2.4 x 3.12 mm^3, 200 vols; TR/TE = 1980/30 ms, FA = 90 deg; iPAT = 2; TA 6:43), dMRI MMDW (47 slices, voxelsize 2.4 x 2.4 x 3.12 mm^3;TR/TE = 8000/113 ms, FA = 90 deg; iPAT = 2; diff.scheme = bipolar; phase encoding A<<P; bvecs = 30 directions, bvals = 0 (n = 1)/1000 (n = 30)/2500 (n = 30) s/mm^2; TA 8:34, 8 PA epi2d diff (47 slices, voxelsize 2.4 x 2.4 x 3.12 mm^3; TR/TE = 7200/113 ms, FA = 90; iPAT = 2; phase encoding P>>A; bvals = 0; TA 0:30. Total examination time ~45 min.

Functional MRI, using blood-oxygenation-level dependent (BOLD) contrast, will be used to record neuronal activity (i.e., neurovascular responses) in both IBS patients and healthy controls. Resting-state fMRI is a special "task-free" variant in which the subject is asked to lie as still as possible in the head coil while being continuously scanned, to investigate spontaneous brain activity (resting state networks).[9–12] Diffusion imaging (dMRI) involves measuring of water diffusion in brain tissue. This provides information on a voxel-by-voxel basis about white matter microstructure, properties of with matter fiber bundles in the brain.[13–15] For this study, we do not plan to use the opportunity of truly simultaneous acquisition of structural and functional information of the same brain regions from both MRI and PET. Our cerebral MRI protocol is a partly compliant with and motivated by the standardized Pain and Interoception Imaging Network protocol.[16]

3.5.2. Psychometric testing. The Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) is a brief, individually administered test-battery designed as a screening instrument to evaluate neuropsychological status of adults, ages 20 to 89 years.[17] The test battery includes 12 subtests giving measures of attention, language, visuospatial/constructional abilities, and immediate and delayed memory function, in addition to a composite score and estimates of intellectual function according to available Scandinavian norms. In the present study, the results will be used for defining the cognitive function of the participants, as a covariate in the statistical analyses, and for establishing norms for patients with IBS.

The Behavior Rating Inventory of Executive Function (BRIEF-A) will be used to assess executive functions (EFs), as they are experienced in the daily life of the participants.[18] The scale includes 86 items in 8 clinical scales (Inhibit, Shift, Emotional Control, Initiate, Working Memory, Plan/Organize, Organization of Materials, Monitor) and 2 validity scales (Inconsistency and Negativity). These scales form 2 global indexes (Behavioral Regulation, Metacognition, Global Executive Composite score) representing overall EF.

The Conners' Continous Performance Test – third edition (CPT-3) is a computerized test wherein the participants are instructed to push the spacebar as soon as a target appears on the screen (a letter), except when the letter is X.[19] The test is characterized by high signal-to-noise ratio by including 90% target stimuli and 10% no-target stimuli (x). The test incudes 360 trials presented across 6 blocks, with 3 subgroups with different interstimulus intervals. The test durance is 14 minutes, and the results generate measures of reaction time, accuracy, variability, consistency, and vigilance. BRIEF and CPT-3 will be used as a measure of attention and EF in patients with IBS, as a predictor of treatment response (baseline); and as an outcome variable (change from baseline to follow-up).

The NEO-FFI-3 is a 60-item version of the NEO-PI-3 providing a quick and reliable measure of the Five-Factor model of personality (Neuroticism, Extraversion, Openness, Agreeableness, and Conscientiousness).[20,21] The instrument is well validated for use in several countries. Measures will include the 5 factors as well as profiles of factors for individual adults. In the present study, the personality assessment will be part of the phenotyping of adults with IBS and controls; one of the predictors of treatment response, a more general predictor of quality of life, pain, and other self-reported challenges associated with IBS, and related to the BGM axis.

3.5.3. Gastric motility and ultrasonography. Gastric motility will be characterized using transabdominal ultrasound and the meal accommodation test (UMAT). The UMAT involves consumption of a standardized 500 mL, pre-boiled, low-caloric soup after which transabdominal ultrasound allows visualization and measurement of the proximal and distal compartments of the stomach.[22–24] We will characterize the patient's gastric accommodation, emptying, and quantify antral contractions. Antral biopsies will be investigated for density of interstitial cells of Cajal, correlated to functional observations during the UMAT.

3.5.4. Vagal activity. Pulse oximetry data will be acquired during the resting-state fMRI sequence in order to collect (heart) interbeat intervals (IBIs), with a photoplethysmograph placed on the right index finger (50 Hz). Pulse oximetry data offers an especially accurate approximation of interbeat intervals and is associated with less artifacts in a MR-environment. In order to control for the effect of respiration in the pulse oximetry data, a chest strain gauge will be applied to measure respiratory frequency. Heart rate variability (HRV) and breathing pattern is registered during resting state, that is, the time the patient is in the MR machine in supine position with spontaneous breathing (approximately 20 minutes). The pulse oximetry data will be used to calculate HRV. In order to estimate the parasympathetic modulation for heart rate, an estimate of fast IBI changes in the time scale of milliseconds will be obtained, that is, faster changes that sympathetic modulation for heart rate. Thus, high frequency HRV > 0.14 Hz and time-domain measures reflecting such fast changes ([-mean-square of successive R-R-interval differences (RMSSD)] provide a readily available, estimate of vagal activity.[25]

3.5.5. Assessment of low-FODMAP dietary response. A subgroup of patients with IBS-D (n = 60) will undergo a strict 12-week low-FODMAP dietary intervention guided by clinical dietitian. Here, patients will exclude FODMAPs from their diet without reintroduction during the entire study period. Patients will meet with their registered dietitian four times (baseline, week 4, 8, and 12). At baseline, the patients will be informed about the diet intervention and the low-FODMAP-diet. They will fill out questionnaires and records of the diet (3 days) are collected at week 2, 4, 6, 8, 10, and 12 (Table 1). The registered dietitian is available for the patients by phone and e-mail throughout the entire study period.

3.5.6. Biological tissue sampling. Routine gastroscopy and sigmoidoscopy will be carried out to exclude other pathologies, and biopsies will be harvested from the antrum, duodenum, and sigmoid colon for histological and molecular biomarker

investigation. Saliva will be sampled for oral microbiota composition. Blood samples (serum, plasma, buffy coat) will be collected for analysis of GI hormones, serotonin levels, gut integrity markers, and pro-inflammatory cytokines and interleukins). Stool samples will be collected for gut microbiota profiling and estimates of abundance and functions.

3.5.7. Questionnaires. During baseline and multiple timepoints throughout the dietary intervention (Table 1), standardized questionnaires on intestinal and extraintestinal symptomatology, sleep, fatigue, and quality of life will be assessed: Irritable Bowel Syndrome Quality of Life Measure (IBS-QOL),[26] Irritable Bowel Syndrome – Symptom Severity Score (IBS-SSS),[8] Hospital Anxiety and Depression scale (HADS),[27] Bristol Stool Scale (BSS),[28] Visceral sensitivity index (VSI),[29] Gastrointestinal Symptom Rating Scale for Irritable Bowel Syndrome (GSRS-IBS),[30] IBS symptom questionnaire (IBS-SQ),[31,32] Chalder fatigue scale (CFQ-11),[33] Wender Utah Rating Scale for ADHD in adults (WURS),[34] Autism-spectrum quotient (AQ),[35] and Bergen Insomnia Scale (BIS),[36] Hospital Anxiety and Depression scale (HADS),[27] Bristol Stool Scale (BSS),[28] Visceral sensitivity index (VSI),[29] Gastrointestinal Symptom Rating Scale for Irritable Bowel Syndrome (GSRS-IBS),[30] IBS symptom questionnaire (IBS-SQ),[31,32] Chalder fatigue scale (CFQ-11),[33] Adult ADHD self-report scale (ASRS),[37] Wender Utah Rating Scale for ADHD in adults (WURS),[34] Autism-spectrum quotient (AQ),[35] and Bergen Insomnia Scale (BIS).[36]

3.5.8. Analysis of microbiota composition, short-chain fatty acids, and shotgun metagenomics. The DNA will be extracted from stool samples using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, with a prior step of bead beating in 2 mL microcentrifuge tubes containing 0.1 mm diameter glass beads, ~ 150 mg faeces, and 1 mL InhibitEX buffer (Qiagen, Hilden, Germany). The faecal DNA concentration will be measured by fluorescence-based methods such as Qubit 3.0 and the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA). Library preparation for shotgun metagenomic sequencing will be performed using the Nextera™ DNA Flex Library Preparation kit (Illumina, San Diego, CA) from 350 to 450 ng of gDNA. A low cycling indexing protocol (5 PCR cycles) and Unique Dual Indices will be used to minimize amplification bias and to prevent index hopping respectively. Every final library will be individually quantified on the Qubit 3.0 fluorometer and run on a Bioanalyzer HS DNA chip (Agilent, Santa Clara, CA) to verify quality and size distribution. The 2 x 150nt paired-end sequencing will be conducted on a NovaSeq 6000 sequencer (Illumina, San Diego, CA, USA). All samples will be accommodated in one S4 flow cell to minimize the bias of batch sequencing. Paired-end fastq files will be used to perform a phylogenetic marker gene-based operational taxonomic units (mOTUs) analyses, enabling the taxonomy profiling of >7700 microbial species.[38] In addition, reconstruction of metagenome assembled genomes (MAGs) will permit assess gut microbiota functionality with different representation across the intervention time points.

Feces will be analysed for short-chain fatty acids (SCFA). About 0.5 g of the fecal material will be homogenised after addition of distilled water containing 3 mmol/L of 2-ethylbutyric acid (as internal standard) and 0.5 mmol/L of H_2SO_4; 2.5 mL of the homogenate will be vacuum distilled, according to the method of Zijlstra et al, as modified by Høverstad et al.[39] The distillate will be analyzed with gas chromatography (Agilent 7890 A, CA), using a capillary column (serial no. USE400345H; Agilent J&W GC columns, CA), and quantified using internal standardization. Flame ionisation detection will be employed. In addition, we will calculate the proportional distribution of individual SCFA to total SCFA.

Analysis of salivary microbiota composition will be performed using PCR. A total of 5 mL unstimulated saliva is collected from each patient and healthy control using a 50 mL sterile Falcon tubes. Each sample is then immediately placed in an ice bag and stored at –80°C until extractions. DNA from the collected saliva samples will be extracted using a TIANamp Bacteria DNA Kit (Tiangen Biotech, Beijing, China) following the manufacturer's instructions. Genomic DNA will be used as the template for bacterial 16S rRNA gene amplification with the barcoded primers 338F (50-ACTCCTACGGGAGGCAGCAG-30) and 806R (50- GGACTACHVGGGTWTCTAAT-30), which target the V3–V4 hypervariable region. A real-time PCR system combination with the Illumina MiSeq platform (16S rRNA) (Illumina, San Diego, CA) will be used for sequencing of oral microbiome profile.[40]

3.5.9. Data management and machine learning. Data will be collected during consultations at Haukeland University Hospital (HUH). Specific patient-reported outcome data (questionnaires: HADS, Rome-III, IBS-SSS) are collected electronically using CheckWare (https://checkware.com), security level 4 (e.g., with BANK-ID). All patient data will be plotted into an electronic database, FileMakerPro 17 Advanced, stored at Haukeland University Hospital Research server (2015–01621). All data are registered using the patient's study-ID, and the study participant-identifying key is stored in a separate location at the research server. No patient data are located in proximity to the study participant-identifying key. Biological material such as GI biopsies, blood (serum, plasma, etc), stool samples, and saliva is collected in 2D barcode labeled tubes (Matrix), scanned, and stored in Biobank #2176 at Haukeland University Hospital.

In this large and heterogeneous data set collected from multiple sources from the individual participant in the study, we will employ exploratory data analysis (EDA) to get insight, and state-of-the-art supervised and unsupervised machine learning techniques for predictions, and detection of IBS subgroups and identification of diagnostic biomarkers (Fig. 1). More specifically, we will use Python (https://www.python.org) and Jupyter notebooks (https://jupyter.org) to support open science and reproducible research.[41,42] For implementing machine learning procedures and pipelines, we will use Pandas Numpy, Scikit-learn (https://scikit-lern.org). For deep learning approaches, especially related to the MRI acquisitions, we will consider the Pytorch (https://pytorch.org) framework and the high-performing fastai (https://docs.fast.ai) libraries using deep learning best practices allowing models to train fast with limited resources. We will address the importance of separate training, validation and test datasets, and employ cross-validation techniques to reduce overfitting and improve generalization to any data from the problem domain. Before employing machine learning techniques, for processing the acquired neuroimaging data, we will consider using Freesurfer (http://surfer.nmr.mgh.harvard.edu), FSL (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/), and AFNI (https://afni.nimh.nih.gov/).[43] Freesurfer will mainly be used for cortical reconstruction and volumetric segmentation to extract the region

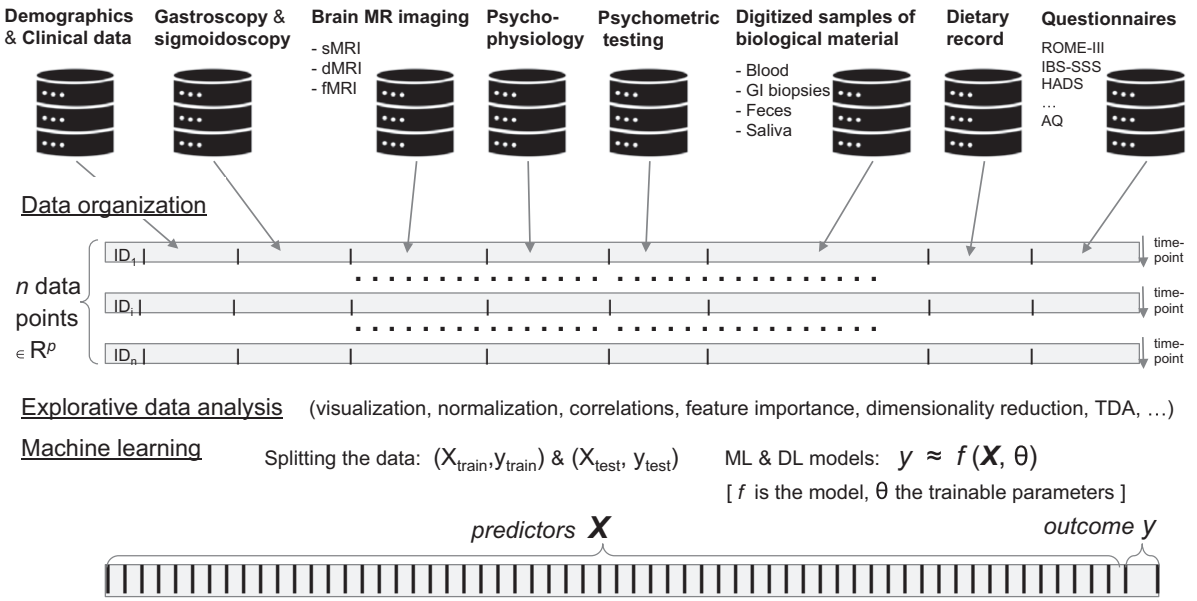

Figure 1. The brain-gut-microbiota spatiotemporal digital biobank. Patients with IBS will be using the same CRF, assessing extrinsic factors, medications, and family history. Blood samples, stool, saliva, and gastrointestinal tissue will be sampled. Established, validated questionnaires to assess GI symptoms, comorbid conditions, and somatization will be collected. Data from multimodal brain imaging examination, detailed measurements of GI function, and psychometric information from tests and questionnaires, dietary records, microbiota-profiles, pain-provocation tests results, and dietary responsiveness will be fed into a spatiotemporal digital biobank for biostatistics integration of data and modelling to identify novel subgroups for personalized treatment.

of interest (ROI). We have planned to used FSL and AFNI to obtain diffusion and functional maps, respectively.

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Author contributions

All authors made a significant contribution to the conception and design of the protocol. BB made major contributions to the design of this study, ethical approval application, development of the protocol, and drafting the manuscript. GAL, EKS, BB, TH, EPT, ERH designed the study and dietary intervention, EKS, OHG, TH designed the UMAT test, BO designed the protocol for vagal activity measures, GAL and IB designed the biochemistry analysis plan, YA, ABP, JV designed the protocol and plan for microbiota analysis, EAM, AG and AL developed and designed the advanced neuroimaging protocol, AL and BHN developed the big data statistical analysis and machine-learning plan. AJL designed the protocol for all psychometric tests. AL, TH, OHG, JGH, GAL, and BB secured funding. All authors contributed to refinement of the study protocol and the final manuscript.

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Changes in serum zonulin in individuals with morbid obesity after weight-loss interventions: a prospective cohort study

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Abstract

Background: Zonulin is a biomarker of impaired intestinal permeability, which has been associated with various disorders. The primary aim was to study serum zonulin (s-zonulin) in individuals with morbid obesity before and after a conservative weight loss intervention followed by bariatric surgery. The secondary aims were to explore predictors of s-zonulin, and the associations between the changes of the predictors and changes in s-zonulin, and to compare the associations in the two treatment periods.

Methods: Individuals with morbid obesity were included. Data before any weight loss interventions, after a 6 months' conservative weight loss intervention, and 6 months after bariatric surgery were used. S-zonulin was measured with an ELISA method from Immundiagnostik AB, Bensheim, Germany. Data were analysed with mixed models.

Results: The mean body mass index was 42.1 kg/m^2 (SD 3.8) at inclusion and was reduced to 38.7 kg/m^2 (SD 3.8) and 29.8 kg/m^2 (SD 3.8) after the conservative treatment and bariatric surgery respectively. S-zonulin was 63 ng/mL (SD 32) at inclusion and was reduced with 19 ng/ml (95% CI 12 to 26, $p < 0.001$) after conservative treatment and 11 ng/ml (95% CI 0 to 21, $p = 0.04$) after bariatric surgery. At inclusion, s-zonulin was significantly associated with factors including p-glucose (B = 2.21, 95% CI 1.09 to 3.33, $p < 0.001$), c-reactive protein (B = 1.02, 95% CI 0.45 to 1.58, $p < 0.001$) and the intake of proteins (B = 0.23, 95% CI 0.08 to 0.38, $p = 0.003$) and non-nutritive sweeteners (B = 0.68, 95% CI 0.19 to 1.17, $p = 0.007$). The reduction in s-zonulin after the conservative weight loss intervention was significantly associated with improvement in diarrhoea (B = 6.6, 95% CI 1.3 to 11.8, $p = 0.02$), HbA1c (B = 9.7, 95% CI 1.1 to 18.3, $p = 0.03$), p-glucose (B = 3.5, 95% CI 1.2 to 5.9, $p = 0.004$) and gamma-GT (B = 0.28, 95% CI 0.09 to 0.47, $p = 0.004$), but not associated with the change in body mass index (B = 0.9, 95% CI − 1.5 to 3.3, $p = 0.46$).

Conclusions: S-zonulin was markedly reduced after the conservative weight loss intervention, and further reduced after bariatric surgery. The reduction in s-zonulin was associated with improvement of diarrhoea, markers of glucose intolerance and liver disease, but not associated with the change in body mass index.

Keywords: Obesity, Intestinal permeability, Zonulin, Bariatric surgery, Diet interventions

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Background

Intestinal permeability is increased in individuals with obesity. Zonulin, a 47 kDa protein, is involved in the regulation of the paracellular permeability in the intestine [1, 2]. The protein is secreted by the liver, the intestine and several other tissues, circulates in the blood and binds to receptors on the enterocytes in the ileum and jejunum [1, 3]. Binding to these receptors leads to reversible modulation of intercellular tight junctions and thereby increased small intestinal paracellular permeability [1, 2]. Zonulin may also impact the blood-brain barrier through similar mechanisms [4]. Zonulin can be measured in the blood and the faeces. High serum zonulin (s-zonulin) has been interpreted as increased intestinal permeability [1, 2, 5]. Measured with the available enzyme-linked immunosorbent assay (ELISA) methods, s-zonulin has been associated with obesity and high energy intake in cross-sectional studies [3, 6–9]. S-zonulin has also been associated with the comorbidities of obesity, such as fatty liver disease and diabetes mellitus [6, 10].

An altered intestinal paracellular permeability may be a mediator between the intestinal environment and metabolic disorders [11]. Zonulin inhibitors can reduce the paracellular permeability, and may become useful as drugs in the future [12]. Studies that go beyond cross-sectional examinations, such as longitudinal studies and intervention studies are needed to explore such relations.

A few longitudinal studies of zonulin before and after conservative weight-loss interventions have been performed, but no studies of the changes after bariatric surgery [5, 13, 14]. If the changes in s-zonulin partly explain the metabolic changes after bariatric surgery, zonulin inhibitors could have an effect on some of the metabolic disorders related to obesity.

The primary aim was to study s-zonulin in individuals with morbid obesity before and after a conservative weight loss intervention followed by bariatric surgery. The secondary aims were to explore predictors of s-zonulin, and the associations between the changes of the predictors and changes in s-zonulin, and to compare the associations in the two treatment periods.

Methods

Study design and setting

All adult individuals referred to an outpatient obesity clinic at Innlandet Hospital Trust in Gjøvik, South-Eastern Norway from December 2012 to September 2014 were invited to participate in a prospective cohort study. The three first visits of the prospective study were used in this substudy; the first visit was before any weight loss interventions, the second visit was after a 6 month conservative weight loss intervention and before bariatric surgery, and the third visit was 6 months after bariatric surgery.

Participants

The inclusion criteria were individuals 18–65 years of age with morbid obesity, defined as body mass index (BMI) above 40 kg/m^2 or BMI above 35 kg/m^2 with comorbidities related to obesity [15]. Exclusion criteria were previous major gastrointestinal surgery, organic gastrointestinal disorders, alcohol and drug addiction, major psychiatric disorders, and serious somatic disorders not related to obesity.

Interventions

The included patients did all follow routine clinical care at the obesity center, no interventions were altered because patients were included in the cohort study.

The conservative weight loss intervention

The conservative weight loss intervention with group counselling and personalised lifestyle interventions was based on the weight loss programs used in most Norwegian obesity clinics in this period of time [16], with some small local changes from the national standard due to availability of personnel. The intervention was organised as a series of outpatient visits [16, 17]. The intervention started with three outpatient consultations of one-hour duration with a nurse, a nutritionist and a physician. These three consultations included registration of the patient's current diet and physical activity and personalised advice about adjustments. A few weeks later, the participants were enrolled in groups with meetings of 4 h duration during seven consecutive weeks. These seven meetings were based on group counselling and were led by nurses specialised in obesity, surgeons, nutritionists, and a psychologist.

The dietary recommendations were based on a reduced daily energy intake and recommendation of food items with a high micronutrient content [17]. The participants were adviced to eat less sugar and fat and more fibre and protein. They were recommended to distribute their food intake into 4–6 daily meals with 2–4 h intervals. The nutritionists gave personalised advice on specific dishes based on what the participants liked to eat and what they were used to eat. At a time point 21 days before the second study visit, the participants were recommended to change to a more standardised "crisp bread diet" with 4200 kJ daily energy intake. During the crisp bread diet, the participants were recommended to eat six units of crisp bread with high-protein, low-fat topping (as fish, cheese or meat) and 0.45 L of milk. They were adviced to eat dishes based on meat or fish with vegetables on small plates for dinner. Participants who preferred meal replacement powder to the crisp bread diet could use meal replacement powder giving 3765 kJ with free amounts of vegetables these 21 days. Concerning the choice of vegetables, corn, olives or avocados were not recommended in this period due to the

energy content. All kinds of beverages without energy content were allowed during the conservative weight loss intervention.

Bariatric surgery

Bariatric surgery was performed as Roux-en-Y Gastric Bypass or Gastric Sleeve. Three experienced surgeons performed all procedures in a standardised manner with a laparoscopic approach [18, 19].

Study visits

The first visit

At the first visit, BMI and waist-hip-ratio were measured and the participant filled in a case report form with assistance from a study nurse with questions about demographics, diseases and health scores. Blood samples were drawn. The participants were asked to bring along a faecal sample and a filled-in food frequency questionnaire (FFQ) at the next visit.

The second visit

The second study visit was scheduled after the conservative weight loss intervention before bariatric surgery. BMI was measured, the participants filled in a case report form, and blood samples were drawn. A faecal test and the FFQ were not included at this visit, however, all participants used a standardised diet at this time point.

The third visit

The third study visit was scheduled 6 months after bariatric surgery. BMI was measured, a case report form was filled out, blood samples were drawn and the participant was asked to bring along a faecal sample test and a filled-in FFQ.

Variables

Anthropometrics

Weight and height were measured to calculate BMI (kg/m^2) at all three visits. The waist circumference was measured at the smallest part of the waist, the hip circumference was measured at the widest parts of the buttocks, and waist-hip-ratio was calculated.

Demographics

Age (years), sex (male/female) and smoking habits (daily smoking/no daily smoking) were registered.

Diseases

Irritable bowel syndrome was diagnosed with the Rome III criteria [20].

Health scores

The participants reported gastrointestinal symptoms on the gastrointestinal symptom rating scale for patients with irritable bowel syndrome (GSRS-IBS). These responses were used to register values for constipation and diarrhoea according to two predefined subscales, GSRS-constipation and GSRS-diarrhoea [21]. These two scales range from 1 to 7, high values indicate many gastrointestinal symptoms.

Blood tests

Serum and EDTA plasma were immediately analysed or stored at − 80 °C for later analysis. C-reactive protein (CRP) and HbA1c were analysed at Innlandet Hospital Trust Gjøvik with a Cobas c501 instrument with the reagents CRPL3 and Tina-quant HbA1C (Roche Diagnostics GmbH, Mannheim, Germany). IL-6 was analysed with Immulite 2000, TNF alpha was analysed with Immulite 1000 (Siemens Healthcare AS, Oslo, Norway). Plasma glucose (p-glucose) and gamma-glutamyl transferase (gamma-GT) were analysed on Cobas 6000, with instruments and reagents delivered by Roche Diagnostics Norway. S-zonulin was analysed by a commercially available ELISA kit (Immundiagnostik, Germany, normal values < 38 ng/ml.)

Faecal tests

The faecal microbiota was assessed with the GA-map dysbiosis test manufactured and marketed by Genetic Analysis, Oslo, Norway. This CE-marked test was created based on selected 16S rRNA gene sequences from bacteria: Probes were selected based on their ability to separate individuals with disease from healthy individuals. Details about the creation process and the test are given in a publication from the manufacturer [22]. The results were given from the manufacturer as a score on a dysbiosis index ranging from 1 to 5, where values above 2 are defined as dysbiosis [22].

Dietary intake

The intake of energy, proteins, fats, carbohydrates, fibres, starch and sugar was registered with a semiquantitative food frequency questionnaire designed for the Norwegian population [23]. The intake of non-nutritive sweeteners (NNS) was estimated from the same questionnaire, one unit was defined as the quantity that corresponds to 100 ml of beverage with NNS, or two tablets for use in coffee or tea [24].

Statistics

The characteristics of all participants at three visits are presented as means with standard deviation or ratio with percentage. To answer the primary aim; the changes in s-zonulin after the two weight-loss interventions, we used a linear mixed model with s-zonulin as the dependent variable, patient as random effect and time point as a three level categorical covariate. To answer the first part of the

secondary aim concerning the predictors of s-zonulin, we added potential explanatory variables one at a time as covariates in the mixed model, including the interaction between the potential explanatory variable and the time point. S-zonulin was dependent variable and patient was random effect. This analysis was done for the whole treatment period and for the three visits separately. To answer the second part of the secondary aim, the associations between the changes of the predictors and changes in s-zonulin, we used mixed models with the change of s-zonulin as dependent variable, patient as random effect and treatment period as a two level categorical covariate. Subsequently, we added changes in the potential explanatory variables one at a time as covariates in the mixed model, including the interaction between the change in the potential explanatory variable and the treatment period. Sex, age and BMI were included as covariates in all mixed model analyses. Normality of residuals was checked by visual inspection of Q-Q plots. Due to multiple hypotheses, p-values between 1 and 5% should be interpreted with caution. A post-hoc exploration of variables independently associated with s-zonulin was performed with backwards elimination of covariates with $p > 0.05$ from a model with all relevant explanatory covariates. The analyses were performed with SPSS 25.

Sample size

The sample size was fixed because the cohort was from a previously performed study and a power calculation was not performed [25].

Ethical approval

The study was approved by the Regional Committees for Medical and Health Research Ethics South East Norway (reference 2012/966) and conducted in accordance with the Declaration of Helsinki including written informed consent from all the participants.

Results

In all, 159 individuals gave informed consent, 16 individuals were later excluded due to organic disease or missing information. Figure 1 shows a flow chart of the individuals. Data from 143 individuals (77% women) with a mean age of 43.0 years (standard deviation (SD) 8.7) and BMI of 42.1 kg/m^2 (SD 3.8) was included in the analyses. Table 1 gives the participants' characteristics. BMI was reduced to 38.7 kg/m^2 (SD 3.8) and 29.8 kg/m^2 (SD 3.8) after the conservative treatment and bariatric surgery, respectively. This corresponded to a mean total weight loss of 36.8 kg (SD 9.4) in the period as a whole.

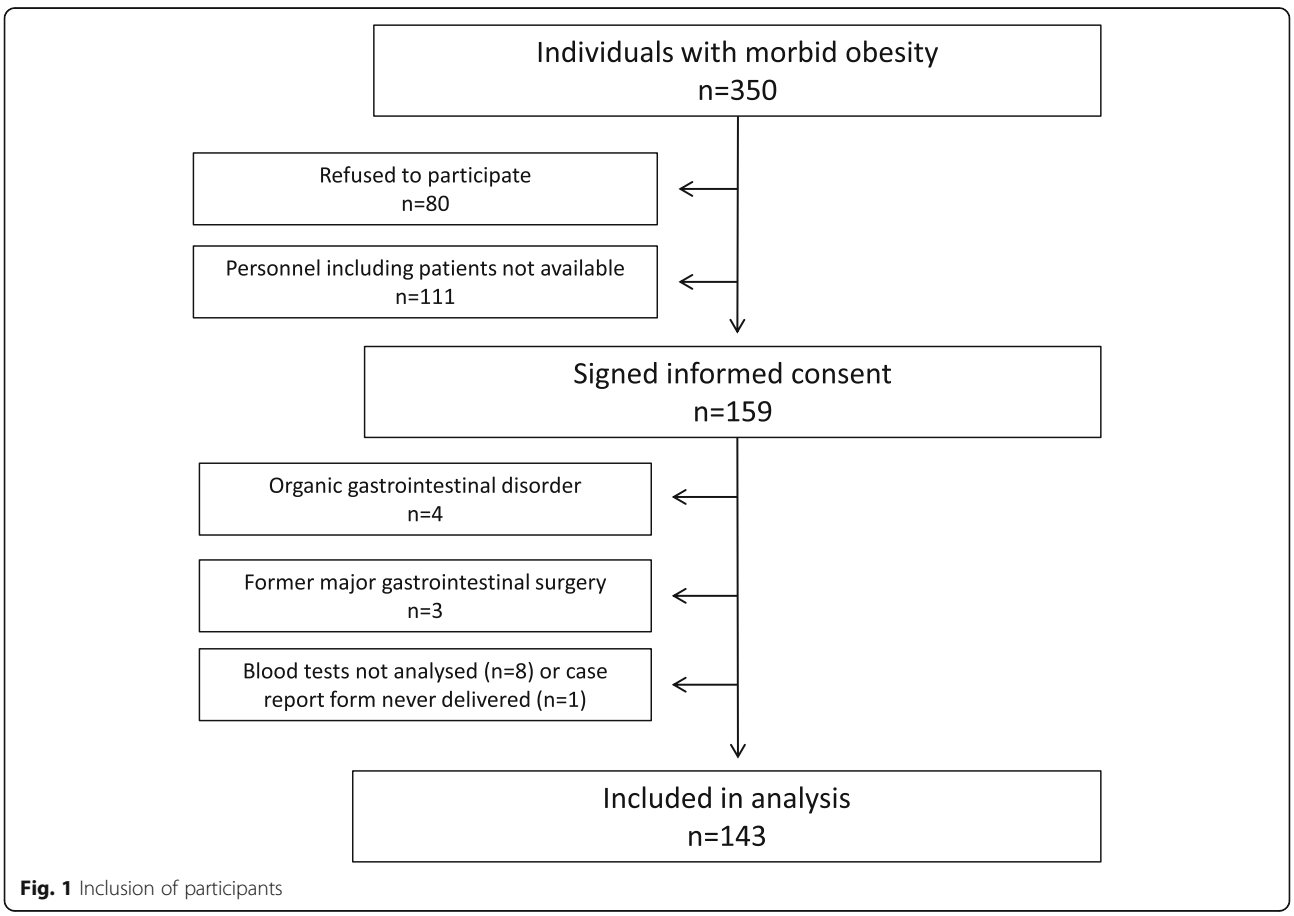

Fig. 1 Inclusion of participants

Table 1 Participant characteristics before and after the conservative and surgical weight loss interventions

	At inclusion		After the conservative weight loss intervention		After bariatric surgery	
	n		N		n	
Sex (%Female)	143	110 (77%)				
Age (years)	143	43.0 (8.7)				
Current smoker	138	25 (18%)				
Weight (kg)	140	123.5 (18.8)	108	114.0 (17.8)	73	87.5 (15.4)
Body mass index (kg/m^2)	140	42.1 (3.8)	107	38.7 (3.8)	72	29.8 (3.8)
Waist-hip-ratio	131	0.98 (0.10)	8	0.98 (0.14)	26	0.94 (0.09)
Irritable bowel syndrome (yes/no)	133	36 (27%)	91	18 (20%)	76	20 (26%)
GSRS-constipation	134	1.5 (0.8)	95	1.6 (0.9)	73	1.8 (0.9)
GSRS-diarrhoea	127	1.8 (1.0)	89	1.5 (0.9)	80	1.6 (0.9)
HbA1c (%)	143	5.9 (1.4)	99	5.5 (0.9)	96	5.2 (0.8)
Plasma glucose (mmol/L)	143	6.6 (3.1)	110	5.8 (1.7)	96	5.4 (1.2)
S-Zonulin (ng/ml)	141	63 (32)	96	43 (7)	94	29 (18)
C-reactive protein (mg/L)	143	6.9 (6.2)	110	4.4 (3.9)	96	1.9 (2.5)
IL-6 (ng/mL)	139	2.8 (2.9)		NA	94	2.2 (1.4)
TNF (ng/mL)	140	12.1 (41.9)		NA	94	10.6 (38.1)
Gamma-GT (U/L)	143	43 (45)	98	32 (24)	96	17 (13)
Nutritional intake of						
Energy (MJ)	96	11 (4)		NA	71	6 (5)
Proteins (g)	96	111 (35)		NA	71	75 (58)
Fats (g)	96	99 (46)		NA	71	58 (49)
Carbohydrates (g)	96	275 (125)		NA	71	158 (110)
Starch (g)	96	134 (50)		NA	71	69 (38)
Fibre (g)	96	34 (10)		NA	71	22 (10)
Sugar (g)	96	47 (75)		NA	71	19 (26)
Non-nutritive sweeteners (units[a])	96	8 (11)		NA	71	6 (7)
Dysbiosis Index[b]	100	2.8 (1.3)		NA	42	4.1 (1.0)

Data are given as mean (standard deviation) or number (percentage)
S-zonulin Serum-zonulin, *GSRS* Gastrointestinal symptom rating scale, *TNF* Tumor necrosis factor, *Gamma-GT* Gamma-glutamyl transferase, *NA* Not available
[a]One unit corresponds to 100 ml of beverage with non-nutritive sweeteners
[b]The dysbiosis index range from 1 to 5, values > 2 indicate gut microbial dysbiosis

The mean s-zonulin at inclusion was 63 ng/ml (SD 32). The value was reduced with 19 ng/ml (95% confidence interval (CI) 12 to 26) after the conservative weight loss intervention and further reduced with 11 ng/ml (95% CI 0 to 21) after the bariatric surgery (Table 2).

At inclusion, high s-zonulin values were associated with a high waist-hip-ratio, irritable bowel syndrome and diarrhoea, and with high values of HbA1c, p-glucose, CRP, gamma-GT and the dysbiosis index. High s-zonulin values were also associated with a high dietary intake of energy, proteins, fats and NNS. S-zonulin was not associated with BMI. At the second and third study visit, s-zonulin was not significantly associated with any of the explanatory variables. Table 3 shows both the overall associations between s-zonulin and the explanatory variables and the associations at each of the three points of time. Table 4 shows associations between changes in s-zonulin and the changes in the explanatory variables. The reduction in s-zonulin after the conservative weight loss intervention was associated with reductions in GSRS-diarrhoea, HbA1c, p-glucose and gamma-GT. No significant associations between the reductions in s-zonulin and the explanatory variables were observed after the surgical weight loss intervention. The difference between the associations after the conservative and after the surgical period was statistically significant for GSRS-diarrhoea, HbA1c and p-glucose.

In the post-hoc analysis, four explanatory variables were independently associated with s-zonulin: P-glucose (B = 2.14, 95% CI 0.46 to 3.81, $p = 0.013$), CRP (B = 1.85, 95% CI 0.88 to 2.83, $p < 0.001$), intake of proteins (B = 0.28, 95% CI 0.14 to 0.42, $p < 0.001$) and intake of NNS (B = 0.50, 95% CI 0.06 to 0.94, $p = 0.03$).

Table 2 Trajectory of s-zonulin before and after the conservative and surgical weight loss interventions

	Estimate (95% confidence interval of the mean)	*P*-values
At inclusion (ng/ml)	62 (57 to 66)	**< 0.001**
Change from baseline to after the surgical intervention (during both interventions) (ng/ml)	−29 (− 17 to −41)	**< 0.001**
Change during the conservative weight loss intervention (ng/ml)	−19 (− 12 to −26)	**< 0.001**
Change during the surgical weight loss intervention (ng/ml)	− 11 (0 to −21)	**0.04**

Analysis with linear mixed models, with zonulin as the dependent variable, patient as random effect and the covariates time, sex, age (centred at age 43 years) and body mass index (measured at three time points, centred at body mass index 39 kg/m^2) as fixed effects
S-zonulin Serum zonulin

Discussion

S-zonulin was high in a group of individuals with morbid obesity referred to a hospital for obesity treatment. High s-zonulin at inclusion was associated with their diet, and with diarrhoea, dysbiosis and biomarkers of metabolic unhealth. S-zonulin was partly normalised after a six-month long conservative weight loss intervention and further reduced 6 months after bariatric surgery. The changes in s-zonulin after the weight-loss interventions were not significantly associated with the changes in BMI.

The mean s-zonulin at baseline was 63 ng/ml, higher than the upper limit of the reference interval. S-zonulin was reduced after both the weight-loss interventions. The change in s-zonulin was most prominent after the conservative weight loss intervention, before the substantial changes in BMI induced by surgery. A reduction in intestinal permeability after a conservative weight loss intervention has also been observed in a study where the permeability was measured with a dual sugar test in individuals with an average BMI around 44 kg/m^2 [13]. On the contrary, an increase in s-zonulin after a dietary intervention was observed in a study where the participants had type 2 diabetes and mean BMI around 30 kg/m^2 [14]. The current study indicates that both conservative and surgical weight loss interventions can lead to reduced s-zonulin in individuals with BMI 42 kg/m^2. Differences between individuals with overweight and severe obesity or differences between the diets used in the different studies might explain the diverging findings.

Several explanatory variables were associated with s-zonulin at the first of the three visits. Abdominal obesity (waist-hip ratio), a marker of fatty liver disease (gamma-GT), reduced glucose tolerance (HbA1C) and increased low-grade inflammation were associated with high levels of s-zonulin, in accordance with earlier studies [3, 5, 6, 8, 26]. S-zonulin was not associated with BMI, but the levels of s-zonulin in the cohort as a whole were much higher than previously observed levels of s-zonulin in individuals without obesity [6, 8].

Gluten and bacteria in the intestinal lumen are among the triggers that can lead to zonulin secretion from the liver and intestine [1]. S-zonulin has also been related to a high intake of energy and proteins [7, 9, 14]. In the current study, the s-zonulin measurements were associated with the intake of proteins, fats, energy and NNS and with gut microbial dysbiosis. Specific peptides in the diet or certain bacteria in the microbiome might stimulate zonulin secretion. NNS have been associated with metabolic disorders in observational studies [27]. That NNS may impact the intestinal permeability through alterations in the gut microbiota is a hypothesis that could be explored further [28].

To explore possible explanations for the changes in s-zonulin, we analysed associations between the changes in s-zonulin after the two weight-loss interventions and the changes in the explanatory variables. After the conservative weight-loss period, the reduction in s-zonulin was associated with improvements in diarrhoea, markers of glucose tolerance and fatty liver disease (gamma-GT). Fatty liver disease is common in individuals with morbid obesity [29] and associated with impaired glucose tolerance and impaired intestinal permeability [13]. The reduction in s-zonulin during the conservative weight loss intervention might be related to amelioration of fatty liver disease [13]. The findings indicate that s-zonulin is associated with diarrhoea and IBS in subjects with morbid obesity. This contrasts to two studies in individuals with lower BMI, which did not find significant associations between s-zonulin levels and gastrointestinal symptoms and disorders [3, 30].

Many covariates were associated with s-zonulin. A post-hoc analysis with inclusion of all the significant covariates was performed to search for independent predictors of s-zonulin during the whole weight-loss period. In the final post-hoc model, four factors remained significantly associated with serum zonulin: S-glucose, CRP, protein intake and NNS intake. Biological links between serum zonulin and glucose tolerance, low-grade inflammation, proteins and NNS in the diet appear probable.

The conservative weight loss intervention used in this study led to an improvement in low-grade inflammation and glucose intolerance [17]. A regulating protein in the zonulin family might be a mediator between the diet and biomarkers. As a zonulin antagonist is available, it is possible to design studies that explore causal relations between s-zonulin and other biomarkers [2, 12]. This

Table 3 Associations between s-zonulin and the explanatory variables overall and at the three points of time

	Overall		At inclusion		After the conservative weight loss intervention		After bariatric surgery	
	B (95% CI)	*p*	B (95% CI)	*p*	B (95% CI)	*p*	B (95% CI)	*p*
Sex (Female)	−1.32 (− 8.05 to 5.40)	0.70						
Age	0.19 (− 0.15 to 0.54)	0.28						
Body mass index	0.39 (−0.40 to 1.18)	0.33						
Waist-hip-ratio	84.2 (18.2 to 150.2)	**0.013**	110.1 (39.1 to 181.1)	**0.003**	3.4 (− 154.7 to 161.5)	0.97	9.1 (− 122.7 to 140.9)	0.89
Irritable bowel syndrome	5.82 (−1.08 to 12.72)	0.10	10.16 (0.70 to 19.61)	**0.04**	1.19 (−11.80 to 14.17)	0.86	1.20 (−12.95 to 15.34)	0.87
GSRS-constipation	−0.23 (−3.56 to 3.09)	0.89	− 0.05 (−5.17 to 5.06)	0.98	− 0.01 (− 5.54 to 5.53)	1.00	− 0.91 (−7.75 to 5.93)	0.79
GSRS-diarrhoea	3.00 (0.06 to 5.95)	**0.046**	5.36 (1.30 to 9.42)	**0.01**	0.49 (−5.45 to 6.43)	0.87	0.42 (−5.50 to 6.33)	0.89
HbA1c (%)	4.44 (1.92 to 6.96)	**0.001**	5.48 (2.54 to 8.42)	**< 0.001**	1.39 (−4.01 to 6.78)	0.61	2.75 (−3.75 to 9.25)	0.41
Plasma glucose (mmol/L)	2.21 (1.09 to 3.33)	**< 0.001**	2.56 (1.30 to 3.81)	**< 0.001**	0.72 (−2.01 to 3.45)	0.60	1.58 (−2.44 to 5.59)	0.44
C-reactive protein (mg/L)	1.02 (0.45 to 1.58)	**< 0.001**	1.17 (0.52 to 1.82)	**< 0.001**	0.64 (−0.56 to 1.85)	0.30	0.37 (−1.67 to 2.42)	0.72
IL-6 (ng/mL)	1.51 (−0.04 to 3.07)	0.06	1.28 (−0.39 to 2.95)	0.13	NA		3.11 (−1.26 to 7.48)	0.16
TNF (ng/mL)	0.02 (−0.07 to 0.11)	0.69	0.03 (−0.08 to 0.14)	0.59	NA		−0.01 (− 0.16 to 0.15)	0.95
Gamma-GT (U/L)	0.16 (0.08 to 0.24)	**< 0.001**	0.18 (0.09 to 0.27)	**< 0.001**	0.02 (−0.18 to 0.22)	0.85	0.14 (−0.29 to 0.58)	0.52
Nutritional intake of								
Energy (kJ)	1.87 (0.43 to 3.31)	**0.01**	2.30 (−0.71 to 3.90)	**0.005**	NA		−0.16 (4.05 to 3.73)	0.94
Proteins (g)	0.23 (0.08 to 0.38)	**0.003**	0.28 (0.11 to 0.44)	**0.001**	NA		0.007 (−0.325 to 0.339)	0.97
Fats (g)	0.18 (0.05 to 0.30)	**0.006**	0.19 (0.06 to 0.32)	**0.003**	NA		−0.06 (−0.47 to 0.35)	0.77
Carbohydrates (g)	0.03 (−0.01 to 0.08)	0.13	0.04 (−0.01 to 0.08)	0.12	NA		0.004 (−0.125 to 0.134)	0.95
Starch (g)	0.54 (−0.59 to 0.17)	0.35	0.07 (−0.05 to 0.19)	0.26	NA		−0.04 (− 0.32 to 0.24)	0.78
Fibre (g)	0.34 (−0.17 to 0.84)	0.19	0.50 (−0.07 to 1.08)	0.09	NA		−0.27 (−1.23 to 0.70)	0.59
Sugar (g)	0.03 (−0.05 to 0.11)	0.46	0.03 (−0.05 to 0.11)	0.48	NA		0.10 (−0.46 to 0.67)	0.72
Non-nutritive sweeteners (u)[a]	0.68 (0.19 to 1.17)	**0.007**	0.76 (0.23 to 1.30)	**0.006**	NA		0.30 (−0.72 to 1.32)	0.56
Dysbiosis index[b]	6.95 (3.11 to 10.80)	**0.001**	6.76 (2.32 to 11.19)	**0.003**	NA		7.54 (−1.35 to 16.43)	0.10

Analyses with linear mixed models with zonulin as dependent variable, and patient as random effect. The fixed effects in the left column are sex, age, body mass index, the explanatory variable in the same row and time (as a three level categorical covariate). In the three other columns, the fixed effects are sex, age, body mass index and time (with the time point given in the heading of the column as reference), the explanatory variable in the same row and the interaction term between this explanatory variable and time

S-zonulin Serum-zonulin, *TNF* Tumour necrosis factor, *GSRS* Gastrointestinal symptom rating scale, *NA* Not available, *Gamma-GT* Gamma-glutamyltransferase

[a]One unit corresponds to 100 ml of beverage with non-nutritive sweeteners

[b]The dysbiosis index range from 1 to 5, values > 2 indicate gut microbial dysbiosis

and previous studies indicate that individuals with morbid obesity could be a suitable group to include in an intervention trial [13].

A large reduction in zonulin was seen after the conservative weight loss intervention, at a time point when the BMI was reduced with only 3.4 kg/m^2. In a previous study from the same cohort, the inflammatory biomarker neopterin, related to cardiovascular disease, showed a comparable pattern [31].. The weight loss after a conservative intervention is usually much smaller than the weight loss after bariatric surgery, but conservative weight-loss interventions do nevertheless have significant effects on morbidity and mortality [32]. The primary purpose of morbid obesity treatment is a reduction of the present and the future comorbidity burden, and the potential effect on BMI is only one of several factors that should be considered when treatment decisions for morbid obesity are made. The current study indicates that individuals with morbid

Table 4 Associations between the changes in s-zonulin and the changes in the factors of interest after the two weight-loss interventions

Change in covariate	Overall change		Change after conservative weight loss intervention		Change after bariatric surgery		Interaction	
	B (95% CI)	*p*-values	B (95% CI)	*p*-values	B (95% CI)	*p*-values	B (95% CI)	*p*-values
Body mass index (kg/m^2)	0.9 (− 1.5 to 3.3)	0.46	3.2 (−0.9 to 7.2)	0.27	−0.4 (− 3.4 to 2.6)	0.80	3.5 (− 1.5 to 8.6)	0.17
Weight (kg)	−0.7 (− 3.9 to 2.5)	0.68	1.1 (− 2.7 to 5.0)	0.56	− 0.4 (− 3.6 to 2.9)	0.83	−1.5 (− 3.2 to 0.2)	0.08
GSRS-constipation	1.2 (− 4.4 to 6.9)	0.67	7.0 (−1.6 to 15.6)	0.11	−3.6 (− 11.1 to 3.8)	0.34	−10.6 (− 22.1 to 0.94)	0.07
GSRS-diarrhoea	6.6 (1.3 to 11.8)	**0.02**	13.6 (6.2 to 20.9)	**< 0.001**	−0.4 (− 7.7 to 6.9)	0.91	14.0 (3.5 to 24.4)	**0.009**
HbA1c (%)	9.7 (1.1 to 18.3)	**0.03**	16.1 (5.9 to 26.3)	**0.002**	−6.0 (−20.9 to 8.9)	0.43	−22.1 (−4.2 to −40.0)	**0.016**
Plasma glucose (mmol/L)	3.5 (1.2 to 5.9)	**0.004**	5.1 (2.4 to 7.7)	**< 0.001**	−2.6 (−7.9 to 2.7)	0.34	−7.6 (−1.7 to 13.6)	**0.012**
C-reactive protein (mg/L)	0.8 (−0.5 to 2.1)	0.22	1.2 (−0.4 to 2.9)	0.15	0.2 (−1.9 to 2.3)	0.88	−1.1 (−3.7 to 1.6)	0.43
Gamma-GT (U/L)	0.28 (0.09 to 0.47)	**0.004**	0.36 (0.15 to 0.58)	**0.001**	−0.02 (− 0.40 to 0.37)	0.93	0.38 (− 0.06 to 0.82)	0.09

Analyses with linear mixed models with zonulin changes as dependent variable, and patient as random effect. The fixed effects are sex, age, body mass index, intervention period (with the intervention period given in the heading of the column as reference), the explanatory variable in the same row and the interaction between this explanatory variable and the intervention period. The interaction terms between the intervention period and the explanatory variable in the same line are also presented separately in the columns to the right with title "Interaction"
TNF Tumour necrosis factor, *GSRS* Gastrointestinal Symptom Rating Scale, *Gamma-GT* Gamma-glutamyl transferase

obesity can reduce the paracellular permeability both through conservative and surgical health interventions.

It has been discussed whether the ELISA measurements of s-zonulin performed by Immundiagnostik AG measure the originally described zonulin pre-haptoglobin 2. The method might measure another protein e.g. properdin, with structural similarities to zonulin [12, 33, 34]. The current study adds to the evidence that the protein measured by Immundiagnostik ELISA is associated with the diet, gut microbial dysbiosis, diarrhoea and several biomarkers of metabolic disease. If the protein is properdin [34] and not the originally described 47 kDa pre-haptoglobin 2 [2] properdin might be an interesting biomarker and therapeutic target.

Strengths and limitations

The strengths of this study include a prospective design with the examination of s-zonulin and a wide range of explanatory variables (diet, bowel symptoms and biomarkers) before and after conservative and surgical weight-loss interventions in individuals with morbid obesity. The explanatory variables were measured with validated instruments. Both the conservative weight loss program and the surgical procedure were effective with a weight loss of approximately ten and 27 k respectively. The study also had limitations: The diet and the microbiome were not measured at the second study visit and the waist-hip-ratio was only measured in a subset of the participants at the second and third visits. Gamma GT was the only liver function test and no radiological examination of the liver was performed. Most of the patients in our study had normal HbA1c, and our results cannot be extrapolated to subjects with diabetes mellitus. A test with a better specification of the gut microbiome would have been preferable. The sample size was limited and there were large variations in SDs for some key variables. As mentioned, s-zonulin was measured with the Immundiagnostik AG ELISA that has been problematised [34]. There were some dropouts from the study, but the missing data were missing at random, and mixed models were therefore considered as an appropriate statistical approach to handle the data set.

Conclusions

S-zonulin, a serum biomarker of impaired intestinal permeability, was elevated above the reference value in individuals with morbid obesity. Out of several variables, p-glucose, CRP, and the dietary intake of protein and NNS were independently associated with s-zonulin. S-zonulin was markedly reduced after the conservative weight loss program, and further reduced after bariatric surgery. The reductions in s-zonulin in the whole period and after the conservative weight loss intervention were associated with improvements in diarrhoea, glucose intolerance and gamma-GT (a marker of liver disease), but not with the reduction in body mass index. The normalisation of s-zonulin may be related to the alleviation of the comorbid conditions.

Abbreviations

BMI: Body mass index; CI: Confidence interval; CRP: C-reactive protein; ELISA: Enzyme linked immunosorbent assay; Gamma GT: Gamma-glutamyl transferase; GSRS: Gastrointestinal symptom rating scale; NNS: Non-nutritive sweeteners; SD: Standard deviation

Acknowledgments

Not applicable.

Authors' contributions

PGF is the guarantor of the project. He designed the study and analysed data in collaboration with MA and SL. SL is responsible for the statistical analysis. MA has recruited participants and prepared the manuscript. All authors have participated in the writing and have approved the last version.

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Availability of data and materials
Case report forms on paper were used for the collection of the clinical data and are all safely stored. The data files are stored by Innlandet Hospital Trust, Brumunddal, Norway, on a server with security according to the rules given by the Norwegian Data Protection Authority, P.O. Box 8177 Dep. NO-0034 Oslo, Norway. The data are available on request to the corresponding author.

Ethics approval and consent to participate
The study was approved by the Norwegian Regional Committee for Medical and Health Research Ethics, PB 1130, Blindern, 0318 Oslo, Norway (reference number 2012/966). Written informed consent was obtained from all participants.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Effects of Dietary Fibres on Acute Indomethacin-Induced Intestinal Hyperpermeability in the Elderly: A Randomised Placebo Controlled Parallel Clinical Trial

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Abstract: The effect of dietary fibres on intestinal barrier function has not been well studied, especially in the elderly. We aimed to investigate the potential of the dietary fibres oat β-glucan and wheat arabinoxylan to strengthen the intestinal barrier function and counteract acute non-steroid anti-inflammatory drug (indomethacin)-induced hyperpermeability in the elderly. A general population of elderly subjects (≥65 years, $n = 49$) was randomised to a daily supplementation (12g/day) of oat β-glucan, arabinoxylan or placebo (maltodextrin) for six weeks. The primary outcome was change in acute indomethacin-induced intestinal permeability from baseline, assessed by an in vivo multi-sugar permeability test. Secondary outcomes were changes from baseline in: gut microbiota composition, systemic inflammatory status and self-reported health. Despite a majority of the study population (85%) showing a habitual fibre intake below the recommendation, no significant effects on acute indomethacin-induced intestinal hyperpermeability in vivo or gut microbiota composition were observed after six weeks intervention with either dietary fibre, compared to placebo.

Keywords: dietary fibres; prebiotics; intestinal permeability; NSAIDs; clinical trial; elderly; intestinal barrier function; gut health

1. Introduction

During the last decades lifespan has increased dramatically due to improved health and health-care. The global ageing phenomenon will have a major impact on health-care systems due to an increased prevalence of age-related diseases [1]. According to the National Board of Health and Welfare, gastrointestinal (GI) disorders are one of the most common side-effects that lead to hospitalizations of elderly persons in Sweden, often due to medications, such as non-steroid anti-inflammatory drugs (NSAIDs), commonly used for pain management [2]. The intestinal barrier has the function to absorb nutrients from the gut lumen while simultaneously acting as a barrier towards foreign substances, such as bacterial components and food derived toxicants. NSAIDs are known to induce an increased intestinal permeability across the epithelium [3]. Hence, therapeutic strategies able to increase the

resistance of the intestinal barrier function against the negative effects of medication, such as NSAIDs, might have the potential to increase wellbeing.

An adequate fibre intake has been emphasized as particularly important for the elderly, and national guidelines underline the necessity to increase the dietary fibre intake among the elderly population [4]. Prebiotics are dietary fibres that are known to have a positive effect on the gut microbiota by increasing the abundance of butyrate-producing bacteria and thus butyrate availability in the colon. Butyrate is the primary nutrient for colonic cells and has been suggested to play a role in maintaining and supporting intestinal barrier function [5–7]. Arabinoxylan, a dietary fibre originating from the wheat endosperm, has been shown to increase the amount of butyrate-producing bacteria in both animal and human studies [5,6,8,9]. Similarly, the dietary fibre oat β-glucan has been shown to increase beneficial bacteria in the gut and to enhance butyrate production in animal models [7,10]. However, the potential of dietary fibres to strengthen the intestinal barrier function in vivo in elderly individuals has, to our knowledge, not been extensively studied previously.

We performed a placebo-controlled parallel clinical trial in a general population of the elderly to investigate whether six weeks of oral supplementation of arabinoxylan or β-glucan could strengthen the intestinal barrier function and counteract a transient acute NSAID-induced hyperpermeability (e.g., indomethacin), using the in vivo multi-sugar permeability test. Furthermore, gut microbiota composition, inflammatory status and self-reported health was evaluated after dietary fibre intervention.

2. Materials and Methods

2.1. Study Participants and Ethics

Community-dwelling individuals aged 65 years and older, representing the general population in the region of Örebro, Sweden, was recruited through advertisements in local and regional newspapers. Notification of interest was made through a web-based survey and information meetings were held at Örebro University. The study protocol was thoroughly presented and the research team was present for individual discussions and questions. Eligible participants were recruited after signing the consent form. A total of 60 eligible free-living elderly individuals were recruited (see inclusion and exclusion criteria in Table 1). The study received approval from the Regional Ethics Board in Uppsala (dnr 2015/357) and was conducted in accordance with the Declaration of Helsinki. The clinical trial has been registered in Clinicaltrials.gov (NCT03336385).

Table 1. Inclusion and exclusion criteria for participation in the clinical trial.

Inclusion criteria
• Informed consent signed by the study participant • Age ≥ 65 years • Mentally and physically fit to complete questionnaires during the study period
Exclusion criteria
• Any known gastrointestinal disease with strictures, malignancies and ischemia • Inflammatory bowel diseases • Participation in other clinical trials in the past three months • Intake of medications known to change the inflammatory status (i.e., proton pump inhibitors, anti-inflammatory medications (including non-steroid anti-inflammatory drugs (NSAIDs))

2.2. Trial Design

The trial was designed as a randomised placebo controlled parallel clinical trial and was performed at Örebro University in Örebro, Sweden during October–December 2015. A schematic overview

of the study design is outlined in Figure 1 and the participant flow is illustrated in the results section. The primary endpoint was change in intestinal barrier function as assessed by the multi-sugar permeability test. As there is limited knowledge regarding the size of the effect from in vivo prebiotic intervention on intestinal barrier function in the elderly, the sample size was based on previous successful studies using the multi-sugar permeability test and set to 20 participants per arm [11,12], giving a total number of 60 participants. The participants were instructed to maintain their normal dietary habits during the study period. Collection of baseline samples (urine, stool and blood) was performed prior to start of intervention at week −2, −1 and 0. After a daily supplementation at breakfast for six consecutive weeks, endpoint samples were collected during the two last weeks; more specifically urine and faecal samples were collected during week 5 (day 30–34) and blood and questionnaire data during week 6 (day 40–42). All samples were collected according to standardized operating procedures.

All participants entered the intervention trial simultaneously. Participants were contacted two times per week during urine sample collection in order to ensure proper handling. In addition, the research team was available for questions through a dedicated study phone open 24/7 for urgent questions or for guidance through unforeseen events. The active study product consisted of Naxus® (arabinoxylan, provided by Bioactor BV, Maastricht, The Netherlands) or Oatwell® (oat β-glucan, provided by Swedish Oat Fibre, Väröbacka, Sweden) to a total weight of 12 g each. The placebo product consisted of maltodextrin with a similar appearance as the active study products, identically packaged and stored. The study products were packed in sachets and labelled by a third-party at Örebro University, Sweden, that had no other involvement in the clinical trial. Active and placebo sachets were packed identically along with the number of the subject and storing instructions. All products were blinded and block-randomised by a third-party not involved in conducting the clinical trial. For the research team, a blinded randomisation list was provided with the subject sequence and subject number. The research team allocated the study participants by randomly assigning them a study ID from the randomisation list of the pre-labelled study product. For each subject enrolled the date of enrolment and ID number of the trial subject was recorded. All other personnel and researchers involved in the study remained blinded to the study treatment until completion of the statistical analysis and discussion of the findings.

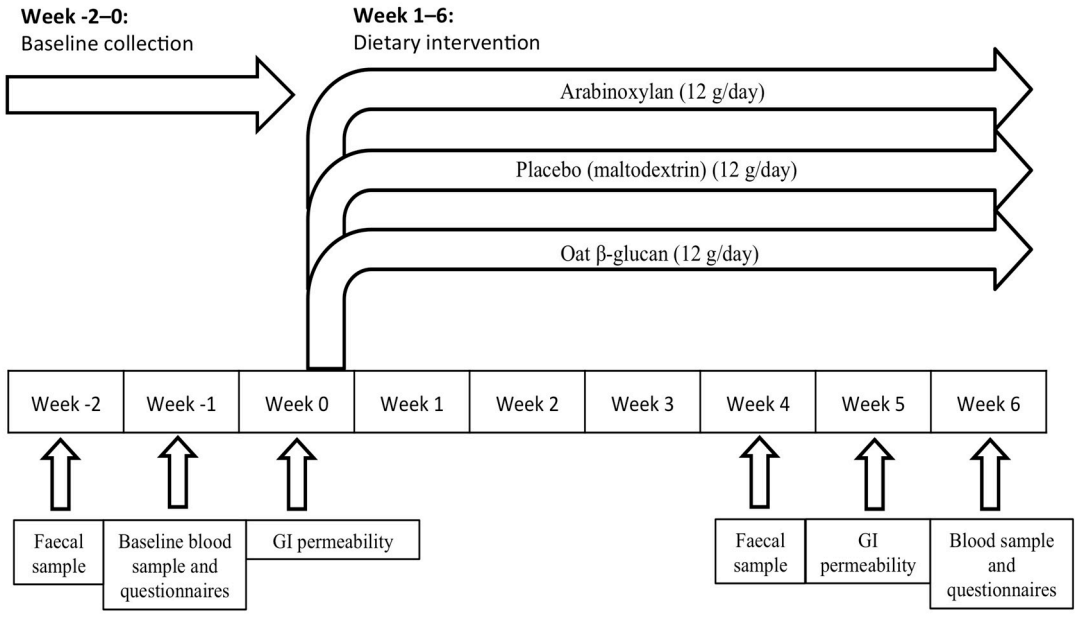

Figure 1. Schematic overview of the randomised clinical trial spanning a total of nine weeks, illustrating the time points for collection of faecal, blood, and urinary samples for gastro–intestinal (GI) permeability, in addition to questionnaire data before and after dietary fibre intervention.

2.3. Data Collection

2.3.1. Demographic Data

The following demographic information was collected: age, sex, BMI, and smoking habits, (see results section, Table 2). Habitual fibre intake was estimated through a food frequency questionnaire (FFQ) completed at study start. The FFQ has previously been validated in a Swedish population [13] and consists of 66 categories of food that evaluates the dietary pattern over the past year.

Table 2. Demographic data of the study cohort.

	Arabinoxylan	**Placebo**	**Oat β–Glucan**	***p*–Value**
	***n* = 17**	***n* = 17**	***n* = 15**	
Male, *n* (%)	9 (55%)	9 (56%)	9 (60%)	ns
Female, *n* (%)	8 (47%)	8 (44%)	6 (40%)	ns
Age, years (median [IQR])	69.0 (66.0–71.5)	70.5 (67.0–78.3)	69.0 (66.0–72.0)	ns
BMI(median [IQR])	24.8 (23.0–29.1)	24.7 (22.1–27.2)	26.2 (22.4–28.0)	ns
Smokers	0	0	0	-
Baseline CRP levels (IQR)	0.7 (0.4–2.7)	0.6 (0.4–1.1)	0.8 (0.2–1.9)	ns
Comorbidities	%	%	%	
Cardiovascular diseases	41.2%	50.0%	33.3%	ns
Gut symptoms	35.3%	33.3%	20.0%	ns
Psychological& neurodegenerative morbidities	0%	11.1%	13.3%	ns
Others (lung, kidney, joints, eyes)	17.6%	50.0%	13.3%	$p = 0.05$
Medications	%	%	%	
Cardiovascular	41.2%	50.0%	33.3%	ns
Anti-inflammation	5.9%	0%	0%	ns
Gut regulating	29.4%	22.2%	20.0%	ns
Antibiotics	17.6%	16.7%	13.3%	ns
CNS-regulating drugs	5.9%	16.7%	6.7%	ns
Others	29.4%	50.0%	26.7%	ns
Multipharmacy (5 or more drugs)	17.6%	11.1%	0%	ns
Other GI regulators (probiotics, fibres etc)	17.6%	16.7%	0%	ns

2.3.2. Medications

Information on prescribed medications taken during the six months preceding the study start was collected through a questionnaire. Current prescription of medications was documented in the case report form (CRF).

2.3.3. Primary Intervention Parameters

GI permeability. A non-invasive previously validated multi-sugar test [3], quantifying 24 h urinary excretion of five different ingested sugars was used to assess gut permeability before (week -2) and after intervention with fibre supplements (week 5, days 32–34). The multi-sugar test estimates the permeability of four segments of the GI tract as outlined in Figure 2. GI permeability was assessed before and after challenge with the NSAID indomethacin (Gorch Fock Apotheke, Kiel, Germany) to investigate if dietary fibre supplementation could strengthen the intestinal barrier function and attenuate the effects of indomethacin. Blood samples were collected prior to study start for assessment of creatinine to ensure normal renal function.

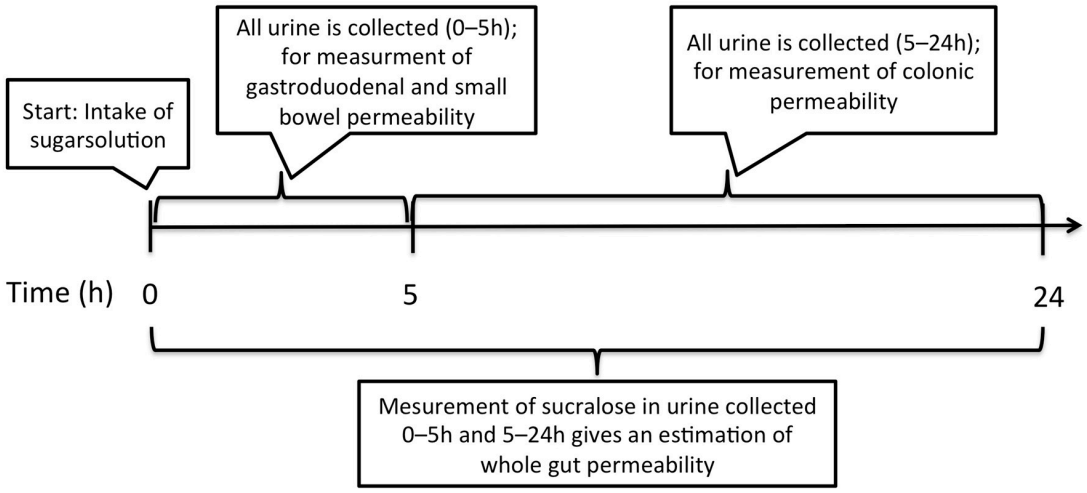

Figure 2. Illustration of the multi-sugar test. Urine is collected in two fractions, 0–5 h and 5–24 h, after intake of the sugar solution. By assessing sucrose in the 0–5 h fraction, gastroduodenal permeability is evaluated. Similarly, measurement of rhamnose and lactulose gives an estimation of small bowel permeability. Analysing sucralose and erythritol in the 5–24 h fraction will estimate the colonic permeability. Measurement of sucralose in both fractions of urine gives an estimation of the whole gut permeability. This study included a challenge to artificially increase intestinal permeability with the NSAID indomethacin.

After an overnight fast, participants were instructed to drink a multi-sugar solution containing five sugars in the morning: 1 g Sucrose (Nordic Sugar, Örtofta, Sweden), 1 g Lactulose (Solactis, Jouy-en-Josas cedex, France), 0.5 g L-rhamnose (BioGaia, Stockholm, Sweden), 1 g Sucralose (Univar, Malmö, Sweden) and 1 g Erythritol (Ingredi, Stockholm, Sweden) dissolved in 150 mL of tap water. Urine was collected in two different fractions: fraction 1 containing the 0–5 h urinary output and fraction 2 the 5–24 h urinary output. The participants were not allowed to ingest any food or drinks except for water throughout the first urinary fraction (0–5 h) collection. During collection of urinary fraction 2 (5–24 h) the participants were allowed to drink and eat as normal, with the exception of caffeine-based products, alcohol, spicy food, drinks or sweets containing the same sugars as in the multi-sugar mix. Gastroduodenal and small intestinal permeability were reflected by the 0–5 h urinary sucrose excretion and the lactulose to L-rhamnose (L/R) ratio, respectively. In fraction 2 (5–24 h) the sucralose to erythritol (S/E) ratio represented colonic permeability. After collection of the 24 h urinary output, the total volume was quantified and urine aliquots were prepared by the participants using a built-in vacuum system in the collection jar (Sarstedt, Sweden) and stored at −20 °C. Before the second multi-sugar test, a 75 mg dose of indomethacin was ingested in the evening (9:30 pm), followed by an overnight fast and ingestion of a second dose of 50 mg at 5:30 am the following morning. The multi-sugar test was conducted 1h after the second dose of indomethacin. Samples were transferred to the lab within one week and stored at −20 °C until further analysis. Urinary sugars were analysed using liquid chromatography-mass spectrometry at Örebro University, Sweden. (See Appendix A for detailed description of the methodology.) Participants with a high creatinine value (women: >90 μmol/L, men: >105 μmol/L), indicating abnormal renal function, were excluded from further analysis (n = 1).

2.3.4. Secondary Parameters

Assessment of gut microbiota composition. Material for collection of faecal samples was provided to each study participant one week prior to sampling and study start (week −1) and during week 5, days 30–34. The sample was collected at the participant's home, instantly frozen at −20 °C and transported in a cooled condition to the laboratory within one week. For analysis of the microbial composition, total DNA was extracted from the faecal samples by using the QIAamp DNA stool mini

kit according to the manufacturer's instructions (Qiagen, Sollentuna, Sweden), coupled with an initial bead-beating step. Polymerase chain reaction (PCR) primers targeting the 16S rRNA gene were used to amplify 1180 base pair fragments containing the variable regions, V3–V9, as previously described by Casen et al. [14]. The PCR fragment was further used in a single-nucleotide extension reaction to label 48 bacterial probes in a panel (Table A1) and two internal controls. Together the 48 bacterial probes assess the presence of approximately 370 bacterial strains. Following hybridisation with barcoded magnetic beads (Luminex MagPlex beads, Luminex Inc., Austin, Texas, USA) the probe signals were detected on a Luminex MagPix.

Questionnaire data. The Gastrointestinal Symptoms Rating Scale (GSRS) is a validated instrument, previously used to assess GI discomfort among the elderly [15,16]. The scale includes 15 questions divided into five symptom domains (e.g., reflux, abdominal pain, indigestion, diarrhoea and constipation). The symptoms are scored from 1 to 7 depending on their severity. Each domain was scored individually to assess the change in single symptoms after intervention while a total GSRS score (the mean score of all five symptom domains) was used to estimate the overall GI discomfort. Data regarding health-related quality of life was assessed through the EuroQol (EQ-5D) [17]. Psychological distress and the experience of stress were estimated through the validated Hospital Anxiety and Depression Scale (HADS) [18] and the Perceived Stress Scale (PSS) [19], respectively. (See Table A2 for more detailed information.) All questionnaires were completed before (during week −1) and at the end of intervention (week 6; day 40–42), as seen in the schematic overview of the randomised clinical trial (RCT) (Figure 1). All questionnaires were completed through a web-based survey with the exception of EQ-5D, which was completed using pen and paper.

Blood biomarkers. After an overnight fast, the participants were instructed not to exercise in the morning, before collection of blood samples. Blood samples were collected before study start (week 0) and after intervention at week 6, day 40–42. Plasma was separated using standard operating procedures used in the daily routine at the Örebro University Hospital. C-reactive protein (CRP), oxidative stress (hydrogen peroxide) levels and cytokine expression were assessed using established methodologies; see Appendix A for detailed description.

2.4. Monitoring of Compliance and Adverse Events

All participants were asked to return any remaining study product after the intervention. Adherence was measured by calculating all remaining sachets. Participants who did not take the supplement on more than two non-consecutive days or consecutive days were considered non-compliant (n = 0). All adverse events following administration of study product and/or indomethacin intake were monitored until the end of the study. No serious adverse events (e.g., events resulting in death, immediately life-threatening events, medically significant events for any reason, events requiring or prolonging hospitalisation or resulting in persistent or significant disability/incapacity) were reported during or after the study.

2.5. Statistical Analysis

Baseline characteristics are displayed as mean (SD) for numerical variables and were compared using independent t-tests and Fisher's exact test for categorical data. The median and interquartile range (IQR) was calculated for analysis of intestinal permeability (+/-indomethacin), inflammatory mediators, GSRS and HADS before and after intervention. An intention-to-treat (ITT) analysis was performed followed by a per-protocol analysis. Differences in intestinal permeability, gut microbiota composition (16S rRNA) and self-reported health after intervention with 12 g arabinoxylan, 12 g oat β-glucan or placebo were assessed using the non-parametric Mann-Whitney *U*-test for between-group analyses after subtraction of the baseline score. Gut microbiota composition before and after intervention was visualised by a Principal Coordinates Analysis (PCoA) based on Bray-Curtis distances between samples using packages vegan and labdsv in R. For assessment of differences within each intervention arm, Wilcoxon matched pair signed rank test was used. GI permeability data was not normally

distributed as assessed by Shapiro-Wilk test and visualisation in histograms. To further explore intestinal permeability in relation to GI symptoms Spearman correlation analysis was performed. In sub-analyses, data were stratified based on a GSRS score > 2, depicting mild to severe GI symptoms. In addition a relief in GI symptoms (a reduction by 0.5 score points) was assessed after intervention, as well as changes in gut microbiota composition. In addition, sub-analyses were performed in order to identify whether participants experiencing a relief in GI symptoms after intervention also displayed an improved intestinal barrier function, as assessed by a reduction in indomethacin-induced intestinal hyperpermeability after intervention.

Gut microbiota composition was analysed in the sub-group focusing on changes in bacteria known from the literature for their butyrate producing capability, as well as bacteria with known probiotic effects (Table A1). Missing values (GSRS, $n = 3$; HADS, $n = 3$; PSS, $n = 3$; EQ-5D, $n = 3$) were replaced with the arithmetic mean of the completed items in the questionnaire, in accordance with the instructions of the questionnaire. All analyses were performed blinded. All data were stored in a common database and statistically analysed using R version 3.0 (New Zeeland) and GraphPad Prism 5 Software (GraphPad Software Inc., La Jolla, CA, USA). Degree of significance was indicated for datasets obtained as follows: p-value < 0.05 (*), p-value < 0.01 (**), p-value < 0.001 (***) or ns (non-significant).

3. Results

3.1. Participant Flow and Baseline Characteristics

A total of 49 participants were included in the final analyses, corresponding to a treatment completion of 82% (17 arabinoxylan, 15 oat β-glucan, 17 placebo); dropouts and excluded participants can be viewed in Figure 3. The baseline characteristics did not differ significantly between the three intervention groups except for the medical category 'others', which contains several subcategories of diverse conditions. These types of conditions were present among 50% of the study participants in the placebo arm and among 15% in the two other arms (Table 2). The distribution of prescribed medications is shown in Table 2.

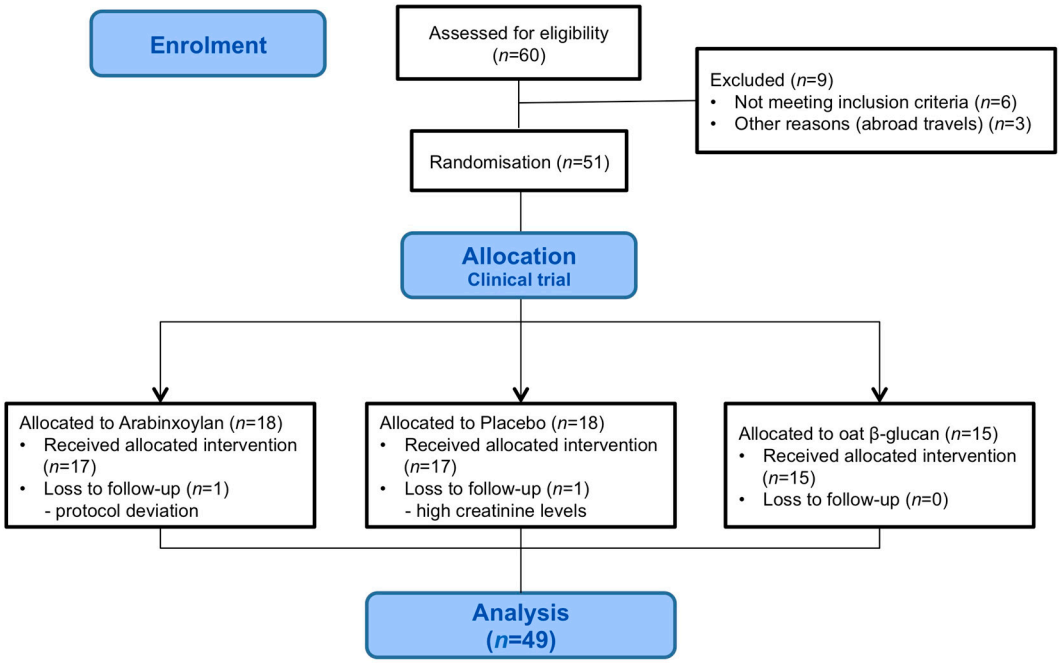

Figure 3. Flow chart showing the general participant flow throughout the study.

3.2. Primary Outcome

Assessment of Intestinal Permeability

Initial analysis confirmed that intake of indomethacin significantly increased gastroduodenal permeability ($p < 0.01$), small intestinal permeability ($p < 0.001$) and colonic permeability ($p < 0.01$) in the whole study group. However, when analysing baseline separately according to the intervention group, indomethacin induced an increased colonic permeability only in the arabinoxylan treatment arm ($p < 0.05$) while small intestinal permeability was significantly increased ($p < 0.05$) in all intervention arms (Table 3). Gastroduodenal permeability was not significantly affected by indomethacin. Intervention with dietary fibres was found not to strengthen the intestinal barrier against indomethacin-induced hyperpermeability, as no significant differences were observed in the two treatment groups when compared to placebo (Table 3). GSRS score (Table 4) was not found to correlate with increased baseline (before indomethacin challenge) intestinal permeability and indomethacin-induced hyperpermeability (data not shown), neither before nor after intervention. In accordance with this observation, stratification of data based on a GSRS score of 2 did not reveal any changes in intestinal permeability after intervention when compared to placebo.

Table 3. Intestinal permeability analysis of multi-sugar probes (μg/mL and ratios) before and after arabinoxylan (AX), placebo, and oat β-glucan (OBG) intervention, respectively, shown as median (IQR). No significant differences were found between the groups when compared to placebo.

	Pre-Intervention			After Intervention		
Study Arm	**Baseline**	**Indomethacin-Challenge**	***p*–Value**	**Baseline**	**Indomethacin-Challenge**	***p*–Value**
AX (*n* = 17)						
Gastroduodenal permeability [sucrose μg/mL]	3.37 (1.35–6.08)	3.86 (1.88–6.78)	ns	3.09 (1.42–8.91)	6.95 (2.67–13.04)	$p < 0.05$
Small intestinal permeability [L/R ratio]	0.038 (0.027–0.048)	0.056 (0.034–0.099)	$p < 0.05$	0.031 (0.020–0.056)	0.055 (0.045–0.125)	$p < 0.05$
Colonic Permeability [S/E ratio]	0.018 (0.009–0.027)	0.033 (0.024–0.04)	$p < 0.01$	0.015 (0.011–0.022)	0.029 (0.020–0.040)	$p < 0.01$
Placebo (*n* = 17)						
Gastroduodenal permeability [sucrose μg/mL]	1.69 (1.04–2.96)	2.26 (1.67–8.10)	ns	1.96 (0.77–6.53)	6.73 (2.03–11.99)	$p < 0.05$
Small intestinal permeability [L/R ratio]	0.029 (0.021–0.05)	0.05 (0.025–0.074)	$p < 0.01$	0.034 (0.019–0.049)	0.064 (0.028–0.098)	$p < 0.01$
Colonic permeability [S/E ratio]	0.023 (0.013–0.04)	0.028 (0.020–0.048)	ns	0.022 (0.017–0.030)	0.029 (0.017–0.046)	ns
OBG (*n* = 15)						
Gastroduodenal permeability [sucrose μg/mL]	2.22 (1.21–3.86)	2.79 (1.79–12.69)	ns	3.28 (1.23–9.18)	4.80 (1.35–13.05)	ns
Small intestinal permeability [L/R ratio]	0.027 (0.021–0.035)	0.056 (0.039–0.077)	$p < 0.001$	0.029 (0.018–0.040)	0.057 (0.042–0.090)	$p < 0.001$
Colonic permeability [S/E ratio]	0.016 (0.010–0.034)	0.029 (0.022–0.039)	ns	0.016 (0.008–0.05)	0.029 (0.014–0.038)	ns

Table 4. Questionnaire scores regarding gut health, psychological distress and stress at baseline and after intervention with arabinoxylan (AX), placebo, and oat β-glucan (OBG). Data presented as median (IQR).

	AX (*n* = 17) *			Placebo (*n* = 17)			OBG (*n* = 15)		
	Baseline	**End of Study**	***p*-value**	**Baseline**	**End of Study**	***p*-Value**	**Baseline**	**End of Study**	***p*-Value**
GSRS - Diarrha	1.5 (1.0–2.8)	1.3 (1.0–1.9)	0.336	1.3 (1.0–2.7)	1 (1.0–2.7)	0.516	1.3 (1.0–2.0)	1.3 (1.0–2.3)	0.45
GSRS - Constipation	1.3 (1.0–2.6)	1.2 (1.0–2.9)	0.242	1.3 (1.2–3.2)	1.3 (1.0–2.8)	0.824	1.3 (1.0–1.7)	1.0 (1.0–2.0)	>0.99
HADS	4 (2–5)	3 (1–5.5)	0.106	5.5 (3.3–9.5)	5.5 (2.3–8)	0.529	3 (2–5)	5 (1–7)	0.55
PSS	8 (4.3–12)	6.5 (4.3–8.8)	0.319	9.5 (6.3–14)	10 (3–14)	0.814	8 (4–12)	6 (3–9)	0.28
EuroQol VAS	85 (76–95)	85 (80–90)	0.621	83 (80–94)	90 (76–95)	0.762	90 (81–95)	90 (80–95)	0.36
EuroQol Index	0.86 (0.79–0.86)	0.86 (0.79–0.86)	0.875	0.86 (0.76–1)	0.80 (0.76–1)	0.594	0.93 (0.86–1)	1 (0.86–1)	0.83

GSRS – Gastrointestinal Symptoms Rating Scale; HADS – Hospital Anxiety and Depression Scale; PSS – Perceived Stress Scale; * 17 included in the group, though there are missing values for HADS ($n = 1$), EuroQol-VAS ($n = 1$) and EuroQol-index ($n = 2$), leaving categories with 16, 16 and 15 participants, respectively.

3.3. Secondary Outcomes

3.3.1. Assessment of Gut Microbiota Composition

By using the 48 labelling probes in the GA-map™ the gut microbiota composition was assessed. The initial ITT analysis showed no significant differences in the gut microbiota composition, i.e., beta diversity, before and after intervention, as visualised by a principal coordinates analysis in Figure 4, illustrating no separation of the three intervention groups after intervention. Neither did we observe any difference of Shannon indices (i.e., alpha diversity) at baseline and after intervention within the separate groups (data not shown).

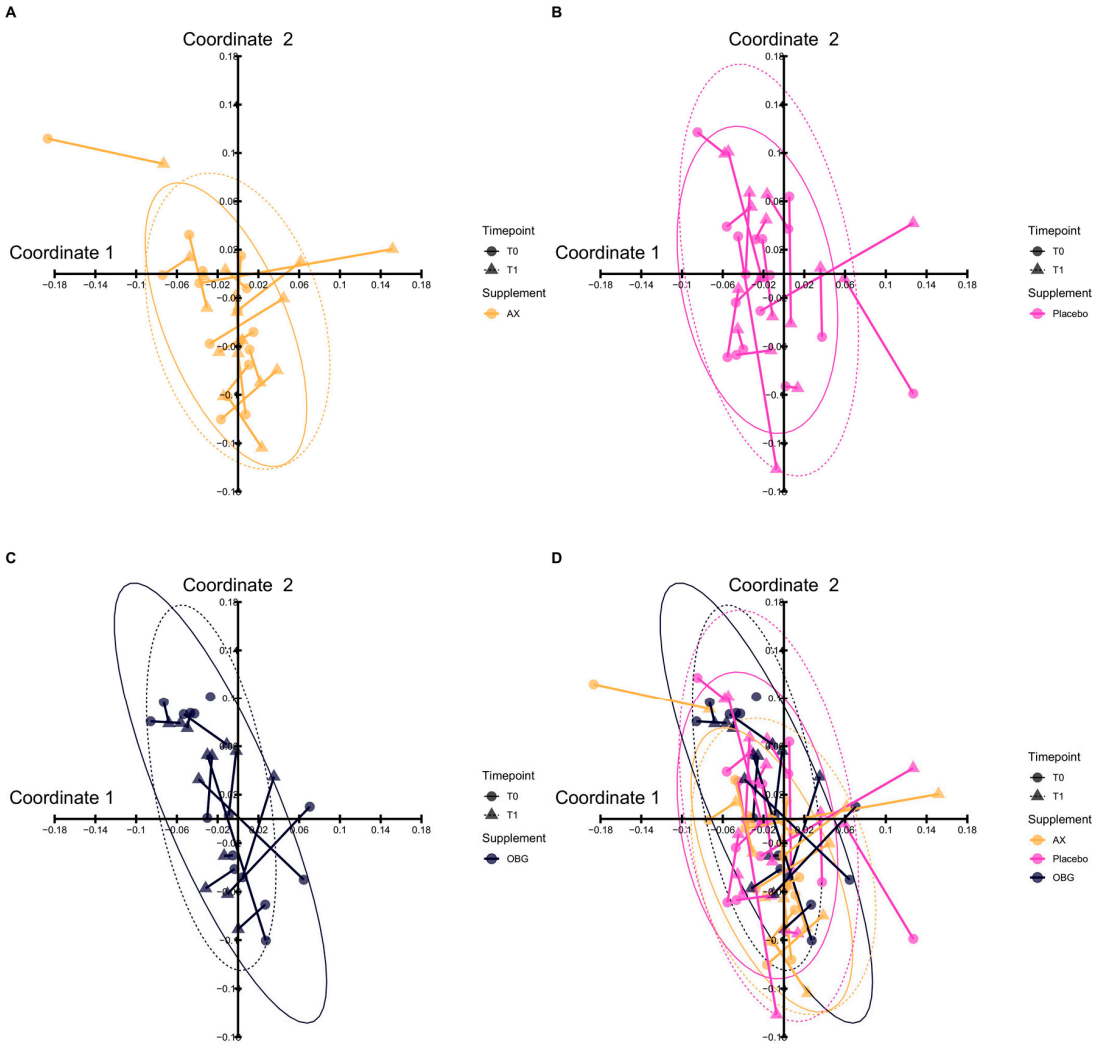

Figure 4. The composition of gut microbiota before and after intervention was visualized by Principal Coordinates Analysis (PCoA). The panels show gut microbial composition before and after intervention with (**A**) AX (Arabinoxylan) (**B**) Placebo and (**C**) OBG (Oat β-Glucan). In panel (**D**) an overlap of all interventions is displayed. The time points of sampling are indicated by shape: circle for T0, baseline and triangle for T1, end of study. To show the composition before and after intervention a line was drawn between samples from the same patient. One outlier per intervention group is not shown in the figure. As illustrated in panel (**D**), no separation of the groups could be observed after intervention.

Moreover, we did not observe any significant changes when investigating participants experiencing a relief in GI symptoms. The generation of butyrate is dependent on the fermentation process of dietary fibres. Hence, as the intake of fibres was low in the study population we specifically assessed

the abundance of 11 butyrate-producing bacteria (Table A1) before and after the dietary intervention. However, no significant changes could be observed, neither between the different dietary fibre intervention groups or when compared to placebo.

3.3.2. Evaluation of Gut Health, Wellbeing and Fibre Intake

No significant differences in GSRS, HADS, PSS or EQ-5D were identified before or after intervention when compared to placebo or within group analysis (Table 4). No differences were observed in any of the subdomains of the GSRS, illustrated in Table 4 by representable data from the two subdomains diarrhoea and constipation. Neither did stratification of data based on GSRS score ≥2 show any reduction in symptoms after intervention when compared to placebo. However, sub-analyses revealed a significant reduction in symptoms of diarrhoea (n = 6), from a GSRS score of 3.2, (IQR 2.23–3.75) to 1.5 (IQR 1.0–2.10) after intervention with arabinoxylan ($p < 0.05$), which failed to reach significance when compared to placebo. FFQ analysis revealed that 85% of those finishing the RCT had an insufficient fibre intake (g/day) at study start, accounting only for a median of 64.6% (IQR 50.6–83.8%) of the recommended intake (25.30 g/day) according to the Nordic Nutrition Recommendations (NNR).

3.3.3. Biomarkers of Inflammation and Oxidative Stress

For all three groups the CRP levels were found to be within the normal range (<2 mg/L) and were not included in further analysis. Similarly, the levels of cytokines and oxidative stress were found to be close to the lower limit for detection (Tables A3 and A4). Samples below detection limit were not included in the analysis.

4. Discussion

The present study focused primarily on the effects of dietary fibres on intestinal barrier function in elderly individuals, with the specific aim to investigate if daily supplementation with arabinoxylan or oat β-glucan could strengthen the intestinal barrier against acute NSAID-induced intestinal hyperpermeability compared to placebo. Before intervention, we observed that indomethacin increased small bowel permeability in all three-intervention arms, while increased colonic permeability was only observed in one of the arms and gastroduodenal permeability in none of the arms. This is in line with previous studies that have shown that indomethacin has a more profound effect on the small intestine [3].

The inability of the dietary fibres to attenuate indomethacin-induced hyperpermeability might be due to the fact that dietary fibres are mainly fermented into butyrate in the colon and might primarily have their effect there [5–7]. Although indomethacin significantly induced colonic hyperpermeability when pooling the pre-intervention colonic permeability data (n = 49), the main effect was seen on small intestinal permeability in the different intervention arms. This is in line with previous research [3,20] but the absence of an effect might be due to the low power in each arm. A cross-over design would have increased the power and reduced the inter-individual variation; however, these types of studies are, according to our experience, more difficult for elderly participants to continue and finish.

Neither did we observe any changes in baseline GI permeability before and after intervention as compared to placebo. This is in line with a recent study that showed no significant differences from baseline intestinal permeability after six weeks of arabinoxylan supplementation (7.5 g/day vs. 15 g/day) among lean and obese individuals [12]. Similarly, the starch pectin was found not to affect the intestinal barrier function in healthy adults and elderly individuals [21].

Furthermore, it is important to consider that only subtle treatment effects might be expected from prebiotics. The indomethacin dosage used in the present study was the standard dose for inducing small intestinal hyperpermeability in clinical studies [22–24]. However, indomethacin is currently not used as treatment in Sweden for any disease, but is widely used in several model systems for epithelial injury.

The low intake of fibres among the elderly further makes the investigation of supplementation with dietary fibres important. However, according to our results supplementation with arabinoxylan or oat β-glucan do not alone have the possibility to affect baseline intestinal permeability, acute induced intestinal permeability or induce changes of the gut microbiota composition in elderly individuals. This is in contrast to previous studies that have shown that arabinoxylan and β-glucan supplementation can induce changes in the gut microbiota and increase the level of butyrate production, in human populations and animal models, respectively [9,10,25,26]. The absence of a significant effect on the gut microbiota after dietary supplementation in this study might be due to the fact that other fibres or a combination of fibres and probiotics might be more suitable for elderly individuals. Even though ageing is associated with a loss of *Faecalibacterium prausnitzii* [27] the non-observed effect might be due to geographical location. It has previously been shown that elderly and adult individuals in Sweden might have a higher abundance of the butyrate producing bacterium *Faecalibacterium prausnitzii* compared to other European countries [28]. Moreover, the gut microbiota composition in the present study was assessed by targeting the 16S rRNA region using 48 labelling probes. Thus, possible differences in the remaining gut microbiota after intervention, including levels of antibiotic resistant bacteria, were not investigated. To thoroughly understand the impact of arabinoxylan and oat-β-glucan on the gut microbiota composition, more advanced technology such as next-generation sequencing needs to be used.

The current study has some limitations. First of all, the collection of samples was not performed simultaneously on follow up out of concern for the elderly participants, and the collection was instead performed step wise; this could potentially have affected the outcome of the study although all samples were collected during a period of ten days for all study participants. Secondly, the study population was comprised of a heterogenous group, with an age range of 65-84 years, experiencing no GI symptoms, hence, potentially making it difficult to observe changes in gut microbiota and GI symptoms. It is therefore likely that the study design would have gained from a larger sample size and/or a more homogenous population regarding age or distribution of GI symptoms. Future studies should validate these results in a larger cohort, where samples are obtained within a shorter identical period in a more homogenous study population of elderly individuals. Moreover, the challenge of indomethacin might be too strong in relation to the modest effects on the intestinal barrier one might expect from intervention with dietary fibres. Hence, future studies should investigate if intake of dietary fibres can counteract the negative effects of long-term intake of milder NSAIDs, such as ibuprofen, amongst the elderly.

5. Conclusions

Despite the negative outcome of the study, our results highlight the importance of investigating dietary fibre supplementation in the elderly. Future clinical studies are needed in order to identify specific fibres able to strengthen the intestinal barrier in the elderly and counteract negative side-effects that are often associated with not only intake of NSAIDs, but also poly-pharmacy in the elderly. To identify cost-effective strategies is particularly important given the growing elderly population.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

GI: Gastrointestinal symptoms, NSAIDs: Non-steroid anti-inflammatory drugs, FFQ: Food frequency questionnaire, PCR: Polymerase chain reaction, GSRS: Gastrointestinal symptoms rating scale, EQ-5D: EuroQol, HADS: Hospital anxiety and depression scale, PSS: Perceived stress scale, CRP: C-reactive protein, IQR: Interquartile range, SD: Standard deviation, ITT: Intention to treat, PCoA: Principal Coordinates Analysis, AX: arabinoxylan, OBG: oat β-glucan.

Appendix A.

Appendix A.1. Measurement of Sugar Probes in Urine Samples

Sample preparation and analysis. 1 mL of urine was centrifuged at 21,000 RCF for 25 min at a temperature of 4 °C. The supernatant was collected and 50 µL was transferred to LC vials to which 5 µL of 13C labelled lactulose and sucrose internal standard (Sigma Aldrich, MO, USA) was added. The urine aliquots were diluted to a volume of 1 mL with a composition of 80:20 acetonitrile: water. Before analysis, all samples were vortexed for 10 seconds and centrifuged for 15 min at 8000 RCF. Analysis was performed on an Acquity UPLC coupled to a Quattro Premier XE UPLC–MS/MS system (Waters Corporation, Milford, USA) with an atmospheric electrospray interface operating in negative ion mode. Analytes were separated on an Acquity BEH Amide column (1.7 µm, 2.1 × 100 mm) (Waters Corporation, Milford, USA). Column temperature was 50 °C, injection volume 10 µL, and flow rate 0.17 mL/min. An isocratic method was used with a mobile phase of 0.1% NH4OH in acetonitrile and water (70:30). Blanks and external standards were frequently injected during the analysis to control for carry over and monitor instrument stability. Mass-analysis was performed in the multiple reaction monitoring (MRM) mode by monitoring two product ions for each sugar analysed (341.16 > 100.80 and 341.16 > 160.80 for lactulose, 163.20 > 59.00 and 163.20 > 103.00 for rhamnose). Lactulose and sucrose were quantified using isotope dilution. Sucralose, erythriol, and rahmnose were quantified using 3-point standard addition curves, since no labelled standard was available for these analytes at the time of analysis. An in-house control sample was included in each batch.

Appendix A.2. Measurement of Immune and Oxidative Markers in Blood

Systemic inflammation. C-reactive protein (CRP) is an acute-phase plasma protein and a marker of inflammation. CRP levels were assessed by the high-sensitivity immunoturbidimetric assay, CardioPhaseTM and analysed on the ADVIA 1800 chemistry system (SIEMENS Healthcare Diagnostics Inc., NY, USA). A normal CRP was defined as ≤2 mg/L according to clinical practice.

Oxidative stress. Oxidative stress was estimated by the validated FORT (Free Oxygen Radicals Test) colorimetric assay (Callegari, Parma, Italy), as previously described (5). Results are expressed as mmol/L H_2O_2.

Quantification of cytokines. Cytokines (IL-1β, IL-6, IL-8, IL-10, IL-2, IL-12p70, IFN-γ and TNF-α) were quantified using the Bioplex Pro™ Assay (BioRad, The Netherlands) according to manufacturer's instructions. Samples were measured in the MagPix reader (BioRad) using MAGPIX xPONENT software (BioRad) and the concentration of the analyses was assessed by the Bio-Plex Manager version 5.0 (BioRad).

Appendix B.

Table A1. List of the bacterial targets of the 48 labelling probes in the GA-map^{TM}.

Bacterial Probe	Phylum	Class	References *,#
Roseburia intestinalis/ hominis *	Firmicutes	Clostridia	Flint HJ et al. 2002
Bacteroides vulgatus	Bacteroidetes	Bacteroidia	
Streptococcus agalactiae/ Eubacterium rectale	Firmicutes	Bacilli/Clostridia	
Firmicutes/Tenericutes/ Bacteroidetes species	Firmicutes/Tenericutes /Bacteroidetes species		
Firmicutes (Bacilli)	Firmicutes	Bacilli	
Enterococcus, Listeria	Firmicutes	Bacilli	

Table A1. *Cont.*

Bacterial Probe	Phylum	Class	References */#
Butyrivibrio crossotus *	Firmicutes	Clostridia	Marius Vita, André Karch & Dietmar H. Pieper. 2017
Prevotella intermedia *	Bacterioidetes	Bacteroida	R. Krajmalnik-Brown et al. 2017
Firmicutes (Negativicutes)	Firmicutes	Negativicutes	
Bacteroides sp 4 3 47FAA/vulgatus	Bacteroidetes	Bacteroidia	
Veillonella, Helicobacter, Clostridia	Firmicutes	Negativicutes/ Epsilonproteobacteria/ Clostridia	
Enterobacter	Proteobacteria	Gamma Proteobacteria	
Ruminococcus champanellensis	Firmicutes	Clostridia	
Proteobacteria	Proteobacteria		
Bacteroides caccae	Bacteroidetes	Bacteroidia	
Clostridium leptum *	Firmicutes	Clostridia	Watada H. 2017
Ruminococcus bromii	Firmicutes	Clostridia	
Subdoligranulum variabile *	Firmicutes	Clostridia	Suzuki et al. 2014
Bacteroides pectinophilus	Bacteroidetes	Bacteroidia	
Coprococcus comes *	Firmicutes	Clostridia	De Vuyst et al. 2016
Parabacteroides johnsonii	Bacteroidetes	Bacteroidia	
Firmicutes (Clostridia) *	Firmicutes	Clostridia	Marius Vita, André Karch & Dietmar H. Pieper. 2017
Akkermansia municiphila #	Verrucomicrobia	Verrucomicrobiae	Cani & de Vos. 2017
Bacterioides fragilis #	Bacteroidetes	Bacteroidia	Zhi et al. 2018
Shigella/Echerichia	Proteobacteria	Gammaproteobacteria	
Prevotella nigrescens	Bacteroidetes	Bacteroidia	
Dorea longicatena	Firmicutes	Clostridia	
Bacteroides sp 2_2_4/ovatus/sp D1 #	Bacteroidetes	Bacteroidia	Chen W et al. 2019
Eubacterium siraeum #	Firmicutes	Clostridia	Putigani et al. 2017
Alistipes putredinis/onderdonki	Bacteroidetes	Bacteroidia	
Megasphaera micronuciformis/ Dialister invisus	Firmicutes	Negativicutes	
Ruminococcus bicirculans	Firmicutes	Clostridia	
Eubacterium ventriosum *	Firmicutes	Clostridia	Kerckhof & Van de Wiele. 2012
Actinobacteria	Actinobacteria	Actinobacteria	
Enterobacter/Aeromonas	Proteobacteria	Gamma Proteobacteria	
Faecalibacterium *	Firmicutes	Clostridia	Flint et al. 2002
Providencia/Klebsiella/Pantoera	Proteobacteria	Gamma proteobacteria	
Lactobacillus #	Firmicutes	Bacilli	Rastall et al. 2019
Collinsella aerofaciens	Actinobacteria	Actinobacteria	
Lachnospiraceae *	Firmicutes	Clostridia	Marius Vita, André Karch & Dietmar H. Pieper. 2017
Prevotella bivia/amnii	Bacteroidetes	Bacterioa	
Bacterioides acidofaciens	Bacterioidetes	Bacterioidetes	
Desulfitobacterium/Blautia	Firmicutes	Clostridia	
Roseburia inulinivorans *	Firmicutes	Clostridia	Marius Vita, André Karch & Dietmar H. Pieper. 2017
Streptococcus salivarius subsp.thermophilus #	Firmicutes	Bacilli	Gibson et al. 2003
Bacteroides / Prevotella	Bacteroidetes	Bacteroidia	
Ruminococcus gnavus	Firmicutes	Clostridia	
Bacteroides dorei/sp. D4/sp. 9_1_42FAA	Bacteroidetes	Bacteroidia	

* Butyrate producing bacteria; # Probiotic bacteria.

Table A2. Overview of questionnaires.

Questionnaire	Variable	Scoring	Cut-off Values
Gastrointestinal Symptoms Rating Scale (GSRS)	Measure of gastrointestinal health, by five symptom domains: diarrhoea, indigestion, constipation, abdominal pain and reflux.	Fifteen questions rated on a 7-point scale using fixed respond alternatives.	0-2, no symptoms >2-3, mild symptoms >3-4, moderate symptoms >5, severe symptoms
Hospital Anxiety and Depression Scale (HADS)	Measure of psychological distress divided into two subscales: depression and anxiety.	Fourteen questions (7 questions per subscale) rated on a 4-point scale using fixed respond alternatives.	>7, signs of depression and/or anxiety
Perceived Stress Scale (PSS)	Measure of perceived stress, providing a total score.	Ten questions rated on a 5-point scale using fixed respond alternatives.	None available, the total score was used to estimate the total stress level
EQ-5D-5L: EQ-Index	Health-related quality of life. Mobility, self-care, usual activities, pain/discomfort and anxiety/depression	5-point scale using fixed respond alternatives.	None available
EQ-VAS	Visual analogue scale (VAS) recording subjective wellbeing	Respondents specify their subjective view on their wellbeing today on a scale from 0 (worst) to 100 (best).	

All questionnaires were completed online as a web survey with the exception of EQ-5D, which was completed using pen and paper.

Table A3. Expression of inflammatory markers analysed before and after intervention with arabinoxylan (AX), placebo, and oat β-glucan (OBG), displayed as median (pg/mL) and interquartile range (IQR).

	AX (*n* = 16)			**Placebo (*n* = 17)**			**OBG (*n* = 15)**		
	Baseline	**End**	***p*-Value**	**Baseline**	**End**	***p*-Value**	**Baseline**	**End**	***p*-Value**
IFN-y	1.0 (0.0–4.7)	0.0 (0.0–7.8)	0.81	9.6 (0.0–44.3)	4.7 (0.0–39.6)	0.26	0.5 (0.0–13.0)	0.0 (0.0–17.7)	0.84
IL-10	4.3 (3.4–6.2)	4.5 (1.0–6.2)	0.75	4.4 (0.0–5.9)	5.0 (0.8–6.4)	0.25	3.9 (1.1–5.6)	4.9 (3.7–7.6)	0.32
IL-12p70	0.1 (0.0–0.5)	0.5 (0.0–1.2)	0.29	0.3 (0.0–0.5)	0.2 (0.0–0.5)	0.51	0.5 (0.1–0.8)	0.5 (0.0–0.5)	0.71
IL-1b	0.0	0.0	>0.99	0.0 (0.0–0.6)	0.0 (0.0–0.5)	0.26	0.0	0.0	0.75
IL-2	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-
IL-6	1.3 (0.1–3.3)	1.4 (1.0–2.7)	0.98	2.1 (1.0–4.0)	1.4 (0.7–4.4)	0.94	1.4 (0.9–3.3)	1.5 (0.2–2.9)	0.76
IL-8	2.3 (1.4–3.7)	4.0 (1.7–6.8)	0.19	1.8 (1.3–3.6)	2.8 (1.7–4.0)	0.37	2.9 (1.6–5.9)	2.0 (1.5–4.0)	0.34
TNF-a	0.0 (0.0–1.9)	0.0 (0.0–2.8)	0.12	3.1 (0.0–10.9)	0.0 (0.0–13.0)	0.76	0.0 (0.0–1.9)	0.0	-
CRP	0.7 (0.4–2.7)	0.4 (0.2–2.3)	0.26	0.6 (0.4–1.1)	0.7 (0.3–1.2)	0.78	0.8 (0.2–1.9)	0.4 (0.2–2.4)	0.12

Table A4. The levels of reactive oxygen species (ROS), H_2O_2 mmol/L, were assessed in blood plasma collected before and after intervention with arabinoxylan (AX), placebo, and oat β-glucan (OBG). Data is shown with/without stratification below or above the Gastrointestinal Systems Rating Scale (GSRS) score 2. Results expressed as median and interquartile range (IQR).

Intervention Group	Baseline	End	*p*-Value
AX (*n* = 13)	2.01 (1.60–2.20)	1.7 (1.52–2.14)	0.106
GSRS diarrhoea /constipation score >2 (*n* = 5)	1.72 (1.52–2.20)	1.7 (1.52–1.96)	0.188
GSRS diarrhoea/constipation score <2 (*n* = 8)	2.07 (1.66–2.21)	1.77 (1.39–2.29)	0.383
Placebo (*n* = 12)	1.94 (1.84–2.19)	1.89 (1.71–2.03)	0.034 *
GSRS diarrhoea /constipation score >2 (*n* = 5)	2.18 (1.89–2.22)	2 (1.81–2.16)	0.437
GSRS diarrhoea /constipation score <2 (*n* = 7)	1.9 (1.8–2.0)	1.73 (1.54–2.0)	0.03 *
OBG (*n* = 13)	2.04 (1.87–2.15)	1.76 (1.57–1.99)	0.0005 ***
GSRS diarrhoea /constipation score >2 (*n* = 5)	2.0 (1.87–2.34)	1.8 (1.57–2.32)	0.125
GSRS diarrhoea /constipation score <2 (*n* = 8)	2.08 (1.67–2.17)	1.75 (1.41–1.93)	0.008 **

- H_2O_2 mmol/L <1.75 indicates no oxidative stress; - H_2O_2 mmol/L 1.75-2.35 indicates intermediate oxidative stress; - H_2O_2 mmol/L between 2.36-3.05 indicates oxidative stress; - H_2O_2 mmol/L 3.05< indicates strong oxidative stress; * = *p*-value < 0.05, ** = *p*-value < 0.01, *** = *p*-value < 0.001.

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ORIGINAL ARTICLE

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Human milk oligosaccharide supplementation in irritable bowel syndrome patients: A parallel, randomized, double-blind, placebo-controlled study

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Abstract

Objectives: Human milk oligosaccharides safely and beneficially impact bifidobacteria abundance in healthy adults, while their effects in patients with irritable bowel syndrome (IBS) are unknown. Hence, we aimed to determine the dose of 4:1 mix of 2'-O-fucosyllactose and Lacto-N-neotetraose (2'FL/LNnT) that increases fecal bifidobacteria abundance without aggravating overall gastrointestinal symptoms in IBS patients in a randomized, double-blind, controlled study. Additionally, the impact of 2'FL/LNnT on the fecal bacterial profile was assessed.

Methods: Irritable bowel syndrome patients diagnosed according to the Rome IV criteria received placebo (glucose), or 5 g or 10 g 2'FL/LNnT for 4 weeks followed by a four-week follow-up period. Gastrointestinal Symptom Rating Scale-IBS was used to assess gastrointestinal symptom severity; fecal microbiota composition was evaluated by GA-map™ Dysbiosis Test.

Results: Of the included 60 patients, two (one placebo and one 10 g) discontinued prematurely. Fecal bifidobacteria abundance was increased at week 4, but not at week 8, in the 10 g group compared to the other groups. Severity of overall or individual gastrointestinal symptoms did not differ between the groups at week 4 or 8, and no symptom deterioration was seen in any of the groups. The 10 g dose influenced overall fecal microbiota composition, and responders—defined as bifidobacteria increase ≥50%—could be discriminated from non-responders based on fecal microbiota modulation.

Conclusions: The 10 g dose of 2'FL/LNnT induced an increase in the beneficial *Bifidobacterium* spp. without aggravating gastrointestinal symptoms in patients with IBS. This approach may be worthwhile to modulate gut microbiota of IBS patients toward a healthier profile.

Lena Öhman and Magnus Simrén are joint senior authors.

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KEYWORDS

2'-O-fucosyllactose, *Bifidobacterium* spp., human milk oligosaccharides, irritable bowel syndrome, Lacto-N-neotetraose

1 | INTRODUCTION

The pathophysiology of irritable bowel syndrome (IBS), a functional bowel disorder characterized by altered bowel habits and abdominal pain, remains ambiguous.[1] However, subgroups of IBS patients show an unbalanced intestinal microbiota profile[2] associated with severity of symptoms.[3,4] Moreover, a recent systematic review of gut microbiota in IBS described a decrease in the genus *Bifidobacterium* in some patients.[5] Potentially, the gut microbiota composition may be modulated by diet, prebiotics, probiotics, synbiotics, and even non-absorbable antibiotics.[6] To date, only a few intervention studies have explored the effect of prebiotics, non-digestible compounds that stimulate specific bacterial growth, in IBS patients, with diverse study outcomes. Trans-galactooligosaccharides have been shown to promote bifidobacteria growth and alleviate gastrointestinal (GI) symptoms[7] and short-chain fructo-oligosaccharides improved digestive comfort.[8] In contrast, another study demonstrated that short-chain fructo-oligosaccharides had no therapeutic value for IBS patients,[9] and a recent meta-analysis of 11 randomized controlled studies concluded that prebiotics did not improve GI symptoms but increased bifidobacteria.[10]

Human milk oligosaccharides (HMO) are unique, complex glycans found in high concentrations (5 - 25 g/L HMO) in human breast milk.[11] They are selective substrates for specific intestinal bacteria, protect against infection by blocking pathogen attachment to epithelial cells, promote immunomodulatory activity, and improve gut barrier function.[12] The most abundant structural classes are fucosylated HMO (35%-50%) and neutral core HMO (42%-55%). 2'-O-Fucosyllactose (2'FL) and Lacto-N-neotetraose (LNnT), respectively, are major component of each class.[13,14] HMO reach the colon intact[15,16] and undergo fermentation by specific bacteria, such as bifidobacteria.[17,18] A 2:1 mix of 2'FL and LNnT has been demonstrated to influence the fecal microbiota composition, including an increase in bifidobacteria in infants.[19] Furthermore, supplementation with either 2'FL or LNnT alone or in a mix was safe and promoted fecal bifidobacteria in healthy adults.[20] However, there are no published studies of the effects of HMO in IBS patients.

We hypothesized that a 4:1 mix of 2'FL/LNnT will be well tolerated and able to beneficially modulate gut microbiota in IBS patients, in particular inducing an increase in *Bifidobacterium* spp. abundance. Hence, we aimed to determine the dose of a 4:1 mix of 2'FL/LNnT which increases fecal bifidobacteria abundance, without negatively influencing GI symptoms. Additionally, we assessed the effects on other IBS-related symptoms and on the general fecal microbiota profile.

Key Points

- Supplementation with a human milk oligosaccharides (HMO) mix of 2'-O-fucosyllactose and lacto-N-neotetraose (2'FL/LNnT) promotes fecal Bifidobacterium spp. and is well tolerated in healthy adults. However, the effects in patients with irritable bowel syndrome (IBS) have not been assessed.
- The 10g dose of 2'FL/LNnT influences overall fecal microbiota composition, specifically induces growth of bifidobacteria, without aggravating gastrointestinal symptoms in IBS patients after a 4-week intervention.
- HMO might be favorable in order to restore altered gut microbiota in IBS.

2 | MATERIAL AND METHODS

2.1 | Study design and population

This was a phase II, parallel, double-blind, randomized, placebo-controlled study in adult male and female IBS patients (n = 61 at randomization). The patients were randomized (1:1:1) into three groups, consuming either active product (two groups) or a placebo product (one group) for four weeks with a four-week follow-up period after the end of the intervention. The design of the study is graphically presented in Figure 1.

The study population comprised patients (18-75 years of age) with IBS symptoms of at least moderate severity (IBS Symptom Severity Scale (IBS-SSS) score ≥175)[21] and diagnosed according to the Rome IV criteria[22] (See Appendix S1 for further description of the main selection criteria). The patients were characterized based on the predominant bowel habit: IBS with diarrhea (IBS-D), IBS with constipation (IBS-C), or IBS with mixed bowel habits (IBS-M) (See Appendix S2.1). Patients were recruited from the outpatient clinic specialized in functional GI disorders at Sahlgrenska University Hospital (Gothenburg, Sweden) or by advertising in the local newspaper. The study followed the principles of Declaration of Helsinki II and was approved by the Regional Ethical Review Board in Gothenburg (Reg. No. 548-16). The study was registered at www.ClinicalTrials.gov (NCT02875847). Written informed consent was obtained from all patients at the screening visit before any study procedures. No major amendments were made after study commencement. The study was funded by Glycom A/S (Hørsholm, Denmark).

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2.2 | Study intervention

Patients were randomized to receive one of the three different interventions for 4 weeks; placebo, 5 g or 10 g doses of 2'FL/LNnT (1:1:1 ratio) stratified on IBS subtypes (IBS-C, IBS-D or IBS-M), using a web-based program developed by Gothia Forum (Gothenburg, Sweden). The active product, a mix of 2'FL/LNnT in a 4:1 mass ratio, was supplied by Glycom A/S and provided as a 5 g or 10 g white, powder in single-serve sachets and distributed in boxes containing 30 sachets, packaged by HB Medical (Hørsholm, Denmark). As placebo, sachets containing 5 g of powdered glucose (Dextropur, Dextro Energy GmbH & Co.) were provided. Both, active product and placebo were similar with regard to appearance, smell, and taste (sweet). All packages were identical except from a unique ID-number. Patients were instructed to dissolve the sachet contents in a minimum of 100 mL of liquid and consume it daily with breakfast. Furthermore, all subjects were advised to keep to their usual diet and medication regimen throughout the study.

After inclusion, patients visited the clinic three times. At visits, the patients completed validated clinical questionnaires electronically to assess the severity of GI and psychological symptoms, including the Gastrointestinal Symptom Rating Scale for IBS (GSRS-IBS),[23] the IBS-SSS,[21] a stool diary based on the Bristol Stool Form scale,[24] and the Hospital Anxiety and Depression Scale (HADS)[25] (See Appendix S2 for more detailed description). Bodyweight (kg), height (cm), adverse events, changes in medication or diet, and compliance were recorded. Patients taking the full dose for ≥24 days during the 28-day supplementation period were considered fully compliant. A physical examination was done by a study physician. Study subjects, investigators, and sponsor were all blinded to treatment allocation until the end of the study.

The patients received equipment for fecal sampling including cooling kits. Samples were collected and kept at −20°C freezers maximum 4 days prior to a visit (visits 2, 3, and 4). Fecal samples were stored at −80°C after each visit.

2.3 | Bifidobacteria and microbiota profiling

The fecal samples were analyzed using a commercially available genome-based microbiota test (GA-map™ Dysbiosis Test, Genetic Analysis AS). Briefly, fecal samples were homogenized prior to mechanical disruption of bacterial cells, total bacterial genomic DNA isolated with magnetic beads, and hypervariable regions V3-V9 amplified by 16S rRNA polymerase chain reaction (PCR), using fifty-four probes, validated to be GI disorder-specific, targeting more than 300 bacterial strains.[26] Probe intensity signal correspondent to fecal bacterial abundance was detected and measured by BioCode 1000A 128-Plex Analyzer (Applied BioCode).

2.4 | Data analyses; primary, secondary, and exploratory endpoints

The primary endpoint was to determine the dose of 2'FL/LNnT that increases *Bifidobacterium* spp. abundance without aggravating GI symptoms, measured by GSRS-IBS. As secondary efficacy endpoints, IBS severity, measured by IBS-SSS, bowel habits (stool consistency), and anxiety and depression were assessed. Moreover, the effect on fecal microbiota and the proportion of responders, defined as a patient with a bifidobacteria abundance increase ≥50% at the end of the intervention period, relative to baseline were explored. All eligible and randomized patients were included in the analysis of the comparisons of clinical questionnaires (intention-to-treat analysis, ITT; n = 60). Statistical analyses were chosen based on normality of distribution determined by boxplots. For the effect on the fecal microbiota profile, patients who completed the intervention were included (per-protocol analysis, PP; n = 58). All analyses were performed after unblinding.

2.5 | Statistical analyses

This study was explorative, and no formal sample size calculation was performed. Instead, it was based on the sample size of a previous study in healthy adults which showed microbiota modulation after supplementation with HMO.[20] For this study, the sample size was slightly increased to address the inherent background noise of microbiota results and the statistical analysis significance. Natural logarithmic transformation was used for the microbiota data to obtain a more homogeneous variance. All tests used an alpha of .05 as cutoff for significance and two-sided confidence intervals.

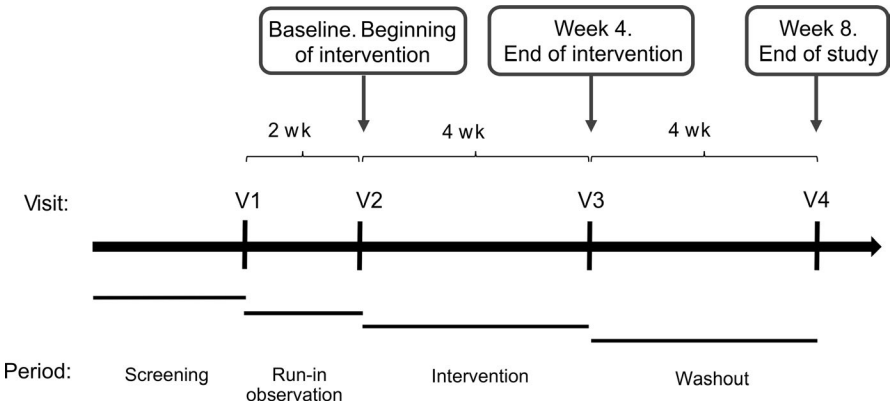

FIGURE 1 Overall study design. V1, Visit 1 (Screening visit); V2, Visit 2 (Baseline); V3, Visit 3 (Week 4. End of intervention); V4, Visit 4 (Week 8. End of study)

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Each primary endpoint of logarithmic *Bifidobacterium* spp. abundance and global GSRS-IBS was analyzed using restricted maximum-likelihood mixed modeling for repeated measurements (SAS, Proc Mixed) with SAS version 9.4, SAS Institute Inc. The model included fixed categorical effects of group dose, visit, and group dose-by-visit interaction. A compound symmetry covariance structure was used to model the within-subject errors. Baseline (visit 2) was included as continued fixed covariate for between-group comparisons and in the categorical effect of visit for within-group comparisons. For the exploratory endpoint, the microbiota profile was assessed by multivariate analysis by using SIMCA® software (version 15.0.2, MKS Umetrics AB). Orthogonal partial least squares-discriminant analyses (OPLS-DA) were performed within placebo, 5 g, and 10 g 2'FL/LNnT groups to identify microbiota profile differences between baseline and week 4 and fold change (week 4/baseline) of responders and non-responders. Additional information about statistical methodology can be found in Appendix S3.

Demographic information at baseline was analyzed based on normality of distribution and compared between the groups. Categorical variables were analyzed with chi-square test, while continuous variables were analyzed by using one-way ANOVA with Bonferroni's correction or Kruskal-Wallis test.

3 | RESULTS

3.1 | Demographics and clinical characteristics

In total, 73 IBS patients were screened for eligibility between January 2017 and April 2018, and of these, 61 patients (41 women and 20 men) were randomized into one of the three intervention groups:

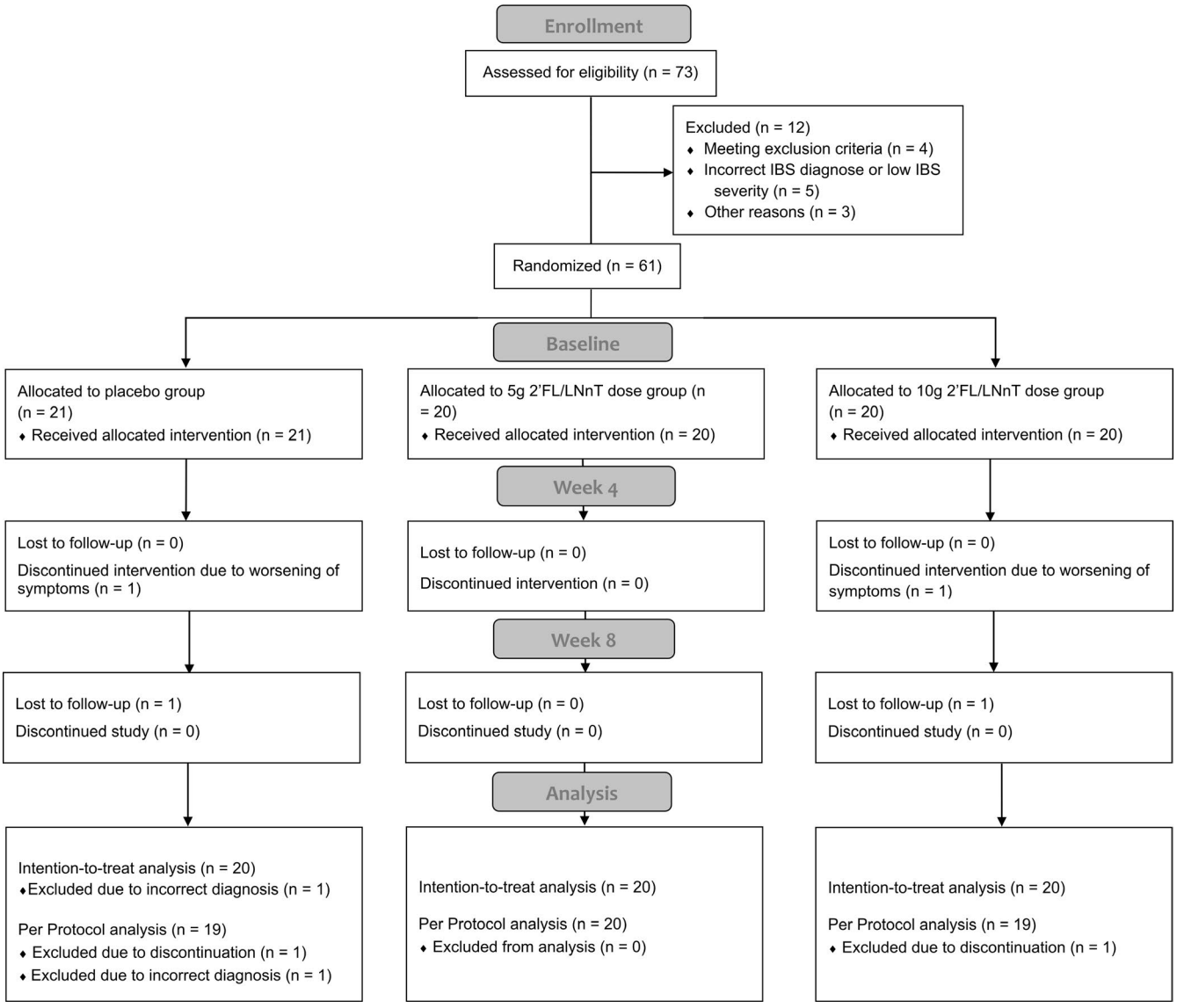

FIGURE 2 CONSORT flow diagram. Patient disposition showing the number of screened, randomized, and completed subjects

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placebo (n = 21), 5 g (n = 20), and 10 g (n = 20) 2'FL/LNnT. Fifty-nine out of 61 patients completed the study to the follow-up visit (week 8). There were no major changes in diet. Fifty-eight patients reported consumption of the study product for ≥24 of the 28 days (Table S1). One subject did not fulfill the compliance criteria but took full dose for 23 days and was compliant to all other study procedures, and was therefore included in all the analysis. Two patients, one from the placebo group and one from the 10 g 2'FL/LNnT group, discontinued prematurely after 2 weeks of intervention due to increased IBS symptoms. Additionally, after completion of the intervention, one patient in the placebo group was diagnosed with Crohn's disease. This patient was excluded from the ITT and PP analyses (Figure 2). No other major protocol violations were reported. Therefore, 40 women and 20 men (mean age 45 (range 19-73) years) of all IBS subtypes (14 IBS-C, 26 IBS-D, 20 IBS-M) were included in the ITT analysis (Table 1). The exploratory analysis (PP analysis) excluded the patients who discontinued the intervention (n = 58). Sex, age, body mass index, and IBS subtype classification did not differ between the three groups at baseline. However, patients in the 5 g 2'FL/LNnT group demonstrated a lower GSRS-IBS total score at baseline compared to the placebo and 10 g 2'FL/LNnT groups (P = .03) (Table 1).

3.2 | Dose effect of 2'FL/LNnT on *Bifidobacterium* spp

The 10 g 2'FL/LNnT group presented with a higher abundance of fecal bifidobacteria after four weeks intervention as compared to placebo

TABLE 1 Demographic data of the randomized patients at baseline

	Placebo (n = 20)	5 g 2'FL/LNnT (n = 20)	10 g 2'FL/LNnT (n = 20)	*P* value
Sex (Female: Male)	14:6	11:9	15:5	.38
Age, y[a]	46 (21-71)	42 (19-67)	46 (26-73)	.55
Body mass index, kg/m^2[b]	24.9 (23.3-26.5)	24.0 (21.7-26.3)	24.3 (22.6-26.0)	.38
IBS subtype, number of patients				
IBS-C	5	5	4	.93
IBS-D	8	9	9	.95
IBS-M	7	6	7	.95
GSRS-IBS total score[c]	50.2 (10.1)	45.2 (8.0)	52.6 (8.4)	**.03**

Abbreviations: 2'FL/LNnT, 4:1 HMO mix of 2'-O-fucosyllactose and Lacto-N-neotetraose; GSRS-IBS, Gastrointestinal Symptoms Rating Score for IBS patients; IBS-C; constipation-predominant; IBS-D, diarrhea-predominant IBS; IBS-M, IBS with mixed loose and hard stools.

[a]Age shown as mean and range (min-max).

[b]Data shown as mean (25th-75th percentile).

[c]Data shown as mean (SD). Differences between groups shown in bold (P < .05).

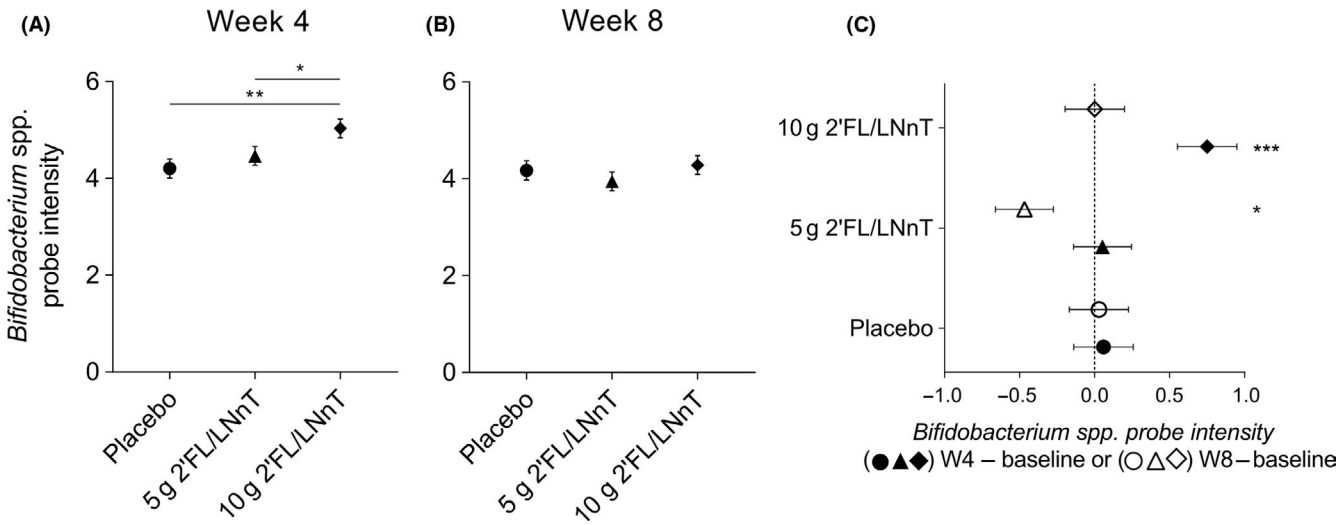

FIGURE 3 Bifidobacteria probe intensity. *Bifidobacterium* spp. probe intensity mean values (±SEM), adjusted to baseline, at (A) week 4 and (B) week 8 in placebo (circle), 5 g 2'FL/LNnT (triangle), and 10 g 2'FL/LNnT (diamond) groups. (C) Change at week 4 (black filled circle, triangle, and diamond) and week 8 (unfilled circle, triangle, and diamond) relative to baseline. Differences (A, B) between groups; and (C) within groups (week 4 or 8 relative to baseline) are shown as asterisks *P < .05; **P < .01; ***P < .001

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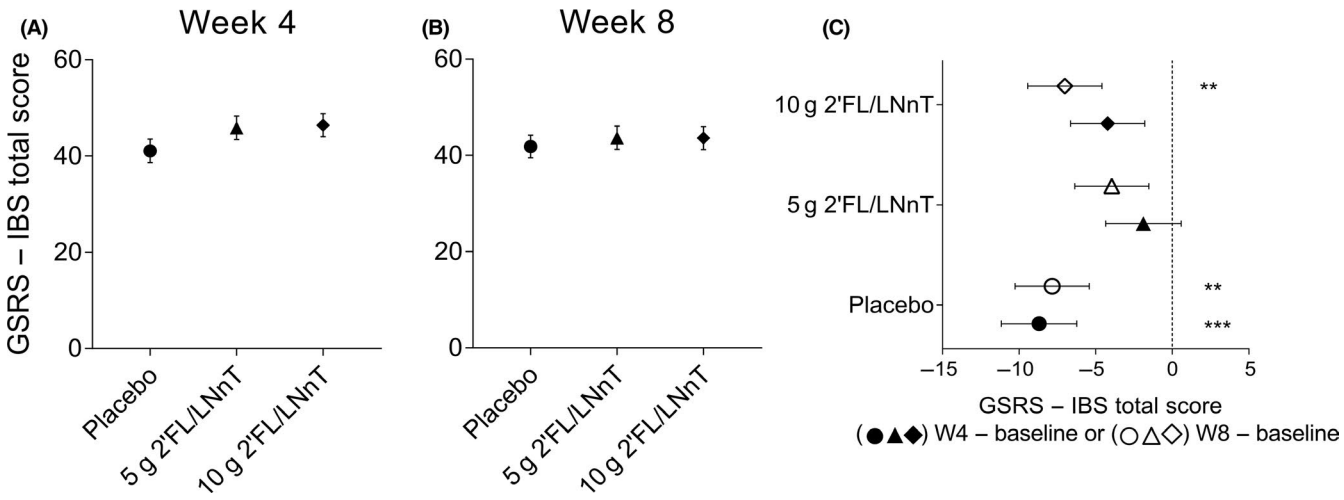

FIGURE 4 Overall gastrointestinal symptom severity (GSRS-IBS total score). GSRS-IBS total scores are shown as mean values (±SEM), adjusted to baseline, at (A) week 4 and (B) week 8 in placebo (circle), 5 g 2'FL/LNnT (triangle), and 10 g 2'FL/LNnT (diamond) groups. (C) GSRS-IBS total score change at week 4 (black filled circle, triangle, and diamond) and week 8 (unfilled circle, triangle, and diamond) relative to baseline. Differences (A, B) between groups and (C) within group are shown as $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$

TABLE 2 Individual domains of GSRS-IBS questionnaire during the study

	Placebo (n = 19)			5 g 2'FL/LNnT (n = 20)			10 g 2'FL/LNnT (n = 19)		
	Baseline	Week 4	Week 8	Baseline	Week 4	Week 8	Baseline	Week 4	Week 8
Abdominal pain score	8.7 (2.0)	7.8 (2.2)	7.6 (1.8)	8.1 (2.7)	7.1 (3.4)	7.4 (2.6)	9.3 (1.7)	8.5 (2.6)	**7.6 (3.1)****
Bloating score	14.3 (3.6)	**11.9 (4.0)***	13.0 (4.8)	12.4 (4.0)	11.6 (4.5)	**11.0 (3.7)****	15.2 (3.1)	14.2 (4.1)	13.7 (4.5)
Constipation score	6.2 (3.5)	5.2 (2.84)	**4.9 (3.6)****	3.7 (2.5)	4.3 (3.4)	4.8 (3.1)	5.5 (4.3)	4.7 (3.3)	4.1 (3.4)
Diarrhea score	15.5 (4.6)	**11.9 (4.2)***	**12.1 (3.6)****	15.4 (4.8)	14.8 (5.0)	13.1 (4.7)	17.3 (5.3)	16.0 (6.9)	15.7 (5.9)
Satiety score	5.6 (3.6)	4.8 (3.4)	4.98 (3.2)	5.7 (2.8)	5.2 (2.7)	5.2 (2.3)	5.5 (3.3)	5.1 (2.7)	4.53 (3.0)

Note: GSRS-IBS, Gastrointestinal Symptoms Rating Score for IBS patients. Data shown as mean (SD).Within-group differences are shown in bold and a symbol (* or $^{**}P < .05$ vs baseline; $^{***}P < .05$ vs week 4).

and 5 g 2'FL/LNnT groups (Figure 3A). However, no differences in fecal bifidobacteria abundance between groups were detected at week 8 (Figure 3B). Within-group comparisons of changes at week 4 and week 8, respectively, relative to baseline, demonstrated an increase in *Bifidobacterium* spp. abundance in the 10 g 2'FL/LNnT group after the intervention but not after the follow-up (Figure 3C). The 5 g 2'FL/LNnT group did not show a change at week 4 but had decreased bifidobacteria abundance at week 8 compared to baseline. No changes were seen in the placebo group at any time point (Figure 3C).

3.3 | Dose effect of 2'FL/LNnT on IBS-related symptoms

The overall mean of GSRS-IBS total score at baseline was 49.29 (9.42), implying that on average patients reported "mild to moderately severe discomfort" on the five main domains of GI symptoms. No differences in overall GI symptom severity (GSRS-IBS total score) between groups were identified at week 4 (Figure 4A) or week 8 (Figure 4B). Within-group comparisons at week 4 and week 8 relative to baseline demonstrated no aggravation of symptoms in any of the groups with the active product. In fact, GI symptoms were improved at week 8, but not week 4, in the 10 g 2'FL/LNnT group (Figure 4C). GI symptoms were improved at week 4 and week 8 in the placebo group, but no changes were seen in the 5 g 2'FL/LNnT group. None of the individual domains of GSRS, that is, abdominal pain, bloating, constipation, diarrhea, and satiety differed between groups at baseline or week 4. Within-groups comparisons detected a decrease in the severity of bloating and diarrhea in the placebo group at week 4, but no differences in the other groups. Additionally, no significant changes were observed at week 8 in the groups, except for a decrease in bloating in the 5 g 2'FL/LNnT group and in abdominal pain in the 10 g 2'FL/LNnT group (Table 2). Furthermore, there were no differences between groups or within groups at week 4 or week 8 regarding IBS symptom severity (IBS-SSS). However, the placebo and 5 g groups demonstrated a tendency toward milder symptoms (See Table S2, Figure S1). Regarding bowel habits, placebo showed a decrease in the proportion of abnormal stool consistency (BSFS 1-2 and BSFS 6-7) at week 4 compared with baseline, which was not seen in the active groups. However, all intervention groups demonstrated improved bowel habits after eight weeks compared to

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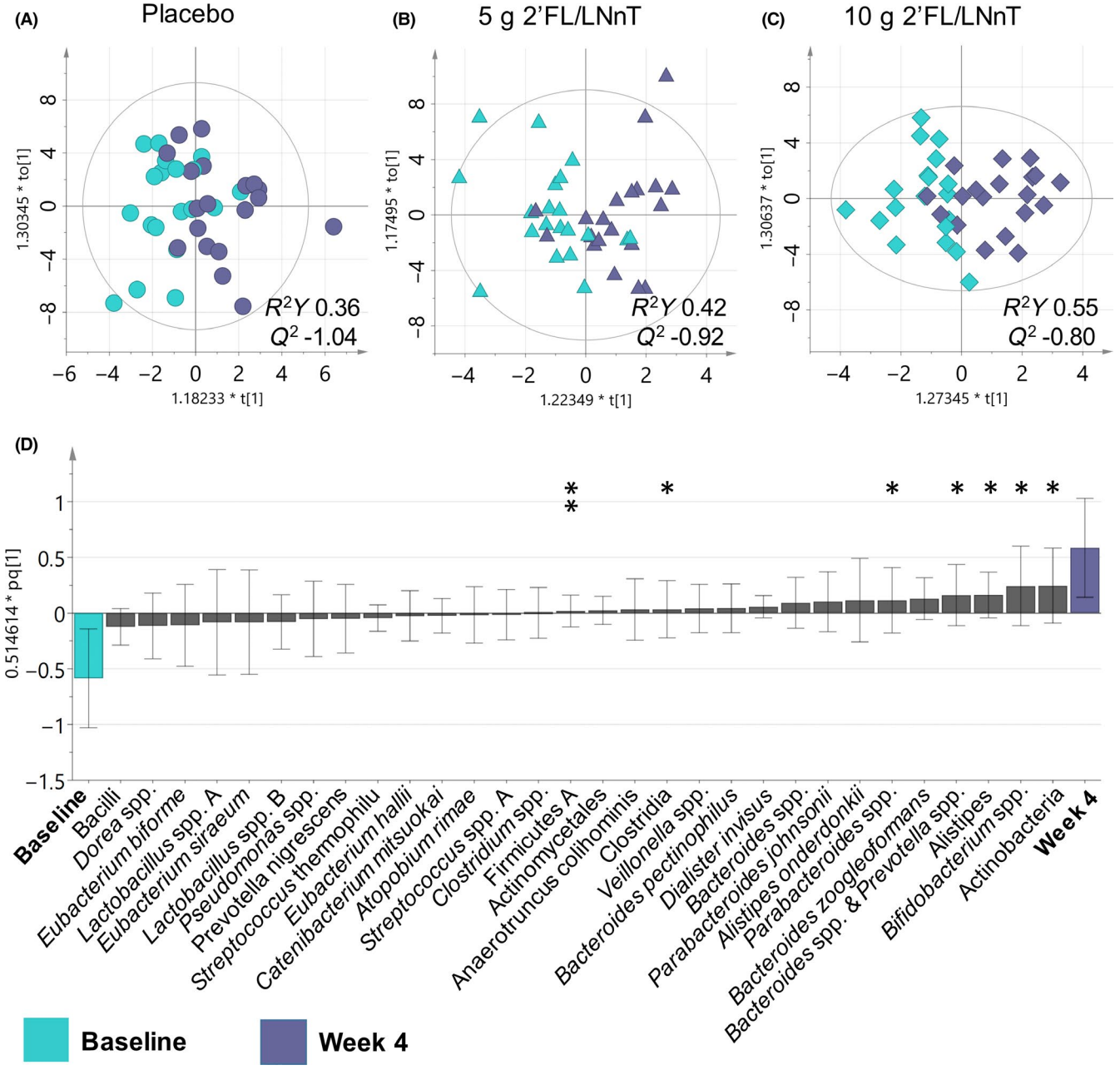

FIGURE 5 Modulation of microbiota profile. (A, B, C) Orthogonal partial least squares-discriminant analysis (OPLS-DA) scatter plots showing the difference between patients at baseline (turquoise) and week 4 (purple) based on the 54 most discriminatory bacterial genera in (A) placebo (circles), (B) 5 g 2'FL/LNnT (triangles), and (C) 10 g 2'FL/LNnT (diamonds) groups. (D) OPLS-DA loading column plot showing the 30 most discriminatory microbiota genera (VIP ≥ 0.8) differentiating the microbiota composition profiles at baseline and week 4 within the 10 g 2'FL/LNnT group. Firmicutes A, *Lactobacillus* spp. A and B, and *Streptococcus* spp. A and B probes target specific families within Firmicutes phylum, and species the genus of *Lactobacillus and Streptococcus*, respectively. A and B probes do not overlap. Error bars correspond to 95% confidence range. Asterisks identify statistically significant bacteria taxa $^{*}P < .05$; $^{**}P < .01$

baseline (See Table S3). Lastly, anxiety and depression subscales did not show any differences between groups at baseline, week 4, or week 8 (See Table S4).

3.4 | Effect of 2'FL/LNnT on microbiota profile

According to multivariate factor discriminant analysis by OPLS-DA, the microbiota profile of patients in the placebo (Figure 5A) and 5 g 2'FL/LNnT (Figure 5B) groups could not differentiate between baseline and week 4, as demonstrated by poor fitness ($R^2Y < 0.5$) and predictability ($Q^2 < 0$). In contrast, the microbiota profile of the 10 g 2'FL/LNnT group was able to differentiate week 4 from baseline ($R^2Y = 0.55$), although the predictability was poor ($Q_2 = -0.80$) (Figure 5C). The bacterial taxa most important for driving the separation in the 10 g 2'FL/LNnT group were Firmicutes A, Clostridia, Actinobacteria, *Bifidobacterium* spp., Alistipes, *Bacteroides* spp., *Prevotella* spp., and *Parabacteroides* spp. which were all increased

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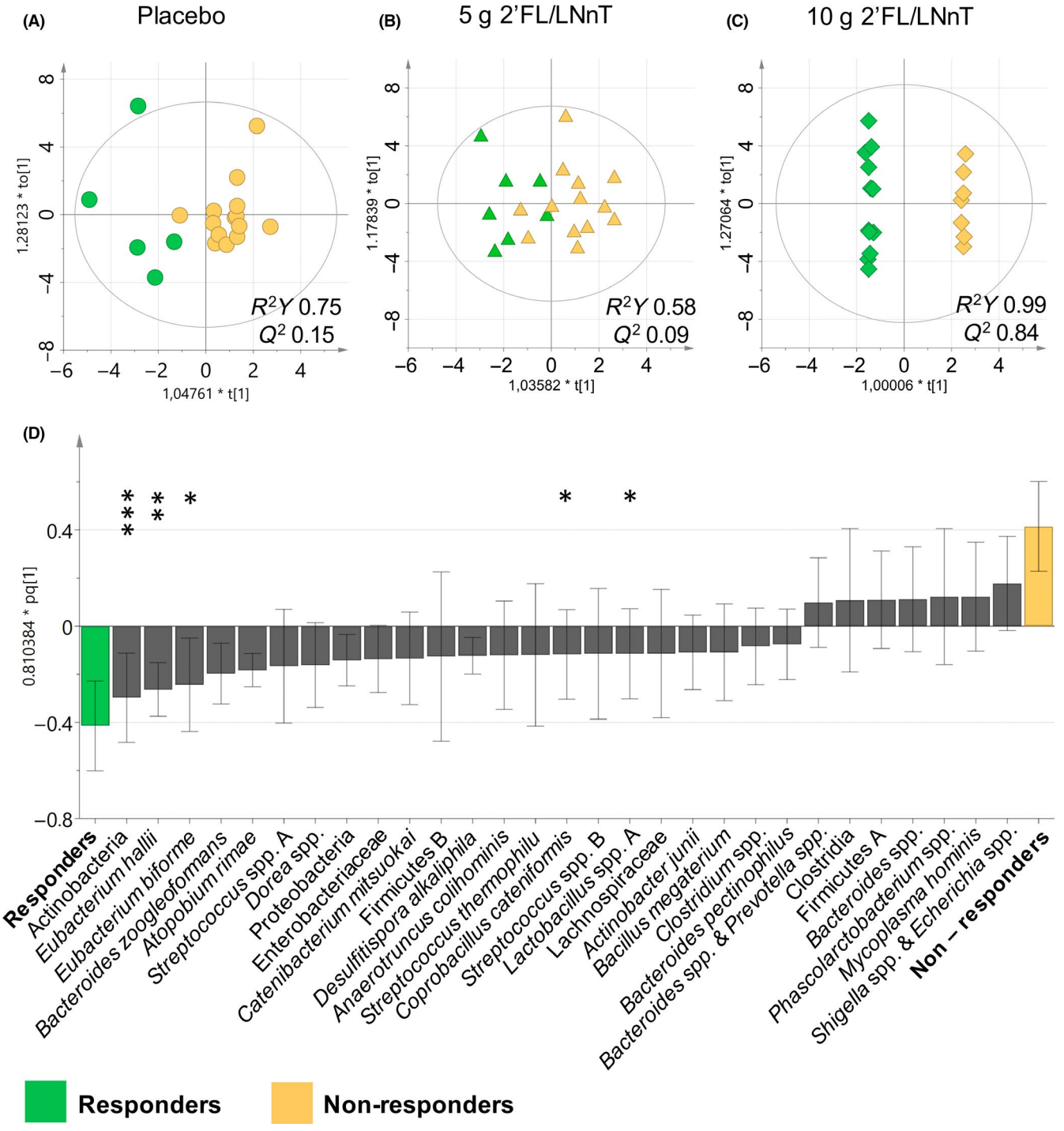

FIGURE 6 Microbiota profile fold change in responders and non-responders. (A, B, C) Orthogonal partial least squares-discriminant analysis (OPLS-DA) scatter plot showing the difference between responders (green) and non-responders (yellow) based on the fold change (week 4/baseline) of the 29 most discriminatory genera (VIP ≥ 0.9) of responders and non-responders in (A) placebo, (B) 5 g 2'FL/LNnT, and (C) 10 g 2'FL/LNnT groups. (D) OPLS-DA loading column plot showing the 29 most discriminatory microbiota genera fold change (week 4/baseline) differentiating the microbiota composition profiles of responders and non-responders in the 10 g 2'FL/LNnT group. *Bifidobacterium* spp. excluded. Firmicutes A and B, *Lactobacillus* spp. A, and *Streptococcus* spp. A and B probes target specific families within Firmicutes phylum, and species the genus of *Lactobacillus and Streptococcus*, respectively. A and B probes do not overlap. Error bars correspond to 95% confidence range. Asterisks identify statistically significant bacteria taxa $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$

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at week 4 as compared to baseline (Figure 5D). Some bacterial taxa showed a significant change during the intervention within the placebo or 5 g group (See Table S5), although these changes were not reflected in modulation of the overall microbiota profile.

3.5 | Microbiota profile in 2'FL/LNnT responders and non-responders

In the full study cohort, 24 patients (41%) met the criteria for response, that is, ≥50% increase of bifidobacteria abundance, whereas 34 patients (59%) were non-responders. The number of responders increased with the dose of 2'FL/LNnT, resulting in five responders in the placebo group (26%), seven responders in the 5 g 2'FL/LNnT (35%), and 12 responders in the 10 g 2'FL/LNnT group (63%). Differentiation of responders and non-responders was determined based on the modulation of the microbiota profile during the 4 weeks of intervention period (fold change; week 4/baseline). *Bifidobacterium* spp. was removed from this analysis to reduce skewness. For the placebo and 5 g 2'FL/LNnT groups, good fitness was achieved, although with poor predictability (Figure 6A,B

). However, the 10 g 2'FL/LNnT group demonstrated good fitness and predictability, that is, good discrimination between responders and non-responders (Figure 6C). The bacterial taxa most important for differentiating responders from non-responders in the 10 g 2'FL/LNnT group were high abundance of Actinobacteria, *Eubacterium halli*, *Eubacterium biforme*, *Lactobacillus* spp. A, and *Coprobacillus cateniformis* (Figure 6D). However, the microbiota profile before the start of the intervention could not predict the treatment response in any of the groups (See Table S6).

4 | DISCUSSION

In this study, daily intake of 10 g 2'FL/LNnT increased abundance of fecal *Bifidobacterium* spp. without negatively influencing GI symptoms in patients with IBS. Further, the product was well tolerated as no worsening of IBS symptoms, bowel habits, anxiety, or depression were detected. Moreover, the dose of 10 g 2'FL/LNnT influenced overall fecal microbiota composition, and responders, defined by bifidobacteria increase ≥50%, could be discriminated from non-responders based on the microbiota composition.

Several factors are considered to influence the pathophysiology of IBS, and growing evidence shows that the intestinal microbiota plays a key role. A well-balanced intestinal microbiota, comprising beneficial members such as bifidobacteria, is crucial to ensure the production of important compounds that improve intestinal health. HMO have been shown to increase bifidobacteria abundance in infants and healthy adults, but their ability to alleviate symptoms in IBS patients is unknown. The study was designed to determine the dose of a 4:1 mix of 2'FL/LNnT that could increase fecal bifidobacteria abundance, without aggravating GI symptoms. The ratio used aimed to reflect the proportion of these two oligosaccharides in human breast milk.[14] The study was not designed to detect improvement and included only lower doses than 20 g,[20] as IBS patients are considered more sensitive than healthy subjects. Similar to healthy adults,[20] four-week intake of 5 and 10 g 2'FL/LNnT in adult IBS patients was well tolerated and did not deteriorate GI symptoms, as assessed by GSRS-IBS total score and individual items of GSRS-IBS. Surprisingly, the placebo group showed a modest tendency to improve GI symptoms. Importantly, the intervention did not increase gas production, a potential risk previously raised due to the non-absorbable properties of other prebiotics.[27,28] Only two patients, one from the placebo group and one from the 10 g 2'FL/LNnT group, discontinued the study due to worsening of GI symptoms. Thus, the low dropout rate together with lack of GI symptom deterioration in IBS patients completing the intervention suggests that the product was well tolerated, as the study was expected to demonstrate.

2'FL and LNnT have been identified as substrates for *Bifidobacterium* spp., in both formula-fed infants and healthy adults.[19,20] Higher levels of bifidobacteria in elderly seem to correlate with healthy status and longevity,[29] whereas reduction in this genus has been associated with several conditions, including IBS, inflammatory bowel diseases, and metabolic disorders.[5,29,30] This study showed, similar to a previous study in a healthy cohort,[20] that a daily intake for four weeks of 10 g, but not 5 g, 2'FL/LNnT increased fecal bifidobacteria abundance in IBS patients. The lack of persistent effects during the washout period may indicate the necessity of continuously supplementation for maintaining increased bifidobacteria abundance.

Consumption of prebiotics induces growth of specific bacteria, but has also been shown to modulate the overall gut microbiota composition.[7,19,31-33] In line with this, the results from the current trial suggest that consumption of 10 g 2'FL/LNnT not only increased *Bifidobacterium* spp. abundance but also modulated the overall gut microbiota profile. In the 10 g 2'FL/LNnT group, increased abundance of Actinobacteria (class that includes *Bifidobacterium* spp.), *Alistipes*, *Bacteroides* spp., and *Prevotella* spp. was seen after four weeks. Interestingly, these bacterial taxa have previously been demonstrated to be in lower abundance in IBS patients relative to healthy individuals,[2] so restoring the abundance of these bacteria may be associated with improved health. However, the study design did not allow for investigating whether these specific bacteria were directly metabolizing 2'FL/LNnT or influenced by cross-feeding. In parallel, despite significant modulation of some bacterial taxa within the placebo and the 5 g 2'FL/LNnT group was observed, these populations might not have been relevant enough to impact the overall gut microbiota profile.

As part of the secondary endpoints, microbiota modulation in responders and non-responders was compared. According to previous experience from determining fecal microbiota composition over time in ulcerative colitis patients in remission (manuscript in preparation), the normal individual monthly variation of *Bifidobacterium*

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spp. abundance is <30%. We therefore chose to define response to the intervention as ≥50% increase (after the intervention period) of relative abundance of *Bifidobacterium* spp. The overall microbiota composition was differently modulated by the HMO mix in responders and non-responders, and the difference was most evident in the 10 g 2'FL/LNnT group. The modulation of microbiota profiles might reflect higher abundance of HMO utilizing bifidobacteria in responders compared to non-responders at the start of the intervention. Despite this, the fecal microbiota could not predict response prior to intervention. Although bifidobacteria was removed from the analysis, the model can have been influenced by Actinobacteria class, including *Bifidobacterium* spp.,[34] but also other bacterial taxa that utilize 2'FL/LNnT as substrate, directly or indirectly.[35,36] In fact, *Eubacterium* spp. has been shown to have feeding interactions with *Bifidobacterium* spp. during HMO degradation.[35] Unexpectedly, a small proportion (five out of 19) of patients fulfilled the responder definition in the placebo group. Studies have suggested that diet impacts on gut microbiota composition,[37] which may account for a bifidogenic effect within some patients within the placebo group. Moreover, since healthy subjects were not included in this study, we cannot evaluate if the microbiota profile of IBS patients responding to the HMO mix changes toward a profile resembling the healthy population. Nevertheless, increased abundance of *Bifidobacterium*, as well as *Eubacterium* spp. and *Lactobacillus* spp., as seen among responders in this study, is often associated with saccharolytic processes and improved gut health.[38-40] Hence, HMO could potentially be used for IBS patients with unbalanced gut microbiota, to re-establish a healthy microbiota profile.

To our knowledge, this is the first study aiming to determine the daily dose of 2'FL/LNnT that increases abundance of *Bifidobacterium* spp. without negatively influencing GI symptoms in IBS patients. Even with its strengths and promising results, there are limitations with the study. Being a truly exploratory study, the size of the study population was small and did not allow for subgroup analyses regarding IBS subtypes, which likely also influenced the fit and predictive ability of microbiota profile analysis models. Moreover, the study did not intend to assess a clinical effect of the intervention but to evaluate the tolerability of the study product. The inclusion of patients of all IBS subtypes, potentially with different disease driving mechanisms, may also have influenced the study outcome. However, since this is the first study exploring effects of the 2'FL/LNnT on IBS patients, we did not want to exclude any IBS subtype. Further, the relatively short intervention period did not allow to study the long-term effect, and this should be considered for future studies, since longer treatment periods may lead to an effect on both bifidobacteria and clinically relevant endpoints. Moreover, the use of GA-map™ technology might have excluded some relevant taxa that could be detected by other techniques, as 16S rRNA gene sequencing. However, the commercially available test used in the study simplified the establishment of gut bacterial profiles and may also be applied in a clinical setting.

In conclusion, four-week daily intake of 10 g 2'FL/LNnT increased the abundance of *Bifidobacterium* spp. without aggravating GI symptoms in IBS patients. This dose was well tolerated and did not induce worsening of IBS symptoms, bowel habits, anxiety, or depression, and most patients completed the four weeks of intervention without significant side effects. Moreover, the 10 g 2'FL/LNnT modulated overall fecal microbiota composition, and responders, defined by bifidobacteria increase ≥50%, could be discriminated from non-responders based on fecal microbiota modulation. Thus, this intervention might be favorable in order to restore gut microbiota of IBS patients toward a healthier profile.

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CONFLICT OF INTEREST

Declaration of personal interests: CI, IA, MKM, and JS do not have conflict of interest to declare. LKV, IDA, BM, and DS are employed at Glycom A/S, Denmark. HT has served as Consultant/Advisory Board member for Almirall, Allergan, Danone, and Shire. LÖ has served as Consultant/Advisory Board member for Genetic Analysis AS, and as a speaker for Ferring Pharmaceuticals, Takeda, AbbVie, and Meda. MS has served as a Consultant/Advisory Board member for AstraZeneca, Danone Nutricia Research, Nestlé, Almirall, Allergan, Albireo, Genetic Analysis AS, Biocodex, Glycom, Arena and Shire, and as a speaker for Tillotts, Takeda, Menarini, Kyowa Kirin, Allergan, Shire, Biocodex, Alimentary Health, AlfaSigma, and Almirall; and has received unrestricted research grants from Danone, and Ferring Pharmaceuticals.

AUTHOR CONTRIBUTIONS

Guarantor of article: MS.

Specific author contributions: CI *involved in* data acquisition, assemblage of database, analyses and interpretation of microbiota database, and drafting the manuscript. HT and IA collected the study subject materials and finalized the manuscript. JS and IDA *involved in* project planning and finalizing the manuscript. MKM interpreted the data, drafted and finalized the manuscript. LKV, DS, and BM *involved in* project planning, interpretation of data, and finalizing the manuscript. LÖ *involved in* project planning, interpretation of data, drafting, and finalizing the manuscript. MS *involved in* project planning, interpretation of data, material acquisition, drafting, and finalizing of manuscript. All authors have approved the final draft submitted.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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RESEARCH ARTICLE Open Access

Fecal microbiota composition is linked to the postoperative disease course in patients with Crohn's disease

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Abstract

Background: The role of the fecal microbiota composition for the postoperative disease course of patients with Crohn's disease (CD) who have undergone ileocecal resection remains to be established. In this study, we investigated if the fecal microbiota composition, determined by a high throughput test quantifying a pre-selected set of bacteria, is associated with the postoperative disease course of CD patients.

Methods: Fecal samples were obtained from healthy subjects as well as from CD patients, 3–10 weeks and 1 year after ileocaecal resection. The fecal microbial composition was analyzed by Genetic Analysis GA-map Dysbiosis test, targeting ≥300 bacteria on different taxonomic levels. Postoperative disease status was assessed endoscopically according to Rutgeerts scoring system 1 year after surgery. Differences in fecal microbiota composition between groups were analyzed by multivariate factor analyses and cluster analysis. Microbial stability over time was determined using Bray-Curtis dissimilarity.

Results: One year after surgery, the fecal microbiota composition differed between CD patients ($n = 21$) and healthy subjects ($n = 7$). At this time point, the microbiota composition of CD patients was associated with disease course, clearly separating patients with disease relapse ($n = 8$) and patients in remission ($n = 13$). Further, the microbial within-patient stability was high during the first year after surgery, irrespective of disease course.

Conclusion: The fecal microbiota composition of CD patients, analyzed by GA-map Dysbiosis test, is subject to little variation over time, and may potentially be used as a non-invasive diagnostic tool for the postoperative disease course.

Keywords: Crohn's disease, Fecal microbiota, Postoperative disease recurrence

Background

Despite increased use of immunosuppressive and anti-tumour necrosis factor drugs, approximately 25% of adult patients with Crohn's disease (CD) are in need of surgery within 10 years of diagnosis [1, 2]. The most common type of surgical procedure for patients that are refractory to medical treatment or develop complications is ileocaecal resection. However, surgical resection is not curative, and approximately 30% of all patients experience postoperative disease recurrence within 5 years of surgery [3–5]. It is not yet known why many patients attain long-term remission while others suffer from an early disease recurrence, and there are currently no reliable non-invasive tools to predict disease postoperative disease course.

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Numerous studies have documented differences in gut microbial profiles between patients with CD and healthy individuals, with a reduced microbial diversity and a lower relative abundance of specific bacteria in CD patients [6–11]. The relationship between gut microbiota profile and CD has further been accentuated by recent studies suggesting that fecal microbiota transplantation from healthy donors may be beneficial for CD patients [12–14]. Efforts have been made to evaluate the link between microbiota of mucosal biopsies and the postoperative disease course of the patients. These studies indicate that distinct profiles of mucosa-associated microbiota at the time of surgery and postoperatively are associated with disease recurrence and remission, respectively, and that these profiles can be distinguished from each other and from the microbiota profiles of healthy individuals [15–21]. However, to the best of our knowledge only two previous studies have addressed the relationship between fecal microbiota composition and postsurgery endoscopic status, although with inconclusive results [7, 15].

The clinical use of previous findings have so far been limited as sequencing microbiome regions of 16 s rRNA with subsequent phylogenetic analysis is a cumbersome task, requiring advanced equipment and in-depth knowledge of data computation and microbiota. Potentially, user-friendly tools, simplifying the analysis of the fecal microbiota, may facilitate the utilization of microbiota profiling in a clinical setting to predict the postoperative disease course in CD. The aim of this study was therefore to determine if the fecal microbiota composition, determined by a high throughput and easy-to-use test, quantifying a pre-selected set of bacteria, is associated with the postoperative disease status of CD patients.

Methods

Study subjects and sample collection

Adult patients, 18–63 years, who had undergone ileocaecal resection for CD, were recruited from four gastroenterology units in southwest Sweden from September 2008 to December 2011 [22]. As previously described [22], exclusion criteria were disease activity beyond the ileocaecal region, malignancy during the last 5 years, severe chronic disease affecting the ability to comply with the study protocol (i.e., renal or liver failure, severe cardiovascular disease, neurological disease, and psychiatric disorder), use of non-steroidal anti-inflammatory drugs (5-aminosalicylic acid not included), drug addiction, and pregnancy at inclusion. All patients were given a single dose of perioperative antibiotic prophylaxis. None of the patients had previously undergone a bowel resection.

Approximately 1 year after surgery, an ileocolonoscopy was performed and the mucosa in neoterminal ileum and ileocolonic anastomosis was assessed according to Rutgeerts scoring system [23]. Five or less aphthoid lesions (i_0-i_1) were considered as remission and more than five aphthoid lesions, lesions confined to the anastomosis or diffuse inflammation (i_2-i_4) were considered as endoscopic disease recurrence.

Fecal samples from CD-patients were collected from the first bowel movement in the morning 2 weeks prior to or 1–4 weeks after the ileocolonoscopy at the follow up 1 year after surgery, and for some patients also at 3–10 weeks after the ileocaecal resection. Fecal samples were stored at − 20 °C until analysis.

Fecal samples were also collected from healthy subjects recruited at Sahlgrenska University hospital, Gothenburg, and stored at − 80 °C until analysis. None of the healthy subjects had any history of gastrointestinal or other chronic disorders or had taken any immunosuppressive agents, antibiotics or any other medications during the last 3 months prior to sample collection.

Microbiota analysis

Fecal microbiota was analyzed by the GA-map Dysbiosis test (Genetic Analysis AS, Oslo, Norway) as previously described [24]. In brief, the test is based on molecular biology techniques whereby bacterial probes, based on the 16S rRNA sequence in seven variable regions (V3–V9), measure the abundance of bacteria according to the strength of fluorescent signal detection (probe intensity). The GA-map test comprises 54 DNA probes targeting ≥300 bacteria on different taxonomic levels [25]. All probes are listed in Supplementary Table 1.

Data analyses

Data analyses for principal component analysis (PCA), Bray-Curtis dissimilarity and clustering were performed in R Studio 1.1.456 (R 3.6.1) using pca3d and vegan packages. Bray-Curtis dissimilarity was used to investigate differences within and between study subjects at different time points. A dissimilarity matrix was calculated using all samples from both time points in the same model. Within-patient analysis over time shows dissimilarities between early and 1-year follow up for the same patients. Between-patient analysis over time show mean dissimilarities between early samples and all non-related samples at 1-year follow up. Between-patient analysis at 1-year follow up show mean dissimilarities between unique patients and all non-related samples (at the same time point). Lower numbers define higher similarity between samples. For post hoc analysis, Kruskal-Wallis test followed by *Dunn's* multiple comparisons test was used (GraphPad Prism; GraphPad Software, La Jolla, CA, USA). Cross-tabulation using Fisher's Exact Test and Spearman correlation analysis were performed in IBM SPSS Statistics version 25.

To examine the difference in fecal microbial composition (X-variables) between CD patients and healthy subjects, as well as between CD patients in remission and with postoperative disease recurrence (Y-variables), multivariate factor analysis was used (SIMCA 15 software; Umetrics, Umeå, Sweden). As previously described [24], orthogonal partial least squares discriminant analysis (OPLS-DA) was employed to correlate X-variables with a selected Y-variable and with each other in linear multivariate models. The quality of OPLS-DA is based on the parameter R2, i.e. the goodness of fit of the model (values ≥0.5 define good discrimination, best possible fit R2 = 1), and Q2, i.e., the goodness of prediction of the model (values ≥0.5 define high predictive ability). In the OPLS-DA loadings column plots and scatter plots, each X variable is shown in relation to Y. The X-variables positioned furthest to the left or right are more closely related to the respective Y variable, and the stronger and more reliable are their contribution to the model. A variable influence on projection (VIP) was used as a variable selection based on discriminatory power since the number of variables exceeded the number of study subjects. To reduce the risk of over fit, post hoc 100 permutation tests of OPLS-DA models were performed and only models with permutation indices fulfilling the post hoc analysis criteria of $R \leq 0.4$ and $Q2Y < 0.05$ were accepted [26]. Differences in bacterial abundance between two groups were further analyzed with Mann-Whitney U test (GraphPad Prism). Statistically significant differences are indicated with asterisks in the OPLS-DA loadings column plots (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$).

Table 1 Demographic and clinical characteristics of the patients

	n = 21	
Characteristics	*n*	(%)
Sex		
Female/Male	7/14	(33/67)
Median age (at inclusion)		
28 years (17–63)		
Median disease duration		
3 years (0–11 years)		
Smoking (at inclusion)		
Never	12	(57)
Past	6	(29)
Present	3	(14)
Disease behavior		
Inflammatory	2	(10)
Stricturing	14	(67)
Perforating	5	(24)
Therapy at time of resection		
No medication	4	(19)
5-ASA[a]	7	(33)
Corticosteroids	13	(62)
Thiopurines	10	(48)
Anti-TNF[b]	1	(5)
Therapy at time of colonoscopy		
No medication	8	(38)
5-ASA	8	(38)
Corticosteroids	2	(10)
Thiopurines	6	(29)

[a]5-aminosalicylic acid
[b]Anti- tumor necrosis factor

Results

Clinical and demographic characteristics of the study subjects

In total, 21 patients were included in the study. Clinical and demographic characteristics for the patients are shown in Table 1. Patients were classed based on disease behavior according to the Montreal classification system (B1-B3). In total, 2 patients had surgery performed due to inflammatory disease behavior (B1), 14 patients due to stricturing disease (B2), and 5 patients due to a perforating disease behavior (B3) (Table 1). To examine the small intestine, a computed tomography and/or a magnetic resonance enterography or small-bowel series was performed in a majority of the patients prior to surgery or at postoperative follow up, as previously described [22]. At surgery, a careful examination of the small intestine was performed in all patients.

Steroid treatment in conjunction to surgery was quickly de-escalted postoperatively, and introduction of any new medical treatments postoperatively, including corticosteroids, was effected on clinical basis throughout the whole follow-up period until the colonoscopy 1 year after surgery. Ileocolonoscopy was performed at follow up, a median time of 52 weeks (41–58 weeks) after ileocaecal resection, to evaluate the inflammatory activity in the colon and to assess the ileocolonic anastomosis and the neoterminal ileum according to Rutgeerts score. The colonic mucosa distal to the anastomotic area was endoscopically normal in all but one patient. This patient had a local oedema in the descendent colon, and a corresponding histological inflammation was detected. According to Rutgeerts scoring system, 13 patients (62%) were considered to be in endoscopic remission (i_0-i_1), whereas 8 patients (38%) had endoscopic disease recurrence (i_2-i_4) at 1-year follow up (Table 2). A graphical flowchart of the study design is shown in Fig. 1.

All patients provided stool samples 1 year after ileocaecal resection (termed 1-year follow up) and eight of the patients also provided samples at 3–10 weeks after ileocaecal resection (termed early follow up). In addition, stool samples were collected from seven healthy subjects (four women) with median age 25 (20–36).

Table 2 Rutgeerts' endoscopic score for recurrence of Crohn's disease 1 year after ileocaecal resection

	n = 21	
Rutgeerts'score	*n*	(%)
i_0	4	(19)
no lesions		
i_1	9	(43)
≤ 5 aphthous lesions		
i_2	7	(33)
> 5 aphthous lesions with normal mucosa between the lesions or skip areas of larger lesions or lesions confined to the ileocolonic anastomosis		
i_3	1	(5)
diffuse aphthous ileitis with diffusely inflamed mucosa		
i_4	0	(0)
diffuse inflammation with already larger ulcers, nodules, and/or narrowing		

Fecal microbiota composition at 1-year follow up discriminate CD patients from healthy subjects

At 1-year follow up, the fecal microbiota composition defined by the GA-map Dysbiosis test differentiated CD patients from healthy subjects (Fig. 2a). A clear discrimination between CD patients and healthy subjects was also observed in the OPLS-DA score scatter plot (Fig. 2b). A majority of the bacterial taxa (31 of 54) showed higher abundance in healthy subjects as compared to CD, while only two were higher among CD patients, i.e. *Ruminococcus gnavus* and *Shigella* spp. & *Echerichia* spp. (Supplementary Table 1). The 21 most important bacteria for the discrimination between CD patients and healthy subjects, all with a higher abundance in healthy subjects, are shown in Fig. 2c.

Fecal microbiota composition at 1-year follow up is associated with disease course

Next, the fecal microbial composition in CD patients at 1-year follow up and the concurrent postoperative disease status, was examined. A separation between CD patients in endoscopic remission and with endoscopic disease recurrence was indicated by the microbiota composition using PCA (Fig. 3a). Cluster analysis based on the microbiota defined two clusters, cluster A with 11 members and cluster B with 10 members (Fig. 3b). As seen by the cross tabulated frequencies in Table 3, there was a significant relationship between the microbiota and the postoperative disease status, $p = 0.008$. The 10 most important bacterial taxa for this discrimination were identified using OPLS-DA (Fig. 3c-d), with *Alistipes* in higher abundance and Actinobacteria and Bifidobacterium spp. in lower abundance in patients in endoscopic remission as compared to patients with disease recurrence (Fig. 3d). *Alistipes*, which was decreased in CD as compared to healthy subjects (Supplementary Table 1), was also found to correlate negatively to Rutgeerts score, r = − 0.659 and $p = 0.001$.

Fecal microbiota composition at early follow up is similar to 1-year follow up

Finally, we examined whether the fecal microbiota composition at early follow up alters until the 1-year visit. Paired samples from 8 CD patients were examined but no separation between the time points could be detected by PCA (Fig. 4a) and no discrimination could be generated using OPLS-DA. Microbial temporal stability was further evaluated for the paired samples in relation to the additional (non-related) 13 samples from the 1-year follow up. First, a PCA analysis was generated to visualize within-patient mobility in the total population (Fig. 4b). Second, differences within and between patients were analyzed by Bray-Curtis dissimilarity and showed that the within-patient microbiota from early follow up to 1-year follow up was more similar than between-patient microbiota over time (Fig. 4c, two upper panels). Within-patient microbiota over time was also more similar than between-patient microbiota at the 1-year follow up (Fig. 4c, top and lower panel). No difference could be detected by Bray-Curtis dissimilarity between patients in endoscopic remission as compared to patients with disease recurrence at 1-year follow up (Fig. 4c, compare open and closed circles within each panel).

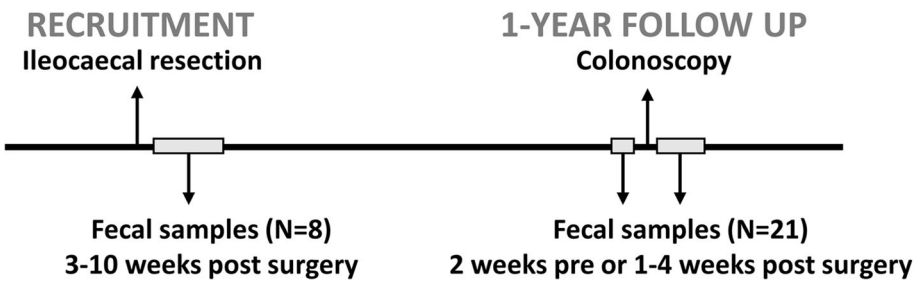

Fig. 1 Graphical flowchart of the prospective study design. Adult patients who had undergone ileocaecal resection for Crohn's disease were included (*N* = 21). Fecal samples were obtained 3–10 weeks after resection (*N* = 8) and 1 year after resection (*N* = 21). Postoperative disease status was assessed by colonoscopy approximately 1 year after surgery

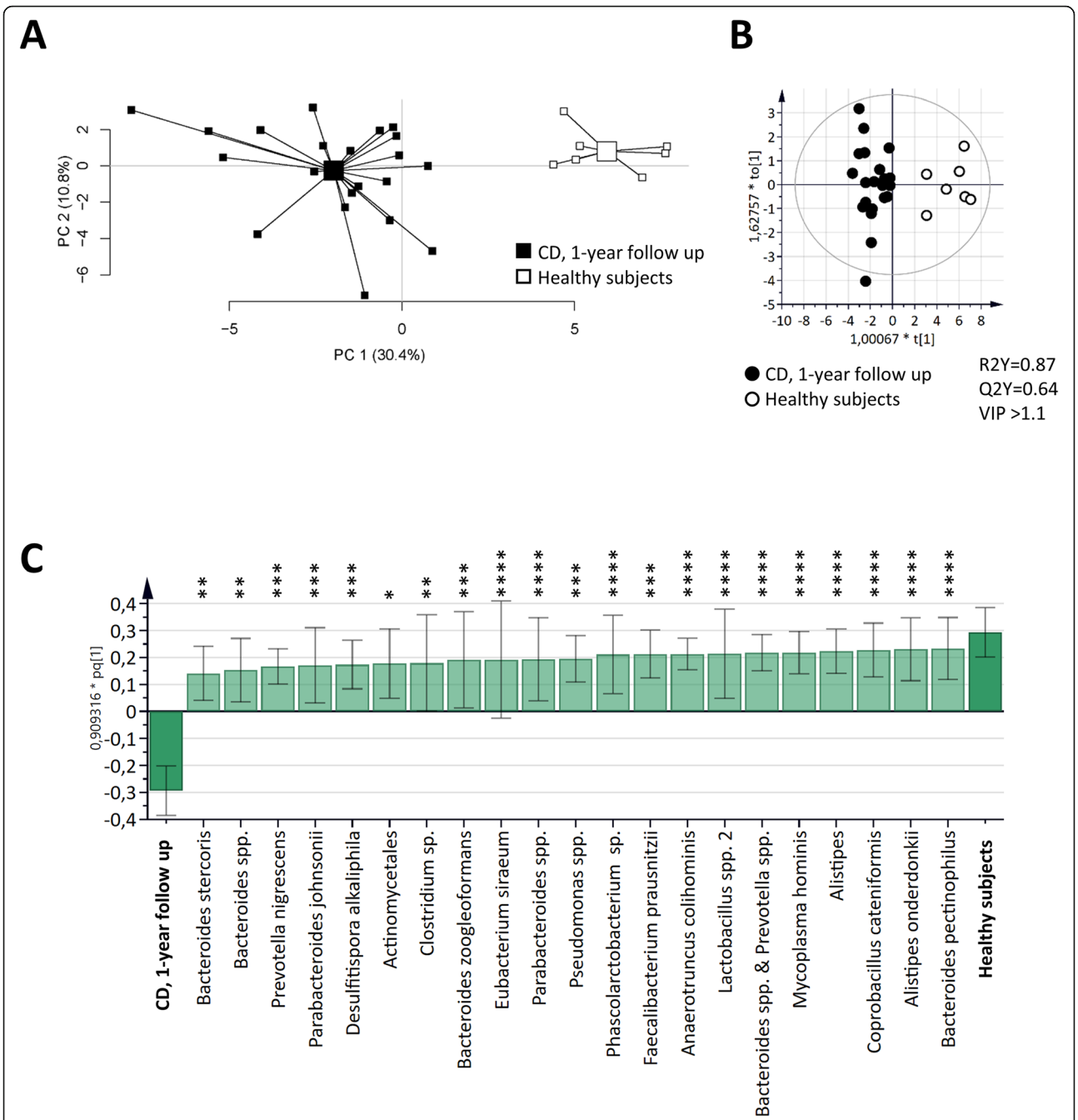

Fig. 2 Differences in fecal microbiota composition in CD patients and healthy subjects (HS). Fecal samples from CD patients 1 year after ileocaecal resection ($n = 21$) and from HS ($n = 7$) were analyzed by the GA-map™ Dysbiosis test. **a** PCA plot of CD patients and HS based on fecal bacterial taxa (X-variables; $n = 54$). Lines to the centroid, showing the group mean, connect each group. OPLS-DA scatter plot (**b**) and loadings column plot (**c**) of CD patients and HS (Y-variables) and fecal bacterial taxa (X-variables; VIP > 1.1; $n = 21$). Statistically significant differences between study groups are indicated with asterisks in the OPLS-DA loadings column plot (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$)

Discussion

It is of clinical importance to identify CD patients at risk of disease recurrence after undergoing surgical resection. In this study, we demonstrate that the fecal microbiota composition of CD patients at 1-year follow up is associated with the postoperative disease status. Further, the microbial temporal stability within-patients was high during the first year after surgery. The analyses of fecal microbiota composition was performed by a user friendly test determining a selected

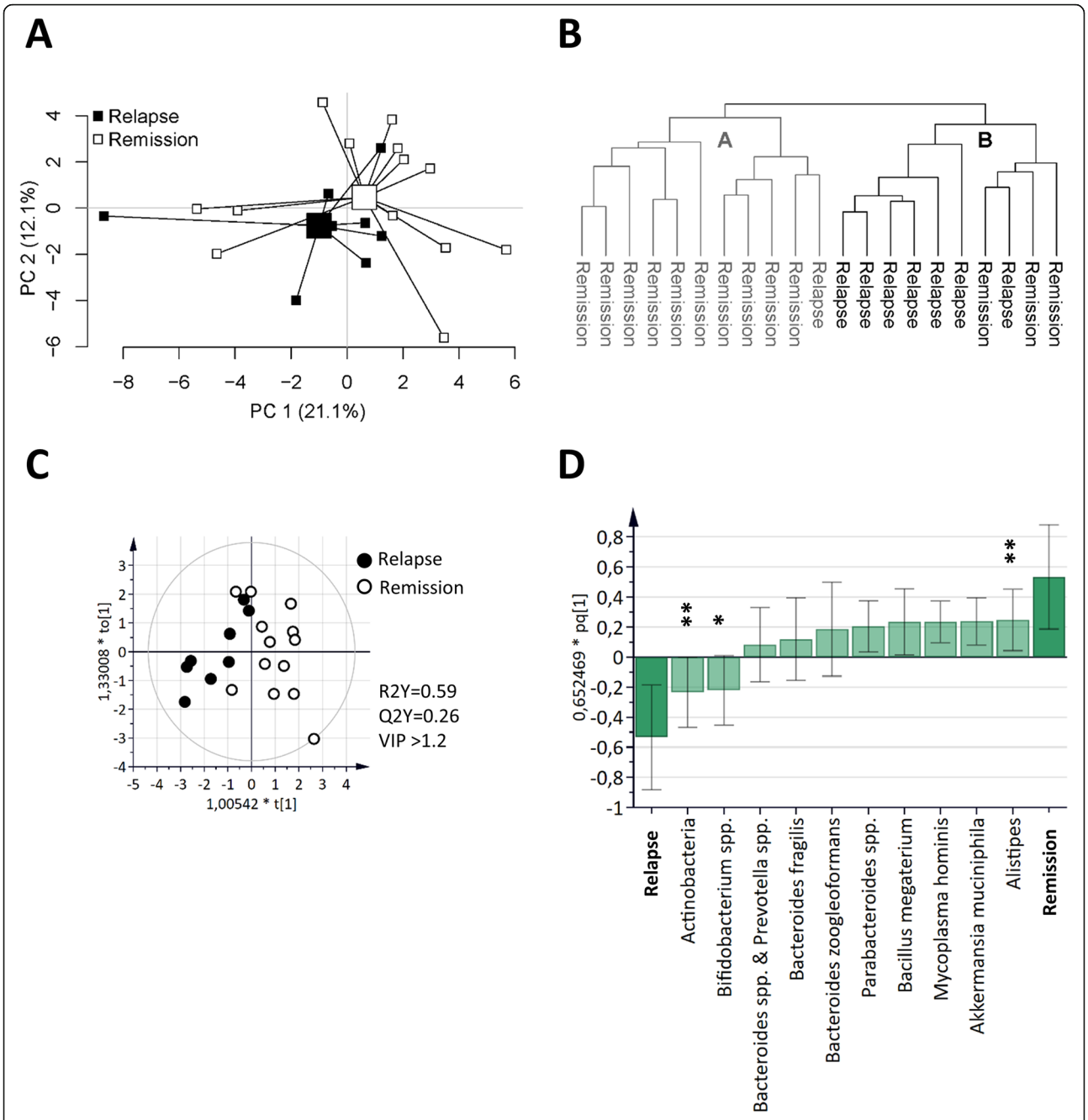

Fig. 3 Associations between fecal microbiota and postsurgical disease course in CD. Fecal microbiota from CD patients with endoscopic disease relapse ($n = 8$) and patients with endoscopic disease remission ($n = 13$) 1 year after ileocaecal resection was analyzed by the GA-map™ Dysbiosis test. **a** PCA plot of CD patients with disease relapse and patients with remission based on fecal bacterial taxa (X-variables; $n = 54$). Lines to the centroid, showing the group mean, connect each group. **b** Cluster analysis of fecal bacterial taxa ($n = 54$) from CD patients with disease relapse and remission 1 year after ileocaecal resection ($n = 21$). OPLS-DA scatter plot (**c**) and loadings column plot (**d**) of postoperative disease course (Y-variables, i.e., disease relapse vs remission) and fecal bacterial taxa (X-variables; VIP > 1.2; $n = 10$). Statistically significant differences between study groups are indicated with asterisks in the OPLS-DA loadings column plot (* $p \leq 0.05$; ** $p \leq 0.01$)

set of bacteria, potentially allowing for microbiota profiles to be used as a diagnostic tool in a clinical setting to identify CD patients in need of more intense treatment regimens after surgery or vice versa, to identify patients at low risk of disease recurrence.

Similar to previous studies, we demonstrate that fecal microbiota profiles differed between CD patients and healthy subjects [7, 27]. The bacterial taxa discriminating CD patients from healthy subjects were found in lower abundance among patients and represented all of the

Table 3 Cross-tabulation of 1-year post-operative disease status and microbiota clusters, $p = 0.008$[a]

Post-operative status	Microbiota cluster[b]		Total
	A	B	
Remission	10	3	13
Relapse	1	7	8
Total	11	10	21

[a]Fisher's Exact Test
[b]Defined by the cluster analysis in Fig. 3b

four major bacterial phyla known to dominate the gut microbiota of humans, i.e., Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria [28, 29]. This suggests that CD is characterized by an impoverished gut microbiota, rather than a shift in abundance of specific species. The importance in using quantitative instead of relative abundance of bacteria has recently been appreciated and similar data have been obtained for CD patients using absolute abundance by estimating the 16S rRNA sequencing data to microbial load defined by flow cytometry [30] and fecal microbial DNA content [31].

Further, the fecal microbiota composition at the 1-year follow up diverged between CD patients who experienced postoperative disease recurrence and those who remained in remission, and a cluster analysis confirmed the relationship between the microbiota and the postoperative disease status. Among the bacterial taxa found to contribute to the separation between the two groups was *Alistipes*, found in higher abundance in patients who remained in remission as compared to patients suffering from subsequent disease recurrence. In a recent meta-analysis of 24 public studies, higher abundance of *Alistipes* was associated with a healthy state [32], likewise we showed that *Alistipes* is decreased in this cohort of CD patients as compared to healthy subjects.

Our results also suggest that the fecal microbiota composition of CD patients was subject to little variation during the first year after surgery. Thus, paired fecal samples from the same individual at early and 1-year follow up had similar microbiota composition, regardless of disease activity. Although fecal microbiota composition of IBD patients has been reported to fluctuate more than that of healthy subjects [7, 10], studies using GA-Map technology demonstrated low fecal microbiota variation over time in IBD patients undergoing anti-TNF therapy regardless of therapy outcome [33] as well as in healthy subjects [25]. The microbial temporal stability within patients together with the relatively strong association between fecal microbiota composition and postsurgical outcome indicates that fecal microbiota profiles may be used to predict disease course even shortly after surgery. Future studies should address whether microbiota profiles of stool samples, obtained before or in close conjunction to surgery indeed may be a biomarker for disease progression.

The GA-Map technology used in this study is a diagnostic tool previously validated for being able to discriminate IBD patients from healthy subjects, based on a selected set of fecal bacteria [25]. In comparison to deep-sequencing and shotgun metagenomic sequencing methods requiring advanced bioinformatics tools and reference datasets, the GA-Map technology allows the individual researcher/clinician to assess fecal microbiota composition without such expertise. Thus, the commercially available GA-map technology used in this study may allow for the realization of gut microbiota profiling as a diagnostic tool in a clinical setting and, in conjunction with the surgical procedure, may provide guidance for the identification of patients at risk of disease recurrence.

Even with its strengths and promising results, there are limitations and weaknesses with our study. The small number of study participants is a significant limitation to the generalizability of the study outcome, and might also have influenced the fit and predictive ability of microbiota profile analysis models. The limited size of the study also prevented us to do further analyses on subpopulations, e.g. with respect to various medical treatments and smoking among the patients. Further limitations are that we did not have access to fecal samples collected before the ileocaecal resection, and that fecal samples from the early follow up were obtained at a relatively broad time span, i.e. 3–10 weeks after resection. A strength, on the other hand, is that the paired fecal samples allowed us to address the microbial temporal stability in the CD patients. Results from these analyses indicated that the within-patient fecal microbiota composition was subject to little variation during the first year after surgery, regardless of disease activity. Thus, although an imminent risk that the perioperative antibiotic treatment would have disrupted the regular microbial flora for the patients at the early follow up, the bacteria detected by the GA-map dysbiosis test were still stable enough to display a similar profile over the first year after surgery.

Conclusion

In summary, the validated GA-Map dysbiosis test allowed us to demonstrate that the fecal microbiota composition of CD patients is associated not only with the disease itself but also with the disease course 1 year after ileocecal resection. In addition, little variation in microbiota composition over time was seen within patients. Although larger studies are needed to confirm these findings our study suggests that fecal microbiota composition may be used as a non-invasive diagnostic tool for the postoperative disease course of CD patients.

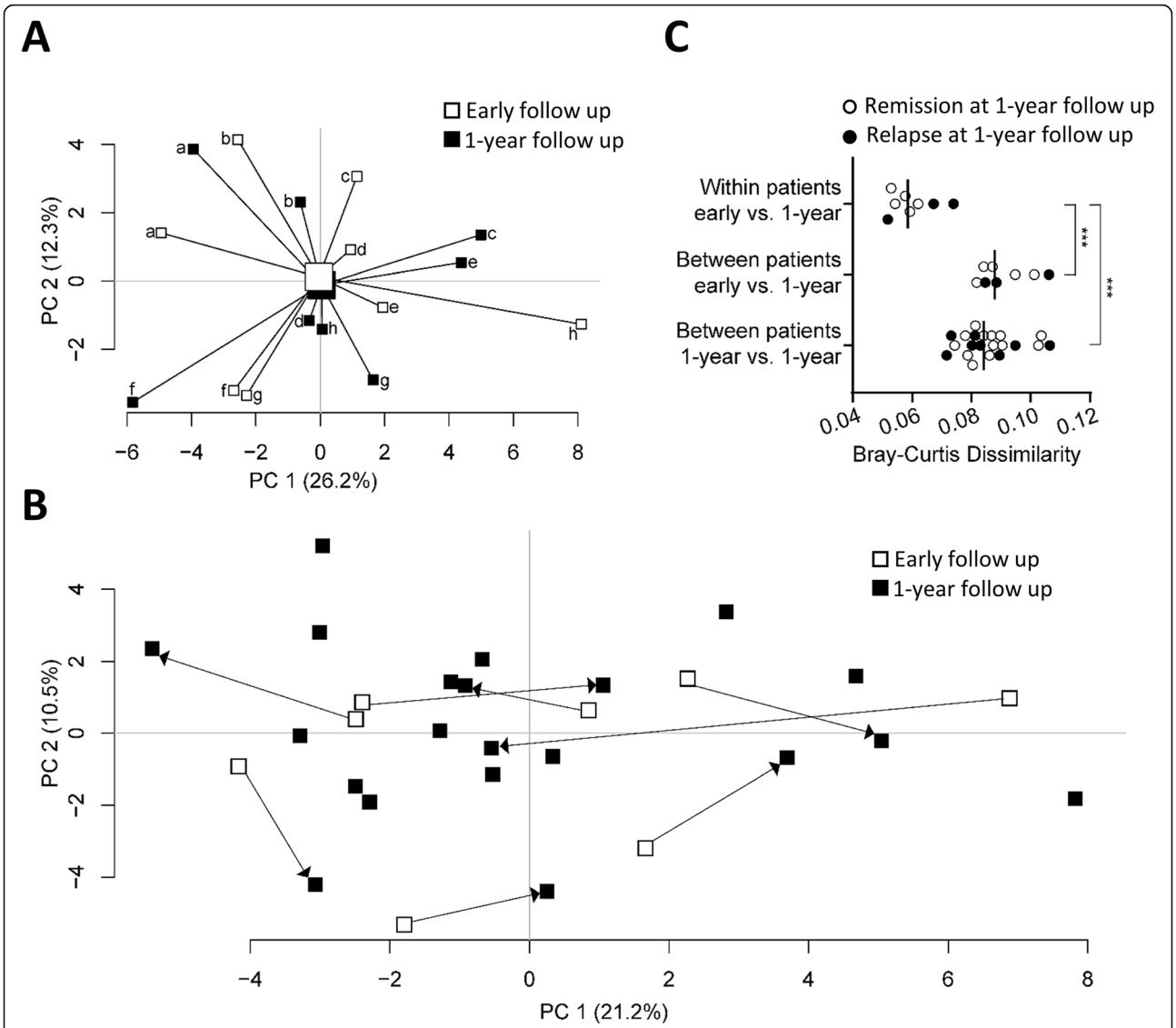

Fig. 4 Stability of fecal microbiota composition the first year after ileocaecal resection. Fecal microbiota from 8 CD patients at early follow up (3–10 weeks after ileocaecal resection) and at the 1-year follow, and from 13 patients at the 1 year follow up was analyzed by the GA-map™ Dysbiosis test. **a** PCA based on fecal microbiota composition (X-variables; $n = 54$) from 8 CD patients at early follow up and from the same patients at the 1-year follow up. Lines to the centroid, showing the group mean, connect each group. Individual patients are marked from a-h for each time point. **b** PCA displaying the within-patient mobility of fecal microbial composition from early follow up to 1-year follow up in relation to the total population at the 1-year follow up ($n = 21$) (paired samples indicated with arrows pointing from early to 1-year follow up, $n = 8$). **c** Within-patient microbial similarity from early follow up to 1-year follow up (top, $n = 8$) in relation to between-patient similarity from early follow up to 1-year follow up (middle, $n = 8$), and to between-patient similarity at 1 year follow up (bottom, $n = 21$) analyzed by Bray-Curtis dissimilarity index. Between-patient dissimilarities show mean values for a unique individual to all non-related patients (mean of $n = 20$). Closed and open circles represent patients with disease relapse and remission, respectively, at the 1-year postsurgical follow-up; *** $p \leq 0.001$

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12876-020-01281-4.

Additional file 1 : **Table S1.** List of the 54 probes included in GA-map™ Dysbiosis Test; bacteria names and taxonomy and comparison of abundance between CD patients at 1-year follow up and healthy subjects (HS).

Abbreviations

CD: Crohn's disease; PCA: Principal component analysis; OPLS-DA: Orthogonal partial least squares discriminant analysis; VIP: Variable influence on projection; rRNA: Ribosomal RNA; IBD: Inflammatory bowel disease

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Not applicable.

Authors' contributions

AS: Study design, analysis and interpretation of data, manuscript preparation. AL, HS, POS: Clinical examinations and data acquisition. JS, MS: Data acquisition. MM: Study design, analysis and interpretation of data, revision of the manuscript. LÖ: Obtained funding, study concept and design, interpretation of data, revision of the manuscript. All authors provided critical revision of the manuscript for important intellectual content and read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All patients gave written informed consent according to the 1975 declaration of Helsinki. The study was approved by the Regional Ethical Review Board at the University of Gothenburg (Ref 268–08, approved 25-06-2008; Ref T227–18, approved 12-03-2018).

Consent for publication

Not applicable.

Competing interests

Lena Öhman is a member of the advisory board of Genetic Analysis. The remaining authors declare that they have no competing interest.

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Changes in Faecal Short-Chain Fatty Acids after Weight-Loss Interventions in Subjects with Morbid Obesity

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Abstract: The gut microbiota and their metabolites, e.g., short-chain fatty acids (SCFA), are associated with obesity. The primary aims were to study faecal SCFA levels and the changes in SCFA levels after weight-loss interventions in subjects with obesity, and secondarily, to study factors associated with the faecal SCFA levels. In total, 90 subjects (men / women: 15/75) with a mean age of 44.4 (SD 8.4) years, BMI 41.7 (SD 3.7) kg/m^2 and morbid obesity (BMI > 40 or > 35 kg/m^2 with obesity-related complications) were included. Faecal SCFA and other variables were measured at inclusion and after a six-month conservative weight-loss intervention followed by bariatric surgery (Roux-en-Y gastric bypass or gastric sleeve). Six months after surgery, the total amount of SCFA was reduced, the total and relative amounts of the main straight SCFA (acetic-, propionic-, and butyric- acids) were reduced, and the total and relative amounts of branched SCFA (isobutyric-, isovaleric-, and isocaproic-acids) were increased. The changes indicate a shift toward a proteolytic fermentation pattern with unfavorable health effects. The amount of SCFA was associated with the diet but not with metabolic markers or makers of the faecal microbiota composition. Dietary interventions could counteract the unfavorable effects.

Keywords: obesity; short-chain fatty acids; bariatric surgery; weight-loss; faecal microbiota

1. Introduction

The gut microbiota and their metabolites, e.g., short-chain fatty acids (SCFA), have health-related effects and have been associated with a wide range of disorders [1,2]. Obesity with comorbidities is one of these microbiota-associated disorders, although a causal relationship has not been documented in humans [3–5]. The microbiota and the metabolites might be both health-promoting and health-damaging. All the individual SCFA are present under physiological conditions and play different roles. An imbalance in the pattern, e.g., in the saccharolytic fermentation characterized by an increase in the main straight SCFA (acetic-, propionic, and butyric- acids) versus the proteolytic fermentation, characterized by an increase in the branched SCFA (isobutyric- isovaleric-, and isocaproic-acids), may signify alterations in the microbial functions that may be associated with either gut health or disease [1,2,6–9]. Knowledge of faecal SCFA in subjects with morbid obesity and the changes after a combined conservative and surgical intervention is limited [4,10–14]. An unbalance in the SCFA pattern before or after weight-reducing treatment might have unfavorable health effects that necessitate interventions.

The primary aims were to study faecal SCFA in subjects with morbid obesity and the changes in SCFA after a combined conservative and surgical treatment, and secondarily, to study associations

between SCFA and the diet, the faecal microbiome composition and some metabolic and inflammatory biomarkers (HbA1c, CRP, and s-zonulin).

2. Materials and Methods

2.1. Study Design

Consecutive subjects with morbid obesity referred to Innlandet Hospital Trust, Gjøvik, Norway for evaluation of bariatric surgery were evaluated for inclusion in this prospective cohort study. After inclusion (T 1) and before bariatric surgery, the subjects completed a six-month conservative treatment period. This is standard procedure, and the conservative weight loss intervention helps the subjects to adapt to lifestyle changes. There was a follow-up visit six months after surgery (T 2).

2.2. Inclusion Criteria

Subjects 18–65 years of age with morbid obesity (defined as BMI > 40 kg/m^2 or > 35 kg/m^2 with obesity-related complications) were available for inclusion. Subjects with previous major gastrointestinal surgery, organic gastrointestinal disorders, alcohol and drug abuse, major psychiatric disorders, and serious somatic disorders not related to obesity were excluded.

2.3. Interventions

The conservative weight-loss intervention period started with three one-hour-long visits separated by one week; consulting a nurse, a nutritionist and a physician. The participants were given individualized dietary advice, physical activity programs and information about the operation and consequences of the operation. Some weeks later, they participated in weekly group meetings for seven weeks chaired by nurses, nutritionists, surgeons and a psychologist. The last three weeks before surgery, they followed a strict "crispbread diet" containing 4200 kJ of energy [15].

Three experienced surgeons performed bariatric surgery with one of two standard methods, either Roux-en-Y gastric bypass or gastric sleeve, chosen at the surgeons' discretion [16,17].

2.4. Variables

The following variables were collected at inclusion (T 1) and six months after bariatric surgery (T 2):

Demographic and anthropometric data including age (years), gender (male/female) smoking habits (daily smoking/ not daily smoking), height (m), body weight (kg) and body mass index (BMI; kg/m^2), and present and previous diseases.

A blood sample was analyzed for a range of haematological and biochemical variables including C-reactive protein (CRP, normal range < 3.0 mg/L; a marker of inflammation), HbA1C (normal range < 5.6%; a marker of metabolic health) and serum zonulin (normal range < 38 ng/mL; a marker of gastrointestinal permeability). CRP and HbA1C were analyzed with a Cobas c501 instrument with the reagents CRPL3 and Tina-quant HbA1C (Roche Diagnostics GmbH, 68305 Mannheim, Germany), and s-zonulin was measured with an ELISA kit (Immundiagnostik, 64625 Bensheim, Germany).

Dietary habits were assessed with a self-reported food frequency questionnaire (FFQ) constructed and validated by the University of Oslo [18]. The University of Oslo calculated the daily intake of nutrients and supplements including non-nutritive sweeteners (NNS) based on the Norwegian food composition table [19]. One unit of NNS was defined as 100 mL of beverages sweetened with NNS, or two tablets/teaspoons of NNS.

The faecal material for the analyses of the microbiota and SCFA was collected by the subjects at home in a "Sample Collection Kit" provided by Genetic Analysis AS, Oslo, Norway, the company that analyzed the microbiota composition, and handled according to their recommendations: *"The kit is designed to ensure hygienic and easy sampling of the faecal material and can be performed at home. No additives are required. The sample should be stored in room temperature and reach the laboratory within 5 days"* [20]. Upon arrival to the hospital, the samples were immediately stored at −80 °C and later transported in

batches for the analyses of the microbiota. Afterwards, the samples were transferred to Unger-Vetlesen Institute, Oslo, Norway, for the analyses of SCFA. All the time from arrival to the hospital to the last analyses had been performed, the samples were stored at −80 °C.

The faecal microbiota composition was analyzed with the commercially available, CE marked, US and European patented, GA-map™ dysbiosis test (Genetic Analysis AS, Oslo, Norway) [21,22]. The test reports the degree of dysbiosis as Dysbiosis Index (DI; range 1–5). Values above 2 indicate a microbiota composition that differs from a reference population. Also, the relative abundance of 39 bacteria at different taxonomic levels are reported as score -3 to 3 relative to the reference population. Twenty-four of the bacteria were from the phylum Firmicutes and eight from Bacteroidetes. The relative abundance of bacteria from the phyla Firmicutes and Bacteroidetes were calculated as the mean of the relative scores from the bacteria in these phyla. Note that the bacteria measured with the actual method do not represent the entire phyla but only parts of the phyla.

Faecal short-chain fatty acids (SCFA) were analyzed as described by Zijlstra et al. and modified by Høverstad et al. [23,24]. The distillate was analyzed with gas chromatography and quantified by using internal standardization. Flame ionization detection was employed. The total amount of all SCFA and the amount of acetic-, propionic-, butyric-, isobutyric-, valeric-, isovaleric-, caproic-, and isocaproic-acids was measured and expressed in mmol/kg wet weight. Some subjects had two analyses of faecal SCFA, the one that was planned six months after surgery and an extra one 12 months after surgery. Some had a test only after 12 months. In subjects with two analyses, there were no significant differences between the results. Therefore, in subjects with only one measurement, the results of the available test 6 or 12 months after surgery were used. In subjects with two analyses, the mean values of the two tests were used.

2.5. Statistics

A linear mixed model was used for the majority of the analyses. The dependent variables appear in the result section. Subject was the random effect. Explanatory variables were the point of time (a two-level categorical covariate), the mean of age and gender, type of operation, and various variables presented in the result section. When appropriate, interaction analyses were performed. Associations between the changes in SCFA and changes in nutrients, biological markers and the microbiota composition were analyzed with linear regression adjusted for age and gender. The analyses were performed with IBM SPSS Statistics for Windows, version 25.0 (IBM Corp., Armonk, NY, USA). P-values < 0.05 were judged as statistically significant. The sample size was fixed by the available study population and no power calculation was performed during the planning of the study.

2.6. Ethics

The study was approved by the Regional Committee for Medical and Health Research Ethics South-East Norway (reference 2012/966) and conducted in accordance with the Declaration of Helsinki. All participants gave written informed consent before inclusion in the study.

3. Results

3.1. Subjects

Out of 239 subjects available for inclusion, 80 refused to participate, 7 with previous or present somatic disorders were erroneously included and later excluded, 21 had no operation, and 41 did not provide faecal samples. In total, 15 (17%) men and 75 (83%) women with a mean age of 44.4 (SD 8.4) years and BMI 41.7 (SD 3.7) kg/m^2 were included in the analyses and 80 had a follow-up visit six months after surgery. At inclusion, BMI was higher in men than in women (difference of 2.99 kg/ m^2 (CI: 0.23 to 3.76; $p = 0.027$)) and decreased 0.15 kg/m^2 per year as age increased (CI: 0.07 to 0.23; $p < 0.001$). The mean reduction in BMI after the interventions was 12.70 kg/m^2. (CI: 12.03 to 13.38; $p < 0.001$). Roux-en-Y gastric bypass was performed in 73 (81%) and gastric sleeve in 17 (19%).

The weight-loss was significantly higher in subjects operated with Roux-en-Y gastric bypass than in those operated with gastric sleeve, difference 1.73 kg/m^2 (CI: 0.03 to 3.42; $p = 0.046$).

3.2. Short-Chain Fatty Acids

Total SCFA levels were reduced after treatment. The absolute and relative amounts of all the SCFA at inclusion and the changes after treatment are given in Table 1. The dependent variables were not associated with the type of operation. The major straight SCFA (acetic-, propionic-, and butyric- acids) changed principally in the same way, as did the branched SCFA (isobutyric-, isovaleric-, and isocaproic-acids). Therefore, in the further analyses they were considered as two groups. The absolute and relative amounts of the straight SCFA were reduced and the absolute and relative amounts of the branched SCFA were increased.

Table 1. The total and relative amounts of short-chain fatty acids (SCFA) at inclusion and changes after the weight-loss interventions. Analyzed with mixed model adjusted for point of time and the means of age and gender.

Dependent Variable	At Inclusion		Change T2 [4] Minus T1 [3]		Statistics (p-Value)
	Mean	95% CI	Mean	95% CI	
Total SCFA [1]	36.96	33.34; 40.59	−5.61	−10.43; −0.79	**0.023**
Acetic acid [1]	20.28	18.37; 21.18	−3.78	−6.33; −1.23	**0.004**
Acetic acid (proportion [2])	55.14	53.76; 56.52	−1.66	−3.70; 0.38	0.109
Propionic acid [1]	6.49	5.73; 7.26	−1.03	−2.05; −0.01	**0.048**
Propionic acid (proportion [2])	17.40	16.49; 18.32	−0.42	−1.58; 0.72	0.461
Butyric acid [1]	7.23	6.35; 8.12	−1.31	−2.50; −0.13	**0.031**
Butyric acid (proportion [2])	18.97	17.89; 20.04	−0.38	−1.77; 1.00	0.582
Valeric acid [1]	1.01	0.86; 1.16	0.01	−0.20; 0.22	0.904
Valeric acid (proportion [2])	2.68	2.42; 2.94	0.56	0.21; 0.91	**0.002**
Caproic acid [1]	0.31	0.23; 0.40	−0.06	−0.17; 0.06	0.353
Caproic acid (proportion [2])	0.79	0.56; 1.02	0.17	−0.14; 0.47	0.281
Isobutyric acid [1]	0.70	0.60; 0.81	0.22	0.08; 0.36	**0.002**
Isobutyric acid (proportion [2])	2.01	1.78; 2.22	0.90	0.55; 1.24	**< 0.001**
Isovaleric acid [1]	1.02	0.87; 1.18	0.36	0.15; 0.57	**0.001**
Isovaleric acid (proportion [2])	2.94	2.60; 3.28	1.41	0.96; 1.86	**< 0.001**
Isocaproic acid [1]	0.00	0.00; 0.00	0.00	−0.00; 0.00	0.753
Isocaproic aicd (proportion [2])	0.00	−0.00; 0.01	0.0	−0.01; 0.01	0.803
Straight SCFA [1,5]	33.93	30.60; 37.26	−6.11	−10.59; -1.63	**0.008**
Straight SCFA [5] (proportion [2])	91.60	90.79; 92.41	−2.77	−3.79; −1.75	**<0.001**
Branched SCFA [1,6]	1.72	1.46; 1.97	0.59	0.25; 0.93	**0.001**
Branched SCFA [6] (proportion [2])	4.95	4.40; 5.50	2.31	1.54; 3.08	**<0.001**

[1] mmol/kg wet weight. [2] The proportion is given as the percentage of total SCFA. [3] T1: At inclusion. [4] T2: 6 months after surgery. [5] The sum of acetic-, propionic-, and butyric- acids. [6] The sum of isobutyric-, isovaleric-, and isocaproic- acids.

3.3. Nutrients, Blood Tests, Type of Surgery and Faecal Microbiota Composition

The energy intake was significantly reduced after the weight-loss interventions. Except for an increase in the relative energy amount of protein and fiber, there was a reduction in all absolute and relative amounts of the nutrients after treatment. The markers of inflammation (CRP), metabolic syndrome (HbA1C) and gut permeability (zonulin) normalized. There was a change in the faecal microbiota composition towards dysbiosis and an increase in the relative amount of Firmicutes. Table 2 gives the details. The type of bariatric surgery was not significantly associated with the changes.

Table 2. The amounts of nutrients (absolute and relative), blood biomarkers and the faecal microbiota at inclusion and changes after the weight-loss interventions. Mixed model adjusted for the means of age and gender.

Dependent Variable	Inclusion		Change T2 [4] Minus T1 [3]		Statistics (*p*-Value)
	Mean	95% CI	Mean	95% CI	
Nutritional variables					
Energy total (KJ)	10662	9647; 11678	−4404	−5359; −3451	**<0.001**
Total food intake (g)	4971	4496; 5447	−1410	−1952; −869	**<0.001**
Protein (g)	112	100; 124	−37	−44; −31	**<0.001**
Protein (energy-%)	18.2	17.5; 19.0	2.0	1.0; 3.0	**<0.001**
Fat (g)	100	89; 111	−44	−54; −34	**<0.001**
Fat (energy-%)	34.2	32.8; 35.6	−0.7	−2.6; 1.1	0.435
Carbohydrates (g)	275	247; 302	−116	−151; - 80	**<0.001**
Carbohydrates (energy-%)	44.1	42.5; 45.8	−1.8	−3.9; 0.4	0.102
Sugar (g)	46	32; 59	−26	−46; −6	**0.011**
Sugar (energy-%)	6.4	5.1; 7.7	−1.9	−3.7; −0.2	**0.032**
Starch (g)	134	124; 145	−65	−78; −53	**<0.001**
Starch (energy-%)	21.9	20.6; 23.1	−2.7	−4.3; −1.0	**0.002**
Fibre (g)	35	32; 37	−12	−15; −10	**<0.001**
Fibre (energy-%)	2.8	2.6; 3.0	0.2	−0.1; 0.4	0.139
NNS (units) [1]	8.0	6.0; 10.0	−2.8	−5.2; −0.5	**0.020**
Blood biomarkers					
CRP	6.9	6.0; 7.8	−5.0	−6.1; −4.0	**<0.001**
HbA1C	6.0	5.7; 6.2	−0.7	−0.9; −0.5	**<0.001**
Zonulin (ng/ml)	65	59; 70	−35	−44; −27	**<0.001**
Microbiota					
Dysbiosis Index (score 1–5)	2.7	2.5; 3.0	1.4	0.9; 1.9	**<0.001**
Firmicutes (mean score) [2]	−0.00	−0.04; 0.04	0.16	0.09; 0.22	**<0.001**
Bacteroidetes (mean score) [2]	0.43	0.37; 0.50	−0.08	−0.19; 0.03	0.151

[1] NNS: Non-nutritive sweeteners. One unit of NNS was 100 mL beverage with NNS or two tablets/teaspoons of NNS. [2] Score range: −3; 3. [3] T1: At inclusion. [4] T2: 6 months after surgery.

3.4. Associations between SCFA Levels and Other Variables

There were significant positive associations between total SCFA and the sum of the straight SCFA and the intake of energy, protein, fat, and starch, but no significant associations with the blood biomarkers and the faecal microbiota composition markers. Table 3 gives the details. Type of surgery was not significantly associated with the SCFA levels, and there were no significant interactions with the point of time.

Out of the associations between changes in total, straight, and branched SCFA on one side (dependent variables) and changes in the nutrients, biological markers and the microbiota composition on the other side, the only significant association was between the change in branched SCFA and the change in the intake of starch. (B: −0.12 (CI: −0.022 to −0.002); partial correlation: −0.344; $p = 0.019$. All results are given in Table 4.

Table 3. Associations between the SCFA levels and the nutrients, biological markers and the microbiota composition markers analyzed with mixed model adjusted for the point of time and the mean of age and gender.

Independent Variables	Dependent Variables					
	Total SCFA (mmol/kg Wet Weight)		Straight SCFA [1] (mmol/kg Wet Weight)		Branched SCFA [2] (mmol/kg Wet Weight)	
	B (95% CI)	*p*-Value	B (95% CI)	*p*-Value	B (95% CI)	*p*-Value
Nutritional variables						
Energy total (KJ) [3]	1.10 (0.14; 2.05)	**0.026**	1.06 (0.18; 1.94)	**0.019**	0.00 (−0.06; 0.08)	0.803
Total food intake (g) [3]	1.55 (−0.16; 3.12)	0.052	1.14 (−0.27; 2.85)	0.054	0.07 (−0.5; 0.18)	0.246
Protein (g)	0.16 (0.06; 0.26)	**0.002**	0.15 (0.06; 0.24)	**0.002**	0.00 (−0.00; 0.01)	0.201
Fat (g)	0.13 (0.04; 0.21)	**0.004**	0.12 (0.04;0.20)	**0.003**	0.00 (−0.00; 0.01)	0.635
Carbohydrates (g)	0.01 (−0.01; 0.04)	0.350	0.01 (−0.01; 0.04)	0.299	−0.00 (−0.00; 0.00)	0.779
Sugar (g)	−0.03 (−0.07; 0.02)	0.305	−0.02 (−0.07; 0.02)	0.325	−0.00 (−0.01; 0.00)	0.379
Starch (g)	0.08 (0.01; 0.15)	**0.027**	0.08 (0.01; 0.14)	**0.018**	0.00 (−0.00; 0.01)	0.960
Fibre (g)	0.23 (−0.08; 0.54)	0.146	0.23 (−0.06; 0.51)	0.120	−0.00 (−0.02; 0.02)	0.984
NNS (units) [4]	−0.14 (−0.50; 0.23)	0.460	−0.11 (−0.45; 0.22)	0.501	−0.00 (−0.01; 0.01)	0.620
Blood biomarkers						
CRP (mg/L)	0.27 (−0.39; 0.92)	0.426	0.25 (−0.35; 0.86)	0.409	0.00 (−0.05; 0.05)	0.977
HbA1C (%)	−1.48 (−3.93; 0.97)	0.234	−1.45 (−3.70; 0.80)	0203	−0.01 (−0.18; 0.17)	0.932
Zonulin (ng/ml)	-0.02 (-0.12; 0.08)	0.672	−0.02 (−0.11; 0.07)	0.669	0.00 (−0.01; 0.01)	0.718
Microbiota						
Dysbiosis Index (score: 1 to 5)	0.27 (−2.14; 2.69)	0.822	0.19 (−2.04; 2.43)	0.864	0.10 (−0.07; 0.27)	0.237
Firmicutes (score: -3 to 3)	−12.4 (−29.8; 4.9)	0.159	−11.2 (−27.2; 4.8)	0.169	−0.80 (−2.00; 0.40)	0.190
Bacteroidetes (score: -3 to 3)	−3.24 (−13.20; 6.72)	0.521	−2.63 (−11.82; 6.56)	0.572	−0.47 (−1.14; 0.21)	0.173

[1] The sum of acetic-, propionic-, and butyric- acids. [2] The sum of isobutyric-, isovaleric-, and isocaproic- acids. [3] The B-values with CI are given as x 10^{-3}.[4] NNS: Non-nutritive sweeteners. One unit of NNS was 100 mL beverage with NNS or two tablets/teaspoons of NNS.

Table 4. Associations between changes in the SCFA levels and changes in nutrients, blood biomarkers and faecal microbiota composition markers (linear regression adjusted for age and gender).

Independent Variables	**Dependent Variables**					
Changes	**Changes in Total SCFA (mmol/kg Wet Weight)**		**Changes in Straight SCFA [1] (mmol/kg Wet Weight)**		**Changes in Branched SCFA [2] (mmol/kg Wet Weight)**	
	B (95% CI)	***p*-Value**	**B (95% CI)**	***p*-Value**	**B (95% CI)**	***p*-Value**
Nutritional variables						
Energy total (KJ)	0.000 (−0.001; 0.002)	0.605	0.001 (−0.001; 0.002)	0.535	0.000 (0.000; 0.000)	0.161
Total food intake (g)	0.001 (−0.002; 0.004)	0.497	0.001 (−0.002; 0.004)	0.495	0.000 (0.000; 0.000)	0.895
Protein (g)	0.168 (−0.083; 0.418)	0.184	0.166 (−0.065; 0.397)	0.155	−0.004 (−0.022; 0.015)	0.681
Fat (g)	0.079 (−0.093; 0.252)	0.359	0.080 (−0.079; 0.239)	0.315	−0.007 (−0.019; 0.006)	0.272
Carbohydrates (g)	−0.001 (−0.046; 0.045)	0.970	0.001 (−0.041; 0.043)	0.965	−0.002 (−0.005; 0.001)	0.205
Sugar (g)	−0.035 (−0.099; 0.029)	0.280	−0.032 (−0.092; 0.027)	0.282	−0.002 (−0.007; 0.003)	0.425
Starch (g)	0.077 (−0.067; 0.221)	0.287	0.086 (−0.046; 0.218)	0.196	−0.012 (−0.22; -0.002)	0.019
Fiber (g)	0.357 (−0.258; 0.972)	0.249	0.369 (−0.199; 0.936)	0.197	−0.002 (−0.067; 0.023)	0.324
NNS (units) [3]	−0.125 (−1.094; 0.844)	0.796	−0.119 (−1.014; 0.776)	0.790	−0.025 (−0.095; 0.046)	0.485
Blood biomarkers						
CRP (mg/L)	0.779 (−0.298; 1.856)	0.153	0.680 (−0.316; 1.675)	0.176	0.059 (−0.017; 0.136)	0.127
HbA1C (%)	0.776 (−4.444; 5.996)	0.766	0.575 (−4.239; 5.389)	0.811	0.165 (−0.205; 0.535)	0.373
Zonulin (ng/mL)	−0.035 (−0.191; 0.120)	0.651	−0.038 (−0.182; 0.105)	0.596	0.005 (−0.006; 0.016)	0.384

[1] Changes in the sum of acetic-, propionic-, and butyric- acids. [2] Changes in the sum of isobutyric-, isovaleric-, and isocaproic- acids. [3] NNS: Non-nutritive sweeteners. One unit of NNS was 100 mL beverage with NNS or two tablets/teaspoons of NNS.

4. Discussion

The main findings were the significant changes in faecal SCFA levels after a conservative weight-loss intervention followed by bariatric surgery. Six months after surgery, the total amount of SCFA was reduced, the absolute and relative sum of the main straight SCFA (acetic-, propionic-, and butyric- acids) was reduced, and the absolute and relative sum of the branched SCFA (isobutyric-, isovaleric-, and isocaproic- acids) was increased. The results are in accordance with reports of other conservative and surgical treatment alternatives [11,12,25]. The design renders the separation of the effects of the two interventions impossible. Other studies have shown that the effect of weight loss on inflammatory biomarkers (e.g., neopterin [26]) and gastrointestinal permeability (submitted by one of the authors) is unrelated to the changes in BMI and could be even higher after conservative rather than surgical treatment of obesity. The separation of the effects of the two interventions is, however, less important than the overall effect since the procedure is a standard treatment combination for subjects with morbid obesity. The reduction of straight SCFA and increase in branched SCFA indicates reduced saccharolytic and increased proteolytic fermentation, respectively [2,9]. A review concludes that the faecal concentrations of the major straight SCFA are elevated in subjects with obesity [4]. Since valid reference values were unavailable for the method used for the analyses, it is unknown if the major straight SCFA were elevated before treatment and then normalized, or were normal and reduced to subnormal amounts after treatment. Possible causes of the changes in SCFA are changes in the diet, the gut microbiota and their host.

There was a significant reduction in food intake. The absolute and relative amounts of all parts of the diet were reduced, except for a significant increase in the relative amount of protein and a minor increase in fiber. Changes in the diet, in particular polysaccharides and proteins, alter the microbiota and their fermentation products such as SCFA [2,8,9,27–30]. There were significant associations between the dietary intake of energy, protein, fat, and starch on one side and the amount of total and straight SCFA, and a significant negative association between the changes in the intake of starch and branched SCFA. The study confirms the associations between the diet and SCFA. The negative association between the changes in the intake of starch and branched SCFA shows the importance of a carbohydrate-rich diet for the reduction of the proteolytic fermentation. The methods measuring the dietary intake and SCFA were judged as valid and reliable. The paper by Tremaroli et al. reports similar changes in SCFA after bariatric surgery and concludes that the changes were not a consequence of the dietary consumption [12]. NNS, which was used in high amounts by a substantial proportion of the participants, were not associated with changes in SCFA. An association was anticipated since NNS induce marked changes in the gut microbiome [31–34]. Separate analyses during the conservative weight-loss period with primarily dietary restrictions could perhaps have shown more explicit associations between changes in the diet and changes in the SCFA. The surgical procedures probably have different and more substantial impacts on the SCFA than the diet. There were, however no associations between the surgical methods and SCFA levels.

At inclusion, the faecal microbiota composition showed a minor deviation from a reference population (a slight degree of dysbiosis) and a further deviation after surgery. Changes in the gut microbiome composition have been reported in several studies in obese subjects, but there is no agreement concerning the type of deviation and causal relations [3,5]. Changes of the microbiota composition after conservative and surgical weight-loss have also been reported [10,13,14]. The increasing degree of dysbiosis after treatment indicates that the treatment does not reset the microbiota, in fact it is the opposite [35]. The method used for the analyses of the microbiota composition did not allow precise characterization of the microbiota and the changes of the microbiota since the method measured only 39 bacteria at different taxonomic levels. The same changes were in a previous study based on the same material judged as unfavorable ("bad" dysbiosis) [34]. In contrast to the review by Wagner el al., this study showed a significant increase in the relative amount of the phylum Firmicutes and a non-significant reduction in Bacteroidetes [5]. An abundance of Firmicutes and a high Firmicutes/Bacteroidetes ratio have been associated with obesity and judged as unfavorable [36–38].

If correct, the changes observed in this study are thus unfavorable. Note that the phyla do not include the complete phyla but only a selection of the microbes present in the phyla. A better characterization of the microbiome composition seems necessary to show associations between the microbiome composition and faecal SCFA levels.

The treatment has several other important health-related impacts on the subjects, such as metabolic and inflammatory changes, changes in physical activity, use of drugs, and gastrointestinal malabsorption and permeability (factors that have an impact on the gut microbiota and their function). In this study, these factors were limited to the study of CRP (a marker of inflammation), HbA1c (a marker of metabolic syndrome), and zonulin (a marker of intestinal permeability) which showed significant normalization after treatment but were not associated with the amount of, or changes in, SCFA levels. In all, the study gives no clear causative explanation of the changes in SCFA levels. The reduction of the total SCFA levels and the major straight SCFA (indicating reduced saccharolytic fermentation) could be due to a reduced intake of nutrients and carbohydrates [29]. The increase in branched SCFA levels (indicating increased proteolytic fermentation) indicates an increase in proteins in the colon that could be due to the increase in the relative amount of protein in the diet or minor protein malabsorption [39].

The microbial fermentation metabolites are markers for health, but the impact of these products on human health is complex, and the clinical consequences of the changes in faecal SCFA levels are not fully understood [1,9]. Low SCFA levels increase energy intake and reduce energy expenditure [40]. The saccharolytic fermentation with the production of acetic-, propionic-, and butyric acids has some health-promoting effects relating to fatty acids, glucose, and cholesterol metabolism, mineral absorption, the regulation of immune and inflammatory responses, as well as being a source for colonocyte energy and tissue repair including the gut barrier function, and having anti-obesogenic, antioxidant and anticancer effects [7,8,28,41,42]. The proteolytic fermentation with an increase in branched SCFA levels is associated with the production of harmful metabolites such as ammonia, phenols and hydrogen sulphides that have clinical relevance for disorders like irritable bowel syndrome, inflammatory bowel diseases and cancer [40,43,44]. In all, the observed alteration from a saccharolytic to proteolytic fermentation after treatment for morbid obesity seems detrimental. Although the clinical relevance is uncertain, a recommendation of a carbohydrate-, fiber-, and polysaccharide-rich diet aiming at a shift toward a saccharolytic fermentation seems reasonable.

The study included consecutive and unselected subjects with morbid obesity referred to the public obesity unit in the region and was performed as part of the daily routine. Data on comorbidity, complications and pharmacotherapy were incompletely registered. The subjects performed a standard combined conservative and surgical intervention. The results were limited to this group in which the majority were females. The validity of the results for men might be reduced, the validity for subjects with less severe obesity is unknown, and the changes after only conservative or surgical treatment might differ [10]. SCFA measured in faeces do not reflect the colonic SCFA production since the majority of SCFA are absorbed within the colon and only a minor proportion (5%–10%) are excreted in faeces. Faecal SCFA are nevertheless commonly used as a marker of colonic SCFA production. The dietary intake was based on a thoroughly prepared food frequency questionnaire and judged as valid, although registration of the nutrient intake is afflicted with uncertainty. A more detailed and complete analysis of the faecal microbiome composition could have given other results. The metabolic and inflammatory changes and changes in other variables were incompletely recorded, and these results are therefore less reliable. The use of antibiotics, which were not registered, might have influenced the microbiota and their metabolites. Because the clinically important results were highly significant, it is unlikely that correcting for multiple testing, which was not performed, would have changed the main conclusions.

5. Conclusions

This study in subjects with morbid obesity showed significant changes in faecal SCFA levels after a combined conservative and surgical weight-loss intervention. The total amount of SCFA was reduced, the total and relative amounts of the main straight SCFA (acetic-, propionic-, and butyric-acids) were reduced, and the total and relative amounts of the branched SCFA (isobutyric-, isovaleric-, and isocaproic- acid) were increased. These changes indicate an alteration in the balance of saccharolytic and proteolytic fermentation toward a proteolytic fermentation pattern with unfavorable health effects. There were significant associations between the amount of total and straight SCFA and the diet. No associations were seen with the metabolic markers and the faecal microbiome composition markers. Although the metabolic changes after bariatric surgery are complex and only partly characterized in this study that also had other limitations, the recommendation of a carbohydrate-rich diet after bariatric surgery in order to augment the saccharolytic- and reduce the proteolytic- fermentation seems to be reasonable clinical advice.

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Clinical Study

Abnormal Uroguanylin Immunoreactive Cells Density in the Duodenum of Patients with Diarrhea-Predominant Irritable Bowel Syndrome Changes following Fecal Microbiota Transplantation

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Altered densities of enteroendocrine cells play an important role in patients with irritable bowel syndrome (IBS). Uroguanylin activates guanylate cyclase-C to regulate intestinal electrolyte and water transport. *Aim*. To quantify uroguanylin immunoreactive cells density in the duodenum of diarrhea-predominant IBS (IBS-D) patients compared to controls and to investigate the effect of fecal microbiota transplantation (FMT) on these cell densities. *Method*. Twelve patients with IBS-D according to Rome III criteria were included. The cause was identified as post infectious (PI, $n = 6$) or idiopathic ($n = 6$). They completed the IBS-symptom questionnaire before and 3 weeks after FMT. Thirty grams of fresh feces donated from healthy relatives were diluted with 60 ml normal saline and instilled via endoscope into the duodenum. Biopsies were taken from the patients' duodenum before and 3 weeks after FMT. Duodenal biopsies taken from eight healthy controls were also included. The biopsies were immunostained for uroguanylin and quantified using computerized image analysis. *Results*. Uroguanylin immunoreactive cells were found both in duodenal villi and crypts in both controls and IBS-D patients. The densities of uroguanylin immunoreactive cells were significantly lower in the villi ($P < 0.0001$) and higher in the crypts ($P < 0.0001$) for the patients than the controls. Following FMT, the densities of uroguanylin immunoreactive cells for the total group and idiopathic subgroup decreased significantly in the duodenal crypts ($P = 0.049$ and 0.04, respectively) but not in the villi. No significant changes were shown in the PI-IBS subgroups. The cells density in only the crypts correlated with diarrhea ($r = 0.97$, $P = 0.001$) and bloating ($r = -0.91$, $P = 0.01$) in the PI-IBS subgroup before FMT and with abdominal pain ($r = 0.63$, $P = 0.03$) in the total group of IBS-D patients after FMT. *Conclusion*. Altered uroguanylin immunoreactive cells density was found in IBS-D patients compared to controls. Changes in these cells density following FMT correlated with IBS symptoms (diarrhea, bloating, and abdominal pain).

1. Introduction

Irritable bowel syndrome (IBS) is a chronic disorder of the gastrointestinal tract with an unclear cause and several contributing factors such as diet [1], infections [2, 3], genetics [4], gut microbiota [5, 6], and gut hormones [7, 8]. Interactions of the above-mentioned contributing factors with each other have certain effects on the gut-brain axis that may contribute to the development of symptoms related to the gastrointestinal [9, 10] and nervous [11, 12] systems. Previous publications have shown the effect of dietary changes on the symptoms [1, 13–16], gut microbiota [17, 18], and enteroendocrine cells [19–21] in IBS patients. In addition, changing the composition of the gut microbiota through fecal microbiota transplantation (FMT) had a positive clinical outcome not only in cases of *Clostridium difficile* [22, 23] but also in IBS [24–28].

The gut neuroendocrine system regulates all the functions of the gastrointestinal tract and consists of enteroendocrine cells and the enteric nervous system [29]. Enteroendocrine

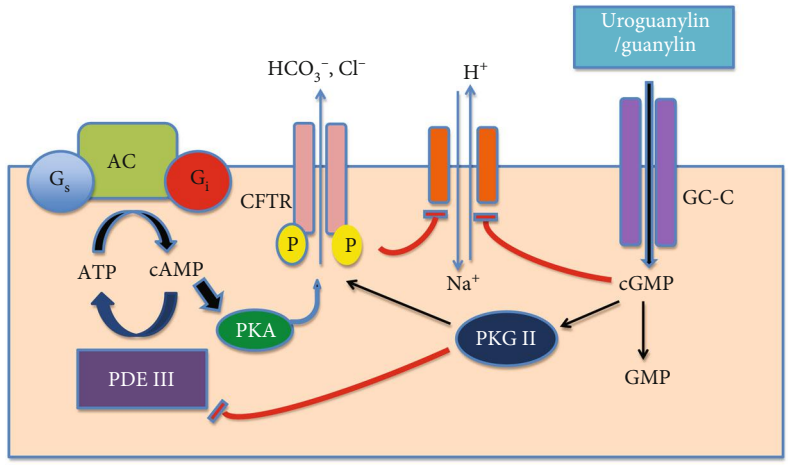


Figure 1: Activation cascade of binding of uroguanylin to guanylate cyclase-C receptor on the intestinal epithelial cell. Binding of uroguanylin to guanylate cyclase-C results in receptor activation, catalyzing the production of cyclic guanosine monophosphate (cGMP). Cyclic GMP can activate cGMP-dependent protein kinase II (PKGII) or inhibit the activity of a cyclic adenosine monophosphate- (cAMP-) specific phosphodiesterase, PDE III, thereby crossactivating cAMP-dependent protein kinase (PKA). PKGII and PKA phosphorylate the cystic fibrosis transmembrane conductance regulator or CFTR, increasing its chloride-secreting activity and preventing the absorption of sodium [37].

cells are specialized cells that spread among the intestinal epithelial cells in both villi and crypts [29]. They have specialized microvilli that project into the gut lumen to act as sensors for the luminal contents and respond to luminal stimuli by releasing hormones that generally target other parts of the digestive system [29]. The guanylin peptide family includes guanylin, uroguanylin, lymphoguanylin, and renoguanylin and is proposed to function as intestinal natriuretic peptides [30]. Uroguanylin, encoded by the *GUCA2B* gene [31, 32], is a 16 amino acid peptide that is secreted by duodenal and proximal small intestinal enterocytes [33]. Uroguanylin acts as an agonist of the guanylyl cyclase receptor guanylate cyclase-C (GC-C) [34–36] by which its activation results in catalyzing the production of cyclic guanosine monophosphate (cGMP) (Figure 1) [37], hence regulating a variety of key processes such as chloride and bicarbonate secretion [37–39], epithelial cell growth, intestinal barrier integrity, and visceral sensitivity [39].

Newly published studies by our group [26, 28] showed that using FMT in patients with diarrhea-predominant IBS (IBS-D) improved their symptoms and changed their gut microbiota profile. Using the same study cohort, the aims of this study were to determine whether there is abnormality in the density of uroguanylin immunoreactive cells in the duodenum of IBS patients compared to healthy controls (controls), to study the effect of changing the gut microbiota through FMT on the density of these cells, and to find the correlations between these cells and IBS symptoms (if any).

2. Methods

2.1. Patients, Donors, and Controls. Patients ($n = 16$) who were referred to the outpatient clinic of gastroenterology, Haukeland University Hospital, Bergen, Norway, met the Rome III criteria for IBS, and scored >175 for the IBS-Symptom Severity Scoring system (IBS-SSS) were included in the current study.

Patients who had a history of inflammatory bowel diseases, GI malignancy, blood in stool, oral thrush, disseminated lymphadenopathy, underwent abdominal surgery, and pregnant or lactating women were excluded. In addition, the exclusion criteria included immunocompromised patients or those taking immunosuppressive medications, had history of opportunistic infections within 1 year, or took probiotics or antibiotics within 1 month prior to fecal transplantation.

Feces donors were healthy family members not complaining of IBS and were 7 males and 9 females with an age range of 20-55 (mean age 35) years. The exclusion criteria for the donors were the same as for the patients.

Biopsy samples from a group of 12 healthy volunteers, 10 females and 2 males with an age range of 20-42 (mean age 39) years, recruited at Stord Hospital, Stord, Norway, by advertising in the local newspapers, were used as controls to study duodenal uroguanylin immunoreactive cells.

The study was performed in accordance with the Declaration of Helsinki [40] and was approved by the Regional Committee for Medical and Health Research Ethics in Western Norway (reference no.: 2013/1497). The current study was registered at ClinicalTrials.gov (ID: NCT03333291). All participants provided written informed consent. All the data concerning uroguanylin immunoreactive cell density for the current study are presented in this article.

2.2. Study Design. As previously explained in details [26], the donors and patients underwent screening one week before FMT and were physically examined and blood tested to exclude contagious infectious agents for the donors (hepatitis A, B, and C; HIV; Epstein-Barr virus; and cytomegalovirus) and organic disorders for the patients according to previously published recommendations [22, 41, 42]. Stool samples from both donors and patients were examined for fecal calprotectin and screened for bacteria, viruses, parasites, and eggs. The patients were instructed not to change their lifestyle or diet

and to report of any change in their life style or use of new medications before the start or during the study.

2.3. FMT Procedure. Thirty grams of fresh feces collected from the donors (<2 hours from production to donation) were manually mixed with 60 ml normal saline and sieved for preparing fecal suspension without any hard particles [22, 41, 42], on the transplantation day. After an overnight fast, 60 ml of fecal suspension followed by 60 ml normal saline were flushed only once through the endoscope into the descending part of the patients' duodenum. Tissue biopsies were taken from the descending part of the duodenum before installation of feces and again after 3 weeks. Fecal samples were collected on the same day of FMT (before the procedure) and 3 weeks after FMT and sent for microbiota analysis using the GA-map™ Dysbiosis Test (Genetic Analysis AS, Oslo, Norway) as previously explained in details [26].

2.4. Questionnaires. The following questionnaires for IBS symptom assessment were completed by the patients before and 3 weeks after FMT: (a) IBS-Symptom Severity Scoring system (IBS-SSS scores < 175 represent mild IBS symptoms, 175–300 represent moderate severity, > 300 represent severe IBS) by which a decrease of 50 points correlates with improvement in clinical symptoms [43] and (b) IBS-symptom questionnaire (IBS-SQ) which reports each of IBS symptoms (nausea, bloating, abdominal pain, constipation, diarrhea, and anorexia) using a severity scale from 0 to 10, where 0 = no symptoms and 10 = severe symptoms [44, 45].

2.5. Gastroscopy and Immunohistochemistry. The patients fasted overnight prior to undergoing a gastroscopy. During the gastroscopy, four tissue biopsies were collected from the descending part of the duodenum, distal to the papilla. The biopsy samples were fixed in formaldehyde and embedded in paraffin. The biopsies were cut into 3 μm thick sections. Antigen retrieval was performed in the PT-Link® system for 20 minutes at 98°C with the Dako® EnVision FLEX Target retrieval buffer at pH 6 (K8005; Dako, Jena, Germany). Peroxidase blocking solution was used for 10 minutes. Thereafter, further blocking with 5% goat serum (X0907; Dako) in 3% bovine serum albumin for 30 minutes was performed. A one-hour incubation with a 1 : 50 dilution of the primary polyclonal rabbit antibody to guanylate cyclase type B was used (GUCA2B, no. LS-C371347, LifeSpan BioSciences Inc., Seattle, WA, USA). The secondary EnVision HRP anti-rabbit (K4011; Dako) antibody was incubated for 30 minutes. The slides were incubated with 3,3′-diaminobenzidine (DAB) solution for 8 minutes followed by counterstaining with hematoxylin.

2.6. Computerized Image Analysis. Uroguanylin immunoreactive cells density was quantified using a light microscope with ×40 objective and a computer software Cell^B imaging program (Olympus, Tokyo, Japan). By keeping the identity of the slides concealed, the quantification was performed by T.M. in 10 nonoverlapping fields. Each field (frame) of epithelial cells represents a tissue area measured at 0.09 mm^2. The density of uroguanylin immunoreactive cells in the duodenal villi was expressed as the number of cells/100 epithelial cells and in duodenal crypts as the number of cells/mm^2 of epithelium.

2.7. Gut Microbiota Analysis. The analysis was previously described in details [26]. Briefly, GA-map™ Dysbiosis Test is based on fecal homogenization and automated total bacterial genomic DNA extraction using magnetic beads. Fifty-four DNA probes were used to target bacterial strains based on their 16S rRNA sequence in seven variable regions (V3–V9). Twenty-six probes detect specific species, 19 probes detect bacteria on the genus level, and 9 probes detect bacteria at higher taxonomic levels. The signal detection was performed by using a BioCode 1000A 128-Plex Analyzer (Applied BioCode, Santa Fe Springs, CA, USA) [46].

3. Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 7 (GraphPad Software, Inc.). Normal distribution of data was tested using the D'Agostino-Pearson omnibus normality test. Kruskal-Wallis with Dunn's post hoc test was used to compare uroguanylin immunoreactive cells density between the controls and the patients before and after receiving FMT. Paired t-test is used to compare between the IBS-SQ symptoms and cell densities of the patients before and after receiving FMT. Mann-Whitney U test was used to compare the gut microbiota profiles of the patients before and after FMT to their respective donors. Correlations were performed using Pearson's test for parametric and Spearman's test for nonparametric datasets. The data are presented as mean ± SEM values. $P < 0.05$ are considered to be statistically significant.

4. Results

Twelve patients (4 females and 8 males, with age range of 20-44 years) completed the study after excluding four patients due to withdrawal of the participation consent for practical reasons ($n = 1$), failed intubation of the endoscope during the gastroscopy session 3 weeks after FMT ($n = 1$), positive stool culture for *Clostridium difficile* ($n = 1$), and being diagnosed with functional dyspepsia ($n = 1$). Six patients suffered from post infectious (PI) IBS and six had idiopathic IBS. They completed the whole study by filling out the questionnaires and delivered stools for microbiota analysis on the same day of undergoing gastroscopy with duodenal biopsies, i.e., the day of FMT, and after 3 weeks. No change in lifestyle, diet, or use of any/new medications has been registered during the study.

4.1. Symptom Questionnaires. The total scores for IBS-SSS and IBS-SQ significantly improved as previously reported [26]. The scores for IBS-SQ showed significant improvements in several of its domains in both groups of IBS as shown in Table 1.

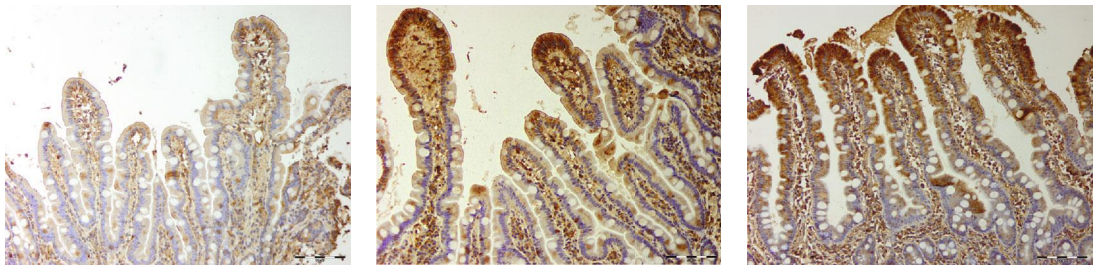
4.2. Gastroscopy, Histopathology, Immunohistochemistry, and Image Analysis. The duodenum of the patients was both macroscopically and microscopically normal.

Uroguanylin immunoreactive cells were found in both the villi (Figure 2) and crypts (Figure 3) of the duodenum,

Table 1: Scores of the irritable bowel syndrome-symptom questionnaire in post infectious and idiopathic irritable bowel syndrome before and after fecal microbiota transplantation.

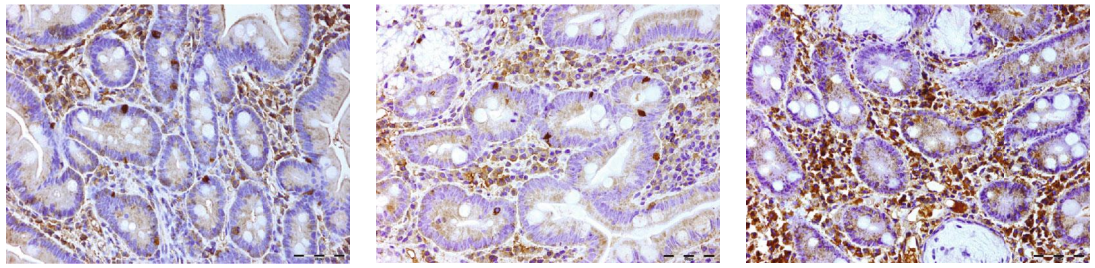
IBS-SQ questionnaire	Post infectious IBS		P value*	Idiopathic IBS		P value**
	Before FMT	3 weeks after FMT		Before FMT	3 weeks after FMT	
Nausea	2 ± 0.7	1 ± 0.6	0.2	5.3 ± 1.4	1.8 ± 0.98	**0.002**
Bloating	7.8 ± 0.5	3 ± 1.2	**0.004**	8 ± 1	3.8 ± 1.3	**0.003**
Abdominal pain	6.3 ± 1.4	4.3 ± 1.2	0.13	6.2 ± 1.2	2.2 ± 1	**0.003**
Constipation	2.7 ± 0.95	1.2 ± 0.8	0.3	5.8 ± 1.9	1.8 ± 1.2	0.1
Diarrhea	5.5 ± 1.5	1.2 ± 0.6	**0.01**	7.3 ± 0.95	1.2 ± 0.5	**0.001**
Anorexia	1 ± 0.5	1 ± 0.5	>0.99	3.5 ± 1.2	0.8 ± 0.5	**0.038**

Comparison: Paired t-test. Data are presented as the mean ± SEM. *Post infectious IBS before vs. after FMT, **idiopathic IBS before vs. after FMT. FMT: fecal microbiota transplantation; IBS-SQ: irritable bowel syndrome-symptom questionnaire.



(a) (b) (c)

Figure 2: Uroguanylin immunoreactive cells in the duodenal villi for patients with irritable bowel syndrome before (a) and after (b) fecal microbiota transplantation and controls (c).



(a) (b) (c)

Figure 3: Uroguanylin immunoreactive cells in the duodenal crypts for patients with irritable bowel syndrome before (a) and after (b) fecal microbiota transplantation and controls (c).

which is consistent with a previous publication [47], in both controls and IBS patients. The staining in the villi was concentrated mainly in the cytoplasm of the epithelial cells. Uroguanylin immunoreactive cells in the crypts, identified as enteroendocrine cells, were either basket- or flask-shaped [48].

At baseline, the densities of uroguanylin immunoreactive cells were significantly lower in the villi ($P < 0.0001$) and higher in the crypts ($P < 0.0001$) for the patients than the controls (Figures 4(a) and 4(b), respectively). Three weeks after FMT, the densities of uroguanylin immunoreactive cells for the total group and idiopathic IBS subgroup decreased significantly in the duodenal crypts ($P = 0.049$ and 0.04, respectively) but not in the villi ($P = 0.5$ and 0.8, respectively, Table 2). No significant changes were shown in either duodenal crypt or villi in the PI-IBS subgroups, Table 2.

4.3. Gut Microbiota. The total changes occurring to the gut microbiota profiles for the donors and the total group of IBS patients before and after FMT were previously described [26, 28]. Briefly, several strains of the gut microbiota, namely, *Ruminococcus gnavus*, *Clostridium sensu stricto*, *Actinobacteria*, *Bifidobacteria*, and *Gardnerella*, for the donors were statistically significantly different from those for the patients before FMT, which normalized for the patients 3 weeks after FMT. No statistically significant differences were found in the bacterial strains when comparing the patients before vs. after FMT.

4.4. Correlations. Using Pearson's test, uroguanylin immunoreactive cells density before FMT correlated positively with diarrhea ($r = 0.97$, $P = 0.001$) and negatively with bloating ($r = -0.91$, $P = 0.01$) in the PI-IBS subgroup (Figures 5(a)

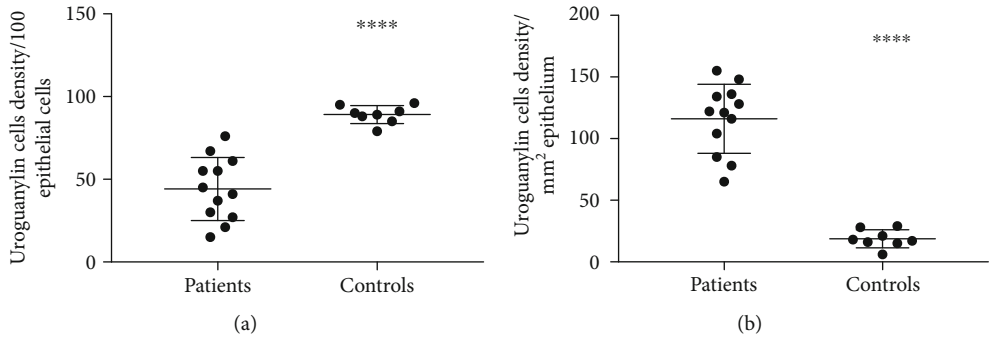


Figure 4: Uroguanylin immunoreactive cells densities in the duodenal (a) villi and (b) crypts, for patients with irritable bowel syndrome and controls.

Table 2: Densities of uroguanylin immunoreactive cells in the duodenum of patients with irritable bowel syndrome before and after fecal microbiota transplantation.

	Uroguanylin immunoreactive cells density		
	Before FMT	After FMT	*P* value
Total IBS group			
Villi (cells/100 epithelial cells)	44 ± 5.5	41 ± 2.3	0.5
Crypts (cells/mm^2)	116 ± 8	96 ± 3	**0.049**
PI-IBS			
Villi (cells/100 epithelial cells)	45.8 ± 7.4	40.5 ± 3.3	0.6
Crypts (cells/mm^2)	108 ± 11.8	102.5 ± 4	0.6
Idiopathic IBS			
Villi (cells/100 epithelial cells)	42.5 ± 8.8	40.7 ± 3.6	0.8
Crypts (cells/mm^2)	124 ± 11	89.5 ± 4	**0.04**

Comparison: Paired *t*-test. Data are presented as the mean ± SEM. IBS: irritable bowel syndrome; PI: post infectious.

and 5(b), respectively). After FMT, uroguanylin cells densitycorrelated positively with abdominal pain ($r = 0.63$, $P = 0.03$) in the total group of IBS patients (Figure 5(c)). No correlations were found between the cells in the villi and IBS symptoms.

Using Spearman's correlation test, significant correlations were found between several bacterial strains and uroguanylin immunoreactive cells density in the duodenal villi and crypts of patients in the total group of IBS patients before and after FMT as shown in Table 3. These cells density also correlated with *Bacteroides fragilis* and *Escherichia*/*Shigella* in the duodenal villi and crypts, respectively, before FMT in the PI-IBS group. No correlations were found between the uroguanylin immunoreactive cells density and gut microbiota in the idiopathic IBS group. The gut microbiota profiles that correlated with the uroguanylin immunoreactive cells density are shown in Table 3.

5. Discussion

The current study describes the differences in the uroguanylin immunoreactive cells density between IBS-D patients and controls. We also investigate how the cells density changes following FMT and correlates to IBS symptoms and gut microbiota before and after FMT.

Gut hormones, which control several functions of the gastrointestinal tract [49, 50], are released by the enteroendocrine cells of the gut for which alterations in the densities of these cells have also been postulated to play an important role in the pathophysiology of IBS [29, 51–55]. To our knowledge, the differences in the uroguanylin immunoreactive cells density between patients with IBS-D and controls have never been described before. Significant differences in the densities of uroguanylin immunoreactive cells were found between IBS-D patients and controls at baseline, and only the density of the cells in the crypts significantly changed following FMT. It is difficult to explain why the changes in the density of uroguanylin immunoreactive cells were specific only to those in the crypts following FMT; however, one may speculate that the cells in the villi may react to different stimuli than those they were exposed to in the current study.

Uroguanylin belongs to the guanylin peptide family that serves as a paracrine/endocrine hormone and acts as agonists of the guanylyl cyclase receptor GC-C, which regulates intestinal electrolyte and water transport [30, 35]. The activation of these receptors by the small heat-stable enterotoxins produced by the enterotoxigenic *Escherichia coli* causes an increase in chloride ion secretion, leading to secretory diarrhea [56, 57]. This explains the correlations between uroguanylin immunoreactive cells density and diarrhea in PI-IBS patients at baseline before FMT. Correlations between uroguanylin and other IBS symptoms such as bloating and abdominal pain are most probably due to the same mechanism of the GC-C and cGMP pathway activation leading to reduced visceral hypersensitivity [58, 59], thus linking it to the gut-brain axis. Plecanatide®, a medication structurally related to uroguanylin, has newly been introduced to the market for the treatment of abdominal pain and constipation in patients with constipation-predominant IBS [60].

Currently, no data is present to explain the correlations between uroguanylin cells and other bacterial strains other than those that produce small heat-stable-like toxins (other *Escherichia coli* species, *Yersinia enterocolitica*, *Vibrio cholerae*, *Salmonella* species, and *Klebsiella pneumoniae*) [61]. However, *Bacteroides fragilis*, which correlates with uroguanylin cells in the crypts, is a bacterium that disrupts tight junctions

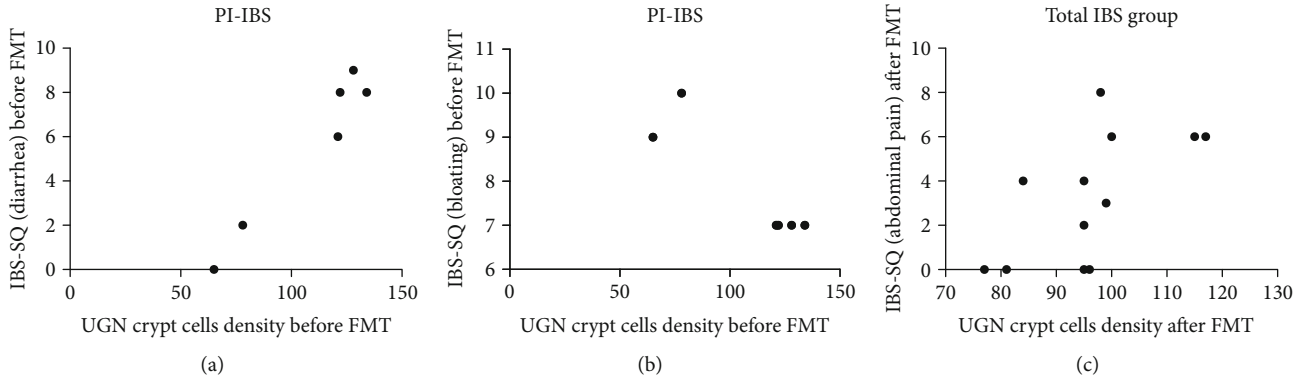


Figure 5: Correlations between scores of irritable bowel syndrome-symptom questionnaire (IBS-SQ) for (a) diarrhea, (b) bloating, and (c) abdominal pain, and uroguanylin (UGN) immunoreactive cells density of the crypt in post infectious (PI-IBS) and total group of patients with irritable bowel syndrome before and after fecal microbiota transplantation (FMT).

Table 3: Gut microbiota profiles that correlated with uroguanylin immunoreactive cells density in the duodenal villi and crypts of patients with irritable bowel syndrome before and after fecal microbiota transplantation.

Bacterial strain	Bacterial signals		P^*	Correlations with uroguanylin cells density (r, P^{**})	
	Before FMT	After FMT		Before FMT	After FMT
Total IBS group					
Bacteroides fragilis	39 ± 23	37 ± 16	0.72	Crypts (-0.81, 0.02)	—
Parabacteroides	7.9 ± 3	14.6 ± 5	0.24	—	Crypts (0.796, 0.01)
Alistipes	107 ± 25	127 ± 27	0.2	—	Crypts (0.71, 0.038)
Streptococcus sanguinis and *S. thermophilus*	171 ± 80	78 ± 42	0.59	—	Villi (0.69, 0.047)
PI-IBS group					
Bacteroides fragilis	65 ± 26	36 ± 28	0.4	Villi (0.94, 0.02)	—
Escherichia/Shigella	141 ± 104	181 ± 135	0.4	Crypts (0.94, 0.02)	—

Comparison: Paired t-test. Data are presented as the mean ± SEM. Spearman's correlation test (r) was used to correlate between the bacterial strain and uroguanylin immunoreactive cells density in the duodenal villi and crypts. *Comparison between the signals before and after FMT; **P values for correlations. FMT: fecal microbiota transplantation; IBS: irritable bowel syndrome; PI: post infectious.

by proteolytic degradation of tight junction proteins using enterotoxin or fragilysin leading to diarrhea [61]. This toxin is more potent when it acts on the basolateral rather than the apical part of the epithelial surface [62].

The relationship between uroguanylin and guanylin and their receptor is not limited to gastrointestinal disorders but also involves renal disorders, colorectal cancer, metabolic syndrome, and mental disorders among others [39]. Recent studies identified a novel role for uroguanylin in obesity by regulating satiety via the gut-brain axis [47].

The current study has several limitations such as the small number of the participants and the lack of a placebo-controlled group from which tissue biopsies could have been taken from. For this exploratory and explanatory study, we aimed to study if there were any differences in the uroguanylin enteroendocrine cells between the different subgroups of IBS-D patients. Despite the small sample size of the different IBS-D subgroups, we found significant differences in uroguanylin immunoreactive cells density before and after FMT; however, further studies with larger groups of participants are required to be able to draw firm conclusions.

6. Conclusions

Altered uroguanylin immunoreactive cells density has been found in IBS-D patients compared to controls. Changes in these cells density following FMT correlated with IBS symptoms (diarrhea, bloating, and abdominal pain). The current study shows that both gut microbiota and the enteroendocrine cells play an important role in the pathophysiology of IBS.

Data Availability

"All the data concerning uroguanylin immunoreactive cells density for the current study are presented in this article." So the data used to support the findings of this study are included within the article. A study protocol is already published as a supplementary in PLOS One with the provided doi: 10.1371/journal.pone.0194904.s00.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

T.M. included the patients, performed gastroscopies and FMT, quantified the enteroendocrine cells, drafted the manuscript, analyzed the data, and planned the study. Ø.E. prepared the slides, supplied the antibodies for immunohistochemistry, performed the immunohistochemistry, and read and commented on the manuscript. G.A.L and T.H. included the patients, performed gastroscopies and FMT, commented on the manuscript, and planned the study.

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OPEN ACCESS

ORIGINAL RESEARCH

Efficacy of faecal microbiota transplantation for patients with irritable bowel syndrome in a randomised, double-blind, placebo-controlled study

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ABSTRACT

Objective Faecal microbiota transplantation (FMT) from healthy donors to patients with irritable bowel syndrome (IBS) has been attempted in two previous double-blind, placebo-controlled studies. While one of those studies found improvement of the IBS symptoms, the other found no effect. The present study was conducted to clarify these contradictory findings.

Design This randomised, double-blind, placebo-controlled study randomised 165 patients with IBS to placebo (own faeces), 30 g FMT or 60 g FMT at a ratio of 1:1:1. The material for FMT was obtained from one healthy, well-characterised donor, frozen and administered via gastroscope. The primary outcome was a reduction in the IBS symptoms at 3 months after FMT (response). A response was defined as a decrease of 50 or more points in the total IBS symptom score. The secondary outcome was a reduction in the dysbiosis index (DI) and a change in the intestinal bacterial profile, analysed by 16S rRNA gene sequencing, at 1 month following FMT.

Results Responses occurred in 23.6%, 76.9% ($p<0.0001$) and 89.1% ($p<00.0001$) of the patients who received placebo, 30 g FMT and 60 g FMT, respectively. These were accompanied by significant improvements in fatigue and the quality of life in patients who received FMT. The intestinal bacterial profiles changed also significantly in the groups received FMT. The FMT adverse events were mild self-limiting gastrointestinal symptoms.

Conclusions FMT is an effective treatment for patients with IBS. Utilising a well-defined donor with a normal DI and favourable specific microbial signature is essential for successful FMT. The response to FMT increases with the dose.

Trial registration
www.clinicaltrials.gov (NCT03822299) and www.cristin.no (ID657402).

Significance of this study

What is already known on this study?
- The intestinal bacterial profile of patients with irritable bowel syndrome (IBS) differs from that of healthy subjects.
- The low bacterial diversity (dysbiosis) in patients with IBS might contribute to the pathophysiology of IBS.
- Faecal microbiota transplantation (FMT) has been investigated in two previous double-blind, placebo-controlled studies. One of those studies found improvement of the IBS symptoms, whereas the other found no effect.

What are the new findings?
- FMT is an effective treatment for IBS that improves abdominal symptoms, fatigue and quality of life.
- The use of a superdonor is necessary for successful FMT.

How might it impact on clinical practice in the foreseeable future?
- This study demonstrates the effectiveness of FMT in the treatment of IBS.
- The use of frozen faeces administered via a gastroscope makes FMT easy to perform in the clinic.

INTRODUCTION

Irritable bowel syndrome (IBS) is a relatively common gastrointestinal disorder with an estimated prevalence of 11.2% in the global population.[1 2] Although IBS does not increase mortality, it reduces the quality of life considerably.[1 2] The aetiology of IBS is not completely understood, and there is no effective treatment for the condition.[1] The gut microbiota in patients with IBS differs from that of the healthy subjects, including exhibiting a low bacterial diversity (dysbiosis).[1 3–5] It is believed that microbiota dysbiosis is one of the factors contributing to the aetiology of IBS.[1]

The application of faecal microbiota transplantation (FMT) in several open-label trials with small cohorts of patients with IBS has produced good results.[1] One recent randomised double-blind, placebo-controlled study of FMT found positive results for FMT, whereas another found no effect.[6 7] The present study was carried out to resolve these contradictory findings.

METHODS

Trial design

The patients included in this single-centre, randomised, double-blind, placebo-controlled, parallel-group study were seen twice: at the baseline and 1 month after transplantation. At the

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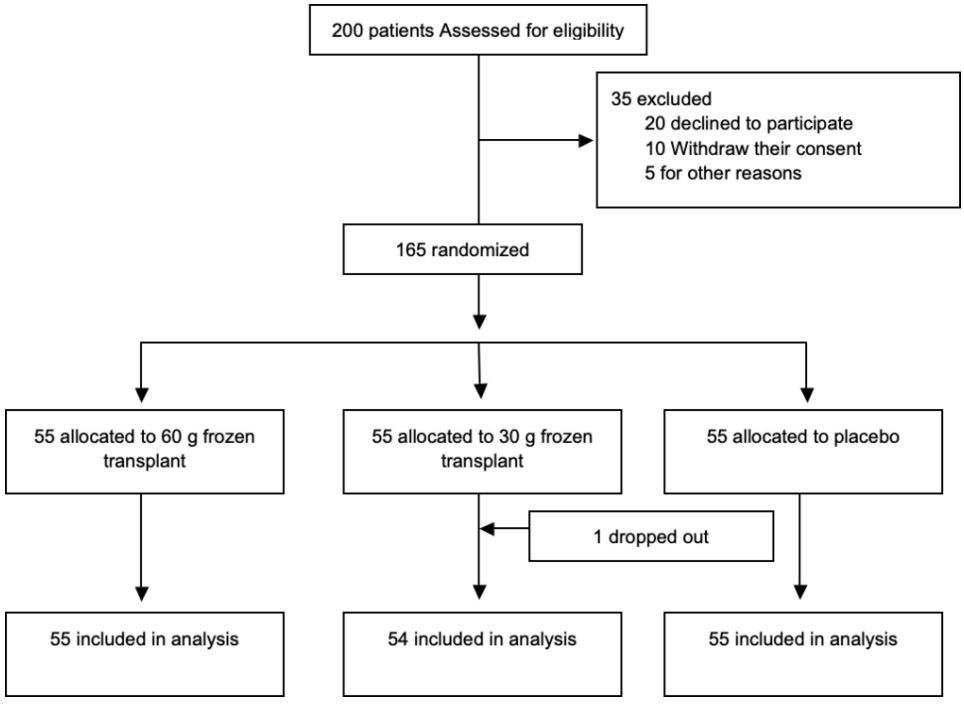

Figure 1 Consolidated standards of reporting trials) diagram showing the enrolment and randomisation of the patients.

baseline visit, the patients provided a faecal sample and were asked to complete five questionnaires to assess their abdominal symptoms, fatigue and quality of life. At the second visit, the patients provided a faecal sample and completed a new set of questionnaires. The patients also completed similar sets of questionnaires at 2 weeks and 3 months after FMT, and returned them by post. The patients were asked to keep a diary to record bowel habits and register any adverse events. Polyethylene glycol and loperamide were allowed during the intervention as rescue medication. The frequency of using these rescue medications in the three intervention groups was not recorded.

Enrolment and randomisation of patients

Randomisation

The patients were randomised to the placebo (30 g of their own faeces), 30 g FMT and 60 g FMT groups at a ratio of 1:1:1 in blocks of six using a Web-based system (http://www.randomization.com) by a nurse who was not involved in the trial (figure 1). The patients delivered two faecal samples at the start of the trial to the same nurse: one was used for gut bacterial analysis in all patients, while the other was either used for transplantation (in patients randomised to placebo) or discarded (in patients randomised to 'superdonor' FMT, as described later). The researcher who prepared the transplant was not aware of the identity of the faecal sample used for transplantation. The patients and researchers involved in the study were blinded to the randomisation. The randomisation key was revealed to the researcher and patients after the trial had ended.

Patients

Patients who fulfilled the Rome IV criteria for the diagnosis of IBS without red flags were recruited from those attending the outpatient clinic at Stord Hospital. All of the patients had been experiencing IBS symptoms for a long time (mean=17 years, range=9–25 years), and the onset of symptoms had not been associated with gastrointestinal or other infections. The medical history was obtained for all patients, and they underwent a complete physical examination as well as blood tests for full blood count, electrolytes and inflammatory markers including faecal calprotectin, liver tests and thyroid function tests. They also underwent a gastroscopy with duodenal biopsies and a colonoscopy with segmental biopsies to exclude other gastrointestinal diseases.

The patients had not previously consumed a low-fermentable oligo-, di-, monosaccharides and polyols diet. All of the recruited patients adhered to a diet consistent with the modified guidelines of the National Institute for Health and Care Excellence (NICE) at least for 3 months, which did not result in a marked improvement in symptoms, including in the bowel habit, abdominal pain or abdominal bloating/distension. These patients were considered as non-responders to a NICE-modified diet,[8] and they stopped consuming that diet on entering the trial. They underwent a 12-hour course of IBS treatment lasting 2 days that provided with information delivered by a gastroenterologist, specialist nurse, psychiatrist, dietitian and physiotherapist.[9] The patients' symptoms improved slightly after participating in the course, which is consistent with previously published data related to a similar course.[9]

The following inclusion criteria were applied:

1. Aged 18–85 years.
2. Moderate-to-severe IBS symptoms, as indicated by a score of ≥175 on the IBS Severity Scoring System (IBS-SSS).

The exclusion criteria were as follows:

1. Presence of systemic disease, immune deficiency or treatment with immune-modulating medication.
2. Pregnant, planning pregnancy or lactating.
3. Having undergone any abdominal surgery, with the exception of appendectomy, cholecystectomy, caesarean section and hysterectomy.
4. Severe psychiatric disorder, or alcohol or drug abuse.
5. Use of probiotics or treatment with antibiotics within 8 weeks prior to study entry.
6. Use of IBS medication within the previous 3 months, with the exception of polyethylene glycol and loperamide.

Donor

A single superdonor was recruited and screened according to the European guidelines for donors for FMT.[1 10] This involved interviewing him to obtain his medical history and lifestyle habits to exclude any exposure to infectious agents or risky social or sexual behaviour such as drug abuse. He also underwent a physical examination as well as blood tests to exclude gastrointestinal, metabolic or neurological disorders (full blood count, blood glucose, electrolytes and inflammatory markers), liver tests and thyroid function tests. Serology screening tests for HIV, syphilis and hepatitis A, B and C were also performed. Stool culturing was performed for pathogenic bacteria (*Shigella* spp., *Salmonella* spp., *Campylobacter* spp., *Yersinia* spp. and toxin-producing *Clostridioides difficile*). Rotavirus and stool ova and parasites were also examined. The findings of all of these tests and examinations were negative.

The donor was an athletic Caucasian male aged 36 years. He was a non-smoker, healthy, not taking any medication and had a BMI of 23.5 kg/m^2. He was not related to any of the patients in the trial. His mother (a medical professional) confirmed that he was born via a vaginal delivery, breastfed and had been treated three times with antibiotics during his life. He trained for 1 hour five times weekly. The MoBa Food Frequency Questionnaire was used to determine his frequency of consuming 225 food items grouped according to the Norwegian meal patterns over the previous 12 months, and his answers were analysed using software for nutrient calculations.[11] The superdonor's diet was within the normal range of those of 35 previously examined healthy subjects.[11] However, the superdonor regularly took dietary supplements rich in proteins, vitamins, fibre and minerals (online supplementary table 1) that made his diet richer than average in those substances.

His microbiota in a faecal sample was analysed using the GA-map Dysbiosis Test (Genetic Analysis, Oslo, Norway),[3] which revealed a dysbiosis index (DI) of 1, indicating normobiosysis. Despite the donor having a DI of 1, deviation from the expected normal abundance was observed in 14 of the 39 bacteria markers. These deviating bacteria belong to the typical commensal bacteria species, and increases or decreases in their abundances are not considered to contribute to dysbiosis. In all, 12 of the bacteria were in the phylum Firmicutes, with one each in the phyla Proteobacteria and Verrucomicrobia. The other 25 more opportunistic bacteria markers that showed abundances similar to normal are important candidates in a dysbiotic condition (figure 2). The donor had donated his faeces in 18 months, and his faeces samples were tested every 3 months. The samples remained normobiotic, with only minor variations in the constituent bacteria (figure 3).

Faecal sample collection, preparation and administration

Faecal samples from the superdonor and the patients were frozen immediately and kept at –20°C until they were delivered frozen to the laboratory on the same day. They were kept at –80°C in the laboratory, and thawed at 4°C for 2 days before transplantation. On the day of transplantation, the faecal samples were weighed, and 30 g and 60 g portions were mixed with 40 mL of sterile saline (0.9 NaCl), filtered through a 110 cm × 10 cm non-woven swab (One Med, Helsinki, Finland), drawn into 50 mL sterile syringes, sealed and kept at 4°C until the time of transplantation. Each transplant was administered to the distal duodenum via the working channel of a gastroscope, which was then flashed with another 40 mL of sterile saline.

Measures

Abdominal symptoms, fatigue and quality of life

Abdominal symptoms were assessed using the IBS-SSS and the Birmingham IBS Symptom (Birmingham IBS-S) questionnaires.[12 13] Fatigue was measured using the Fatigue Assessment Scale (FAS).[14] Quality of life was measured using the IBS Quality of Life (IBS-QoL) and Short-Form Nepean Dyspepsia Index (SF-NDI) questionnaires.[15–17] Higher IBS-QoL and lower SF-NDI scores indicate a better quality of life.

Patients whose total IBS-SSS score decreased by ≥50 points after FMT were considered responders. A decrease of ≥175 points in the IBS-SSS total score, a decrease of ≥4 points in the FAS score and an increase of ≥14 points in the IBS-QoL score were considered to indicate significant clinical improvements

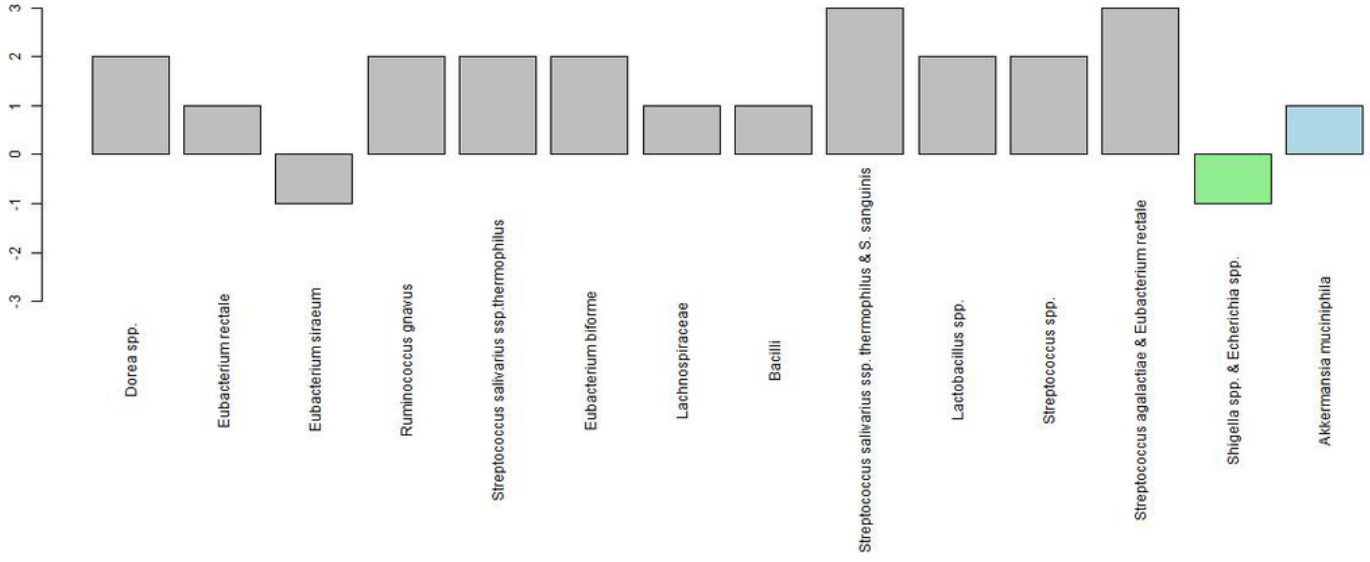

Figure 2 Although the superdonor was normobiotic, his bacterial profile deviated from the expected normal abundance in 14 of the 39 bacteria markers. The deviating bacteria belong to the typical commensal bacteria species that do not contribute to dysbiosis. In all, 12 of these bacteria belong to the phylum Firmicutes (grey), one to the phylum Proteobacteria (light green) and one to the phylum Verrucomicrobia (light blue).

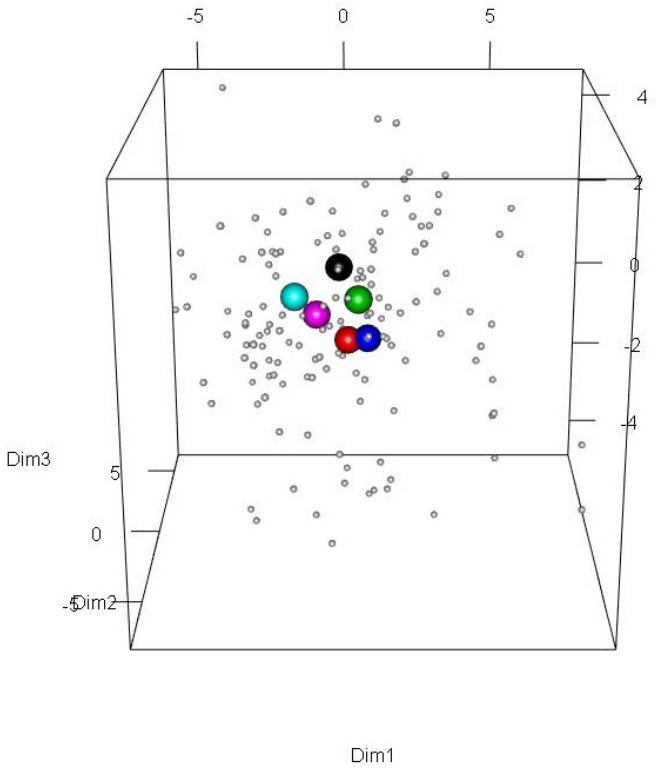

Figure 3 Scaled principal component analysis plot of faecal samples from the superdonor and patients before transplantation. The patient samples are indicated by small grey circles. The superdonor samples are indicated by the larger circles of different colours that indicate the sampling times: Black, 3 months; red, 6 months; green, 9 months; blue, 12 months; light blue, 15 months; and pink, 18 months. All of the superdonor samples are grouped closely together and remain in very similar positions over time.

in abdominal symptoms, fatigue and quality of life, respectively.[12 16 18] The responses were also analysed according to the European Medicines Agency and (EMA) and the Food and Drug Administration (FDA) using a composite responder endpoint.[19 20] According to EMA, a responder should fulfil the response criteria for at least 50% of the observation time. The response criteria for IBS-D are an improvement at least 30% in the abdominal pain score and a 50% reduction in the number of days with at least one stool that that has consistency of 6 or seven as compared with the base line. The response criteria for IBS-C are a reduction at least 30% in the abdominal pain score and an increase of at least one complete spontaneous bowel movement per week as compared with the base line. In IBS-M, a responder is a patient who has at least 30% improvement in the abdominal pain score and a subjects global assessment of efficacy scale of the highest two improvement grades in a 7-point scale, or of the highest improvement grade in a 5-point scale as compared with the base line.

Faecal bacterial analysis

The faecal bacteria were analysed with the GA-map Dysbiosis Test using a method described in detail elsewhere.[3 4] In brief, the test uses the 16S rRNA gene to determine both the bacterial profile and DI. It detects bacteria within five phyla (Firmicutes, Proteobacteria, Bacteroidetes, Tenericutes and Verrucomicrobia) that cover 10 bacterial classes, 36 genera and 32 species,[3] which means that the test assesses >300 bacteria at different taxonomic levels.[4] The DI is measured on a 5-point scale from 1 to 5 (severe dysbiosis), where DI values>2 indicate the presence of dysbiosis.[3]

The GA-map Dysbiosis Test was also used to analyse the changes in the intestinal bacterial profile following transplantation, in which the bacterial abundance was quantified from –3 to +3, where 0 represents the normal value based on a previous analysis of faecal samples of 297 healthy subjects.[3] In addition, principal component analysis (PCA) was used to plot scaled

Table 1 Characteristics of patients at the trial baseline

	Overall	Placebo	30 g FMT	60 g FMT	P
All patients	164	55	54	55	
Age, years	39.9±9.0	41.2±13.7	39.2±12.4	39.3±13.2	0.672
Sex, female/male	133/32	47/8	40/14	46/9	0.189
IBS-D	63	21	22	20	
IBS-C	62	22	20	20	0.989
IBS-M	39	12	13	14	
IBS-SSS score	313.4±80.3	315.2±77.1	311.8±76.8	313.9±87.3	0.975
Birmingham IBS-S score	24.3±7.1	23.2±8.1	26.5±6.0	25.2±6.8	0.050
IBS-QoL score	113.5±21.8	117.8±19.7	109.1±22.7	113.4±22.4	0.117
SF-NDI score	30.2±7.7	29.9±1.6	29.7±7.1	30.9±8.4	0.728
FAS score	31.1±4.9	30.6±4.9	31.4±5.1	31.3±4.8	0.634
DI	2.8±1.1	2.7±1.1	2.8±1.0	2.9±1.0	0.781
Patients with dysbiosis	(64)	(67)	(57)	(67)	0.578
PPI medication	59 (35.8)	21 (38.2)	20 (36.4)	18 (32.7)	0.810
Birth-control medication	84 (50.9)	25 (45.5)	29 (52.7)	30 (54.5)	0.601
Antimigraine medication	12 (7.3)	3 (5.5)	5 (9.1)	4 (7.3)	0.764
Medication against asthma/allergies	18 (10.9)	6 (10.9)	7 (12.7)	5 (9.1)	0.829
Medication with levothyroxine	3 (1.8)	1 (1.8)	0 (0)	2 (3.6)	0.361
Medication with heart/vascular drugs	6 (3.6)	3 (5.5)	2 (3.6)	1 (1.8)	0.595

Data are mean±SD, n, n/n, (%) or n (%) values.
DI, dysbiosis index; FAS, Fatigue Assessment Scale; FMT, faecal microbiota transplantation; IBS, irritable bowel syndrome; IBS-QoL, IBS Quality of Life; IBS-SSS, IBS Severity Scoring System; PPI, proton-pump inhibitor; SF-NDI, Short-Form Nepean Dyspepsia Index.

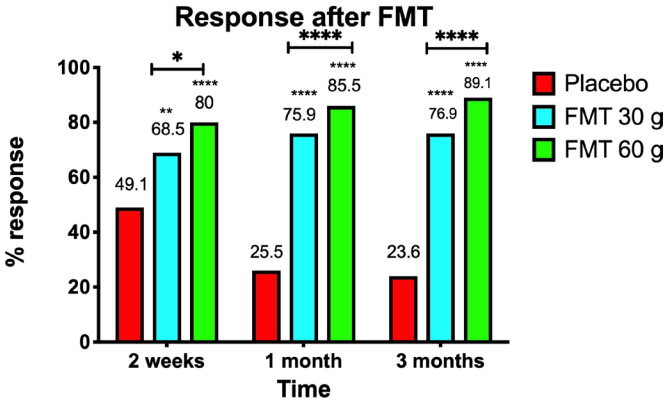

Figure 4 Responses of patients with IBS to placebo, 30 g FMT and 60 g FMT at different intervals after transplantation. **, p<0.001; ****, p<0.0001 compared with placebo. *, p<0.001; ***, p<0.0001 for 30 g FMT compared with 60 g FMT. IBS, irritable bowel syndrome; FMT, faecal microbiota transplantation.

Table 3 FAS scores in placebo and FMT-treated patients

Time	Group	Total score	Physical fatigue	Mental health
0	Placebo	30.6±4.9	15.8±2.6	14.8±2.6
	30 g FMT	31.4±5.1	15.9±3.0	15.5±2.8
	60 g FMT	31.3±4.8	15.9±2.8	15.4±2.6
2 weeks	Placebo	30.4±5.7	15.8±3.2	14.6±2.9
	30 g FMT	28.1±5.5	14.6±3.2	13.9±2.9
	60 g FMT	28.4±6.0	14.5±3.0	13.9±3.4
1 month	Placebo	30.8±6.0	16.1±2.9	14.7±3.4
	30 g FMT	27.5±6.7*	14.3±3.8*	13.3±3.1*
	60 g FMT	27.8±6.2*	14.5±3.4*	13.4±3.2
3 months	Placebo	29.8±4.6	15.2±2.6	14.5±2.7
	30 g FMT	27.1±5.8*	13.4±3.5*	13.6±3.0
	60 g FMT	27.0±6.3*	14.1±3.4	13.1±3.1*

Data are mean±SD values.
*p<0.05 compared with placebo.
FAS, Fatigue Assessment Scale; FMT, faecal microbiota transplantation.

probe signals inside an ellipse that covered 80% of the samples within a group. The differences in the signals were analysed using the lmFit function in the limma package.[21]

Outcomes

The primary endpoint was a reduction in the IBS-SSS total score of ≥50 points at 3 months following transplantation,[22] which the secondary endpoint was a change in the DI and intestinal bacterial profile.

Ethics

All subjects provided both oral and written consents to participate.

Statistical analysis

The sample size required in each arm of the trial was calculated by assuming that a placebo effect was 40% and an effect response was 80%. The total sample size was estimated to be 60 patients, with 20 in each arm ($\alpha=0.05$, $1-\beta=0.80$). We intended to assess 200 patients for eligibility mainly because a previous study employing a similarly calculated sample size failed to show any benefit of FMT, and also to allow for drop-outs.[7] Differences between the placebo, 30 g FMT and 60 g FMT groups in age and in IBS-SSS, Birmingham IBS-S, FAS, IBS-QoL and SF-NDI scores were analysed using one-way analysis of variance with Tukey's multiple-comparisons test as a post-test. The differences between the placebo, 30 g FMT and 60 g FMT groups in sex, overall responses, numbers of included IBS subtypes, IBS-subtype responses, IBS-subtype dysbiosis and IBS-subtype medications, and differences in responses with sex and IBS duration were analysed using the χ^2 test. The correlations between the changes in bacterial profile and the IBS-SSS and FAS scores were analysed using the non-parametric Spearman test. The paired t-test was used to calculate the differences in the bacterial profile and DI in the placebo, 30 g FMT and 60 g FMT groups between before and 1 month after transplantation. Wilcoxon's test was applied in PCA to test for differences in the placebo, 30 g FMT and 60 g FMT before and 1 month after transplantation. These analyses were performed using GraphPad Prism (version 8, La Jolla, CA, USA). All authors had access to the study data and reviewed and approved the final manuscript.

Table 2 IBS-SSS total scores and scores for the four items of the scale in placebo and FMT-treated patients following transplantation

Time	Group	Total score	1	2	3	4
0	Placebo	315.2±77.1	107.5±46.1	55.5±24.7	76.1±20.7	75.4±19.8
	30 g FMT	311.8±76.8	111.1±42.8	51.0±25.8	76.4±19.2	75.6±17.8
	60 g FMT	313.3±87.3	106.5±45.8	55.0±25.4	78.6±18.1	75.6±20.8
2 weeks	Placebo	278.7±124.7	88.1±57.0	51.2±30.5	62.6±28.5	64.5±27.5
	30 g FMT	244.0±98.5**	84.4±49.3	35.0±24.8**	53.3±25.7**	54.4±25.2*
	60 g FMT	184.6±96.3***	58.9±41.9**†	32.7±26.0***	43.6±26.0***	48.2±26.4**
1 month	Placebo	299.5±106.1	102.0±51.2	53.5±27.4	70.3±25.1	69.1±26.3
	30 g FMT	213.4±100.1***	97.7±47.8*	36.9±22.8***	49.4±28.9***	50.9±29.1***
	60 g FMT	186.8±107.0***	66.8±50.8***	33.7±24.6***	40.4±28.4***	46.4±26.8***
3 months	Placebo	307.0±87.1	112.0±69.7	56.8±31.8	73.1±22.3	71.7±22.0
	30 g FMT	186.3±109.0***	69.4±48.5***	30.5±21.7***	44.6±29.7***	45.3±30.9***
	60 g FMT	166.8±117.9***	56.5±47.5***	30.4±26.4***	35.6±26.2***	40.2±27.1***

IBS-SSS items: 1, abdominal pain; 2, abdominal distension; 3, dissatisfaction with bowel habits; 4, interference with quality of life.
Data are mean±SD values.
*p<0.05;**p<0.01; ***p<0.001 compared with placebo.
†p<0.05 compared with 30 g FMT.
FMT, faecal microbiota transplantation; IBS, irritable bowel syndrome; IBS-SSS, IBS Severity Scoring System.

Table 4 IBS-QoL total scores and scores in the eight domains of the scale in placebo and FMT-treated patients

Time	Group	Total score	1	2	3	4	5	6	7	8
0	Placebo	117.8±19.7	27.3±5.8	23.6±5.8	11.9±2.7	10.6±2.6	7.2±2.6	15.0±3.1	7.8±1.7	11.2±2.5
	30 g FMT	109.1±22.7	26.2±6.9	20.0±5.9**	11.2±3.4	10.3±2.8	5.9±2.8	13.6±3.5	7.5±2.1	10.8±2.6
	60 g FMT	113.4±22.4	27.4±6.7	21.4±5.5	11.5±2.8	10.6±2.7	6.7±2.8	14.1±3.4	7.7±1.7	10.8±2.8
2 weeks	Placebo	122.4±28.1	29.1±6.5	23.0±4.9	12.3±3.33	11.3±2.7	8.0±3.4	14.7±4.6	7.6±2.6	11.1±3.5
	30 g FMT	118.0±23.0	28.6±6.7	20.4±5.8*	13.3±3.2	11.3±3.1	7.1±3.1	14.3±3.4	7.8±2.0	10.9±2.1
	60 g FMT	124.5±25.1	31.1±6.6	23.3±5.9†	13.4±3.7	12.0±2.7	8.3±2.8	15.5±3.5	8.1±1.9	11.7±2.7
1 month	Placebo	122.9±25.4	29.2±7.7	23.1±4.8	12.7±4.2	12.3±2.4	8.6±2.9	14.7±3.4	7.0±2.3	11.4±2.8
	30 g FMT	121.6±23.9	29.5±7.1	21.8±4.8	13.2±3.7	11.6±2.5	7.2±2.9*	15.8±3.1	7.9±1.6	11.5±2.4
	60 g FMT	127.7±25.5	31.1±6.7	23.0±5.1	13.9±3.5	12.1±2.4	8.6±3.3†	15.1±3.9	8.2±2.0**	11.7±2.8
3 months	Placebo	113.0±24.3	27.2±6.3	21.2±4.6	12.1±3.0	12.8±6.3	7.1±3.0	13.6±3.8	7.3±2.3	10.4±2.5
	30 g FMT	131.5±21.6***	32.1±6.9**	24.1±5.2**	14.0±3.0**	12.6±2.9	8.8±3.2*	16.1±2.6***	8.2±1.6*	12.6±1.8****
	60 g FMT	132.0±24.8***	32.5±6.7**	24.0±4.7**	14.5±3.4***	12.2±2.2	9.6±3.6***	15.7±3.5**	8.3±1.6*	12.1±2.6***

IBS-QoL domains: 1, dysphoria; 2, interference with daily activities; 3, body image; 4, health worries; 5, food avoidance; 6, social reaction; 7, sexual function; 8, impact on relationships.
*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 compared with placebo.
†p<0.05 compared with 30 g FMT.
FMT, faecal microbiota transplantation; IBS, irritable bowel syndrome; IBS-QoL, IBS Quality of Life.

RESULTS

Patients and responses

In all, 200 patients were initially assessed for eligibility, of which 164 patients completed the study (figure 1). The characteristics of these patients are summarised in table 1. Responses occurred after 3 months in 23.6%, 76.9% and 89.1% of the patients in the placebo, 30 g FMT and 60 g FMT groups, respectively (figure 4). There were significant response differences between 30 g FMT and 60 g FMT after 2 weeks, 1 month and 3 months (figure 4). The response to FMT did not differ between the IBS subtypes. However, IBS-C (constipation-predominant IBS) and IBS-M (mixed-diarrhoea-and-constipation IBS) patients in the placebo group exhibited significantly larger responses after 1 and 3 months (online supplementary table 2), which could have been at least partially due to the rescue medication used for constipation (polyethylene glycol) being more effective than that used for diarrhoea (loperamide). The responses in the placebo, 30 g FMT and 60 g FMT groups did not differ with sex (p=0.9, 0.5 and 0.6, respectively) or between patients with IBS for ≥10 and<10 years (p=0.9, 0.8 and 0.7, respectively). The total IBS-SSS scores for diarrhoea-predominant IBS (IBS-D), IBS-C and IBS-M patients in the 30 g FMT group at the endpoint of the trial (after 3 months) were 157.7±83.2 (mean±SD), 207.9±114.7 and 204±125.5, respectively, with corresponding values in the 60 g FMT group of 156.4±113.3, 189.2±123.0 and 158.9±127.1. The total IBS-SSS score did not differ between the IBS-subtype patients who received 30 g FMT and 60 g FMT (p=0.260 and 0.652, respectively).

Abdominal symptoms, fatigue and quality of life

Abdominal symptoms as measured using IBS-SSS and Birmingham IBS-S were significantly improved after 3 months for both 30 g FMT and 60 g FMT compared with placebo (table 2 and online supplementary table 3), as was fatigue as assessed using FAS (table 3) and the quality of life as assessed using IBS-QoL and SF-NDI (table 4 and online supplementary table 4). In more detail, there were clinical improvements in abdominal symptoms in 5.5%, 35.2% and 47.3% of the patients in the placebo, 30 g FMT and 60 g FMT groups, respectively, in fatigue in 21.8%, 53.7% and 52.7% of them, and in the quality of life in 7.3%, 61.1% and 58.2% of them (online supplementary table 5). The responses according to EMA/FDA composite responder endpoint, 3 months after FMT were 16.7%, 50% and 70.9% in the placebo, 30 g and 60 g groups, respectively. The responses in 30 g and 60 g groups versus placebo group were significant (p<0.0001, both). There was a significant difference in response between 30 g and 60 g groups (p=0.004).

Bacterial analysis

Some of the tubes containing faecal samples shattered during transportation to the laboratory for analysis, which resulted in the bacterial analyses of faecal samples before and after transplantation only being performed on 47, 42 and 39 patients in the placebo, 30 g FMT and 60 g FMT groups, respectively. The DI values before transplantation were 2.6±1.1, 2.8±1.1 and 2.7±0.9 in the placebo, 30 g FMT and 60 g FMT groups, respectively; the corresponding values after transplantation were 2.6±1.1, 2.6±1.1 and 2.4±1.1 (p=0.087, 0.508 and 0.262). Dysbiosis was present in 57%, 55% and 61% of the patients in the placebo, 30 g FMT and 60 g FMT groups before transplantation, respectively, in 53%, 50% and 39% of them after transplantation (p=0.836, 0.828 and 0.108, respectively).

The analysis of the faecal bacterial profiles obtained using the GA-map Dysbiosis Test showed significant changes in the abundance of bacteria in the 30 g FMT and 60 g FMT groups but not in the placebo group (online supplementary table 6). *Alistipes* spp. were increased for both 30 g FMT and 60 g FMT. *Bacteroides* and *Prevotella* spp. increased in the 30 g FMT group while *Eubacterium hallii* decreased, and *Firmicutes* spp. and *Akkermansia muciniphila* increased in the 60 g FMT group, while *Dorea* spp. decreased.

PCA and the differences in signals analysed using the lmFit function in the limma package showed changes in the bacterial profiles after transplantation in the placebo, 30 g FMT and 60 g FMT groups as well as in the responder and non-responder groups (figure 5). The same approach showed that the responders in both the 30 g FMT and 60 g FMT groups had higher signals for *Eubacterium biforme*, *Lactobacillus* spp. and *Alistipes* spp. after transplantation, and lower signals for *Bacteroides* spp. (online supplementary figure 1). These changes occurred in responders in the 30 g FMT and 60 g FMT groups but not in the placebo group (online supplementary figures 2–5).

The IBS-SSS score was significantly correlated with the concentrations of *Lactobacillus* spp. (p=0.002, r=−0.3) and

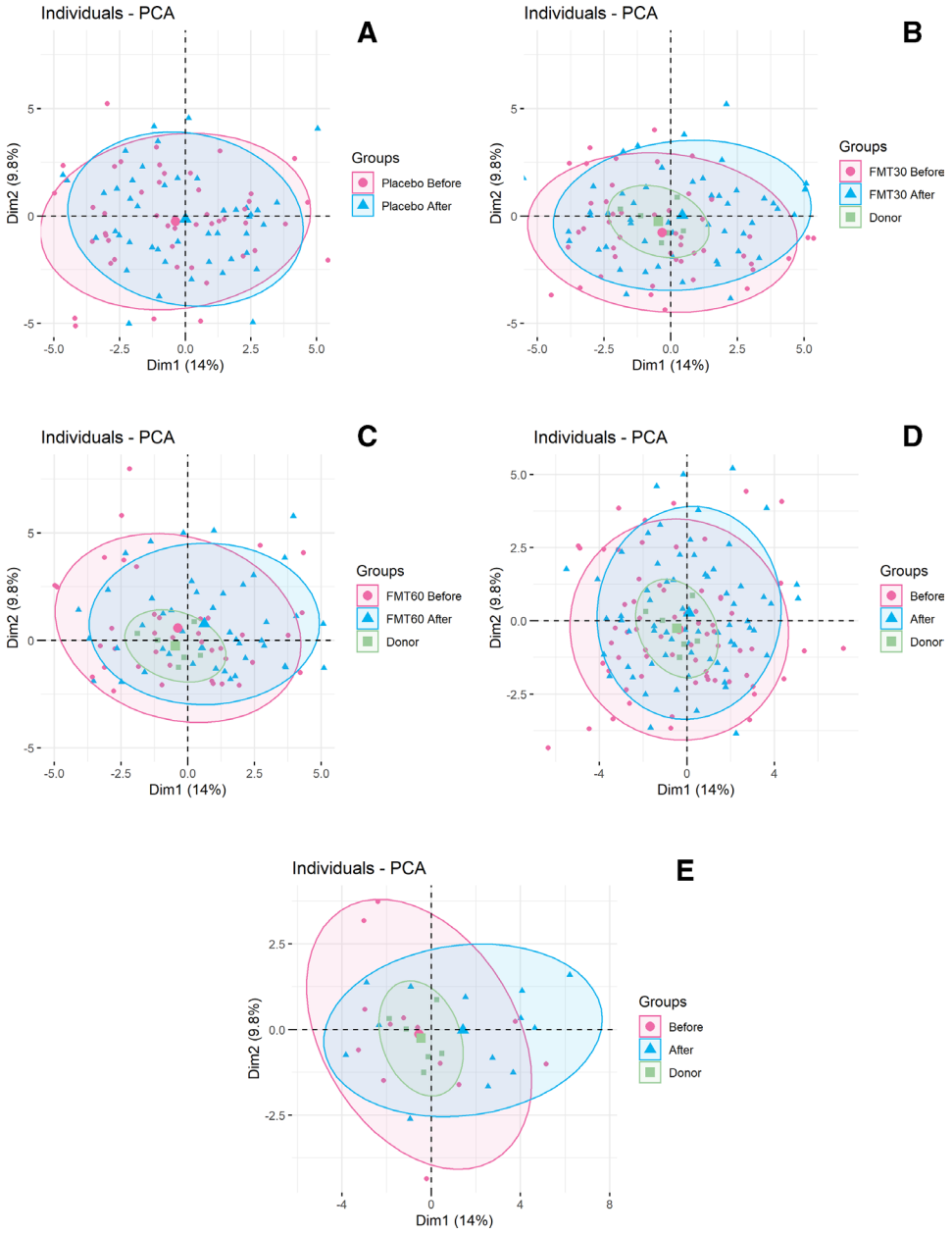

Figure 5 Scaled PCA plot of faecal samples before and after transplantation for placebo (A), 30 g FMT (B), 60 g FMT (C), responders (D) and non-responders (E). Faecal samples before and after transplantation are indicated by pink circles and blue triangles, respectively. The ellipses cover 80% of the samples within a group. FMT, faecal microbiota transplantation; PCA, principal component analysis.

Table 5 Adverse events following transplantation in patients with IBS.

Adverse event	Placebo	FMT total	30 g FMT	60 g FMT
Nausea	9 (16.3)	17 (15.6)	8 (14.8)	9 (16.4)
Abdominal pain/cramping/ tenderness	0 (0)	21 (19.3)*	11 (20.4)	10 (18.2)
Diarrhoea	2 (3.6)	26 (23.9)*	14 (25.9)	12 (21.8)
Constipation	1 (1.8)	24 (22.0)*	13 (24.1)	11 (20)
Diverticulitis	0 (0)	2 (1.8)	2 (1.8)	0 (0)

Data are n (%) values.
*, p<0.001 compared with placebo.
FMT, faecal microbiota transplantation; IBS, irritable bowel syndrome.

Alistipes spp. (p=0.001, r=–0.3) but not with that of *Eubacterium biforme* (p=0.754, r=0.03) or *Bacteroides* spp. (p=0.458, r=0.06). The FAS score was correlated with the concentration of *Alistipes* spp. (p=0.007, =–0.2) but not that of *Eubacterium biforme* (p=0.137, r=0.1), *Lactobacillus* spp. (p=0.829, r=–0.02) or *Bacteroides* spp. (p=0.174, r=0.1).

Adverse events

Mild intermittent abdominal pain, diarrhoea or constipation occurred in the first 2 days after FMT (table 5). Two patients (a 52-year-old male and a 55-year-old female) developed diverticulitis at 2 and 3 months after FMT. Both had diverticulosis as verified by colonoscopy and had experienced several diverticulitis attacks before FMT.

DISCUSSION

The findings of this study show that FMT is an effective treatment for IBS that improves both the symptoms and quality of life regardless of the IBS subtype. About half of the patients experienced significant clinical improvements in abdominal symptoms, fatigue and quality of life. These improvements were accompanied by changes in the bacterial faecal profile but not in the DI. The clinical relevance of the dysbiosis has been questioned recently.[4 23] It is notable that the scores in all IBS-QoL domains except for health worries improved after the patients received FMT. This is probably because the patients attended an IBS course prior to the trial, which is reported to improve the perceived knowledge of IBS.[9] The response to FMT increased with the dose. The adverse events of FMT were mild self-limiting gastrointestinal symptoms. It is noteworthy that a cohort of patients with IBS stated that they would give up an average of 15 years of their life (corresponding to 25% of their remaining lifespan) while 14% would risk a 1-in-1000 chance of death to receive a treatment that would make them free of IBS symptoms.[24]

At 1 month following the FMT, higher concentrations of *Eubacterium biforme*, *Lactobacillus* spp. and *Alistipes* spp. and a lower concentration for *Bacteroides* spp. were observed in this study in both the 30 g FMT and 60 g FMT groups. The concentrations for *Alistipes* spp. and *Lactobacillus* spp. were inversely correlated with the IBS-SSS score, while the signal for *Alistipes* spp. was inversely correlated with the FAS score. These findings indicate an association between the clinical improvement and the changes found in the bacterial profile following FMT in the present patients.

The finding that frozen faeces samples were effective in FMT confirms previous observations for FMT in *Clostridioides difficile and* in patients with IBS.[6 25] Furthermore, administering the transplants via the upper gastrointestinal route with the aid of a gastroscope seems to work well. These observations would eliminate the logistical problems associated with both FMT involving fresh faeces and the bowel preparation needed for administering such a transplant to the colon. Moreover, it might be possible to establish faeces banks for the routine clinical use of FMT.

It is difficult to compare the findings of the present study with those of previously reported randomised placebo-controlled trials.[6 7] due to differences in the sizes of the patient cohorts, the forms and amounts of the transplants, the routes of administration and the donors used. Johnsen *et al* investigated 83 patients with either IBS-D or IBS-M in whom 50–80 g of a mixture of the faeces from two donors was introduced into the colon.[6] Halkjaer *et al* included 51 patients with all IBS subtypes except unclassified IBS who received 50 g of a mixture of faeces from four donors in a capsule form for 12 days (totalling 600 g).[7] In the present study, the response rate in the placebo group at 2 weeks after transplantation was 49.1%, which decreased to 24.5% after 1 month and 23.6% after 3 months following transplantation. The placebo-group response in this study at 3 months after transplantation was slightly lower than that of 30% reported by Johnsen *et al*.[6] This difference could be due to the present placebo group containing almost twice as many patients as in that previous study. Moreover, the patients in the study of Johnsen *et al* were subjected to bowel preparation and colonoscopy, which is often painful and takes more time than the gastroscopy applied in the present study. This aspect could have affected the expectations of the patients and increased the placebo effect. Halkjaer *et al*[7] used a reduction in the IBS-SSS score as the primary end point rather than a response based on a definition of such a reduction in IBS-SSS score, which makes it difficult to compare the response in their placebo group with the present observations.

The clinical efficacy of FMT is not affected by the choice of the donor in patients with *Clostridioides difficile*,[5 26] whereas the success of FMT in inflammatory bowel disease and other disorders is donor-dependent.[5] A new definition of superdonor has emerged for someone who is normobiotic and has a positive microbial signature, but an attempt to use stool pooling to produce a superdonor was not successful.[5] Two randomised double-blind, placebo-controlled studies of FMT in patients with IBS produced conflicting results, which might have been due to variability in the donor stools used.[6 7]

What constitutes a positive microbial signature in an IBS superdonor remains unclear.[5] Therefore, when choosing the superdonor in the present study, we had to consider the factors that might positively affect intestinal microbiota. Smoking/smoking cessation affects the gut microbiota negatively.[27 28] To be born by caesarean section and/or be formula-fed affect the gut microbiota profile and reduce the bacterial diversity.[29–32] Frequent treatment with antibiotics and/or a regular intake of non-antibiotic drugs have negative effects on the gut microbiota.[33–35] Regular exercise and consuming a sport-specific diet are known to be associated with a favourable gut microbiota.[36–38] Furthermore, the superdonor should not be a first-degree relative of any of the patients in a trial since the intestinal microbiota is affected by the genetic composition, and a superdonor and recipient with greater genetic similarity may also have greater similarities in their faecal microbiota.[39 40] Thus, among the several candidate donors that we screened, we chose a donor who was a non-smoker, not taking any medication and had been treated only a few times with antibiotics. He was born via a vaginal delivery and breastfed, he trained regularly, consumed a sport-specific diet rich in protein and fibre, and was not related to any of the patients in the trial.

The present study utilised a single well-defined donor who was normobiotic with a bacterial signature that included an abundance of *Streptococcus*, *Dorea*, *Lactobacillus* and *Ruminococcaceae* spp. These four genera of bacteria have been reported to constitute favourable bacteria for a donor.[5 41–43] Further studies involving well-defined donors are needed to identify their favourable bacterial signatures.

One strength of this clinical trial is that it involved a relatively large cohort of patients with IBS that included three of the IBS subtypes (including IBS-C) and used a single well-defined donor. Furthermore, it confirmed that frozen faeces samples obtained from a donor are effective in FMT, which facilitates their use in the clinic. However, the study also had limitations: it did not investigate the entire intestinal bacterial contents or the long-term effects of FMT, and it did not record the frequency of using rescue medication in the intervention groups. A simultaneous weakness and strength of the study was that it investigated a cohort of patients that had moderate-to-severe IBS symptoms despite adhering to a diet consistent with the modified NICE diet. This is a weakness because the outcome cannot be applied to the entire IBS population, while it is a strength since it showed that FMT succeeded when diet failed.

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Contributors MES designed the study, obtained the funding, administrated the study, recruited the patients, performed FMT, collected, analysed, and interpreted the data and drafted the manuscript, ABK analysed the bacterial profiles using PCA and critically revised of the manuscript for important intellectual content. OHG, JGH and TH contributed to the design of the study, to the analysis and

interpretation of the data, and critically revised of the manuscript for important intellectual content.

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Competing interests None declared.

Patient consent for publication Not required.

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Arthritis Research & Therapy

RESEARCH ARTICLE Open Access

A distinct gut microbiota composition in patients with ankylosing spondylitis is associated with increased levels of fecal calprotectin

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Abstract

Background: Ankylosing spondylitis (AS) shares many characteristics with inflammatory bowel disease (IBD). Intestinal microbiota most likely plays an important role in the development of IBDs and may also be involved in the pathogenesis of AS. We aimed to define and compare the fecal microbiota composition in patients with AS, ulcerative colitis (UC), and healthy controls (HC) and to determine relationships between fecal microbiota, fecal calprotectin, and disease-related variables in AS.

Methods: Fecal microbiota composition was assessed with GA-map™ Dysbiosis Test (Genetic Analysis, Oslo, Norway), which also reports the degree of deviation of the microbiota composition compared with a healthy control population, a Dysbiosis Index (DI) score 1–5. The AS patients were assessed with questionnaires, back mobility tests, fecal calprotectin, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP).

Results: Totally, 150 patients with AS (55% men, median age 55.5 years, median BASDAI 3.2), 18 patients with UC (56% men, median age 30.5 years), and 17 HC (65% men, median age 22 years) were included. Principal component analysis showed highly separate clustering of fecal microbiota from the patients with AS, UC, and HC. Compared with HC, fecal microbiota in AS was characterized by a higher abundance of *Proteobacteria*, *Enterobacteriaceae*, *Bacilli*, *Streptococcus* species, and *Actinobacteria*, but lower abundance of *Bacteroides* and *Lachnospiraceae*.
Further, fecal microbiota composition differed between patients with normal (≤ 50 mg/kg, $n = 57$) and increased (≥ 200 mg/kg, $n = 36$) fecal calprotectin. Patients with increased fecal calprotectin had lower abundance of bacteria with anti-inflammatory properties such as *Faecalibacterium prausnitzii* and *Clostridium* and higher abundance of the genus *Streptococcus*. No association was found between the fecal microbiota composition and HLAB27 status, disease activity, function, or medication. Dysbiosis (defined as DI ≥ 3) was found in 87% of AS patients.

Conclusions: Patients with AS have a distinct fecal microbiota signature, which is linked to fecal calprotectin levels, a marker of intestinal inflammation, but not to other clinical parameters. These findings suggest a local interplay between intestinal microbiota and gut inflammation in AS.

Trial registration: ClinicalTrials.gov, NCT00858819. Registered March 9, 2009.

Keywords: Ankylosing spondylitis, Spondyloarthritis, Microbiota, Intestinal inflammation, Inflammatory bowel disease

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Background

Ankylosing spondylitis (AS) is a chronic inflammatory disease that shares several clinical, pathogenetic, and pathophysiologic characteristics with the inflammatory bowel diseases (IBD), ulcerative colitis (UC), and Crohn's disease (CD). Besides chronic inflammation of the spine, sacroiliac joints, entheses, and peripheral joints, AS is characterized by microscopic intestinal inflammation, which has been demonstrated in 40–60% of the patients [1–3]. AS patients also have an increased risk of developing IBD, especially CD [4–6]. The histopathology of the chronic form of intestinal inflammation in AS resembles CD, with presence of granulomas, activation of Paneth cells, and increased production of anti-microbial peptides [7–9]. Interleukin (IL) 23 and IL17, which are key cytokines in AS, are produced in the inflamed gut, both in AS and in IBD [10]. Active intestinal inflammation has been associated with increased disease activity in AS, more pronounced bone marrow edema of the sacroiliac joints in non-radiographic axial spondyloarthritis (nr-axSpA), and higher risk of development of AS from nr-axSpA [2, 5, 6, 11, 12]. This indicates a link between the inflammation in the gut and the locomotor system.

The gastrointestinal tract is the home of more than 1000 species of bacteria, but also fungi and viruses, which coexist with the host in a reciprocal relationship. The gut microbiota is necessary for the development and shaping of the immune system, and the host genetics play a role in the establishing and shaping of the gut microbiota [13]. Intestinal microbiota most likely play a role in initiating and triggering the immune system in individuals who are genetically susceptible for IBD, leading to the typical gut inflammation of CD and UC [14]. Aberrations in the gut microbiome, dysbiosis with decreased bacterial diversity, expansion of potentially pro-inflammatory bacteria, and reduction of potentially anti-inflammatory, protective bacteria have repeatedly been shown in IBD [15–17]. However, it is still unclear whether the dysbiosis in IBD is a cause or a consequence of the gut inflammation.

In a cohort of AS patients followed for 5 years, we have previously shown that two thirds of the patients had elevated fecal calprotectin levels, which was predictive of the development of CD [6]. The aims of the present study were to evaluate differences in fecal microbiota composition between patients with AS, patients with UC, and healthy controls. Further, we aimed to determine potential relationships between fecal microbiota composition, intestinal inflammation measured indirectly by fecal calprotectin, and disease-related variables in the AS patients.

Methods

Subjects of the study

Patients with AS

Patients with a diagnosis of AS according to the modified New York criteria were recruited from three rheumatology clinics in the west of Sweden [18]. Exclusion criteria were psoriasis, diagnosis of IBD, pregnancy, and difficulties in understanding Swedish language. All patients fulfilling the study criteria were invited to participate. In total, 204 patients were included in 2009, and the same patients were invited to a 5-year follow-up in 2014. The current study is based on data from the 5-year follow-up. At the 5-year follow-up, all patients were assessed by the same physician (AD) for swollen and tender joints count and back mobility. Back mobility, disease activity, and function were assessed with the Bath Ankylosing Spondylitis Metrology Index (BASMI), Ankylosing Spondylitis Disease Activity Score ($ASDAS_{CRP}$), Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), Bath Ankylosing Spondylitis patient Global score (BAS-G), and the Bath Ankylosing Spondylitis Functional Index (BASFI) [19]. Blood samples were analyzed for hemoglobin, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) using standard laboratory techniques, and the patients were asked to send in a stool sample. The presence of the HLAB27 antigen was assessed by HLA typing with sequence-specific oligonucleotide primers (PCR-SSO) by LABType® (One Lambda, Inc., CA, USA) and use of the Luminex platform. In total, 150 patients provided a stool sample at the 5-year follow-up with enough material to be used for microbiota and fecal calprotectin analyses.

Patients with UC

Eighteen treatment-naïve patients with newly diagnosed UC were recruited from Sahlgrenska University Hospital (Gothenburg) and Södra Älvsborgs Hospital (Borås). The UC diagnosis was based on endoscopic and histological findings. The patients had not received any antibiotics during the month before inclusion. The disease activity of the patients with UC was evaluated using the Mayo score, which contains four variables: stool frequency, rectal bleeding, endoscopic findings, and the physician's global assessment. Each variable is graded from 0 to 3, and the maximum total score is 12 [20]. The extent of disease was classified into proctitis, left-sided colitis, or extensive colitis (beyond the left colonic flexure) according to the Montreal classification [21].

Healthy controls

Seventeen healthy controls with no prior history of gastrointestinal or other chronic disorders were recruited at Sahlgrenska University Hospital (Gothenburg). None of the healthy controls had any gastrointestinal

complaints during the last week prior to inclusion, assessed using a standardized questionnaire. Further, none of the healthy controls had taken any immunosuppressive agents, antibiotics, or any other medication during the last 3 months prior inclusion.

Ethical approval

All patients and healthy controls in the study gave their written informed consent. The research protocol was approved by the Regional Ethics Committee in Gothenburg and carried out in accordance with the Helsinki Declaration.

Stool samples and fecal calprotectin

Stool samples were collected and sent in to the laboratory by the patients and healthy controls. The samples were immediately frozen and stored at − 20 °C.

The stool samples were analyzed for fecal calprotectin using an enzyme-linked immunosorbent assay (ELISA) kit (Bühlmann Laboratories AG, Schönenbuch, Switzerland). Calprotectin, which is a cytosolic protein abundant in neutrophils and belonging to the calcium-binding calgranulins or S100 proteins, is a marker of intestinal inflammation, but its concentration in feces is also increased by, for example, the use of non-steroidal anti-inflammatory drugs (NSAIDs) [22]. A fecal calprotectin ≤ 50 mg/kg was defined as normal, and a value ≥ 200 mg/kg was defined as increased. The threshold 200 mg/kg was chosen since it was considered a reasonable level on which to initiate further endoscopic investigation in a patient [23].

Analysis of fecal microbiota

Microbiota analysis of fecal samples from the patients with AS, patients with UC, and healthy controls was performed using the GA-map™ Dysbiosis Test (Genetic Analysis, Oslo, Norway), which consists of 54 DNA probes targeting ≥ 300 bacteria on different taxonomic levels. The probes have been selected based on the ability to distinguish between healthy controls, irritable bowel syndrome (IBS), and IBD patients [24]. The results are given as abundances of bacteria denoted as probe signal intensity (PSI). The test also algorithmically assesses fecal bacterial abundance and profile in comparison with a healthy reference group at the laboratory. A deviation in the microbiome from normobiosis is summarized in a Dysbiosis Index (DI) score (1–5). DI ≥ 3 indicates a microbiota that differs from the healthy reference group. The bacterial profile used to create the DI score is based on 15 different bacteria (defined by Genetic Analysis AS): *Ruminococcus albus/bromii*, *Ruminococcus gnavus*, *Faecalibacterium prausnitzii*, *Lactobacillus*, *Streptococcus sanguinis* and *Streptococcus salivarius thermophilus*, *Dialister invisus*, *Akkermansia muciniphila*, *Bacteroides fragilis*, *Alistipes*, *Shigella*/*Escherichia*, *Bifidobacterium*, *Bacteroides*/*Prevotella*, *Firmicutes* (*Bacilli*), *Firmicutes* (*Clostridia*), and *Proteobacteria*. The normobiotic reference in the DI was based on fecal samples collected from 165 healthy donors in Sweden and Norway, with no clinical signs or symptoms of gut disorder [24]. The GA-map™ Dysbiosis Test has been used in studies on IBD, IBS, scleroderma, Sjogren's syndrome, and obesity, but never before in AS, to the best of our knowledge [25–29].

Statistical analyses

Statistical analyses were made using SPSS Statistics version 25 (IBM, Chicago, USA). Descriptive statistics are presented as median and interquartile range (IQR). In comparisons between two groups, the Mann-Whitney *U* test was used for continuous variables and the chi-square test or Fisher's exact test for categorical variables. Correlations were calculated using Spearman's correlation (r_s). All tests were two-tailed. A Bonferroni corrected p value of < 0.0009 was considered statistically significant.

Multivariate factor analysis (SIMCA-P+ software; Umetrics, Umeå, Sweden version 15) was used to examine the relationship between categorical variables (*Y*-variables) and detection levels of bacteria (*X*-variables). The microbiota composition in the patients with AS, patients with UC, and healthy controls was analyzed with principal component analysis (PCA). Orthogonal partial least squares discriminant analyses (OPLS-DA) were used to correlate a selected *Y*-variable and multiple *X*-variables with each other in linear multivariate models to further investigate the differences between groups and to determine which variables had the largest discriminatory power. The following *Y*-variables were explored with OPLS-DA: (1) patients with AS compared with healthy controls, (2) AS patients with normal (≤ 50 mg/kg, $n = 57$) vs increased (≥ 200 mg/kg, $n = 36$) fecal calprotectin, (3) HLAB27 positive (84.7%, $n = 127$) vs. negative (15.3%, $n = 23$) AS patients, and (4) dichotomized levels (below vs. above median value and first vs. fourth quartile) of indices of disease activity, back mobility, and function in the AS patients, i.e., BASDAI, ASDAS-CRP, BASMI, BASFI, CRP, and ESR.

The quality of the OPLS-DA was based on the parameters *R*2, i.e., the goodness of fit of the model (values of ≥ 0.5 define good discrimination, best possible fit, R2 = 1), and Q2, i.e., the goodness of prediction of the model (values of ≥ 0.5 or no more than 0.3 lower than the R2 value, define predictive ability). To reduce the risk of overfitting, CV-ANOVA tests and post hoc 100 permutation tests of OPLS-DA models were performed. Models with $p < 0.05$ and permutation indices fulfilling

the post hoc analysis criteria of intercepts of R2Y ≤ 0.4 and Q2Y < 0.05 were accepted [30].

Results

Clinical characteristics of the patients

The characteristics of the 150 patients with AS, 18 patients with UC, and 17 healthy controls are demonstrated in Table 1. Notably, in contrast to the patients with AS, the healthy controls and patients with UC were younger and not taking any NSAID or immunosuppressant. There was also a discrepancy in disease duration between the patients with AS and UC.

Among the patients with UC, the median total Mayo score was 7 (IQR 5.7–8.3). Three (16.7%) patients presented with proctitis, 3 (16.7%) patients presented with left-sided colitis, and 12 (66.7%) suffered from extensive colitis.

The microbiota composition in patients with AS compared with patients with UC and healthy controls

Based on the fecal microbiota composition, patients with AS, patients with UC, and healthy controls clustered separately in a PCA, indicating major differences in the microbiota profile between the groups (Fig. 1).

To further define the differences in microbiota composition between AS and healthy controls, an OPLS-DA was performed, which showed excellent discrimination and predictive ability (R2 = 0.958, Q2 = 0.935, $p < 0.0001$) (Fig. 2a, b). Permutation test showed that the model was well fitted (intercepts: R2Y = 0.205 and Q2Y = − 0.388). In comparison with healthy controls, the fecal microbiota in AS was characterized by a higher abundance of *Proteobacteria*, *Enterobacteriaceae*, *Bacilli*, *Streptococcus* species, and *Actinobacteria*, but lower abundance of *Bacteroides* and *Lachnospiraceae.* A complete list of the bacteria is shown in Additional file 1: Table S1.

Fecal microbiota composition and association to fecal calprotectin in the AS patients

The fecal microbiota composition of the AS patients with normal fecal calprotectin (≤ 50 g/kg, $n = 57$) was compared to patients with increased fecal calprotectin (≥ 200 mg/kg, $n = 36$).

An OPLS-DA demonstrated that the fecal microbiota composition of patients with normal fecal calprotectin levels discriminated from patients with increased fecal

Table 1 The characteristics of the patients with ankylosing spondylitis (AS), ulcerative colitis (UC), and healthy controls (HC)

	AS ($n = 150$)	UC ($n = 18$)	HC ($n = 17$)
Women/men, *n* (%)	68 (45.3)/82 (54.7)	8 (44.4)/10 (55.6)	6 (35.5)/11 (64.7)
Age, years	55.5 (46–67)	30.5 (27–39)	22 (21–31)
AS symptom duration, years	28.5 (18–39)		
HLAB27 positive, *n* (%)	127 (84.7)		
BAS-G, score	2.8 (1.5–5.9)		
ASDAS-CRP, score	2.1 (1.3–1.7)		
BASDAI, score	3.2 (1.8–5.2)		
BASFI, score	2.3 (1.1–4.1)		
BASMI, score	3.4 (2.4–4.6)		
ESR, mm/h	8 (4–14)		
CRP, mg/L	3 (1–6)	5 (3.5–16.5)	1 (1–1)
Fecal calprotectin, mg/kg	80 (0–190)	606 (29–10,320)	All < 15
Patients on NSAIDs, *n* (%)	115 (76.7)	0	0
- Daily use of NSAIDs	61 (40.7)		
- On-demand use of NSAIDs	54 (36.0)		
On TNFi all, *n* (%)	35 (23.3)	0	N.A.
- TNFi in monotherapy	16 (10.7)		
- TNFi + methotrexate	19 (12.7)		
On DMARD monotherapy	16 (10.7)	0	N.A.
- Methotrexate	9 (6.0)		
- Sulfasalazine	7 (4.7)		

Data is presented as median (interquartile range) or number (%)

ASDAS-CRP Ankylosing Spondylitis Disease Activity Score based on CRP, *BASDAI* Bath Ankylosing Spondylitis Disease Activity Index, *BAS-G* Bath Ankylosing Spondylitis patient Global score, *BASFI* Bath Ankylosing Spondylitis Functional Index, *BASMI* Bath Ankylosing Spondylitis Metrology Index, *CRP* C-reactive protein, *DMARD* disease modifying anti-rheumatic drug, *ESR* erythrocyte sedimentation rate, *NSAID* non-steroidal anti-inflammatory drug, *TNFi* tumor necrosis factor inhibitor

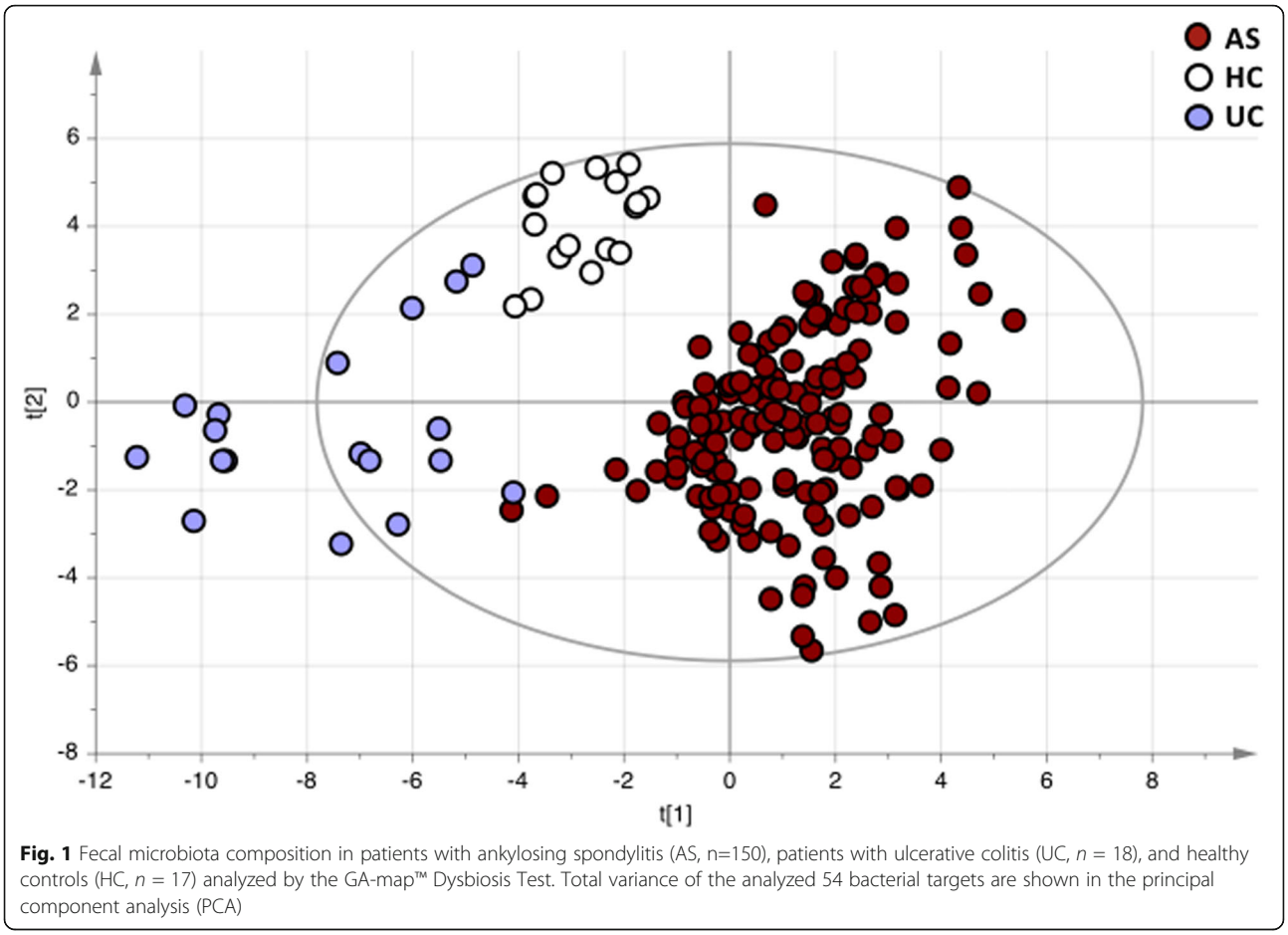

Fig. 1 Fecal microbiota composition in patients with ankylosing spondylitis (AS, n=150), patients with ulcerative colitis (UC, *n* = 18), and healthy controls (HC, *n* = 17) analyzed by the GA-map™ Dysbiosis Test. Total variance of the analyzed 54 bacterial targets are shown in the principal component analysis (PCA)

calprotectin levels, although the predictive ability was modest (R2 = 0.513, Q2 = 0.205, p = 0.0004) (Fig. 3a, b). Permutation test showed that the model was well fitted (intercepts: R2Y = 0.307, Q2Y = – 0.331).

The AS patients with normal fecal calprotectin levels had higher abundance of *Bacteroides*, *Clostridium*, *Prevotella*, *Actinomycetales*, and *Faecalibacterium prausnitzii*, whereas patients with increased fecal calprotectin levels had a higher abundance of *Bacilli* class, *Streptococcus* genus, and *Lactobacillus* genus. A complete list of the bacteria which differed between patients with normal respective increased calprotectin is shown in Additional file 1: Table S2. Correlations between fecal calprotectin and bacteria in all the AS patients (n = 150) are shown in Additional file 1: Table S3, and scatterplots between calprotectin and bacteria in users and non-users of NSAIDs are shown in Additional file 2: Figure S1.

Fecal calprotectin was associated with several clinical parameters. Fecal calprotectin was higher in NSAID users compared with non-users (median (IQR) 88 (43–220) vs. 36 (19–120) mg/kg; p = 0.005) and weakly positively correlated with ASDAS-CRP (r_S = 0.191, p = 0.019), CRP (r_S = 0.252, p = 0.002), and BASMI (r_S = 0.203, p = 0.013). No association was found between fecal calprotectin and reported gastrointestinal symptoms.

Fecal microbiota in relation to HLAB27, disease activity, function, and medication in the AS patients

Weak correlations (Spearman's rho) were found between PSI values for a few bacteria and measures of disease activity (Additional file 1: Table S3). None of these correlations however reached a p value of < 0.0009, which was the threshold for statistical significance after the Bonferroni correction. Further, there was no significant difference (p value < 0.0009) in the PSI value of any bacteria between users and non-users of NSAIDs, TNFi or csDMARDs, or between smokers and non-smokers. No association was found between fecal microbiota composition and reported gastrointestinal symptoms.

Multivariate analysis of the microbiota composition using OPLS-DA could not discriminate between HLAB27 positive or negative patients or between patients with

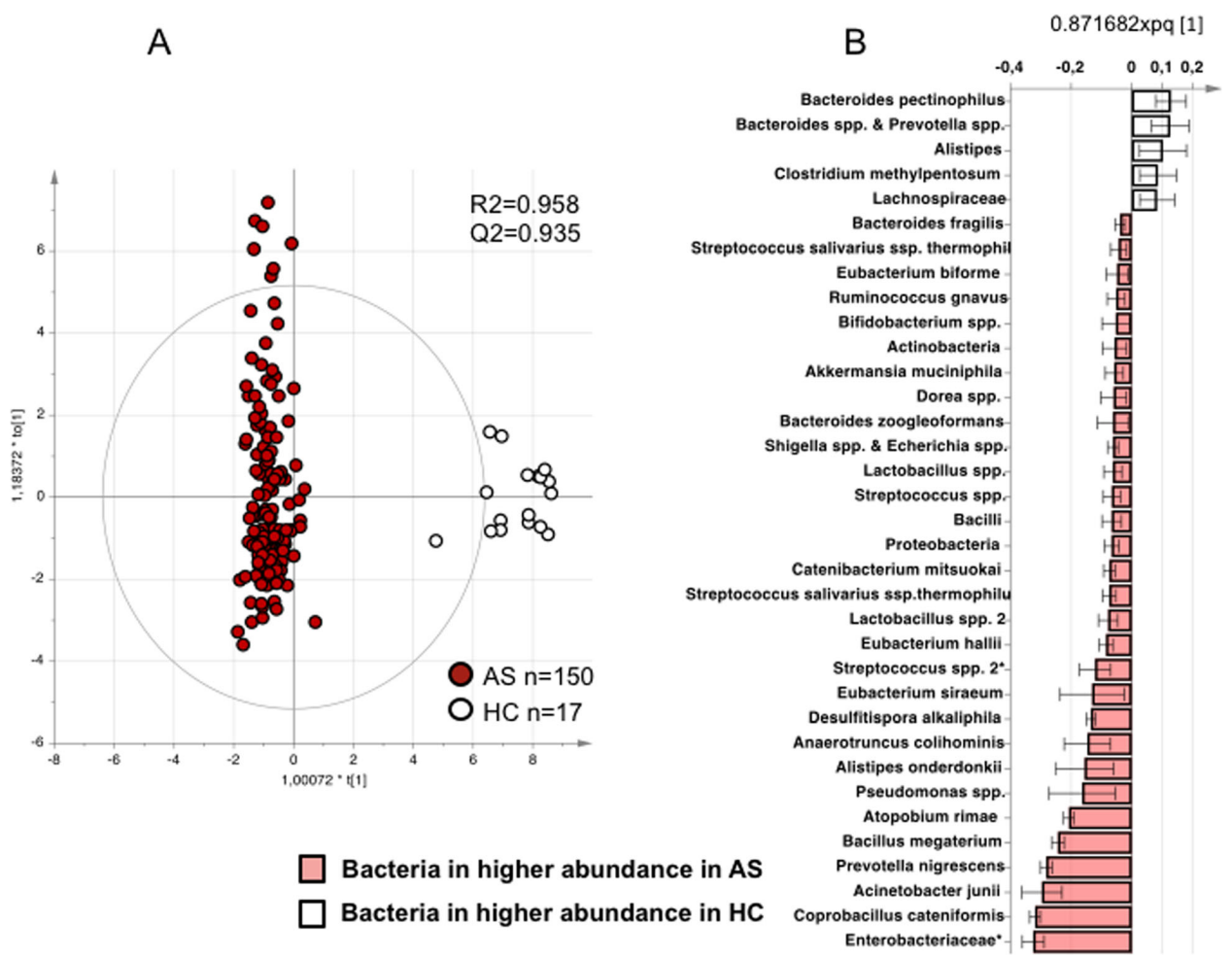

Fig. 2 Comparison of fecal microbiota composition in patients with ankylosing spondylitis (AS) and healthy controls (HC). Fecal samples were analyzed by the GA-map™ Dysbiosis Test and evaluated using orthogonal partial least squares discriminant analysis (OPLS-DA). AS ($n = 150$) and HC ($n = 17$). **a** Score scatter plot from the OPLS-DA showing the separation between patients with AS (red circles) and HC (white circles). R2 defines the goodness of fit, and Q2 the goodness of prediction. **b** Loading column plot from the OPLS-DA. White columns represent bacteria in higher abundance in HC, and red columns represent bacteria in higher abundance in AS patients. All bacteria were included in the analysis, but the loading column plot shows only bacteria which abundance differed between the groups. Error bars represent 95% confidence interval. All analyzed bacteria are listed in Additional file 1: Table S1

dichotomized levels (below vs. above median value and first vs. fourth quartile) of parameters reflecting disease activity or function (BASDAI, ASDAS-CRP, BASMI, BASFI, CRP, and ESR) (Additional file 1 Table S4).

Dysbiosis Index score in patients with AS, patients with UC, and healthy controls

Fecal microbial dysbiosis, defined as a Dysbiosis Index (DI) score ≥ 3, was found in 131 (86.7%) of the patients with AS, in 17 (94.4%) of the patients with UC, and in 4 (23.5%) of the healthy controls [24]. The distribution of the DI score among the AS patients was similar to that of the UC patients ($p = 0.8$) but differed significantly from that of the healthy controls ($p < 0.001$) (Fig. 4).

The AS patients with the most pronounced dysbiosis, DI = 5 (36.7%), had significantly higher fecal calprotectin than the patients with DI < 5 (63.3%) (170 (58–360) mg/kg vs. 58 (27–120) mg/kg; $p < 0.001$ (Fig. 5). DI was also positively correlated with fecal calprotectin ($r_S = 0.303$; $p < 0.001$). No association was found between DI and medication. Boxplots of the DI among users and non-users of NSAIDs and TNFi are shown in Additional file 3: Figure S2 and Additional file 4: Figure S3.

Discussion

We studied the fecal microbiota composition in patients with AS, patients with UC, and healthy controls and found evidence for a distinct fecal microbiota signature in AS, which differed significantly from the patients with UC and healthy controls in the study. The fecal microbiota composition of the AS patients showed association with fecal calprotectin, but not with other clinical

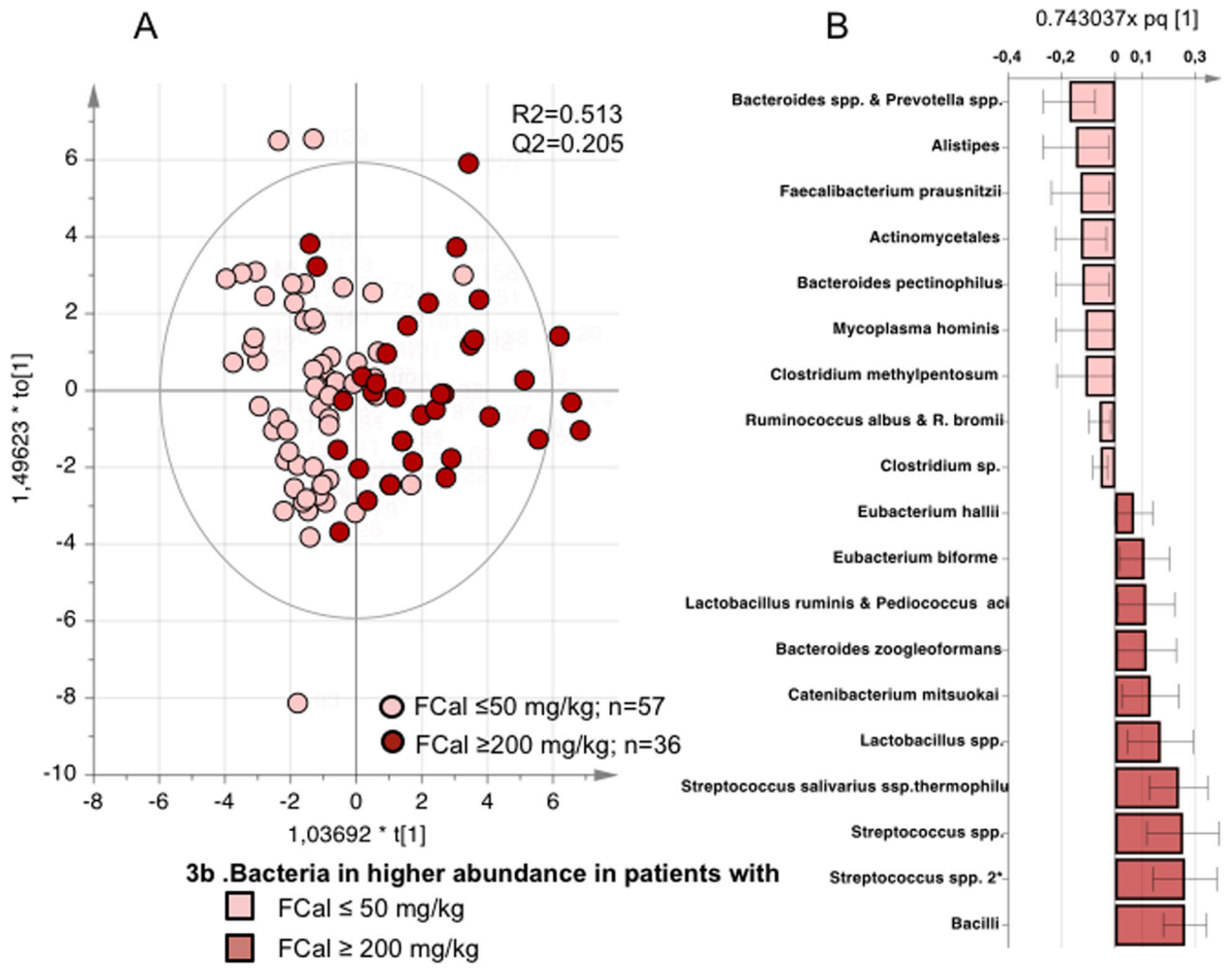

Fig. 3 Fecal microbiota composition in relation to levels of fecal calprotectin. Comparison of the fecal microbiota composition in ankylosing spondylitis (AS) patients with normal (≤ 50 mg/kg, $n = 57$) vs. increased (≥ 200 mg/kg, $n = 36$) fecal calprotectin (Fcal). Orthogonal partial least squares discriminant analysis (OPLS-DA) was used to define fecal microbial differences between the groups. **a** Score scatter plot from the OPLS-DA showing the separation between patients with normal Fcal (pink circles) and increased Fcal (red circles). R2 defines the goodness of fit, and Q2 the goodness of prediction. **b** Loading column plot from the OPLS-DA. Pink columns represent bacteria in higher abundance in AS patients with normal fecal, and red columns represent bacteria in higher abundance in AS patients with increased Fcal. All bacteria were included in the analysis, but the loading column plot shows only bacteria which abundance differed between the groups. Error bars represent 95% confidence interval. All analyzed bacteria are listed in Additional file 1: Table S2

parameters. Thus, no clear association was found between the overall fecal microbiota composition and HLAB27 status, disease activity, physical function, medication, or smoking status. Dysbiosis was found in 88% of the AS patients, and an increased dysbiosis was associated with elevation of fecal calprotectin.

Several of our findings indicate that there are similarities in the aberrations of the gut microbiota in IBD and AS. We found higher abundance of the phylum *Proteobacteria*, especially the family *Enterobacteriaceae* and the genus *Shigella* and *Escherichia* among the AS patients compared with healthy controls. *Proteobacteria* is a phylum, consisting of Gram-negative staining bacteria containing pro-inflammatory lipopolysaccharides (LPS) in their cell membrane, which is overrepresented in the gut in several conditions characterized by chronic inflammation [31]. Similar to the findings of the present study, *Enterobacteriaceae*, belonging to the *Gammaproteobacteria*, have repeatedly been found to be enriched in the gut in UC and CD [17, 32–34]. Adherent-invasive *Escherichia coli* (AIEC), belonging to the family of *Enterobacteriaceae*, which can persist and replicate inside epithelial cells and macrophages are increased in the ileal mucosa in CD [35–37]. The presence of adherent and invasive bacteria, mainly *Escherichia coli* and *Prevotella*, has also been reported in AS in association with gut inflammation and damage of the intestinal mucosal barrier [38]. An increase in the *Gammaproteobacteria*

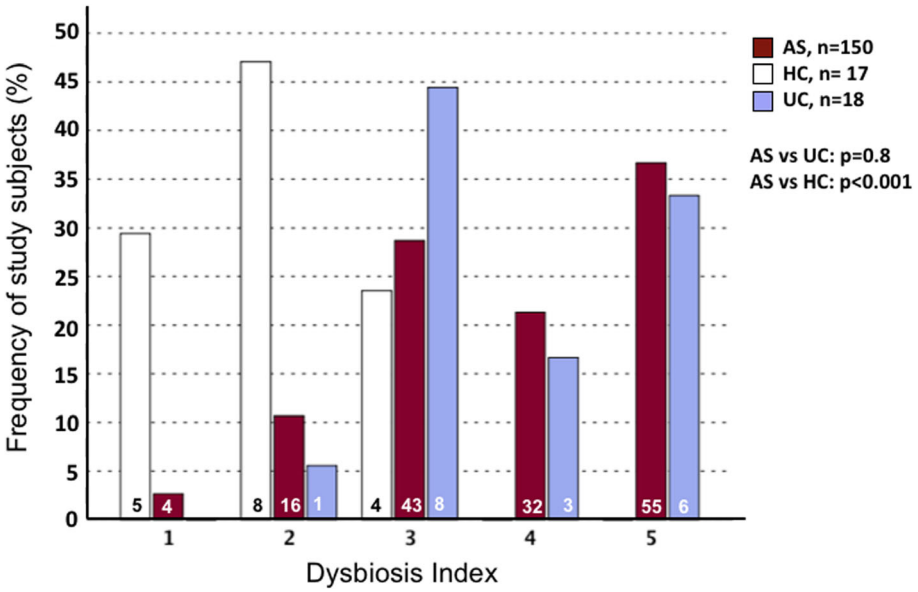

Fig. 4 The distribution of the Dysbiosis Index (DI) score among patients with ankylosing spondylitis (AS, *n* = 150), patients with ulcerative colitis (UC, *n* = 18), and healthy controls (HC, *n* = 17). Fecal samples were analyzed by the GA-map™ Dysbiosis Test. DI is scored between 1 and 5, where a score of 1 and 2 signifies normobiosis and 3–5 dysbiosis of increasing severity. Digits inside the columns represent the number of subjects within each column

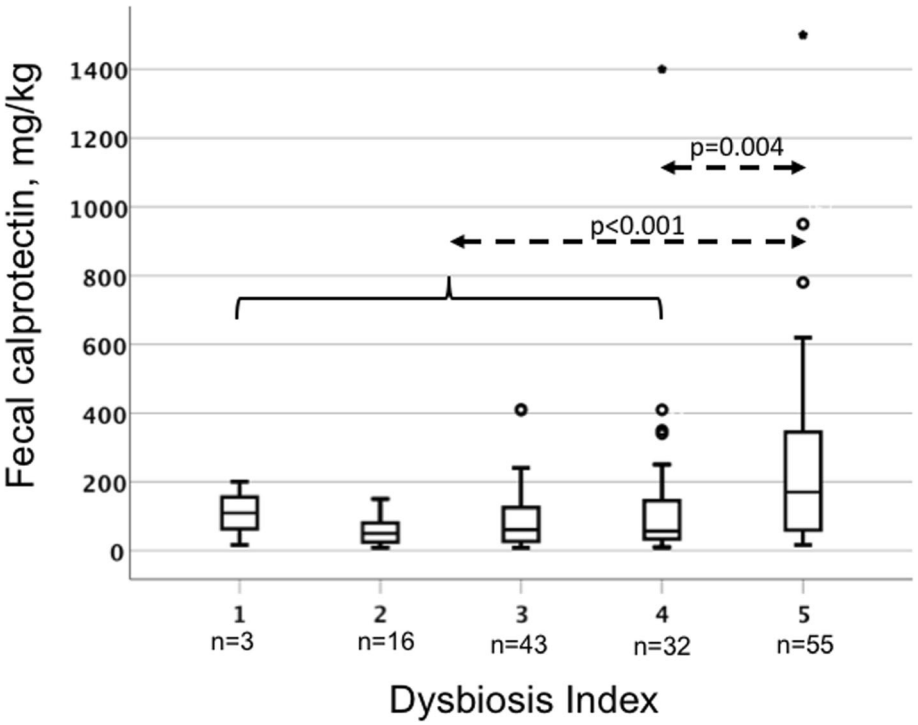

Fig. 5 Fecal calprotectin (Fcal) in relation to dysbiosis index (DI) in patients with ankylosing spondylitis (AS). Boxplots represent the Fcal concentration within each DI score. Values are the medians (horizontal line), interquartile range (box), and range (whiskers). Outliers: circles show cases with values between 1.5 and 3.0 box lengths and stars (extremes) values more than 3 box lengths from the upper or lower edge of the box. Fcal was compared between groups of patients with different DI score using the Mann-Whitney *U* test. AS patients with the most pronounced dysbiosis (DI = 5) had significantly higher Fcal than patients with a DI of 4 ($p = 0.004$) and patients with a DI 1–4 ($p < 0.001$)

Erwinia and *Pseudomonas* and a decrease in *Lachnospiraceae* have also been shown in reactive arthritis [39].

The AS patients with an elevated fecal calprotectin (≥ 200 mg/kg) had a relative decrease in the genus *Clostridium* and the species *Faecalibacterium prausnitzii* and *Bacteroidetes*. Both *F. prausnitzii* and *Clostridium* have been shown to have immune-suppressive effects [40]. Decreased levels of *Clostridiales* and *F.prausnitzii* have been found in CD and UC, and low abundance of the bacteria is associated with higher recurrence of CD after surgery and poorer effect of treatment with infliximab in CD and UC [28, 32, 40, 41]. *F.prausnitzii* produces the short-chain fatty acid (SCFA) butyrate, an important nutrient for epithelial cells. The bacterium has been found to have immune-suppressive effects on peripheral blood mononuclear cells in vitro, to produce a protein which inhibits the NF-κB pathway, to stimulate the production of IL-10 and to be able to inhibit experimental colitis in BALB/c mice [40, 42]. An earlier study on the fecal microbiota in children with enthesitis-related arthritis reported findings similar to ours with lower abundance of *F. Prausnitzii* and the family *Lachnospiraceae* among the patients [43]. The AS patients with a fecal calprotectin ≥ 200 mg/kg in the current study had an increase of the genus *Streptococcus*. Interestingly, a gain in *Streptococcus* in stool samples has also been found in new-onset CD and has been associated with higher recurrence of CD after surgery [32, 41, 44]. Thus, several of the bacteria which we found to be increased or decreased respectively in AS have previously been reported to be increased and decreased in studies on IBD, with an extra strong resemblance with early CD. The findings suggest that similar microbial mechanisms may be involved in the pathogenesis of gut inflammation in the diseases and give further food for thought that subclinical gut inflammation in AS could be viewed as a preclinical CD. Yet, the fecal microbiota of the AS patients differed greatly from the UC patients in the current study, which may be explained by the much more inflamed state of the gut mucosa of the UC patients.

A large proportion (77%) of the AS patients of this study were using NSAIDs, and intestinal bacteria play a role in NSAID enteropathy [45, 46]. Further, NSAID use may alter the gut microbiota composition [47, 48]. In the present study, the microbiota composition did however not discriminate between users and non-users of NSAIDs.

There are earlier studies on the gut microbiota in AS or axial SpA, which have all found significant differences in the fecal microbiota composition in AS or SpA compared with healthy controls [49–52]. Tito et al. examined ileal and colonic biopsies in patients with newly diagnosed AS or nr-axSpA in relation to gut histology and found differences in the microbiota composition between patients with or without microscopic gut inflammation [50]. The study also reported a positive correlation between the abundance of the genus *Dialister* and ASDAS and BASDAI. Breban et al. studied the microbiota in fecal samples from patients with SpA, rheumatoid arthritis, and healthy controls and reported an increased abundance of the species *Ruminococcus gnavus* in SpA, especially in SpA patients with a history of IBD, and a positive correlation between *Ruminococcus gnavus* and BASDAI [51]. The current study confirms the findings of a distinct microbiota composition in AS and supports the prior report of differences in the microbiota between AS patients with or without subclinical gut inflammation. We also found that the abundance of *Ruminococcus gnavus* was higher in AS than in healthy controls. Conversely, we found no associations between the fecal microbiota composition and disease activity. There are differences between the studies regarding the methods used for microbiota analyses and sampling niche, mucosal biopsies vs. feces, which may have affected the results. Active gut inflammation has been associated with increased disease activity in AS [2, 5, 6, 12]. Our results indicate that there may be an interaction between intestinal bacteria and inflammation in the gut in AS, but we found no evidence for a direct link between the intestinal microbiota composition and other AS-related disease activity measures.

Strengths of the present study were the well-characterized cohorts and a large number of patients with AS. Limitations of the study were that the microbiota analysis was based on a defined set of bacterial probes instead of metagenomic sequencing and that the patients were assessed with fecal calprotectin, but not with endoscopy. A major limitation of the study was also the discrepancy between the patients with AS, UC, and healthy controls in regard to age, number of participants, medication, and disease duration, which may have affected the results. The study also lacked a control group with CD.

Conclusions

We have demonstrated a distinct fecal microbiota signature in the patients with AS, which differed from the patients with UC and healthy controls. In the AS patients, fecal microbiota signature was linked to fecal calprotectin levels, a marker of intestinal inflammation, but not to other clinical parameters. This suggests that the intestinal microbiota may be involved in an interplay with subclinical gut inflammation in AS.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13075-019-2018-4.

Additional file 1: Table S1. Comparison of the bacterial composition in patients with ankylosing spondylitis (AS, $n = 150$) and healthy controls

(HC, $n = 17$). **Table S2.** Comparison of the bacterial composition in patients with ankylosing spondylitis (AS) with normal (≤ 50 mg/kg) versus increased (≥200 mg/kg) fecal calprotectin. **Table S3.** Correlations (Spearman's Rho) between Probe Signal Intensity (PSI) of fecal bacteria and fecal calprotectin and parameters reflecting disease activity and function in 150 patients with ankylosing spondylitis. All correlations with a *p*-value ≤0.05 are shown. A Bonferroni corrected p-value of < 0.0009 was considered statistically significant (marked with *). **Table S4.** Comparison of the fecal microbiota composition in groups of ankylosing spondylitis patients with dichotomized levels (below vs. above median value and first vs. fourth quartile) of indices of disease activity, back mobility and function. Comparisons were also made between users vs. non-users of medication and smokers vs. non-smokers. Orthogonal partial least squares discriminant analysis (OPLS-DA) was used to define fecal microbial differences between the groups. The quality of OPLS-DA was based on the parameters R2, i.e., the goodness of fit of the model (values of ≥0.5 define good discrimination, best possible fit, R2 = 1), and Q2, i.e., the goodness of prediction of the model (values of ≥0.5 define high predictive ability).

Additional file 2: Figure S1. Scatterplots of Probe Signal Intensity (PSI) of fecal bacteria (Y-axis) and fecal calprotectin (mg/kg) (X-axis) in AS patients. Patients on NSAIDs are represented by circles, patients not on NSAIDs represented by squares.

Additional file 3: Figure S2. Boxplot showing the distribution of the Dysbiosis Index among non-users, on-demand users and daily users of non-steroidal anti-inflammatory drugs (NSAIDs).

Additional file 4: Figure S3. Boxplot showing the distribution of the Dysbiosis Index among non-users and users of tumour necrosis factor inhibitors (TNFi).

Abbreviations

AIEC: Adherent-invasive *Escherichia coli*; AS: Ankylosing spondylitis; ASDAS-CRP: Ankylosing Spondylitis Disease Activity Score based on CRP; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; BAS-G: Bath Ankylosing Spondylitis patient Global score; BASMI: Bath Ankylosing Spondylitis Metrology Index; CD: Crohn's diseases; CRP: C-reactive protein; cs/bDMARDs: Conventional synthetic or biologic disease modifying anti-rheumatic drugs; DI: Dysbiosis Index; DMARD: Disease modifying anti-rheumatic drug; ELISA: Enzyme-linked immunosorbent assay; ESR: Erythrocyte sedimentation rate; IBD: Inflammatory bowel disease; IBS: Irritable bowel syndrome; IL: Interleukin; IQR: Interquartile range; LPS: Lipopolysaccharides; nr-axSpA: Non-radiographic axial spondyloarthritis; NSAID: Non-steroidal anti-inflammatory drug; OPLS-DA: Orthogonal partial least squares discriminant analyses; PCA: Principal component analysis; PSI: Probe signal intensity; SpA: Spondyloarthritis; TNFi: Tumor necrosis factor inhibitor; UC: Ulcerative colitis

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Authors' contributions

EK participated in the design of the study, collected the data, made the statistical analyses, and drafted the article. MKM participated in the design of the study, collected and interpreted the data, and made the statistical analyses. HS participated in the design of the study, and the collection and interpretation of data. AD examined the patients and participated in the design of the study, and the collection and interpretation of data. AS was responsible for the fecal analyses and interpretation of data. JS participated in the design of the study, and the collection and interpretation of data. MS participated in the design of the study, and the collection and interpretation of data. HC participated in the design of the study and the interpretation of data. LÖ participated in the design of the study, and the collection and interpretation of data. HF supervised the study and was responsible for the study design and interpretation of data. All authors have critically reviewed the manuscript, approved the final version to be published, and agreed to be accountable for all aspects of the work.

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Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by the Regional Ethics Committee in Gothenburg and carried out in accordance with the Helsinki Declaration. All participants gave their written informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Research Article

Are Nonnutritive Sweeteners Obesogenic? Associations between Diet, Faecal Microbiota, and Short-Chain Fatty Acids in Morbidly Obese Subjects

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Obesity has been associated with changes in the gut microbiota and its metabolites. The study explored changes in the faecal microbiota and short-chain fatty acids (SCFA) associated with the diet (including nonnutritive sweeteners (NNSs)) and evaluated metabolic consequences in subjects with morbid obesity. The diet was assessed with a validated food frequency questionnaire. One unit of NNSs was 100 mL beverage with NNSs or 2 tablets/teaspoons of NNSs. The faecal microbiota was assessed with GA-map® dysbiosis test and SCFA with gas chromatography and flame ionisation detection. Fourteen men and 75 women with a mean age of 44.6 (SD 8.7) years, BMI 41.8 (SD 3.6) kg/m^2, and intake of NNSs 7.5 units/day (SD 3.2; range 0–43) were included. Faecal butyric acid was positively and negatively associated with the intake of starch (partial correlation = 0.264; $p = 0.015$) and NNSs (partial correlation = −0.274; $p = 0.011$), respectively. NNSs were associated with changes in four out of 39 bacterial groups. Butyric acid has antiobesogenic effects, reduces insulin resistance, and improves dyslipidaemia. Since the weight-reducing effect of NNSs on obese adults trying to lose weight is dubious, it seems imprudent to use NNSs that might counteract the favourable effects of butyric acid.

1. Introduction

Obesity, which has nearly tripled worldwide since 1975, has health-related consequences such as increased risk of cardiovascular diseases, metabolic syndrome with diabetes type 2, musculoskeletal disorders, and cancer [1–3]. The high and increasing prevalence of obesity, estimated to 13% of the world population and mentioned as the global obesity epidemic [4], has been linked to alterations in the diet with increased intake of high fat, energy-dense food, and reduced physical activity [1]. The dietary alterations affect the gut microbiota (induce dysbiosis) and the microbiota's metabolites (e.g., straight and branched short-chain fatty acids, referred to as short-chain fatty acids (SCFA) in this paper) [5]. Evidence indicates associations between alterations of the gut microbiota and their metabolites and obesity [6–9]. Propionic and butyric acids have been ascribed antiobesogenic effects, and a high *Firmicutes/Bacteroidetes* ratio has been associated with obesity [10–16]. Although these changes have been observed in both animals and humans, a causal role of the gut microbiome in the pathogenesis of obesity has not yet been proven in humans [6, 17].

Dietary factors like fibre and starch, intake of nonnutritive sweeteners (NNSs), and use of drugs like metformin alter the gut microbiome and their metabolic products [5, 18–26]. This study aimed to explore associations

between the diet and drugs, and changes in the gut microbiota and SCFA in subjects with morbid obesity, and to evaluate the metabolic consequences. Because our previous research has indicated unfavourable effects of NNSs, the study focused on the effects of NNSs [25, 27]. A secondary aim was to study direct and indirect effects (mediated via the faecal microbiota) of NNSs on SCFA.

2. Materials and Methods

2.1. Study Design. The design was a cross-sectional study in subjects with morbid obesity (MO) referred for evaluation of bariatric surgery. Results from this cross-sectional study have been reported in previous papers, and this study followed the methods of Farup and Valeur [28].

2.2. Participants. Consecutive subjects aged 18–65 years with MO (defined as BMI >40 or >35 kg/m^2 with obesity-related comorbidity), referred to the Unit for Obesity at Innlandet Hospital Trust–Gjøvik, Norway, in the period from December 2012 to September 2014 were informed about the study and were asked to participate. Exclusion criteria were organic gastrointestinal disorders, major psychiatric disorders, severe somatic disorders not related to obesity, alcohol or drug addiction, and previous obesity surgery or other major abdominal surgery.

2.3. Accomplishment. In all participants, a medical history was taken, a physical examination was performed, and blood and faecal samples were collected. The doctors, the study nurse, and the participants filled in paper-based questionnaires. Supplementary examinations were performed at the doctors' discretion.

2.4. Variables

2.4.1. Participants' Characteristics. The following variables were registered:

(i) Gender, age (years), height (m), weight (kg), BMI (kg/m^2), coffee (cups/day), smoking (daily, previously, and never), and previous and present diseases.

(ii) Use of metformin and other drugs (yes/no).

(iii) The diet was assessed with a food frequency questionnaire based on the official Norwegian food composition table and validated by the University of Oslo [29]. The amount of NNSs was calculated. One unit of NNSs was defined as 100 mL NNS-containing beverage or two NNS tablets/teaspoons for use in tea or coffee.

2.4.2. Faecal Samples. The producer of the microbial test, Genetic Analysis AS, Oslo, Norway, provided kits for collecting the faecal specimens. The participants collected faecal material in the kits at home and stored it at room temperature for a maximum of five days before bringing the specimen to the hospital where it was kept at minus 80°C until it was analysed [30].

(1) Faecal Microbiota. GA-map® dysbiosis test (Genetic Analysis AS, Oslo, Norway) was used for the analyses of the faecal microbiota [30]. The test is CE marked and has a US (Patent No. 9243297) and a European patent (Patent No. 2652145) for its technology [31]. The test is based on advances in DNA profiling using probes targeting variable regions (V3 to V7) of the bacterial 16S rRNA gene to characterise and identify bacteria present at different taxonomic levels.

The overall result is given as the Dysbiosis Index (DI) with scores 1 to 5; values above 2 indicate a microbiota profile that differs from the producer's reference population (i.e., dysbiosis). The test also reports the relative abundance of 39 bacteria at different taxonomic levels compared with a reference population (score −3 to 3) of Actinobacteria, Actinomycetales, *Bifidobacterium* spp., *Alistipes*, *Alistipes onderdonkii*, *Bacteroides fragilis*, *Bacteroides* spp. and *Prevotella* spp., *Bacteroides stercoris*, *Bacteroides zoogleoformans*, *Parabacteroides johnsonii*, *Parabacteroides* spp., Firmicutes, Bacilli, *Catenibacterium mitsuoka*, Clostridi a, *Clostridium* sp., *Dialister invisus*, *Dialister invisus* and *Megasphaera micronuciformis*, *Dorea* spp., *Eubacterium biforme*, *Eubacterium hallii*, *Eubacterium rectale*, *Eubacterium siraeum*, *Faecalibacterium prausnitzii*, Lachnospiraceae, *Lactobacillus ruminis* and *Pediococcus acidilactic*, *Lactobacillus* spp., *Phascolarctobacterium* sp., *Ruminococcus albus* and *R. bromii*, *Ruminococcus gnavus*, *Streptococcus agalactiae and Eubacterium rectale*, *Streptococcus salivarius* ssp. *thermophiles* and *S. sanguinis*, *Streptococcus salivarius* ssp. *Thermophilus*, *Streptococcus* spp., *Veillonella* spp., Proteobacteria, *Shigella* spp. and *Escherichia* spp., *Mycoplasma hominis*, and *Akkermanasia muciniphilia*. The dysbiosis scores are the producer's commercial secret.

An Alternative dysbiosis index (ADI) (scores −14 to 14), which we have claimed to separate favourable dysbiosis (positive scores) from unfavourable dysbiosis (negative scores), was also calculated [25]. The ADI is based on the relative abundance of the bacteria Alistipes, Proteobacteria and *Shigella* spp. and *Escherichia* spp., and the relative scarcity of *Bacteroides fragilis*, *Ruminococcus gnavus*, *Bacteroides* spp. and *Prevotella* spp., and *Dialister invisus.*

(2) Faecal Short-Chain Fatty Acids. 0.5 g of the faecal samples and distilled water containing 3 mmol/L of 2-ethylbutyric acid (as internal standard) and 0.5 mmol/L of H_2SO_4 were homogenized. 2.5 mL of the homogenate was vacuum distilled according to the method of Zijlstra et al. and modified by Høverstad et al. [32, 33]. The distillate was analysed with gas chromatography (Agilent 7890 A; Agilent, CA, USA) using a capillary column (serial no. USE400345H, Agilent J&W GC columns; Agilent, CA, USA) and quantified while using internal standardisation. Flame ionisation detection was employed. The total amount of all SCFA and the amount of acetic, propionic, butyric, i-butyric, valeric, i-valeric, caproic, and i-caproic acids expressed in mmol/kg wet weight were measured, and two indices were calculated:

(i) Index A (saccharolytic fermentation) was the concentration of acetic minus propionic and butyric acid divided by the total amount of SCFA. The index reflects the fermentation of carbohydrates and the proinflammatory effect of SCFA. It was constructed as a balance between the proinflammatory effects of acetic acid and the anti-inflammatory effects of butyric and propionic acids [34].

(ii) Index B (proteolytic fermentation) was the sum of i-butyric and i-valeric acids. The index reflects the fermentation of proteins and the anti-inflammatory effects of SCFA [34].

2.5. Statistics. Descriptive statistics are given as number and proportion (%), mean with standard deviation (SD), or median with range. The mediation analyses were carried out as follows: first, linear regression analyses were used with each SCFA as the dependent variable, one at a time, and NNSs, starch, metformin, age, and gender as independent variables to identify SCFA with a statistically significant total effect of NNSs. Second, linear or ordinal logistic regression analyses were used with each of the 39 candidate microbiota and the dysbiosis indices, one at a time, as the dependent variable and the aforementioned independent variables, to identify the microbiota with statistically significant association with NNSs. Third, for the combinations of SCFA microbiota, mediation analyses were carried out as described by Hayes AF et al. [35, 36], to estimate direct and indirect effects (mediated through the bacterial groups at different taxonomic levels) of NNSs on SCFA, with age, gender, starch, and metformin as covariates. Bootstrap confidence intervals based on 5000 bootstrap replications was calculated for the indirect effect. The method does not allow the calculation of p values for the indirect effects. Figure 1 shows a directed acyclic graph of the mediation model. p values <0.05 were judged as being statistically significant. To adjust for multiple testing, Benjamini–Hochberg false discovery rate-adjusted q values were calculated in R for the associations between NNSs and the individual SCFA and reported for p values below 0.05. Other analyses were performed with IBM SPSS Statistics for Windows, version 25.0 (IBM Corp., Armonk, NY, USA).

2.6. Ethical Approval. The study was approved by the Norwegian Regional Committees for Medical and Health Research Ethics (reference number 2012/966) and was performed in accordance with the Declaration of Helsinki. All the participants gave written informed consent before inclusion.

3. Results

3.1. Subject Characteristics. Eighty-nine out of 350 consecutive subjects with morbid obesity in the period from December 2012 to September 2014 were included in the study. Reasons for exclusions were as follows: study nurse was unavailable (no = 111), refused participation (no = 80), erroneously included (no = 17), and did not fill in the food frequency questionnaire or provide faecal samples (no = 53). Table 1 gives the characteristics of the 89 participants.

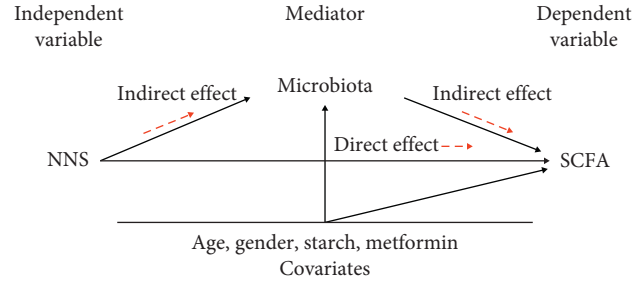

Figure 1: A directed acyclic graph of the direct and indirect effects (mediated via the microbiota) of nonnutritive sweeteners (NNSs) on short-chain fatty acids (SCFA) adjusted for age, gender, and intake of starch and metformin.

3.2. Short-Chain Fatty Acids. Table 2 gives the associations between SCFA and starch, NNSs, and metformin adjusted for age and gender. NNSs were negatively associated with butyric acid and valeric acid. Figure 2 shows the associations between butyric acid and starch and NNSs adjusted for the means of age, gender, metformin, and/or NNSs/starch.

3.3. Faecal Microbiota. NNSs were positively associated with the DI and negatively with the ADI, and associated with four out of the 39 bacteria reported by the commercially available test. Table 3 gives the statistically significant associations between NNSs and the faecal microbiota adjusted for starch, metformin, age, and gender.

3.4. Direct and Indirect Effects of NNSs on SCFA. NNSs were associated with butyric acid and valeric acid, and with the dysbiosis indices and four of the bacteria groups. Table 4 gives the direct and indirect effects of NNSs on butyric acid and valeric acid. The effects are shown only for the bacteria associated with NNSs. No statistically significant indirectly mediated effects were seen.

4. Discussion

This study in subjects with morbid obesity showed significant effects of the diet and drugs on the faecal microbiota and SCFA. The most important finding was the association between the use of NNSs and reduced butyric acid. Similar effects of NNSs have been observed in mice [37].

4.1. Physiological Effects of Butyric Acid and NNSs. Butyric acid has multiple potential beneficial effects of particular importance for subjects with obesity [38]. Butyric acid reduces appetite, induces sustained satiety, promotes energy expenditure and fat oxidation by activation of brown adipose tissue, reduces insulin resistance, and improves dyslipidaemia [11, 12, 39, 40]. In mice, butyric acid prevents dietary-induced weight gain and induces significant weight

Table 1: Characteristics of the 89 subjects (the number of patients is given in brackets if less than 89).

Subject characteristics	Number (%) (mean and/or median)	SD and/or range
Gender (male/female)	14 (15.7%)/75 (84.3)	
Age (years)	44.6	8.7
Height (cm)	170	8.1
Weight (kg)	121.1	16.4
BMI (kg/m^2)	41.8	3.6
Coffee (cups/day)	3.0	2.3
Smoking (daily/previously/never)	11 (12.4%)/42 (47.2%)/36 (40.4%)	
Diabetes (no 86) (yes/no)	20 (23.3%)/66 (76.7%)	
Metformin use (yes/no)	16 (18.0%)/73 (82.0%)	
Protein (g/day)	110 (median 106)	36 (40 to 213)
Fat (g/day)	97 (median 87)	47 (21 to 283)
Carbohydrates (g/day)	273 (median 246)	128 (65 to 903)
Sugar (g/day)	45 (median 25)	75 (0.8 to 632)
Starch (g/day)	132 (median 122)	51 (24 to 336)
Fibre (g/day)	35 (median 35)	11 (12 to 72)
Nonnutritive sweeteners (units*/day)	7.5 (median 3.2)	10 (0 to 43)
Dysbiosis (yes/no)	58 (65%)/31 (35%)	
Dysbiosis Index (score 1–5)	3.0 (median 3.0)	1.3 (1 to 5)
Alternative dysbiosis index (−14–14)	−0.5 (median 0.0)	2.7 (−8.0 to 7.0)
SCFA total (mmol/kg wet weight)	36.7 (median 28.7)	21.6 (5.9 to 149.2)
Acetic acid (mmol/kg wet weight)	19.9 (median 16.4)	10.8 (2.9 to 67.9)
Propionic acid (mmol/kg wet weight)	6.4 (median 5.2)	4.2 (1.3 to 25.6)
Isobutyric acid (mmol/kg wet weight)	0.7 (median 0.7)	0.6 (0.0 to 5.2)
Butyric acid (mmol/kg wet weight)	7.2 (median 5.6)	5.4 (1.0 to 34.5)
Isovaleric acid (mmol/kg wet weight)	1.1 (median 0.9)	1.0 (0.0 to 7.8)
Valeric acid (mmol/kg wet weight)	1.0 (median 0.8)	0.9 (0.0 to 5.1)
Isocapronic acid (mmol/kg wet weight)	0.0 (median 0.0)	0.0 (0.00 to 0.08)
Capronic acid (mmol/kg wet weight)	0.32 (median 0.06)	0.53 (0.00 to 3.10)
Index A[†]	0.19 (median 0.19)	0.10 (−0.11 to 0.44)
Index B[#]	1.84 (median 1.55)	1.61 (0.00 to 13.04)

*One unit = 100 mL beverage with nonnutritive sweeteners or 2 tablets/teaspoons of nonnutritive sweeteners for coffee or tea. [†]Index A: saccharolytic fermentation, i.e., the concentration of acetic minus propionic and butyric acid divided by the total amount of SCFA. [#]Index B: proteolytic fermentation, i.e., the sum of concentrations of i-butyric and i-valeric acid.

Table 2: Linear regression analyses with the SCFA one at a time as the dependent variable and starch, nonnutritive sweeteners, metformin, gender (not shown), and age (not shown) as simultaneous independent variables.

Dependent variable	Starch[#]			Nonnutritive sweeteners			Metformin		
	B (95% CI)	p value	Partial corr.	B (95% CI)	p value	Partial corr.	B (95% CI)	p value	Partial corr.
Total SCFA	0.108 (0.009, 0.206)	0.032	0.232	−0.488 (−0.986, 0.009)	0.054	−0.210	11.1 (1.9, 24.0)	0.09	0.184
Acetic acid	0.057 (0.008, 0.106)	0.024	0.244	−0.207 (−0.455, 0.042)	0.102	−0.179	5.3 (−1.1, 11.8)	0.106	0.177
Propionic acid	0.017 (−0.002, 0.036)	0.084	0.189	−0.066 (−0.164, 0.032)	0.183	−0.146	2.1 (−0.4, 4.7)	0.104	0.178
Isobutyric acid	0.001 (−0.002, 0.004)	0.503	0.074	−0.011 (−0.026, 0.004)	0.139	−0.162	0.4 (0.0, 0.8)	0.045	0.218
Butyric acid	0.030 (0.006, 0.054)	0.015	0.264	−0.159 (−0.280, −0.037)	0.011*	−0.274	2.1 (−1.1, 5.2)	0.200	0.140
Isovaleric acid	0.001 (−0.003, 0.006)	0.529	0.069	−0.016 (−0.038, 0.007)	0.170	−0.150	0.6 (0.0, 1.2)	0.046	0.217
Valeric acid	0.001 (−0.003, 0.005)	0.603	0.057	−0.022 (−0.043, −0.002)	0.029*	−0.237	0.5 (−0.02, 1.0)	0.061	0.204
Isocapronic acid	0.000 (0.000, 0.000)	0.746	0.036	0.000 (0.000, 0.000)	0.438	0.085	0.01 (0.00, 0.02)	0.002	0.327
Capronic acid	0.000 (−0.002, 0.003)	0.819	0.025	−0.008 (−0.020, 0.004)	0.205	−0.139	0.11 (−0.22, 0.43)	0.513	0.072
Index A	0.000 (−0.001, 0.000)	0.705	−0.042	0.001 (−0.001, 0.004)	0.251	0.126	0.005 (−0.06, 0.07)	0.873	0.018
Index B	0.002 (−0.005, 0.010)	0.517	0.071	−0.027 (−0.063, 0.010)	0.156	−0.155	0.98 (0.02, 1.95)	0.045	0.218

*False discovery rate-adjusted q values for butyric and valeric acids were 0.088 and 0.116, respectively. [#]Fibre and starch were significantly correlated (Pearson's $r = 0.60$, $p < 0.001$). Since fibre was not significantly associated with either butyric acid or valeric acid, starch was used in the analyses.

loss [12, 39]. These effects are, without exception, favourable for subjects with obesity. The effects are in part mediated via gut-brain neural circuits [12, 41]. The discrepant sensory and metabolic signals (taste-calorie uncoupling) to the intake of NNSs modify the brain response to food and could have long-term consequences for food intake [42]. The adverse physiological effects associated with NNSs (such as metabolic changes with glucose intolerance, increased appetite and weight gain, weaker caloric compensation, and neurophysiological and brain dysfunction) are similar to those

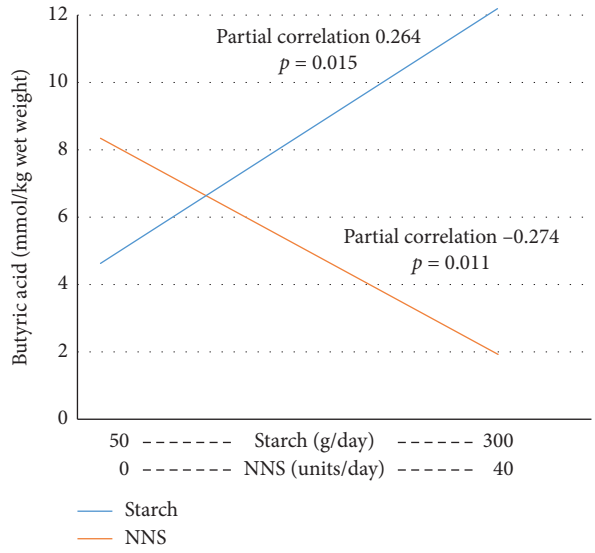

Figure 2: Regression lines for the associations between starch and NNSs (on the *x*-axis) and butyric acid (on the *y*-axis) calculated at the mean of the covariates age, gender, use of metformin, and/or NNSs/starch.

associated with butyric acid depletion and could in part be due to butyric acid reduction.

4.2. Clinical Effects of NNSs. The clinical usefulness of NNSs for weight control is uncertain. Intake of NNSs, often in large amounts, combined with an attempt to reduce intake of food rich in sugar are common in subjects with obesity aiming at weight reduction. In this study, intake of 20–43 units/day (corresponding to 2.0–4.3 litres of carbonated beverages with NNSs) was rather common. NNSs have been associated with an unhealthy lifestyle [27]. The clinically relevant favourable and unfavourable effects of NNSs in humans are still under debate despite comprehensive research. A recently published systematic review and meta-analyses concluded that no evidence of any effect of NSSs was seen on overweight and obese adults trying to lose weight, and that potential harms could not be excluded [43].

4.3. Comparisons of the Clinical and Physiological Effects of NNSs. The clinical and physiological effects of NNSs seem discordant. The favourable effects of a modest reduction of the intake of sugar by subjects using NNSs could be counteracted by the unfavourable physiological effects. The clinical relevance and physiological effects of reduced valeric acid associated with NNSs are unknown.

4.4. The Diet and the Faecal Microbiota. The faecal microbiota and its metabolites are highly influenced by the diet [5, 19]. NNSs were associated with an unfavourable dysbiosis, reduced amounts of *Faecalibacterium prausnitzii* and *Bacteroides fragilis*, and increased amounts of *Ruminococcus gnavus* and *Streptococcus* spp. Changes in the gut microbiota are common in subjects with obesity, and an increased Firmicutes/Bacteroidetes ratio has often been reported [6, 16, 17]. Increased *Ruminococcus gnavus* and *Streptococcus* spp. that are part of the Firmicutes and reduced *Bacteroides fragilis* that is part of the Bacteroidetes could indicate an increase in Firmicutes/Bacteroidetes ratio. *Faecalibacterium prausnitzii* has been ascribed important health-related effect also in subjects with obesity [14, 44, 45].

4.5. The Diet and Drugs and Butyric Acid. The positive association between intake of starch and faecal butyric acid was expected. The microbes use resistant nondigestible carbohydrates and fibre such as slowly digestible and resistant starch for production of SCFA, in particular butyric and propionic acids [19, 46, 47]. Low-carbohydrate diets such as a low content of rapidly digestible starch reduce faecal butyric acid [19]. The antiobesity effect of slowly digestible and resistant starch, which is due to increased energy expenditure and not reduced caloric intake, is likely mediated by increased microbial butyric acid production [48]. The intake of fibre and starch was significantly correlated, and associations between fibre and SCFA were expected but not found. Therefore, starch was used in the analyses. No associations were seen between metformin and the SCFA. Since metformin was used by nearly all subjects with diabetes, the effects of diabetes and metformin are difficult to separate. Metformin was used in this study because other studies have shown that metformin and not diabetes is the main contributor to the microbial alterations [21, 49].

4.6. The Microbiota and SCFA. Several microbes, of which *Faecalibacterium prausnitzii* has been mentioned as the most important one, have the capability for production of butyric acid by different metabolic pathways [45, 47]. Four groups of microbes including *Faecalibacterium prausnitzii* were associated with intake of NNSs. It was unexpected that neither *Faecalibacterium prausnitzii* nor any of the other bacterial groups were significant mediators of the negative association between NNSs and butyric acid. Since a direct effect of NNSs on butyric acid is unlikely, bacterial groups not specified by the commercially available microbial test probably mediated the effect.

4.7. Other Effects of the Microbiota and SCFA. In a previous study with the same methods, we reported the associations between the microbiota and SCFA and psychobiological comorbidity [28]. In contrast to this study with clear and significant associations between dietary factors and butyric acid with possible clinical consequences, the previous study showed a high number of significant and partly divergent associations and revealed no straightforward gut-brain communication pathways.

4.8. Strengths and Limitations. The participants with morbid obesity in need of weight reduction and therefore having a high intake of NNSs and referred for evaluation of bariatric surgery were well suited for the study of health-related

Table 3: Linear and ordinal regression analyses with the dysbiosis indices one at a time, and the six out of 41 faecal bacterial species/groups that were statistically significantly associated with nonnutritive sweeteners as the dependent variable, and starch, nonnutritive sweeteners, metformin, age (not shown), and gender (not shown) as independent variables.

Dependent variable	Starch		Nonnutritive sweeteners		Metformin	
	B (95% CI)	p value	B (95% CI)	p value	B (95% CI)	p value
*Dysbiosis Index**	−0.002 (−0.007, 0.003)	0.455	0.049 (0.022, 0.077)	0.001	0.834 (0.122, 1.546)	0.022
*Alternative Dysbiosis Index**	−0.007 (−0.018, 0.004)	0.198	−0.090 (−0.143, −0.036)	0.001	1.676 (0.279, 3.074)	0.019
Faecalibacterium prausnitzii†	−0.003 (−0.012, 0.007)	0.583	−0.056 (−0.103, −0.009)	0.019	−1.142 (−2.312, 0.028)	0.056
Bacteroides fragilis†	−0.003 (−0.013, 0.008)	0.610	0.074 (0.025, 0.122)	0.003	0.416 (−0.899, 1.730)	0.536
Ruminococcus gnavus†	0.005 (−0.009, 0.018)	0.520	0.069 (0.009, 0.128)	0.024	0.793 (−0.928, 2.514)	0.367
Streptococcus spp.†	−0.009 (−0.023, 0.004)	0.156	0.093 (0.036, 0.150)	0.001	−0.658 (−2.143, 0.826)	0.385

*Linear regression analyses. †Ordinal regression analyses.

Table 4: The total, direct, and indirect (mediated) effects of NNSs on faecal SCFA. The results are presented for the SCFA that were statistically significantly associated with NNSs, and the indirect (mediated) effects are shown for the bacterial groups and species significantly associated with NNSs.

Dependent variable	Mediator	Total effect of NNS B (95% CI), p value	Direct effect of NNS B (95% CI), p value	Indirect effect via mediator B (95% CI)
Butyric acid	Dysbiosis Index	−0.159 (−0.281 to −0.037), 0.011	−0.148 (−0.279 to −0.016), 0.029	−0.011 (−0.061 to 0.033)
Butyric acid	Alternative Dysbiosis Index	−0.159 (−0.281 to −0.037), 0.011	−0.159 (−0.289 to −0.029), 0.016	0.000 (−0.040 to 0.049)
Butyric acid	*Faecalibacterium prausnitzii*	−0.159 (−0.281 to −0.037), 0.011	−0.176 (−0.303 to −0.049), 0.007	0.017 (−0.022 to 0.073)
Butyric acid	*Bacteroides fragilis*	−0.159 (−0.281 to −0.037), 0.011	−0.149 (−0.281 to −0.017), 0.027	−0.010 (−0.051 to 0.024)
Butyric acid	*Ruminococcus gnavus*	−0.159 (−0.281 to −0.037), 0.011	−0.162 (−0.292 to −0.031), 0.016	0.003 (−0.023 to 0.048)
Butyric acid	*Streptococcus* spp.	−0.159 (−0.281 to −0.037), 0.011	−0.152 (−0.282 to −0.023), 0.022	−0.006 (−0.034 to 0.018)
Valeric acid	Dysbiosis Index	−0.022 (−0.043 to −0.002), 0.029	−0.025 (−0.046 to −0.003), 0.027	0.002 (−0.004 to 0.010)
Valeric acid	Alternative Dysbiosis Index	−0.022 (−0.043 to −0.002), 0.029	−0.022 (−0.044 to −0.001), 0.045	−0.001 (−0.008 to 0.008)
Valeric acid	*Faecalibacterium prausnitzii*	−0.022 (−0.043 to −0.002), 0.029	−0.029 (−0.050 to −0.009), 0.006	0.007 (−0.001 to 0.018)
Valeric acid	*Bacteroides fragilis*	−0.022 (−0.043 to −0.002), 0.029	−0.022 (−0.044 to −0.001), 0.046	−0.000 (−0.008 to 0.008)
Valeric acid	*Ruminococcus gnavus*	−0.022 (−0.043 to −0.002), 0.029	−0.020 (−0.041 to 0.002), 0.069	−0.003 (−0.010 to 0.006)
Valeric acid	*Streptococcus* spp.	−0.022 (−0.043 to −0.002), 0.029	−0.023 (−0.045 to −0.002), 0.032	−0.001 (−0.003 to 0.007)

effects of NNSs. The external validity is, however, restricted to this group. Measurement of SCFA in faeces and not in proximal parts of colon might be a limitation. Faecal concentration of SCFA is a poor estimate of colonic SCFA production, but the study did not aim to quantify colonic SCFA production. However, SCFA present in faeces is highly dependent on colonic SCFA production. The microbial test measured only the amount of 39 bacterial groups at different taxonomic levels relative to a reference population and on a coarse scale. A precise measurement of more bacterial groups could have improved the results. The effects might differ between various NNSs, and information about types of NNSs was not available. The results are based on a high number of statistical tests, mostly correlations, and type I errors cannot be excluded. Adjustment for multiple testing was performed for the main findings, which were the associations between NNSs and the individual SCFA. Multiplicity adjustment is a field of much research and controversy. The influential epidemiologist Kenneth Rothman argues against multiplicity adjustment in many settings [50]. In this study, adjustment for multiple testing was added to the other analyses for the most important findings. The analyses, which give very conservative results, showed a clear trend for an association between NNSs and butyric acid and strengthened the findings. The presence of unknown confounders cannot be excluded.

5. Conclusions

Faecal butyric acid was positively and negatively associated with the use of starch, which has been claimed to have antiobesogenic effects, and NNSs, respectively. The measured bacterial groups did not mediate these effects. Lack of butyric acid has weight-inducing effects and metabolic consequences that are unfavourable for subjects with obesity. The negative association between NNSs and butyric acid could indicate an obesogenic effect of NNSs. Since there is no evidence for a weight-reducing effect of NNSs on subjects with obesity and NNSs might counteract the favourable effects of butyric acid, it seems imprudent to use NNSs for weight reduction.

Data Availability

Case report forms (CRFs) on paper were used for collection of the clinical data, and all the CRFs are safely stored. The data were transferred manually to SPSS for statistical analyses. The data files are stored by Innlandet Hospital Trust, Brumunddal, Norway, on a server dedicated to research and with security according to the rules given by the Norwegian Data Protection Authority, P.O. Box 8177 Dep, NO-0034 Oslo, Norway. The data are available on request to the authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this article.

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nutrients

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Article

Increasing the Dose and/or Repeating Faecal Microbiota Transplantation (FMT) Increases the Response in Patients with Irritable Bowel Syndrome (IBS)

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Abstract: Background: Faecal microbiome transplantation (FMT) appears to be an effective method for treating irritable bowel syndrome (IBS) patients. However, it is not clear if a high transplant dose and/or repeating FMT are/is needed to ensure a response. The present study was undertaken to clarify this matter. Methods: Ten IBS patients who did not respond to a 30-g transplant subsequently received a 60-g transplant into the duodenum via a gastroscope. The patients provided faecal samples before and 1 month after FMT. They completed five questionnaires measuring symptoms, fatigue and quality of life at baseline and then at 2 weeks, 1 month and 3 months after FMT. The dysbiosis index (DI) was measured using the GA-map Dysbiosis Test®. Results: Seven patients (70%) responded to the 60-g transplant, with significant clinical improvements in the abdominal symptoms, fatigue and quality of life in 57%, 80% and 67% of these patients. The 60-g transplant also reduced the DI. Conclusion: FMT is an effective treatment for IBS. A high-dose transplant and/or repeated FMT increase the response rate and the intensity of the effects of FMT.

Keywords: abdominal symptoms; dysbiosis; faecal microbiota transplantation; fatigue; IBS; quality of life

1. Introduction

Irritable bowel syndrome (IBS) is a chronic gastrointestinal disorder affecting 12.1% of the world population [1,2]. There are no biochemical or radiological tests for the diagnosis of IBS, and the diagnosis of IBS is based on symptom criteria such as the Rome criteria [3]. IBS affects the patients' quality of life in the same degree as major chronic diseases such as diabetes, liver and kidney failure [3]. There is no effective treatment for the condition, and the current treatment in the clinic is symptomatic [3].

The aetiology of IBS appears to involve multiple factors, including genetics, environment, social learning, dietary intake, intestinal microbiota, low-grade inflammation and disturbances in the endocrine cells of the gastrointestinal tract [1]. The intestinal bacterial composition differs between IBS patients and healthy subjects [4–17]. The role of the intestinal microbiome in several diseases and disorders including IBS has received considerable attention recently [15–18]. The composition and proportions of microbial fractions in healthy subjects are affected by several factors, including genetic factors, diet, social behaviours and other environmental factors [18]. The ideal microbiome composition and bacteria species that are important in the pathophysiology of IBS remain unclear [15,18,19].

Moreover, the impact of low diversity of intestinal bacteria (dysbiosis) in IBS patients is still debated [16–18].

Faecal microbiome transplantation (FMT) has been performed in patients with IBS with the aim of restoring their intestinal microbiota to a healthy condition [18,20,21]. Applying FMT to a small cohort of IBS patients in open-label studies produced positive results [18,20,21]. However, conflicting results have been reported for two recently published randomised double-blind placebo-controlled studies [22,23].

The outcome of FMT seems to be donor-dependent, and the utilization of a so-called superdonor appears to be essential for successful FMT [24]. A superdonor has been defined as a healthy subject who is normobiotic and has a favourable bacterial signature [24]. We recently conducted a randomised, double-blind placebo-controlled study using a single superdonor, and found that the response rate was higher and the effect was stronger in patients who received 60-g transplants than in those who received 30-g transplants [25]. The present study was conducted to establish whether the non-responders to a 30-g transplant in the previous study would respond to a 60-g transplant.

2. Material and Methods

2.1. Study Design

Patients who did not respond to a 30-g transplant in the previous randomised double-blind placebo-controlled study were enrolled in the present open-label study to receive a 60-g transplant.

The patients provided a faecal sample at the baseline and completed five questionnaires to measure their symptoms, fatigue and quality of life. The patients provided another faecal sample at 1 month after FMT, and they completed a further set of questionnaires at 2 weeks, 1 month and 3 months after FMT, which were sent by post. Polyethylene glycol and loperamide were allowed during the intervention as rescue medication.

2.2. The Donor

The donor used has been described in detail elsewhere [25]. In summary, he was a 36-year-old male with a body mass index (BMI) of 23.5 kg/m^2. He was non-smoker and did not take any medication. He was born by vaginal delivery and breastfed. He was screened according to the standard guidelines for FMT donors [26]. In summary, he was interviewed and his medical history and lifestyle habits were reviewed to exclude any exposure to infectious agents or risky social or sexual behaviour such as drug or alcohol abuse. In addition, gastrointestinal, metabolic, and neurological disorders were excluded through physical examination and blood tests. He had also undergone serological tests and stool cultures/examinations in order to avoid any risk of transmitting an infectious disease to the recipients.

The donor regularly consumed dietary supplements rich in protein, vitamins, fibre and minerals. Before using his faeces, an analysis of his faecal bacteria showed a dysbiosis index (DI) of 1, indicating normobiosis. The donor bacterial profile deviated from the normal abundance in 14 out of the 39 bacteria markers tested. Twelve of the deviating bacteria belonged to phylum Firmicutes, one in the phylum Proteobacteria and one in the phylum Verrucomicrobia. Of the bacteria belonging to the Firmicutes phylum, *Lactobacillus*, *Streptococcus* and *Dorea* spp. were higher than the normal levels.

2.3. Patients

Ten IBS patients who did not respond to a 30-g transplant in the previous study [24] were included in the present study, and received a 60-g transplant at 3–4 months after the first transplant. They had IBS according to the Rome IV criteria without alarming symptoms. The medical history was obtained for all of the patients, and they were examined physically. In addition, they were examined by gastroscopy and colonoscopy to exclude other gastrointestinal diseases. All of the patients adhered to a modified NICE (National Institute for Health and Care Excellence) diet. They comprised eight females and two

males aged 39.1 ± 9.0 years (mean ± SD), and three of them had diarrhoea-dominated IBS (IBS-D), two had constipation-dominated IBS (IBS-C) and five had mixed IBS (IBS-M).

2.4. Collection, Preparation and Administration of Faecal Samples

All faecal samples were frozen immediately and kept at −20 °C until they were delivered frozen to hospital on the same day, where they were kept at −80 °C. The faecal samples of the donor were thawed at 4 °C for 2 days before FMT. On the day of the FMT, the faecal samples were weighed, and each 30 g of the faeces was mixed with 40 mL of sterile saline, filtered through a 110 × 10 cm non-woven swab (One Med, Helsinki, Finland), drawn into 50-mL sterile syringes, sealed and kept at 4 °C until the time of the FMT. To achieve a 60-g transplant, 80 mL of this solution was injected through the working channel of a gastroscope into the distal duodenum. The working channel of the gastroscope was then flushed with another 40 mL of sterile saline.

2.5. Questionnaires

Abdominal symptoms were assessed by the IBS Symptom Severity Score (IBS-SSS) and Birmingham IBS symptom questionnaires [27,28]. Some of the items of these two questionnaires overlap, but they were both completed since the IBS-SSS lacks items about diarrhoea and constipation, and the Birmingham IBS symptom questionnaire does not contain an item about abdominal distension. Fatigue was measured using the Fatigue Assessment Scale (FAS) [29]. Quality of life was measured by the IBS Quality of Life (IBS-QoL) and the Short-Form Dyspepsia Index (SF-NDI) questionnaires [30–32]. These questionnaires are complementary since the SF-NDI contains a specific item about work/study that the IBS-QoL questionnaire lacks. In contrast to the IBS-QoL, which measures the quality of life in such a way that an increase in the score indicates an improvement in the quality of life, the SF-NDI measures the reduction in the quality of life, with a decrease in the score indicating an improvement in the quality of life.

A reduction of the total IBS-SSS of ≥50 points after the FMT was considered a response. Furthermore, significant clinical improvements in abdominal symptoms, fatigue and quality of life were considered to have occurred in patients with a decrease of ≥175 points in the total IBS-SSS, a decrease of ≥4 points in the FAS score and an increase of ≥14 points in the IBS-QoL score, respectively [27,31,33].

2.6. Dysbiosis Index

The DI was measured using a commercially available test (GA-map Dysbiosis Test®, Genetic Analysis, Oslo, Norway). The method has been described in detail elsewhere [34,35].

2.7. Ethics

This study was approved by the Regional Committee for Medical and Health Research Ethics West, Bergen, Norway (approval no. 2017/1197/REK vest). All subjects provided oral and written consent to participate. The study was registered at www.clinicaltrials.gov (NCT03822299).

2.8. Statistical Analysis

The paired *t*-test and Kruskal–Wallis test with Dunn's multiple-comparisons test as a post-test were used as applicable. A probability value of $p < 0.05$ was considered significant.

3. Results

Seven of the ten patients responded to the 60-g transplant (70%). The three non-responders belonged to the IBS subtypes IBS-D ($n = 1$), IBS-C ($n = 1$) and IBS-M ($n = 1$).

3.1. Questionnaires

The abdominal symptoms as assessed by the IBS-SSS and Birmingham IBS symptom questionnaires were significantly reduced following the FMT with the 60-g transplant (Figure 1; Figure 2), as was the fatigue score (Figure 3). The quality of life of the patients improved after they received the 60-g transplant, as indicated by their scores for the IBS-QoL and SF-NDI questionnaires (Figures 4 and 5).

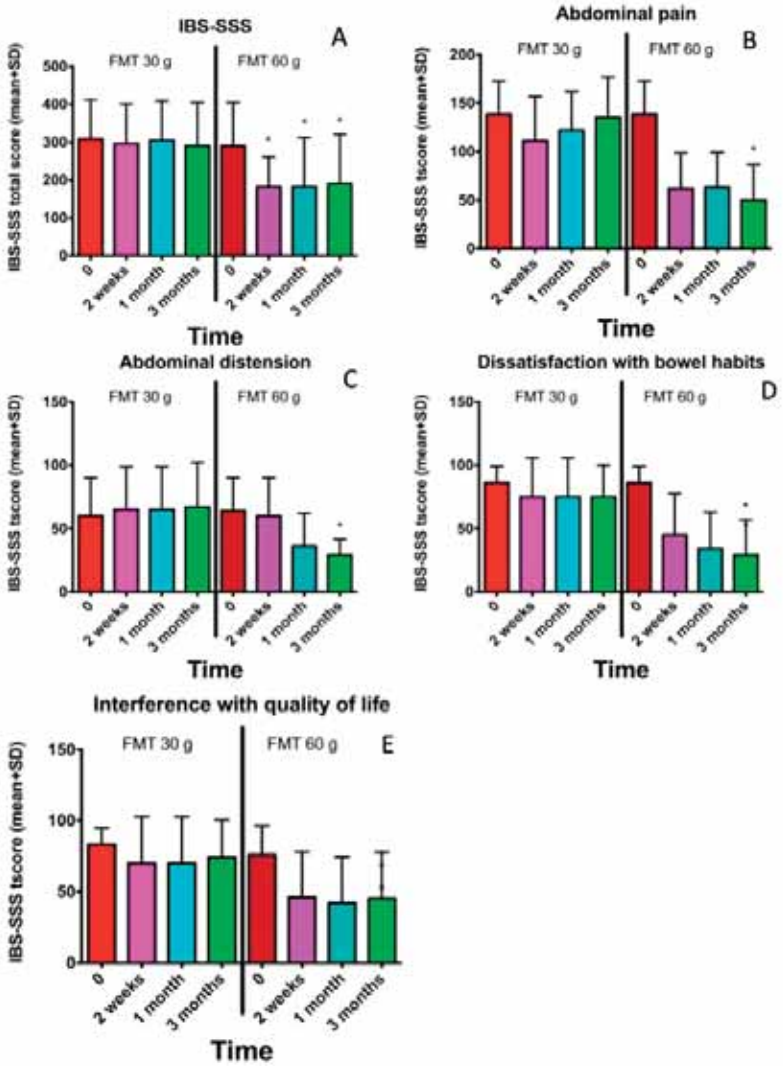

Figure 1. The Irritable Bowel Syndrome Symptom Severity Score (IBS-SSS) at different intervals after receiving 30-g and 60-g transplants for the total score (**A**), abdominal pain (**B**), abdominal distension (**C**), dissatisfaction with bowel habits (**D**) and interference with quality of life (**E**). * $p < 0.05$ compared to baseline. FMT: faecal microbiome transplantation.

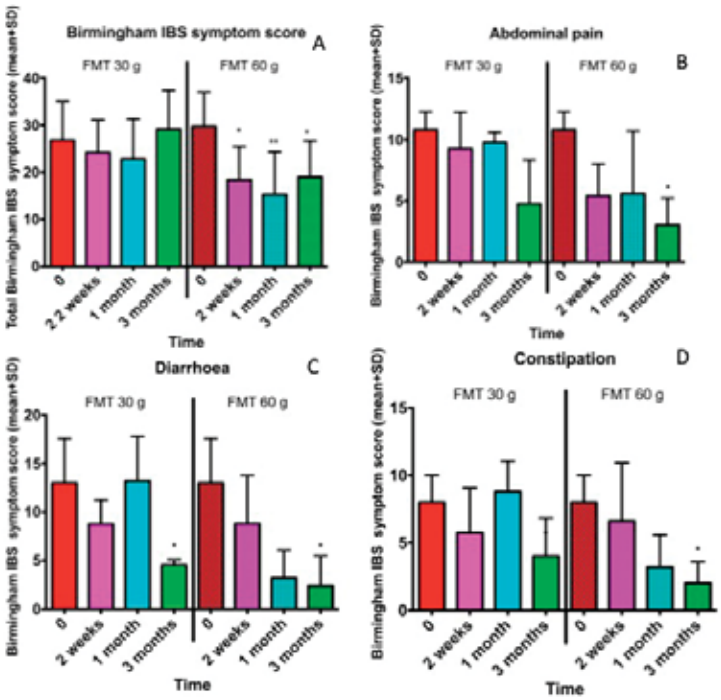

Figure 2. The total Birmingham IBS symptom score and its three items in IBS patients who initially received a 30-g transplant that was subsequently followed by a 60-g transplant for the total score (**A**), abdominal pain (**B**), diarrhoea (**C**) and constipation (**D**). * $p < 0.05$; ** $p < 0.01$ compared to baseline.

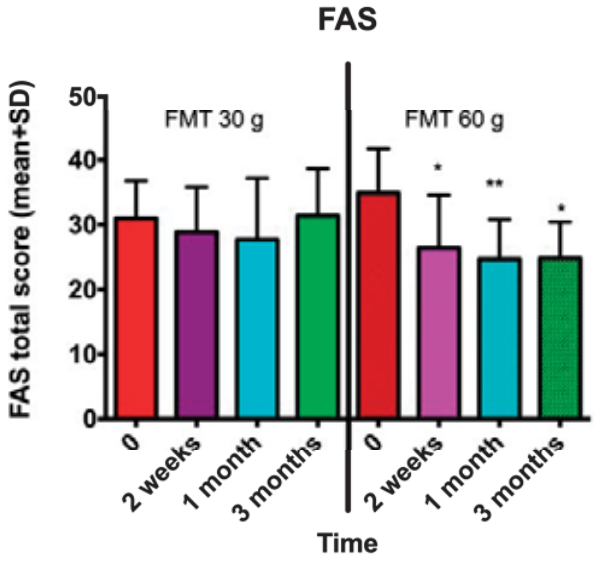

Figure 3. Fatigue as measured by the Fatigue Assessment Scale (FAS) questionnaire was reduced in IBS patients after they received a 60-g transplant but not when they received a 30-g transplant. * $p < 0.05$; ** $p < 0.01$ compared to baseline.

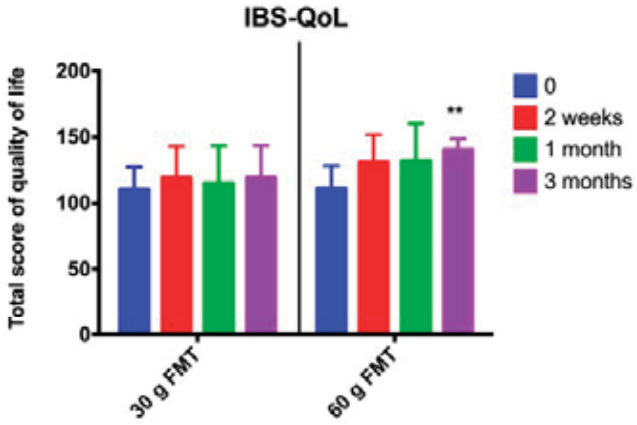

Figure 4. The quality of life as assessed by the IBS Quality of Life (IBS-QoL) questionnaire after FMT with 30-g and 60-g transplants. ** $p < 0.01$ compared to baseline.

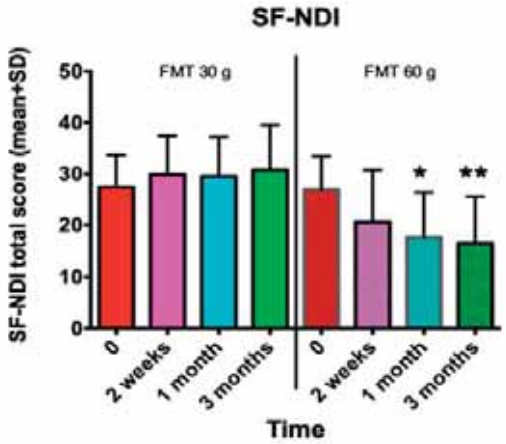

Figure 5. The change in the quality of life as measured by the Short-Form Dyspepsia Index (SF-NDI) questionnaire in IBS patients following FMT with 30-g and 60-g transplants. * $p < 0.05$; ** $p < 0.01$ compared to baseline.

3.2. Dysbiosis Index

The DI decreased significantly following FMT with a 60-g transplant but not with a 30-g transplant (Figure 6).

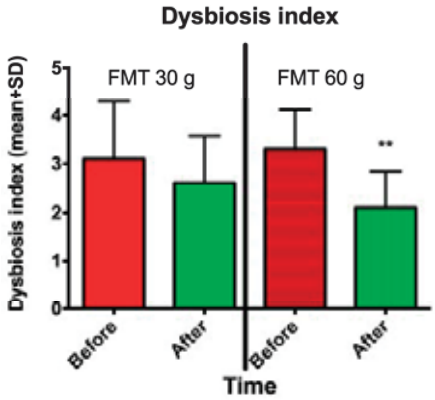

Figure 6. The dysbiosis index (DI) in IBS patients at baseline and 1 month after FMT with 30-g and 60-g transplants. ** $p < 0.01$ compared to baseline.

4. Discussion

As mentioned previously, the intestinal bacterial profile of IBS patients differs from that of healthy subjects and shows a low diversity (dysbiosis) [4–17]. Among the important factors that determine the composition of gut microbiota are genetic factors, diet and drug intake, especially antibiotics [18]. Genetic factors explain only 5–10% of the intestinal bacterial composition [24]. Thus, environmental factors appear to be of great importance in determining the intestinal bacterial profile [24,36]. Despite the gene(s) responsible for IBS not being known yet, IBS seems to be hereditary [1,3] and this can at least partially explain the abnormalities in intestinal microbiota. Moreover, IBS patients avoid certain food items that they believe trigger their symptoms [37]. This might affect their intestinal bacterial profile. Furthermore, proton pump inhibitors (PIPs) have been reported to affect the intestinal bacterial composition [38]. In a recent study on a large cohort of IBS patients, 66% of the patients suffered from gastro-oesophageal reflux, 97% had endoscopic-verified erosive oesophagitis, and most of the patients used PIPs.

FMT appears to be effective at improving the symptoms and quality of life of patients with IBS, and also at reducing their fatigue [18,20–22,25]. The apparent efficacy of frozen faeces facilitates the use of FMT in the clinic by simplifying the logistical problems associated with FMT and makes it possible to establish faeces banks [22,25,39]. The outcome of FMT is dependent on the donor, and utilising a superdonor who is normobiotic and has a favourable specific bacterial signature is essential for success, although what constitutes a "favourable" bacterial signature remains somewhat unclear [24]. We recently identified that such a signature should include an abundance of *Streptococcus*, *Dorea*, and *Lactobacillus* spp. and bacteria belong to the Ruminococcaceae family. There are several issues that need to be clarified before FMT can be applied routinely to IBS patients in the clinic, such as the route of transplant administration, the transplant dose and whether the transplantation should involve a single or repeated dose.

FMT can be achieved by administering the transplant to the duodenum via a nose catheter or the working channel of a gastroscope, or to the colon via the working channel of a colonoscope or as an enema [26]. It has been shown that administering the transplant to the duodenum is effective [20,25]. This makes FMT easier to perform in the clinic, since a gastroscopy takes a shorter time to perform than a colonoscopy and does not require bowel preparation. Regarding the transplant dose needed for a successful FMT, we have shown recently that the response rate is higher and the effect occurs earlier and is more intense for a 60-g transplant than for a 30-g transplant.

The question addressed by the present study was whether the IBS patients who did not respond to a 30-g transplant in our previous study would respond to a 60-g transplant. The present findings showed that 70% of these patients responded to the FMT, and that increasing the dose of the transplant and/or repeating FMT improved their abdominal symptoms, fatigue, quality of life and dysbiosis.

The present study showed further that increasing the dose of the transplant and/or repeating FMT led to the complete remission of abdominal symptoms in 57% of the responders and to clinically significant improvements in fatigue and quality of life in 80% and 67% of the patients, respectively [27,31,33]. However, the present study had the following limitations: it investigated a small cohort of patients and did not study the long-term effects of FMT. Furthermore, the patients enrolled in the study adhered to a NICE-modified diet and therefore the outcomes cannot be extrapolated to the whole IBS population.

5. Conclusions

FMT is an effective treatment for patients with IBS. The success of FMT with frozen faeces administered via the upper gastrointestinal tract makes this treatment easy to perform in everyday clinical work. However, the availability of a superdonor and high-dose and/or repeating FMT are required.

Author Contributions: M.E.-S. designed the study, obtained the funding, administrated the study, recruited the patients, performed FMT, collected, analysed, and interpreted the data and drafted the manuscript, J.G.H., and T.H. contributed to the design of the study, to the analysis and interpretation of the data, and critically revised of the manuscript for important intellectual content.

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Clinical and Experimental Gastroenterology

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ORIGINAL RESEARCH

Fecal microbiota profiles in treatment-naïve pediatric inflammatory bowel disease – associations with disease phenotype, treatment, and outcome

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Purpose: Imbalance in the microbiota, dysbiosis, has been identified in inflammatory bowel disease (IBD). We explored the fecal microbiota in pediatric patients with treatment-naïve IBD, non-IBD patients with gastrointestinal symptoms and healthy children, its relation to IBD subgroups, and treatment outcomes.

Patients and methods: Fecal samples were collected from 235 children below 18 years of age. Eighty children had Crohn's disease (CD), 27 ulcerative colitis (UC), 3 IBD unclassified, 50 were non-IBD symptomatic patients, and 75 were healthy. The bacterial abundance of 54 predefined DNA markers was measured with a 16S rRNA DNA-based test using GA-Map™ technology at diagnosis and after therapy in IBD patients.

Results: Bacterial abundance was similarly reduced in IBD and non-IBD patients in 51 of 54 markers compared to healthy patients (P<0.001). Only *Prevotella* was more abundant in patients (P<0.01). IBD patients with ileocolitis or total colitis had more *Ruminococcus gnavus* (P=0.02) than patients with colonic CD or left-sided UC. CD patients with upper gastrointestinal manifestations had higher *Veillonella* abundance (P<0.01). IBD patients (58%) who received biologic therapy had lower baseline Firmicutes and *Mycoplasma hominis* abundance (P<0.01) than conventionally treated. High Proteobacteria abundance was associated with stricturing/penetrating CD, surgery (P<0.01), and nonmucosal healing (P<0.03). Low *Faecalibacterium prausnitzii* abundance was associated with prior antibiotic therapy (P=0.001), surgery (P=0.02), and nonmucosal healing (P<0.03). After therapy, IBD patients had unchanged dysbiosis.

Conclusion: Fecal microbiota profiles differentiated IBD and non-IBD symptomatic children from healthy children, but displayed similar dysbiosis in IBD and non-IBD symptomatic patients. Pretreatment fecal microbiota profiles may be of prognostic value and aid in treatment individualization in pediatric IBD as severe dysbiosis was associated with an extensive, complicated phenotype, biologic therapy, and nonmucosal healing. The dysbiosis persisted after therapy, regardless of treatments and mucosal healing.

Keywords: dysbiosis, Crohn's disease, ulcerative colitis, Proteobacteria, biologic therapy, *Faecalibacterium prausnitzii*

Plain language summary

- Studies have shown a disturbed gut bacterial composition in chronic inflammatory diseases such as inflammatory bowel disease (IBD) (Crohn's disease and ulcerative colitis).
- In children, it might be difficult to diagnose IBD. Symptoms are often nonspecific, such as abdominal pain and altered bowel movements.

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- Dr Olbjørn et al investigated whether the bacterial composition from stool samples can help to diagnose and treat IBD in children.
- They used advanced DNA profiling to identify and quantify bacteria. They compared the bacterial composition in stool from children with IBD with healthy children and children with gastrointestinal symptoms but without inflammation.
- The researchers report that the bacterial composition in patients with IBD was very different than in healthy children. The differences persisted after treatment.
- The bacterial composition in patients with gastrointestinal symptoms but no inflammation was similarly disturbed as in IBD patients.
- The degree of disturbances in the bacterial composition in children with IBD correlated with the disease course and later therapy. Patients with higher numbers of "bad" bacteria, such as Proteobacteria, were more likely to need aggressive treatment and surgery.
- In children with IBD, testing the bacterial composition in the stool before treatment can help physicians in targeting and individualizing treatments.

Introduction

The pathogenesis of the inflammatory bowel diseases (IBD), Crohn's disease (CD), and ulcerative colitis (UC) is not fully understood, but IBD is thought to occur due to an exaggerated immune response to luminal microbial contents in the gastrointestinal tract in genetically susceptible individuals.[1] A rising incidence of IBD, especially in the pediatric population, has been demonstrated, and the influence of environmental changes, including diet and gut microbiota on the disease pathogenesis, is increasingly recognized.[2,3] The gut microbiota is thought to play an important role not only in IBD but also in functional gastrointestinal disorders such as irritable bowel syndrome, which may display similar symptoms representing a differential diagnosis to IBD.[4,5] Studies of the gut microbiota in IBD and functional gastrointestinal disorders have shown an imbalance, dysbiosis, with compositional changes, including decreased bacterial diversity and abundance.[6–8] The shift in the gut microbiota seems to be associated with a depletion of beneficial vs a relative increase of pro-inflammatory bacteria.[9,10] The diagnostic and prognostic significance of fecal microbiota profiles in children with gastrointestinal symptoms and IBD is not fully explored.

We hypothesized that the fecal microbiota composition could be helpful in diagnosing pediatric IBD patients and in predicting their prognosis. We aimed to assess differences in the abundance of fecal microbiota in treatment-naïve pediatric IBD patients at the time of diagnosis compared to healthy controls and pediatric non-IBD patients with gastrointestinal symptoms. We further explored the value of microbiota abundance in differentiating between IBD phenotypes, subsequent need of biologic therapy, surgery and treatment outcomes, and whether the microbiota changes with therapy.

Patients and methods

IBD patients, non-IBD symptomatic patients, and healthy controls

Patients enrolled in the present study were recruited from the catchment areas of two university hospitals in three population-based prospective epidemiological studies of treatment-naïve pediatric IBD in South-Eastern Norway (IBSEN II),[11,12] Early IBD (in preparation), and EU IBD Character.[13] The inclusion periods for these three multicenter trials were from 2005 to 2015, all with identical protocols and inclusion criteria. Pediatric patients under 18 years, referred during the inclusion periods and believed to have IBD based on symptoms, were included. IBD was diagnosed in accordance with the Porto criteria.[14] Patients who did not meet the diagnostic criteria for IBD were included as non-IBD symptomatic controls. These patients had a macroscopically and histologicallly normal mucosa and normal MRI examinations. Healthy children and adolescents between the age of 2 and 18 years and recruited during the period of 2013–2014 from the same catchment areas as the patients delivered fecal samples and were included as healthy controls. They had no chronic diseases, no IBD in the family, followed a normal diet (children on exclusion diets, gluten-free, cows milk protein-free, vegetarian/vegan, were excluded), had not traveled outside Europe or used antibiotics within the last 6 months, had no recorded gastrointestinal complaints, did not use proton pump inhibitors, and had normal fecal calprotectin levels (<50 mg/kg).[15]

Clinical, endoscopic, radiological, and laboratory data

Age, gender, symptoms, disease activity index scores, disease, and family history of the IBD and non-IBD symptomatic patients were registered as previously described.[11,12,16] The Paris classification was used to characterize disease distribution and behavior.[17] In patients, feces were sampled at home in three designated containers without additives on the day before endoscopy, kept refrigerated or frozen, and brought to the hospital the next day. Fecal sample from one container was analyzed for calprotectin (FeCal-test, Bühlmann, Basel, Switzerland), the second for pathogenic bacteria, and the third

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container with feces was frozen at –80°C for later microbiota analysis. The healthy controls received two designated fecal sampling kits at home for handling of samples before deliverance to Genetic Analysis AS, Oslo, Norway. One sample was analyzed for fecal calprotectin (FeCal-test, Bühlmann); the other was frozen at –80°C and stored for later microbiota analysis. For all samples, the maximum time interval until frozen at –80°C was 3 days; thereafter the samples were kept frozen and not thawed until analysis.

Microbiota analysis

The microbiota was analyzed using the GA-Map™ technology (Genetic Analysis AS), a PCR, and 16S RNA-based analysis. The method uses a targeted approach to detect predefined bacteria believed to be important in identifying and characterizing gut bacteria dysbiosis.[18] The test measures relative bacterial abundance based on the fluorescence signal strength (FSS) of bacterial DNA markers. The markers are targeting variable regions V3–V7 of the bacterial 16S rRNA gene. The method utilizes 54 bacterial markers (Table S1), covering more than 300 bacteria at different taxonomic levels: 26 species specific, 19 detect genus specific, and 9 bacteria at higher taxonomic levels (phyla, class, and family). All samples were analyzed at the same time point. The laboratory was blinded for the diagnosis of IBD, non-IBD, or healthy.

IBD treatment

Treatment was decided individually, prospectively, at the discretion of the treating pediatrician. Initial treatment options to induce remission were exclusive enteral nutrition in CD and corticosteroids and/or 5-aminosalicylic acids in CD and UC patients. Maintenance therapy with azathioprine or methotrexate was in general started simultaneously (Table 1). The indication for surgery or treatment with biologic therapy (TNF blockers) was failure to induce remission with conventional treatments or relapse after primary induction.

Statistical analyses

Data were described using counts and percentages for categorical data and medians and ranges for continuous data. To explore the ability of all 54 bacterial markers to distinguish between IBD, non-IBD symptomatic patients, and healthy controls, we performed principal component analysis. The FSSs from the 54 markers were added for each patient, and the sum illustrated a relative abundance, denoted the total signal strength. Crude comparisons between groups were performed using Mann–Whitney Wilcoxon tests and Wilcoxon signed-rank tests (before and after treatment) for continuous variables and chi-squared tests for categorical data.

Table 1 Disease extent and behavior at diagnosis according to the Paris classification and treatments in pediatric IBD patients

IBD diagnosis	n (%)
CD	80 (73)
UC	27 (25)
IBDU	3 (3)
CD behavior	
Inflammatory	53 (66)
Stricturing	12 (15)
Penetrating	15 (19)
CD distribution	
Ileal	5 (6)
Colonic	24 (30)
Ileocolonic	47 (59)
Upper gastrointestinal	54 (68)
Perianal	17 (21)
UC/IBDU disease extent	
Proctitis	5 (17)
Left sided colitis	8 (27)
Extensive/total colitis	17 (57)
Treatment	
Immunomodulators	98 (89)
Biologic therapy	64 (58)
Surgery	17 (15)

Abbreviations: CD, Crohn's disease; IBD, inflammatory bowel disease; IBDU, inflammatory bowel disease unclassified; UC, ulcerative colitis.

Areas under the curves were calculated and receiver operating characteristic analysis conducted to evaluate the performance of selected bacterial abundances in distinguishing IBD phenotypes and treatments. All tests were two-sided. *P*-values <0.05 were considered statistically significant. We regarded our study exploratory; therefore, we did not correct for multiple testing. However, in order to validate our results, each observation was randomized into a test set or a training set so that the number of observations was equal in both sets. Only the statistically significant differences confirmed in the training set are reported. All analyses were performed using SPSS, statistical software, version 24 (SPSS Inc., Chicago, IL, USA) and Stata, version 9.

Ethical considerations

The study was conducted with informed patients and parental/guardian written consent as appropriate and with full ethical approval, in accordance with the Declaration of Helsinki, and with approval by the Regional Committee for Medical Research Ethics, South-Eastern Norway, reference no. REK S-04209.

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Results

Of the 235 included children and adolescents, IBD was diagnosed in 110 patients (80 CD, 27 UC, and 3 IBDU) (Table 1), 50 patients were included as non-IBD symptomatic patients, and 75 healthy children served as controls. None of the non-IBD symptomatic patients have developed IBD as of December 1, 2018. IBD, non-IBD, and healthy controls were comparable concerning all demographic variables except for more females among the non-IBD patients and a slightly lower median age in the healthy controls (Table 2).

The bacterial abundances were compared between the three pediatric groups, healthy controls, IBD patients and non-IBD symptomatic patients, as well as between subgroups of IBD and after treatment in 31 of the IBD patients. To investigate the impact of antibiotics on microbiota profiles of the IBD patients, they were grouped according to whether they had received antibiotics within 3 months prior to diagnosis or not, and analyzed separately. Eight of the 110 IBD patients had received antibiotics, and these patients had significantly lower abundance of *Faecalibacterium prausnitzii* (P=0.001) compared to IBD patients without prior antibiotic therapy (Figure 1). However, excluding these patients from the statistical analyses did not impact the other results presented in the material.

Microbiota in relation to age

We found significant differences in microbiota abundance when comparing healthy children below (n=38) and above (n=37) 10 years of age. Healthy children aged <10 years had lower abundance of *Clostridiales* and higher abundance of *Bifidobacterium*, both P<0.01. These differences were not replicated in the patients as we did not find any differences in bacterial profiles between high and low age groups in the IBD and non-IBD symptomatic patient groups. Additional post hoc analysis with an age matched selection of controls

Table 2 Demographics and laboratory tests of IBD, non-IBD patients, and healthy controls at baseline

Variable	CD	UC	IBD (CD + UC + IBDU)	Non-IBD	Healthy
Patients, n (%)	80 (100)	27 (100)	110 (100)	50 (100)	75 (100)
Age in years, median (range)	13 (0.74–17.9)	11.5 (4–17)	12.5 (0.74–17.9)	12 (3.7–18)	10 (2–17.9)
Males, n (%)	43 (54)	11 (41)	56 (51)	18 (36)	34 (45)
PCDAI/PUCAI, median (range)	20 (0–62.5)	40 (0–75)	–	N/A	N/A
Fecal calprotectin mg/kg, median (range)	589 (20–8,625)	987 (11–6,123)	701	47 (9–1,260)	15 (0–50)
Fecal calprotectin >1,000 mg/kg, n (%)	31 (39)	12 (48)	43 (40)	2 (4)	0

Abbreviations: CD, Crohn's disease; IBD, inflammatory bowel disease; IBDU, inflammatory bowel disease unclassified; N/A, not applicable; PCDAI, pediatric Crohn's disease activity index; PUCAI, pediatric ulcerative colitis activity index; UC, ulcerative colitis.

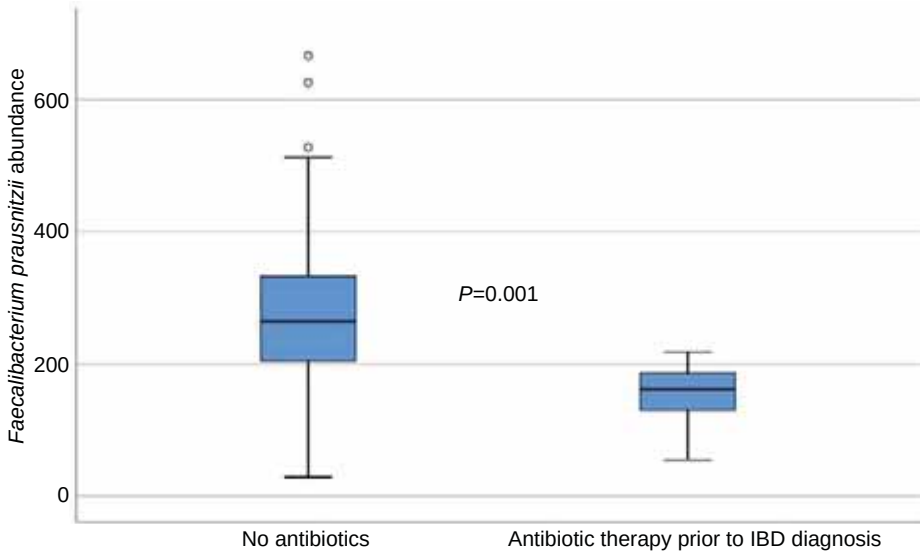

Figure 1 *Faecalibacterium prausnitzii* abundance in IBD patients according to whether they had received antibiotics prior to the diagnosis (measured in fluorescence signal strength in 1,000 units).
Abbreviation: IBD, inflammatory bowel disease.

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did not influence outcome/differences between patients and healthy.

Microbiota in IBD and non-IBD vs healthy

In all symptomatic patients, regardless of IBD or non-IBD status, the total signal strength, measured as the sum of the 54 FSSs, was significantly lower compared to healthy controls, illustrating that the patients had lower abundance of the predefined bacterial markers. Patients had reduced bacterial abundances in 51/54 markers, $P<0.001$ (Figure 2). The only bacterial marker that was more abundant in patients (IBD and non-IBD) compared to healthy controls was *Prevotella* ($P<0.01$). The abundances of *Lachnospiraceae* and *Bacteroides* were similar in all groups. The principal component analysis plot visualizes how the microbiota composition differs between IBD, non-IBD, and healthy and overlaps between IBD and non-IBD symptomatic patients (Figure 3).

Microbiota in IBD vs non-IBD

The bacterial abundances were similarly dysbiotic in IBD and non-IBD symptomatic patients; however, one marker targeting the Firmicutes phylum was significantly less abundant in IBD patients compared to non-IBD patients ($P<0.01$), as well as *Eubacterium rectale* ($P<0.01$), *Eubacterium biforme*/*Streptococcus agalactiae* ($P=0.04$), *Parabacteroides*, and *Bifidobacterium* species (both $P=0.02$).

Microbiota in IBD patients

The fecal microbiota abundances did not differ between UC and CD, except that CD patients had lower abundance of *Mycoplasma hominis* ($P<0.02$).

Microbiota related to disease distribution and behavior in IBD patients

IBD patients with extensive disease, ileocolitis in CD or extensive colitis in UC, had higher abundance of *Ruminococcus gnavus* ($P=0.02$) compared to CD patients with isolated colonic disease and UC patients with limited disease distribution (left-sided colitis or proctitis). CD patients with upper gastrointestinal involvement had higher *Veillonella* abundance ($P<0.01$) compared to patients without upper gastrointestinal lesions.

CD patients with a high abundance of Proteobacteria were more likely to have complicated disease behavior, stricturing or penetrating disease, compared to patients with lower levels of these bacteria, $P<0.01$ (Figure 4).

Microbiota and association with treatment

IBD patients who were treated with biologic therapy, 64 (58%), had lower abundance of Firmicutes ($P=0.015$) and *M. hominis* ($P=0.009$) compared to conventional treated patients (Figure 5). Seventeen (15%) of the IBD patients required surgery, and mucosal healing (assessed by ileocolonoscopy) was not achieved in 40 (36%) of the patients

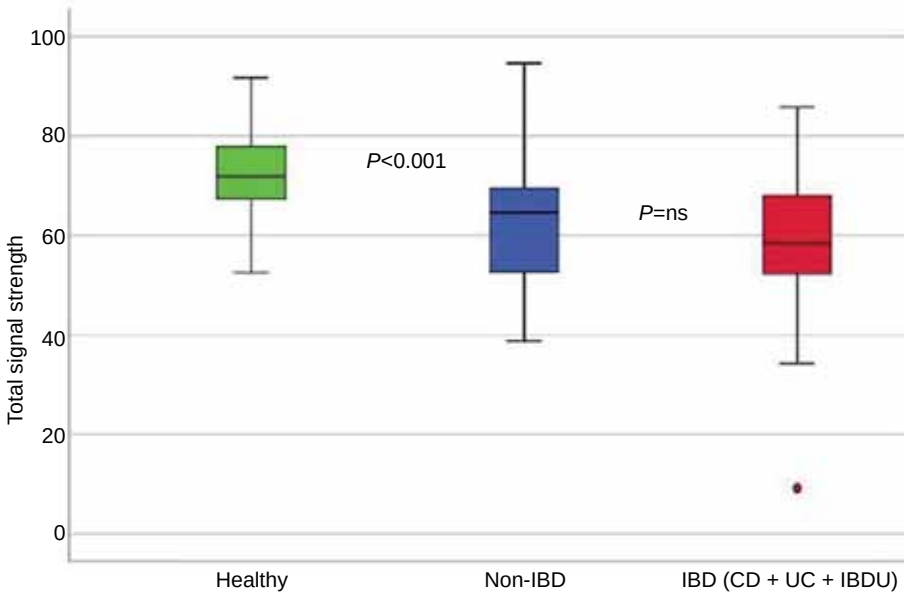

Figure 2 Boxplot illustrating the differences in the total fluorescence signal strength measured in 1,000 units between IBD, non-IBD symptomatic patients, and healthy controls.

Abbreviations: IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; IBDU, inflammatory bowel disease unclassified; ns, not significant.

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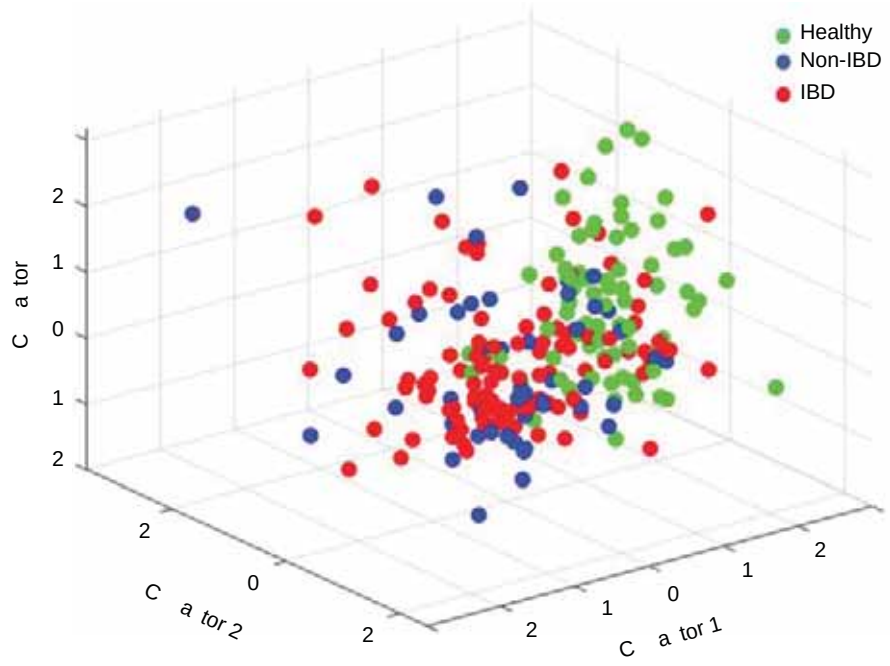

Figure 3 Principal component analysis, illustrating the difference in microbiota abundance of all 54 bacterial probes between IBD, non-IBD symptomatic patients, and healthy controls.
Notes: Each dot represents one individual. The units represent the total item loadings on each of the extracted factors.
Abbreviations: IBD, inflammatory bowel disease; PCA, principal component analysis.

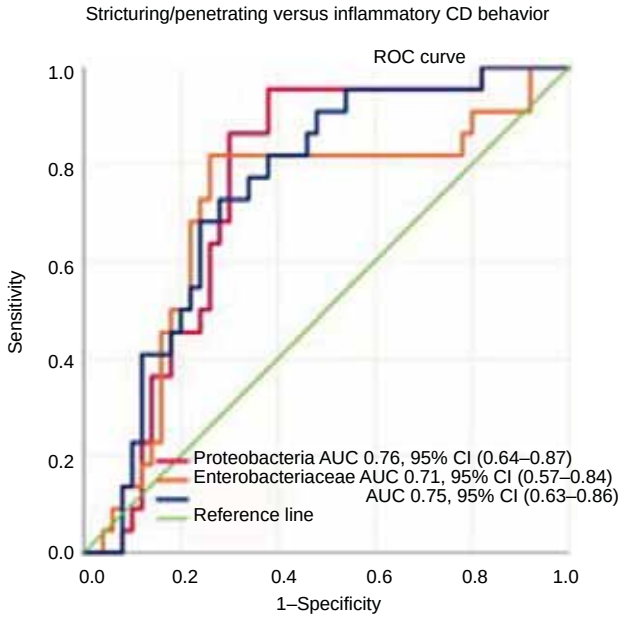

Figure 4 Sensitivity and specificity of Proteobacteria, *Enterobacteriaceae,* and *Shigella/Escherichia* abundance in differentiating Crohn's disease phenotypes (stricturing/penetrating vs inflammatory disease behavior) using the area under the receiver operating characteristics curve analysis.
Abbreviations: CD, Crohn's disease; AUC, area under the curve.

despite medical therapy. Surgery and lack of mucosal healing were associated with higher abundance of Proteobacteria (P=0.002 and P=0.011) (Figure 6) and lower baseline abundance of *F. prausnitzii* (P=0.02 and P=0.017), respectively, compared to nonoperated IBD patients and patients with mucosal healing.

Of the IBD patients (22 CD and 9 UC) with repeated microbiota analysis at follow-up 18 months after treatment,

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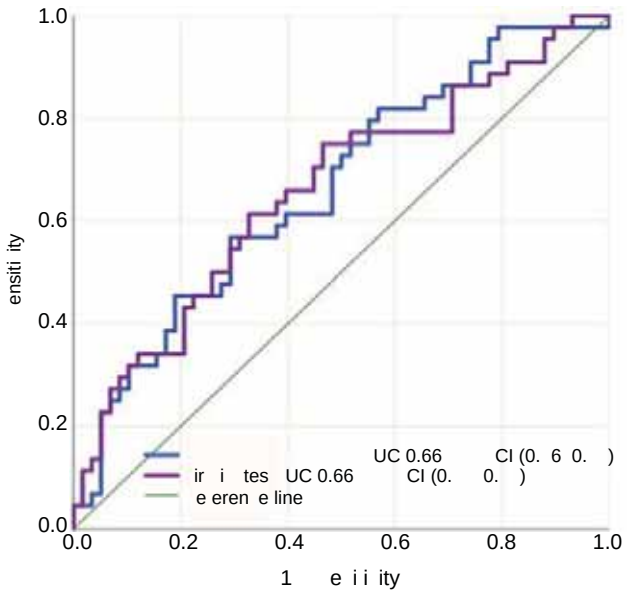

Figure 5 Sensitivity and specificity of Firmicutes and *Mycoplasma hominis* abundance in differentiating conventional- vs biologic therapy-treated IBD patients using the area under the receiver operating characteristics curve analysis.
Abbreviations: IBD, inflammatory bowel disease; CD, Crohn's disease; AUC, area under the curve.

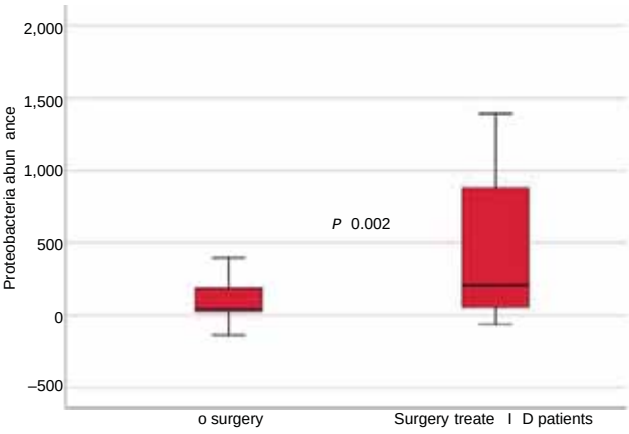

Figure 6 Proteobacteria abundance in IBD patients according to whether they needed surgery or not (measured in fluorescence signal strength in 1,000 units).
Abbreviation: IBD, inflammatory bowel disease.

15 (48%) patients had received biologic therapy and 18 (58%) were in remission with mucosal healing. The microbiota composition and bacterial profiles were unchanged for 53 of 54 markers after treatment, regardless of treatment modality received and remission status. One marker targeting *Eubacterium hallii* species was less abundant after treatment, *P*=0.03.

Microbiota and association with fecal calprotectin

IBD patients with fecal calprotectin levels above 1,000 mg/kg (31 CD, 12 UC) had significantly higher abundance of Proteobacteria (*P*=0.012) and *Prevotella* (*P*=0.011) than patients with lower levels (<1,000 mg/kg) of fecal calprotectin (Table 2). Fecal calprotectin over 1,000 was associated with subsequent biologic therapy, *P*=0.001, but not with later surgery.

Discussion

In the present prospective study of newly diagnosed children and adolescents with IBD, we demonstrated dysbiosis in both treatment-naïve pediatric IBD and non-IBD symptomatic patients. Their fecal microbiota differed significantly from the microbiota of healthy children with lower bacterial abundances measured with the GA-Map technology. Our non-IBD symptomatic patients consisted of pediatric patients admitted to the hospital due to symptoms and findings suspicious of IBD, but without evidence of inflammation during workup. Some of these patients may have had preclinical/latent IBD or other conditions such as disturbed permeability and motility influencing the study results. We believe most of these non-IBD symptomatic patients have functional gastrointestinal disorders. Ideally we should have further characterized and subtyped these patients with the use of Rome criteria for functional gastrointestinal disorders. However, due to the limited sample size of 50 non-IBD symptomatic patients, further subclassification would reduce the statistical power to reveal clinical significant differences between the groups.

There was a similar dysbiotic profile with reduced microbial abundance in IBD and non-IBD compared to healthy individuals in the present study; thus the bacterial profiles provided by the GA-Map technology performed less well than fecal calprotectin in detecting inflammation and discriminating IBD from non-IBD symptomatic patients. However, the finding of dysbiosis in non-IBD symptomatic patients may confirm the relevance of their symptoms and discomfort. Presence and characterization of dysbiosis enables the physician to diagnose "functional" disease in a positive manner.

Within the group of patients diagnosed with IBD, we found that bacterial abundances at baseline seemed to be associated with disease extension, phenotype, biologic therapy, surgery, and mucosal healing. At follow-up, after treatment, the dysbiosis was still present and its status mainly unchanged in IBD patients.

We found reduced abundances of beneficial *Eubacterium* and *Bifidobacterium* species in IBD and non-IBD symptomatic patients compared to healthy children, in agreement with previous adult[6,19,20] and pediatric studies.[21–23] *Eubacteria* and *Bifidobacterium* are known to inhibit the growth of potentially pathogenic species[24] and produce short-chain fatty acids (SCFAs) through fermentation of dietary fiber. SCFAs are important energy sources for enterocytes and contribute to

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homeostasis of colonic regulatory T cell populations.[25] The reduction of protective commensal microbes and concomitant loss of their protective function can have an influence on development of IBD and the disease course. As expected, *Bifidobacterium* was more abundant in healthy children below 10 years of age than in the healthy adolescents.[26] We found no difference in bacterial abundance between age groups for our IBD and non-IBD symptomatic patients. This may be due to disease state being a stronger driver of the microbiota composition than age.

Patients with IBD have an expansion of pro-inflammatory bacteria such as *Prevotella*,[27,28] *R. gnavus*,[29] and *Veillonella*.[22,28] V*eillonella* was enriched in our CD patients with upper gastrointestinal involvement. *R. gnavus*, a bacterium that expresses beta-glucuronidase activity, which may cause local inflammation, was associated with more extensive IBD distribution in our patients. *Prevotella*, *R. gnavus*, and Proteobacteria have been found to correlate with markers of disease activity and inflammation,[28,30] which were reproduced in the present study. Proteobacteria are pathobionts, meaning that they may expand as a result of a microbial imbalance and exert pathogenic effects on the host and are consistently reported enriched in IBD.[31–33] Our CD patients with a complicated phenotype had high abundance of Proteobacteria, in accordance with the previous reports. Proteobacteria enrichment has been associated with early relapse after induction of remission with exclusive enteral nutrition in pediatric CD,[34] and in our patients, high abundance was associated with the need for surgery and lack of mucosal healing. These findings implicate that Proteobacteria abundance might be a marker for an aggressive disease course with a higher risk of treatment failure.

F. prausnitzii, a highly abundant human gut microbe, is reported to be reduced in both adult and pediatric patients with IBD.[6,13,35,36] It acts as a protective factor for the intestinal mucosa, enhances barrier function, and can exert anti-inflammatory effects.[20,37,38] Our IBD patients who needed surgery and who did not achieve mucosal healing with therapy, as well as patients treated with antibiotics before the IBD diagnosis, had the lowest abundance of *F. prausnitzii*. This is in line with observations that low abundance of *F. prausnitzii* may predict nonresponse to anti-TNF therapy in UC[39] and relapse after infliximab termination in CD patients.[40] Studies have found baseline microbiota to be associated with treatment responses,[34,36,39] but how the microbiota composition and abundances change with treatment is less studied. The IBD patients in our sample with repeated fecal microbiota analysis displayed persistent, unchanged dysbiosis after treatment, regardless of treatment modalities and remission status. Similar results have been reported in another pediatric study, where the dysbiosis improved, but nonetheless persisted despite mucosal healing.[41] Lewis et al found that effective exclusive enteral nutrition and TNF blocker therapy reduced but failed to eliminate the dysbiosis of pediatric CD patients.[42] Others have found the fecal microbiota to become more dysbiotic with dietary treatment such as exclusive enteral nutrition.[43–45] Perhaps sustained and deep remission requires normalization of the gut dysbiosis, or maybe it is not possible to reverse the dysbiosis once the gut homeostasis is perturbed as fundamentally as it is in IBD. Measuring relative fecal microbiota abundance might not be an optimal method as it is not suited to determine the effects of dysbiosis, giving no information about the functional consequences. As a prognostic tool, fecal microbiota profiles may still be of value, also in established IBD patients on treatment, as the dysbiosis remained despite treatment and remission. However, due to the small number of patients with repeated sampling, firm conclusions cannot be drawn.

Regarding fecal microbial differences between CD and UC, the literature has been conflicting. Similarly, as in our report, some previous studies did not find major differences in bacterial profiles between active CD and active UC.[23,36]

The strength of our study is the extensive workup, characterization, and classification of our IBD patients. All non-IBD symptomatic patients underwent the same procedures as the IBD patients. Upper and lower endoscopies as well as MRI of the small intestine were performed, and for patients included in the IBSEN II cohort these investigations were repeated after 1–2 years of follow-up. The fact that none of the non-IBD symptomatic patients have been diagnosed with IBD despite several (minimum 3, maximum 13) years of follow-up makes misclassifications and undiagnosed IBD less likely.

The healthy controls were not investigated in the same manner as the patients, as invasive tests in healthy children are considered unethical. Even though children with gastrointestinal complaints, recent antibiotic exposure, and elevated fecal calprotectin were excluded as healthy controls, some could have had conditions that may have influenced the study results, as there is substantial evidence that diseases outside of the gastrointestinal tract influence the gut microbiota.[46]

Dietary patterns and smoking are known to influence the microbiota;[45] therefore, we excluded patients on exclusion diets. None of our adolescents admitted to smoking.[47]

The selection of microbes in the GA-map™ technology is based on literature studies and contain gut bacteria whose

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profiles are known to define dysbiosis in adults, with the inherent risk of not including bacteria that could be important in children and adolescents. Bacterial 16S sequencing of all microbes would give additional results, but is more expensive. The same is true for shotgun metagenomic sequencing, encompassing all DNA of bacteria, viruses, and fungi. Together with an altered bacterial composition, studies have revealed that IBD patients have fungal dysbiosis as well as alterations in the intestinal virome, which we have not investigated in our study.[48,49] Deep sequencing and shotgun metagenomic sequencing methods need bioinformatics tools and reference datasets that are still under development and not yet readily available for clinical practice. The GA-Map technology provided us with a commercially available and clinically validated (in adults) tool.

Our study has several limitations. First, the sample size is limited, reducing the statistical power to detect differences in microbiota composition as statistically significant. We did not adjust for multiple testing as we considered this study to be exploratory, increasing the risk for accepting false-positive associations. However, we validated our results by splitting our data into a training and a test set, and most associations estimated in the whole cohort remained statistically significant. The positive relationship between inflammation, increased abundance of pathobionts and concomitant loss of beneficial bacteria, is reassuring as it is in line with previous research reports.[50]

Another limitation is the difference in storage time of the fecal samples, which may have influenced outcomes. Also, theoretically, the representativeness of the samples could have deteriorated during the timespan from collection until frozen. Based on previous experience and in vitro examinations,[18] the microbial material collected in different cohorts was not considered to be affected. Since repeated thawing is known to influence the microbiota, the samples were kept frozen until analysis.

We acknowledge that the GA-Map technology test measures the abundance of bacteria without giving information about the functional importance and highly abundant bacteria might not be functionally active.[51] Additionally, in the present study, we explored the fecal microbiota only. One study comparing mucosal associated microbiota with fecal microbiota reported that the ileal mucosa followed by the rectal mucosa obtained the best performance in classifying CD and that stool samples performed less well.[22] Mucosa associated microbiota must be sampled by invasive methods. In this study however, we wanted noninvasive methods to associate microbiota with disease state. Our findings show promise for microbiota profiles and abundance to be used in conjuncture with other prognostic factors and known biomarkers in an attempt to risk stratify and individualize treatments in pediatric IBD.

Conclusion

Fecal microbiota profiles similarly differentiated IBD and non-IBD symptomatic children from healthy children. Microbiota profiles with relative enrichment of Proteobacteria and low abundance of *F. prausnitzii* in newly diagnosed pediatric IBD seem to be associated with complicated disease phenotypes, subsequent need of biologic therapy, surgery, and nonmucosal healing. The dysbiosis persisted after therapy, regardless of treatments and remission status. The relative abundances of selected bacteria might be of value as prognostic markers in stratifying pediatric IBD into subgroups and aid in patient selection for early aggressive therapy in an effort to prevent a complicated disease course.

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Author contributions

All authors have made substantial contributions in conception and study design, acquisition of data, or analysis and

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interpretation of data and taken part in drafting the article or revising it critically for important intellectual content. All authors have seen and approved the final version of the manuscript submitted to the journal and agree to be accountable for all aspects of the work. CO and GP contributed in planning the study, collecting data, analyzing, and interpreting the results and drafting the article. MCS, BN, ETE, and MHV contributed in planning the study, analyzing and interpreting the results, and drafting the article.

Disclosure

Christine Olbjørn is a member of the advisory board of AbbVie and has received speaker honoraria from AbbVie, Nutricia, Norgine, Tillotts Pharma, and Mead Johnson. Morten H Vatn has been an advisor for Genetic Analysis and organizer of the International Advisory Board of Genetic Analysis, a member of the advisory board for Tillotts Pharma, and has received speaker honoraria from AstraZeneca, AbbVie, MSD, and Falk. Gøri Perminow is a member of the advisory board of AbbVie and is a member of the steering committee in the IBSEN III study. The IBSEN III study has received an Investigator Initiated Research Grant from Takeda and nonrestricted research grants from Ferring Pharmaceuticals and Tillotts Pharma. Christina Casén and Magdalena K Karlsson are employed by Genetic Analysis. The authors report no other conflicts of interest in this work.

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Supplementary material

Table S1 List of phyla and bacterial names of the GA-Map™ technology markers

Bacteria number	Phylum	Name
100	Actinobacteria	*Actinobacteria*
101	Actinobacteria	*Actinomycetales*
102	Actinobacteria	*Atopobium rimae*
103	Actinobacteria	*Bifidobacterium* spp.
201	Bacteroidetes	*Alistipes*
202	Bacteroidetes	*Alistipes onderdonkii*
203	Bacteroidetes	*Bacteroides fragilis*
204	Bacteroidetes	*Bacteroides pectinophilus*
205	Bacteroidetes	*Bacteroides* spp.
206	Bacteroidetes	*Bacteroides* spp. and *Prevotella* spp.
207	Bacteroidetes	*Bacteroides stercoris*
208	Bacteroidetes	*Bacteroides zoogleoformans*
209	Bacteroidetes	*Parabacteroides johnsonii*
210	Bacteroidetes	*Parabacteroides* spp.
211	Bacteroidetes	*Prevotella nigrescens*
300	Firmicutes	*Firmicutes*
301	Firmicutes	*Anaerotruncus colihominis*
302	Firmicutes	*Bacilli*
303	Firmicutes	*Bacillus megaterium*
304	Firmicutes	*Catenibacterium mitsuokai*
305	Firmicutes	*Clostridia*
306	Firmicutes	*Clostridium methylpentosum*
307	Firmicutes	*Clostridium* sp.
308	Firmicutes	*Coprobacillus cateniformis*
309	Firmicutes	*Desulfitispora alkaliphila*
310	Firmicutes	*Dialister invisus*
311	Firmicutes	*Dialister invisus* and *Megasphaera micronuciformis*
312	Firmicutes	*Dorea* spp.
313	Firmicutes	*Eubacterium biforme*
314	Firmicutes	*Eubacterium hallii*
315	Firmicutes	*Eubacterium rectale*
316	Firmicutes	*Eubacterium siraeum*
317	Firmicutes	*Faecalibacterium prausnitzii*
318	Firmicutes	*Lachnospiraceae*
319	Firmicutes	*Lactobacillus ruminis* and *Pediococcus acidilactici*
320	Firmicutes	*Lactobacillus* spp.
321	Firmicutes	*Lactobacillus* spp. 2
322	Firmicutes	*Phascolarctobacterium* sp.
323	Firmicutes	*Ruminococcus albus* and *Ruminococcus bromii*
324	Firmicutes	*Ruminococcus gnavus*
325	Firmicutes	*Streptococcus agalactiae* and *Eubacterium rectale*
326	Firmicutes	*Streptococcus salivarius* ssp. *thermophilus* and *sanguinis*
327	Firmicutes	*Streptococcus salivarius* ssp. *thermophilus*
328	Firmicutes	*Streptococcus* spp.
329	Firmicutes	*Streptococcus* spp. 2
330	Firmicutes	*Veillonella* spp.
331	Firmicutes/Tenericutes/Bacteroidetes species	*Firmicutes* (various)
500	Proteobacteria	Proteobacteria
501	Proteobacteria	*Acinetobacter junii*
502	Proteobacteria	*Enterobacteriaceae*
503	Proteobacteria	*Pseudomonas* spp.
504	Proteobacteria	*Shigella* spp. and *Echerichia* spp.
601	Tenericutes	*Mycoplasma hominis*
701	Verrucomicrobia	*Akkermansia muciniphila*

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RESEARCH ARTICLE Open Access

Separating "good" from "bad" faecal dysbiosis – evidence from two cross-sectional studies

Per G. Farup[1,2*], Martin Aasbrenn[3,4] and Jørgen Valeur[5]

Abstract

Background: Faecal dysbiosis associated with the use of metformin has been conceived as a favourable ("good") dysbiosis and that with intake of non-nutritive sweeteners (NNS) as unfavourable ("bad"). The study aimed to construct an alternative dysbiosis index (ADI) for the separation of the dysbioses into "good" and "bad", and to validate the ADI.

Methods: Subjects with morbid obesity were included. Use of NNS and drugs were noted, IBS was classified according to the Rome III criteria and the severity measured with the Irritable bowel severity scoring system (IBSSS). Faecal dysbiosis was tested with GA-Map ™ Dysbiosis test (Genetic Analysis AS, Oslo, Norway). The result was given as Dysbiosis Index (DI) scores 1–5, score > 2 indicates dysbiosis. An ADI was constructed and validated in subjects with IBS at another hospital.

Results: Seventy-six women and 14 men aged 44.7 years (SD 8.6) with BMI 41.8 kg/m^2 (SD 3.6) were included. Dysbiosis was associated with the use of NNS and metformin, but not with IBS or IBSSS. An ADI based on differences in 7 bacteria was positively and negatively associated with the "good" metformin dysbiosis and the "bad" NNS dysbiosis respectively. The ADI was also negatively associated with IBSSS (a "bad" dysbiosis). The negative associations between ADI and IBS and IBSS were confirmed in the validation group.

Conclusions: The new ADI, but not the DI, allowed separation of the "good" and "bad" faecal dysbiosis. Rather than merely reporting dysbiosis and degrees of dysbiosis, future diagnostic tests should distinguish between types of dysbiosis.

Keywords: Dysbiosis, Irritable bowel syndrome, Metformin, Microbiota, Non-nutritive sweeteners; obesity

Background

The gut microbiota interferes with the mucosal immune system, the cytokine secretion, the intestinal permeability, the secretion of mucus, antimicrobial peptides and IgA, and the production of metabolites and other unknown factors. Gut dysbiosis, defined as an imbalance or deviation from the normal composition of the microbiota, might be either beneficial (good) due to improved immune system, increased anti/pro inflammatory cytokine ratio etc., or deleterious (bad). Dysbiosis has been associated with and mentioned as a causal factor for obesity in humans [1, 2]. Dysbiosis has also been suggested as a causal factor for insulin resistance, glucose intolerance and type 2 diabetes, which are common comorbidities in in subjects with morbid obesity [1, 3]. These types of dysbiosis are "bad".

Both the diet and drugs influence the faecal microbiota [4–7]. Metformin has anti-hyperglycemic and weight-reducing effects, which are beneficial in subjects with obesity [8–10]. The effects depend in part on the altering of the gut microbiome [11–13]. The metformin-induced dysbiosis, therefore, contributes to the therapeutic effects and is referred to as "good" dysbiosis.

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To prevent weight gain and facilitate weight reduction, subjects with obesity have a high intake of non-nutritive sweeteners (NNS) [14]. NNS induce glucose intolerance by altering the gut microbiota and has been linked to obesity by the obesity-associated metabolic changes [15–17]. Therefore, the dysbiosis associated with NNS seems to be unfavourable and is henceforth denoted as "bad" dysbiosis.

Irritable bowel syndrome (IBS), a common comorbidity in subjects with morbid obesity, is one of many disorders associated with alterations in the gut microbiota ("bad" dysbiosis) [18–21]. In all, dysbiosis is associated with various disorders. According to the recently proposed "Anna Karenina principle" for animal microbiomes, the microbiome varies more in dysbiotic than in healthy subjects, and such variations might be separated into "good" and "bad" dysbioses [22].

Today's knowledge about dysbiosis is limited, the diagnostic tests are complicated and expensive, and the clinical utility is questionable. Knowledge of "good" and "bad" dysbioses might have clinical implications, such as normalising or preventing the "bad" dysbioses and preserving the "good" ones.

A simplified test for faecal dysbiosis based upon 54 DNA probes targeting gut bacteria has been marketed in Europe and USA (GA-map™ Dysbiosis Test, manufactured by Genetic Analysis, Oslo, Norway) [23, 24].

In this study, the primary aims were to assess the commercially available dysbiosis test's ability to detect faecal dysbiosis in subjects with morbid obesity and to detect dysbiosis associated with other variables, primarily metformin, NNS, diabetes, IBS and gastrointestinal symptoms. Based on the hypothesis that the dysbioses associated with the use of metformin and NNS differed [22], the secondary aims were to use the results of the dysbiosis test to explore alternative scoring algorithms to detect differences between the dysbiosis associated with metformin and NNS. The alternative scoring was validated in a new cross-sectional study.

Methods

Study design

Exploratory analyses were performed in one cross-sectional study (the test group) and validated in another cross-sectional study (the validation group).

In the test group, the dysbiosis test's ability to detect dysbiosis related to obesity, diabetes, IBS, the severity of gastrointestinal symptoms and use of NNS and metformin were studied. If dysbiosis was detected, explorative analyses were performed to detect differences between the dysbioses related to metformin (the "good" dysbiosis) and NNS (the "bad" dysbiosis) and to work out an Alternative Dysbiosis Index that distinguished between the "good" and "bad" dysbioses. Some of the results were validated in the validation group.

Subjects

From December 2012 to September 2014, consecutive subjects aged 18–65 years with morbid obesity (defined as BMI ≥ 40 kg/m^2 or ≥ 35 kg/m^2 with obesity-related complications) were included in the test group at Innlandet Hospital Trust, Gjøvik, Norway. At Lovisenberg Diaconal Hospital's outpatient clinic for gastrointestinal disorders, consecutive subjects above 18 years of age with IBS were from April 2013 to October 2014 included in the validation group. At both centres, a medical history was taken, paper-based questionnaires were filled in by the patients, a physical examination was performed, and blood and faecal samples were collected. Supplementary examinations were performed at the doctors' discretion. Subjects with serious somatic and psychiatric disorders (if judged as unrelated to obesity in the test group) were excluded because they could confound the evaluation of dysbiosis, and subjects with previous major abdominal surgery including bariatric surgery were excluded to ascertain the diagnosis of IBS. In addition, subjects not delivering faecal samples, subjects with incompletely filled in food frequency questionnaires (FFQ) were excluded from the test group, and subjects using antibiotics the last month or with a ^{13}C-D-Xylose breath test indicating malabsorption were excluded from the validation group. At both centres, trained personnel was responsible for the care of the patients and the practical work.

Variables

Gender, age (years), body weight (kg), height (meter), body mass index (BMI, kg/m^2), smoking habits (never / previously / daily smokers), and present or previous somatic disorders including hypertension, diabetes, and hypothyroidism (yes / no) were noted. Irritable Bowel Syndrome (IBS) was diagnosed with a validated Norwegian translation of the Rome III criteria, and the degree of gastrointestinal complaints with Irritable Bowel Severity Scoring System (IBSSS) [25]. The use of metformin, statins, and thyroxin was recorded. A range of haematological and biochemical blood tests including vitamins and minerals were analysed.

The dietary intake of micro- and macro nutrients, energy, and NNS were assessed with an FFQ prepared, validated and analyzed by the Department of Nutrition at the University of Oslo, Norway. The analyses were performed with their in-house calculation program (KBS, version 7.3, food database AE-14) based on the official Norwegian food composition table from 2016 [26]. The intake of NNS was calculated pragmatically since the FFQ did not specify the type or amount of NNS in the

beverages. One unit of NNS was defined as 100 ml NNS-containing beverage (divided into carbonated and non-carbonated beverage) which was considered equal to the sweetening of sugar-containing beverages with 10% of sugar (10 g/100 ml). Two NNS tablets/teaspoons for use in tea or coffee were judged as equal to 100 ml NNS in beverages. The unit (100 ml beverages or two tablets/teaspoons) could easily be calculated since the subjects reported the intake in litre and glasses, and the unit is easily understood. Intakes of NNS from other sources than beverages and tablets/teaspoons used in beverages were not recorded. Sugar alcohols and naturally-derived sweeteners not defined as NNS were not included. In addition to the associations between dysbiosis and NNS, the associations between dysbiosis and sugar-containing beverages and the absolute and relative amounts of macronutrients were analysed.

The faecal microbiota was analysed with the CE marked GA-map™ Dysbiosis Test (Genetic Analysis AS, Oslo, Norway) [23]. The test has both a US (Patent No. 9243297) and European patent (Patent No. 2652145) for its technology governing the oligonucleotide probe set and methods of microbiota profiling [24]. It uses 54 oligonucleotide probes targeting the 16S rRNA gene at different bacterial taxonomic levels and scores the relative abundance of each bacteria compared to the distribution in a reference population (score −3 to 3). The overall result is given as the Dysbiosis Index (DI) with scores 1 to 5, where values above 2 indicate a microbiota profile that differs from the reference population (i.e. dysbiosis). Exploratory analyses were performed to show differences between metformin and NNS in the relative abundance of one or more of the bacteria measured on the score from −3 to 3. The detected differences were summarised in the ADI.

Statistics

The results have been reported as mean (SD), median (range), and number (proportion in percentage). Comparisons between groups were analysed with chi-square tests, t-test, Mann-Whitney U-test, Pearson's and Spearman's correlation analyses depending on type and distribution of the data. Independent predictors of dysbiosis were assessed with linear regression analyses including gender, BMI and all variables significantly associated with dysbiosis in the univariable analyses followed by stepwise forward regression analyses. The results of the linear regression analyses are given as B-value with 95% confidence interval (CI), p-value and partial correlation (pc). The analyses were performed with IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp. p-values < 0.05 were judged as statistically significant.

Results

The test group

Out of 350 consecutive subjects visiting the obesity unit, 90 (76 women and 14 men with a mean age of 44.7 years (SD 8.6) and BMI 41.8 kg/m2 (SD 3.6)) were included in the test group. The reasons for the exclusion of 260 subjects are given in Fig. 1. Table 1 gives the participants' characteristics in detail divided into subjects with and without dysbiosis. Dysbiosis was present in 59 (66%) of the subjects; the mean DI score was 3.0 (SD 1.3). The DI scores 1–5 were present in 16 (18%), 15 (17%), 30 (33%), 13 (14%), and 16 (18%) subjects respectively. The main finding was the associations between dysbiosis and diabetes, metformin and NNS (all p-values < 0.01). There were no significant associations with either IBS or IBSSS. Table 1 gives all the associations except for the associations with the relative amounts of the macronutrients since there were no significant associations with these variables. Figure 2 shows the associations between the DI and the use of NNS and metformin and IBSSS.

Explorative analyses revealed significant differences between the dysbiosis related to NNS and metformin. Compared to NNS, the dysbiosis related to metformin was characterised by a relative abundance of the bacteria Alistipes, Proteobacteria and Shigella spp. & Escherichia spp., and a relative scarcity of *Bacteroides fragilis*, *Ruminococcus gnavus*, Bacteroides spp. & Prevotella spp., and *Dialister invisus*. The signs of the scores for the bacteria with a relative scarcity were changed. Then the scores for the seven bacteria were summed up and adjusted to the Alternative Dysbiosis Index (ADI) with scores from −14 to 14; positive scores were associated with the use of metformin (the "good" dysbiosis) and negative scores with the use of NNS (the "bad" dysbiosis). The mean ADI score was −0.8 (SD 2.8). Table 1 gives all associations between the patients' characteristics and the ADI. Figure 3 shows the positive association between the ADI and metformin and the negative associations with NNS and IBSSS, which were the main and statistically significant findings.

Multivariable analyses were used to study independent predictors of DI and ADI. These analyses included gender and BMI and all variables with a significant association with either DI or ADI. Diabetes and metformin were highly correlated ($r = 0.80$). Because the associations between metformin and DI and ADI were significantly higher than between diabetes and DI and ADI, diabetes was excluded from the analyses. Separate multivariable analyses (not shown) of the associations between DI and ADI on one side and total energy intake and the absolute and relative intake of macronutrients on the other side showed that the absolute intake of starch was the only independent predictor of DI and ADI. Therefore, starch was the only nutrient included in the multivariable analyses.

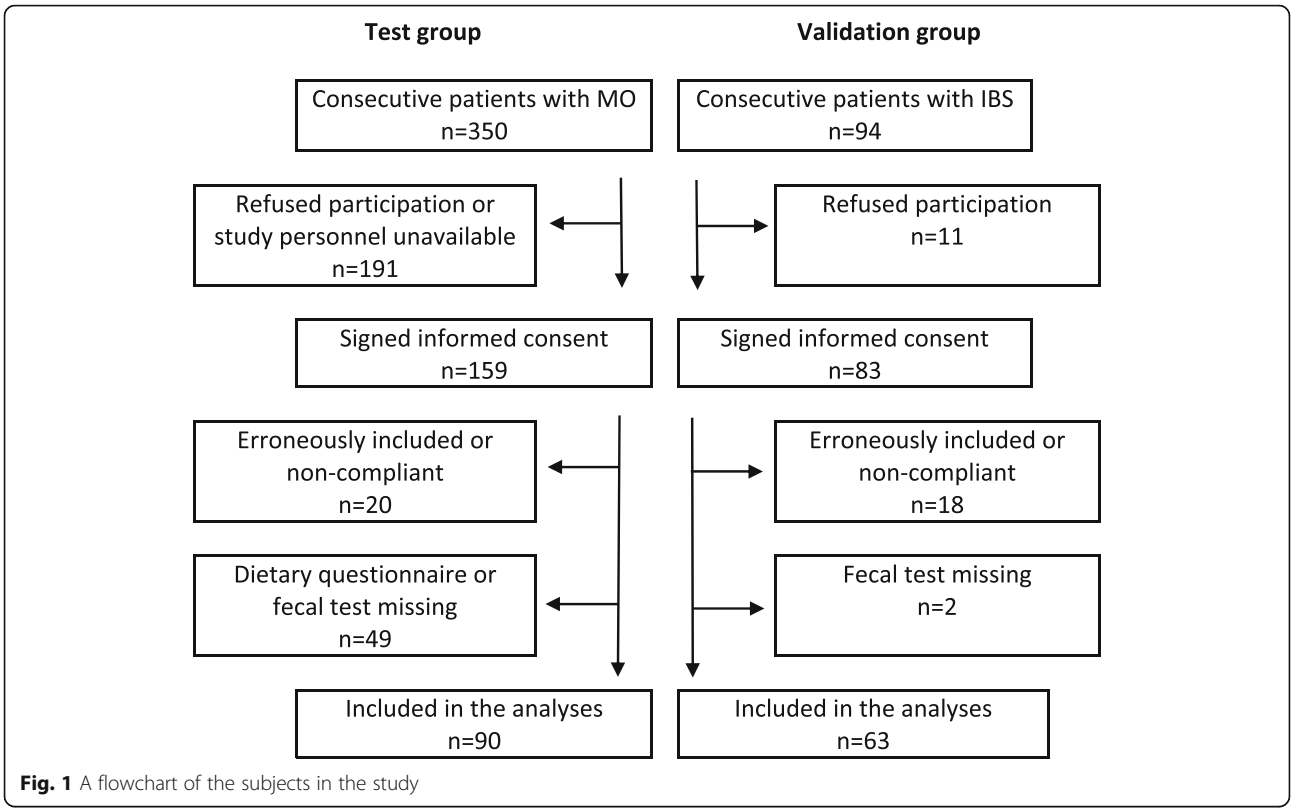

Fig. 1 A flowchart of the subjects in the study

The results of the multivariable analyses with all variables in the equation and the stepwise forward analyses are given in Table 2. The main findings were the positive associations between DI and use of metformin and NNS, the positive association between ADI and metformin (the "good" dysbiosis), and the negative associations between ADI and NNS and IBSSS (the "bad" dysbiosis).

The validation group

Fifty-six women and seven men with a mean age of 38.8 (SD 12.4) years were included in the validation group. The mean ADI and IBSSS scores were −1.68 (SD 2.26) and 287 (SD 79). No one used metformin, and information about NNS was not available. Table 3 gives the associations between the ADI and IBS and IBSSS in the test group and the validation groups with comparisons between the groups. IBS and IBSSS were associated with negative ADI scores. The significant associations between ADI and IBSSS in the two groups were of the same order.

Discussion

Based partly on the same data material and the same dysbiosis test, we have previously published that dysbiosis was prevalent in subjects with morbid obesity and not associated with IBS [18]. The new findings in this study were that dysbiosis measured with the producer's DI was associated with the use of metformin and NNS, but not with the severity of gastrointestinal symptoms measured as IBSSS. Dysbiosis associated with metformin and NNS have been reported in other studies with more complex, resource demanding, and costly methods [4, 5, 11, 12, 15, 27]. Another new finding was that alternative analyses of the producer's results allowed separations of types of dysbioses; one type was associated with the use of metformin ("good" dysbiosis) and one with IBS and the use of NNN ("bad" dysbiosis). Today's lack of knowledge about the clinical significance of dysbiosis measured with this test, and the test's seemingly inability to differentiate between types of dysbioses nearly eliminates its clinical usability. Hopefully, further research will clarify the clinical consequences of dysbiosis and types of dysbioses measured with this test.

Dysbiosis has been attributed a causal role of obesity in animals. The clinical significance of dysbiosis in humans with obesity and for obesity associated disorders such as insulin resistance, glucose intolerance and type 2 diabetes is less clear [1–3, 28]. The relatively weak associations between obesity and dysbiosis and the large interpersonal variation hamper the interpretation of the results [28]. The variations might indicate different types of dysbiosis, e.g. "good" and "bad". Theoretically, one type of dysbiosis might have favourable and unfavourable effects referring to different outcomes.

Table 1 The characteristics of the participants in the study and associations with dysbiosis

Participants' characteristics[a]	Dysbiosis		Dysbiosis	Association with DI [b]	Association with ADI [b]
	Yes (no 59)	No (31)	*p*-values		
Gender (female/male)	49 (83%) / 10 (17%)	27 (87%) / 4 (13%)	0.76 [(1)]	2.9 (1.3) / 3.4 (1.5); $p = 0.17$ [(2)]	−0.9 (2.8) / −0.5 (2.7); $p = 0.66$ [(2)]
Age (years)	44.0 (8.6)	46.0 (8.6)	0.31 [(2)]	$r = -0.078$; $p = 0.47$	$r = -0.375$; *$p < 0.001$*
Body weight (kg)	120.5 (15.2)	122.1 (18.3)	0.66[(2)]	$r = -0.064$; $p = 0.55$	$r = 0.009$; $p = 0.93$
BMI (kg/m2)	42.1 (3.7)	41.2 (3.2)	0.30 [(2)]	$r = 0.022$; $p = 0.84$	$r = -0.186$; $p = 0.08$
Smoking (never/previously /daily)	19 (32%) / 31 (53%) / 9 (15%)	17 (55%) / 12 (39%) / 2 (6%)	*0.046*[(3)]	$r = 0.195$; $p = 0.07$	$r = -0.230$; *$p = 0.030$*
Coffee (cups/day)	2.6 (2.3)	3.5 (2.4)	0.08 [(2)]	rho = −0.240; *$p = 0.026$*	rho = 0.163; $p = 0.13$
Food intolerance (65)	33/43 (77%)	17/22 (77%)	1.00 [(1)]	3.0 (1.3) / 3.0 (1.5); $p = 0.95$ [(2)]	−1.1 (2.5) / −1.7 (3.0); $p = 0.45$ [(2)]
Diabetes (87)	18/56 (32%)	2/31 (7%)	*0.007*[(1)]	3.7 (1.1) / 2.8 (1.3); *$p = 0.005$*[(2)]	0.5 (3.3) / −1.1 (2.5); $p = 0.057$ [(2)]
Hypothyreosis (86)	7/56 (13%)	3/30 (10%)	1.00 [(1)]	3.5 (1.3) / 2.9 (1.3); $p = 0.19$ [(2)]	−0.1 (2.8) / −0.8 (2.8); $p = 0.46$ [(2)]
IBS (88)	17/59 (29%)	8/29 (28%	1.00 [(1)]	3.1 (1.3) / 3.0 (1.4); $p = 0.60$	−1.6 (2.8) / −0.4 (2.7); $p = 0.063$ [(2)]
IBSSS (86)	120 (0–389)	99 (0–339)	0.43 [(4)]	rho = 0.110; $p = 0.31$	rho = −0.304; *$p = 0.004$*
Metformin	15/59 (25%)	1/31 (3%)	*0.009*[(1)]	3.9 (1.0) / 2.8 (1.3); *$p = 0.002$*[(2)]	1.1 (3.1) / −1.2 (2.5); *$p = 0.002$*[(2)]
Thyroxin (89)	5 / 59 (9%)	2/30 (7%)	1.00 [(1)]	3.6 (1.3) / 2.9 (1.3); $p = 0.23$ [(2)]	0.1 (2.9) / −0.9 (2.8); $p = 0.33$ [(2)]
Statins (88)	8 / 58 (14%)	5 / 13 (17%)	0.76 [(1)]	2.5 (1.3) / 3.1 (1.3); $p = 0.19$ [(2)]	−0.3 (2.3) / −0.9 (2.8); $p = 0.47$ [(2)]
CRP (88)	6 (0–27)	5 (1–28)	0.34 [(4)]	rho = 0.076; $p = 0.48$	rho = −0.034; $p = 0.75$
Zonulin (ng/ml) (85)	71 (36)	60 (30)	0.14 [(2)]	$r = 0.179$; $p = 0.10$	$r = -0.125$; $p = 0.25$
Total energy (kJ)	10,387 (3875)	10,777 (4324)	0.66 [(2)]	$r = -0.044$; $p = 0.68$	$r = -0.236$; *$p = 0.025$*
Water (unit)	44 (21)	42 (19)	0.65 [(2)]	$r = 0.030$; $p = 0.78$	$r = -0.251$; *$p = 0.017$*
Carb. beverages w/ sugar (unit[c])	0.0 (0–7.9)	0 (0–2.6)	0.99 [(4)]	rho = −0.016; $p = 0.89$	rho = −0.220; *$p = 0.037$*
Total NNS (unit[c])	4.9 (0–43)	1 (0–22)	*0.002*[(4)]	rho = 0.387; *$p < 0.001$*	rho = −0.353; *$p = 0.001$*
Carb. beverages w/NNS (unit[c])	1.1 (0–40)	0 (0–20)	*0.002*[(4)]	rho = 0.378; *$p < 0.001$*	rho = −0.268; *$p = 0.011$*
Non-carb. Beverages w/NNS (unit[c])	0.4 (0–32)	0 (0–8)	*0.026*[(4)]	rho = 0.270; *$p = 0.010$*	rho = −0.240; *$p = 0.023$*
Coffee/tea w/NNS (unit[c])	0.0 (0–27)	0 (0–14)	0.73 [(4)]	rho = −0.047; $p = 0.66$	rho = −0.151; $p = 0.16$
Protein (g/day)	110 (36)	110 (36)	0.94 [(2)]	rho = −0.053; $p = 0.62$	rho = −0.156; $p = 0.14$
Fat (g/day)	95 (42)	103 (56)	0.45 [(2)]	rho = −0.048; $p = 0.66$	rho = −0.235; *$p = 0.026$*
Carbohydrates (g/day)	273 (119)	280 (150)	0.80 [(2)]	rho = 0.016; $p = 0.88$	rho = −0.200; $p = 0.058$
Starch (g/day)	133 (54)	131 (46)	0.83 [(2)]	rho = 0.035; $p = 0.74$	rho = −0.263; *$p = 0.012$*
Sugar (g/day)	25 (1–268)	32 (5–632)	0.99	rho = 0.012; $p = 0.91$	rho = −0.221; *$p = 0.037$*

DI Dysbiosis Index, *ADI* Alternative Dysbiosis Index, *NNS* Non-Nutritive Sweeteners. The results are given as mean (SD); median (range); number (%). [a]The number of subjects is given in brackets if less than 90. [b]The order of the results are yes/no.[c] One unit is 100 ml beverages with NNS or 2 tablets NNS for coffee/tea

[(1)]: Fisher's exact test; [(2)] t-test; [(3)] chi-square, linear-by-linear; [(4)] Exact Mann-Whitney; rho: Spearman's rho; r = Pearson Correlation test

Italicized *p*-values are statistically significant

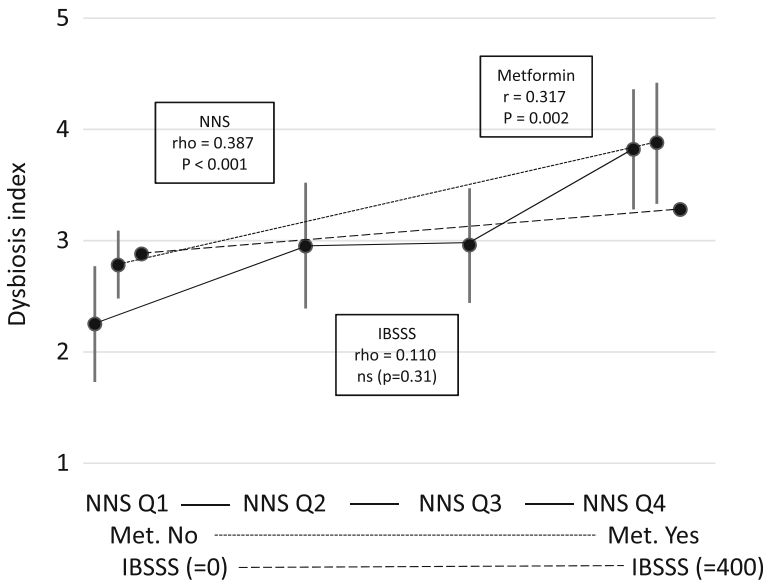

Fig. 2 Associations between the Dysbiosis Index and the main variables. NNS Q1, NNS Q2, NNS Q3, NNS Q4: Intake of Non-Nutritive Sweeteners divided into quartiles. Met: Metformin. IBSSS: Irritable Bowel Severity Scoring System. The results for NNS and Met are given as mean with 95% CI. The associations are given as Pearson's and Spearman's correlation coefficients (r and rho) and significance value (*p*-value)

Neither has the clinical significance of dysbiosis associated with the diet and use of drugs been clarified [4–7]. Metformin is a drug of particular interest in subjects with morbid obesity because of the anti-hyperglycemic, insulin sensitising, and weight-reducing effects [8, 9]. The drug's effect on the faecal microbiota is well established [4, 5, 11, 12].The mechanisms by which metformin exerts its effects have until recently been uncertain [8, 29]. Importantly, intravenous administration has no effect in either non-diabetic subjects or subjects with type 2 diabetes [30, 31]. The glucose tolerance improved in germ-free mice given faeces from metformin-treated mice, indicating that the effect in part depends on alteration of the gut microbiome [11, 12]. The metformin-induced dysbiosis is, therefore, "good" for the effect of metformin.

The favourable and unfavourable effects of NNS on body weight, lifestyle, and metabolism is continuously discussed, and the literature is probably heavily biased

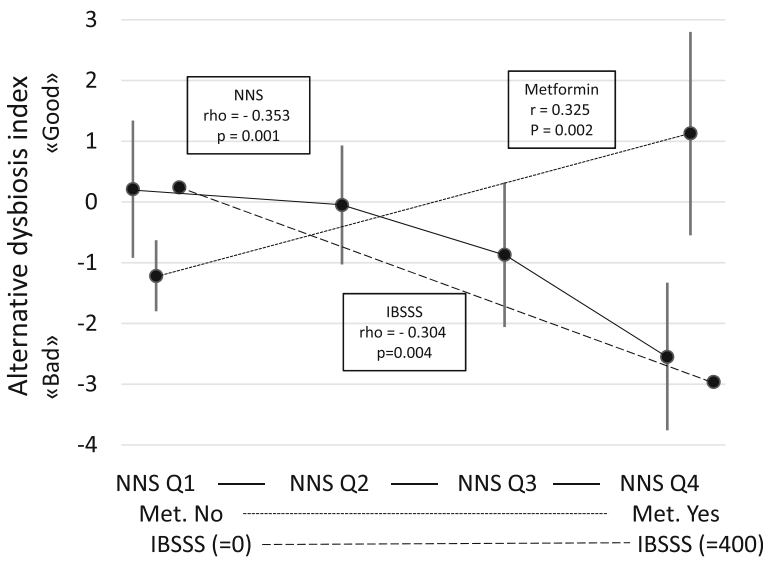

Fig. 3 Associations between the Alternative Dysbiosis Index and the main variables. NNS Q1, NNS Q2, NNS Q3, NNS Q4: Intake of Non-Nutritive Sweeteners divided into quartiles. Met: Metformin. IBSSS: Irritable Bowel Severity Scoring System. The results for NNS and Met are given as mean with 95% CI. The associations are given as Pearson's and Spearman's correlation coefficients (r and rho) and significance value (*p*-value)

Table 2 Predictors of Dysbiosis Index and Alternative Dysbiosis Index (linear regression analyses)

Independent predictors	Dependent: DI (all variables)			Dependent: DI (stepwise forward)			Dependent: ADI (all variables)			Dependent: ADI (stepwise forward)		
	B (95% CI)	*p*-value	pc	B (95%CI)	*p*-value	pc	B (95% CI)	*p*-value	pc	B (95%CI)	*p*-value	pc
Gender (female/male)	1.01 (0.17; 1.85)	0.19	0.275				−0.96 (−2.61; 0.70)	0.25	−0.137			
Age (years)	−0.01 (−0.05; 0.02)	0.46	−0.088				0.05 (−0.02; 0.13)	0.15	0.172	0.07 (0.01; 0.13)	*0.033*	0.241
BMI (kg/m2)	−0.00 (−0.08; 0.08)	0.95	−0.007				−0.07 (−0.23; 0.09)	0.38	−0.105			
Smoking (never/ previously /daily)	0.36 (−0.08; 0.80)	0.10	0.193				−0.67 (−1.53; 0.19)	0.12	−0.183			
Coffee (cups/day)	−0.02 (−0.16 to 0.13)	0.79	−0.032				0.02 (−0.26; 0.31)	0.87	0.020			
IBSSS (86)	0.00 (−0.002; 0.004)	0.47	0.086				−0.006 (−0.01; 0.00)	*0.039*	−0.244	−0.01 (−0.01; −0.00)	*0.022*	−0.258
Metformin	0.99 (0.22; 1.75)	*0.012*	0.295	1.11 (0.42; 1.80)	*0.002*	0.339	2.53 (1.04; 4.03)	*0.001*	0.374	2.32 (0.93; 3.71)	*0.001*	0.354
Water (unit [a])	0.00 (−0.02; 0.02)	0.98	−0.002				−0.01 (−0.04; 0.03)	0.76	−0.037			
Carb. bev. w/ sugar (unit [a])	0.20 (−0,05; 0.45)	0.12	0.186				0.13 (−0.37; 0.63)	0.60	0.062			
Total NNS (unit [a])	0.035 (−0.00; 0.07)	0.054	0.228	0.04 (0.01; 0.07)	*0.005*	0.311	−0.08 (−0.15; −0.01)	*0.024*	−0.266	−0.10 (−0.16; −0.04)	*0.001*	−0.362
Starch (g/day)	−0.00 (−0.01; 0.00)	0.36	−0.109				−0.00 (−0.02; 0.01)	0.53	−0.076			

DI Dysbiosis Index, *ADI* Alternative Dysbiosis Index, *IBSSS* Irritable Bowel Severity Scoring System, *NNS* Non-Nutritive Sweeteners, *pc* partial correlation, *Carb.* bev: Carbonated beverages. [a]One unit is 100 ml beverages with NNS or 2 tablets NNS for coffee/tea

Gender and BMI and all variables significantly associated with either DI of ADI in the univariate analyses (age, smoking habits, coffee, IBSSS, Metformin, total NNS, starch) were included as predictors of DI and ADI in the regression analyses. The reasons for exclusion of diabetes and some nutrients are explained in the text. All the variables were included in the first step, and then stepwise forward regression analyses were performed

Italicized *p*-values are statistically significant

Table 3 ADI scores and associations with IBS and IBSSS with comparisons between the groups

Variables	ADI	ADI	Statistics
	Test group	Validation group	*p*-value
	(IBS no/yes: no 63/25)	(IBS yes: 63)	
IBS (no / yes)	−0.41 (2.75) / −1.64 (2.77) ($p = 0.11$)†	−1.68 (2.26)†	0.013*
IBSSS	rho = −0.304 ($p = 0.004$)	rho = −0.249 ($p = 0.049$)	0.86 #

ADI Alternative Dysbiosis Index, *IBS* Irritable Bowel Syndrome, *IBSSS* Irritable Bowel Severity Score System
*One-Way ANOVA with comparisons between the three groups
† Post hoc comparisons (Tukey) between the validation group and subjects with and without IBS in the test group were $p = 1.00$ and 0.016 respectively
# Univariate analysis of variance with ADI as the dependent variable and IBSSS and group as independent variables. The *p*-value is the interaction between IBSSS and group and indicates no significant difference between the correlations

[14, 32–35]. The dysbiosis caused by NNS induces glucose intolerance and has been linked to obesity by the obesity-associated metabolic changes [15–17]. Therefore, the NNS associated dysbiosis is probably "bad" for subjects with morbid obesity.

IBS is one among many disorders that has been associated with alterations in the gut microbiota [18–21]. The dysbiosis in subjects with IBS is a "bad" dysbiosis since faecal microbiota transplantation may normalise the microbiota and improve symptoms [36]. In all, there are several types of dysbiosis that might be separated into "good" and "bad".

The producer's test response does not differentiate between types of dysbiosis. The ADI based on simple explorative analyses of available results in the producer's report could easily separate the "good" metformin-type dysbiosis from the "bad" NNS-type dysbiosis. The ADI-score was adjusted so that "good" and "bad" dysbiosis had positive and negative scores respectively. If the results are reproducible, and the dysbiosis test allows construction of other clinically relevant dysbiosis indexes, the potential usefulness of the test increases markedly.

The ADI was not constructed to explore dysbiosis associated with IBS and gastrointestinal complaints. The negative correlations between ADI and IBS and gastrointestinal symptoms were therefore new and interesting findings, which were confirmed with unadjusted and adjusted analyses in the validation group. The findings are in accordance with other reports indicating associations between IBS and dysbiosis [19–21]. The ADI could be a test for detection of "bad" dysbiosis in subjects with IBS and gastrointestinal complaints and replace complex, resource demanding and costly 16S gene sequencing.

Further research, aiming at enlarging the producer's test response with the specification of the type of dysbiosis related to dietary factors, drugs, disorders and diseases (e.g. metformin- or NNS-like, or "good" or "bad") is desirable. Specified results might predict response to treatment, e.g. antibiotics and other drugs, probiotics, prebiotics, diet, and faecal microbiota transplant. Treatment aiming at prevention or normalising of a "bad" dysbiosis or induction of a "good" dysbiosis could change the treatment of a range of disorders [37].

Strengths and limitation

The test group and the validation group were consecutive subjects representative of subjects referred to outpatient clinics for morbid obesity and gastrointestinal complaints respectively. Because the ADI was constructed to detect differences between metformin and NNS, the significant differences between the ADI scores for metformin and NNS were expected. It was nevertheless pleasing that the ADI could be constructed so easily. The most impressive findings were the associations between IBS and IBSSS and the negative ADI score. The ADI was not constructed to find these differences, and they were not detected with the producer's result report. It was a strength that these findings were confirmed in the validation group, which substantiates that a negative ADI indicates a "bad" NNS- or IBS-like dysbiosis.

The external validity could be questioned since the ADI was based on results from subjects with morbid obesity who might have a high prevalence of dysbiosis also without having gastrointestinal comorbidity and use of metformin and NNS. The exclusion of subjects using antibiotics the last month might have been a too short period.

Conclusions

A commercially available test for faecal dysbiosis showed a high prevalence of dysbiosis in subjects with morbid obesity, particularly in users of metformin and NNS, but no association with gastrointestinal complaints. An ADI based on explorative analyses of the results from the test could differentiate between the "good" dysbiosis associated with metformin and the "bad" dysbiosis associated with NNS. The "bad" dysbiosis was also associated with gastrointestinal symptom severity. The associations between IBS and gastrointestinal symptom severity were confirmed in an independent validation group, indicating that ADI might be a valid diagnostic tool for the diagnosis of IBS-associated dysbiosis. Rather than merely reporting dysbiosis and degrees of dysbiosis, diagnostic tests for faecal dysbiosis should separate between types of dysbiosis.

Abbreviations

ADI: Alternative Dysbiosis Index; BMI: Body Mass Index; CI: Confidence interval; DI: Dysbiosis Index; FFQ: Food Frequency Questionnaire; IBS: Irritable

Bowel Syndrome; IBSSS: Irritable Bowel Severity Scoring System; NNS: Non-Nutritive Sweeteners; pc: partial correlation; SD: Standard Deviation

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Availability of data and materials
Case report forms (CRFs) on paper were used for collection of the clinical data, and all the CRFs are safely stored. The data were transferred manually to SPSS for statistical analyses. The data files are stored by Innlandet Hospital Trust, Brumunddal, Norway, on a server dedicated to research and with security according to the rules given by The Norwegian Data Protection Authority, P.O. Box 8177 Dep. NO-0034 Oslo, Norway. The data are available on request to the authors.

Authors' contributions
PGF is the guarantor of the project. He designed the main study, was responsible for the practical implementation, performed the statistical analyses, wrote the manuscript and is responsible for the integrity of the work. MA recruited subjects to the test group and prepared the data files for the statistical analyses. JV was responsible for the collection and preparation of all information from the validation group. All authors have contributed to the interpretation of the results, given valuable comments on the manuscript and approved the last version.

Ethics approval and consent to participate
The study was approved by the Norwegian Regional Committees for Medical and Health Research Ethics, PB 1130, Blindern, 0318 Oslo, Norway (reference number 2012/966 and 2013/454) and performed in accordance with the Declaration of Helsinki. Written informed consent was given by all participants before inclusion.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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RESEARCH ARTICLE

The kinetics of gut microbial community composition in patients with irritable bowel syndrome following fecal microbiota transplantation

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Abstract

Background

Gut microbiota alterations are important in irritable bowel syndrome (IBS). The aim was to investigate the effect of fecal microbiota transplantation (FMT) on gut microbiota and the symptoms in patients with IBS.

Material and methods

The study included 13 IBS patients according to Rome III criteria and 13 healthy donors. Freshly donated feces were administered to the descending part of the duodenum via a gastroscope. Feces were collected from donors and patients before FMT, and from the patients at 1, 3 and 12 weeks and donors and patients at 20/28 weeks after FMT. Microbiota analysis was performed using GA-map Dysbiosis test (Genetic Analysis AS, Oslo, Norway). The patients completed the following questionnaires before and at the aforementioned weeks after FMT: IBS Symptom Questionnaire (IBS-SQ), IBS-Symptom Severity Scoring system (IBS-SSS), Short Form of Nepean Dyspepsia Index (SF-NDI), Bristol stool form scale, the Eysenck Personality Questionnaire-Neuroticism and Hospital Anxiety and Depression.

Results

Donors and IBS patients had significantly different bacterial strain signals before FMT (*Ruminococcus gnavus*, *Actinobacteria* and *Bifidobacteria*) that became non-significant after 3 weeks following FMT. The changes in gut microbiota were similar between donors and patients at 20/28 weeks after FMT. Thus, patients' microbiota profiles became more-or-less similar to donors.

The scores of all the questionnaires were significantly improved at all time points following FMT. No reported adverse effects.

Conclusions

FMT was associated with a change in gut microbiota and improvement in IBS symptoms and quality of life lasting for up to 28 weeks.

Trial registration

ClinicalTrials.gov ID: NCT03333291

Introduction

Irritable bowel syndrome (IBS) is a common chronic gastrointestinal (GI) disease, affecting 10–20% of the adult population leading to significant morbidity and huge costs for the society [1]. The pathogenesis of IBS is unclear, but it is believed to be multifactorial; and includes altered gut microbiota, [2, 3] abnormal enteroendocrine cells of the GI tract [4], mucosal low-grade inflammation, [5, 6] genetic predisposition [7] and diet [8, 9]. Some reports describe that postinfectious IBS (PI-IBS) occurs in 10–30% of patients following acute gastroenteritis, suggesting that alterations in the gut microbiota may play a role in the pathogenesis of this type of IBS [10–12].

Gut microbiota play an important role in maintaining health, regulating cellular immunity and energy metabolism [10]. Recent studies have shown that the gut microbiota are involved in GI and non-GI disorders (e.g. obesity, atherosclerosis and type II diabetes mellitus) [13–15]. The important role of alterations in the gut microbiota in IBS [2, 3] has led to increased interest in probiotic [16] and antibiotic [17] treatment approaches.

Fecal microbiota transplantation (FMT), the infusion of a fecal preparation from a healthy donor into the GI tract of a human recipient may alter the gut microbiome (the bacterial gene content) of the new host by re-establishing the balance in the gut microbiota [10]. It is speculated that human feces from a healthy donor may constitute "the ultimate human probiotic" [10], thus proposing FMT as a treatment option for conditions where an altered gut microbiota has been detected, including IBS [10, 18, 19]. FMT was first reported to be used for treatment of pseudomembranous colitis caused by *Micrococcus pyogenes (Staphylococcus)* in 1958 [20] and then in 1983 for *Clostridium difficile* infection [21]. Currently, FMT is widely accepted as the recommended treatment for recurrent *Clostridium difficile* enterocolitis [22]. Two new studies have shown that FMT improves the symptoms of recipient patients with IBS [23, 24] and one case report shows that the stool microbiome of the recipient resembled that of the donor following FMT [25]. Other reports about the use of FMT in selective cases of ulcerative colitis [26, 27], chronic fatigue syndrome [10] and autism [28] have resulted in positive outcomes [10].

The aims of the current study were to investigate the effect of FMT on i) the characteristics and kinetics of the gut microbiota in IBS patients, and ii) the symptoms and quality of life in IBS patients.

Material and methods

Eligible patients

A recipient group (n = 16) included both male and female patients, aged between 18–70 years, who met Rome III criteria for the diagnosis of IBS with moderate to severe abdominal

symptoms as defined by IBS-Symptom Severity Scoring system (IBS-SSS) score >175 [29] and were referred to the gastroenterology outpatient clinic, Haukeland University Hospital, Bergen, Norway. The exclusion criteria included history of inflammatory bowel diseases, GI malignancy, blood in stool, an immunocompromised state, a history of opportunistic infections within 1 year prior to FMT, oral thrush, or disseminated lymphadenopathy. Patients who were scheduled for abdominal surgery, pregnant or lactating women and patients taking probiotics or antibiotics within 4 weeks prior to fecal installation were also excluded.

Donors

A donor group of healthy family members, males and females who were over 18 years of age was included. The exclusion criteria of the donors were pregnancy, history of inflammatory bowel diseases, IBS, chronic abdominal pain, GI malignancy, diarrhea, blood in stool, antibiotic and probiotic use within 4 weeks prior to FMT, an immunocompromised state, history of opportunistic infections within 1 year prior to FMT, oral thrush and disseminated lymphadenopathy.

The study was performed in accordance with the Declaration of Helsinki [30] and was approved by the Regional Committee for Medical and Health Research Ethics in Western Norway (reference no.: 2013/1497). All participants provided written informed consent. According to the Norwegian legislation, clinical trials concerning fecal transplantation are not regarded as drug clinical trial. When the study started we were unfortunately not aware of the requirements of registering non-drug clinical trials. Hence this trial was registered retrospectively at ClinicalTrials.gov (ID: NCT03333291).

Study design

The FMT procedure was done only once and fecal samples were analyzed across several time points before FMT at screening (week -1) and FMT day (week 0), and then after FMT at weeks 1, 3, 12 and 28 weeks. The scheduled study visits are outlined in Table 1. The donors and patients completed several questionnaires and delivered fresh stool samples soon (a couple of hours) after defecation at screening and during visits after FMT as outlined in Table 1. The patients received special containers to preserve their stool in and were informed to place them in the refrigerator (4˚C) if it will take longer than a couple of hours before delivery. The patients were informed not to apply any changes to their diet or life style and to report any bout of new infections and/or use of new medications during the study.

Screening

Screening of the donors and the patients was scheduled one week before FMT. All of the donors and patients filled out symptom questionnaires (Table 1), received physical examinations and were screened (in blood and stool) for previous exposure to contagious infectious agents, inflammation and other organic diseases. Screening of the donors' blood included serologic testing for hepatitis A, B, C, human immunodeficiency virus (HIV), Epstein-Barr virus and cytomegalovirus. The blood from the patients was tested and included: Hemoglobin, leucocytes, platelets, creatinine, aspartate transaminase (AST), alanine transaminase (ALT), International Normalized Ratio (INR), electrolytes and chromogranin A. Stool samples from the donors and patients were examined for fecal calprotectin, cultured for enteric bacterial pathogens and screened for viruses and parasites.

Table 1. The intervention plan and timing of the visits.

Participants	Screening (-1 week)	FMT-day (week 0)	Visit 1 (week 1)	Visit 2 (week 3)	Visit 3 (week 12)	Visit 4 (week 20/28)
Patients						
	IBS-SSS	IBS-SSS	IBS-SSS	IBS-SSS	IBS-SSS	IBS-SSS
	IBS-SQ		IBS-SQ	IBS-SQ		
	History and physical examination	Bristol stool form scale	Bristol stool form scale	Bristol stool form scale	Bristol stool form scale	Bristol stool form scale
	Blood tests	SF-NDI		SF-NDI		SF-NDI
	Stool tests	EPQ-N-12		EPQ-N-12		EPQ-N-12
		HAD		HAD		HAD
		Fresh stool for storage (-80˚C) and analysis	Fresh stool for storage (-80˚C) and analysis	Fresh stool for storage (-80˚C) and analysis	Fresh stool for storage (-80˚C) and analysis	Fresh stool for storage (-80˚C) and analysis
		Gastroscopy for installation of freshly donated feces				
Donors						
	History and physical examination	Fresh stool for donation				Fresh stool for storage (-80˚C) and analysis
	IBS-SSS					
	IBS-SQ					
	Blood tests					
	Stool tests					

IBS-SSS: Irritable bowel syndrome-symptom severity scale; IBS-SQ: IBS symptom questionnaire; SF-NDI: short form-Nepean dyspepsia index; EPQ-N-12: The Eysenck Personality Questionnaire-Neuroticism; HAD: hospital anxiety and depression.

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The FMT procedure

On FMT day, the patients brought >60 g of fresh feces from their donors along with 60 g of their own feces before transplantation. Only 30 g of donor feces were used to prepare the fecal suspension by mixing them with 60 ml of normal saline. The remaining donor feces and feces from the patients were stored at -80˚C until they were analyzed for microbial analysis. The patients completed several questionnaires before FMT (Table 1). Gastroscopy was performed on the patients (after an overnight fast) to install 60 ml of fecal suspension followed by 60 ml of normal saline in the descending part of the duodenum distal to the papilla Vateri. All of the gastroscopies were performed by an endoscopist (T.M., G.A.L. or T.H.) at the gastrolab, Haukeland University Hospital, Bergen, Norway. The second visit was planned at week 3, instead of week 4 as outlined in the original protocol, due to practical reasons, and only 30 g of donor feces was used only once, as outlined in the original protocol (S1 File), in accordance with previous recommendations [10, 31].

Gut microbiota analysis

Gut microbiota analysis was performed using the GA-map Dysbiosis test (Genetic Analysis AS, Oslo, Norway) by algorithmically assessing fecal bacterial abundance and profile (dysbiosis index, DI), and potential deviation in the microbiome from normobiosis. [32] Briefly, GA-map Dysbiosis test is based on fecal homogenization, mechanical bacterial cell disruption and automated total bacterial genomic DNA extraction using magnetic beads. DI is based on 54 DNA probes targeting more than 300 bacterial strains based on their 16S rRNA sequence in seven variable regions (V3–V9). Twenty-six bacteria probes are species specific, 19 detect

bacteria on genus level, and 9 probes detect bacteria at higher taxonomic levels. Probe labeling is by single nucleotide extension and hybridization to complementary probes coupled to magnetic beads, and signal detection by using BioCode 1000A 128-Plex Analyzer (Applied BioCode, Santa Fe Springs, CA, USA). A DI above 2 shows a microbiota profile that differs from that of the normobiotic reference collection (DI 1–2: non-dysbiosis, DI 3: moderate, DI 4–5: severe dysbiosis) [32].

Questionnaires

Gastrointestinal symptoms and bowel habits were evaluated using IBS-SSS [29] in which a decrease of 50 points in IBS-SSS score using a visual assessment scale (VAS) from baseline (before FMT) correlated with improvement in clinical symptoms, and IBS symptom questionnaire (IBS-SQ) [33, 34] that was completed on the day of screening and then daily for 20 days after FMT. Responders and late-responders were patients who achieved a reduction of >50 points in IBS-SSS score after 1 and 3 weeks following FMT, respectively [29]. Non-responders were those who achieved <50 points in IBS-SSS score following FMT at any time period compared to baseline.

Stool consistency was evaluated using Bristol stool form scale [35], which ranges from 1 (constipation) to 7 (diarrhea). Quality of life (QoL) was assessed using Short Form of Nepean Dyspepsia Index (SF-NDI) questionnaire [36]. Psychometric evaluation was performed using the Eysenck Personality Questionnaire-Neuroticism (EPQ-N-12) with a cut-off value of 4 [37], and Hospital Anxiety and Depression (HAD) where scores >8 in either subscale were considered to indicate anxiety or depression, respectively [38, 39].

Statistical analysis

Graphpad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) was used for all analysis. Kruskal-Wallis non-parametric test with Dunn's post test and Mann-Whitney U test were used to analyse the data between the donors and patients before and after FMT. One-way ANOVA with repeated measures and Paired *t*-test was used to analyze the data for the patients before FMT and each visit after FMT. Multiple *t*-test corrected using Holm-Sidak method, was used to compare between the bacterial signals of responders and non-responders. $P < 0.05$ is considered to indicate a statistically significant difference. Cluster analysis and principal component analyses (PCA) were used to visualize the microbiota data, showing the extent to which microbial communities share branch length.

Results

Participants

The recipients group included 16 patients with IBS and the donors group included 16 healthy subjects. Participants of both groups were recruited after fulfilling the inclusion's criteria and, most importantly, none has used antibiotics during the past 6 months prior to inclusion in the study (Fig 1). Three, originally recruited, patients were excluded after withdrawing their consent to participate for practical reasons ($n = 1$), being diagnosed with functional dyspepsia ($n = 1$) and finding *Clostridium difficile* in stool culture ($n = 1$). Hence, 13 patients (9 males and 4 females, mean age of 32 years and age range of 20–44 years) and 13 donors (6 males and 7 females, mean age of 33 years and age range of 20–42 years) completed the whole study, and filled out the questionnaires and delivered fecal samples as previously explained. All of the patients had IBS mostly diarrhea-predominant, in which six patients had PI-IBS (after a local Giardia outbreak in Bergen in 2004 [40]) and seven patients had idiopathic IBS. The last visit

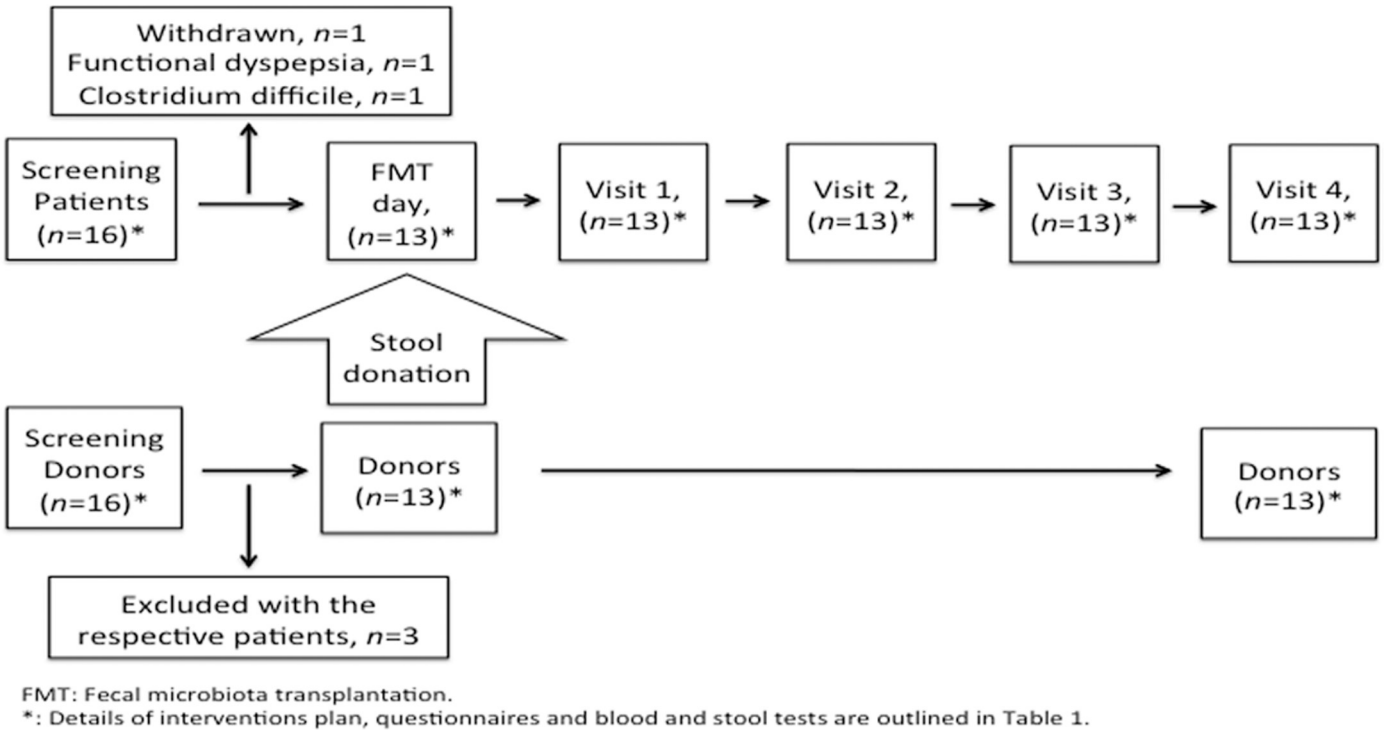

Fig 1. Study flow chart.

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was originally scheduled at 28 weeks after FMT, but 4 patients and also their respective donors were scheduled for a last visit at 20 weeks instead of 28 weeks after FMT due to practical reasons. The blood tests and stool cultures of both donors and patients were normal prior FMT and control blood tests for the patients were also normal at the end of the study. Detailed health and symptom questionnaires were provided to both groups at screening day and only to the patients following FMT throughout the study. We asked both the patients and donors to report any changes in their diet, life style, medications or health history during the whole study. Neither group reported any such changes during study participation.

Gut microbiota

At baseline, the patients had significantly higher DI than the donors (4±0.5 and 2.6±0.2, respectively, $P = 0.046$, Fig 1). Following FMT, the DI for the patients gradually decreased to 3.9±0.4 at week 1, then 3.3±0.3 at week 3 and 2.9±0.2 at week 12 but then increased again to 3.5±0.3 at week 20/28. The changes in the DI comparing between the patients following FMT and the donors were not statistically significant (Fig 2).

Donors and IBS patients had significantly different bacterial signals before FMT, namely, signals for *Ruminococcus gnavus*, *Actinobacteria* and *Bifidobacteria*, which became non-significantly different after 3 weeks following FMT (Table 2). At weeks 12 and 20/28, new bacterial strains in the patients feces; namely *Bacteroides/Prevotella*, *Alistipes*, *Actinobacteria* and *Bifidobacteria* became significantly different from that of the donor at the beginning of the study, as shown in Table 2, but not statistically different from that of the donors at the end of the study at week 20/28 ($P = 0.09$, 0.08, 0.6, 0.14 and 0.9, respectively). The signal levels of *Actinobacteria* and *Bifidobacteria* increased significantly towards the levels measured for the donors and lasted for 12 weeks after FMT but then significantly decreased at week 20/28. The PCA scores

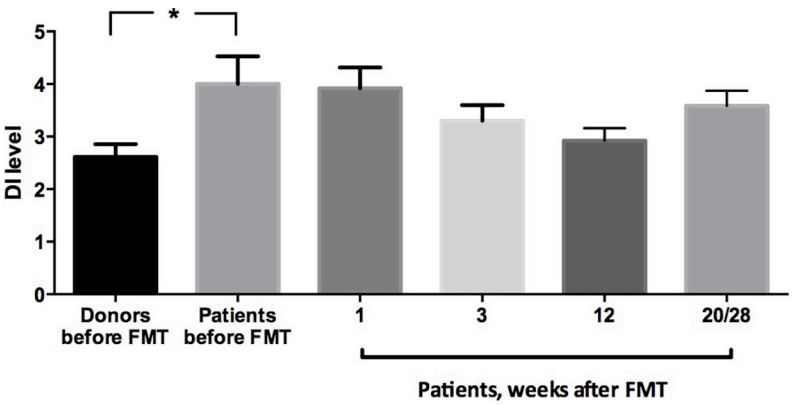

Fig 2. Dysbiosis index of the donors before fecal microbiota transplantation (FMT) and for the patients before FMT (day 0) and following FMT (weeks 1, 3, 12 and 20/28).

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plot of the gut microbiota profiles corrected for sample differences showed a gradual shift of the gut microbiota profile over time (Fig 3).

Comparing the microbiota between the groups, responders (n = 8) and non-responders (n = 5), which were defined based on achieving >50 or <50 IBS-SSS points, respectively, at week 20/28 compared to baseline, showed significant differences (adjusted P-values) in the *Bacteroides* signals between the donors at the beginning and the end of the study (P<0.0001 and 0.23, respectively) and between the patients at weeks 0, 1, 3, 12 and 20/28 (P<0.0001, <0.0001, 0.23, 0.08 and 0.08, respectively), Fig 4A. The respective values for *Desulfitispora* signals between the donors at the beginning and the end of the study (P = 0.0005 and 0.98, respectively) and between the patients at weeks 0, 1, 3, 12 and 20/28 (P = 0.0002, <0.0001, 0.0005, 0.0025 and <0.0001, respectively), Fig 4B, and for *Megasphaera/Dialister* signals between the donors at the beginning and the end of the study (P = 0.54 and 0.53, respectively) and between the patients at weeks 0, 1, 3, 12 and 20/28 (P = 0.13, 0.029, 0.24, 0.013 and 0.029, respectively), Fig 4C. As for *Bifidobacteria*, differences in the bacterial signals between responders and non-responders were noted, however, they were statistically not significant, Fig 4D.

Questionnaires

The score of IBS-SSS (mean±SEM) for the donors was 18±8.9 and for IBS-SQ is 0.7±0.3, which indicated asymptomatic status. The score of IBS-SSS of 11 patients were considered severe (IBS-SSS score >300) and only two patients had moderate severity (IBS-SSS score = 176–300) before FMT. The score of IBS-SSS (mean±SEM) for the patients at screening day was 333.6±20. IBS-SSS scores at FMT-day and at weeks 1, 3, 12 and 20/28 after FMT and comparisons between FMT-day and at each week are presented in Table 3. Using paired t test showed no significant difference in the aforementioned IBS-SSS scores for the patients between screening and FMT-day (P = 0.45), however, a significant reduction was noted in IBS-SSS scores of the patients between screening and weeks 1, 3, 12 and 20/28 (P = 0.003, 0.0004, 0.0095 and 0.012, respectively). No significant differences were observed by comparing the scores in weeks 1, 3, 12 and 20/28 interchangeably between each other, Fig 5. Four out of the 13 patients did not achieve >50 points reduction in IBS-SSS scores from baseline at week 1. However, two out of these four patients were late responders and achieved >50 points reduction in IBS-SSS scores from baseline at week 3. Therefore, a total of 9, 11, 9 and 8 out of the 13 patients achieved >50 points reduction in IBS-SSS scores from baseline at weeks 1, 3, 12 and 20/28 following FMT, respectively, and were considered as responders.

Table 2. Characteristics of the fecal bacterial signals between the donors at the beginning and end of the study, and patients in weeks 0, 1, 3, 12 and 20/28. The left part of the table shows the bacterial signals given for donors and recipients at different time points before and after FMT and the directionality towards or away from that of the donors at baseline. The right part of the table shows the *P*-values when comparing the bacterial signals for the recipients at different time points to that for the donors at baseline.

Bacteria strain	Donors, Beginning of study, *n* = 13	Patients					Donors, End of study, *n* = 10	P[a] Before FMT	P[b] After 1 week	P[c] After 3 weeks	P[d] After 12 weeks	P[e] After 20/28 weeks	P[f] Donors
		FMT day, *n* = 9	After 1 week, *n* = 12	After 3 weeks, *n* = 9	After 12 weeks, *n* = 13	After 20/28 weeks, *n* = 12							
Ruminococcus gnavus	4.6±1.1	40±15.6	8±2.3	15.3±9	25±17	8.1±1.8	8.8±2	**0.015**	>0.9	0.15	0.44	>0.9	0.19
Bacteroides	27.2±4.1	38.9 ±8.3	47.1 ±16.6	30.9±3.7	55.4±12.7	49.3±11	42±5.2	>0.9	>0.9	>0.9	**0.02**	0.1	0.097
Bacteroides/ Prevotella	483±51.4	634 ±28.5	599 ±25.8	551±63.9	783±49.7	731±52	788±58	0.7	>0.9	>0.9	**0.005**	**0.02**	**0.009**
Alistipes	100.5±16.8	100.5 ±23	119 ±17.4	140±26.8	186±11.9	208±9.6	188±15.5	>0.9	>0.9	0.9	**0.011**	**0.0006**	**0.03**
Parabacteroides	7.6±1.8	7.9±2.9	8.3±2	14.6±5.4	11.8±1.8	15.7±3.5	19.5±3.8	>0.9	>0.9	>0.9	0.4	0.3	**0.03**
Actinobacteria	287±45	66.6±13	95±23	197±54	138±29	92±23	204±57	**0.0010**	**0.007**	0.7	0.2	**0.003**	**0.018**
Bifidobacteria	324±57	65±13	97±25	205±57	150±34	92.5±25	241±72	**0.0011**	**0.008**	0.6	0.2	**0.004**	**0.017**
Proteobacteria	27±7.8	195 ±121.6	565±162	63.8 ±28.5	105±70	56.5±40	17±1.9	>0.9	**0.03**	>0.9	0.9	>0.9	0.5
Shigella/ Escherichia	62±23	260 ±124	578±128	116±45	188±77	90.9±65	41±14	>0.9	**0.002**	0.8	>0.9	>0.9	0.8

Data are presented as the mean±SEM. Comparison: Kruskal-Wallis multiple comparisons test with Dunn's post test

[a] Donors at the beginning of the study vs. patients on FMT day before fecal installation.

[b] Donors at the beginning of the study vs. patients 1 week after FMT.

[c] Donors at the beginning of the study vs. patients 3 weeks after FMT.

[d] Donors at the beginning of the study vs. patients 12 weeks after FMT.

[e] Donors at the beginning of the study vs. patients 20/28 weeks after FMT.

Paired *t* test

[f] Donors at the beginning vs. end of the study.

FMT: fecal microbiota transplantation.

https://doi.org/10.1371/journal.pone.0194904.t002

The scores for the following questionnaires are presented in Table 3: Bristol stool form scale, total SF-NDI scores, EPQ-N-12 and HAD. Bristol stool form scale showed significant changes in the stool form from diarrhea type before FMT to normal following FMT (weeks 1 and 3, $P = 0.07$ and 0.04, respectively), however, no significant differences were noted between weeks 1, 3, 12 and 20/28 when comparing them interchangeably with each other. The total SF-NDI scores showed a significant improvement in the QoL following FMT that lasted to the end of the study and no significant difference was found between weeks 3 and 20/28. The scores for EPQ-N-12 and HAD showed only significant improvement in HAD scores (anxiety and depression) 3 weeks after FMT ($P = 0.016$ and 0.038, respectively). The scores for the different domains of IBS-SQ during the first 3 weeks (20 days) showed significant improvements (except for anorexia) after receiving FMT, as presented in Table 4.

Post-FMT complications

No complications were reported during and following FMT until the end of the study.

Discussion

In this study of the kinetics of gut microbial community composition after FMT in IBS patients, the gut microbiota profile of the patients, which differed significantly from the donors

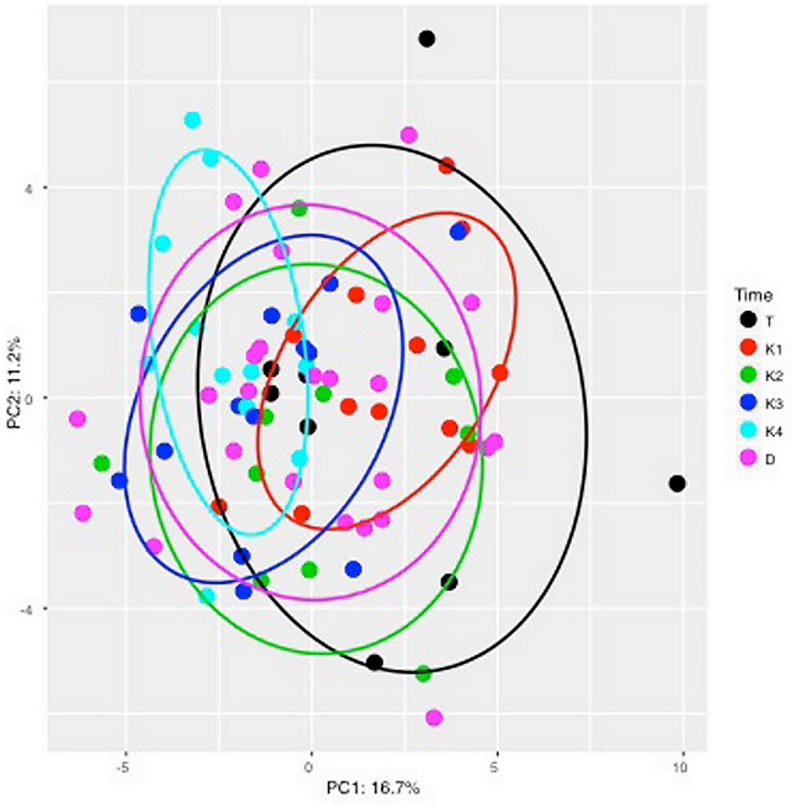

Fig 3. Scores for the first two principal component analysis (PCA) of fecal microbiota in donors at the beginning of the study and patients with irritable bowel syndrome after fecal microbiota transplantation (FMT, *n* = 13). Data have been centered within each donor series to remove donor differences for this analysis. Data are shown as donor (D: pink dots), FMT day (T: black dots), visit at week 1 (K1: red dots), visit at week 3 (K2: green dots), visit at week 12 (K3: dark blue dots) and visit at week 20/28 (K4: light blue dots). Each dot represents data from one patient for one visit. The first two PCs account for 27.9% (sum of PC1 and PC2) of the variation. The colored ellipses demonstrate the 68% confidence interval for PC1 and PC2. The PCA scores show that FMT seem to have an effect on the gut microbiota as systematic change is going forward.

https://doi.org/10.1371/journal.pone.0194904.g003

before FMT, have shown some dynamic changes during the 28 weeks of follow up period post FMT. In addition, the patients complained of severe IBS symptoms and low QoL before FMT. The change in the gut microbiota in IBS patients during the course of the study parallels a rapid improvement in the patients' symptoms and QoL that lasts up to 28 weeks.

The DI of the patients changed from severe dysbiosis before FMT to moderate dysbiosis after 12 weeks following FMT and maintained its new status throughout the course of the study. This showed that administrating the FMT via gastroscope in to the duodenum did not cause/worsen dysbiosis, on the contrary, it helped change the gut microbiota towards normobiosis. In general, dysbiosis in IBS is characterized by a decrease in *Actinobacteria*, *Bifidobacteria* and *Lactobacillus* [41], and an increase in *Bacteroidetes*, *Firmicutes* and *Proteobacteria* in the feces [41, 42]. Increased *Proteobacteria* in diarrhea-predominant IBS (IBS-D) including *E. coli* is associated with increased inflammation [41]. *Bifidobacteria* count is either decreased [41, 43] or increased in IBS patients [44]. In the current study, the bacterial signals for *Actinobacteria* in general and especially in *Bifidobacteria* in the total IBS group and the subgroups of IBS patients are significantly reduced compared to the donors' group at the beginning of the study.

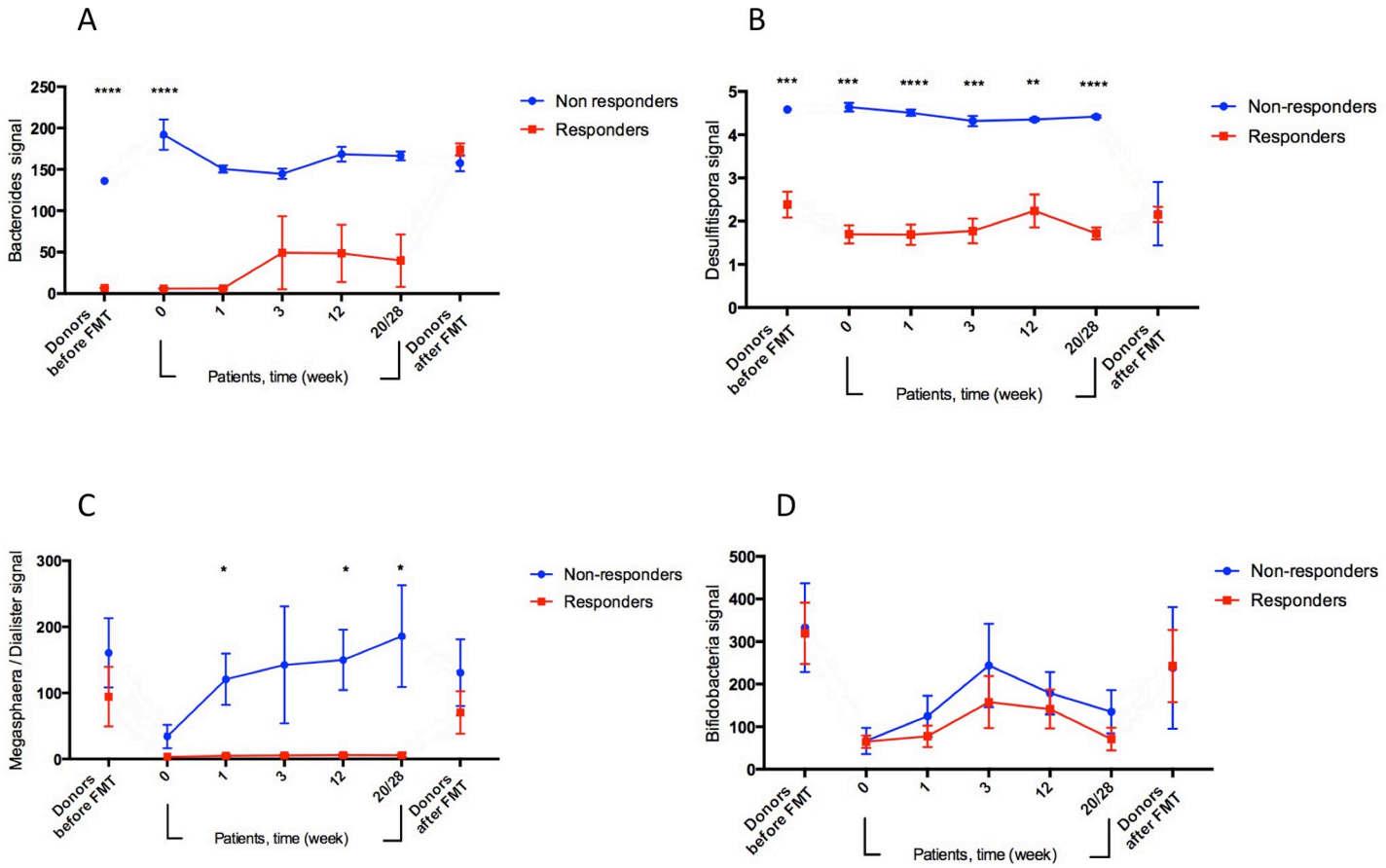

Fig 4. The characteristic differences of gut microbiota between responders and non-responders groups before and after FMT. The bacterial signals of (A) *Bacteroides*, (B) *Desulfitispora*, (C) *Megasphaera/Dialister* and (D) *Bifidobacteria*, in the responders and non-responders groups between the donors at the beginning of the study (before FMT), and between the patients in weeks 0, 1, 3, 12 and 20/28.

https://doi.org/10.1371/journal.pone.0194904.g004

The gut microbiota profile in patients with IBS-D has a significant increase in bacteria belonging to the *Bacteroidetes* phylum and in *Ruminococcus* species [41, 45]. Increased *Ruminococcus* species may cause degradation of the mucus layer that allows infiltration of *Streptococcus* species causing low-grade inflammation [41]. In contrast to a previous study [24] that found no significant difference in the gut microbiota in IBS patients compared to donors other than a significant increase in *Streptococcus* counts in donors, we found significant differences in several bacterial signals in our IBS patients compared to their donors including a significant increase in bacterial signals for *Actinobacteria* and *Bifidobacteria* in the donors' group. *Actinobacteria* and *Bifidobacteria* are important for gut mucosal barrier to keep pathogens from crossing over [41]. These alterations in the gut microbiota profile, especially that of *Ruminococcus gnavus*, *Proteobacteria* and *Shigella/Escherichia* might have contributed to the mechanism of low-grade inflammation in PI-IBS and IBS-D. *Proteobacteria* and *Shigella/Escherichia* signals in the recipients were significantly higher than that of the donors before FMT. *Proteobacteria* and *Shigella/Escherichia* signals further increased during the first week following FMT but then changed (decreased) towards values of the donors. No inflammatory changes were clinically noted among the recipients but one cannot exclude that a low-grade inflammation occurs in patients with IBS even from before FMT. The PCA scores show that FMT seems to have an effect on the gut microbiota as systematic change occurred from baseline before FMT and over a period of 28 weeks.

Table 3. Scores of the patients' questionnaires before and after fecal microbiota transplantation.

Questionnaire	FMT-day (week 0)	Week 1	Week 3	Week 12	Week 20/28)	P[a]	P[b]	P[c]	P[d]	P[e]
IBS-SSS	328.8±20.7	219.1±30.2	236.4±31.1	247.9±37.1	250.8±35.9	**0.008**	**0.002**	**0.0003**	**0.014**	**0.015**
Bristol stool form scale	4.8±0.5	3.7±0.4	3.9±0.4	4.2±0.3	3.8±0.5	**0.07**	**0.042**	**0.0075**	0.17	0.16
SF-NDI	34.9±2.0	-	27.5±2.3	-	28.6±2.4	**0.0045**	-	**0.0039**	-	**0.0068**
EPQ-N-12	4.8±0.8	-	3.7±0.8	-	4.7±1.3	0.36	-	0.07	-	0.6
HAD, anxiety	7.2±1.1	-	5.3±1.0	-	5.9±1.5	0.18	-	**0.016**	-	0.24
HAD, depression	5.3±0.98	-	3.5±0.8	-	5.3±1.2	0.08	-	**0.038**	-	>0.9

Data are presented as the mean±SEM. Comparison

[a] one-way ANOVA with repeated measures; and Paired *t* test

[b] FMT day (week 0) vs. week 1

[c] FMT day (week 0) vs. week 3

[d] FMT day (week 0) vs. week 12

[e] FMT day (week 0) vs. week 20/28.

FMT: Fecal microbiota transplantation.

IBS-SSS: Irritable bowel syndrome-symptom severity scale.

SF-NDI: short form-Nepean dyspepsia index.

EPQ-N-12: The Eysenck Personality Questionnaire-Neuroticism.

HAD: Hospital Anxiety and Depression.

https://doi.org/10.1371/journal.pone.0194904.t003

In a comparison of the gut microbiota profiles between the responders and non-responders groups, the changes in the bacterial signals of *Bacteroides*, *Desulfitispora* and *Megasphaera/Dialister* between the patients during the study are similar to or tend to change toward those

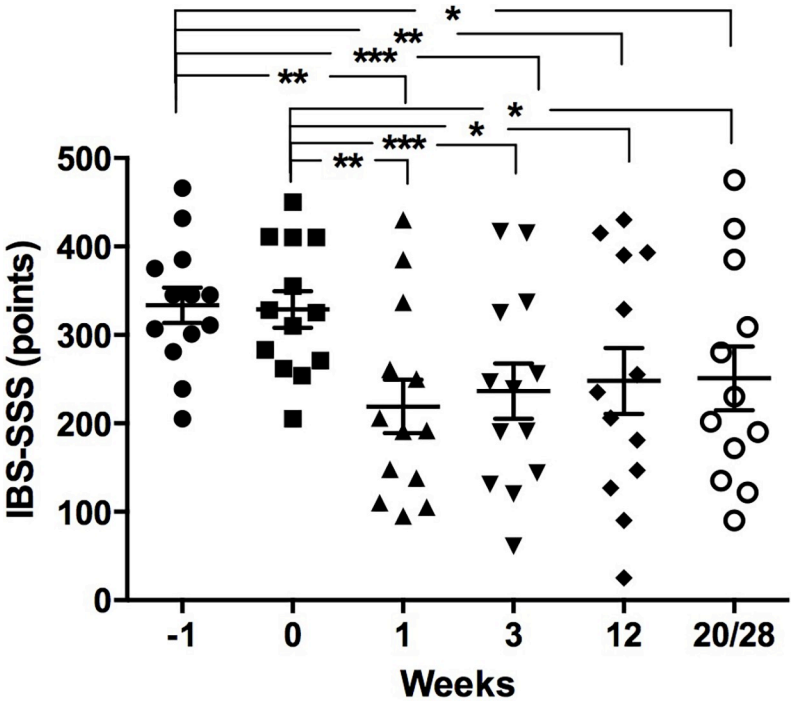

Fig 5. IBS-SSS scores of the patients one week before fecal microbiota transplantation (FMT, week -1), at FMT day (0), and following FMT (weeks 1, 3, 12 and 20/28).

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Table 4. Total score and scores of the six domains of the Irritable Bowel Syndrome-Symptom Questionnaire (IBS-SQ) in patients with IBS before and after fecal microbiota transplantation.

IBS-SQ	Screening	After FMT					P[a]	P[b]	P[c]	P[d]	P[e]	P[f]	P[g]
		Day 1	Day 2	Day 3	Week 1	Week 3							
Total	30.9±3.0	19.2±2.8	15.4±3	12.1±2.8	13±2.2	11.7±1.9	**<0.0001**	**0.0046**	**0.0003**	**<0.0001**	**<0.0001**	**<0.0001**	0.5
Nausea	3.5±0.8	2.8±0.8	1.3±0.5	1.9±0.6	2.2±0.7	1.3±0.5	0.07	0.4	**0.01**	0.08	**0.047**	**0.0013**	0.15
Bloating	7.9±0.5	4.9±1	4.7±0.8	3.1±0.8	3.5±0.9	3.2±0.8	**<0.0001**	**0.002**	**0.0002**	**<0.0001**	**<0.0001**	**<0.0001**	0.5
Abdominal pain	6.5±0.9	4.5±0.9	4.2±0.9	2.7±1	2.8±0.8	3.5±0.8	**0.0017**	**0.03**	**0.02**	**0.0009**	**0.0005**	**0.0005**	0.4
Constipation	4.2±1.0	2.6±0.8	2.6±1	1.4±0.8	1.5±0.6	1.4±0.6	**0.03**	0.3	0.07	**0.01**	**0.009**	**0.027**	0.8
Diarrhea	6.5±0.8	6.5±0.8	2.5±0.8	2.1±0.8	1±0.5	1.5±0.5	**0.0001**	**0.0018**	**0.0016**	**0.0004**	**<0.0001**	**<0.0001**	0.4
Anorexia/ loss of appetite	2.1±0.7	1.9±0.7	1.5±0.6	1.6±0.6	1.5±0.5	0.8±0.3	0.3	0.9	0.6	0.6	0.5	0.09	0.08

Data are presented as the mean±SEM. Comparison

[a] one-way ANOVA with repeated measures; and Paired *t* test

[b] Screening vs. day 1 post FMT.

[c] Screening vs. day 2 post FMT

[d] Screening vs. day 3 post FMT

[e] Screening vs. week 1 after FMT

[f] Screening vs. week 3 after FMT

[g] Week 1 vs. Week 3.

IBS-SQ: IBS symptom questionnaire.

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measured for the donors at the beginning of the study. This may be explained by the fact that the donors and the patients are relatives and/or living in the same environment and also by the impact the donors' gut microbiota may have on the patients' gut microbiota. Another interesting observation is that the signals for *Bifidobacteria* in the responders group are lower than those in the non-responders group. Low *Bifidobacteria* signals have also been observed in IBS patients following a low-FODMAP diet as shown in a previous study [46].

The changes in the patients' gut microbiota following FMT may have contributed to the subsequent improvement in their symptoms and thus QoL [25]. Similar observation has been recently described after using FMT to restore the bacterial diversity and resolve the dysbiosis in patients with recurrent *Clostridium difficile* infection [47]. Some of the bacterial strains changed towards the end of the study (weeks 12 and 20/28) without significantly worsening the symptoms of IBS and/or QoL during the same period. This may suggest that the changes in the symptoms and QoL may be related to the collective changes in the gut microbiota rather than individual bacterial change. The change in the gut microbiota profile of the patients at weeks 12 and 20/28 following FMT compared to the donors before FMT (Table 2) resembled that of the donors at the end of the study compared to the donors at the beginning of the study, which may have contributed to the increase in the DI towards the end of the study (Fig 2). The changes towards the end of the study are quite interesting as they raise a question to whether other factors may have influenced these changes such as the participants' milieu, dietary (which were not changed according to the metadata) or hereditary factors; since the donors and the patients are relatives either living in the same environment (for example: spouse) or sharing the same genes (for example: a parent or a sibling) or both. Another explanation may have been due to the imposed changes to the patients' gut microbiota following FMT rendering them susceptible to the same changes occurring to the donors' gut microbiota.

FMT was associated in time with rapid improvement in IBS-SSS score (>50 points reduction from baseline) [29]. In the current study, 70% of the patients has improved IBS-SSS scores during the first week, 85% by 3 weeks, 70% by 12 weeks and 62% over 20 weeks towards the

end of the study. The long lasting effect of FMT on IBS symptoms is in line with previous studies [23, 24]. In addition to an immediate improvement (during the first 3 days after FMT) in IBS-SQ–total and specific–symptom scores, namely; bloating, abdominal pain and diarrhea, a gradual but statistically significant improvement in all of the IBS symptoms as assessed by IBS-SQ (except for anorexia/loss of appetite) was observed on daily basis during the first 3 weeks following FMT. The improvements in IBS related QoL, abdominal pain and bloating during the course of the study is consistent with a previous report [24].

Currently, dietary manipulation is one of the methods for the management of IBS symptoms. [48–50] The response rate of an elimination diet ranges between 15 and 71% [51], and of low FODMAP diet is up to 86% [46, 48], with a high placebo response rate reaching to 40% [48]. One can suggest that FMT may serve as an alternative method for managing IBS instead of the dietary manipulation due to similar high response rate, easy to apply, and long lasting improvements in the symptoms and QoL up to one year [23, 24]. However, head to head comparison studies must be further conducted.

The route of administration of feces, either via gastroscopy [23] or colonoscopy [24] in to the upper or lower GI tract, respectively, have reported similar effects on IBS symptoms [23, 24]. In a systematic review of FMT in recurrent *Clostridium difficile* infection, the use of gastroscopy as an administration route had a response rate of 76% vs. 88% for colonoscopy [52]. In this study, we have chosen to use gastroscopy because it is an easy and a fast route of administration and because patients with IBS often have dilation of small bowel segments giving small intestinal bacterial overgrowth, SIBO, [53], which would have escaped the suspension had we chosen to use colonoscopy for FMT. The procedure is considered to be safe [31]. No complications were reported during and following FMT until the end of the study. In other studies, short-term adverse events occurred after FMT such as abdominal cramps, belching and nausea but they were self-limited and transient [23, 54]. Long-term, follow-up studies (3–68 months after FMT) have found FMT to be relatively free of adverse effects [55].

The strength of the study is the usage of validated methods to study the kinetics in the gut microbiota and validated questionnaires to assess the changes in the stool form, symptoms and QoL. The main limitations of the study are its design as an open-label trial, not placebo- or sham-controlled, and the small sample size. However, our main focus was to study the changes in the gut microbiota in the patients compared to the gut micobiota of the donors, which are supposed to have more-or-less stabile profiles during the study. Nevertheless, significant results were obtained despite the small sample size. Larger double-blinded, placebo-controlled studies are necessary to address applicability of FMT in IBS and are currently running else where in Norway (NCT02154867).

Conclusions

This is the first study to investigate the kinetics of microbial community composition in IBS patients following FMT. FMT was associated with a rapid change in the alterations in the signals for several strains of the gut microbiota making it statistically not significantly different from the donors after 3 weeks following FMT. The gut microbiota profile at the end of the study was similar to the profile of the donors taken at the same time. The symptoms and QoL have improved significantly quite soon after FMT and lasted up to 28 weeks.

Supporting information

S1 File. FMT protocol-2013.
(PDF)

S2 File. Trend checklist.
(PDF)

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Article

Faecal Microbial Markers and Psychobiological Disorders in Subjects with Morbid Obesity. A Cross-Sectional Study

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Abstract: Morbidly obese subjects have a high prevalence of comorbidity and gut microbial dysbiosis, and are thus suitable for the study of gut-brain interactions. The aim was to study the associations between the faecal microbiota's composition and function and psychobiological comorbidity in subjects with BMI > 40 kg/m^2 or >35 kg/m^2 with obesity-related complications. The faecal microbiota was assessed with GA-Map dysbiosis test ™ (Genetic Analysis, Oslo Norway) and reported as dysbiosis (yes/no) and degree of dysbiosis, and the relative abundance of 39 bacteria. The microbiota's function was assessed by measuring the absolute and relative amount of faecal short chain fatty acids. Associations were made with well-being, mental distress, fatigue, food intolerance, musculoskeletal pain, irritable bowel syndrome, and degree of abdominal complaints. One hundred and two subjects were included. The results confirmed the high prevalence of comorbidity and dysbiosis (62/102; 61%) and showed a high prevalence of significant associations (41/427; 10%) between the microbiota's composition and function and the psychobiological disorders. The abundant, but in part divergent, associations supported the close gut-brain interaction but revealed no clear-cut and straightforward communication pathways. On the contrary, the study illustrates the complexity of gut-brain interactions.

Keywords: gut-brain axis; faecal microbiota; faecal short chain fatty acids; morbid obesity; psychobiological disorders; well-being; mental distress; irritable bowel syndrome

1. Introduction

Obesity is a worldwide health problem that has nearly tripled since 1975 and it affects 13% of the adult population. It is associated with a wide range of comorbidities, such as cardiovascular diseases, diabetes, musculoskeletal disorders, cancer, and psychobiological disorders, and an increased risk of death [1].

Faecal dysbiosis has been defined as an imbalance in the faecal microbiota. It is common in subjects with morbid obesity (MO) and it has been mentioned as a causal factor for obesity and the comorbidities such as insulin resistance, glucose intolerance and diabetes type II, as well as psychiatric and functional disorders [2–6].

The gut-brain axis is a bidirectional link between the gut and the brain and of importance for various psychobiological disorders, such as anxiety, depression, fatigue, stress reactions, pain syndromes, and functional gastrointestinal disorders [7–9]. The absolute or relative amounts of the gut microbes per se and the microbes' metabolic products are possible mediators of the gut-brain

effects. Faecal short chain fatty acids (SCFA), which are products of bacterial fermentation, have been proposed as mediators of the health-related effects [10,11].

The primary aim of this study in subjects with morbid obesity was to explore associations between the faecal microbiota's composition and metabolic products and a selection of psychobiological disorders. The secondary aims were to compare the subjects' microbiota with that of healthy reference populations. The high prevalence of psychobiological disorders and faecal dysbiosis in subjects with morbid obesity makes this group of special interest for the study of gut-brain interactions.

2. Materials and Methods

2.1. Study Design

A cross-sectional study in subjects with morbid obesity. The microbial composition was analysed with a commercially available test, and the results were compared with the test producer's reference population [12]. The faecal SCFA were compared with the faecal samples from healthy volunteers, as previously published [13].

2.2. Participants

Consecutive subjects aged 18–65 years with MO (defined as BMI > 40 or >35 kg/m^2 with morbidity related comorbidity), referred to the Unit for Obesity at Innlandet Hospital Trust–Gjøvik, Norway in the period from December 2012 to September 2014 were eligible for the study. Subjects with organic gastrointestinal disorders, major psychiatric disorders, severe not obesity-related somatic disorders, alcohol or drug addiction, and previous obesity surgery or other major abdominal surgery were excluded.

The healthy volunteers were healthcare workers and students from Haukeland University Hospital, Bergen, Norway who considered themselves healthy.

2.3. Accomplishment

In all morbidly obese subjects, a medical history was taken, a physical examination was performed, and blood and faecal samples were collected. The information was collected on paper-based questionnaires that were filled in by the doctors, the study nurse, and the participants. Other examinations were performed at the doctors' discretion. Except for some demographic data, no information was available about the healthy volunteers.

2.4. Variables

2.4.1. Participants' Characteristics

- Gender, age (years), height (m), weight (kg), BMI (kg/m^2), coffee (cups/day), smoking (daily, previously, never), and previous and present diseases.
- Physical activity was the sum of two questions: Easy activity (not sweaty/breathless): None; <1 h; 1–2 h; >3 h/week (score 0–3). Strenuous activity (sweaty/breathless): none; <1 h; 1–2 h; >3 h/week (score 0, 3, 4, 5). Sum score physical activity 0–8.
- Use of Metformin and other drugs (Yes/No)
- Use of Non-Nutritive Sweeteners (NNS). One unit of NNS was defined as 100 mL NNS-containing beverage or two NNS tablets/teaspoons for use in tea or coffee. A validated food frequency questionnaire that is based on the official Norwegian food composition table was used for the calculation [14].

2.4.2. Psychobiological Disorders

- WHO-5 Well-being index (score 0–100; scores $\leq$ 50 indicate low mood and scores $\leq$ 28 indicate likely depression) [15]

- Hopkins symptom checklist 10, (score 1–4; scores $\geq$ 1.85 indicate mental distress) [16]
- Fatigue (Score 9–63; scores $\geq$ 36 indicate further evaluation). The diagnose was based on a validated Norwegian translation of the Fatigue Severity Scale [17].
- Musculoskeletal pain from six parts of the body (score 0–12).
- Food intolerance (yes/no) as reported by the participants.
- Irritable Bowel Syndrome (IBS) (yes/no) was diagnosed with a validated Norwegian translation of the Rome III criteria [18].
- Abdominal complaints were scored with IBS Severity Score system (IBS-SSS) (score 0–500) [19]. All of the subjects with abdominal complaints, and not only those with IBS, filled in the questionnaire.

2.4.3. Faecal Microbiota

The CE marked GA-map™ Dysbiosis Test (Genetic Analysis AS, Oslo, Norway) was used for the analyses of the faecal microbiota [12]. The test has a US (Patent No. 9243297) and a European patent (Patent No. 2652145) for its technology governing the oligonucleotide probe set and methods of microbiota profiling [20]. It uses 54 oligonucleotide probes targeting the 16S rRNA gene at different bacterial taxonomic levels.

The overall result is given as the Dysbiosis Index (DI) with scores 1 to 5; values above 2 indicate a microbiota profile that differs from the producer's reference population (i.e., dysbiosis). The results are also given as the relative abundance compared to a reference population (score −3 to 3) of 39 bacteria at different taxonomic levels (Actinobacteria, Actinomycetales, *Bifidobacterium* spp., Alistipes, *Alistipes onderdonkii*, *Bacteroides fragilis*, *Bacteroides* spp. & *Prevotella* spp., *Bacteroides stercoris*, *Bacteroides zoogleoformans*, *Parabacteroides johnsonii*, *Parabacteroides* spp., Firmicutes, Bacilli, *Catenibacterium mitsuoka*, Clostridi a, *Clostridium* sp., *Dialister invisus*, *Dialister invisus* & *Megasphaera micronuciformis*, *Dorea* spp., *Eubacterium biforme*, *Eubacterium hallii*, *Eubacterium rectale*, *Eubacterium siraeum*, *Faecalibacterium prausnitzii*, Lachnospiraceae, *Lactobacillus ruminis* & *Pediococcus acidilactic*, *Lactobacillus* spp., *Phascolarctobacterium* sp., *Ruminococcus albus* & *R. bromii*, *Ruminococcus gnavus*, *Streptococcus agalactiae & Eubacterium rectale*, *Streptococcus salivarius* ssp. *thermophiles* & *S. sanguinis*, *Streptococcus salivarius* ssp. *Thermophilus*, *Streptococcus* spp., *Veillonella* spp., Proteobacteria, *Shigella* spp. & *Escherichia* spp., *Mycoplasma hominis*, and *Akkermanasia muciniphilia).* The test is a commercial and patented product—hence the dysbiosis scores are the producer's secret.

In addition, an Alternative Dysbiosis Index (ADI) that is based on the relative abundance of the bacteria Alistipes, Proteobacteria and *Shigella* spp. & *Escherichia* spp., and the relative scarcity of *Bacteroides fragilis*, *Ruminococcus gnavus*, *Bacteroides* spp. & *Prevotella* spp., and *Dialister invisus* was calculated. The ADI has been claimed to separate the favourable dysbiosis (positive scores) from the unfavourable one (negative scores) [21].

2.4.4. Faecal Short Chain Fatty Acids

The subjects with morbid obesity collected the faecal material at home in kits that were provided by the producer of the microbial test and stored it at room temperature for maximum five days before freezing at minus 70 °C [12].

Distilled water containing 3 mmol/L of 2-ethylbutyric acid (as internal standard) and 0.5 mmol/L of H_2SO_4 was added to 0.5 g of the faecal content and homogenized. According to the method of Zijlstra et al. as modified by Høverstad et al. 2.5 mL of the homogenate was vacuum distilled [22,23]. The distillate was analysed with gas chromatography (Agilent 7890 A; Agilent, CA, USA) using a capillary column (serial no. USE400345H, Agilent J&W GC columns; Agilent, CA, USA) and quantified while using internal standardisation. Flame ionisation detection was employed. The total amount of SCFA and the total and relative amount of acetic, propionic, n-butyric, i-butyric, n-valeric, i-valeric, n-caproic, and i-caproic acids expressed in mmol/kg wet weight and proportion (percentage) were measured and reported.

The following variables were also calculated:

- Index A (saccharolytic fermentation), which was the concentration of acetic minus propionate minus butyrate divided by the total amount of SCFAs [24].
- Index B (proteolytic fermentation), which was the sum of concentrations of isobutyrate and isovalerate [24].
- The ratio "Propionic acid/Butyric acid". A high ratio has been proposed as unfavourable [25].

In principle, the analyses of SCFA were performed with identical methods in the subjects with morbid obesity and the healthy volunteers. However, since the analyses were performed in different laboratories and with slightly different preanalytical handling of the samples, only the relative amounts of the SCFA were compared between the groups to avoid bias in the measuring of the total amounts of SCFA.

2.5. Statistics

Student *t*-test was used for comparisons between groups, Wilcoxon sign-rank test for comparisons with a reference standard, and linear and logistic regression analyses for the study of associations. In each analysis, all of the cases with data on the relevant variables were included ("available case analysis"). p-values < 0.05 were judged as being statistically significant. The analyses were performed with IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY, USA: IBM Corp.

2.6. Ethics

The study was approved by the Norwegian Regional Committees for Medical and Health Research Ethics, (reference numbers 2012/966 and 030.08) and was performed in accordance with the Declaration of Helsinki. All the participants gave written informed consent before inclusion.

3. Results

3.1. Subject Characteristics

Out of 350 consecutive subjects with morbid obesity, 111 were excluded because the study nurse was unavailable, and 80 refused participation. Out of 159 subjects included in the study, 17 were erroneously included or non-compliant and 40 did not provide faecal samples. Table 1 gives the characteristics of the 102 subjects that were included in this study.

Table 1. Subject characteristics.

Subject Characteristics	Number (%) Mean and/or Median	SD and/or Range
Gender (male/female)	15 (14.7%)/87 (85.3)	
Age (years)	44.2	8.6
Height (cm)	170	7.8
Weight (kg)	120.8	16.1
BMI (kg/m^2)	41.8	3.6
Coffee (cups/day)	3.2	2.5
Smoking (daily/previously/never)	14 (13.7%)/46 (45.1%)/42 (41.2%)	
Physical activity (0–8)	4.5	2.3
Diabetes (yes/no)	23 (23.2%)/76 (76.8%)	
Metformin use (yes/no)	16 (18.0%)/73 (82.0%)	
Non-nutritive sweeteners (units *)	7.5 (median 3.3)	10.1 (0–43)
WHO-5 (0–100)	60.4 (median 60)	16 (12–92)
HSCL-10 (1–4)	1.58 (median 1.4)	0.54 (1.0–3.2)
HSCL-10 Mental distress (yes/no)	26 (26.5%)/72 (73.5%)	
Fatigue (6–63)	35.9	14.8
Musculoskeletal pain (0–12)	4.4	2.9
Food intolerance (Yes/No)	55 (55.6%)/44 (44.4%)	
IBS (Yes/No)	27 (27%)/73 (73%)	
IBS Severity scoring system (0–500)	103	0–389

* One unit = 100 mL beverage with non-nutritive sweeteners or 2 tablets/teaspoons for coffee of tea. WHO-5: WHO Well-being index. HSCL-10: Hopkin Symptom Check List 10. IBS: Irritable Bowel Syndrome.

The healthy volunteers that were used for comparisons of the SCFA were four men and eleven women with a mean age of 32.1 years (range 22–68) and BMI 23.7 (range 20.1–27.8 kg/m^2).

3.2. Dysbiosis Test

Dysbiosis [Dysbiosis Index (DI) > 2] was present in 62/102 (61%). The mean DI and Alternative Dysbiosis Index (ADI) scores were 2.8 (1.3) and −0.4 (2.6), respectively. When compared with producer's reference population [12], the relative amount of 22 bacteria were significantly elevated (p-values < 0.05, of which 12 bacteria with $p < 0.001$) and the relative amount of 10 bacteria was significantly reduced (p-values < 0.05, of which 5 bacteria with $p < 0.001$) The most marked deviations from the reference population were: *Bacteroides* spp. & *Prevotella* spp.: Score 1.59 (1.27) ($p < 0.001$). Bacteroides fragilis: Score 0.54 (0.89) ($p < 0.001$). Bacteroides stercoris: Score 0.44 (0.74) ($p < 0.001$). Eubacterium hallii: Score −0.54 (0.54) ($p < 0.001$). Faecalibacterium prausnitzii: −0.49 (0.71) ($p < 0.001$).

3.3. Short Chain Fatty Acids

Total amount of SCFA was 35.99 (SD 21.24) mmol/kg wet weight. Table 2 gives the results in the subjects with morbid obesity and the healthy volunteers with comparisons between the relative amounts of SCFA in the two groups.

Table 2. Short chain fatty acids (SCFA) in subjects with morbid obesity and healthy volunteers. The results are given as mean (SD).

SCFA	Subjects with Morbid Obesity		Healthy Volunteers	MO vs. HV Relative Amounts
	mmol/kg Wet Weight	Relative Amount (%)	Relative Amount (%)	p-Value
SCFA total	35.99 (21.24)			
Acetic acid	19.57 (10.72)	55.1 (6.4)	76.9 (9.6)	<0.001
Propionic acid	6.25 (4.16)	17.3 (4.4)	8.5 (3.7)	<0.001
Iso-butyric acid	0.72 (0.61)	2.1 (0.9)	1.4 (0.7)	0.006
Butyric acid	7.13 (5.28)	19.2 (5.3)	9.5 (4.6)	<0.001
Iso-valeric acid	1.05 (0.93)	3.0 (1.5)	2.0 (1.2)	0.017
Valeric acid	0.96 (0.84)	2.6 (1.2)	1.3 (0.8)	<0.001
Iso-capronic acid	0.00 (0.01)	0.0 (0.0)	0.0 (0.0)	0.163
Capronic acid	0.29 (0.51)	0.7 (1.0)	0.4 (0.5)	0.187
Index A	0.19 (0.11)			
Index B	1.77 (1.53)			
Pro/But ratio	1.01 (0.53)	1.01 (0.53)	1.0 (0.4)	0.864

SCFA: Short chain fatty acids. MO: Subjects with morbid obesity. HV: Healthy volunteers. Pro/But ratio: The ratio Propionic acid/Butyric acid.

3.4. Associations between the Psychobiological Disorders and the Microbial Markers

Out of 427 analysed associations between the faecal microbiota and the psychobiological disorders, 41 (10%) were statistically significant. Table 3 gives all of the statistically significant associations between the psychological disorders and the microbiota, and Table 4 provides all of the statistically significant associations between the functional somatic disorders and the microbiota.

Table 3. The significant associations between the psychological disorders and the faecal microbiota and SCFA. Regression analyses with the psychological variables as dependent variables.

Microbiota	WHO-5		HSC-10		Fatigue	
	B; *p*-Value *	**B; *p*-Value †**	**B; *p*-Value ***	**B; *p*-Value †**	**B; *p*-Value ***	**B; *p*-Value †**
Dysbiosis Index	−2.86; 0.024					
ADI			−0.056; 0.011		−1.98; 0.001	−1.81; 0.002
Alistipes					−5.42; 0.022	
Bacteroides spp. & *Prevotella* spp.	−3.43; 0.010				2.84; 0.021	
Bacteroides stercoris			0.174; 0.019	0.159; 0.028		
Bacilli	4.86; 0.039					
Dorea spp.	12.18; 0.014	11.44;0.016				
Faecalibacterium prausnitzii	6.37; 0.007	5.65; 0.013	−0.205; 0.011	−0.191; 0.015		
Phascolarctobacterium sp.					−6.77; 0.005	−5.94; 0.009
SCFA total	−0.179; 0.019					
Acetic acid	−0.342; 0.024					
Propionic acid	−0.890; 0.022					
Butyric acid	−0.681; 0.026	−0.675; 0.020				

* Linear regression analyses with the psychological variable as dependent variable and one-by-one of the microbiota variables adjusted for gender, age and BMI; † Stepwise forward linear regression analyses. All the significant variables in the one-by-one analyses were included adjusted for gender, age and BMI.

Table 4. The significant associations between the functional somatic disorders and the faecal microbiota and SCFA. Regression analyses with the functional somatic disorders as dependent variables.

Microbiota	Food Intolerance		Musculoskeletal Pain		IBS		IBS-SSS	
	OR; *p*-Value *	OR; *p*-Value †	B; *p*-Value *	B; *p*-Value †	OR; *p*-Value *	OR; *p*-Value †	B; *p*-Value *	B; *p*-Value †
ADI							−10.86; 0.010	−10.86; 0.010
Actinomycetales			1.34; 0.034					
Bifidobacterium spp.			1.22; 0.012	0.94; 0.039				
Alistipes	0.34; 0.019	0.34; 0.019					−40.3; 0.012	
Alistipes onderdonkii	0.52; 0.041							
Bacteroides stercoris			1.25; 0.001	1.07; 0.004				
Bacteroides zoogleoformans					4.64; 0.026	15.55; 0.009		
Parabacteroides johnsonii								
Parabacteroides spp.					2.10; 0.037	3.31; 0.007		
Firmicutes					2.30; 0.037			
Dia/ister invisus					1.95; 0.026	2.91; 0.008		
Eubacterium rectale			−1.63; 0.023					
Phascolarctobacterium sp.			−1.016; 0.030	−0.85; 0.049				
Proteobacteria			−1.026; 0.050					
Shigella spp. & *Escherichia* spp.			−0.71; 0.049	−0.75; 0.030				
SCFA total					0.967; 0.049			
Acetic acid					0.935; 0.033			
Iso-butyric acid					0.080; 0.006			
Iso-valeric acid					0.213; 0.006			
Valeric acid					0.217; 0.012	0.14; 0.003		
Iso-capronic acid			−67.1; 0.034					
Index B					0.379; 0.005			
Valeric acid Pct					0.623; 0.029			
Iso-capronic acid Pct			−27.57; 0.034					
Propionic acid Pct					1.14; 0.021			

* Linear and logistic regression analyses with the functional disorders as dependent variable and one-by-one of the microbiota variables adjusted for gender, age and BMI; † Stepwise forward linear and logistic regression analyses. All the significant variables in the one-by-one analyses were included adjusted for gender, age and BMI.

4. Discussion

The study demonstrated the numerous significant associations between the faecal microbiota's composition and function and the psychobiological disorders that are challenging to interpret. There is no simple and straightforward explanation and understanding of the gut-brain pathway. The numerous associations indicate complex connections that follow several pathways that are dependent on the trigger and psychobiological disorder.

4.1. Associations between the Faecal Microbial Composition and Psychobiological Disorders

A connection between the faecal microbiota's composition and function and psychological disorders seems to be established, but it is poorly understood [7,9]. The connection is not explained by one or a few species or genus [8]. The multiple associations that are seen in this study indicate a complex regulation of the gut-brain connection. Neither was the DI, a general marker of microbial imbalance, a suitable predictor of all the psychological disorders. The associations between the microbiota and the psychological disorders varied between the disorders. It is unlikely that the three variables that were measured in this study (WHO-5, HSCL-10, and fatigue) are specifically associated with different microbes. HSCL-10 and fatigue were negatively associated with ADI, i.e., associated with an unfavourable dysbiosis. Dysbiosis indices that are based on a combination of microbes might prove to be the best suited predictors of psychological disorders. Similar microbial abnormalities in psychological and functional somatic disorders, indicating common aetiological factors, have been shown in other studies but they were not demonstrable in the current study [26,27].

Some bacteria, such as Faecalibacterium prausnitzii and Proteobacteria, have attracted particular attention [28–31]. Faecalibacterium prausnitzii was associated with improved well-being (WHO-5) and less mental distress (HSCL-10), supporting the importance of this bacterium. This study did not support previous reports, indicating an association between the phylum Proteobacteria and epithelial dysfunction and risk of disease. Shigella spp. & Escherichia spp. and Proteobacteria were negatively associated with musculoskeletal pain, but not with other psychobiological disorders.

4.2. Associations between the Faecal SCFA and Psychobiological Disorders

Faecal SCFA have been associated with behavioural, psychological and functional somatic disorders and response to treatment [10,11,13,25,32,33]. Like the microbial composition, the results are divergent and in part contradictory and non-reproducible. The favourable effects of butyric acid on brain function were not confirmed in the current study in which total SCFA, acetic acid, propionic acid, and butyric acid were negatively associated with well-being [33]. Previous studies have demonstrated higher levels of faecal SCFA in obese as compared to lean subjects [34,35]. However, the "obesogenic" effect of SCFA remains to be investigated [36].The most noteworthy finding was the associations between IBS and low amounts of total SCFA, acetic acid, iso-butyric acid, iso-valeric acid and valeric acid (both total and relative amount), and reduced proteolytic fermentation. Butyrate has shown favourable effects on visceral sensitivity in healthy volunteers [37]. The local effects of SCFA on the gut seem to be more pronounced than the systemic and centrally mediated ones. The proposed ratio proprionic/butyric acid as a biomarker of IBS was not confirmed [25].

4.3. Faecal Microbial Composition and Obesity

As expected, and in accordance with other studies with different methods, the prevalence of faecal dysbiosis measured with the commercially available test was high (61%) [2,3]. Thirty-two out of 39 bacterial groups (82%) deviated significantly from the producer's reference population. Since diabetes, the use of Metformin and consumption of NNS, which are associated with dysbiosis, were common in the studied population, dysbiosis might have been related to these factors and not to obesity per se [38–41]. Of note, Faecalibacterium prausnitzii, which has been associated with obesity, was significantly reduced [30].

4.4. Faecal SCFA and Obesity

In subjects with morbid obesity, the relative proportions of six out of eight SCFA (75%) deviated significantly from the group of healthy volunteers. In particular, the functions of butyric acid have been studied and seem to be contradictory [42]. In this study, the relative amount of butyric acid was high in the subjects with morbid obesity when compared with the healthy volunteers. In mice, butyric acid reduces appetite and food intake via a central appetite regulation and has a positive influence on the energy balance and diet-induced overweight [43]. If these results are transferable to humans, butyrate has a weight-reducing effect. We are not aware of such studies in humans. Our results did not support this effect. Studies have reported other and opposite effects of butyrate, which in part, could be explained by differences in the metabolic background and dosage [33,42,44].

4.5. Strengths and Limitations

The study population was representative of subjects with morbid obesity referred for evaluation of bariatric surgery, and was well suited for this study because of the high prevalence of faecal dysbiosis and psychobiological comorbidity.

Out of the 427 associations between the microbiota and psychobiological disorders (microbiota's composition: 41 variables; SCFA: 20 variables; and, psychobiological disorders: seven variables), 41 (10%) were statistically significant. The number is higher than expected to occur by chance (type I error). Significant associations do not mean causality, and type II errors are also likely. In such studies, there are numerous unknown confounders, colliders, and mediators, and the analyses were not adjusted for such factors. Therefore, the results of this and similar studies should be interpreted with caution.

The test used for the microbiota's composition, measuring an undefined dysbiosis index and the relative amount of "only" 39 bacteria at different taxonomic levels might have been inaccurate or incomplete for the purpose of this study. More precise and detailed analytical methods could have given other results.

Since carbohydrates and fibre are major substrates for the microbial SCFA production, the lack of dietary data is another limitation of the study. In subjects with obesity, reduced intake of carbohydrates has been associated with low concentrations of butyrate and butyrate-producing bacteria in faeces [45]. Dietary differences between the subjects with obesity and the healthy volunteers, and not only the differences in BMI, could thus explain the differences in SCFA between the groups. In the gut-brain communication, it is likely that SCFA, which are dependent on the diet, are the mediators of the psychobiological disorders.

The lack of information about the psychobiological disorders in the healthy volunteers, which is a limitation, render analyses of the associations between the faecal markers and psychobiological disorders in this group impossible. The study, therefore, confines itself to a description of the differences in the SCFA profiles between the groups and discusses associations with obesity without mentioning the psychobiological disorders.

Comparisons of the total amount of SCFA, and not only the relative amounts, with the healthy volunteers, could have strengthened the study.

5. Conclusions

The current study in subjects with morbid obesity showed a wide range of associations between the faecal microbial markers and psychobiological comorbidity, and thus confirmed the important gut-brain interaction. The study did not clarify simple communication pathways. On the contrary, the study indicated complex and multifactorial relations that often seem contradictory and that need further studies to clarify clinical implications.

Author Contributions: P.G.F. has been responsible for accomplishing the study in subjects with morbid obesity, for collecting and analysing the data, and drafting the manuscript. J.V. has been responsible for the group of healthy volunteers and the analyses of SCFA. Both authors have contributed to the interpretation of the results and approved the last version.

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Abbreviations

MO	Morbid Obesity
SCFA	Short Chain Fatty Acids
BMI	Body Mass Index
HV	Healthy Volunteers
DI	Dysbiosis Index
ADI	Alternative Dysbiosis Index
NNS	Non-nutritive sweeteners
OR	Odds Ratio
B	Unstandardized coefficient in the linear regression analyses
WHO-5	WHO Well-being index
HSCL-10	Hopkin Symptom Checklist 10
IBS	Irritable Bowel Syndrome
IBS-SSS	Irritable Bowel Severity Scoring System

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ORIGINAL ARTICLE

Multivariate modelling of faecal bacterial profiles of patients with IBS predicts responsiveness to a diet low in FODMAPs

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ABSTRACT

Objective The effects of dietary interventions on gut bacteria are ambiguous. Following a previous intervention study, we aimed to determine how differing diets impact gut bacteria and if bacterial profiles predict intervention response.

Design Sixty-seven patients with IBS were randomised to traditional IBS (n=34) or low fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) (n=33) diets for 4 weeks. Food intake was recorded for 4 days during screening and intervention. Faecal samples and IBS Symptom Severity Score (IBS-SSS) reports were collected before (baseline) and after intervention. A faecal microbiota dysbiosis test (GA-map Dysbiosis Test) evaluated bacterial composition. Per protocol analysis was performed on 61 patients from whom microbiome data were available.

Results Responders (reduced IBS-SSS by ≥50) to low FODMAP, but not traditional, dietary intervention were discriminated from non-responders before and after intervention based on faecal bacterial profiles. Bacterial abundance tended to be higher in non-responders to a low FODMAP diet compared with responders before and after intervention. A low FODMAP intervention was associated with an increase in Dysbiosis Index (DI) scores in 42% of patients; while decreased DI scores were recorded in 33% of patients following a traditional IBS diet. Non-responders to a low FODMAP diet, but not a traditional IBS diet had higher DI scores than responders at baseline. Finally, while a traditional IBS diet was not associated with significant reduction of investigated bacteria, a low FODMAP diet was associated with reduced *Bifidobacterium* and Actinobacteria in patients, correlating with lactose consumption.

Conclusions A low FODMAP, but not a traditional IBS diet may have significant impact on faecal bacteria. Responsiveness to a low FODMAP diet intervention may be predicted by faecal bacterial profiles.

Trial registration number NCT02107625.

Significance of this study

What is already known on this subject?

- Dietary intervention is effective at reducing IBS symptom severity, but not all patients respond to the intervention.
- Subgroups of patients with IBS have an altered gut microbiota composition.
- Gut bacteria have been demonstrated to be affected by alterations in dietary composition.

What are the new findings?

- Low fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs), but not traditional IBS diet might influence faecal bacterial composition.
- Low FODMAP, but not traditional IBS diet responders could be discriminated from non-responders before the intervention based on faecal bacterial profiles.
- Dysbiosis Index scores were higher in patients after 4 weeks of low FODMAP diet, but not after traditional IBS diet.
- Non-responders to low FODMAP diet had higher Dysbiosis Index scores than responders at baseline.

How might it impact on clinical practice in the foreseeable future?

- The potential ability of faecal bacteria composition to predict response to a low FODMAP diet in IBS may help in selecting patients for this intervention.

INTRODUCTION

Affecting approximately 10–15% of the Western world,[1] IBS is a functional GI disorder characterised by abdominal discomfort or pain associated with altered bowel habits.[2] IBS is heterogeneous in aetiology with the underlying mechanism not yet fully elucidated. However, host–microbe interactions are suggested to play a role in symptom manifestation in a subgroup of patients with IBS.[3]

In healthy individuals, bacteria live in symbiosis with each other and the host, which is often referred to as normobiosis. Disturbance of the intestinal bacteria may cause a permanent imbalance to occur, known as dysbiosis.[4] Dysbiosis is suggested to evoke maladies of the GI tract[5] and previous studies propose an altered bacterial composition,[6] interpretable as dysbiosis,[7] to be present in a subset of patients with IBS.

Among factors such as pathogenic bacterial infection[8] and antibiotic use,[9] diet is an additional factor with potential to alter gut bacterial composition.[10] Nutrients such as dietary fibres, which have not been directly absorbed by the host, become a

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food source for bacterial species of the gut, for example, *Bifidobacteria*.

Dietary intervention has since long been one of the basic treatment options in IBS. Based on the dietary recommendations from the National Institute for Health and Care Excellence (NICE) and the British Dietetic Association,[11 12] the 'traditional' IBS diet encourages 'healthy eating', with a regular meal plan, minimising portion size and reducing fats, caffeine and excessive fibre intake, together with avoidance of soft drinks and gas-producing foods such as cabbage, beans and onions. Furthermore, patients are advised to eat in a calm manner and to chew thoroughly.[11 13 14] Recently, fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) have been suggested as symptom-generating factors in IBS. FODMAPs have osmotic activity, causing increased luminal water retention,[15 16] and promote luminal distension by rapid gas production through bacterial fermentation.[17] In previous studies by our group and others, it has been demonstrated that symptoms may be alleviated in some patients with IBS after traditional and low FODMAP diet intervention.[14 18] However, a diet low in FODMAPs has marked effects on gut bacteria[19 20] and ramifications are still under deliberation. Further, it is still unknown why some patients with IBS respond favourably to dietary changes, while others show no or minimal response.

In this secondary analysis of patients from a study previously published by our group,[14] we hypothesised that gut bacteria profiles of patients with IBS are altered through dietary intervention and that patient responsiveness to intervention may be linked to gut bacteria composition. Using multivariate analysis, we aimed to determine if dietary interventions affect gut bacteria and if bacterial profiles of responders to intervention can be discriminated from non-responders for use to predict efficacy of dietary intervention therapy.

MATERIALS AND METHODS

Study cohort

The study cohort has been described in detail in a previously conducted study by Böhn *et al*.[14] Briefly, patients meeting the ROME III criteria for IBS[2] were recruited through outpatient clinics in Gothenburg and Stockholm, Sweden. Exclusion criteria included other GI diseases such as IBD or coeliac disease or presence of severe liver, neurological, cardiac or psychiatric disease. Furthermore, patients were not allowed to be on excessively nutrient restrictive diets prior to the study. Patients consuming probiotics and/or on a lactose-reduced diet were allowed to continue their practices as long as they were unaltered during the study.

Study design

At the beginning of the 10-day screening period, verbal and written information was provided to patients about the study and all participating patients gave written informed consent. During screening, participants completed a stool diary based on the Bristol Stool Form scale [2] used to subgroup patients according to bowel habits. Additionally, food intake was recorded in food diaries and assessed both during screening (baseline) and after the intervention as described in detail in Böhn *et al*.[14] After screening and at the end of the diet intervention, patients completed an IBS Symptom Severity Score (IBS-SSS) questionnaire.[21] Only patients with IBS-SSS ≥175 at baseline, that is, reporting moderate or severe IBS symptoms, were eligible for study inclusion. Patients were randomised 1:1 through an external contact research organisation computer-generated web-based program to follow either a traditional IBS diet or a low FODMAP diet for 4 weeks. Faecal samples were collected once during the screening period and once during the last week of the diet intervention, as detailed in the online supplementary material. Patients that reduced IBS-SSS by ≥50 were defined as responders since this indicates a clinically meaningful improvement.[21] The study was registered at ClinicalTrials.gov ID NCT02107625.

Intervention diets

The traditional IBS diet

Based on the British Dietetic Association and NICE dietary recommendations,[11 13] the traditional IBS diet focused on portion control and frequency. Patients on this diet were instructed to eat three meals and three snacks during the day and to do so in a relaxed manner, chewing thoroughly and to a comfortable degree of fullness. Fibres were advised to be eaten distributed evenly over the day with a reduction in spicy and fatty foods, alcohol, coffee, onions, cabbage and beans. Soft drinks, carbonated beverages and sweeteners that end with -ol (frequently found in chewing gums) were to be avoided. Further details of the traditional IBS diet can be found in Böhn *et al*.[14]

The low FODMAP diet

Patients following this diet had restricted intake of foods containing FODMAPs. Examples of food items with high FODMAP content are foods containing wheat, barley and rye, as well as onion and certain legumes, all rich in fructans and galacto-oligosaccharides. Additionally, lactose-containing products, foods with fructose in excess of glucose, for example, apples, pears, asparagus, watermelon and honey; food items rich in mannitol, maltitol, sorbitol and xylitol, for example, peaches, apricots and artificially sweetened products were also excluded. Further details of the low FODMAP diet can be found in Böhn *et al*.[14]

Food intake assessment

All patients completed a 4-day food diary once during the screening period and once during the last week of the 28-day intervention. Average daily intakes were calculated in DIETIST XP V.3.1 (Kostdata.se, Stockholm, Sweden) for energy, dietary fibres, lactose, monosaccharides and FODMAPs as described in detail in Böhn *et al*.[14]

Symptom assessment

Bowel habits were recorded in a stool diary each day during the 10-day screening period and the 28-day intervention period. IBS symptom severity was assessed on days 0, 14 and 29 of the intervention using the IBS-SSS questionnaire. Details are found in the online supplementary material. An extensive comparison of the diets regarding clinical response was performed in our previous publication.[14]

Gut bacterial analysis

Gut bacterial analysis was performed by using a commercially available test, GA-map Dysbiosis Test[22] (Genetic Analysis AS, Oslo, Norway), which is described in detail in the online supplementary material. Briefly, the GA-map Dysbiosis Test[22] output is a bacterial profile and a Dysbiosis Index (DI) score. A DI >2 (maximum 5) indicates a bacteria composition that differs from a healthy reference group and are as such considered to be dysbiotic.[22]

Data and statistical analysis

Univariate analysis

Statistical analysis was performed using both GraphPad Prism V.6.04 (GraphPad Software, California, USA) and SPSS statistical

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package, V.21.0 (SPSS, Chicago, Illinois, USA). The Mann-Whitney U test was applied when comparing two groups for significant differences in continuous data. Results in text, tables and figures are presented as median followed by range shown as 25th and 75th percentile. In this study, while significance was denoted as a p value of <0.05, if significance did not hold true after correction for multiple comparisons using the classical one-stage method in order to account for false-positive results, presented as q values, they were thus described as tendencies.

Multivariate analysis

Multivariate factor orthogonal partial least squares discriminant analysis (OPLS-DA) (SIMCA V.13.0.3.0, Umetrics AB) was implemented to examine if responders could be discriminated from non-responders (Y variable observations) based on totality of signal intensities from 54 DNA probes targeting ≥300 bacteria on different taxonomic levels (X variables) measured through GA-map Dysbiosis Test analysis (details described in the online supplementary material). Evaluators were not blinded to responder status when analysing the microbiota data. This analysis aims to identify which of the multiple bacterial targets are associated with and drives either of the multiple outcomes that is, responder or non-responder. By using multivariate analysis, the complexity of analysing >50 variables can be reduced down to a more interpretable model. The goodness of fit of the OPLS-DA is represented by the R^2 parameter with the best possible fit being $R^2=1$, indicating that the model explains the data perfectly. When considering heterogeneous biological variables, a model would be considered to have a good fit with an $R^2 \geq 0.5$.[23] Internal cross-validation of the model was performed whereby the data were remodelled seven times and each time a portion consisting of 1/7 of the samples was kept out until all samples had been kept out once. During this, the ability of the model to predict the class of each patient was tested. Once completed, the predictive robustness of the models ability to predict a future patient's class was defined and represented by the Q^2 value. A Q^2 value >0.4 is considered satisfactory with biological variables;[23] furthermore, the difference between the Q^2 and R^2 values should not exceed 0.2–0.3 since this indicates presence of many irrelevant model terms.[23 24] To further refine the models derived, extreme outliers which exceed the Hotelling's T2 95% ellipses of critical distance for classification and have potential to skew the model were identified and given priority for exclusion before remodelling. Then, moderate outliers exceeding the critical distance in DModX which are not powerful enough to shift the model but do not fit the model well were subsequently excluded. Finally, variable influence on projection (VIP) was used as a variable selection based on discriminatory power. While variables with a VIP >1 are most influential for the model and are most relevant for explaining the Y observations (patients with IBS), a VIP of 0.7–0.8 is commonly used.[24] In this study, a VIP cut-off of 0.7 was implemented.

RESULTS

Clinical characteristics of the study cohort

Of the 67 patients with IBS who completed the full diet intervention, two patients did not provide faecal samples, while faecal samples from four patients (two non-responders and one responder to the traditional IBS diet and one non-responder to the low FODMAP diet) failed to pass quality control during the GA-map Dysbiosis Test,[22] as described in the online supplementary material. Briefly, three samples had abnormally low levels of hybridisation (as described in detail in Casén *et al*[22]) and were justified as being atypical and thus considered outliers. The fourth failed due to technical circumstances. Hence, 61 patients following a traditional IBS diet (n=30) or a low FODMAP diet (n=31), with faecal samples obtained before and after intervention, were included in this study (figure 1). In total, 35 patients were responders to the dietary interventions when following either the traditional IBS diet (n=16, 53%) or the low FODMAP diet (n=19, 61%). Distribution of IBS subgroups according to bowel habit and IBS symptom severity within the cohort is demonstrated in table 1. Good adherence to the dietary advice was recorded among all patients as previously demonstrated.[14]

Faecal bacterial profiles discriminate between responders and non-responders before low FODMAP but not traditional IBS dietary intervention

OPLS-DA was performed on faecal bacterial profiles of responders and non-responders to diet therapy before and after the 28-day traditional IBS or low FODMAP dietary intervention.

Before intervention, bacterial profiles of responders and non-responders to a traditional IBS diet did not differ between the groups. The OPLS-DA fitted model for the traditional diet before the intervention comprised of one predictive component after exclusion of strong and moderate outliers and subsequent focus on variables with a VIP >0.7. The fit of the model was poor ($R^2=0.46$) with no predictive ability ($Q^2=-0.04$) and an unsatisfactory level of irrelevant model terms ($\Delta R^2Q^2=0.5$) (figure 2A). After the traditional IBS diet, the similarity in responder and non-responder bacterial profiles was unaltered as evident by the indices of the one predictive component OPLS-DA model ($R^2=0.58$, $Q^2=-0.41$ and $\Delta R^2Q^2=0.99$). Although model fit had improved, the predictability of the model and thus the difference between R^2 and Q^2 had worsened (figure 2B). Thus, bacterial profiles of responders and non-responders to a traditional IBS diet did not differ between the groups before or after the intervention.

Before the low FODMAP intervention, bacterial profiles of responders and non–responders were effectively discriminated against each other in an OPLS-DA model comprising one predictive component. The model had adequate robustness ($R^2=0.65$) and moderately high predictive ability ($Q^2=0.54$) to identify responders and non-responders to low FODMAP intervention ($\Delta R^2Q^2=0.11$) (figure 2C). After the low FODMAP intervention, this discrepancy between faecal bacterial profiles of responders and non-responders persisted as depicted in a one-component OPLS-DA model ($R^2=0.55$, $Q^2=0.26$ and $\Delta R^2Q^2=0.3$). The lower Q^2 indicates that the responder and non-responder profiles had become more similar. The model was however suitably robust for classifying responders and non-responders to a low FODMAP diet based on the faecal bacterial profiles after diet intervention (figure 2D).

However, an OPLS-DA model built of bacterial profiles obtained before intervention of all patients randomised to follow either a traditional IBS or low FODMAP diet revealed the profiles to be similar before their respective interventions as indicated by the model having a poor model fit ($R^2=0.35$) and no predictive ability ($Q^2=-0.025$). Univariate analysis of the abundance of each investigated bacteria, that is, the relative amount of bacterial DNA respective for each bacterial probe covered in the GA-map Dysbiosis Test, presented no difference before intervention between patients destined to follow a traditional IBS diet or a low FODMAP diet (data not shown).

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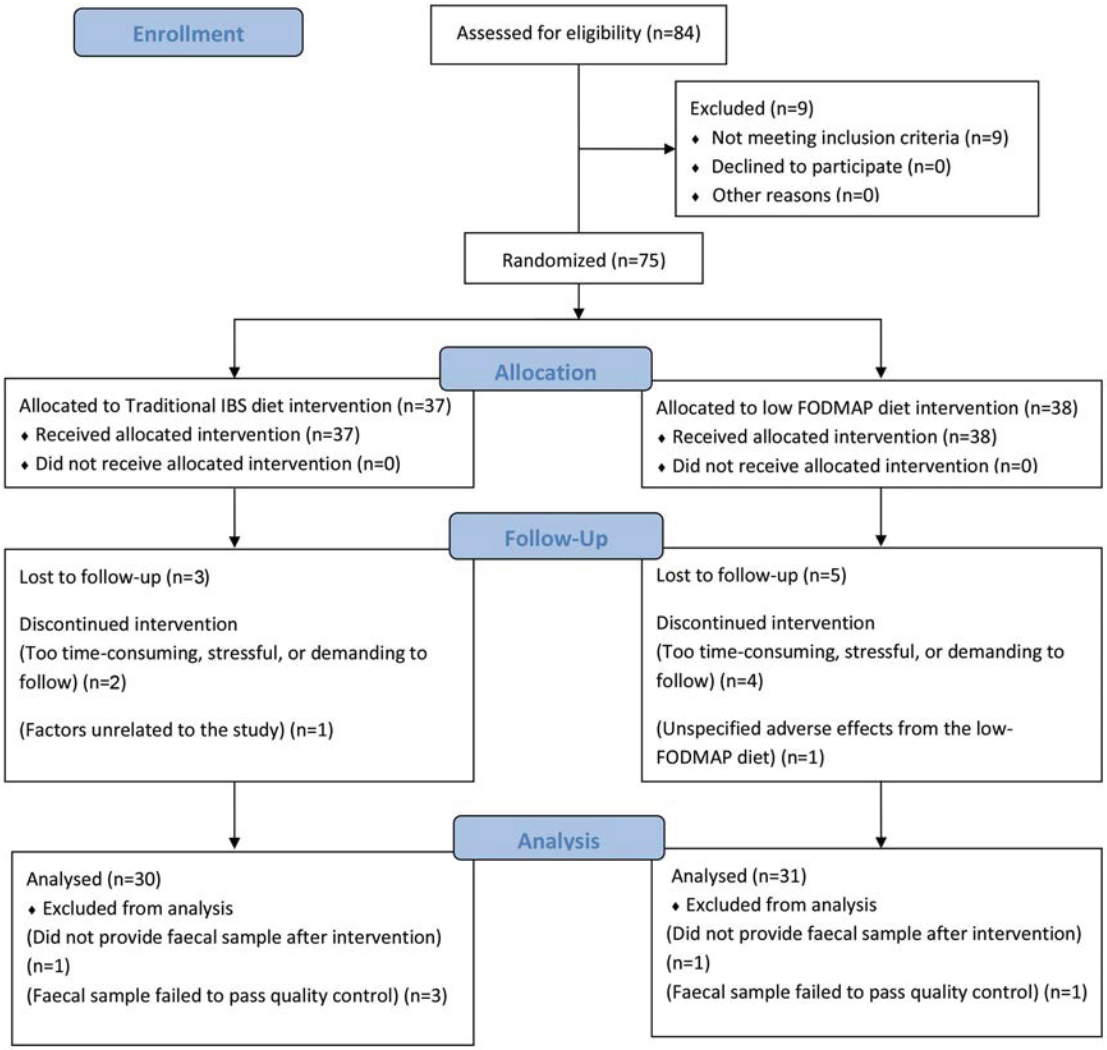

Figure 1 CONSORT flow diagram. Flow chart depicting patient numbers during the different phases of the study. FODMAP, fermentable oligosaccharides, disaccharides, monosaccharides and polyols.

Table 1 Demographics of cohorts during diet interventions

	Low FODMAP diet		Traditional IBS diet	
	Responders (n=19)	Non-responders (n=12)	Responders (n=16)	Non-responders (n=14)
Sex (F/M)	(18/1)	(8/4)	(15/1)	(10/4)
Age, years*	51 (37–63)	40 (27–59)	35 (24–49)	50 (30–63)
Body mass index, kg/m^2*	24 (21–25)	24 (21–30)	24 (21–26)	22 (21–28)
IBS subtype during screening period (number of patients)				
IBS-C	4	4	2	7
IBS-D	7	4	2	3
IBS-nonCnonD	8	4	12	4
IBS severity based on IBS-SSS during screening period (number of patients)				
Moderate	6	4	6	7
Severe	13	8	10	7

*Data shown as median (25–75th percentile).
FODMAP, fermentable oligosaccharides, disaccharides, monosaccharides and polyols; IBS-C, constipation-predominant IBS; IBS-D, diarrhoea-predominant IBS; IBS-nonCnonD, IBS with mixed loose and hard stools (IBS-M) or unsubtyped IBS (IBS-U); IBS-SSS, IBS Symptom Severity Score.

Limited bacterial discrepancies between responders and non-responders to a traditional IBS diet intervention

Bacterial profile composition of responders and non-responders to the traditional IBS diet intervention were compared through univariate analysis. Although abundance of many bacteria was comparable between responders and non-responders before traditional IBS diet intervention, the probe signal, indicative of bacterial abundance, of some bacteria was dissimilar. Before diet

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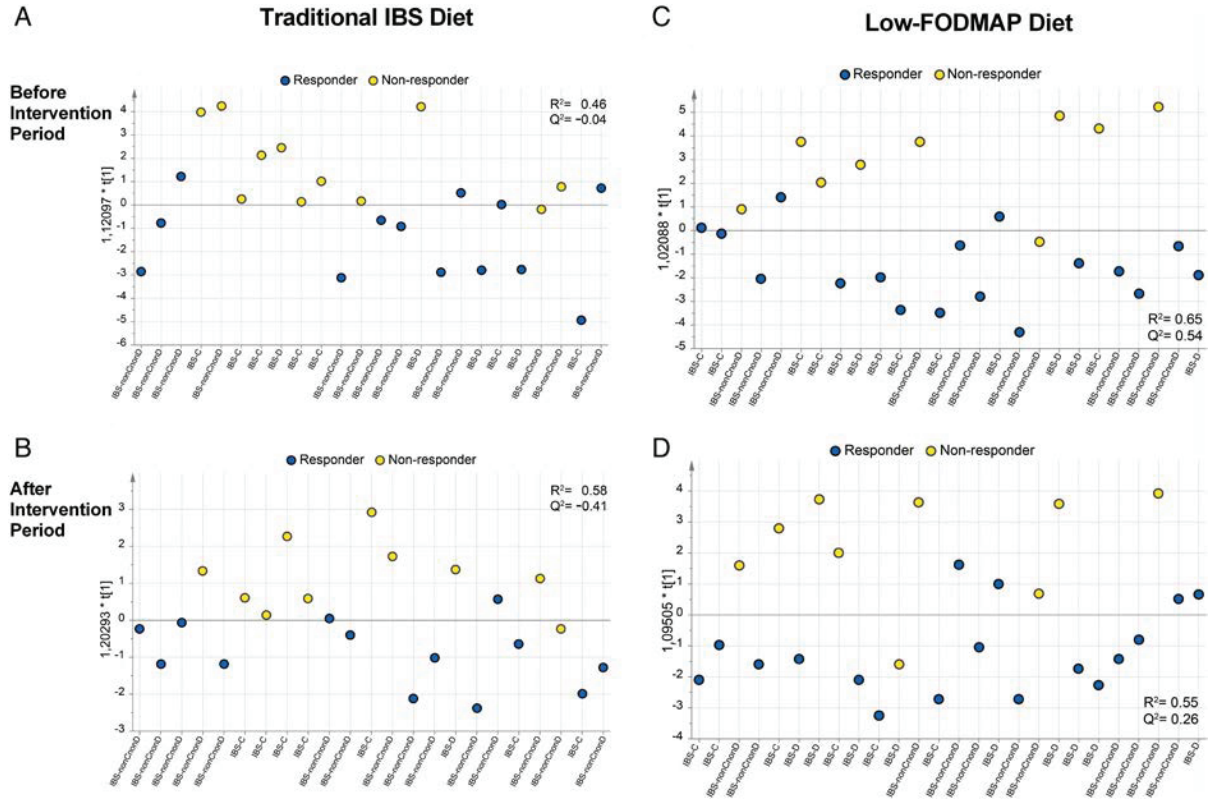

Figure 2 Bacterial profile analysis of non-responders and responders to dietary intervention. The GA-map Dysbiosis Test[22] analysing signals from 54 probes targeting ≥300 bacteria on different taxonomic levels was used to create bacterial profiles for patients undergoing either traditional IBS diet (n=30) or low fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAP) (n=31) intervention. Any class discriminations made are depicted along the Y axis with each individual patient plotted along the X axis. (A) Multivariate discriminate analysis (orthogonal partial least squares discriminant analysis (OPLS-DA)) scatter plot showing the discrimination between non-responders (yellow dots) (one strong and two moderate outliers excluded) and responders (blue dots) (three moderate outliers excluded) (n=24) before traditional IBS dietary advice based on all bacterial probes (n=54), R^2=0.46, Q^2=−0.04. (B) OPLS-DA showing discrimination between non-responders (four strong outliers excluded) and responders (three strong outliers excluded) after traditional IBS dietary advice (n=23), R^2=0.58, Q^2=−0.41. (C) OPLS-DA scatter plot showing discrimination between non-responders (two strong and one moderate outliers excluded) and responders (one strong and one moderate outliers excluded) before a low FODMAP diet (n=26), R^2=0.65, Q^2=0.54. (D) OPLS-DA scatter plot showing discrimination between non-responders (two strong outliers excluded) and responders (three moderate outliers excluded) after low FODMAP diet (n=26), R^2=0.55, Q^2=0.26. An R^2 value representing the goodness of fit shows the ability for a model to explain the data; while an R^2 of 1 is the best possible fit, an R^2>0.5 is acceptable for biological data.[23] Q^2 represents the predictive robustness of a model and is derived after leave one out validation. Like the R^2 value, the higher the Q^2 value the stronger its predictive ability with values >0.4 to be acceptable for biological data.[23] IBS-C, constipation-predominant IBS; IBS-D, diarrhoea-predominant IBS; IBS-nonCnonD, IBS with mixed loose and hard stools (IBS-M) or unsubtyped IBS (IBS-U); IBS-SSS, IBS Symptom Severity Score.

intervention *Phascolarctobacterium* tended to be more abundant in responders compared with non-responders while abundance of Firmicutes (*Bacilli* and *Clostridia*) tended to be higher in non-responders than responders (table 2). After intervention, only *Eubacterium* tended to be more abundant in non-responders compared with responders (76 (72–131) probe signal intensity (PSI) vs 69 (67–74) PSI; p=0.01, q=0.8). Analysis of bacterial changes after the traditional diet intervention in responders and non-responders, respectively, showed no significant alterations. Bacterial profile composition of all patients, irrespective of responsiveness, did not change after traditional IBS diet intervention (table 3). Data on bacterial abundance and dietary parameters for each patient both before and after the intervention were pooled and used to investigate how the dietary parameters, energy, protein, fat, carbohydrates, monosaccharides, glucose, dietary fibre, alcohol, fructose, galacto-oligosaccharides, fructans, polyols, lactose and overall FODMAP consumption impacted bacterial abundance. Correlations which held significance after correcting for multiple comparisons are presented (table 4).

Non-responders exhibit increased abundance of certain bacteria compared with responders both before and after low FODMAP dietary intervention

Of all bacteria investigated, seven tended to be more abundant after correcting for multiple comparisons in non-responders to low FODMAP intervention compared with responders, both before and after intervention (table 2). These included *Bacteroides stercoris*, *Pseudomonas*, *Acinetobacter* and the sulfur-reducing anaerobic genus *Desulfitispora*. Additionally, six bacteria tended to be more abundant in non-responders compared with responders but only prior to the intervention (table 2). These included *Streptococcus*, *Dorea* and *Ruminococcus gnavus*. Comparing bacterial composition of all patients in the low FODMAP diet group, irrespective of response, the species *Mycoplasma hominis* tended to be lower while the genus *Bifidobacterium* and the phyla Actinobacteria were both significantly lower after the intervention as compared to before, as denoted by lower probe signal intensity (table 3).

All dietary intake parameters were significantly reduced after the low FODMAP intervention and we thus investigated if this

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Table 2 Bacterial targets differing between responders and non-responders before traditional and low FODMAP diets

	Bacterial target of labelling probe before intervention	Responders (PSI)	Non-responders (PSI)	p Value†	q value‡
Traditional IBS diet	*Phascolarctobacterium**	25 (0–207)	0 (0–20)	0.03	0.8
(n=30)	Firmicutes (*Bacilli* and *Clostridia*)*	109 (87–173)	168 (132–221)	0.03	0.8
Low FODMAP diet	*Acinetobacter*	201.8 (199.7–203)	204 (202–206)	0.005	0.07
(n=31)	*Bacteroides stercoris*	27 (23.4–30.7)	33.4 (27.6–41)	0.005	0.07
	Parabacteroides	0 (0–0)	17 (0–30)	0.02	0.1
	Bacillus	15.7 (0–18.5)	20 (16.2–27.5)	0.04	0.2
	Pseudomonas	20.3 (16.9–23)	28 (22.9–31)	0.002	0.07
	Desulfitispora	0 (0–18)	18.8 (16–24.4)	0.009	0.07
	Salmonella, Citrobacter, Cronobacter, Enterobacter	39 (34–41)	41.8 (40–50)	0.02	0.1
	*Dorea**	28 (25–30)	40 (30–56)	0.004	0.07
	*Ruminococcus gnavus**	17 (15–26)	33 (18–118)	0.01	0.1
	*Clostridium**	75 (72–78)	82 (76–86)	0.009	0.07
	*Coprobacillus**	33 (32–35)	36 (33–39)	0.04	0.2
	Firmicutes (Clostridia)*	318 (266–347)	379 (330–441)	0.04	0.2
	*Streptococcus**	0 (0–0)	19 (4–25)	0.008	0.07

*No difference in abundance between responders and non-responders after intervention.
†Mann-Whitney U test. Data shown as median (25–75%).
‡Correction for multiple comparisons, using classical one-stage method.
FODMAP, fermentable oligosaccharides, disaccharides, monosaccharides and polyols; PSI, probe signal intensity indicating bacterial abundance in sample.

Table 3 Bacterial targets significantly altered during dietary intervention irrespective of patient responsiveness

	Bacterial target of labelling probe	Before (PSI)	After (PSI)	p Value*	q value†
Traditional IBS diet (n=30)	None	n/a	n/a	n/a	n/a
Low FODMAP diet (n=31)	*Mycoplasma hominis*	66 (26–110.4)	40 (22.7–96.8)	0.02	0.3
	Bifidobacterium	152 (45.7–270)	32.8 (25.4–122.4)	0.0005	0.02
	Actinobacteria	120 (57.5–197.4)	59.6 (47.9–102.4)	0.001	0.02

*Mann-Whitney U test. Data shown as median (25–75%).
†Correction for multiple comparisons, using classical one-stage method.
FODMAP, fermentable oligosaccharides, disaccharides, monosaccharides and polyols; PSI, probe signal intensity indicating bacterial abundance in sample. n/a, not applicable.

Table 4 Correlations between bacteria and dietary parameters over the course of the intervention calculated using pooled data from before and after dietary advice for each subject

	Bacterial target of labelling probe	Dietary parameter (g)	ρ Value*	p Value†	q Value‡
Traditional IBS diet (n=30)	*Staphylococcus*	Protein	0.421	0.0009	0.001
	Dialister		0.4	0.002	0.003
	Bacteroides	Alcohol	−0.463	0.0002	0.001
	Firmicutes (*Bacilli* and *Clostridia*)	Polyols	0.441	0.0005	0.001
Low FODMAP diet (n=31)	*Eubacterium*	Carbohydrates	−0.413	0.0009	0.05
	Eubacterium	Monosaccharides	−0.560	0.001	0.0001
	Dorea		−0.400	0.002	0.03
	Eubacterium	Glucose	−0.488	0.0009	0.003
	Dorea		−0.444	0.002	0.008
	Streptococcus		−0.371	0.003	0.05
	Bacteroides		−0.365	0.004	0.05
	Eubacterium	Fructose	−0.501	0.0009	0.002
	Bifidobacterium	Lactose	0.410	0.0009	0.05
	Actinobacteria		0.390	0.002	0.05

*Spearman's rank correlation coefficient.
†Mann-Whitney U test depicting statistical significance of the correlation.
‡Correction for multiple comparisons, using classical one-stage method.
FODMAP, fermentable oligosaccharides, disaccharides, monosaccharides and polyols.

correlated with bacterial abundance. Although many correlations were found, after correction for multiple comparisons, only *Eubacterium*, *Dorea*, *Streptococcus* and *Bacteroides* were found to be negatively correlated with dietary parameters such as monosaccharides and glucose, while *Bifidobacterium* and Actinobacteria were positively correlated with lactose (table 4).

Response to traditional IBS dietary advice, but not low FODMAP, improves DI

The GA-map Dysbiosis Test provides a DI analysis of study samples denoted by a DI score of 1–5. Patients following traditional IBS dietary advice were associated with reduced DI scores, whereas a low FODMAP diet was associated with

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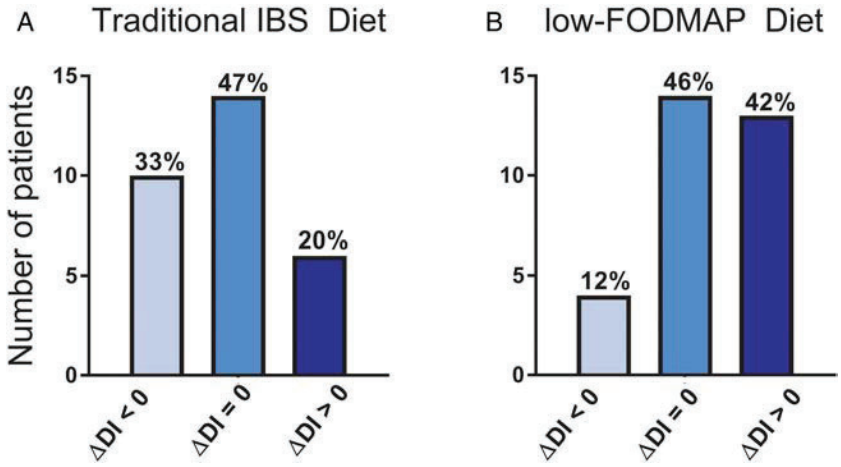

Figure 3 Change in Dysbiosis Index scores from before to after the traditional IBS diet or low fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAP) intervention period. The composition of faecal bacteria was determined by the GA-map Dysbiosis Test[22] in patients following traditional IBS dietary advice (n=30) low FODMAP (n=31) for 4 weeks. The difference in Dysbiosis Index scores was compared between patients following the two intervention diets. An improvement in dysbiosis and thus reduction in dysbiotic score was denoted as a negative change while a worsening and thus increase in dysbiotic score was indicated as a positive change. No change in Dysbiotic Index score was denoted by a 0.

Table 5 Overview of responder and non-responder change in dysbiosis during intervention

		Frequency of patients who had a change in Dysbiosis Index (%)		
Distribution of patients		**Improved**	**No change**	**Worsened**
Traditional IBS (n=30)	Responders	31	56	13
	Non-responders	28	36	36
Low FODMAP (n=31)	Responders	16	42	42
	Non-responders	8	50	42

FODMAP, fermentable oligosaccharides, disaccharides, monosaccharides and polyols.

increased DI scores irrespective of their intervention response (figure 3). However, when taking intervention responsiveness into consideration for both diets, approximately 50% of responders and non-responders were not associated with any change of their DI score after the intervention (table 5). However, while the frequencies of patients who were associated with higher DI scores after following a low FODMAP diet were equal among responders and non-responders, the frequency of non-responders to the traditional IBS diet who were associated with higher DI score was greater than responders (table 5). Moreover, responders and non-responders to the traditional IBS diet had similar DI scores both before (figure 4A) and after (figure 4B) the intervention. A similar comparison performed before (figure 4C) and after (figure 4D) the low FODMAP diet revealed that non-responders had consistently higher DI scores than responders. Additionally, comparing the responders before and after traditional IBS dietary advice showed no difference in DI scores (3 (3–3) vs 3 (3–3); p=0.26). This similarity in DI scores was also true for non-responders to traditional IBS diet (3 (2–4) vs 3 (2–4); p=0.56), as well as for responders (3 (2–4) vs 3 (2–4); p=0.56) and non-responders (3 (3–4) vs 3.5 (3–4); p=0.33) to the low FODMAP intervention.

DISCUSSION

This is the first study to demonstrate that gut bacteria profiles of adult patients with IBS responding to a low FODMAP dietary intervention differ from non-responders before starting the intervention. Additionally, the low FODMAP diet was associated with altered faecal bacteria and increased DI scores. Furthermore, patients who did not respond to the low FODMAP diet were characterised by higher DI scores before the intervention than those who responded. None of these findings was seen in the group following a traditional IBS diet.

In-depth analysis of gut bacteria generates a large amount of data and has a multitude of interlinked variables to consider. Furthermore, patients with IBS are notoriously heterogeneous as a cohort. Since this study focuses on both, multivariate statistical OPLS-DA was chosen and implemented as the best means to handle this large and complicated data set. We demonstrated that before a traditional IBS dietary intervention, faecal bacterial profiles of responders and non-responders were similar and thus had poor predictability/validation Q^2 indices in an OPLS-DA model. This was not the case for responders and non-responders to a low FODMAP dietary intervention, whereby an OPLS-DA model's R^2 and Q^2 indices were high enough to validate the class separation. Thus, our data suggest that multivariate analysis of gut bacterial profiles might be used to predict responsiveness to dietary intervention and that severity of dysbiosis as defined by DI scores is associated with responsiveness to low FODMAP intervention in IBS. If this can be confirmed in future studies, only patients with a higher chance of treatment response should be selected for this rather restrictive exclusion diet after analysis of faecal bacterial composition.

To elucidate bacteria potentially driving differentiation between responders and non-responders, a deeper analysis was performed on bacterial profiles before and after the interventions. We demonstrated that before a low FODMAP intervention non-responders tended to exhibit a greater number of more abundant bacteria than responders including *Streptococcus* and *Dorea*, previously shown to be elevated in IBS[25] and *R. gnavus*, a species suggested to be a potential biomarker for IBS.[25] This is in contrast to a previous study in children with IBS demonstrating a greater number of abundant bacterial taxa in responders compared with non-responders before a low FODMAP intervention.[26] The method used by Chumpitazi *et al*, identifying all sequences of the V3–V5 region, compared with the GA-map Dysbiosis Test which determines preidentified sequences of the V3–V7 regions of the 16S gene, might explain the differences between studies. In our study, a large proportion of bacteria tended to be more abundant both before and after the intervention in non-responders, suggesting potentially pathogenic or non-beneficial species from these genera might be hindering the

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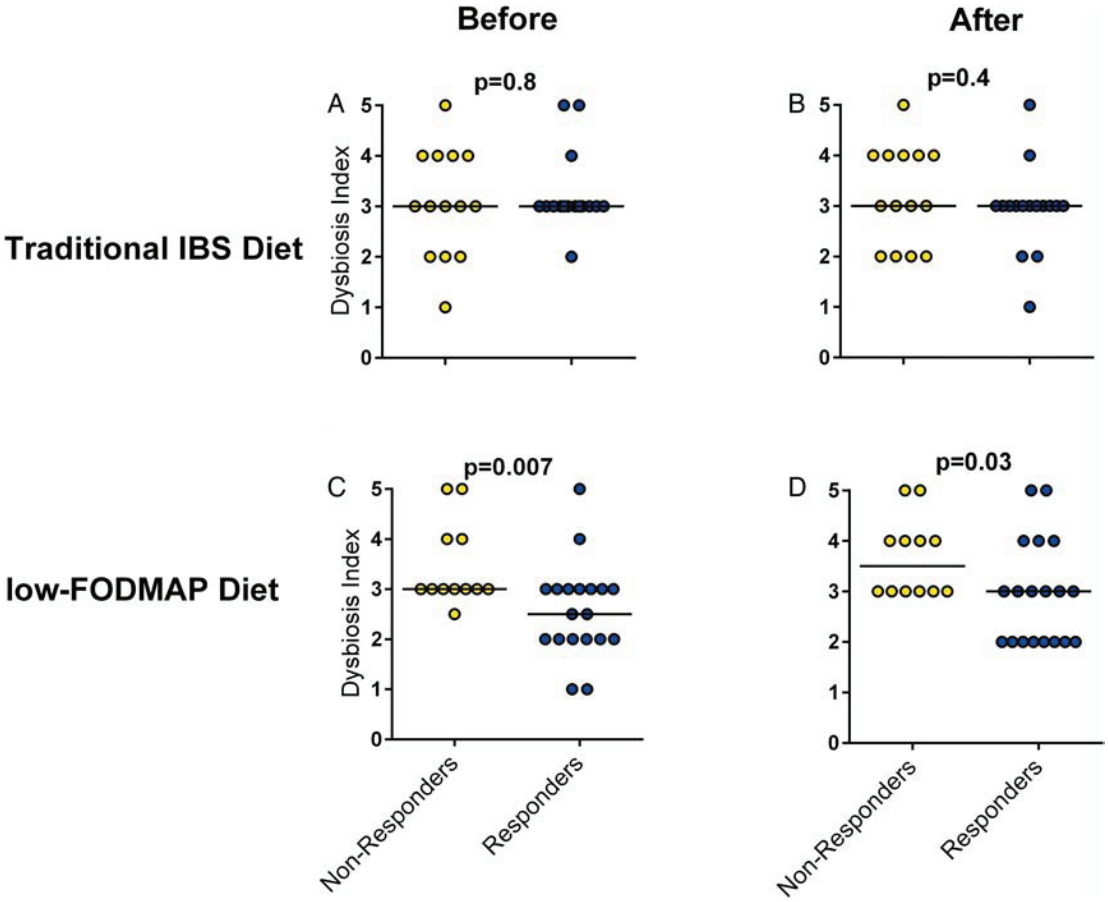

Figure 4 Comparison of Dysbiosis Index between non-responders and responders both before and after the respective intervention diets. The composition of faecal bacteria was determined by the GA-map Dysbiosis Test[22] in patients following traditional IBS dietary advice (n=30) or low fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAP) diet (n=31) for 4 weeks. Dysbiosis Index is scored between 1 and 5, where a score of 1 and 2 signifies normobiosis, 2.5 is on the boundary to being dysbiotic and 3–5 is dysbiotic of increasing severity. The number of patients within each Dysbiosis Index score group of non-responders and responders (A) before and (B) after traditional IBS dietary advice and (C) before and (D) and after low FODMAP diet. Y axis depicts absolute dysbiosis index values.

responsiveness to a low FODMAP diet. The ability to classify responders from non-responders after a low FODMAP diet through multivariate analysis demonstrates that even after following the same dietary advice for 4 weeks the bacterial profiles are dissimilar. This leads to the hypothesis that there may be something more fundamentally different between the two groups which requires further investigation. Before traditional IBS dietary intervention, although *Phascolarctobacterium* and Firmicutes (*Bacilli and Clostridia*) tended to be more abundant in responders and non-responders, respectively, the lack of any other bacterial discrepancies corroborated with the multivariate bacterial model which depicted poor profile discrimination. We therefore conclude that there is most likely no major difference in faecal bacterial profiles between responders and non-responders to traditional dietary intervention.

Short-term interventions, like a traditional IBS diet, have previously been reported to only moderately impact gut bacteria[27 28] contrary to a more restrictive intervention like a low FODMAP diet.[29–31] This was also demonstrated in our study whereby microbial profiles of patients following traditional IBS dietary advice were not associated with any change after the intervention irrespective of responsiveness. In contrast, patients following the low FODMAP diet were associated with a lower abundance of *Bifidobacteria* and its taxonomic phyla Actinobacteria, which use FODMAPs in their metabolism.[20] Interestingly, an extensive study by McIntosh *et al*[32] comparing the impact of high and low FODMAP diets on gut bacteria found decreased *Bifidobacteria* yet increased Actinobacteria richness after a low FODMAP diet compared with a high FODMAP diet. Although not all strains of *Bifidobacteria* have documented beneficial effects, *Bifidobacterium* strains, for example, *Bifidobacterium infantis* 35624 and *Bifidobacterium animalis* DN-173 010, have been demonstrated to alleviate IBS symptoms in some patients with IBS upon supplementation.[33–35] Thus, a reduction in abundance of this probiotic genera may be why symptom improvement was not seen in all patients following a low FODMAP diet. Furthermore, reduction of a whole phylum of bacteria will likely impact the bacterial community[36] and may reduce other populations of bacteria, as indicated by the higher DI scores observed in some patients following the low FODMAP diet.

Dysbiosis has been suggested to be present in at least subsets of patients with IBS as indicated by altered bacterial abundance compared with healthy subjects.[7] We used a DI to evaluate if intervention diets were associated with any shift of the bacterial profiles of patients with IBS. While the DI itself is not a tool for identifying changes in specific bacteria, it provides a numeric score of how the composition of a bacterial profile is in relation to that of healthy subjects. In patients following the traditional IBS dietary advice, DI scores were associated with a marginal

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decrease after the intervention, but the majority of those who had increased DI scores were non-responders. As previously mentioned, while the composition of a traditional IBS diet is not so different from a normal diet, the inherent regularity of the small meals may account for decreased DI scores found in the patients.

Patients following the low FODMAP diet, experiencing a more drastic dietary change, were associated with a general increase in DI scores. The measurable depletion in the abundance of certain bacteria[20] and increase in DI scores after the low FODMAP intervention, irrespective of responsiveness, is corroborative data not only from a clinical perspective but also from a microbiologist and dietician perspective. However, the change in DI scores only occurred in 50% of the subjects following the low FODMAP diet. The explanation for this was neither bad compliance nor a habitual diet low in FODMAPs since significant decrease in FODMAP consumption during the intervention was documented.[14] Also, a prior diet excessively restricting specific nutrients (eg, low in FODMAPs, gluten-free, vegan diet) was an exclusion factor in the study. Nevertheless, we cannot rule out that the influence of other factors, unrelated to ingestion of carbohydrates, not controlled for in this study, may have affected the study outcome.

The finding that non-responding patients tended to have higher DI scores than responding patients among both intervention groups is indeed interesting and suggests that some non-responding patients might be 'too dysbiotic' for dietary intervention and may benefit more from other therapies. Although speculative, a gut bacterial composition shifted too far from the healthy norm whereby even a change in diet fails to bring the composition back to that of a healthy one is plausible but requires further investigation.

Although many correlations were identified between dietary parameters and bacteria, many did not hold true after performing statistical correction for multiple comparisons. Nevertheless, *Eubacterium* was shown to have a negative correlation with carbohydrates, monosaccharides, fructose and glucose, respectively. This is contrary to prior findings reporting a reduction in *Eubacterium* in non-IBS obese patients following diets low in carbohydrates indicating a positive correlation.[37 38] Importantly, correlations between bacterial groups and different sugar molecules may be a secondary finding due to alteration in abundance in other bacteria and/or their products of metabolism which are not included in the GA-map Dysbiosis Test or found primarily in the small intestine. Interestingly, *Bifidobacterium* correlating with lactose is in line with a previous study using culture supernatants whereby *Bifidobacterium* was observed to prefer lactose over glucose as the primary carbon source.[39] Moving forward, it would thus be interesting to investigate if a less restrictive low FODMAP diet can reduce IBS symptoms while retaining consumption of lactose in order to preserve the beneficial *Bifidobacterium* populations of the gut.

This study of course has limitations. The cohort size was relatively small when taking into account the subgrouping of patients into the respective dietary intervention groups and then subsequently as responders and non-responders. Further, while patients were advised to follow the instructions of the respective diets and we were able to track their eating habits with food diaries, these were kept only during the first and last four days of the screening and intervention period and since we did not provide all the food during the course of the intervention we were unable to check for compliance during the remaining days of the intervention period. However, this is a common feature in most dietary intervention studies and would likely have occurred in both responders and non-responders. Furthermore, although the GA-map Dysbiosis Test has limitations since it is bound to determine abundance of preidentified sequences as previously discussed, it was specifically developed to analyse abundance of gut bacteria and has a straightforward process which translates to a clinical setting creating standardised patient bacterial profiles. Ideally, other means to identify gut dysbiosis such as through whole 16S bacterial sequencing which also delivers α and β diversity values are needed to confirm our findings yet these are less optimal for envisioned clinical interpretation. The diets of the healthy Nordic (Norwegian and Swedish) control reference group used in the creation of the GA-map Dysbiosis Test are unknown,[22] but we can assume that they were eating standard Scandinavian diets similar to that of the Swedish subjects included in our study before the interventions. Therefore, there is little concern that differing diets between the two cohorts has interfered with the DI scores recorded. Finally, factors other than the effects of a lowered intake of FODMAPs per se, such as non-specific effects from being included in a study and other physiological effects of dietary changes not associated with microbiota composition, likely helped in the improvement of symptoms in responders. Additionally, while baseline variables such as age and severity of IBS were not adjusted for, they were similar between responders and non-responders and any impact of these is described in detail in our previous study.[14] Finally, since some findings of this study are not from the randomised phase, for example, correlation of diet with bacterial abundance, potential confounding bias inherent to studies of this nature must be acknowledged. Even after considering these limitations and even the possibility of chance, we are confident that the study design has a high likelihood of fulfilling the aim of this study, which was to record the impact of dietary change on gut bacteria and determine if bacterial profiles predict intervention response. Identification and a detailed description of the mechanism behind symptom improvement after dietary changes in IBS are still lacking.

In summary, our study may demonstrate that before a low FODMAP, but not a traditional IBS dietary intervention, non-responders have faecal bacterial profiles distinct from patients responding favourably. Moreover, altered bacteria contributing to profile discrimination tended to be more abundant in low FODMAP non-responders compared with responders. Additionally, low FODMAP, but not traditional, dietary advice was associated with a reduced abundance of some bacteria and increased DI scores in patients after intervention irrespective of responsiveness. Finally, although future studies are required to test the robustness of our findings, our study suggests the potential ability to identify responders to a low FODMAP diet through faecal bacterial profile multivariate analyses.

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approved the final draft submitted. SS, TL and LC: data acquisition. Have approved the final draft submitted. PL and HT: data acquisition and manuscript finalisation. Has approved the final draft submitted. LÖ: project planning, interpretation of data, drafting and finalising of manuscript. Has approved the final draft submitted. MS: project planning, interpretation of data, material acquisition, drafting and finalising of manuscript. Has approved the final draft submitted.

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ORIGINAL ARTICLE

Exploring Gut Microbiota Composition as an Indicator of Clinical Response to Dietary FODMAP Restriction in Patients with Irritable Bowel Syndrome

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Abstract

Background A diet low in fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAPs) may relieve symptoms of irritable bowel syndrome (IBS). However, nutritional counseling is resource-demanding and not all patients will benefit.

Aims To explore whether gut microbial composition may identify symptom response to a low-FODMAP diet in patients with IBS.

Methods Patients were recruited consecutively to participate in a 4-week FODMAP-restricted diet. Response to diet was defined as ≥ 50% decrease in IBS symptom severity scores (IBS-SSS) compared to baseline. Fecal microbiota were analyzed by a commercially available method (the GA-map™ Dysbiosis Test), assessing 54 bacterial markers targeting more than 300 bacteria at different taxonomic levels.

Results Sixty-one patients (54 F; 7 M) were included: 32 (29 F; 3 M) classified as responders and 29 (25 F; 4 M) as non-responders. Ten of the 54 bacterial markers differed significantly between responders and non-responders. Based on median values (used as cutoff) of responders for these 10 bacterial markers, we constructed a Response Index (RI): Each patient was given a point when the value for each selected bacterial marker differed from the cutoff. These points were summed up, giving an RI from 0 to 10. Patients with RI > 3 were 5 times more likely to respond (OR = 5.05, 95% CI [1.58; 16.10]), and the probability to respond was 83.4%, 95% CI [61.2–94%].

Conclusions Gut microbial composition, assessed by using a new RI, may constitute a tool to identify patients that are likely to respond to dietary FODMAP restriction.

Keywords Clinical nutrition · Functional gastrointestinal disorders · Gut microbiome · Irritable bowel severity scoring system

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Introduction

Irritable bowel syndrome (IBS) is a common condition, affecting at least 10% of the general population [1]. The disorder is characterized by abdominal pain, bloating, and disturbed bowel habits, despite a lack of structural or biochemical abnormalities as detected by routine investigations. The etiology is obscure, but the pathogenesis seems to involve a disturbance in one or more of the control systems that regulate bowel function: the central nervous system, the enteric nervous system, the enteroendocrine system, the gut immune system, and the gut microbiota [2]. During recent years, the interplay between diet, host, and microbes has emerged as an important pathophysiological basis for symptom generation [3]. In particular, restricting dietary intake of fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAPs) may relieve the symptoms [4]. Such a dietary intervention is associated with profound alterations of gut microbiota composition [5] and function [6]. Although the long-term health consequences of adherence to a low-FODMAP diet remain to be established, the change in the colonic microenvironment is conceivably detrimental [7, 8]. In addition, not all patients will respond favorably, and dietary counseling is cumbersome, costly, and time-consuming. Proper selection of patients that will benefit from a low-FODMAP diet would therefore be valuable. We examined a previously investigated patient population [6], aiming to explore whether assessment of gut microbiota composition may be used to differentiate between responders and non-responders of dietary FODMAP restriction.

Methods

Patients

Patients with IBS were recruited consecutively from a secondary care outpatient clinic (Lovisenberg Diaconal Hospital, Oslo, Norway) between April 2013 and October 2014, as previously described [6]. In brief, all patients fulfilled the Rome III criteria for IBS [9], and were thoroughly examined by the same experienced gastroenterologist (AR) to exclude organic diseases. Of note, all patients underwent a ^{13}C-D-xylose breath test to exclude small intestinal malabsorption, and only patients with high levels of $^{13}CO_2$ excretion (i.e., compared to a healthy control group, as described by Tveito et al. [10]) following ^{13}C-D-xylose ingestion were included. All patients gave written informed consent, and the study was conducted according to the revised Declaration of Helsinki, and approved by the Regional Committee for Medical Research Ethics (REK Sør-Øst, reference number 2013/454).

Dietary FODMAP Restriction

All patients were referred to nutritional counseling by an experienced clinical dietician (TK), educated within the low-FODMAP concept. Baseline diets were carefully evaluated to ensure that none of the patients had a particularly restricted diet compared to an average Norwegian diet before entering the study. Of note, the low-FODMAP diet was not well known in Norway at the time of inclusion. The patients were then instructed to strictly eliminate all foods containing excessive amounts of FODMAPs, according to the principles given by the Monash University (Melbourne, Australia) [11]. Thus, the patients were instructed to avoid foods containing galacto-oligosaccharides (e.g., beans, lentils, and peas), fructans (e.g., wheat, cabbage, and onion), lactose (e.g., milk, yoghurt, and dairy products), and polyols (e.g., mushrooms, cauliflower, and apricots—including foods sweetened with polyols), as well as foods containing fructose in excess of glucose (e.g., apples, pears, and dried fruits). Food items with low content of FODMAPs, such as oranges, bananas, rice, oats, meat, fish, eggs, and lactose-free dairy products, were suggested as alternatives to food items with high content of FODMAPs. The duration of the dietary intervention was 4 weeks. Throughout the study, adherence to the diet was ensured by close follow-up by the clinical dietician, including personal consultations and telephone and e-mail correspondence, and dietary compliance was assessed by evaluation of food diaries that the patients were requested to fill in.

Evaluation of Symptoms and Definition of Response to Diet

Severity of abdominal symptoms was assessed before and after the dietary intervention by using the irritable bowel severity scoring system (IBS-SSS), according to Francis et al. [12]. The maximum achievable score of this inventory is 500 points, allowing grading of symptom severity as follows: mild (75–175 points), moderate (175–300 points), and severe (> 300 points). As recommended by the Rome Design of Treatment Trials Committee [13], responders of the dietary intervention were defined as patients reporting ≥ 50% decrease on IBS-SSS. Accordingly, non-responders were defined as patients reporting < 50% decrease on IBS-SSS.

In addition to abdominal symptom assessment, severity of extra-intestinal symptoms was evaluated at baseline, using the Hospital Anxiety and Depression Scale (HADS) for evaluation of anxiety and depression [14], and the Fatigue Impact Scale (FIS) for evaluation of chronic fatigue [15].

Analysis of Gut Microbiota Composition

Patients collected fecal samples before and after the dietary intervention by using designated containers (Genetic Analysis, Oslo, Norway). They were carefully instructed to freeze the samples immediately at − 20 °C at home and bring the frozen containers to the hospital as soon as possible. The specimens were thereafter stored at − 80 °C and not thawed until analysis. To assess gut microbial composition, we used the GA-map™ Dysbiosis Test (Genetic Analysis, Oslo, Norway), which is a targeted approach to evaluate gut bacterial profiles [16]. In total, 54 bacterial markers, based on the 16S rRNA sequence in seven variable regions (V3–V9), measure relative abundance of bacteria according to the strength of fluorescent signals detection. Twenty-six bacterial markers are species-specific, 19 detect bacteria at genus level, and 9 bacterial markers detect bacteria at higher taxonomic levels. In total, more than 300 species are covered by this technology. The GA-map™ technology utilizes fecal homogenization, mechanical and enzymatic bacterial cell disruption, and automated total bacterial genomic DNA extraction using magnetic beads. Bacterial DNA labeling is by single-nucleotide extension and hybridization to complementary DNA strands coupled to beads, and signal detection by using BioCode 1000A 128-Plex Analyzer (Applied BioCode, Santa Fe Springs, CA, USA). The company (Genetic Analysis) provided the results both as relative abundances of bacteria according to the 54 targeted bacterial markers, measured as "fluorescence signal strength," and as a Dysbiosis Index (DI; range 0–5), where DI > 2 is denoted as "dysbiotic" [16].

Statistical Methods

Due to a limited sample size, all continuous variables were compared between responders and non-responders by using nonparametric tests: Mann–Whitney–Wilcoxon test when comparing unrelated variables and Wilcoxon signed-rank test when measurements before and after treatment were compared. Possible associations between pairs of categorical variables were assessed using Chi-square test or McNemar test when comparing proportions before and after treatment. Correlation was computed using the Spearman's rho. To explore discrimination ability of all the measured bacterial markers to distinguish between responders and non-responders, we performed principal component analysis (PCA) using a covariance matrix. The probability of being a responder was calculated using logistic regression, and the results were expressed as odds ratios (OR) with 95% confidence intervals (CI). In addition, probabilities given selected covariates were calculated. Positive predictive value (PPV) was calculated as described by Altman [17]. Due to a limited sample size, we were not able to divide our data into a training set and a test set, so model evaluation was performed using fivefold cross-validation (CV) [18]. The accuracy was computed as a mean score from CV with a 95% CI. Since our analyses were considered exploratory, no correction for multiple testing was performed and p values < 0.05 were considered statistically significant. All analyses were performed using SPSS version 22 and R (programming language), version 3.3.2.

Results

Subject Characteristics: Responders and Non-responders

The recruitment process has been described in detail previously [6]. In brief, 63 patients were initially enrolled; however, fecal samples for gut microbiota composition analysis from 2 patients were missing, reducing the total number of participants to 61. Based on the responder definition (≥ 50% decrease on IBS-SSS [13]), 32 patients were classified as responders and 29 patients as non-responders of the dietary intervention. Responders and non-responders did not differ significantly regarding clinical baseline characteristics (Table 1). The gender distribution was similar in both groups, with a large majority being females. Body mass index (BMI) and distribution of IBS subtypes were also similar; however, responders tended to be younger than non-responders.

There were no differences between responders and non-responders regarding any of the IBS-SSS measurements. The distribution of IBS-SSS categories was also very similar in both groups, and none of the patients were categorized as having mild IBS-SSS before treatment. However, after treatment 13 of the 32 responders reported mild IBS-SSS, while only one of the non-responders was in this category after treatment. Following treatment, both groups reported statistically significantly lower scores of IBS-SSS compared to baseline (both $p < 0.01$).

Gut Microbiota Profiling

We compared bacterial profiles between responders and non-responders based upon data using 54 bacterial markers. The overall ability of all measured bacterial markers to distinguish between responders and non-responders was assessed using the PCA method. The two-factor solution is depicted in Fig. 1. The responders had significantly higher levels for the following bacterial markers (Table 2): *Bacteroides fragilis*, *Acinetobacter*, *Ruminiclostridium*, *Streptococcus*, and *Eubacterium* (all $p < 0.05$). Furthermore, the responders had significantly lower levels for the following bacterial markers (Table 2): Clostridia/Negativicutes/Bacilli, Actinomycetales, *Anaerotruncus*, Clostridiales, and *Shigella/Escherichia* (all $p < 0.05$). For the remaining 44 bacterial markers, our data

Table 1 Comparison of baseline characteristics of IBS patients classified as responders ($n = 32$) and non-responders ($n = 29$) to a 4-week FODMAP-restricted diet

Variable	Responders ($n = 32$)	Non-responders ($n = 29$)	P value
Females, *n* (%)	29 (91)	25 (86)	0.70
Age, years, median (range)	32.5 (19–67)	39 (25–66)	0.05
BMI, kg/m^2, median (range)	25.3 (17.7–35.8)	23.4 (18.2–30.2)	0.18
IBS subtype, *n* (%)			0.93
IBS-D	16 (50)	16 (55)	
IBS-C	5 (16)	5 (17)	
IBS-M	11 (34)	8 (28)	
IBS-SSS, median (range)			
Total IBS-SSS score	294 (174–449)	281 (105–459)	0.15
Pain score	107 (0–173)	87 (0–196)	0.07
Bloating score	64 (25–100)	52 (0–97)	0.19
Bowel habit score	72.5 (34–100)	69 (34–100)	0.49
Life interference score	69 (44–99)	69 (35–99)	0.42
IBS severity, *n* (%)			0.87
Mild	0 (0)	0 (0)	
Moderate	17 (53)	16 (55)	
Severe	15 (47)	13 (45)	
HADS, median (range)			
Total HADS score	14.5 (5–31)	13 (0–31)	0.55
Anxiety score	10 (2–18)	8 (0–18)	0.68
Depression score	4.5 (0–13)	3 (0–13)	0.23
FIS, median (range)	69.5 (12–155)	75 (1–147)	0.57

BMI body mass index, *IBS* irritable bowel syndrome, *IBS-D* diarrhea-predominant IBS, *IBS-C* constipation-predominant IBS, *IBS-M* IBS with mixed bowel habits, *IBS-SSS* irritable bowel severity scoring system, *HADS* Hospital Anxiety and Depression Scale, *FIS* Fatigue Impact Scale

did not reveal any differences between responders and non-responders. The differences between responders and non-responders remained statistically significant also after treatment for 8 of the above-described 10 bacterial markers. The two bacterial markers that were not statistically significant after diet were targeted at *Bacteroides fragilis* and *Acinetobacter*, as levels of these declined following treatment in the responder group (from median 27.4 to 24.2, $p = 0.16$ and from median 188.9 to 183.6, $p = 0.19$, for *Bacteroides fragilis* and *Acinetobacter*, respectively).

Dysbiosis Index (DI)

About half of the tested individuals were classified as "dysbiotic" (DI > 2) before treatment, and the proportions of responders and non-responders being "dysbiotic" were similar, 50% (16/32) and 48% (14/29), respectively. These proportions increased numerically but not statistically significantly after treatment and remained very similar for responders and non-responders, 56% (18/32) and 59% (17/29), respectively. However, many patients among both responders and non-responders changed their DI classification after treatment. For non-responders, 7 became "dysbiotic" and 5 "non-dysbiotic." The number of such patients was slightly smaller in the responders group, in which 5 became "dysbiotic" and 3 had a normal value of DI after treatment. When measured on a scale from 1 to 5, DI scores remained unchanged for both responders and non-responders after the treatment. The median (range) was 3 (1–4) for responders and 3 (1–5) for non-responders, both before and after treatment. When analyzing changes in DI from before to after the treatment for both responders and non-responders, we were unable to identify any patterns (Fig. 2).

Response Index (RI)

Of the 54 bacterial markers used to assess gut microbial composition before treatment, 10 were significantly different between responders and non-responders (as described above). Based on median values of responders for these markers, we constructed a Response Index (RI) as follows:

1. The responder's median values for the 10 selected bacterial markers were used as cutoff levels.
2. Each patient was given a point when his/her value for each selected marker differed from the cutoff value. For bacteria that were less abundant in responders than in non-responders, the patients were given a point when the

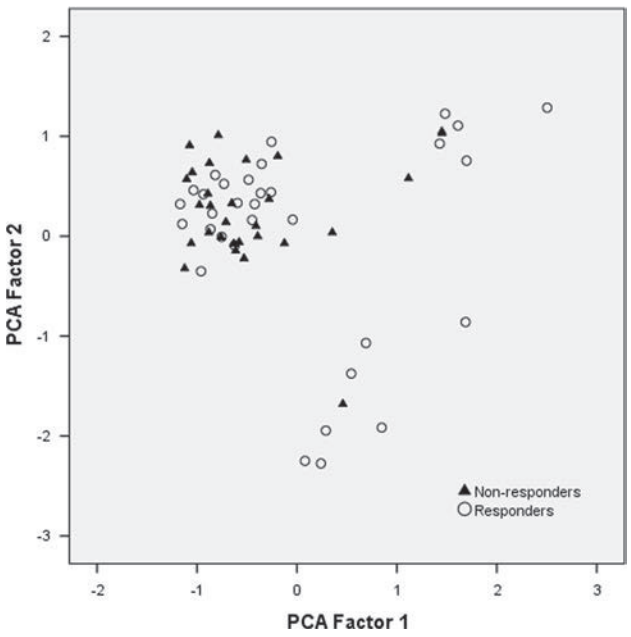

Fig. 1 Principal component analysis (PCA) showing a decomposition of gut microbiota markers using two-factor solution, as assessed by the GA-map™ Dysbiosis Test. The PCA confirmed that it is possible to distinguish between patients with IBS classified as responders ($n = 32$, depicted with open circles) and non-responders ($n = 29$, depicted with black triangles) to a 4-week FODMAP-restricted diet

bacterial marker level was lower than the cutoff level. For bacteria that were more abundant in responders than in non-responders, the patients were given a point when the bacterial marker level was higher than the cutoff level.

3. The points were summed up, giving a number from 0 to 10 (RI sum score). This sum was further dichotomized: Patients who scored 3 points and lower were assigned to value 0 (negative response) and patients who scored 4 or more points were assigned to value 1 (positive response).
4. Finally, the performance of RI was validated and accuracy computed as the mean score: 0.72, 95% CI [0.63; 0.81].

Although there was a high diversity in our results before treatment, responders reached higher RI sum scores compared to non-responders (median 4.9 for responders and 2.6 for non-responders, range 0–10 for both). Furthermore, there was a statistically significant, however, only low to moderate correlation between the RI sum score and percent decrease on IBS-SSS (Spearman's rho = 0.44, $p < 0.001$; Fig. 3). A majority of responders reached high RI sum scores also after their treatment.

RI Before Treatment

In total, 60% (19/32) of the responders scored 4 points or higher on the RI sum score, whereas only 21% (6/29) of the non-responders had a positive RI. Responders were younger and had a slightly higher BMI, and further analyses were thus adjusted for these possible confounders. When adjusted for age and BMI, only being a responder remained strongly statistically associated with positive RI ($p < 0.004$). However, we kept age in the final model. Patients with a positive RI were 5 times more likely to be responders compared to those who scored lower (OR = 5.05, 95% CI [1.58; 16.10]). Younger patients were more likely to be responders ($p = 0.04$). The probability to respond for patients having a positive RI was 83.4%, 95% CI [61.2–94%]. Furthermore, we calculated the probability that a patient will respond to FODMAP diet given a positive RI, i.e., the positive predictive value: PPV = 76.0, 95% CI [61.1–86.9].

Table 2 Bacterial abundance, as assessed by the GA-map™ Dysbiosis Test, in baseline fecal samples collected from responders and non-responders to a low-FODMAP diet

Microbial target	Responders ($n = 32$)	Non-responders ($n = 29$)	p value*
Bacteroides fragilis [s]	27.4 (11.1–58.6)	8.0 (5.2–45.7)	0.04
Acinetobacter [g]	188.9 (178.9–195.7)	177.4 (172.5–189.1)	0.02
Ruminiclostridium [g]	51.3 (46.2–63.2)	45.7 (42.9–50.7)	0.01
Clostridia [cl], Negativicutes [cl], Bacilli [cl]	486.3 (385.6–597.0)	622.5 (450.3–694.4)	0.02
Streptococcus III [g]	13.8 (7.9–51.9)	8.5 (5.8–11.9)	0.03
Actinomycetales [o]	5.8 (1.2–9.6)	10.0 (4.2–20.9)	0.02
Anaerotruncus [g]	75.6 (63.7–90.4)	83.7 (77.9–90.4)	< 0.01
Clostridiales [o]	275.4 (248.4–300.0)	285.5 (275.3–298.2)	< 0.01
Eubacterium II [g]	32.5 (11.9–61.0)	19.4 (10.7–61.4)	0.03
Shigella [g], *Escherichia* [g]	12.2 (8.2–21.6)	15.3 (10.7–22.9)	0.04

The results are listed as median intensity signal with 25–75 percentiles of the bacterial DNA markers

S species, *g* genus, *o* order, *cl* class

*Mann–Whitney–Wilcoxon test

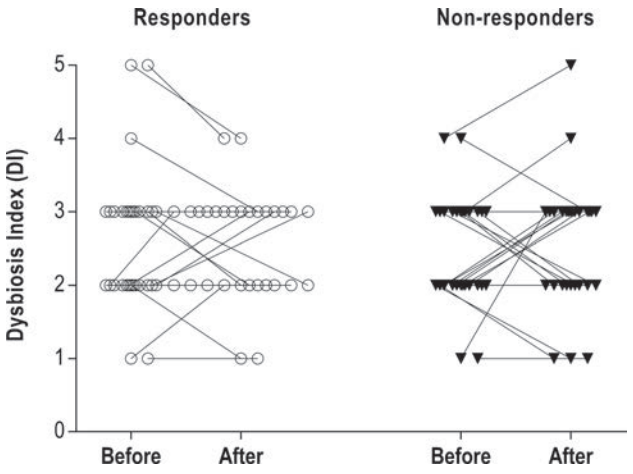

Fig. 2 Dysbiosis Index (DI), as provided by the producer of the GA-map™ Dysbiosis Test, assessed before and after a 4-week FODMAP-restricted diet in IBS patients defined as responders ($n = 32$) and non-responders ($n = 29$). Each line represents one individual

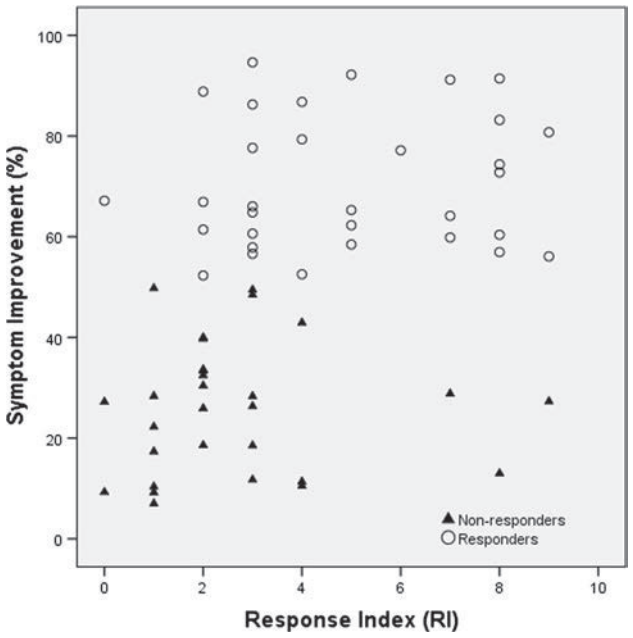

Fig. 3 Association between Response Index (RI), based on gut microbiota composition results, and symptom response, as assessed as reduction in IBS-SSS in percent from baseline values. There was a statistically significant, however, low to moderate correlation between the variables (Spearman's rho = 0.44, $p < 0.001$)

RI After Treatment

A majority of responders had a positive RI also after treatment. In total, 56% (18/32) of the responders scored positively compared to 14% (4/29) of the non-responders. When adjusted for age and BMI, responders were more than 7 times more likely to score positive using the RI compared to non-responders (OR = 7.31, 95% CI [1.90–28.23], $p = 0.004$).

Discussion

We aimed to explore possible associations between gut microbiota composition and clinical response to a low-FODMAP diet in patients with IBS, and demonstrated distinct differences of bacterial DNA profiles between responders and non-responders. The results indicate that gut microbiota composition may influence symptom response to dietary FODMAP restriction, and suggest that assessment of gut microbial composition, by using our new RI, should be investigated further as a clinical tool to identify patients that are likely to respond to nutritional management.

During the last decade, increasing evidence that the gut microbiota plays an important role in IBS pathogenesis has emerged [19]. Whereas mucosa-associated microbiota mainly seems to influence the host via regulatory control system located within the gut wall, luminal microbiota mainly seems to exert effects through fermentation, yielding gas, and other metabolites [20]. Both compartments seem to be disturbed in patients with IBS, and such alterations may be involved in symptom generation [21]. In the present study, we evaluated the fecal microbiota composition by assessing bacterial DNA markers. Patients classified as responders had a different profile of such markers as compared to non-responders. Whether this constellation represents distinct disturbances of pathophysiological significance cannot be inferred from the present study. However, some of the bacterial taxa may be associated with alterations of host immunology. In our study, responders had higher levels of *Streptococcus* than non-responders. Of note, increased levels of *Streptococcus* spp. have previously been associated with increased levels of the pro-inflammatory cytokine IL-6 [22], and dietary FODMAP restriction may reduce IL-6 levels in patients with IBS [23]. Furthermore, we have recently shown that dietary FODMAP restriction is accompanied with decreased saccharolytic and increased proteolytic fecal fermentation, i.e., important microbial processes that may have consequences for symptom generation [6]. It is tempting to speculate that saccharolytic bacteria are more dominant in responders than in non-responders, as has also been suggested in a recent pediatric study [24]. Interestingly, both *Bacteriodes fragilis* and *Acinetobacter* were more abundant in IBS patients classified as responders in our study, and the levels were reduced following dietary intervention. This may imply a possible pathophysiological role of these bacteria in generating "FODMAP-dependent" symptoms. However, deducing microbial functions from purely compositional analyses has many pitfalls [25].

Although a mechanistic relationship between gut microbiota composition and IBS symptom generation cannot be ascertained from the present study, the results suggest that our approach of using microbial DNA markers as an indicator of treatment response may be of value in a clinical setting. Indeed, the use of gut microbiota composition as a prognostic biomarker seems to be an emerging concept for several diseases. For instance, previous studies suggest that gut microbiota composition may predict treatment response to antibiotic therapy in patients with *Clostridium difficile* infection [26] and to anti-TNF therapy in patients with ulcerative colitis [27]. Importantly, Bennet et al. [28] have recently reported results from a similar project as our study, using the same methodology (the GA-map™ Dysbiosis Test) to evaluate microbiota composition. Although their findings are similar to ours, e.g., showing increased abundance of bacterial markers targeting Clostridia at different taxonomic levels in non-responders compared to responders, there are also important discrepancies. Contrary to Bennet et al., who found increased levels of certain bacteria only in the non-responder group, our data revealed higher levels of *Bacteroides fragilis*, *Acinetobacter*, *Ruminiclostridium*, *Streptococcus*, and *Eubacterium* in the responder group compared to the non-responder group. Furthermore, Bennet et al. demonstrated that non-responders were more "dysbiotic" than responders at baseline and that a 4-week low-FODMAP diet, but not a traditional IBS diet, was associated with increased DI scores. In contrast, we did not detect any differences in "Dysbiosis" (defined as DI > 2), between our groups of responders and non-responders at baseline, and the proportions of patients classified as "dysbiotic" did not change significantly following the dietary intervention in neither group. The above-discussed differences between our study and the study of Bennet et al. may in part be due to differences in patient selection. For instance, whereas both patient populations were thoroughly investigated to exclude organic diseases, our patient group was also selected based upon a ^{13}C-D-xylose breath test to exclude small intestinal malabsorption [10]. Thus, only patients with high levels of $^{13}CO_2$ excretion following ^{13}C-D-xylose ingestion were included. Such differences in selection criteria may potentially account for differences in gut microbiota composition, but this remains to be established. Different definitions of responder status may also have impacted the results: Bennet et al. used a 50-point reduction in IBS-SSS as a cutoff level to distinguish between responders and non-responders, whereas we used a 50-percent improvement definition. We acknowledge that such definitions are debatable [13], but would argue that a 50-percent improvement definition is probably stricter, which we consider important in a study evaluating symptom response with an open design.

Our study was of exploratory character, and as such has limitations. The number of participants was relatively small, however, comparable to other studies involving resource-demanding procedures such as dietary counseling, stool collection, and microbiota analyses. The duration of the diet was probably sufficient to detect effects upon the gut microbiota [29], but may be too short to evaluate sustainable symptom relief. Based upon a previously validated method to evaluate the gut microbiota composition [16], we developed a new RI to discriminate between responders and non-responders of dietary FODMAP restriction. Although this index seemed to perform well in the present setting, we acknowledge that it was constructed by using explorative statistical methods, and that it remains to be validated in an independent patient cohort. Future studies should preferably include patients from other countries than Sweden and Norway, since the potentially distinct nature of the Scandinavian diet and gut microbiota may limit the applicability of the test results.

To conclude, our data suggest that pre-treatment levels of selected gut microbial DNA markers may be associated with higher probability to respond favorably to dietary FODMAP restriction. In the present study, we incorporated such markers into a score to construct an index, denoted RI. Further work is needed to validate our findings on new data and to determine the usefulness of RI as a clinical tool in IBS management.

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Author's contribution JV conceived and designed the study, performed the data collection, and wrote the manuscript. MCS performed the statistical analyses and wrote the manuscript. TK guided the patients throughout the dietary intervention. GAL interpreted the data and contributed to critical revision. AR conceived and designed the study, and recruited the patients. All authors reviewed and approved the final version of the manuscript to be published.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in the study were in accordance with the ethical standards of the Regional Committee for Medical Research Ethics (REK Sør-Øst, Reference No. 2013/454) and with the 1964 Helsinki Declaration and its later amendments.

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ORIGINAL ARTICLE

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Evaluation of a faecal dysbiosis test for irritable bowel syndrome in subjects with and without obesity

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ABSTRACT

Biomarkers for irritable bowel syndrome (IBS) are demanded. An altered faecal microbiome has been reported in subjects with IBS and could be a valuable biomarker. This study evaluated the diagnostic properties of a new test for faecal dysbiosis, designed to distinguish IBS from healthy volunteers and compared the prevalence rates of dysbiosis related to IBS and morbid obesity. Subjects with and without morbid obesity and IBS were included. The faecal microbiota was assessed with GA-map™ Dysbiosis Test (Genetic Analysis AS, Oslo, Norway). The test result was given as dysbiosis (yes/no). Comparisons were made between four groups: subjects with IBS and morbid obesity (IBS+/MO+); subjects without IBS and with morbid obesity (IBS−/MO+); subjects with IBS and without morbid obesity (IBS+/MO−); and healthy volunteers (IBS−/MO−).The prevalence rates of dysbiosis in the groups IBS+/MO+, IBS-/MO+, IBS+/MO- and IBS-/MO- were 18/28 (64%), 45/71 (63%), 31/63 (49%) and 38/91 (42%). Dysbiosis was more prevalent in subjects with morbid obesity, both in those with and without IBS, than in healthy volunteers (*p* values .04 and .006). Used as a diagnostic test for IBS in subjects without morbid obesity, the positive and negative likelihood ratios (LR) were 1.18 (0.83–1.67) and 0.87 (0.65–1.18), respectively, and in subjects with morbid obesity the LR were 1.01 (95% CI: 0.73–1.41) and 0.98 (0.54–1.75) respectively. The dysbiosis test was unsuitable as a diagnostic test for IBS. Dysbiosis was statistically significantly associated with morbid obesity, but not with IBS.

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Introduction

The pathophysiology of irritable bowel syndrome (IBS) includes changes in the gut microbiota and mucosal immune activation [1]. There is a need for biomarkers that allow a positive diagnosis of IBS, but despite extensive research, no biomarker has yet been judged as clinically useful [2,3].

The microbes that collectively inhabit a given ecosystem are called a microbiota [4], dysbiosis has been defined as a microbiota that 'differs from what is found in a healthy gut' [5]. Dysbiosis has been associated with both intestinal and extra-intestinal disorders such as IBS, diabetes and obesity [6–10]. A unique faecal microbiota profile in subjects with IBS or subgroups of IBS could become a valuable biomarker for the diagnosis of IBS or a useful treatment guide.

A test for faecal dysbiosis based upon 54 DNA probes that target gut bacteria has been marketed in Europe. The paper presenting the method and the first results said that the test 'is intended to be used as a gut microbiota DNA analysis tool to identify and characterise dysbiosis' and that the probes 'were selected based on their ability to distinguish between samples isolated from healthy individuals and IBS patients' [11]. The sensitivity and specificity of the test for the diagnosis of IBS were 73% and 84%, respectively [11]. The test has not been validated for the use in daily practice but is never the less commercially available in several countries and used for research [12]. The present study is the first manufacturer-independent evaluation of the test's diagnostic accuracy. The test has not been used in subjects with obesity, who in studies with other methods have been shown to have changed faecal microbiota [7,13].

The aims were to evaluate the diagnostic properties of a new test for faecal dysbiosis for the diagnosis of IBS and to compare the prevalence rates of faecal dysbiosis in subjects with and without IBS and morbid obesity (i.e. four groups of subjects).

Methods

Study design and setting

The study included consecutive subjects with morbid obesity at the unit for morbid obesity, Innlandet Hospital Trust, Gjøvik, Norway, and subjects with IBS and without morbid obesity at the gastroenterological outpatient clinic, Lovisenberg Diaconal Hospital, Oslo, Norway. At inclusion, demographics were noted, a medical history was taken, a clinical examination was performed, a laboratory screen was assessed, faecal samples were collected and endoscopic

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examinations and other investigations were conducted when indicated. All subjects filled in questionnaires for the classification of functional bowel disorders and evaluation of abdominal symptoms. Subjects at Innlandet Hospital Trust and Lovisenberg Diaconal Hospital were recruited from December 2012 to September 2014 and from April 2013 to October 2014, respectively.

Participants

At Innlandet Hospital Trust, the inclusion criteria were age 18–65 years and morbid obesity, defined as body mass index (BMI) $>40\,kg/m^2$ or BMI $>35\,kg/m^2$ with obesity-related comorbidity [14]. At Lovisenberg Diaconal Hospital, the inclusion criteria were age >18 years and IBS. At both centres, exclusion criteria were pregnancy/lactation, major psychiatric disorders, alcohol and drug addiction, organic gastrointestinal disorders, former obesity surgery and other major abdominal surgery. Use of antibiotics the last month or a ^{13}C-D-xylose breath test that indicated malabsorption were additional exclusion criteria at Lovisenberg Diaconal Hospital.

We had no data from healthy volunteers. For comparisons with our results, we therefore used a summary of all results in healthy volunteers tested for faecal dysbiosis with the new test and published until 30th June 2017 [11,15]. The data used for the creation of the test [11] were excluded.

The study consisted of four groups: Subjects with IBS and morbid obesity (IBS+/MO+); subjects without IBS and with morbid obesity (IBS−/MO+); subjects with IBS and without morbid obesity (IBS+/MO−); and healthy volunteers (IBS−/MO−).

Variables

Demographics

Age, gender and BMI were registered on all subjects.

Abdominal complaints

IBS and the subtypes IBS with diarrhoea (IBS-D), IBS with constipation (IBS-C), mixed IBS (IBS-M) and unsubtyped IBS (IBS-U) were defined according to the Rome III criteria [16]. The severity was evaluated with IBS severity scoring system (IBS-SSS) that ranges from 0 to 500 (mild: 75–175; moderate 175–300; and severe >300) [17].

Faecal dysbiosis test

Faecal dysbiosis was assessed with GA-map™ Dysbiosis Test (dysbiosis test), manufactured by Genetic Analysis, Oslo, Norway. The test, which is CE-marked, was based on the characterization of selected 16 S rRNA gene sequences from bacteria and created to profile the intestinal microbiota and to identify and characterise dysbiosis. Fifty-four probes were selected based on their ability to separate patients with IBS from healthy individuals [11]. The results were given as dysbiosis yes/no and a dysbiosis index (DI) with scores 1–5. High DI scores indicate more severe dysbiosis and scores ≤2 indicate no dysbiosis. Only data on dysbiosis yes/no were available for the healthy volunteer group. All tests were performed by the manufacturer of the test.

The faecal material for the dysbiosis test was collected by the subjects in kits provided by the producer and handled according to the producer's protocol which said that the samples could be stored at room temperature until five days before analysis or freezing [11]. For logistical reasons, the time until freezing was shorter at Lovisenberg Diaconal Hospital than at Innlandet Hospital Trust, but always within the producer's recommendations.

Statistical analysis

Student's *t*-test, Fisher's Exact test, Pearson chi-squared test, ANOVA or Kruskal–Wallis test were used for comparisons depending on the type of data and normality, and the results are presented as mean (standard deviation), median (range) and proportion (percentage) according to the type and distribution of data. The diagnostic properties of the dysbiosis test for the diagnosis of IBS were reported as sensitivity, specificity, accuracy, positive and negative predictive values (PPV and NPV), and positive and negative likelihood ratios (LR + and LR-). The DI was analysed with the receiver operating characteristics curve (ROC-curve) with the calculation of the area under the curve. The results have been reported with 95% confidence intervals (CI). Associations between dysbiosis and subtypes of IBS among subjects with IBS were analysed with logistic regression analyses with dysbiosis as the dependent variable and adjusted for age and recruitment centre. All tests were 2-tailed, and p values <.05 were judged as statistically significant. The data analyses were performed with IBM SPSS Statistics for Windows, Version 21.0 (Armonk, NY).

Ethics

The study was approved by the Regional Committee for Medical and Health Research Ethics South East Norway (reference numbers 2012/966 and 2013/454) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants before inclusion in the study.

Results

Ninety-nine out of 350 consecutive subjects with morbid obesity were included in the study, of whom 28 had IBS. Sixty-three out of 94 consecutive subjects with IBS without morbid obesity were included (Figure 1 shows the details). Ninety-one healthy subjects reported in published papers were used as controls [11,15]. The subjects' characteristics are given in Table 1.

The prevalence of dysbiosis in the four groups varied from 42% to 64% (Table 2). The differences in prevalence rates between the groups are given in Table 3. Dysbiosis was significantly more common in the two groups with morbid

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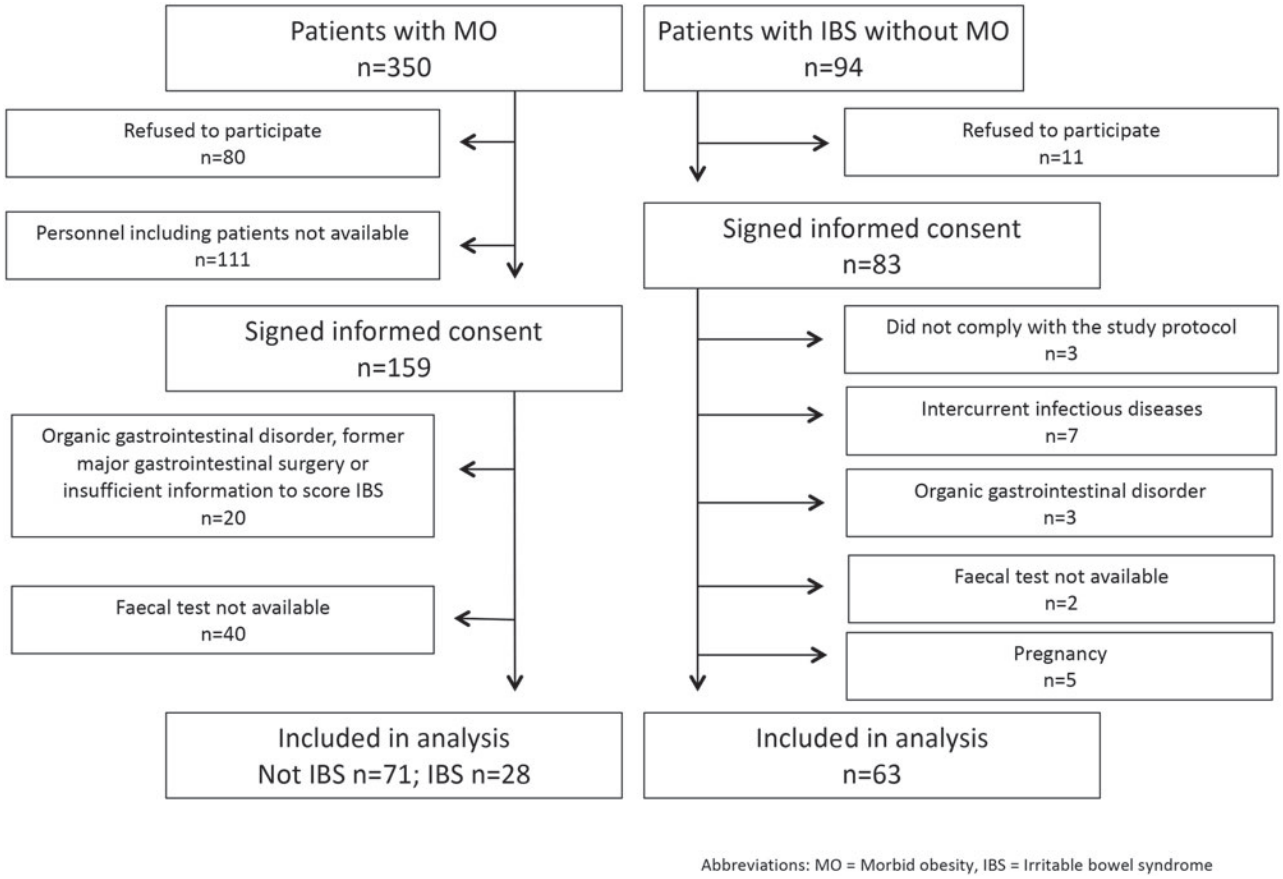

Figure 1. Flow chart depicting inclusion of subjects.

Table 1. Subjects' characteristics.

	IBS+/MO+ ($n=28$)	IBS-/MO+ ($n=71$)	IBS+/MO− ($n=63$)	p value
Age (years)	44 (8)	45 (9)	39 (12)	.003
Gender (% male)	2/28 (7%)	12/71 (17%)	7/63 (11%)	.37#
BMI (kg/m^2)	42 (3)	42 (4)	24 (4)	<.001
IBS severity scoring system	224 (73)		288 (79)	<.001*
IBS with constipation	4/28 (14%)		10/63 (16%)	1.00**
IBS with diarrhoea	8/28 (29%)		33/63 (52%)	.04**
IBS with mixed symptoms	15/28 (54%)		20/63 (32%)	.06**
Unsubtyped IBS	1/28 (4%)		0/63 (0%)	.31**

BMI: body mass index; IBS: irritable bowel syndrome; IBS+/MO+: subjects with irritable bowel syndrome and morbid obesity; IBS−/MO+: subjects without irritable bowel syndrome with morbid obesity; IBS+/MO−: subjects with irritable bowel syndrome without morbid obesity; MO: morbid obesity.
Statistical analysis with ANOVA, Pearson chi-squared test (marked with #), Student's t-test (marked with *) or Fisher's exact test (marked with **).

Table 2. Dysbiosis and the dysbiosis index score in the groups of subjects with and without irritable bowel syndrome (IBS) and morbid obesity (MO).

	Prevalence of dysbiosis	Dysbiosis index score (1–5)#
IBS+/MO+	18/28 (64%)	3 (1–5)
IBS-/MO+	45/71 (63%)	3 (1–5)
IBS+/MO-	31/63 (49%)	2 (1–5)
IBS-/MO-	38/91 (42%)	n.a

IBS+/MO+: Subjects with irritable bowel syndrome and morbid obesity; IBS−/MO+: Subjects without irritable bowel syndrome with morbid obesity; IBS+/MO−: Subjects with irritable bowel syndrome without morbid obesity; IBS−/MO−: Subjects without irritable bowel syndrome and without morbid obesity.
#Statistics: $p=.13$ (Kruskal–Wallis test).

obesity than in healthy volunteers (Table 3). There were no significant differences in the prevalence of dysbiosis between the groups with and without IBS stratified for morbid obesity (Table 3). Among the subjects with IBS, the prevalence of dysbiosis was higher in the subgroup IBS-D than in subjects without IBS-D (odds ratio 2.48 (95% confidence interval 1.01–6.09), $p=.047$).

In subjects with morbid obesity, the sensitivity, specificity, LR+ and LR− of the dysbiosis test for the diagnosis of IBS were 64%, 37%, 1.01 and 0.98, respectively (Table 4). Data on the DI were available in three groups. In these groups, there were no significant differences in the DI (Table 2). In subjects with morbid obesity, the area under the ROC curve for the DI was 0.54 (95% CI 0.42–0.67, $p=.50$). In subjects without morbid obesity, the sensitivity, specificity, LR+, and LR− were 49%, 58%, 1.18 and 0.87 respectively (Table 4).

Table 3. Differences between the prevalence rates of dysbiosis in the groups with and without irritable bowel syndrome (IBS) and morbid obesity (MO).

	Difference between the groups	p value
IBS+/MO + vs. IBS-/MO+	1% (−21% to 22%)	.93
IBS+/MO + vs. IBS+/MO−	15% (−9% to 36%)	.18
IBS+/MO + vs. IBS−/MO−	22% (0% to 41%)	**.04**
IBS+/MO − vs. IBS−/MO+	−14% (−31% to 4%)	.10
IBS−/MO + vs. IBS−/MO−	21% (4% to 36%)	**.006**
IBS+/MO − vs. IBS−/MO−	7% (−10% to 23%)	.36

IBS+/MO+: subjects with irritable bowel syndrome and morbid obesity; IBS−/MO+: subjects without irritable bowel syndrome with morbid obesity; IBS+/MO−: subjects with irritable bowel syndrome without morbid obesity; IBS−/MO−: subjects without irritable bowel syndrome and without morbid obesity. The results are given as differences (95% confidence interval).
Statistical analysis with Pearson chi-squared tests.

Table 4. Diagnostic properties of the dysbiosis test for the diagnosis of irritable bowel syndrome.

	Sensitivity	Specificity	PPV	NPV	Accuracy	DOR	LR+	LR−
Morbid obesity	64% (44%–81%)	37% (26%–49%)	29% (18%–41%)	72% (55%–86%)	44% (34%–54%)	1.04 (0.41–2.59)	1.01 (0.73–1.41)	0.98 (0.54–1.75)
Without morbid obesity	49% (36%–62%)	58% (47%–69%)	n.a	n.a	n.a	1.35 (0.71–2.58)	1.18 (0.83–1.67)	0.87 (0.65–1.18)

DOR: diagnostic odds ratio; LR+: positive likelihood ratio; LR−: negative likelihood ratio; NPV: negative predictive value; PPV: positive predictive values. All values are given with 95% confidence intervals.

Discussion

The prevalence of dysbiosis was above 40% in all four groups of subjects examined in this study. The subjects with IBS had a lower prevalence and the healthy volunteers had a higher prevalence of dysbiosis than previously reported [11,18]. The association between dysbiosis and obesity with this dysbiosis test was in accordance with studies using other methods [7,13].

IBS and dysbiosis were not significantly associated, either among subjects with or without morbid obesity. This was unexpected, as the dysbiosis test was created by selecting the probes that best could separate subjects with and without IBS [11]. The high prevalence of dysbiosis in subjects with IBS-D could be explained by the association between stool consistency and the microbiota [19].

The dysbiosis test was unsuitable as a diagnostic test for IBS. The upper and lower limits of the 95% confidence intervals for the LR + and LR- were below 2 and above 0.5 respectively, which have been described as the limits for unimportant effects [20]. Other cut-off values on the dysbiosis index did not improve the diagnostic usefulness.

Standard DNA-based methods for the study of microbiota produce huge data sets and are expensive [4]. The faecal dysbiosis test evaluated in this paper uses 54 probes and analyses only a small proportion of the genetic material. The test, therefore, gives limited knowledge about the entire faecal microbiota, though high overlap between the dysbiosis test and metagenomic sequencing has been reported for some bacteria [11,21]. For the use in clinical practice, knowledge about tests that give a summary of the genetic material is nevertheless very important, as such tests are easily available and less expensive.

Prevalence rates of dysbiosis as measured by the dysbiosis test appear to vary widely between different groups of healthy volunteers as well as between groups of subjects with the same disorder. A large number of confounding factors are relevant, including but not restricted to diet, geography and stool consistency [19]. This study reminds us that biomarkers based on a limited number of subjects and promoted by the producer demand validation in daily practice and in larger and different groups of subjects before taken into regular use.

The imperfect properties of the test for the diagnosis of IBS do not exclude other areas of application. The symptom-based diagnosis of IBS defined by the Rome III criteria may represent several disorders with similar symptoms. Subjects with and without dysbiosis or with different degrees of dysbiosis might be subgroups of IBS with different aetiology, pathophysiology, severity or prognosis and profit from different treatment alternatives. For instance, the effect of fermentable oligosaccharides, disaccharides and monosaccharides and polyols (FODMAP) reduced diet has been shown to be best in subjects without dysbiosis [12]. Such areas of application of the test deserve further evaluation. The test has also been related to the prognosis of inflammatory bowel disease [22].

The microbiota may be a modifiable cause of obesity-related metabolic comorbidity [23]. Subjects with obesity and subjects with a metabolic risk profile have been shown to be identifiable by less than ten bacterial species from faecal samples [7]. If the dysbiosis test or other tests based on the same technology can characterise obesity-related changes in the microbiota, they may become useful for the management of subgroups of subjects with obesity and metabolic disorders.

Strengths and limitations

To achieve high quality, the manufacturer's laboratory performed all the faecal analyses. In the two groups of patients with morbid obesity, the laboratory was blinded to the diagnosis of IBS; and for all subjects, the laboratory was blinded to symptom severity. The diagnosis of IBS was based on a validated Norwegian translation of the Rome III criteria [16]. The study size resulted in narrow confidence intervals that excluded the test as a diagnostic tool for IBS. All the faecal samples were handled according to the producer's recommendations. The small difference in preanalytical handling at the two centres was judged as unimportant. A limitation of the study was that the data in the healthy volunteers were based on previously published data and not our own data.

Selection bias is unlikely. The main reason for the inadequate inclusion of subjects with morbid obesity was the absence of a study nurse 2–3 days a week (Figure 1). In the group with IBS without morbid obesity, consecutive subjects were included. As expected, symptoms were more severe in the group with IBS without obesity than in the group with obesity, because subjects referred to a gastroenterological outpatient clinic for abdominal complaints are likely to have more severe symptoms than subjects referred for obesity and asked about abdominal complaints (Table 1).

Conclusions

The dysbiosis test was inappropriate as a diagnostic test of IBS. Dysbiosis was more common in subjects with morbid

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obesity than in healthy volunteers. There were no significant differences in the prevalence rates of dysbiosis in subjects with and without IBS, either in subjects with or without morbid obesity. IBS-D was associated with dysbiosis.

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Disclosure statement

The authors report no conflicts of interest.

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Arthritis Research & Therapy

RESEARCH ARTICLE Open Access

Severe intestinal dysbiosis is prevalent in primary Sjögren's syndrome and is associated with systemic disease activity

Thomas Mandl[1,7*], Jan Marsal[2,3,4], Peter Olsson[1], Bodil Ohlsson[5] and Kristofer Andréasson[6]

Abstract

Background: Altered microbial composition of the intestine, commonly referred to as dysbiosis, has been associated with several autoimmune diseases including primary Sjögren's syndrome (pSS). The aims of the current study were to study the intestinal microbial balance in pSS and to identify clinical features associated with dysbiosis.

Methods: Forty-two consecutive pSS patients and 35 age-matched and sex-matched control subjects were included in the study in an open clinic setting. Stool samples were analyzed for intestinal dysbiosis using a validated 16S rRNA-based microbiota test (GA-map™ Dysbiosis Test; Genetic Analysis, Oslo, Norway). Dysbiosis and severe dysbiosis were defined in accordance with the manufacturer's instructions. Patients were evaluated with regard to disease activity (European League Against Rheumatism (EULAR) Sjögren's Syndrome Disease Activity Index (ESSDAI) and Clinical ESSDAI (ClinESSDAI)). In addition, patients were examined for laboratory and serological features of pSS as well as fecal calprotectin levels.
Furthermore, patients were investigated regarding patient-reported outcomes for pSS (EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI)) and irritable bowel syndrome (IBS)-like symptoms according to the Rome III criteria.

Results: Severe dysbiosis was more prevalent in pSS patients in comparison to controls (21 vs 3%; $p = 0.018$). Subjects with pSS and severe dysbiosis had higher disease activity as evaluated by the ESSDAI total score (13 vs 5; $p = 0.049$) and the ClinESSDAI total score (12 vs 5; $p = 0.049$), lower levels of complement component 4 (0.11 vs 0.17 g/L; $p = 0.004$), as well as higher levels of fecal calprotectin (110 vs 33 μg/g; $p = 0.001$) compared to the other pSS patients. In contrast, severe dysbiosis among pSS patients was not associated with disease duration, IBS-like symptoms, or the ESSPRI total score.

Conclusions: Severe intestinal dysbiosis is a prevalent finding in pSS and is associated both with clinical and laboratory markers of systemic disease activity as well as gastrointestinal inflammation. Further studies are warranted to elucidate a potential causative link between dysbiosis and pSS.

Keywords: Primary Sjögren's syndrome, Microbiome, Gastrointestinal, Dysbiosis, Disease activity

Background

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease, characterized by lymphocytic infiltration of exocrine glands resulting in exocrine hypofunction and sicca symptoms. The disease may also result in various extraglandular manifestations (EGM) such as arthritis, pulmonary involvement, renal disease, vasculitis, and neuropathy [1, 2]. The gastrointestinal (GI) tract may also be involved and esophageal dysmotility [3, 4], gastroparesis [5], atrophic gastritis [6], and pancreatic insufficiency [6] are commonly encountered in pSS.

Increased levels of fecal calprotectin (F-calprotectin), a validated marker for GI inflammation, have been found in a subgroup of pSS patients and were associated with concomitant organic GI disease [7]. GI complaints are common in pSS and a large proportion of pSS patients

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exhibit symptoms typically observed in irritable bowel syndrome (IBS) or dysmotility [5, 8, 9].

Recently, increasing interest has been directed against the importance of the GI microbiota and its influence on autoimmune disease [10]. In both health and disease, there is a continuous interaction between the microbiota, the epithelium, and the immune cells of the GI mucosa. This interaction has been suggested to have profound effects on both the local and systemic immune system [11], and may both curtail and amplify local and systemic inflammatory disease [12]. Because the GI epithelium is affected in pSS, through the inflammation of the exocrine glands as well as through diminished secretions, there are decreased levels of both protective and proliferative factors [6, 13, 14]. Consequently, the integrity of the epithelium and its barrier function may be compromised in pSS patients, possibly resulting in a perturbed microbiota–host immune system interaction.

An altered microbiota composition, commonly referred to as dysbiosis, has been shown to induce and modulate systemic inflammation in experimental animal models of rheumatic diseases [15], including pSS [16]. In the clinical setting, intestinal dysbiosis has been associated with several autoimmune diseases including rheumatoid arthritis, systemic lupus erythematosus, ankylosing spondylitis, celiac disease, autoimmune hepatitis, systemic sclerosis, and pSS [10, 15, 17–21]. Recently, de Paiva et al. demonstrated that intestinal dysbiosis may worsen experimental pSS in mice. They also reported an association between pSS and intestinal dysbiosis in a small number of patients ($n = 10$) [16].

The aims of this study were to explore intestinal microbial balance in pSS and to relate these findings to clinical features of disease.

Methods

Patients

Forty-two consecutive outpatients (median age 62, range 24–80 years; 40 females) diagnosed with pSS according to the American–European Consensus Group (AECG) criteria [22], but also fulfilling the American Congress of Rheumatology–European League Against Rheumatism (EULAR) criteria for pSS [23], seen at the Department of Rheumatology, Skane University Hospital, Malmö, Sweden, were included in the study. Concurrent inflammatory bowel disease (IBD) and antibiotic treatment during the last 3 months served as exclusion criteria. Patients' characteristics are presented in Table 1.

Table 1 Patient characteristics

	pSS patients ($N = 42$)
Age (years)	62 (51; 68)
Sex (males/females)	2/40
Disease duration (years)	16 (7; 23)
Current/prior/never smokers (%)	20/41/39
Anti-SS-A antibody seropositives (%)	76
Anti-SS-B antibody seropositives (%)	48
ANA seropositives (%)	79
RF seropositives (%)	52
IgG (g/L)	13.1 (10.0; 17.1)
Complement component 3 (g/L)	1.00 (0.89; 1.15)
Complement component 4 (g/L)	0.16 (0.12; 0.21)
Lip biopsy—focus score ≥ 1 (*N* positives/*N* studied (%))	28/33 (85)
ESSDAI total score	6 (1; 12)
ESSPRI total score	6 (5; 8)
Fecal calprotectin (μg/g)	38 (20; 123)
Irritable bowel syndrome-like symptoms (%)	42
Using proton pump inhibitors (%)	36
Using NSAIDs (%)	38
Using glucocorticoids (%)	36
> 5 mg prednisolone daily	7
5 mg prednisolone daily	24
< 5 mg prednisolone daily	5
Using anti-malarials (%)	29
Using other DMARDs (%)	2

Demographic and clinical characteristics of the patients with primary Sjögren's syndrome (*pSS*). Results presented as median (interquartile range) or percentage of subjects

DMARD disease-modifying anti-rheumatic drug, *ESSDAI* EULAR Sjögren's Syndrome Disease Activity Index, *ESSPRI* EULAR Sjögren's Syndrome Patient Reported Index, *EULAR* European League Against Rheumatism, *NSAID* nonsteroidal anti-inflammatory drug, *ANA* antinuclear antibodies, *RF* rheumatoid factor

Control group

Thirty-five age-matched and sex-matched control subjects (median age 62, range 39–78 years; 33 females) consisting of hospital workers, their relatives, and their friends were invited and accepted for this study. Subjects with any rheumatologic or IBD diagnosis, as well as concurrent antibiotic treatment, were excluded.

Clinical assessment

The patients were evaluated for systemic disease manifestations by the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) [1] and the Clinical ESSDAI (ClinESSDAI) [24]. Patient-reported outcomes, including symptoms of sicca, pain, and fatigue, were evaluated by the EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI) [25]. In addition, the presence of IBS-like symptoms, as defined by the Rome III criteria, was evaluated by a validated questionnaire on GI complaints [26]. Concomitant use of proton pump inhibitors (PPI), nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids

(GCs), anti-malarials (AMA), and other disease-modifying anti-rheumatic drugs (DMARDs) was noted.

Laboratory analyses

Laboratory testing included measurement of levels of IgG, as well as analysis of autoantibodies including anti-SS-A and anti-SS-B antibodies, ANA, and RF. Complement levels were assessed by measurement of complement component 3 (C3) and complement component 4 (C4). F-calprotectin, a marker of GI inflammation, was measured with a commercially available enzyme-linked immunosorbent assay using a monoclonal antibody (Bühlmann Laboratories, Schönenbuch, Switzerland). All analyses were performed at the Department of Laboratory Medicine, Skane University Hospital.

Evaluation of intestinal dysbiosis

The GA-map™ Dysbiosis Test (Genetic Analysis, Oslo, Norway) has been developed in order to identify and grade intestinal dysbiosis by analysis of microbial genes in a stool sample. The test makes use of 54 bacterial 16S rRNA probes specific for various intestinal bacterial species or clades to generate data on the intestinal microbiota composition. Using a defined algorithm, these data are subsequently translated into a Dysbiosis Index Score (DIS) ranging from 1 to 5. The test has been compared with MiSeq Illumina sequencing-based protocols and proven successful in identifying dysbiosis [21, 27–29]. In a previous study of a population consisting of young healthy adults, 84% exhibited DIS 1–2 and 16% exhibited DIS ≥ 3 [27]. In the current study, dysbiosis was defined as DIS ≥ 3 and severe dysbiosis as DIS 5, in accordance with the manufacturer's instructions.

Ethics

The study was approved by the Regional Ethics Review Board, Lund, Sweden (LU 2011/596). All patients and controls gave written informed consent and the study was performed in accordance with the declaration of Helsinki.

Statistical analyses

The Mann–Whitney *U* test was used when comparing continuous variables and the chi-square test or Fisher's exact test for comparing discrete variables and frequency distribution analysis. Correlations were evaluated by Spearman rank correlation. Values are presented as median and interquartile range (IQR) or number and percentage of subjects. $p < 0.05$ was considered statistically significant.

Results

Eubiosis and moderate dysbiosis were common in both the pSS group and the control group. However, the frequency distribution among the pSS patients was different from that of the controls ($p = 0.045$) (Fig. 1). A large subgroup of pSS patients exhibited severe dysbiosis (DIS 5) which was not the case among controls (21 vs 3%; $p = 0.018$) (Fig. 1). Consequently, we chose to analyze this pSS subgroup in relation to the other pSS subjects.

Patients with severe dysbiosis had higher disease activity, as evaluated by the ESSDAI, compared to the other pSS patients (13 (5; 16) vs 5 (1; 10); $p = 0.049$) (Fig. 2a). Similar findings were found for the ClinESSDAI (12 (4; 16) vs 5 (0; 9); $p = 0.049$). In line with these findings, hypocomplementemia was more pronounced amongst pSS patients with severe dysbiosis as reflected by lower serum levels of C4 (0.11 (0.07; 0.14) vs 0.17 (0.14; 0.21) g/L; $p = 0.004$) (Fig. 2b). pSS patients with severe dysbiosis had significantly higher F-calprotectin (110 (61; 330) vs 33 (20; 74) μg/g; $p = 0.001$) (Fig. 2c) compared to the other patients. The associations remained when excluding patients with milder dysbiosis and comparing patients with DIS 5 only to patients with DIS 1–2 (data not shown).

Neither disease duration, smoking, classical symptoms of pSS (as evaluated by the ESSPRI), IgG, IgM, or IgA levels, or serological features of pSS was associated with dysbiosis or severe dysbiosis in pSS patients. IBS-like symptoms were not more common in pSS patients with severe dysbiosis (Table 2).

Usage of GCs was more common among patients with DIS 5 (67 vs 27%; $p = 0.049$). However, concomitant use of other medications (including the use of PPI, NSAIDs, and AMA) were not associated with severe or moderate dysbiosis in pSS patients (Table 2).

Subanalysis of the GA-map™ Dysbiosis Test made it possible to explore the abundance of 15 separate clades or species of bacteria. A significantly higher fraction of pSS patients exhibited decreased levels of bacteria from the genera *Bifidobacterium* (38 vs 3%; $p < 0.001$) and *Alistipes* (19 vs 3%; $p = 0.017$) in comparison to control subjects. There was also a tendency toward lower levels of *Faecalibacterium prausnitzii* among the pSS subjects, but this difference was not statistically significant ($p = 0.061$).

Discussion

In the current study, we found that severe intestinal dysbiosis was a prevalent finding in pSS, affecting 21% of studied patients. In this cohort of consecutive pSS patients, severe intestinal dysbiosis was associated with both clinical and laboratory signs of systemic disease activity as well as with laboratory signs of GI tract inflammation, as evaluated by F-calprotectin.

Eubiosis and mild or moderate dysbiosis (DIS 1–4) were prevalent both in the pSS group and the control group. The relatively high fraction of intestinal dysbiosis

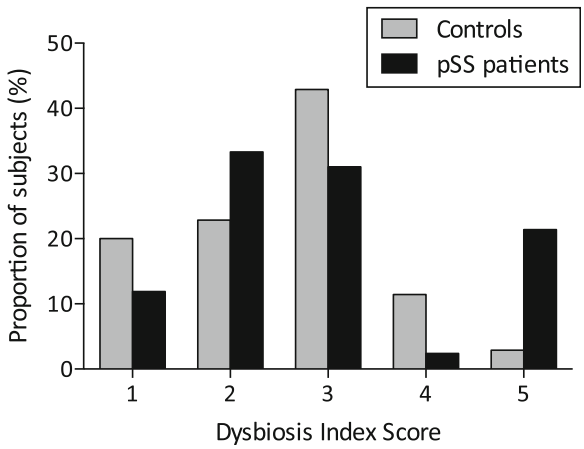

Fig. 1 Dysbiosis Index Score in primary Sjögren's syndrome and age-matched and sex-matched control subjects. A subgroup of patients with severe dysbiosis (DIS 5) can be identified among the subjects with primary Sjögren's syndrome (pSS) but not in the control group

in the control group differs from a previous report where only 16% of healthy adults exhibited DIS ≥ 3 [27]. However, the controls described previously were younger (mean age 41 years) [27] than the age-matched and sex-matched controls of the current study (mean age 60 years). Also, in our control group we had few exclusion criteria (ongoing GI or rheumatic disease), which is in contrast to the previous report. However, severe intestinal dysbiosis was significantly more common in pSS patients (21%) than in age-matched and sex-matched controls (3%), indicating an altered intestinal microbial balance in a subgroup of pSS patients.

Intestinal dysbiosis has been found to be a feature of several rheumatic diseases, including rheumatoid arthritis, systemic lupus erythematosus, systemic sclerosis, and pSS [10, 16, 20], as well as IBD [30]. Intestinal dysbiosis has hitherto been studied most extensively in IBD patients, in whom a decreased diversity in gut microbiota, increased numbers of bacteria driving inflammatory activity, as well as decreased numbers of bacteria with immunoregulatory effects have been demonstrated [30]. In rheumatic diseases as well as in IBD, it is still a matter of debate whether intestinal dysbiosis is a primary or secondary phenomenon of disease [31]. In animal models of IBD, loss of immunoregulatory bacteria and addition of disease-promoting bacteria contribute to disease activity, supporting a primary disease-driving role for intestinal dysbiosis in IBD [32]. Recently, two small studies reported oral [19] as well as intestinal [16] dysbiosis in pSS patients. In the prior study, the bacterial diversity was found to be lower in pSS patients, with normal salivary flow [19], thus implying that oral dysbiosis can occur irrespective of whether salivary flow was decreased or not. In the latter

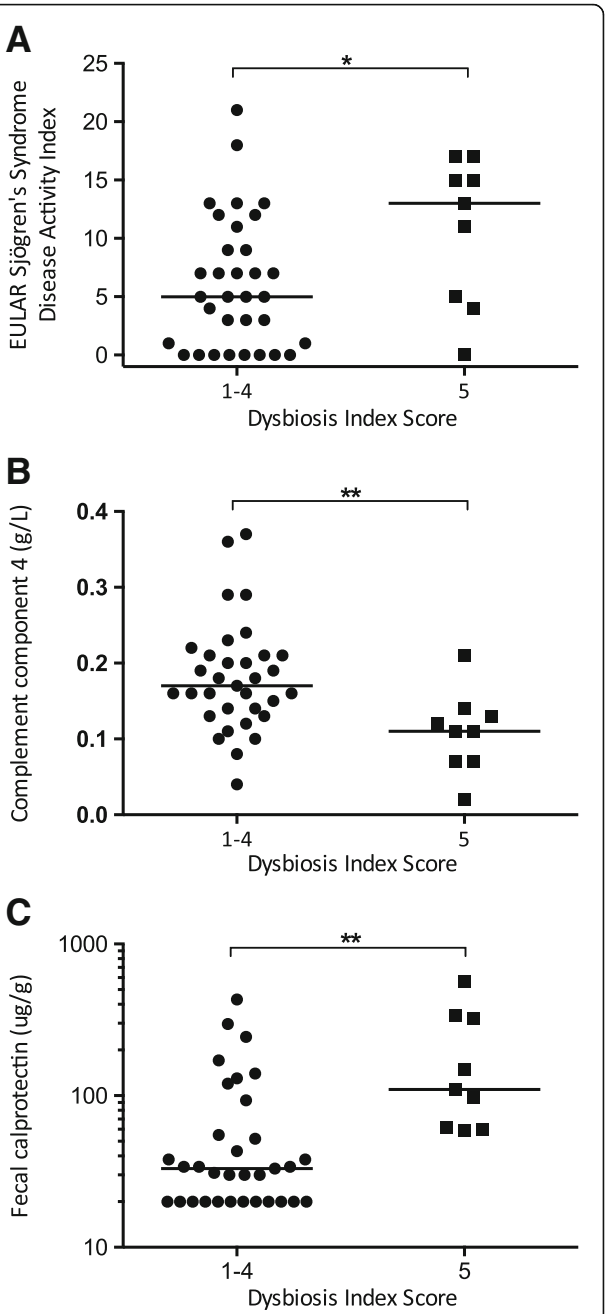

Fig. 2 Disease activity (**a**), hypocomplementemia (**b**), and intestinal inflammation (**c**) in pSS subjects with and without severe dysbiosis, defined as a Dysbiosis Index Score 5. Patients with intestinal dysbiosis have higher ESSDAI scores (**a**), lower C4 levels (**b**), and higher levels of F-calprotectin (**c**) compared to other patients. Dot plot, median values marked by a horizontal line. *$p < 0.05$, **$p < 0.005$. EULAR European League Against Rheumatism

study, signs of dysbiosis were found both in the oral cavity and in stool, and intestinal dysbiosis was associated with disease severity [16].

In the current study, we found that clinical disease activity, as evaluated by the ESSDAI and the ClinESSDAI,

Table 2 Clinical features in patients with primary Sjögren's syndrome with and without severe dysbiosis

	Dysbiosis Index Score 1–4 (*N* = 33)	Dysbiosis Index Score 5 (*N* = 9)	*p* value
Age (years)	61 (50; 68)	62 (58; 71)	0.534
ESSDAI total score	5 (1; 10)	13 (5; 16)	0.049*
ClinESSDAI total score	5 (0; 9)	12 (4; 16)	0.049*
ESSPRI total score	6 (5; 8)	7 (6; 8)	0.224
IgG (g/L)	13.2 (10.0; 17.1)	12.4 (10.2; 22.0)	0.718
Complement component 3 (g/L)	1.01 (0.90; 1.21)	0.98 (0.79; 1.10)	0.608
Complement component 4 (g/L)	0.17 (0.14; 0.21)	0.11 (0.07; 0.14)	0.004**
Anti-SS-A antibody seropositives (%)	78	76	1.000
Anti-SS-A antibody seropositives (%)	45	56	0.714
Lip biopsy—focus score ≥ 1 (%)	85	86	1.000
Irritable bowel syndrome-like symptoms (%)	39	57	0.425
Fecal calprotectin (μg/g)	33 (20; 74)	110 (61; 330)	0.001**
Using glucocorticoids (%)	27	67	0.049*
Using anti-malarials (%)	24	44	0.406
Using proton pump inhibitors (%)	33	44	0.698
Using NSAIDs (%)	39	33	1.00

Comparison of clinical characteristics in primary Sjögren's syndrome patients with severe dysbiosis (Dysbiosis Index Score 5) vs subjects with eubiosis or mild to moderate dysbiosis (Dysbiosis Index Score 1-4). Results presented as median (interquartile range) or fraction of patients with abnormal findings (%)
ClinESSDAI Clinical EULAR Sjögren's Syndrome Disease Activity Index, *ESSDAI* EULAR Sjögren's Syndrome Disease Activity Index, *ESSPRI* EULAR Sjögren's Syndrome Patient Reported Index, *EULAR* European League Against Rheumatism, *NSAID* nonsteroidal anti-inflammatory drug
*$p < 0.05$
**$p < 0.01$

as well as laboratory signs of disease activity were associated with severe intestinal dysbiosis. These findings are reminiscent of the findings in the study by de Paiva et al. [16] showing an inverse correlation between fecal microbial diversity and combined ocular and systemic disease activity in pSS patients. In our study, patients with severe dysbiosis more often required treatment with low-dose GCs, which we suggest reflects systemic disease activity, but we cannot exclude that GC treatment causes dysbiosis. Indeed, some animal studies have indicated a direct effect of GCs on the microbial balance in the intestine [33, 34]. Of interest, treatment with PPI, NSAIDs, or AMA was not associated with severe intestinal dysbiosis in our study, reminiscent of findings in previous studies [35, 36].

F-calprotectin, a marker of GI inflammation, was significantly increased in pSS patients with severe intestinal dysbiosis, which is similar to findings in patients with systemic sclerosis [21], and suggests that an imbalance in microbiota in the gut of pSS patients coincides with GI inflammation. However, a potential causality remains to be determined and it can only be speculated whether intestinal dysbiosis drives the inflammatory process in pSS or is merely a consequence of systemic disease affecting the GI tract [37]. One hypothesis, however, is that an imbalance in the gut microbiota in the genetically susceptible individual leads to an increase in T_H17 cells in the gut [38], which in turn enter the circulation and migrate to the exocrine glands, and possibly other tissues, to drive inflammation and development of both exocrine disease and EGMs in pSS.

We report low levels of bacteria from the genera *Alistipes* and *Bifidobacterium* in pSS patients. Interestingly, low levels of *Alistipes* have been reported previously in psoriatic arthritis and Crohn's disease [39, 40] and low levels of *Bifidobacterium* have been reported previously in both rheumatoid arthritis and Crohn's disease [41, 42]. It remains to be explored why these specific alterations are found in clinically disparate diseases.

In contrast to patients with elevated F-calprotectin, patients with IBS-like symptoms did not display more severe dysbiosis than other patients. Instead, severe dysbiosis was as common among these subjects as in the control group. This finding is in contrast to previous reports in comparing IBS with healthy controls. The etiology of IBS and IBS-like symptoms is unknown, but is supposed to be multifactorial. Our findings may indicate that functional bowel symptoms associated with pSS have a different etiology compared to idiopathic IBS [9, 27].

To better understand the impact and importance of intestinal dysbiosis in pSS, future studies should investigate intestinal dysbiosis in larger samples of pSS patients. Ideally, we would like to study the intestinal microbiome longitudinally and in relation to factors likely to affect the intestinal microbiota (e.g., probiotics,

prebiotics, antibiotics, specific diets, and anti-inflammatory medication). To further elucidate the important question of causality, further experimental animal studies in line with the previous publication by Paiva et al. [16] are warranted.

Conclusions

Severe intestinal dysbiosis is prevalent in pSS and associated with both clinical and laboratory markers of disease activity as well as with laboratory signs of GI inflammation.

Abbreviations

AECG: American–European Consensus Group; AMA: Anti-malarials; C3: Complement component 3; C4: Complement component 4; ClinESSDAI: Clinical EULAR Sjögren's Syndrome Disease Activity Index; DIS: Dysbiosis Index Score; DMARD: Disease-modifying anti-rheumatic drug; EGM: Extraglandular manifestations; ESSDAI: EULAR Sjögren's Syndrome Disease Activity Index; ESSPRI: EULAR Sjögren's Syndrome Patient Reported Index; EULAR: European League Against Rheumatism; F-calprotectin: Fecal calprotectin; GC: Glucocorticoid; IBD: Inflammatory bowel disease; IBS: Irritable bowel syndrome; NSAID: Nonsteroidal anti-inflammatory drug; PPI: Proton pump inhibitors; pSS: Primary Sjögren's syndrome

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Availability of data and materials

The datasets of the current study are available from the corresponding author on reasonable request.

Authors' contributions

TM and KA designed and organized the study, acquired and analyzed data, and prepared the manuscript. JM, PO, and BO contributed to analyzing data and reviewing the manuscript for intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Regional Ethics Review Board, Lund, Sweden (LU 2011/596). All patients and controls gave written informed consent according to the declaration of Helsinki.

Consent for publication

Not applicable.

Competing interests

The authors of the study declare that they have no competing interests.

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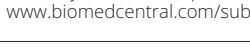

The Mucosal Antibacterial Response Profile and Fecal Microbiota Composition Are Linked to the Disease Course in Patients with Newly Diagnosed Ulcerative Colitis

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Background: The clinical disease course of ulcerative colitis (UC) varies substantially between individuals and can currently not be reliably predicted. The gut microbiota and the host's immune defense are key players for gut homeostasis and may be linked to disease outcome. The aim of this study was to determine fecal microbiota composition and mucosal antibacterial response profile in untreated patients with newly diagnosed UC and the impact of these factors on disease course.

Methods: Stool samples and intestinal biopsies were obtained from therapy-naive newly diagnosed patients with UC. Patients were defined to have mild or moderate/severe disease course assessed by disease activity during the 3 years follow-up. Fecal microbiota was analyzed by the GA-map Dysbiosis test (n = 18), and gene expression in intestinal biopsies was analyzed by RT^2 Profiler polymerase chain reaction array (n = 13) and real-time polymerase chain reaction (n = 44).

Results: At the time of diagnosis of UC, the fecal microbiota composition discriminated between patients with mild versus moderate/severe disease course. Also, the mucosal antibacterial gene expression response profile differed between patients with mild versus moderate/severe disease course with bactericidal/permeability-increasing protein (BPI) being most important for the discrimination. Mucosal bactericidal/permeability-increasing protein gene expression at diagnosis was higher in patients with mild versus moderate/severe disease course when confirmed in a larger patient cohort ($P = 0.0004$, n = 44) and was a good predictor for the number of flares during the 3 years follow-up ($R^2 = 0.395$, $P < 0.0001$).

Conclusions: In patients with newly diagnosed UC, fecal microbiota composition and mucosal antibacterial response profile, especially bactericidal/permeability-increasing protein, are linked to disease course.

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Key Words: ulcerative colitis, antibacterial response, microbiota

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) characterized by mucosal inflammation of the colon. Disease extent, from localized to extensive, and disease course, from mild to aggressive, are highly variable between patients. Some studies have categorized patients into different groups according to the disease course: prolonged remission (decline in symptoms after initial flare), increasing severity (after initial low activity), chronic intermittent disease, and chronic continuous disease.[1,2] Among these, prolonged remission and chronic intermittent disease were the most prevalent.[2]

There have been attempts to identify biomarkers predicting disease outcome at the time for diagnosis, and a factor associated to a more severe disease outcome is young age (<40 yrs) at diagnosis.[3] In contrast to this, older age (>40 yrs) has also been identified as a marker for a more aggressive disease course.[4] In the 10-year follow-up of the IBSEN study, relapse rate was lower in

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patients diagnosed at >50 years of age as compared with <30 years of age; also extensive colitis, elevated erythrocyte sedimentation rate, anemia, and fever at diagnosis were associated with an increased risk of colectomy.[2] Other factors associated to severe disease course have been extensive disease[4,5] and high serum interleukin 17A levels[6] at diagnosis. Further variables, such as serologic, fecal, and genetic markers, have been evaluated without sufficient predictive ability.[7]

Recently, the role of the microbiota in the pathogenesis of various intestinal diseases has been assessed and patients with UC demonstrate dysbiosis with reduced microbial diversity.[8–10] In close interplay with the microbiota is the anti-inflammatory response of the host, orchestrated by innate immune cells and cells lining the gut epithelium. These cells express different antimicrobial peptides to keep bacteria secluded from the epithelial lining. Among the antimicrobial peptides, bactericidal/permeability-increasing protein (BPI) is one of our most potent natural antibiotics and is produced not only by epithelial cells[11] but also by neutrophils during inflammation.[12] BPI is a small cationic peptide with high affinity for lipopolysaccharide (LPS) from the cell wall of gram-negative bacteria.[13] Apart from its bactericidal and neutralizing abilities, it also minimizes the inflammatory response of the host by directing BPI-coated gram-negative bacteria and free LPS to neutrophils for removal via phagocytosis, thus circumventing the pro-inflammatory CD14/TLR4 activation in macrophages.[14,15]

Increased mucosal levels of BPI have been reported previously during inflammation in UC.[16,17] It has also been shown that patients with UC have increased serum levels of antineutrophil cytoplasmic antibodies against BPI, which may hamper the antimicrobial effects.[18,19]

The unpredictable disease course of UC at disease onset prevents individualized treatment, which is currently not a valid option in clinical practice. In this study, we hypothesized that the fecal microbiota and the host's innate immune response at the time of diagnosis are associated with the disease course. Therefore, we determined fecal microbiota composition and mucosal antibacterial response pattern in patients with newly diagnosed UC. Data obtained were correlated to the disease outcome during 3 years follow-up in patients defined as having a mild or a moderate/severe disease course.

MATERIALS AND METHODS

Study Subjects and Sample Specimens

Forty-eight patients with newly diagnosed UC among patients referred to the inpatient and outpatient clinics and the endoscopy units at Sahlgrenska University Hospital (Gothenburg, Sweden) and Södra Älvsborgs Hospital (Borås, Sweden) were included into the study. The inclusion criteria of the study were newly diagnosed patients with UC (18–75 yrs), based on endoscopic and histological findings, and without medical treatment for IBD. Exclusion criteria were other severe diseases, such as heart, lung, or neurological disease, active malignancies, and antibiotic use during the month before inclusion. The extent of the disease and the endoscopic disease activity were established with colonoscopy or sigmoidoscopy. The disease activity was determined by Mayo score, which included the score from the endoscopic examination.[20] The extent of disease was classified into proctitis, left-sided colitis, or extensive colitis (beyond the left colonic flexure) according to the Montreal classification.[21] Serum and biopsy samples were obtained from all patients (n = 48) and stool samples from 18 patients. Serum samples were stored at −80°C. Biopsies were collected in RNAlater (Ambion, Austin, TX) and kept at −80°C until RNA extraction or in Histocon (Histolab Products AB, Göteborg, Sweden) and subsequently placed in plastic forms (Cryomold; Miles Inc., Elkhart, IN) filled with O.C.T. Compound (Miles Inc.), snap frozen in isopentane in liquid N_2 for approximately 60 seconds, and then stored at −80°C until processed further. Stool samples were stored at −20°C until analysis.

Additionally, rectal biopsies were obtained from 7 individuals undergoing colonoscopy for other reasons than inflammation (polyps, weight loss). In addition, 4 healthy subjects were recruited for in vitro analysis of peripheral blood mononuclear cells (PBMCs). None of the noninflamed subjects in the study were taking any medications known to affect the gastrointestinal tract, gut microbes, or the immune system.

Subgrouping of Patients with Newly Diagnosed UC

From the time point of diagnosis of UC, patients were followed clinically for 3 consecutive years, during which the disease severity and the colonic disease extent were assessed yearly. The patients were defined to have a *mild disease course* or a *moderate/severe disease course* based on the numbers and severity of flares during the years (excluding the flare at diagnosis). A flare was defined as an episode of at least 2 weeks where symptoms exceeded baseline severity of symptoms and the majority of flares (>95%) persisted for 2 to 8 weeks. The severity of the flares were graded as mild, moderate, or severe based on the physician's global assessment with the help of symptoms (stool frequency, rectal bleeding) and, if existing, endoscopy. All patients with ≤2 flares and patients with 3 to 4 flares of no more than mild severity grade during the years were defined to have a *mild disease course*. All patients with ≥5 flares and patients with 3 to 4 flares where any of the flares had a severity grade of moderate or severe during the years were defined to have a *moderate/severe disease course*.

Microbiota Analysis

Microbiota analysis of fecal samples was performed using the GA-map Dysbiosis test (Genetic Analysis AS, Oslo, Norway). The GA test is based on molecular biology techniques, comprising human fecal sample homogenization and mechanical bacterial cell disruption; automated total bacterial genomic DNA extraction using magnetic beads; 16S rRNA polymerase chain reaction (PCR) DNA amplification covering V3–V9; probe labelling by single nucleotide extension; hybridization to complementary probes coupled to magnetic beads; and signal detection using

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BioCode 1000A 128-Plex Analyzer (Applied BioCode, Santa Fe Springs, CA).[10] The GA test consists of 54 DNA probes targeting ≥300 bacteria on different taxonomic levels and generates a bacterial profile based on 15 different bacteria (defined by Genetic Analysis AS): *Ruminococcus albus/bromii*, *Ruminococcus gnavus*, *Faecalibacterium prausnitzii*, *Lactobacillus*, *Streptococcus sanguinis* and *Streptococcus salivarius thermophilus*, *Dialister invisus*, *Akkermansia muciniphila*, *Bacteroides fragilis*, *Alistipes*, *Shigella/Echerichia*, *Bifidobacterium*, *Bacteroides/Prevotella*, *Firmicutes* (*Bacilli*), *Firmicutes* (*Clostridia*), and *Proteobacteria*. The model algorithmically assesses fecal bacterial abundance and profile, and potential clinically relevant deviation in the microbiome from normobiosis and the output is a bacterial profile and a Dysbiosis index score. Dysbiosis indexes >2 (maximum 5) indicates a microbiota that differs from the healthy reference group.

Messenger RNA Extraction from Mucosal Biopsies

Total messenger RNA (mRNA) from mucosal biopsies was extracted using Nucleospin DNA, RNA, and protein purification Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. RNA concentration and purity were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware) with 260/280 and 260/230 ratios of ~2 and 2.1 to 2.2, respectively. Samples from 4 out of 48 patients had insufficient RNA quantity for the study.

RT^2 Profiler PCR Array

Complementary DNA was prepared using the RT^2 First Strand Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The RT^2 Profiler PCR array for "Antibacterial response" (PHAS-148Z; Qiagen) was analyzed in a CFX384 Touch Real-Time (RT) PCR Detection System (BioRad, Hercules, CA) by the use of RT^2 qPCR SYBR Green MasterMix (Qiagen). Data were analyzed in the CFX Manager software (BioRad) and the RT^2 Profiler PCR Array Data Analysis version 3.5 (Qiagen). All samples except two passed the quality checks for PCR Array reproducibility, RT efficiency, and genomic DNA contamination. *GAPDH* and *HPRT1* were chosen as housekeeping genes. A complete list of the genes in the array is shown in Table 1, Supplemental Digital Content 1, http://links.lww.com/IBD/B501.

Quantitative RT-PCR Analysis

Complementary DNA was prepared using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. Complementary DNA was then used for RT-PCR using Taqman Universal PCR MasterMix (Applied Biosystems, Foster City, CA) and Taqman Gene Expression assays (Applied Biosystems) according to the manufacturer's protocol. Expression of *BPI* (Hs01552756_m1), conserved helix-loop-helix ubiquitous kinase (*CHUK*, Hs00989502_m1), and chemokine (C-X-C motif) ligand 2 (*CXCL2*, Hs00601975_m1) was determined. Amplification was carried out using a 7500 RT-PCR system (Applied Biosystems), and all samples were run in triplicate. The results were normalized to the expression level of *GAPDH* (Hs03929097_g1) and *HPRT* (Hs02800695_m1) and expressed as $2^{-(\text{Target-Housekeeping})}$.

PBMC Cultivation Assay and Flow Cytometry Analysis

PBMCs were isolated from heparinized venous blood from healthy volunteers by density gradient centrifugation using Ficoll-Paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Cells were cultured in Iscove's medium supplemented with 100 μg/mL gentamicin, 3 μg/mL L-glutamine (all from Sigma-Aldrich, St Louis, MO), and 10% fetal bovine serum (Gibco by Thermo Fisher Scientific, Waltham, MA). Cell cultures from each subject were stimulated with a final concentration of 1 ng/mL LPS (Sigma-Aldrich) or 10 μg/mL peptidoglycan (tlrl-pgnb2; Invivogen, San Diego, CA) with and without addition of 0.02, 0.2, or 2 μg/mL BPI (SRP6307; Sigma-Aldrich) in flat-bottomed 96-well plates (Nunc, Roskilde, Denmark). Before addition to the cells, LPS or peptidoglycan were preincubated with or without BPI at 37°C for 15 minutes. The preincubation step was performed at 10× concentration in a total volume of 20 μL 0.1 M citric acid buffer (pH 5.5). Subsequently, 180 μL supplemented media was added to generate a 1× solution, the mixture was added to the cells, and the plates were incubated for 18 hours at 37°C. Cell culture supernatants were collected and stored at −80°C at the end of the cell culture. Cultured cells were stained for flow cytometry analysis using the following antibodies: anti-HLADR-Alexa700, anti-CD14-PeCy7, anti-CD80-APC, lineage markers (anti-CD3-PE-CF594, anti-CD19-PE-CF594, anti-CD56-PE-CF594) (all from BD Biosciences, San Jose, CA). 7-Aminoactinomycin D (BD Biosciences) was used according to the manufacturer's protocol to exclude nonviable cells. Flow cytometry analysis was performed using an LSR II flow cytometer (BD Pharmingen, San Jose, CA). The data were analyzed using Flow Jo software (Treestar Inc., Ashland, OR).

Analysis of CXCL2 Protein and Anti-BPI antibodies

Serum samples were analyzed by enzyme-linked immunosorbent assay for levels of CXCL2 protein (Human MIP2 Enzyme-Linked Immunosorbent Assay Kit; Abcam, Cambridge, MA) and anti-BPI-IgG antibodies (Demeditec Diagnostics GmbH, Kiel, Germany) according to the manufacturers' instructions. In vitro PBMC cultivation supernatants were analyzed for levels of CXCL2 protein (Abcam).

Immunohistochemistry

Frozen sections were prepared (7 μm) on microslides with a cryostat (Leitz, Wetzlar, Germany), fixed in 100% ice-cold acetone, blocked in phosphate-buffered saline with 1% bovine serum albumin and 5% goat serum, and stained with polyclonal rabbit-anti-BPI-IgG (PA5-26069, diluted 1:200; Thermo Fisher Scientific) followed by goat-anti-rabbit-AlexaFluor488 (Molecular Probes A11008, diluted 1:50; Invitrogen). Mounting was performed with 4′, 6-Diamidino-2-Phenylindole Dihydrochloride (DAPI)-UltraCruz mounting medium (sc-24941; Santa Cruz Biotech,

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Santa Cruz, CA). Fluorescence was visualized on a Zeiss AX10 Imager Z2 microscope at ×20 magnification.

Data Analyses

To examine the relationship between patients with mild and moderate/severe disease course (Y variable) and various mRNA levels or bacteria (X variables), multivariate factor analysis (SIMCA-P+ software; Umetrics, Umeå, Sweden) was used. Orthogonal partial least squares discriminant analyses (OPLS-DA) were implemented to correlate a selected Y variable and multiple X variables with each other in linear multivariate models. The quality of OPLS-DA was based on the parameters R2, i.e., the goodness of fit of the model (values of ≥0.5 define good discrimination, best possible fit, R2 = 1), and Q2, i.e., the goodness of prediction of the model (values of ≥0.5 define high predictive ability). In the OPLS-DA loading scatter plots, each X variable is shown in relation to Y. The X variables positioned furthest to the left or right are more closely related to the respective Y variable and thus contribute most to the model.

The Mann–Whitney U test and the chi-square test were used to evaluate differences between 2 groups, Spearman's rank correlation coefficient was used to test the association between 2 variables, and Friedman's test was used to test the difference between several related samples. Linear regression analyses were used to model relationships between a dependent variable and explanatory variables. All values were logarithmized before use in regression analyses. Validation of the regression analyses was performed for (1) normally distributed residuals, (2) homoscedasticity of residuals, (3) linearity of regression function, (4) independence of error terms (Durbin–Watson test), and (5) outlier observations. Defined by Cook's distance and leverage values, data from one patient were considered as being an outlier and were excluded from further analyses.

All statistical analyses were performed using IBM SPSS Statistics 23; P values <0.05 were considered statistically significant. Data are shown as median (range) or median (interquartile range), as defined in the text. Power analysis to estimate size of patient cohorts was not included in the experimental design because it was an exploratory study.

Ethical Considerations

The study was performed after receiving written informed consent from all subjects, and the protocol was approved by the Regional Ethical Review Board at the University of Gothenburg. Evaluators of the disease course were blinded to laboratory data, and evaluators of the laboratory analyses were blinded to the disease course of the individual patients.

RESULTS

Clinical and Demographic Characteristics of Patients with Newly Diagnosed UC

Among the 48 patients with newly diagnosed UC, 19 were defined to have a mild disease course and 29 were defined to have a moderate/severe disease course during the 3 years follow-up, according to our definition. Among the patients with a mild disease course, 10 patients had ≤2 flares, 4 patients had 3 flares, and 3 patients had 4 flares during the 3 years and all flares were graded as mild. Among the patients with a moderate/severe disease course, 5 patients had 3 flares, 4 patients had 4 flares, and 20 patients had ≥5 flares during the 3 years and 1 patient was colectomized during year 3. The patient groups showed no differences in gender distribution, age, smoking habits, disease extension, total Mayo score, endoscopic Mayo score, body mass index, C-reactive protein, or calprotectin at the time of UC diagnosis (Table 1).

There was a strong agreement between the definitions of a mild or a moderate/severe disease course concerning medical care for gastrointestinal symptoms and medical therapy during the 3 years follow-up. Patients with a moderate/severe course were more health care seeking, more often hospitalized, and more frequently used 5-aminosalicylic acid and thiopurines during years 1, 2, and 3 as compared with the mild disease group (Table 2). Corticosteroid use was more frequent for patients with a moderate/severe disease course during year 1, whereas during years 2 and 3, corticosteroid use was similar between the groups (Table 2).

Fecal Microbiota Composition in Patients with Newly Diagnosed UC Is Associated with Disease Course

To investigate if the fecal microbiota was associated with disease course, the microbial composition of stool samples

TABLE 1. Demographics of the Newly Diagnosed Patients with UC

	Mild Disease Course[a]	Moderate/Severe Disease Course[b]	P
Total no. patients	19	29	
Male/female	11/8	16/13	0.85[c]
Age, median (range)	39 (22–60)	32 (21–54)	0.32[d]
Smoking (yes/no)	3/16	7/22	0.49[c]
Mayo score, median (range)	9 (3–11)	8 (4–12)	0.58[d]
Endoscopic Mayo score, median (range)	2 (1–3)	2 (1–3)	0.72[d]
Disease extension, extensive/left sided/ proctitis	12/5/2	14/12/3	0.55[c]
BMI	22.1 (20.0–31.1)	22.9 (17.8–33.4)	0.63[d]
CRP, median (range)	7 (3–109)	5 (3–114)	0.90[d]
Calprotectin, μg/g	1550 (130–4560)	560 (30–10,320)	0.22[d]

[a]Patients with ≤2 flares and patients with 3 to 4 flares of no more than severity grade 1 during the years.
[b]Patients with ≥5 flares and patients with 3 to 4 flares where any of the flares had severity grade ≥2 during the years.
[c]Chi-square test.
[d]Mann–Whitney U test.
BMI, body mass index; CRP, C-reactive protein.

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TABLE 2. Health Care Use and Medical Treatment During the 3 Years Follow-up

	Mild Disease Course (n = 19)	Moderate/Severe Disease Course (n = 29)
Health care seeking during the year[a], n (%)		
Year 1 (≥4 times/1–3 times)	1 (5)/5 (26)	10 (34)/10 (34)
Year 2 (≥4 times/1–3 times)	0 (0)/2 (11)	3 (10)/14 (48)
Year 3 (≥4 times/1–3 times)	0 (0)/5 (26)	2 (7)/14 (48)
Hospitalized during the year[a], n (%)		
Year 1	2 (11)	9 (31)
Year 2	0 (0)	1 (3)
Year 3	0 (0)	2 (7)
5-ASA use during the year[b], n (%)		
Year 1	14 (74)	24 (83)
Year 2	12 (63)	25 (86)
Year 3	11 (59)	24 (83)
Thiopurine use during the year, n (%)		
Year 1	0 (0)	4 (14)
Year 2	0 (0)	5 (17)
Year 3	0 (0)	11 (38)
Corticosteroid use during the year[b], n (%)		
Year 1	1 (5)	7 (24)
Year 2	1 (5)	3 (10)
Year 3	2 (11)	1 (3)

[a]For gastrointestinal symptoms.
[b]Excluding treatment during the first flare.
5-ASA, 5-aminosalicylic acid.

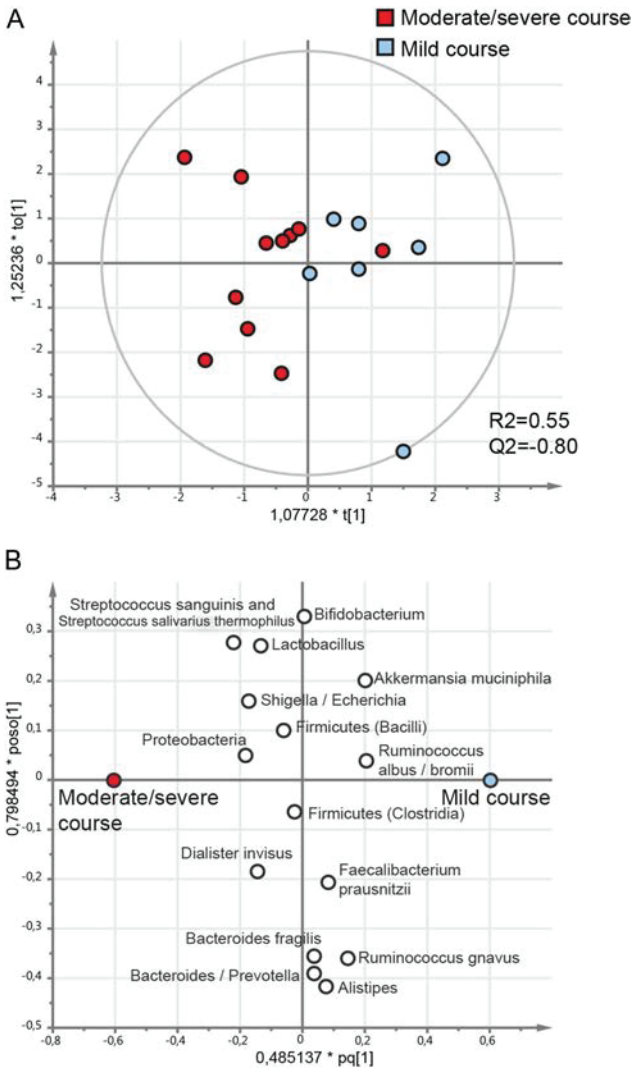

FIGURE 1. Patients with a mild and a moderate/severe disease course differ at diagnosis of disease with respect to their fecal microbiota composition. Fecal samples obtained from patients with newly diagnosed UC were analyzed by the GA-map Dysbiosis test (mild disease n = 7, moderate/severe disease n = 11). A, Multivariate discriminant analysis (OPLS-DA) scatter plot showing separation between patients with mild disease course (blue circles) and moderate/severe disease course (red circles). R2 defines the goodness of fit and Q2 the goodness of prediction. B, OPLS-DA loading scatter plot showing associations between fecal bacterial groups and disease outcome. The multivariate analysis was performed with bacterial groups as X variables (n = 15) and disease course (mild disease and moderate/severe disease) as Y variables.

obtained from 18 patients with newly diagnosed UC was analyzed using the GA-map Dysbiosis test. Stool samples obtained at the time of UC diagnosis were provided by 7 patients who had a mild disease course and 11 patients with a moderate/severe disease course during the 3 years follow-up. All patients, except one, had dysbiosis (Dysbiosis index > 2), but no differences in Dysbiosis index were found between patients with a mild and a moderate/severe disease (median [range], 3 [3–5] versus 5 [2–5], $P = 0.30$). Multivariate analysis revealed that fecal microbiota composition at the time of UC diagnosis differed between patients who had a mild versus a moderate/severe disease course, including the total set of bacteria (Fig. 1A). The model showed a good R2 value (0.55), defining a good separation between the groups, but a low Q2 value (−0.80), showing low predictive ability.

The OPLS-DA loading scatter plot showed that no single bacteria at the diagnosis of disease defined either patients with a mild disease course or patients with a moderate/severe disease course because all X variables (bacteria) were distributed relatively close to the mid-vertical line (Fig. 1B). Nevertheless, a bacterial composition with higher abundance of *Proteobacteria* and *Streptococcus* was seen in patients with a moderate/severe disease course, whereas a composition including higher abundance of *Ruminococcus* and *Akkermansia* was demonstrated in patients with a mild disease course (Fig. 1B). Together, these data suggest that there are differences in the fecal microbial composition, but without strong prognostic value, between patients with newly diagnosed UC who will have a mild or a moderate/severe disease course over a 3-year follow-up period.

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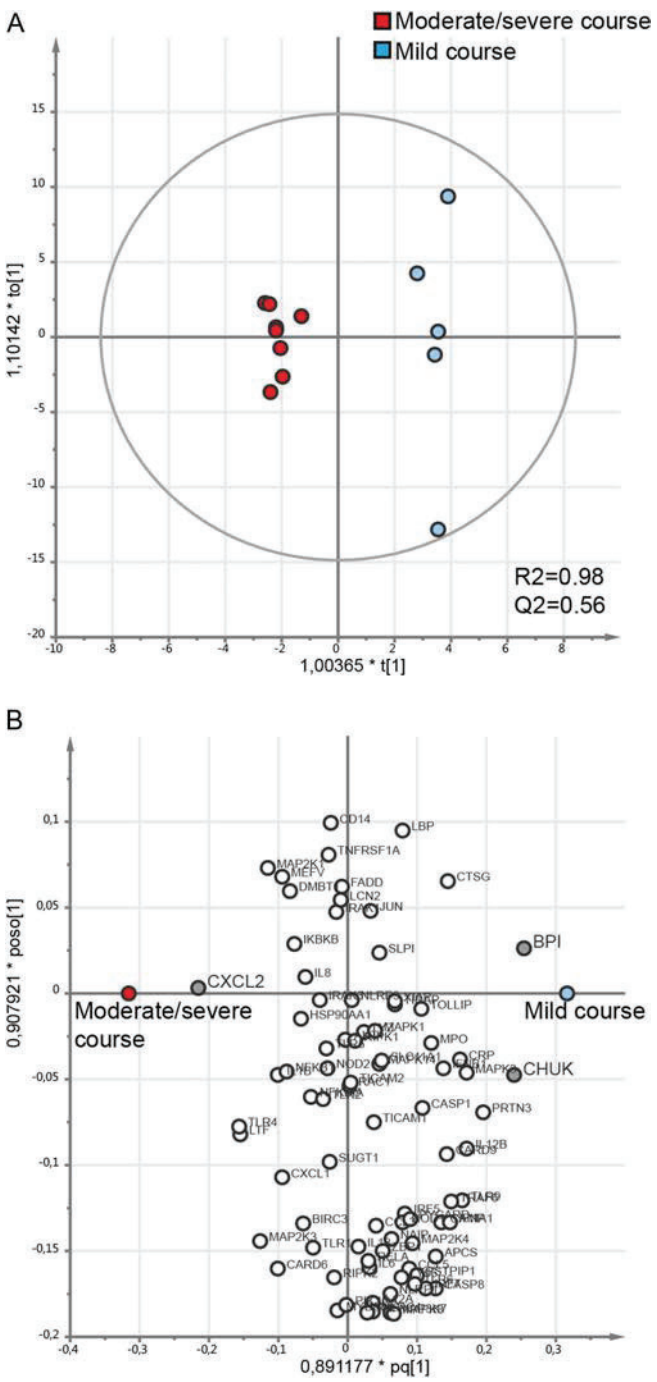

FIGURE 2. Patients with a mild and a moderate/severe disease course display different mucosal antibacterial response profiles at diagnosis of disease. Rectal mucosal biopsies obtained from patients with newly diagnosed UC were analyzed using a PCR array for 84 genes involved in antibacterial response (mild disease n = 5, moderate/severe disease n = 8). A, OPLS-DA scatter plot showing the separation between patients with mild disease course (blue circles) and moderate/severe disease course (red circles). R2 defines the goodness of fit and Q2 the goodness of prediction. B, OPLS-DA loading scatter plot depicting the association between disease course and antibacterial gene expression. The multivariate analysis was performed with antibacterial response gene expression as X variables (n = 84) and disease course (mild disease and moderate/severe disease) as Y variables. Gene expression was normalized to the housekeeping genes *GAPDH* and *HPRT*.

Mucosal Antibacterial Response Profile in Patients with Newly Diagnosed UC Is Associated with Disease Course

The composition of the gut microbial milieu develops in symbiosis with the host's innate immune response. Thus, an exploratory mRNA array was performed to study the mucosal antibacterial response profile in biopsies obtained from the patients at diagnosis. Samples from the patients with gut microbiota profiling were intended for inclusion in these analyses, but 2 samples did not pass quality control for the array (mild n = 1, moderate/severe n = 1) and 3 samples had insufficient RNA quantity (mild n = 1, moderate/severe n = 2), which resulted in 13 patients for these analyses. The overall mucosal antibacterial response profiles at the time of UC diagnosis showed excellent discrimination and high predictive ability when comparing patients with a mild and a moderate/severe disease course, respectively (Fig. 2A, R2 = 0.98, Q2 = 0.56). The most important nominators for the discrimination between the groups were higher expression of *CXCL2* for patients with a moderate/severe disease course, whereas patients with a mild disease course had higher expression of *BPI* and *CHUK* (Fig. 2B). Among the 84 genes, 25 had a variable influence on projection larger than 1 and were thus the most important genes for the explanation of the different mucosal antibacterial response profiles between the 2 patient groups (see Fig. 1, Supplemental Digital Content 2, http://links.lww.com/IBD/B502). A multivariate factor analysis combining data from fecal microbiota and mucosal antibacterial response (X variables, n = 99) and disease course (Y variable, mild n = 5, moderate/severe n = 8) did not result in an improved discrimination between the groups (R2 = 0.89, Q2 = 0.16, data not shown).

Next, mucosal gene expression of *BPI*, *CXCL2*, and *CHUK* at the time of UC diagnosis was evaluated by RT-PCR in the full patient cohort (n = 44), and it was confirmed that patients with a mild disease course had higher levels of *BPI* and lower levels of *CXCL2* compared with patients with a moderate/severe disease course (Fig. 3A). In contrast, no differences in gene expression were detected when patients were subgrouped according to high (≥9) versus moderate (≤8) Mayo score (Fig. 3B) or according to disease extent at the time of UC diagnosis (Fig. 3C). *BPI* expression was also analyzed in noninflamed control patients (n = 7, median age 42 yrs; 4 males/3 females) who showed higher *BPI* gene expression compared with patients with newly diagnosed UC (n = 44) (median [interquartile range], 0.00024 [0.00020–0.00067] versus 0.00015 [0.00009–0.00021], $P = 0.01$). Protein expression of BPI was confirmed by immunohistochemistry, where BPI expression was detected in the epithelial cell layer and crypts (Fig. 4). However, no quantifiable differences in BPI protein expression could be detected by immunohistochemistry between noninflamed controls and UC patients with a mild or a moderate/severe disease course (data not shown).

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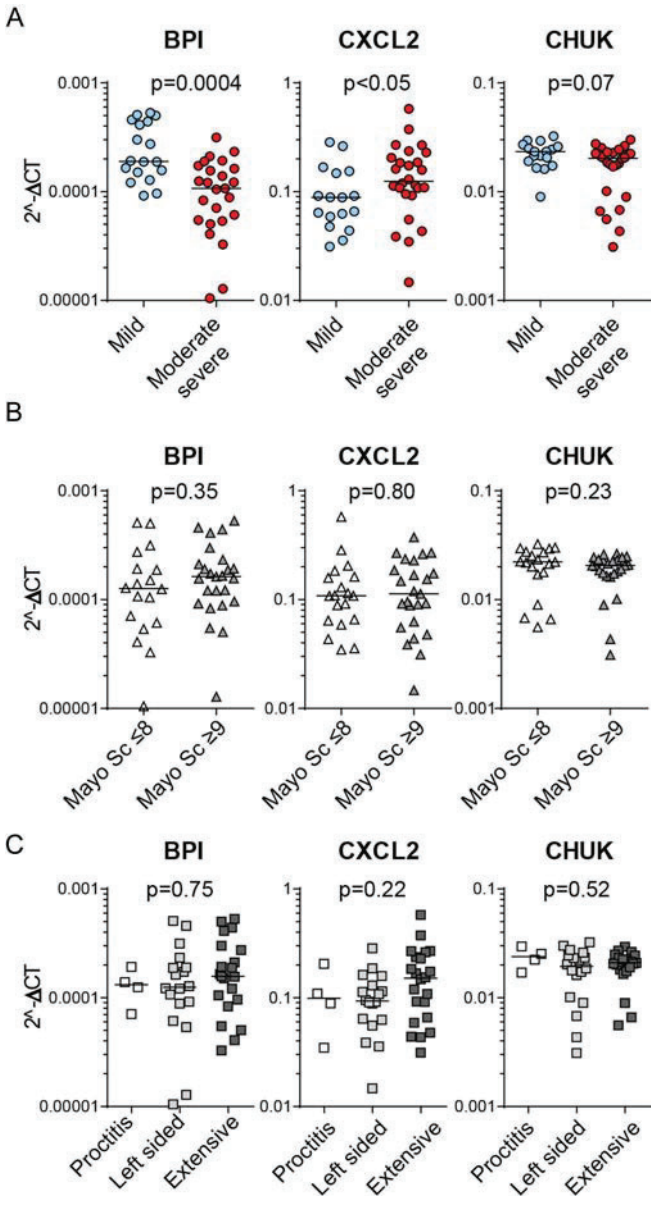

FIGURE 3. Mucosal expression of BPI, CHUK, and CXCL2 in patients with newly diagnosed UC is associated with disease course. Rectal mucosal biopsies obtained were analyzed for mRNA expression of *BPI*, *CXCL2*, and *CHUK* using RT-PCR. For patients with newly diagnosed UC, gene expression was compared between patients who presented with a mild (blue circles) and a moderate/severe disease course (red circles), respectively (A), between patients with a Mayo score of ≤8 and ≥9 (B), and between patients with proctitis, left-sided colitis, or extensive colitis (C) at diagnosis. Gene expression was normalized to the housekeeping genes *GAPDH* and *HPRT*. Each symbol represents one individual, and horizontal lines indicate median of the group (n = 44).

In patients with newly diagnosed UC, no differences were detected in levels of circulating CXCL2 protein (median [range], 612 pg/mL [196–2454] versus 458 pg/mL [159–2493], $P = 0.19$) or for BPI autoantibodies (median [range], 39 U/mL [20–510] versus 34 U/mL [23–182], $P = 0.53$) between patients with a mild and a moderate/severe disease course.

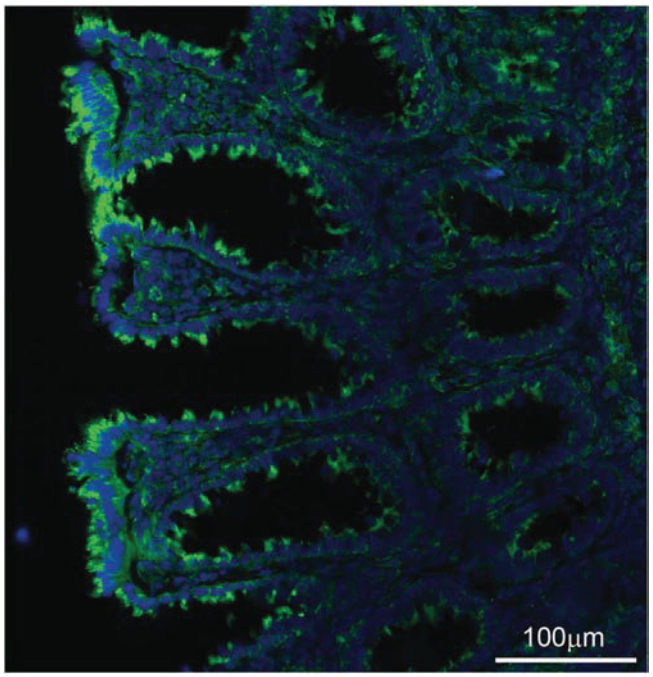

FIGURE 4. Localization of BPI in the epithelial cell layer and crypts. Biopsy specimens from rectum stained with polyclonal rabbit-anti-BPI-IgG followed by goat-anti-rabbit-AlexaFluor488. Sections were mounted with 4′, 6-Diamidino-2-Phenylindole Dihydrochloride (DAPI) (chromosome stain) and visualized at ×20 magnification, green = BPI stain, blue = nuclear stain (DAPI). A representative staining from a patient with newly diagnosed UC is shown.

BPI Decreases Release of CXCL2 from LPS-stimulated Monocytes

Because higher mucosal expression of *BPI* and lower mucosal expression of *CXCL2* in patients with newly diagnosed UC were associated with the disease course during the 3 years follow-up, the link between BPI and CXCL2 was investigated in a set of in vitro experiments. BPI dose dependently inhibited CXCL2 release from LPS-stimulated monocytes from healthy donors (n = 4, median age 44 yrs; 3 males/1 female) (Fig. 5A, left). In addition, the expression of the costimulatory molecule CD80 was decreased with increasing concentrations of BPI (Fig. 5A, right). No effects of BPI on CXCL2 release could be detected when monocytes were stimulated with peptidoglycan with and without BPI (Fig. 5B). Taken together, this indicates that high levels of BPI can reduce CXCL2 expression and aid in dampening the immune response to gram-negative bacteria.

Mucosal BPI Expression as a Predictor for the Number of Flares During the 3 Years Follow-up

To determine the relationship between mucosal antibacterial gene expression in patients with newly diagnosed UC and the

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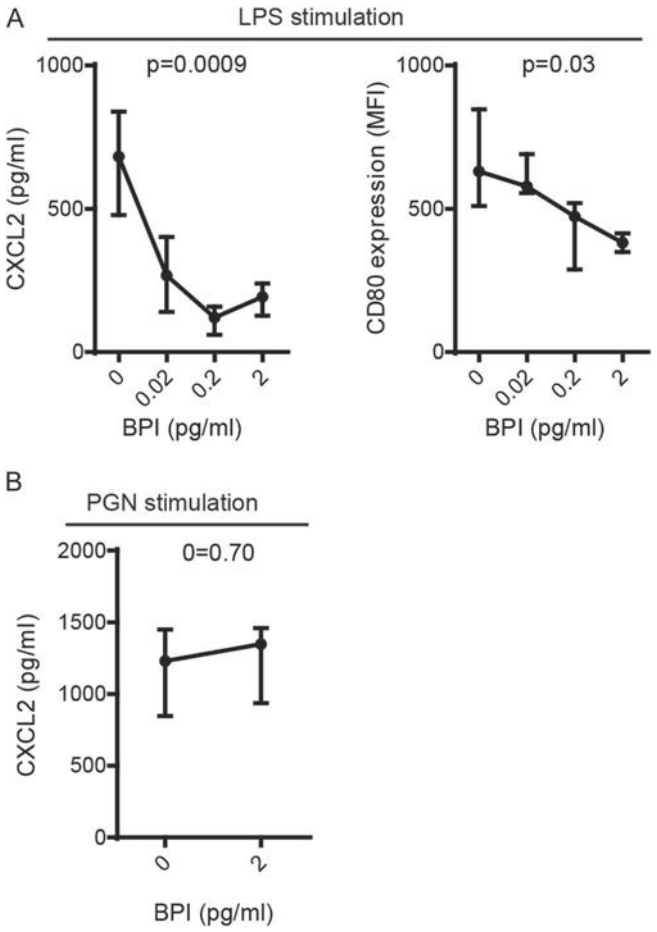

FIGURE 5. BPI reduces CXCL2 secretion from monocytes during LPS stimulation. PBMCs from healthy controls were stimulated with (A) LPS or (B) peptidoglycan (PGN) without and with increasing concentrations of BPI for 18 hours. Secretion of CXCL2 into the media was determined by enzyme-linked immunosorbent assay. Median fluorescent intensity (MFI) of CD80 was determined by FACS analysis on monocytes defined as 7-aminoactinomycin $D^-lin^-HLADR^+CD14^+$ cells. Data show median (range) (n = 4).

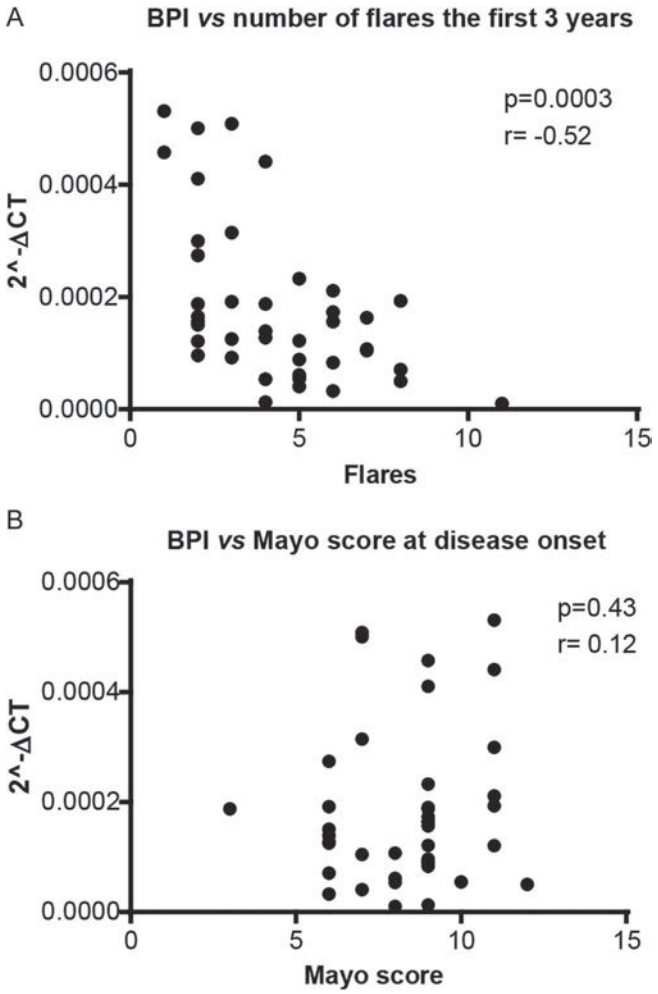

FIGURE 6. Mucosal gene expression of BPI in patients with newly diagnosed UC correlates to the number of flares during the 3 years follow-up. Rectal mucosal biopsies obtained from patients with newly diagnosed UC (n = 44) were analyzed for mRNA expression of *BPI* using RT-PCR. Gene expression was correlated to the total number of flares during the 3 years follow-up (A) and Mayo score at diagnosis of disease (B). Gene expression was normalized to the housekeeping genes *GAPDH* and *HPRT*. Each symbol represents one individual.

number of flares during the 3 years follow-up, correlation and linear regression analyses were performed. Mucosal *BPI* expression at the time of UC diagnosis was negatively correlated to the number of flares (Fig. 6A), whereas *CXCL2* and *CHUC* did not correlate with the number of flares ($r = 0.26$, $P = 0.087$, and $r = -0.27$, $P = 0.082$, respectively) during the 3 years follow-up. Furthermore, no correlations were detected between mucosal expression of *BPI* and Mayo score (Fig. 6B) or fecal calprotectin levels ($r = 0.06$, $P = 0.79$) at the time of UC diagnosis.

Linear regression analyses identified *BPI* as a strong predictor, *CHUK* as a weak predictor, and *CXCL2* without predictive ability at the time of UC diagnosis for the number of flares during the 3 years follow-up (Table 3). Multiple linear regression analyses combining the parameters did not result in a better fit of the model, probably because of collinearity as judged by Eigenvalues of <0.005 and condition indexes of >25 when combining more than one independent variable. As defined by the highly significant R^2 value (Table 3), almost 40% of the variance in the number of flares during the 3 years follow-up could be explained by the mucosal *BPI* expression at diagnosis of disease. Thus, the relation between the predictor ($x = BPI$) and the outcome ($y =$ number of flares during the 3 years follow-up) can be calculated by the regression equation:

$$y = -1.119 - 0.439\ (x)$$

DISCUSSION

In this study, we have shown that the mucosal antibacterial response profile and the fecal microbiota composition differed

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TABLE 3. Linear Regression Analysis of the Association Between BPI, CXCL2, and CHUK with the Number of Flares the Coming 3 Years in the Newly Diagnosed Patient Cohort[a]

	R^2	β	*P*
BPI	0.395	−0.621	<0.0001
CXCL2	0.045	0.212	0.173
CHUK	0.110	−0.332	0.029

[a]Defined by Cook's distance and leverage values, one patient was considered as outlier and was excluded from analyses.

between untreated newly diagnosed UC patients with a mild and a moderate/severe disease course. Furthermore, the mucosal antibacterial response profile, but not the fecal bacterial microbiota composition, had high predictive ability when comparing patients with a mild and a moderate/severe disease course. Additionally, the mucosal gene expression of *BPI* was found to be a good predictor of the disease course; the higher the mucosal expression of *BPI* at the time of UC diagnosis, the fewer flares during the 3 years follow-up.

The fact that we coexist with our microbiota is well established, and it is also known that numerous diseases, including IBD, are associated with an altered microbiota of the gut.[22–24] Whether this is the cause or the consequence of the disease and how the microbiota is affected by disease duration and various treatments are currently unknown. To correct for treatment as a confounding factor, we decided to analyze samples from patients with newly diagnosed UC naive to IBD-related medical treatment. Disease duration is more difficult to correct for as patients may have had their symptoms during varying periods of time before seeking health care, but at least all patients had their first flare needing medical care. The fecal microbial composition analysis was limited by the low number of fecal samples obtained, but even though predictability was low, substantial differences between the groups were detected. The microbiota composition has been analyzed previously for treatment-naive UC patients; in the IBSEN II study, untreated UC patients showed only minor fecal microbial differences compared with a noninflamed control group,[25] whereas Shah et al[26] reported that the mucosal microbiome in untreated pediatric patients with UC showed a decrease of *Verrucomicrobia* at the phylum level and *Roseburia* at the genus level as compared with non-IBD controls. Concerning microbiota and disease status, a large pediatric cohort of Crohn's disease (the RISK cohort) defined mucosal dysbiotic features correlating with clinical disease severity.[27] In the same cohort, the interaction between the mucosal microbiota and the ileal transcriptome was analyzed resulting in a model where baseline *APOA1* expression together with *Blautia* and *Veillonella* abundance could predict the 6-month clinical outcome.[28] The microbiota data obtained in the abovementioned studies[25–27] were all based on 16S rRNA gene sequencing, whereas the analysis used here was based on a defined set of bacterial probes. Although not directly comparable, we can conclude that our data extend beyond previous findings and demonstrate that the altered fecal microbiota profile at diagnosis of disease can also be linked to the clinical outcome in patients with UC.

We were also interested in the link between the microbiota composition and the antimicrobial defense of the host and used an mRNA array to determine the mucosal antibacterial response profile. The global gene expression pattern identified clear differences in the bacterial innate immunity between patients with a mild versus a moderate/severe disease course, especially in *BPI*, *CHUK*, and *CXCL2* expression. CHUK, also known as inhibitor of nuclear factor kappa-B kinase subunit alpha (IKK-α or IKK1), is a protein kinase having an important role in the release of nuclear factor kappa-B to the nucleus.[29] Because CHUK in itself needs to be phosphorylated before exerting its effects[30] and no material for western blot analysis was available, we could not explore this finding further. Regarding CXCL2, we were able to demonstrate that efficient neutralization of LPS via BPI reduced CXCL2 secretion from monocytes. However, as compared with *BPI*, both *CHUK* and *CXCL2* turned out to be of less importance in relationship to the disease course. We are aware of the fact that gene expression may not always reflect protein expression; therefore, we also analyzed protein expression by immunohistochemistry. BPI protein expression was detected in the epithelial layer and crypts, but the method did not allow for discrimination between noninflamed controls and UC (mild and moderate/severe). For the relationship between BPI and number of flares, the regression analysis showed that mucosal *BPI* gene expression was a strong predictor for the number of flares during the 3 years follow-up and thus identifies BPI as an important component in gut homeostasis for patients with UC. It is known that BPI is an effector molecule at the mucosal surface with a high affinity (nanomolar) for LPS, acting to neutralize and circumvent cell activation by LPS in polymorphonuclear leukocytes.[31,32] Higher levels of BPI may therefore help to reduce inflammation and enhance the ability for patients to enter, and maintain, remission.

So far, no clinically validated biomarker predicting disease outcome in patients with newly diagnosed UC has been identified, irrespective of biological source (blood, intestinal biopsy, or stool sample). Here, we found that mucosal gene expression of BPI is closely linked to disease outcome, and as much as 40% of the variance in the number of flares could be explained by mucosal *BPI* gene expression. Also, via the regression equation, the numbers of flares during the 3 years follow-up can be estimated by the mucosal gene expression level of *BPI*. In contrast, circulating factors like serum CXCL2 and BPI autoantibodies did not reflect future disease activity. Antineutrophil cytoplasmic antibodies and BPI autoantibodies have been considered possible serological markers for IBD,[33,34] and we therefore speculated that high levels of BPI autoantibodies could reflect the disease course, but no differences could be detected between the groups. Presumably, it can be difficult to discover a successful circulating biomarker

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for predicting disease course of patients with IBD because the gut ecology, including the microbiota and the immune defense, is shaped and, to a large extent, maintained locally.

There are some limitations of this study, and the low numbers of fecal samples have already been mentioned. In addition, only the microbiota in fecal samples was analyzed, and mucosal microbial composition may yield different results. Furthermore, mucosal samples were only obtained from the inflamed rectum, and it would be of interest to study BPI expression throughout the colon to assess the correlation with disease extent and activity. The most important factor is, however, how a mild versus a moderate/severe disease course was defined. Others have used different strategies to subgroup patients into mild/moderate/severe or mild/relapsing groups based on therapy and incidence of colectomy,[3,4] relapse rate, need for admission/surgery and extraintestinal manifestations,[7] or numbers of relapses during the first 3 years.[35] Because it has been shown that a majority of patients with UC experience initial high activity followed by remission or disease with flares of mild severity,[2] we made use of both the number and severity of flares for subgrouping. Patients with few flares ($\leq$2) or many flares ($\geq$5) were automatically assigned to the groups of mild or moderate/severe disease course, respectively, whereas the severity of the flares was taken into account for patients with 3 to 4 flares. The follow-up concerning health care–seeking patients and medical use showed strong agreement to the group definition. Use of biological therapy was not reported because most of the patients were included before biological therapy was introduced as clinical routine in Sweden. For the regression analysis, the total number of flares during the 3 years follow-up (irrespective of flare severity) was used. Importantly, both the subgrouping of patients according to disease activity together with the number of flares and the number of flares alone highlighted the importance of BPI as a predictive factor, which strengthens the results.

In summary, we have shown that the fecal microbiota composition and the mucosal antibacterial response profile in patients with newly diagnosed UC differ for patients with a mild or a moderate/severe disease course, respectively. Also, the overall mucosal antibacterial response profiles at the time of UC diagnosis showed high predictive ability when comparing patients with a mild and a moderate/severe disease course. Most importantly, mucosal *BPI* expression is a strong predictor for total number of flares in newly diagnosed patients with UC during the first 3 years of the disease. The predictive value of BPI needs to be validated in larger cohorts of patients and during a longer follow-up period, before strongly claiming its clinical impact. However, our finding demonstrates an important relationship between a specific molecule of the innate immune system and disease outcome in patients with newly diagnosed UC.

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ORIGINAL ARTICLE

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Effects of varying dietary content of fermentable short-chain carbohydrates on symptoms, fecal microenvironment, and cytokine profiles in patients with irritable bowel syndrome

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Abstract

Background: A diet low in fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAPs) is increasingly recommended for patients with irritable bowel syndrome (IBS). We aimed to investigate the effects of a blinded low-FODMAP vs high-fructo-oligosaccharides (FOS) diet on symptoms, immune activation, gut microbiota composition, and short-chain fatty acids (SCFAs).

Methods: Twenty patients with diarrhea-predominant or mixed IBS were instructed to follow a low-FODMAP diet (LFD) throughout a 9-week study period. After 3 weeks, they were randomized and double-blindly assigned to receive a supplement of either FOS (FODMAP) or maltodextrin (placebo) for the next 10 days, followed by a 3-week washout period before crossover. Irritable bowel syndrome severity scoring system (IBS-SSS) was used to evaluate symptoms. Cytokines (interleukin [IL]-6, IL-8, and tumor necrosis factor alpha) were analyzed in blood samples, and gut microbiota composition (16S rRNA) and SCFAs were analyzed in fecal samples.

Key Results: Irritable bowel syndrome symptoms consistently improved after 3 weeks of LFD, and significantly more participants reported symptom relief in response to placebo (80%) than FOS (30%). Serum levels of proinflammatory IL-6 and IL-8, as well as levels of fecal bacteria (Actinobacteria, Bifidobacterium, and *Faecalibacterium prausnitzii*), total SCFAs, and n-butyric acid, decreased significantly on the LFD as compared to baseline. Ten days of FOS supplementation increased the level of these bacteria, whereas levels of cytokines and SCFAs remained unchanged.

Conclusions and Inferences: Our findings support the efficacy of a LFD in alleviating IBS symptoms, and show changes in inflammatory cytokines, microbiota profile, and SCFAs, which may have consequences for gut health.

KEYWORDS

FODMAPs, gut microbiota, irritable bowel syndrome, proinflammatory cytokines, short-chain fatty acids

The abstract form of this paper will be orally presented and published at the United European Gastroenterology Week (UEGW) in Vienna, 2016.

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1 | INTRODUCTION

Irritable bowel syndrome (IBS) is a functional gastrointestinal (GI) disorder, characterized by recurrent abdominal pain or discomfort and altered bowel habits, often accompanied by abdominal bloating and/or distension.[1] The etiology of IBS remains unknown, but several pathogenetic factors seem to be involved, including enhanced visceral sensitivity and altered intestinal motility. Irritable bowel syndrome is increasingly viewed as a dysfunction of the "gut-brain axis" that should be considered in a broader biopsychosocial context.[2,3]

Although about three quarters of IBS patients state that food triggers symptoms, dietary treatments have, until recently, not been a central part of therapy. There is, however, an increasing interest in using diet in the management of IBS, and emerging evidence supports the efficacy of a diet with reduced content of fermentable, short-chain carbohydrates.[4] The so-called low-FODMAP (fermentable oligosaccharides, disaccharides, monosaccharides, and polyols) diet (LFD) is a pathophysiologically based and promising approach to dietary management in IBS.[5–7] The general strategy is to avoid foods high in FODMAPs with the intention of limiting the delivery of osmotically active and readily fermentable substrates to the intestine, thereby minimizing luminal distension.[8,9] In addition, it has been proposed that the mechanisms by which symptom response is mediated, may partly be through inducing changes in the gut microbiota, in GI endocrine cells, in immune function, and/or in the intestinal barrier.[5,10–12] However, due to a lack of placebo-controlled studies and studies conducted over longer periods of time, there is a current uncertainty regarding sustained effects on symptom relief and long-term health consequences.[13] Fermentable carbohydrates provide an energy source for the GI bacteria, and fermentation products such as short-chain fatty acids (SCFAs) provide nutrition for the colonic mucosa, and interact with the immune system. Hence, a LFD might elicit alterations in the GI microbiota and its fermentation products.[14]

In the present randomized, double-blinded, placebo-controlled crossover study, we aimed to investigate the effects of a low-FODMAP vs high-fructo-oligosaccharides (FOS) diet on symptoms, immune activation, gut microbiota composition, and SCFAs in patients with IBS.

Key Points

- A low-FODMAP diet is a widespread management strategy in irritable bowel syndrome, but biological consequences are only partly known, and an important placebo effect on symptom relief has not been excluded.
- In a double-blinded interventional study, we found that restricting the ingestion of fermentable carbohydrates significantly reduced the severity of IBS symptoms, as well as additional gastrointestinal and comorbidity symptoms.
- A low-FODMAP diet reduced the levels of selected pro-inflammatory cytokines, altered gut bacterial profile, and reduced fecal levels of short-chain fatty acids, which may have consequences for gut health.

2 | MATERIAL AND METHODS

2.1 | Participants

Twenty consecutive patients with diarrhea-predominant IBS (IBS-D) or mixed IBS (IBS-M) according to Rome III criteria[1] and moderate to severe symptoms (IBS Severity Scoring System [IBS-SSS][15] score of >175) were recruited in the trial. The recruitment took place in the autumn of 2015 via the outpatient clinic at the section of gastroenterology at Haukeland University Hospital, Bergen, and through a list of patients waiting for "IBS school" participation (a 2-day interdisciplinary approach about living with IBS). Exclusion criteria comprised pregnant or lactating women, patients with constipation-predominant IBS (IBS-C) or an IBS-SSS score of <175, patients using probiotics or antibiotics, and patients presenting with organic diseases explaining their GI symptoms. Laxatives and antidiarrheal agents were not allowed during the trial.

2.2 | Study protocol

The study used a randomized, double-blinded, placebo-controlled, crossover design to compare a low-FODMAP vs high-FOS diet. Participants were instructed by Hustoft TN, both in oral and written form, to implement and follow a LFD for 9 weeks. After 3 weeks they were randomized according to a computer-generated list to receive a supplement of either A (16 g of FOS [FODMAP] per day for 10 days), a 3-week washout, and then B (16 g of maltodextrin [placebo] per day for 10 days), or the reverse sequence (hence, AB or BA). The allocation sequence was double blinded to minimize placebo and nocebo effects on symptom alterations. Test substances were carbohydrates given as powders, identical in appearance, color, and taste. Participants were instructed to reconstitute the powder in 500 mL water and distribute the drink into three meals daily (morning, midday, and evening). It was stressed that the drinks should be taken with meals rather than drinking on empty stomachs. The latter was important as the physiological response to FODMAPs delivered in liquid might be different from their delivery within foods, and we wanted to reflect a realistic intake of FODMAPs (ie, mainly through the ingestion of foods).[16] Preliminary testing on FOS was performed on six healthy subjects (five women and one man) to ensure that the amount of 16 g was not too high. This was of importance due to the risk of producing a biased result showing benefits of a LFD, as most people experience uncomfortable GI symptoms when ingesting large quantities of undigested carbohydrates.[5] The test period lasted for 2 days and the drinks were well-tolerated by all except for the experience of mild to moderate bloating and increased flatulence (well-known and often accepted side effects of fermentation[17]). The respective times for data and sample collection were as the following: at baseline, after 3 weeks of LFD, after 10 days with supplementation (A/B), after the 3-week washout period, and finally, after 10 last days with supplementation (A/B).

The study protocol was approved by the Regional Ethic Committee of Northern Norway (2015/594), and performed according to the Declaration of Helsinki and the BMJ guidelines. All participants provided written informed consent prior to study commencement.

2.3 | Symptom assessment

Changes in symptoms were assessed using the validated IBS-SSS questionnaire.[15] Irritable bowel syndrome-SSS consists of five items that measure, on a 100-point visual analog scale (VAS), the severity of abdominal pain, frequency of abdominal pain, severity of abdominal distension, dissatisfaction with bowel habits, and interference with life in general, over the preceding 10 days.[15] Each question generates a maximum score of 100, leading to a possible overall score of 0–500 with a higher score indicating more severe symptoms.[18] The overall score is used to classify IBS severity and to evaluate response to treatment, of which a reduction of at least 50 points is considered as a significant treatment response.[15,18] The questionnaire was also supplemented with a "global" question about satisfaction with symptom relief with regard to the last 7 days.

In addition to IBS-SSS, participants were asked about selected additional GI complaints and comorbidity symptoms. These included nausea and/or vomiting, early satiety, headache, backache, tiredness, belching and/or passing gas, heartburn, frequent or sudden urge to urinate, pain in the thighs, and pain in the muscles and joints. These respective questions were also measured using a 100-point VAS.

2.4 | Blood samples

Blood samples were centrifuged for at least 30 minutes within 2 hours after phlebotomy and serum was frozen for later analyses at −80°C. The Cytokine Human Ultrasensitive Magnetic Luminex® platform (Thermo Fisher Scientific, Life Technologies AS, Oslo, Norway) was used for quantifying proinflammatory cytokines, interleukin 6 (IL-6), interleukin 8 (IL-8), and tumor necrosis factor alpha (TNF-α) in serum.

2.5 | Fecal samples

At each visit, the participants were given a sample container for feces collection prior to the next appointment, and were told to store it in a refrigerator for a maximum of 3 days before delivery. Afterward, the fecal samples were kept in a freezer at −80°C until analysis for microbiota composition and metabolites (SCFAs).

2.6 | Gut microbiota

Microbiota analysis was performed using the GA-map™ DysbiosisTest (Genetic Analysis AS, Oslo, Norway). The GA test is based on regular molecular biology techniques comprising fecal sample homogenization and mechanical bacterial cell disruption, combined with chemical cell lysis, automated total bacterial genomic DNA extraction using magnetic beads, 16S rRNA PCR DNA amplification covering the variable regions V3–V9, probe labeling by single nucleotide extension, hybridization to complementary probes coupled to magnetic beads, and signal detection using BioCode 1000A 128-Plex Analyzer (Applied BioCode, Santa Fe Springs, CA, USA).[19] A dysbiosis index (DI) above 2 (maximum 5) indicate a microbiota that differs from the reference group (DI 1–2: non-dysbiosis, DI 3: moderate, DI 4–5: severe dysbiosis).[19]

2.7 | Short-chain fatty acids

The fecal material (0.5 g) was homogenized after addition of distilled water containing 3 mmol/L of 2-ethylbutyric acid (as internal standard) and 0.5 mmol/L of H_2SO_4; 2.5 mL of the homogenate was vacuum distilled, according to the method of Zijlstra et al.,[20] as modified by Hoverstad et al.[21] The distillate was analyzed with gas chromatography (Agilent 7890 A; Agilent, CA, USA) using a capillary column (serial no. USE400345H, Agilent J&W GC columns; Agilent, CA, USA) and quantified using internal standardization. Flame ionization detection was employed. The following SCFAs were analyzed: major SCFAs (acetic, propionic, and n-butyric acids) and minor SCFAs (i-butyric, n-valeric, i-valeric, n-caproic, and i-caproic acids). The results were expressed in mmol/kg wet weight.

2.8 | Statistical analyses

Statistical analyses were performed using SPSS Statistics version 23.0 (IBM, Armonk, NY, USA) and GraphPad Prism version 6.0 (GraphPad Software, Inc., San Diego, CA, USA). Comparisons were assessed by the use of Student's *t* test (mean) or Wilcoxon's signed rank test (median) in the cases of non-parametric distributions. McNemar's test was used for categorical data and correlations were assessed with Pearson's and Spearman's correlation coefficients. Cluster analysis and principal component analyses (PCA) were used to visualize the microbiota data, showing the extent to which microbial communities share branch length. *P* values of less than or equal to 5% were regarded as statistically significant. Reported *P* values and 95% CI are based on two-sided tests unless otherwise specified.

3 | RESULTS

3.1 | Demographic and clinical data

Baseline characteristics are presented in Table 1. Twenty patients were included of whom 75% were women. Mean age was 34.6 years (range 18–52 years). Eleven patients presented with IBS-D (55%) and nine with IBS-M (45%). Duration of IBS symptoms was from 1.5 to 40 years (mean 15.8 years). Four patients had moderate IBS (IBS-SSS score of 175–300) and 16 had severe IBS (IBS-SSS score of >300). Eleven patients were overweight (BMI: >25 kg/m^2) and nine were normal weight (BMI: 18.5–25 kg/m^2). One patient was in need of several pharmacological agents with potential GI side effects, and another used loperamide. However, both patients held their respective dosages constant throughout the study period. All 20 study participants completed the trial.

TABLE 1 Demographic and clinical data in the study population

Participants	*n*=20
Male/Female	5/15
Mean age (range), years	34.6 (18–52)
Mean duration of symptoms (range), years	15.8 (1.5–40)
IBS severity	
Mild	*n*=0
Moderate	*n*=4
Severe	*n*=16
IBS subtype	
IBS diarrhea predominant	*n*=11
IBS mixed	*n*=9
Median Bristol Stool Form (range)	4.8 (1.5–6.5)
Mean BMI (range)	26.9 (19.5–37.2)
IBS-associated disorders	
Migraine	*n*=6
Fibromyalgia	*n*=2
Reflux	*n*=3
Depressed mood, depression, and/or anxiety (present or history)	*n*=6
Other disorders	*n*=9
A history of gastroenteritis	*n*=8

3.2 | Symptoms

There was a significant improvement in all IBS symptoms after 3 weeks of LFD (Figure 1) with a mean reduction in IBS-SSS total score of 163.8 (one-sided 95% CI: 135.7–500). All participants had an overall reduction of at least 50 (range 57–275). When supplementing the LFD with FOS or placebo, significantly more participants reported symptom relief in response to placebo (80%) than FOS (30%; *P*=.013). The mean overall IBS-SSS score and 4 of 5 subscores were significantly higher in response to FOS compared with placebo (Figure 1), with no evidence of a carryover or a period effect. There was a large intersubject variability in the responses to FODMAP provocation (FOS vs placebo) as compared to FODMAP reduction (baseline vs LFD; Figure 1).

Seven of the 10 additional GI complaints and comorbidity symptoms were significantly improved from baseline to 3 weeks of LFD, with the greatest mean VAS reduction observed for belching and/or passing gas (39.4; *P*<.001), followed by nausea and/or vomiting (24.3; *P*<.001) and tiredness (21.2; *P*=.001). Early satiety, heartburn, and pain in the thighs were not improved at this time, but a significant relief was found for the two first symptoms after 7 weeks dietary adherence. When comparing symptom severity between the FOS and placebo periods, VAS scores of nausea and/or vomiting, headache, and belching and/or passing gas were significantly higher in response to FOS (*P*=.002, *P*=.04, and *P*<.001, respectively).

3.3 | Proinflammatory cytokines

Baseline median (range) values of IL-6, IL-8, and TNF-α were 0.11 pg/mL (0.00–1.87), 3.30 pg/mL (0.00–15.70), and 0 pg/mL (0.00–1.15), respectively. Levels of IL-6 and IL-8, but not TNF-α, decreased significantly after 3 weeks of LFD, with a median reduction in 0.065 pg/mL (*P*=.001) and of 2.95 pg/mL (*P*<.001), respectively (Figure 2). The levels did, however, not change in response to FOS supplementation.

3.4 | Gut microbiota composition

At baseline, 50% of the patients were dysbiotic, and this was increased to 60% after LFD and decreased to 55% after FOS supplementation. Dysbiosis index was mainly 3 (moderately dysbiotic; *n*=6 at baseline and *n*=9 after LFD). A PCA scores plot of the microbiota profiles corrected for sample differences indicates that the LFD treatment systematically shifts the microbiota of a number of patients. The following FOS treatment again changes this shift and takes the microbiota back to levels more similar to the baseline measurement (Figure 3).

Significant reductions from baseline to 3 weeks of LFD were found for Clostridium (*P*=.002), *Faecalibacterium prausnitzii* (*P*=.009), Bifidobacterium (*P*=.008), Megasphaera (*P*=.044), Pediococcus (*P*=.024), and Actinobacteria (*P*=.015), while a significant increase was found for Dorea (*P*=.027). Moreover, significant increases from LFD to FOS were found for Bacteroides (*P*=.019), Bifidobacterium (*P*=.009), Actinobacteria (*P*=.004), *F. prausnitzii* (*P*=.030), and Firmicutes (*P*=.048), while a significant reduction was found for Proteobacteria (*P*=.036) and *Mycoplasma hominis* (*P*=.048). Hence, *F. prausnitzii*, Actinobacteria, and Bifidobacterium abundance were significantly altered in both dietary interventions (Figure 4).

3.5 | Short-chain fatty acids

Levels of total SCFAs and n-butyric acid decreased following a LFD as compared to baseline (*P*=.035 and *P*=.014, respectively), but SCFA levels were otherwise not significantly altered when comparing values from samples obtained at baseline, following LFD, and after FOS supplementation (Figure 5).

3.6 | Correlations

There was no correlation between reductions in the levels of proinflammatory cytokines, Bifidobacterium, Actinobacteria, *F. prausnitzii*, or SCFAs with symptom relief from baseline to LFD, nor was there any correlation between the levels of *F. prausnitzii* and n-butyric acid.

4 | DISCUSSION

In the present randomized, double-blinded, placebo-controlled crossover study, we observed that dietary FODMAP content was related to symptom severity, as well as levels of proinflammatory cytokines and

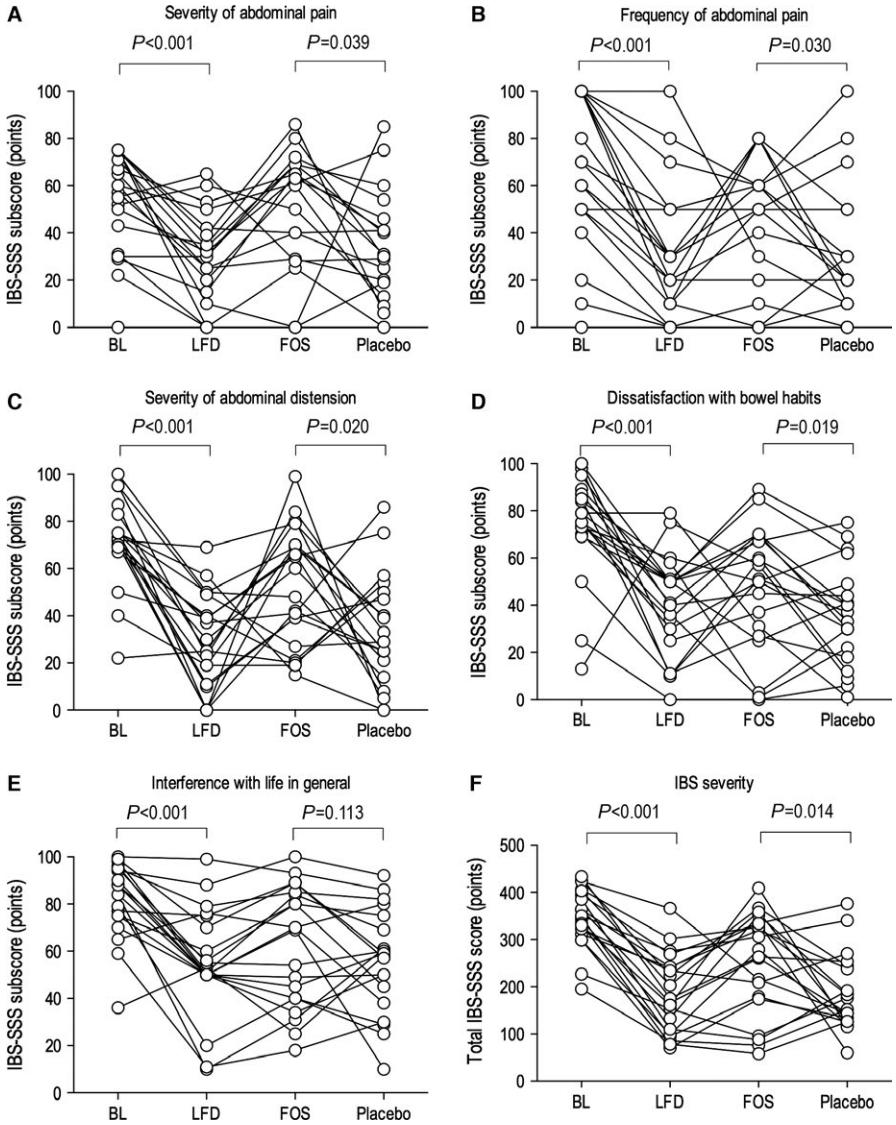

FIGURE 1 Individual responses in IBS-SSS subscores (A–E) and total score (F) registered at baseline (BL), following a 3-week low-FODMAP diet (LFD), and after a 10-day fructo-oligosaccharides (FOS) and placebo intervention in patients with irritable bowel syndrome (*n*=20). There was a significant improvement in all symptoms after implementing the LFD, and four of the five symptoms (A–D) were significantly worsened in response to FOS compared with placebo. The responses to a global restriction of FODMAPs (BL vs LFD) were much more consistent among participants compared to a 10-day provocation of a single FODMAP

microbiota composition and function. Although a small study population, all IBS-SSS symptoms significantly improved on the LFD, and four of the five symptoms were significantly worsened in response to FOS compared with placebo. The differences observed were irrespective of the order and time the interventions were given. Hence, the efficacy of a LFD seems to be beyond placebo effects, and symptom relief is thus likely to be sustainable. In the following, we will discuss possible implications of these findings.

After 3 weeks of restricting high-FODMAP foods, there was an average reduction in the overall IBS-SSS score of 164, considerably greater than the 50 points reduction considered to be a meaningful treatment response. Visual analog scale scores were reduced to a level that arguably is considered good symptom control, confirmed through an overall patient satisfaction of 85%. These results are consistent with those reported in the current literature, albeit somewhat higher.[5,12,22] Although IBS symptoms were significantly worsened in response to FOS, the severity was not comparable to the symptom level observed at baseline. As participants were following a LFD when taking the supplement, their ingestion of the other respective FODMAPs were kept low. The symptom worsening observed was therefore an effect related to a high-FOS content in the diet, while baseline measurements reflected a broader high-FODMAP diet. The different response to these two diets supports the notion that a collective restriction has greater and more consistent effect than a limited restriction.[22] Despite a statistically significant difference in symptoms between the FOS and placebo periods, the mean differences were not very large numerically, probably explaining the lack of a significant difference in symptoms' interference with life in general. There was also a large intersubject variability in response to the two supplements, in contrast to a much more consistent response from baseline to LFD. These observations may support the view of performing a reintroduction phase to individualize the diet.

Baseline severity of symptoms not included in the diagnostic criteria but frequently present in IBS patients were surprisingly high. Interestingly, restricting high-FODMAP foods improved several of these symptoms. An improvement in belching and/or passing of gas was not a surprising finding as a restriction of FODMAPs leads to a reduced production of intestinal gases. However, the explanations

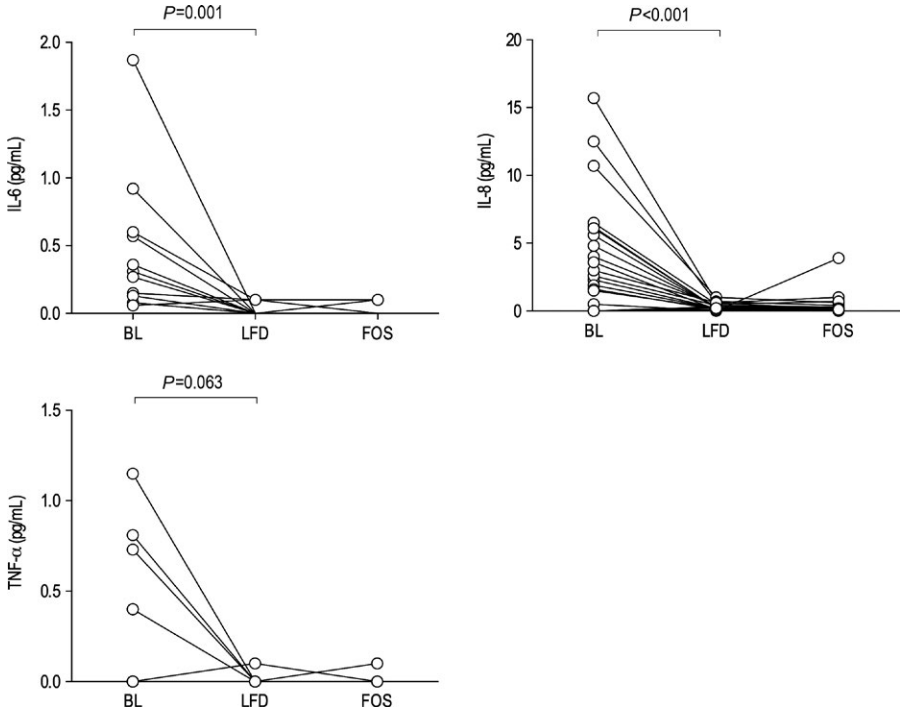

FIGURE 2 Concentrations (pg/mL) of proinflammatory cytokines in blood samples collected at baseline (BL), following a 3-week low-FODMAP diet (LFD), and after a 10-day fructo-oligosaccharides (FOS) intervention in patients with irritable bowel syndrome (*n*=20). There was a significant decrease in IL-6 and IL-8, but not TNF-α, after 3 weeks of LFD

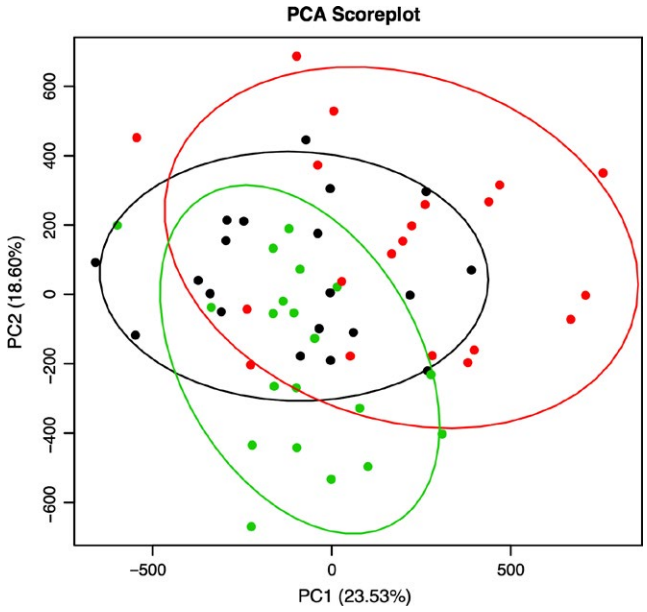

FIGURE 3 Scores for the first two principal components analyses (PCA) of fecal microbiota in a group of patients with irritable bowel syndrome (*n*=20). Data are shown at baseline (BL; black dots), after 3 weeks on a low-FODMAP diet (LFD; green dots), and after a 10-day fructo-oligosaccharides (FOS) provocation (red dots). Each dot represents data from one patient. The first two PCs account for 42.1% (sum of PC1 and PC2) of the variation. The colored ellipses demonstrate the 95% confidence interval for PC1 and PC2. The PCA scores show that both LFD and FOS supplementation seem to have an effect on the gut microbiota; the cohort shifts down during LFD and back up after FOS intervention.

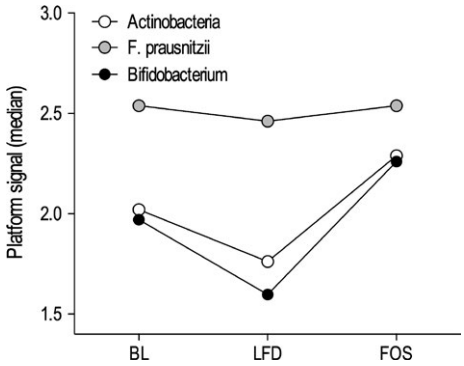

FIGURE 4 Abundance of luminal Actinobacteria, Bifidobacterium, and *Faecalibacterium prausnitzii* in stool samples collected at baseline (BL), following a 3-week low-FODMAP diet (LFD), and after a 10-day fructo-oligosaccharides (FOS) provocation in patients with irritable bowel syndrome (*n*=20; BL vs LFD: Actinobacteria, *P*=.015; Bifidobacterium, *P*=.008; and *F. prausnitzii*, *P*=.009; and LFD vs FOS: Actinobacteria, *P*=.004; Bifidobacterium, *P*=.009; and *F. prausnitzii*, *P*=.03)

behind the effects observed in upper GI symptoms such as heartburn and nausea, as well as comorbidities such as headache and tiredness, remain unknown. Earlier studies have also evaluated benefits of a LFD beyond the relief in GI IBS symptoms, but the results have been inconsistent.[6,16,23]

With respect to the gut microbiota, we observed that most patients were moderately dysbiotic at baseline, as consistent with a previous study.[19] Furthermore, it was observed that patients tended to become more dysbiotic on the LFD. The phylum Firmicutes is known to contain several fermenting bacteria, and it was expected to find a reduction in fermenting bacteria after implementing the LFD as these are being starved. An increase in other bacteria might also be expected because when some species are reduced, other bacteria may occupy their metabolic niches. In our study Clostridium, *F. prausnitzii*, Megasphaera, and Pediococcus, which are all members of the phylum

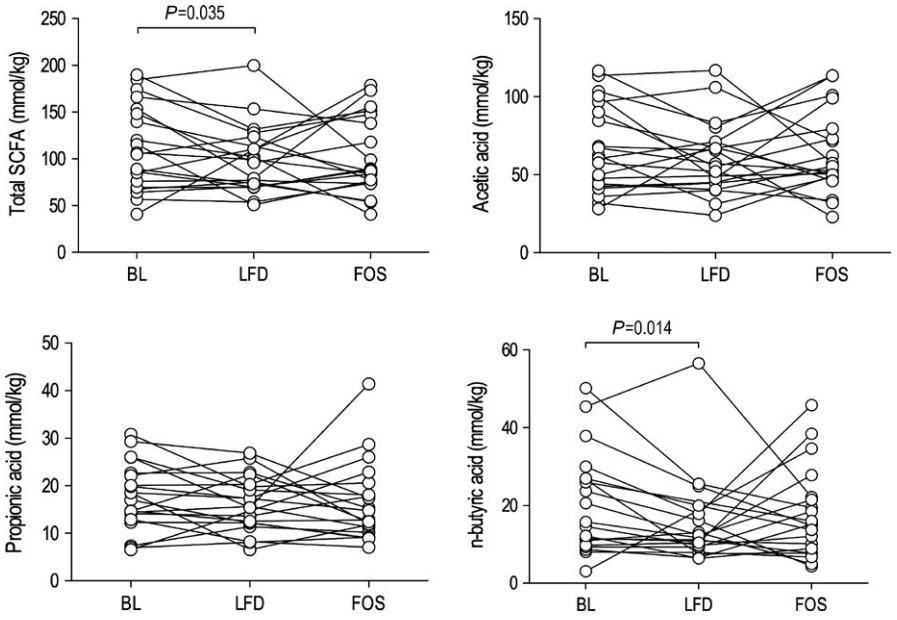

FIGURE 5 Concentrations (mmol/kg) of major short-chain fatty acids (SCFAs) in fecal samples collected at baseline (BL), following a 3-week low-FODMAP diet (LFD), and after a 10-day fructo-oligosaccharides (FOS) provocation in patients with irritable bowel syndrome (*n*=20)

Firmicutes, decreased significantly from baseline to 3 weeks of LFD. Interestingly, Dorea, another member of this phylum increased significantly. Moreover, a significant reduction in Bifidobacterium was observed from baseline to LFD, with a subsequent increase in response to FOS supplementation. The latter was, however, accompanied by symptom relief and worsening, respectively, a paradoxical finding considering studies showing beneficial effects of Bifidobacteria probiotics.[24,25] This observation has already been seen in other studies.[14,26] However, despite a simultaneous reduction in levels of Bifidobacterium and symptom severity, this does not establish a relationship between cause and effect. In fact, levels of Bifidobacterium did not correlate with symptom severity, and whether a Bifidobacteria probiotic in addition to LFD enhances symptom response is yet to be determined.[14] Reduced levels of Bifidobacterium and *F. prausnitzii* after 3 weeks of LFD are the effects most likely due to a reduced availability of prebiotic fructans (including FOS) and galacto-oligosaccharides.[14] Increased levels of these bacteria in response to FOS supplementation support this supposition. Considering promising beneficial effects of both Bifidobacterium and *F. prausnitzii*,[27,28] consequences of possible reduced levels when following a LFD need to be investigated. Moreover, as diet-induced changes in the microbiota are known to be rapidly reversed, it should be evaluated if potential changes persist once FODMAPs are reintroduced to tolerance.[14] In contrast to the reduced level of Actinobacteria observed in the present trial, McIntosh et al.[26] reported a higher richness and diversity of this phylum when comparing low- and high-FODMAP diets. Interestingly, the latter was observed despite a decrease in Bifidobacteria relative abundance.

We observed significantly reduced levels of total SCFAs and n-butyric acid after 3 weeks of LFD. The latter may indicate a reduced level and/or activity of butyrate-producing bacteria. *Faecalibacterium prausnitzii* is a major commensal butyrate producer, and has been labeled as a biomarker of intestinal health in adults.[28] Despite a simultaneous reduction from baseline to LFD in the levels of n-butyric acid and this bacterium, the changes were not significantly correlated. In contrast to the present study, neither Halmos et al.[29] nor Staudacher et al.[14] observed reduced levels of SCFAs when restricting FODMAPs. When supplementing LFD with FOS, n-butyric acid did not increase significantly. This finding contrasts with a recently published study on healthy adults by Clarke et al.,[30] using fructan supplementation in a similar amount as in the present study. However, different duration of supplementation (10 days vs 28 days) may explain this discrepancy. Interestingly, animal studies have shown that rectal instillation of n-butyric acid may induce visceral hypersensitivity,[31] whereas studies in humans are conflicting.[32,33] It remains to be evaluated if a restriction of FODMAPs leads to a reduction in the level of n-butyric acid, and whether this plays a part in the symptom relieving effect by reducing visceral sensitivity. More important, it should be evaluated if a reduced level of n-butyric acid is detrimental considering putative beneficial effects on colonic health and diseases.[33,34]

Several studies have suggested imbalanced cytokine signaling as an important pathogenetic factor of IBS, but the alterations found have not been consistent.[35,36] Prior research has especially suggested elevated systemic IL-6, IL-8, and TNF-α in IBS patients relative to healthy controls.[36–41] Our data do not support this finding, as baseline values did not seem to deviate from those measured in the general population. Interestingly, 3 weeks of LFD reduced serum levels of proinflammatory IL-6 and IL-8. To our knowledge, no previous studies have evaluated effects of a LFD on cytokine profiles. However, McIntosh et al.[26] also reported changes in immune activation in response to a LFD, using histamine as a measurement of inflammatory state. Proinflammatory cytokines and histamine are both synthesized and secreted by inflammatory cells, suggesting a reduced level and/or activity of these cells when restricting FODMAPs. Paradoxially, in the present study, both n-butyric acid and selected bacteria associated with anti-inflammatory properties were reduced concomitantly with a reduced level of immune activation.[34,42]

We acknowledge that there are limitations in the present study design. Due to the lack of a control group when comparing baseline

diets with LFD, we cannot conclude that the effects observed were exclusively due to dietary changes. Despite the use of a double-blinded design, it was difficult to ensure a 100% successful blinding, as participants were likely to be influenced by changes in symptoms. Participants were initially told that they were about to receive powders both high and low in FODMAPs, an informative message likely to create expectations. With regard to the low-FODMAP powder, there was no significant placebo response as only minimal differences, in both directions, were observed between the LFD and placebo periods. However, as discussed by Shepherd et al.,[16] the placebo response rate is typically higher when reduction rather than induction of symptoms is the endpoint of interest. While a LFD restricts all FODMAPs collectively, we chose only one of them (FOS) to represent a high-FODMAP diet. However, using only one FODMAP enabled us to evaluate FOS effects alone on the measured parameters and demonstrated differences in FOS sensitivity among IBS patients. It would have been interesting to do the same provocation with other isolated FODMAPs to consider which of them matter most for the individual patient. With regard to the performed correlation analyses, one should be aware of potential unreliable results due to small sample size.

In conclusion, the results from the present controlled study support the efficacy of a LFD in reducing functional GI symptoms in patients with IBS-D or IBS-M, as significantly more participants reported symptom relief in response to placebo (80%) than FOS (30%). The changes observed in proinflammatory cytokines IL-6 and IL-8, microbiota profile with alterations in Actinobacteria, Bifidobacteria, and *F. prausnitzii*, as well as decreased fecal levels of SCFAs may have consequences for gut health. Gastroenterologists should consider the LFD as a strategy to alleviate IBS symptoms, although the implications of long-term changes in microbiota composition and function require further elucidation.

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DISCLOSURE

None.

AUTHOR CONTRIBUTION

TH, JGH, TNH, GAL, and SOY designed the project; TNH conducted the research; KAB, TNH, JV, GAL, and Genetic Analysis AS, Oslo, analyzed the data; JGH, TH, TNH, GAL, and JV wrote the paper. All authors read and approved the final manuscript.

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RESEARCH ARTICLE Open Access

Intestinal dysbiosis is common in systemic sclerosis and associated with gastrointestinal and extraintestinal features of disease

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Abstract

Background: Recent evidence suggests a link between autoimmunity and the intestinal microbial composition in several rheumatic diseases including systemic sclerosis (SSc). The objective of this study was to investigate the prevalence of intestinal dysbiosis in SSc and to characterise patients suffering from this potentially immunomodulatory deviation.

Methods: This study consisted of 98 consecutive patients subject to in-hospital care. Stool samples were analysed for intestinal microbiota composition using a validated genome-based microbiota test (GA-map™ Dysbiosis Test, Genetic Analysis, Oslo, Norway). Gut microbiota dysbiosis was found present as per this standardised test. Patients were examined regarding gastrointestinal and extraintestinal manifestations of SSc by clinical, laboratory, and radiological measures including esophageal cineradiography, the Malnutrition Universal Screening Tool (MUST), levels of plasma transthyretin (a marker of malnutrition) and faecal (F-) calprotectin (a marker of intestinal inflammation).

Results: A majority (75.5%) of the patients exhibited dysbiosis. Dysbiosis was more severe ($r_s = 0.31$, $p = 0.001$) and more common ($p = 0.013$) in patients with esophageal dysmotility. Dysbiosis was also more pronounced in patients with abnormal plasma levels of transthyretin ($p = 0.045$) or micronutrient deficiency ($p = 0.009$). In 19 patients at risk for malnutrition according to the MUST, 18 exhibited dysbiosis. Conversely, of the 24 patients with a negative dysbiosis test, only one was at risk for malnutrition. The mean ± SEM levels of F-calprotectin were 112 ± 14 and 45 ± 8 μg/g in patients with a positive and negative dysbiosis test, respectively. Dysbiosis was more severe in patients with skin telangiectasias ($p = 0.020$), pitting scars ($p = 0.023$), pulmonary fibrosis ($p = 0.009$), and elevated serum markers of inflammation ($p < 0.001$). However, dysbiosis did not correlate with age, disease duration, disease subtype, or extent of skin fibrosis.

Conclusions: In this cross-sectional study, intestinal dysbiosis was common in patients with SSc and was associated with gastrointestinal dysfunction, malnutrition and with some inflammatory, fibrotic and vascular extraintestinal features of SSc. Further studies are needed to elucidate the potential causal relationship of intestinal microbe-host interaction in this autoimmune disease.

Keywords: Systemic sclerosis, Microbiome, Gastrointestinal, Dysbiosis

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Background

Systemic sclerosis (SSc) is an autoimmune systemic disease of unknown etiology. Genetic factors may only partly explain the pathobiology, and as yet uncharacterised environmental factors have been suggested to have a major influence on the development of SSc [1]. The number of bacteria in the human gastrointestinal (GI) tract has been estimated to 10^{14}, reaching a biomass of around 2 kg [2]. In both health and disease, these microbiota are in continuous interaction with the epithelium and immune cells of the GI mucosa, and have profound effects on the host's local and systemic immune system [3]. Maintenance of a balanced bidirectional interaction has been suggested to be essential in preventing development and progression of autoimmune diseases [4].

Altered microbiota composition, commonly referred to as dysbiosis, has been shown to induce and modulate systemic inflammation in animal models of rheumatic diseases and other immune-mediated inflammatory diseases (IMIDs) [5–7]. In the field of rheumatology, intestinal dysbiosis has been associated with rheumatoid arthritis (RA), systemic lupus erythematosus, Sjögren's syndrome and ankylosing spondylitis [7–11]. A randomised double-blind placebo-controlled clinical trial in RA patients indicated that disease activity may be sensitive to modulation of gut microbiota through ingestion of probiotics [12]. In contrast, a similar trial did not show any significant differences between probiotics and placebo [13].

In SSc, small intestinal bacterial overgrowth is a well-described complication associated with GI dysmotility, GI discomfort, and malnutrition [14, 15]. Successful treatment of small intestinal bacterial overgrowth in SSc leads to improvement in GI symptoms [14]. Recently, alterations also in the colonic microbial composition in SSc have been reported [16].

Assessment of GI disease in SSc is challenging. Esophageal cineradiography has been suggested as the gold standard in the objective assessment of GI SSc [17]. Others and we have suggested that faecal calprotectin (F-calprotectin) constitutes a feasible tool in the evaluation of GI SSc [15, 18]. Malnutrition is one facet of GI disease that has been linked not only to morbidity and decreased quality of life, but also to increased mortality [19]. The Malnutrition Universal Screening Tool (MUST) is a validated method for identifying SSc patients at risk for malnutrition [20]. Decreased plasma levels of transthyretin, also known as prealbumin, represent a biomarker of malnutrition that also predicts mortality in SSc [19, 21].

The objective of this study was to examine the prevalence of dysbiosis in SSc. Furthermore, we aimed at exploring how intestinal dysbiosis relates to extraintestinal as well as gastrointestinal manifestations of SSc, including malnutrition.

Methods

Patients

Consecutive patients fulfilling the American Congress of Rheumatology/European League Against Rheumatism (ACR/EULAR) 2013 classification criteria for SSc and subject to planned in-hospital care due to SSc at the Skane University Hospital in Lund, Sweden between April 2014 and October 2015, were invited to this study. Out of 226 patients, 100 subjects both agreed to participate and were able to provide a fresh stool sample during their in-hospital stay. Patients with inflammatory bowel disease (IBD), intestinal malignancy, and/or colostomy were excluded (n = 2). In total, the study cohort consisted of 98 patients.

Ethics

The study was approved by the Regional Ethics Review Board, Lund, Sweden, reference number 2011/596. Informed written consent was obtained from all patients before study inclusion and the study conformed to the ethical guidelines of the Declaration of Helsinki.

Clinical assessment

The following data were collected: age, sex, and disease duration (defined both as years since onset of Raynaud's phenomenon [RP] and years since the first non-RP manifestation). Patients were classified as having either diffuse cutaneous SSc (dcSSc) or limited cutaneous SSc (lcSSc) [22]. Esophageal function was assessed by cineradiography and evaluated by a radiologist, as previously described [23]. The cineradiograms were obtained by recording the swallowing of barium contrast in upright and prone positions using a high-speed camera. Esophageal motility dysfunction was categorised as absent, mild, moderate, or severe. Skin involvement was assessed using the modified Rodnan skin score (mRSS) [24]. The presence or absence of skin telangiectasia and pitting scars were noted. Pulmonary function was evaluated using a body plethysmograph (Erich Jaeger GmbH, Hoechberg, Germany). Lung fibrosis was identified by high-resolution computed tomography. Echocardiography was performed on all patients, and pulmonary arterial hypertension (PAH) was diagnosed by means of right heart catheterisation.

Assessment of medical records

Medical records were systematically studied. Height and weight were noted as well as weight change during the last 12 months. Individual MUST scores were calculated as previously described [20]. A MUST score of 0 represents low risk for malnutrition, a score of 1 medium risk, and a score of ≥2 high risk. Patients' usage of prescribed drugs including proton pump inhibitors (PPIs), antibiotics, glucocorticoids and immunosuppressive agents were noted.

Assessment of intestinal symptoms

All patients were systematically questioned regarding the following GI symptoms: heartburn (dyspepsia), dysphagia, diarrhea, and/or constipation. These were recorded as present or not.

Laboratory examinations

Blood tests included measurements of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), haptoglobin, orosomucoid, α_1-antitrypsin, immunoglobulin (Ig)G, IgM, IgA, vitamin B_{12}, folic acid, ferritin, iron, transferrin iron-binding capacity (TIBC) and transthyretin. Subjects with an iron/TIBC ratio < 0.16 were considered to be iron deficient [25]. F-calprotectin was measured using a commercially available enzyme-linked immunosorbent assay (ELISA, Calpro, Lysaker, Norway). The lower limit of the ELISA was 30 μg/g and values below this cutoff were estimated as 20 μg/g. In accordance with published data and recommendations from the manufacturer, we considered F-calprotectin levels < 50 μg/g to be within normal range [26].

Assessment of gut dysbiosis

The GA-map™ Dysbiosis Test (Genetic Analysis, Oslo, Norway) has been developed and validated in relation to a Scandinavian control population to identify dysbiosis in adults by genetic analysis of a stool sample. The test makes use of 54 bacterial ribosomal RNA probes specific for various intestinal bacterial species or clades to generate genomic data on the intestinal microbiota composition. Using a defined algorithm, these data are subsequently translated into a Dysbiosis Index Score ranging from 1 to 5 (grades 1–2 are defined as eubiosis and 3–5 as dysbiosis). The test has been compared with MiSeq Illumina sequencing-based protocols and proven successful in identifying dysbiosis [6, 27]. In a healthy control population, 84% exhibited eubiosis and 16% dysbiosis [27]. In the current study, gut microbiota eubiosis and dysbiosis were delineated as per the standardised GA-map™ Dysbiosis Test results.

Statistical analyses

The Mann-Whitney *U* test was used to compare the degree of dysbiosis and the $\chi 2$ test to compare the frequency of dysbiosis in patients with and without various manifestations of SSc. Spearman correlation coefficient (r_s) was used to correlate the Dysbiosis Index Score with other continuous variables.

Results

Study population characteristics and levels of dysbiosis

Systemic sclerosis patients (n = 98) were examined for an array of characteristics and assessed for intestinal dysbiosis analysing their stools using the GA-map™ Dysbiosis Test. Patient characteristics are presented in Table 1. A majority (75.5%) of the patients exhibited dysbiosis to some degree (score 3–5), and a significant proportion (24.9%) suffered from severe dysbiosis (score 5, Fig. 1).

Dysbiosis was associated with gastrointestinal manifestations of systemic sclerosis

A majority of the patients (84%) exhibited esophageal dysfunction, and dysbiosis was significantly more common in this group ($p = 0.013$; Fig. 2). The degree of dysmotility correlated with intestinal dysbiosis (Table 2). Malnutrition was frequent; 53% of the patients exhibited

Table 1 Patient characteristics

	n	(%)
Systemic sclerosis subtype		
limited cutaneous SSc	77	(78)
diffuse cutaneous SSc	21	(22)
Autoantibodies		
ANA-positive	87	(89)
ACA-positive	33	(34)
ARA-positive	10	(10)
ATA-positive	11	(11)
Smoking		
smoker	11	(11)
ex-smoker	43	(44)
non-smoker	44	(45)
Telangiectasias	39	(40)
Pulmonary arterial hypertension[a]	13	(13)
Pitting scars, current	23	(23)
Lung fibrosis[b]	35	(36)
Pathological cineradiography	82	(84)
Regular PPI usage	78	(80)
Immunosuppressive therapy		
mycophenolate mofetil	23	(23)
methotrexate	5	(5)
azathioprine	10	(10)
no immunosuppressive therapy	60	(61)
	median	interquartile range
Modified Rodnan skin score		
limited cutaneous	2	(0, 4)
diffuse cutaneous	10	(4, 22)
Disease duration, years[c]	6	(2, 16)
Prednisolone, daily intake (mg)	0	(0, 4)

ANA anti-nuclear antibodies, *ACA* anti-centrome antibodies, *ARA* anti-RNA polymerase III antibodies, *ATA* anti-topoisomerase1 antibodies, *PPI* proton pump inhibitor

[a]As determined by right heart catheterisation

[b]As determined on high-resolution computed tomography

[c]Years since first non-Raynaud's phenomena symptom

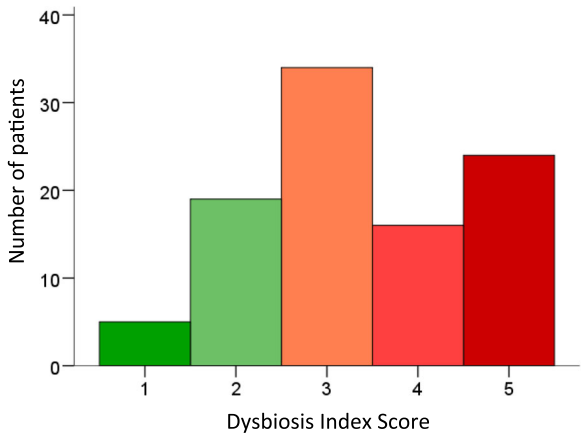

Fig. 1 Dysbiosis is common in patients with systemic sclerosis.A majority of the study population suffers from dysbiosis, as defined by the GA-map™ Dysbiosis Test, with 25% exhibiting pronounced dysbiosis

deficiency of folic acid, vitamin B12, and/or iron. Nineteen patients had a MUST score of ≥ 1, of which 18 exhibited dysbiosis, and 17 patients had pathological levels of P-transthyretin of which 15 exhibited dysbiosis. Patients with these malnutrition-associated characteristics (any deficiency, MUST ≥ 1, and/or abnormal transthyretin levels) displayed a higher degree of dysbiosis (Fig. 2) compared to the other subjects. Similarly, patients with any self-reported GI symptoms (Fig. 2) and patients using PPIs had a higher degree of dysbiosis compared to the other subjects ($p = 0.019$ and $p = 0.002$, respectively). Subanalysis of different types of self-reported GI symptoms did not reveal any significant associations. A majority of the SSc subjects exhibited abnormal F-calprotectin levels which were associated with the degree of dysbiosis (Fig. 2, Table 2). The mean ± SEM levels of F-calprotectin were 112 ± 14 and 45 ± 8 μg/g in patients with a positive and negative dysbiosis test, respectively.

Dysbiosis was associated with certain extraintestinal manifestations of systemic sclerosis

The degree of dysbiosis was analysed in reference to major fibrotic and vascular extraintestinal manifestations of SSc. Dysbiosis frequencies and severity did not differ between patients with dcSSc and lcSSc (Fig. 2), and the degree of dysbiosis did not correlate with the extent of skin disease (Table 2). However, dysbiosis was more pronounced among patients with pulmonary fibrosis (Fig. 2). We were unable to identify any association between the degree of dysbiosis and vital capacity or carbon monoxide diffusing capacity, ($r_s = -0.126$, $p = 0.216$, n = 98; $r_s = -0.172$, $p = 0.232$, n = 96). Among the 98 patients, 13 (13.3%) suffered from PAH. Dysbiosis was not more common or more severe among these ($p = 0.316$). However, dysbiosis was more severe among the 39 patients exhibiting skin telangiectasia, and among the 23 patients with pitting scars (Fig. 2). Dysbiosis was not more severe or prevalent among subjects with antibodies against centromere, topoisomerase 1, or RNA polymerase III. The degree of dysbiosis did not correlate with usage of glucocorticoids ($r_s = 0.15$, $p = 0.139$) and was not associated with usage of immunosuppressive therapy or antibiotics ($p = 0.344$ and $p = 0.684$, respectively).

Dysbiosis was associated with laboratory markers of inflammation

Routine blood tests addressing systemic inflammation were assessed and correlated with the degree of dysbiosis. The grade of dysbiosis correlated with levels of CRP, haptoglobin, orosomucoid, and α_1-antitrypsin, but not with the levels of ESR, IgG, IgM or IgA (Table 2). Of note, all three patients with IgA levels above reference levels had a Dysbiosis Index Score of 5 ($p = 0.059$).

Dysbiosis was common also in patients with early systemic sclerosis

Disease duration was defined by two different measures and subsequently correlated with the degree of dysbiosis. The Dysbiosis Index Score did not correlate either with disease duration defined as years since RP debut or disease duration defined as years since first non-RP symptom or age (Table 2). Dysbiosis was prevalent among patients with less than 2 years since the debut of RP or first non-RP symptom (73% and 72%, respectively), similarly to patients with more long-standing disease (76% and 76%, Fig. 2).

Analysis of specific bacterial genera

In a secondary analysis, we examined the frequency of specific bacterial genera and species previously associated with SSc, included in the GA-map™ Dysbiosis Test. A large proportion of patients with SSc exhibited low levels of *Faecalibacterium prausnitzii* (66/98; 67.3%) and/or *Clostridiaceae* (25/98; 25.5%) compared to eubiotic individuals. Also, relatively high levels of *Lactobacillus* (31/98; 31.6%) but not *Bifidobacterium* (6/98; 6.1%) were common among our subjects.

Discussion

In this cross-sectional study encompassing 98 SSc patients we show that intestinal dysbiosis is common in SSc and is related to GI manifestations of disease. Also, we show that dysbiosis is associated with certain extraintestinal SSc features of inflammatory, vascular, and fibrotic type. We present data showing that intestinal dysbiosis is already present early in the course of SSc, indicating that dysbiosis may precede initial signs of fibrosis.

Several IMIDs have been associated with alterations in the microbial composition in the intestine, including

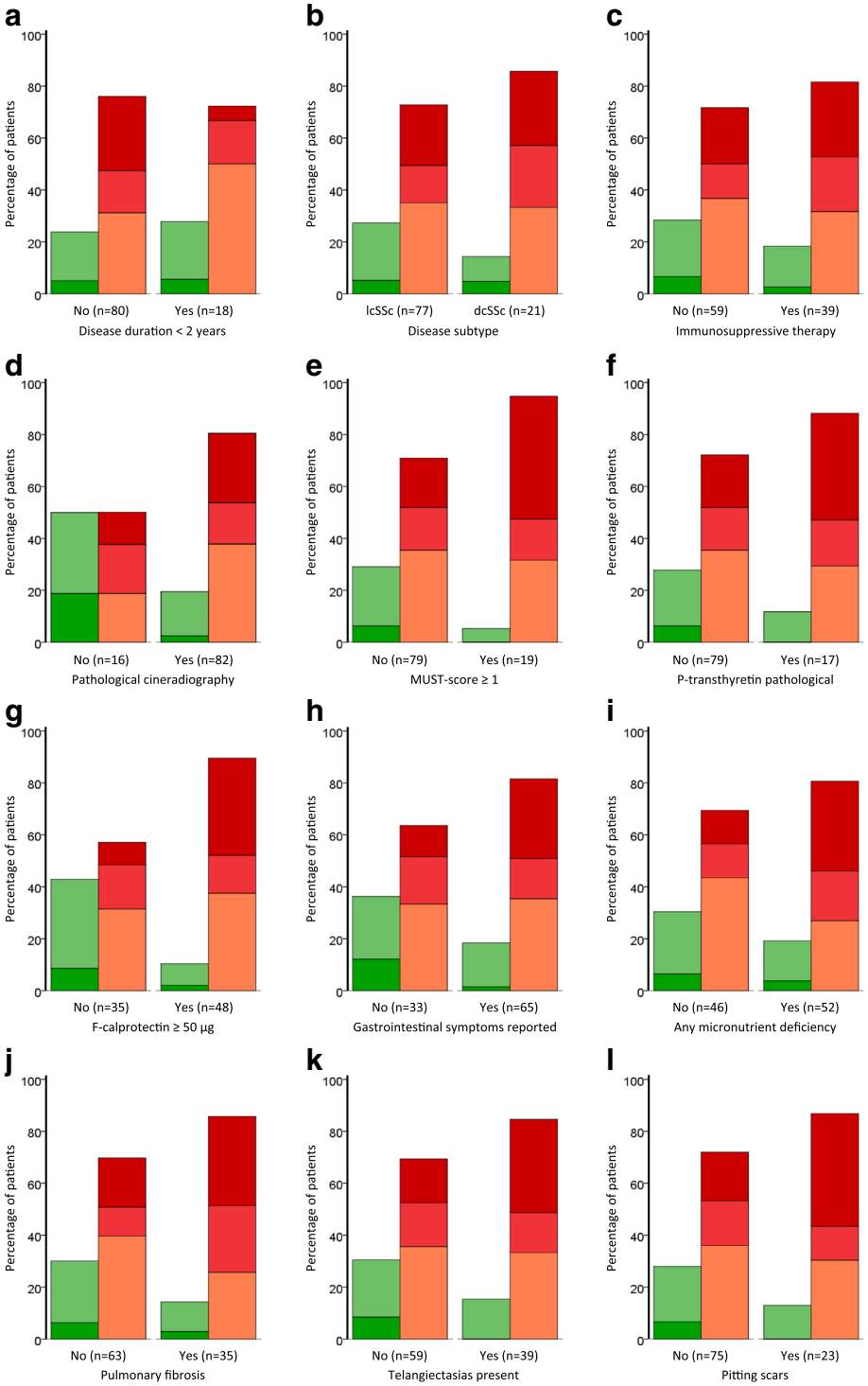

Fig. 2 Dysbiosis correlates with gastrointestinal and some extraintestinal manifestations of SSc, but not disease subtype or immunosuppressive therapy. Dysbiosis was prevalent in patients with both short and long disease duration (**a**), lcSSc and dcSSc (**b**) as well as in patients with and without immunosuppressive therapy (**c**), with no significant differences between groups. Dysbiosis was more pronounced in patients with gastrointestinal manifestations of SSc including pathological oesophageal function, $p = 0.036$ (**d**); at risk for malnutrition, $p = 0.005$ (**e**); low levels of P-transthyretin, $p = 0.045$ (**f**); increased levels of F-calprotectin, $p < 0.001$ (**g**); gastrointestinal symptoms present, $p = 0.019$ (**h**) or micronutrient deficiency $p = 0.009$ (**i**). Also, patients with pulmonary fibrosis, $p = 0.009$ (**j**); telangiectasias, $p = 0.020$ (**k**); or pitting scars, $p = 0.023$ (**l**) had more pronounced dysbiosis compared to other patients. *dcSSc* diffuse cutaneous SSc, *F-calprotectin* faecal calprotectin, *lcSSc* limited cutaneous SSc, *MUST* Malnutrition Universal Screening Tool

Table 2 Correlation between the Dysbiosis Index Score and laboratory markers of inflammation, and disease characteristics, respectively

	n	Spearman's correlation coefficient (r_s)	*p* value
Laboratory markers of inflammation			
C-reactive protein	98	0.35	<0.001
Haptoglobin	98	0.34	<0.001
Orosomucoid	98	0.39	<0.001
α_1-antitrypsin	98	0.27	0.007
Erythrocyte sedimentation rate	98	0.16	0.156
IgA	98	0.13	0.266
IgM	98	−0.05	0.654
IgG	98	−0.05	0.632
Faecal calprotectin	83	0.38	<0.001
Disease characteristics			
Years since onset of RP	94	−0.07	0.501
Years since the first non-RP symptom	89	0.09	0.383
Patient's age at dysbiosis analysis	98	0.08	0.413
modified Rodnan skin score	98	0.05	0.659
Dysmotility of oesophagus	97	0.31	0.002

Ig immunoglobulin, *RP* Raynaud's phenomenon

RA, systemic lupus erythematosus, Sjögren's syndrome, and IBD [7, 8, 11, 28]. Among human IMIDs, dysbiosis has been most extensively studied in IBD. These patients display decreased diversity in their gut microbiota, increased numbers of bacteria driving inflammatory activity, and decreased numbers of bacteria with immunoregulatory effects [28]. An important question is whether IBD-associated dysbiosis is a primary or secondary phenomenon. In animal models of IBD both loss of immunoregulatory and addition of disease-promoting bacteria have been shown to contribute to disease activity, supporting a primary disease-driving role for dysbiosis [29]. In IBD patients, various strategies for manipulating the gut microbiota, including exclusive enteral nutrition, prebiotics, probiotics, postbiotics, and faecal microbiota transplantation have shown mixed but overall promising results [30].

Molecular analyses have revealed some similarities between the process of IBD-associated intestinal fibrosis and SSc-associated skin fibrosis, including transforming growth factor beta (TGF-β) and peroxisome proliferator-activated receptor-dependent pathways resulting in collagen I production by fibrocytes and fibroblasts [31, 32]. Furthermore, while inflammation can be treated by immunosuppressive therapy, these fibrotic processes are resilient also to modern therapy in both diseases. In IBD as well as SSc, elevated F-calprotectin levels are common, indicating intestinal inflammation. Similar to data presented in this study, increased F-calprotectin levels have been associated with dysbiosis also in IBD [33].

Volkmann et al. recently reported altered microbial colonic mucosal composition in 17 SSc patients [16]. Our study comprising 98 SSc patients corroborates this finding, as we show a high incidence of dysbiosis in our patients. We also report low levels of the immunoregulatory bacteria *Faecalibacterium prausnitzii,* which is in agreement with studies in IBD [34]. In accordance with Volkmann et al., we report high levels of *Lactobacillus* in SSc patients, which contrasts this disease from several other IMIDs [4]. As previously suggested, this finding might raise novel questions regarding the usage of *Lactobacilli*-containing probiotics in SSc.

Our report is based on faecal analyses and not analyses on colonic lavage or intestinal biopsies. Consequently, a weakness of our approach is the inability to specifically focus on bacteria prevalent in the interface between the colonic mucosa and the intestinal lumen. It is noteworthy that even though different methodologies were used, our major finding is consistent with Volkmann's report.

Objective evaluation of GI disease in SSc is challenging. SSc can affect the GI tract in several different ways including dysmotility, malnutrition, inflammation, and fibrosis. In our study, we evaluated the GI tract by assessment of esophagus motility using barium cineradiography which has previously been suggested as the gold standard in objective evaluation of this disease [17]. We investigated malnutrition by laboratory markers including P-transthyretin, and anthropometric data using the MUST [19, 20]. While malnutrition in SSc has been suggested to be caused by malabsorption [35], additional mechanisms are likely to be involved including cachexia caused by the chronic inflammatory process [36].

A majority of our patients were prescribed PPI and usage of this medication was interestingly enough associated with dysbiosis. However, previous studies have failed to show that PPI usage per se causes significant aberrations in colonic microbiota composition [37]. In agreement with a previous study and interpretation by Krause et al. [36], we suggest that regular use of PPI primarily is an unspecific marker of symptomatic GI SSc. In this study, all patients were questioned about GI symptoms, and indeed, dysbiosis was more common in patients with GI symptoms indicating that a validated questionnaire, such as the UCLA SCTC GIT 2.0 should be included in future studies [38].

The primary aim of this study was to study the prevalence of dysbiosis in SSc. Unlike whole-genome sequencing studies, we have only limited data on specific bacterial genera. Furthermore, we do not have data on intestinal metabolic pathways used by the different microbiomes

our patients harbor. Further studies encompassing such analyses are needed to further elucidate the intricate relationship between the host and the microbiome in SSc [39].

We can only speculate on the mechanisms behind the associations between dysbiosis and GI or extraintestinal manifestations of SSc. It can be hypothesised that several of these manifestations are indirect markers of severe disease. However, we were unable to identify an association between the mRSS, disease subtype, autoantibody profile, and immunosuppressive therapy. Taking this into consideration, we are therefore inclined to suggest that the relationship between the intestinal microbiome and SSc is multifactorial and related to factors independent of disease severity or autoantibody status. We note that dysbiosis is associated with increased serum levels of markers of inflammation and we suggest that further studies are warranted to elucidate the impact of GI dysbiosis on the immune system in SSc.

Conclusions

Examining a large cross-sectional cohort of SSc patients we report that intestinal dysbiosis is prevalent in early as well as late disease, and associated with both GI and extraintestinal manifestations of SSc. Given our current knowledge from other IMIDs, we suggest that an aberration of the intestinal microbiota may contribute to the development of systemic inflammation and fibrosis, although causal relationships remain to be established.

Abbreviations

ACA: anti-centromeric antibodies; ACR/EULAR: American Congress of Rheumatology/European League Against Rheumatism; ANA: anti-nuclear antibodies; ARA: anti-RNA-polymerase III antibodies; ATA: anti-topoisomerase antibodies; CRP: C-reactive protein; dcSSc: diffuse cutaneous SSc; ELISA: enzyme-linked immunosorbent assay; ESR: erythrocyte sedimentation rate; F-calprotectin: faecal calprotectin; GI: gastrointestinal; IBD: inflammatory bowel disease; Ig: immunoglobulin; IMID: immune-mediated inflammatory disease; lcSSc: limited cutaneous SSc; mRSS: modified Rodnan skin score; MUST: Malnutrition Universal Screening Tool; PAH: pulmonary arterial hypertension; PPI: proton pump inhibitor; RA: rheumatoid arthritis; RP: Raynaud's phenomenon; SSc: systemic sclerosis; TGF-β: transforming growth factor beta; TIBC: total iron-binding capacity

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Availability of data and materials

The data sets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KA conceived and designed the study, analysed and interpreted the data and drafted the manuscript. ZA analysed and interpreted the data and reviewed the manuscript for intellectual content. AP acquired the data and organised the study and reviewed the manuscript for intellectual content. GJ analysed and interpreted the data and reviewed the manuscript for intellectual content. JM conceived the study, analysed and interpreted the data and helped drafting and revising the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study was approved by the Regional Ethics Review Board, Lund, Sweden, reference number 2011/596. Informed written consent was obtained from all patients before study inclusion and the study conformed to the ethical guidelines of the Declaration of Helsinki.

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Note

Bead-beating artefacts in the *Bacteroidetes* to *Firmicutes* ratio of the human stool metagenome

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ABSTRACT

We evaluated bead-beating cell-lysis in analysing the human stool metagenome, since this is a key step. We observed that two different bead-beating instruments from the same producer gave a three-fold difference in the *Bacteroidetes* to *Firmicutes* ratio. This illustrates that bead-beating can have a major impact on downstream metagenome analyses.

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1. Background

The ratio between *Bacteroidetes* and *Firmicutes* has been identified as the most important ratio in microbiome studies. A decade ago, this ratio explained the relation between obesity and the microbiota (Ley et al., 2006, Turnbaugh et al., 2006). More recently, bacterial ratios involving *Bacteroidetes* and *Firmicutes* have been used in the establishment of different enterotypes of the human gut microbiota (Arumugam et al., 2011), and have been associated with type 2 diabetes (Greenhill, 2015). Although there has been an increasing awareness of irreproducibility in microbiome studies (Hanage, 2014), the issue of sample preparation has not received much attention. Particularly, the key bead-beating step lacks proper evaluation. A recent study has shown that omitting the bead-beating step entirely can have dramatic effect on the determined microbiome composition (Walker et al., 2015). However, knowledge about the different bead-beating approaches commonly used are still lacking.

2. Main text

The aim of our work was to conduct a highly controlled study of only one factor, namely the use of bead-beating instruments in the sample preparation. This is a key step affecting all downstream metagenome analyses, including both shotgun and amplicon. We evaluated two widely used instruments (FastPrep-24™ and FastPrep-96™) from one producer (MP Biomedicals, Santa Ana, California, USA), both operated under recommended conditions using Lysing Matrix E (MP Biomedicals Inc.) and 2 × 40 s cell disruption time, with speed of 6.0 m/s and 1800 RPM, respectively. All other factors were kept as constant as possible (see Supplement for methodological and experimental details). Eight aliquots from each of six individuals were processed in this study (four using FastPrep-96 and four using FastPrep-24).

We used 16S rRNA gene sequencing (Illumina Inc., San Diego, CA, USA) and the more targeted GA-map™ Dysbiosis Test (Genetic Analysis, Oslo, Norway) to evaluate the effect of bead-beating on the resolution of stool metagenome (Casen et al., 2015). For both methods we found that the diversity across bead-beating instruments was nearly at the level of differences between individuals, while the diversity within the same bead-beating approach was much lower (Fig. 1A). The targeted GA-map test also revealed major differences in abundance at the species level between the two bead-beating approaches (Fig. 1B). Furthermore, Illumina sequencing showed a three-fold difference in the *Bacteroidetes* content between the bead-beating instruments used (Fig. 2A), in which all *Bacteroidetes* Operational Taxonomic Units (OTUs) showed an over-representation with the FastPrep-96™ method as determined by the ratio of number of sequences (Fig. 2B). In contrast, *Firmicutes* OTUs were mainly underrepresented when using this method (Fig. 2B). Collectively, our results illustrate a dramatic effect of different bead-beating methods on the generated microbiome profile, particularly on the *Bacteroidetes* to *Firmicutes* ratio. A shift in this ratio depending on sample preparation method has also previously been demonstrated with respect to freezing and thawing (Bahl et al., 2012). Our samples, however, have only been subjected to the same number of freezing and thawing

Abbreviation: OTU, Operational Taxonomic Unit.

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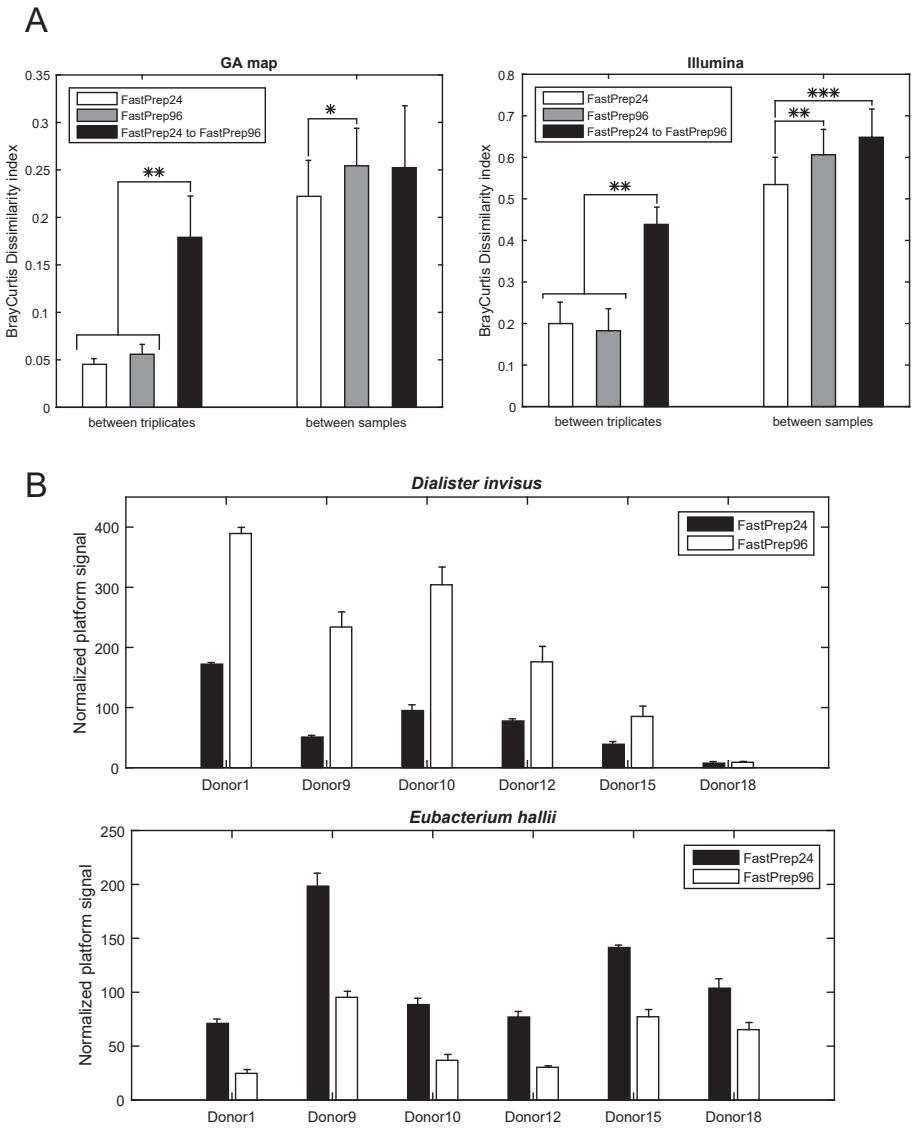

Fig. 1. Effect of bead-beating on (A) microbiota beta diversity and (B) species composition. (A) Mean Bray-Curtis beta diversity within and between sample categories are shown. Error bars represent standard error of the mean. The significance levels are designated by asterisks: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. (B) Differences in a normalized signal between bead-beating instruments for two species, as determined by GA-map™ Dysbiosis Test (mean with standard error of the mean as error bars). p-Value adjusted *t*-test of 6.6×10^{-10} and 2.4×10^{-6}, respectively, for the two species, comparing the bead-beating instruments.

cycles with no systematic differences in sample handling. Therefore, our results are probably a reflection of varying efficiency in bacterial disruption, as some organisms are more easily disrupted than others. Selection of a specific technique may thus lead to alterations in apparent community composition.

3. Conclusion

Our results highlight the challenges imposed by lack of standardization with regards to sample handling and preparation in the microbiome field, as profiles obtained using slightly different methods will be difficult to compare. Although sample preparation seems trivial and of low scientific impact, ignoring these issues can have detrimental consequences for large-scale multicenter microbiota studies. Therefore, we recommend it is key for the scientific community to take a step back to ensure that their protocols are properly optimized and harmonized. If standardization is not possible, then at least reference material with sufficient biological variation should be included to detect differences obtained using the different protocols.

Availability of data and materials

Data is available at the MiDiv Lab homepage: www.nmbu.no/en/about-nmbu/faculties/vetbio/departments/ikbm/research/midivlab/archive.

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Competing interests

KR is a board member and owner of Genetic Analysis. MKK and LF are employees in Genetic Analysis.

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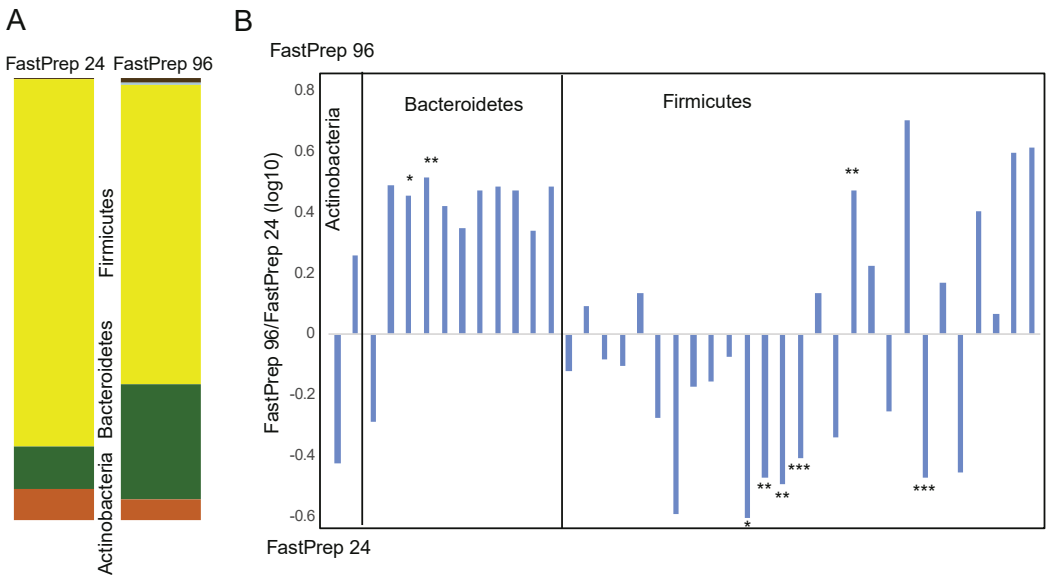

Fig. 2. Effect of bead-beating on microbiota taxonomic composition as determined by Illumina sequencing. (A) Effect on phylum level and (B) on OTU level (for OTU's that have an abundance of >1% on average in the dataset) as determined by Illumina sequencing. The ordinate represents the average number of sequences for FastPrep 96 divided by the average number of sequences for FastPrep 24 for each of the OTU's represented on the abscissa. Significant OTU's after False Discovery Correction are marked with $^{*}p < 0.05$, $^{**}p < 0.01$ and $p < 0.001$.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2016.08.005.

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Anti-TNF Therapy Response in Patients with Ulcerative Colitis Is Associated with Colonic Antimicrobial Peptide Expression and Microbiota Composition

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Abstract

Background and Aims: Anti-tumour necrosis factor [TNF] therapy is used in patients with ulcerative colitis [UC], but not all patients respond to treatment. Antimicrobial peptides [AMPs] and the gut microbiota are essential for gut homeostasis and may be important for treatment outcome. The aim of this study was to determine AMP and microbiota profiles in patients with UC before anti-TNF therapy start and correlate these data to treatment outcome.

Methods: Serum and biopsies were obtained from UC patients naïve to biological therapy [$n = 56$] before anti-TNF therapy start [baseline]. Fecal samples were taken at baseline and Weeks 2 and 6. Quantitative proteomic analysis was performed in mucosal biopsies. Expression of AMPs and cytokines was determined in biopsies and serum. Microbiota analysis of fecal samples was performed using GA-map™ Dysbiosis Test and real-time quantitative polymerase chain reaction [rtPCR]. Treatment response was evaluated 12–14 weeks after baseline.

Results: At baseline, proteomic analysis of biopsies showed that treatment responders and non-responders had differential expression of AMPs. Eleven AMP and AMP-related genes were analysed by rtPCR in mucosal biopsies and could together discriminate responders from non-responders at baseline. The most important nominators for response were increased expression of defensin 5 and eosinophilic cationic protein. Microbiota analysis revealed lower dysbiosis indexes and higher abundance of *Faecalibacterium prausnitzii* in responders compared with non-responders at baseline. Also, abundance of *F. prausnitzii* increased during induction therapy in responders.

Conclusions: Anti-TNF therapy responders and non-responders display distinctly separate patterns of mucosal AMP expression and gut microbiota before treatment start. This indicates that intestinal antimicrobial/microbial composition can influence treatment outcome.

Key Words: Anti-TNF; microbiota; antimicrobial peptides

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1. Introduction

Ulcerative colitis [UC] is a chronic inflammatory bowel disease [IBD] thought to be caused by an overreaction against the microbiota in the intestine of genetically susceptible individuals. The fact that the microbiota is of importance for IBD has been established in several studies concerning fecal stream diversion and antibiotic treatment as well as experimental mouse models.[1,2,3,4,5]

Within the gut there is a delicate interplay between the microbiota and the host. It has been shown that patients with IBD have altered microbial composition [dysbiosis]. In particular, patients with IBD show decreased microbial diversity [α diversity] and increased levels of bacteria in close contact with the mucosa.[6,7,8,9,10] Up till now no specific pathogen has been identified driving the diseases, but several studies have identified alterations in bacterial families, genuses, or phyla during inflammation. Enriched bacteria detected in IBD have for example been members of Enterobacteriaceae, Fusobacterium, and Proteobacteria which may be involved in potentiation of disease.[11,12,13,14,15,16] In contrast, a bacterium repeatedly shown to be underrepresented in IBD is *Faecalibacterium prausnitzii*.[16,17,18,19] Interestingly, it has been established that *F. prausnitzii* can exert anti-inflammatory effects via metabolites, i. short-chain fatty acids [SCFAs], acting as inhibitors of histone deacetylases [HDACs].[18,19,20] Consequences of HDAC inhibition, either via bacterial metabolites, free SCFAs, or HDAC inhibitors, have been shown to be up-regulation of transcription factors such as the proto-oncogene cFOS [cFOS] and early growth response protein 1 [EGR-1] and down-regulation of pro-inflammatory cytokines and activation markers.[21,22,23,24,25,26,27] In correlation to this, SCFAs, as well as medium-chain fatty acids, have been shown to be reduced among IBD patients compared with healthy individuals.[28,29,30]

Due to the massive load of microbes resident in our intestines, the body is equipped with a sophisticated defence system. An important feature of this system is the expression of antimicrobial peptides [AMPs], regarded as endogenous antibiotics.[31] A broad range of AMPs exists, such as defensins, cathelicidins, and lysozymes, some of which are expressed constitutively whereas others are induced by inflammation.[32] Studies have shown that patients with Crohn's disease [CD] have reduced antimicrobial activity and decreased expression of alpha-defensins and cathelicidin.[33,34,35] It has also been shown that the dysregulation of AMPs in the inflamed IBD mucosa can be widely restored in patients responding to infliximab [IFX] therapy.[36]

In order to dampen the inappropriately activated immune reaction against the commensal flora in UC, patients can be offered treatment with anti-tumour necrosis factor [anti-TNF] agents, most frequently IFX or adalimumab [ADA].[37,38,39] However, it has been shown that only 50–70% of patients with UC respond to IFX [37,40] and the reason for this is incompletely known. Recent data show that dose escalation can overcome incomplete primary response in some patients.[41,42] This correlates well with studies showing that non-responders have a more severe pro-inflammatory cytokine profile before treatment start, with higher mucosal mRNA expression of tumour necrosis factor [TNF], interleukin [IL]-1β, IL-17A, IL-6, and interferon [IFN]-γ as compared with responders.[43,44]

Many studies have focused on either the gut microbiota or the defence mechanisms of the mucosa. However, few have studied these factors together and, at the same time, linking them to treatment outcome. In this study we hypothesised that the functional composition between the microbiota and the host defence in the gut is of importance for anti-TNF therapy outcome in patients with UC. To investigate this, we analysed factors expressed by the mucosa in response to the microbiota with focus on AMPs, AMP-regulating genes, cytokines, and transcription factors. Also, we analysed fecal dysbiosis and the fecal microbiota profile before treatment start and examined the levels of *F. prausnitzii* before and during induction therapy. All data were related to anti-TNF therapy outcome.

2. Materials and Methods

2.1. Patient population

Patients with UC [n = 56], naïve to any biological therapy, who were starting anti-TNF therapy between September 2010 and April 2014, were consecutively recruited and included in the study at the outpatient clinics at: Sahlgrenska University Hospital, Gothenburg; Kärnsjukhuset in Skövde; and Södra Älvsborg Hospital, Borås, Sweden. Clinical and demographic characteristics are presented in Table 1. The disease activity was determined by Mayo score[45] before treatment started [baseline] and at Week 14. All patients had active mucosal inflammation at inclusion, with a total Mayo score of ≥ 3 and an endoscopic Mayo score of 1–3 [Table 1]. All patients had failed increased dosing of 5-ASA and/or thiopurines and were either corticosteroid-refractory or corticosteroid-dependent.

Treatment response was defined as a decrease in total Mayo score with ≥ 3 compared with baseline, a definition used in the Applied Clinical Trials [ACT] 1 and 2[37] and by us previously. [46,47] Responders had a reduction of at least 1 in endoscopic Mayo score from 2 [1–3] at baseline to 0 [0–2] at evaluation, $p < 0.0001$. Only one responding patient had an endoscopic Mayo score of 2 at evaluation; all the others reached mucosal healing with an endoscopic Mayo score of 0–1. Non-responders showed no improvement of the endoscopic Mayo score, being 2 [1–3] at baseline and 2 [1–3] at evaluation, $p > 0.99$. Numbers show median [range].

2.2. Sample specimens

At baseline, serum samples were obtained from all included patients, four biopsy specimens from inflamed sigmoid colon were obtained from 30 of the patients [responders n = 18 and non-responders n = 12], and stool samples for calprotectin analysis were obtained from 45 of the patients [responders n = 22 and non-responders n = 23]. Stool samples for microbiota analysis were obtained from 7 patients at baseline [responders n = 4 and non-responders n = 3], 15 patients at Week 2 [responders n = 8 and non-responders n = 7], and 13 patients at Week 6 [responders n = 8 and non-responders n = 5]. Comparison of patient characteristics as in Table 1 between responders and non-responders for analysis of biopsies [n = 30] and microbiota [baseline n = 7, Week 2 n = 15, and Week 6 n = 13] showed no differences between the groups [data not shown]. Serum samples were stored at -80°C and stool samples at -20°C. All biopsies were taken from inflamed rectosigmoid junction and stored at -80°C. Two biopsies were collected in RNA later for gene expression analysis and two were collected and cultured for 24 h in Iscove´s medium supplemented with 5% human AB$^+$ serum, 100 µg/ml gentamicin [Sigma, St Louis, MO] and 3 µg/ml L-glutamine [Sigma]. At the end of the cell culture, the biopsies were collected and pooled and stored at -80°C.

2.3. Proteomic analysis

Proteomic analysis using mass spectrometry [MS] was performed at the Proteomics Core Facility at Sahlgrenska Academy, University of Gothenburg, Sweden, as previously described.[47] In brief, cultured mucosal biopsies from three responders and three non-responders were lysed, sonicated, homogenised, and tryptically digested.

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Table 1. Demographics of UC patients before anti-TNF therapy start.

	Responders[a]	Non-responders[b]	*p*-Value[c]
Total number of patients	31	25	
Male/female	21/10	19/6	0.56
Age, median [range]	36 [18–66]	37 [21–67]	0.44
Treatment [IFX/ADA]	28/3	22/3	> 0.99
Smoking habit [active/ex-smoker/never]	2/8/21	1/2/20[d]	0.17
Disease duration [years], median [range]	4 [1–27]	2 [1–24]	0.46
Mayo score, median [range]	8 [3–11]	8 [3–11]	0.63
Endoscopic Mayo score [score 1/2/3]	5/19/7	3/15/7	0.63
Colonic area involved, left side/extensive	7/24	5/20	> 0.99
Calprotectin [µg/g], median [range]	1250 [230–18000]	1400 [270–15400]	0.46
C-reactive protein, median [range]	5 [< 1–73]	4 [< 1–110]	0.96
Other treatments than anti-TNF:			0.55
Corticosteroids, 5-ASA, thiopurines	5	1	
Corticosteroids, 5-ASA	3	6	
Thiopurines, 5-ASA	7	5	
Corticosteroids, thiopurines	0	1	
5-ASA	11	7	
Corticosteroids	3	1	
Thiopurines	2	2	
No treatment	0	1	
Antibiotics	0	0	

TNF, tumour necrosis factor; UC, ulcerative colitis; IFX, infliximab; ADA, adalimumab; 5-ASA, 5-aminosalicylic acid.

[a]Defined as a decrease in Mayo score ≥ 3, 12–14 weeks after therapy start.

[b]Defined as a decrease in Mayo score of ≤ 2, 12–14 weeks after therapy start.

[c]Mann-Whitney test.

[d]Data from two patients are missing.

Protein concentration was determined and peptides were labelled with TMT [tandem mass tag] following the manufacturer's instructions [Thermo Scientific Inc.] for an Isobaric Mass Tagging Kit. Two groups of labelled samples were produced [one group comprised cultured biopsy samples from three responders and the other comprised biopsies from three non-responders]. The complexity of each set was reduced by fractionation using strong cation exchange chromatography. Each fraction was then desalted and subjected to nano-liquid chromatography-MS/MS using an LTQ-Orbitrap Velos [Thermo Fisher Scientific, Inc., Waltham, MA, USA] mass spectrometer interfaced with an Easy-nLC autosampler [Thermo Fisher Scientific, Inc., Waltham, MA, USA] and a nano-LC column, 200 x 0.075 mm, packed in-house with 3 µm Reprosil-Pur C18-AQ particles [Dr Maisch, Ammerbuch, Germany]. The fractionated sample was analysed twice, excluding identified peptides from the first run in the second analysis. All resulting spectra per TMT set were merged for protein identification using Proteome Discoverer version 1.3 [Thermo Fisher Scientific, Inc., Waltham, MA, USA] with the Mascot search engine [Matrix Science Ltd, London, UK] using the human Swissprot Database version 2.3 from October 2010. The peptide threshold was set to 1% false discovery rate by searching against a reversed database, and identified proteins were grouped by sharing the same sequences to minimise redundancy.

2.4. Quantitative real-time polymerase chain reaction analysis of mucosal biopsies

Total mRNA from mucosal biopsies was extracted using Nucleospin® DNA, RNA, and protein purification kit [Macherey-Nagel, Düren, Germany] according to the manufacturer's protocol. RNA concentration and purity were measured using a NanoDrop ND-1000 spectrophotometer [NanoDrop Technologies, Wilmington, Delaware, USA] with 260/280 and 260/230 ratios of ~2 and 2.1–2.2, respectively. Also, RNA integrity of five randomly picked RNA samples was analysed on a 1.5% agarose gel stained with ethidium bromide. cDNA was prepared using the QuantiTect Reverse Transcription kit [Qiagen, Hilden, Germany] according to the manufacturer's protocol. cDNA was then used for real-time polymerase chain reaction [RT-PCR] using Taqman Universal PCR Master Mix [Applied Biosystems, Foster City, CA] and Taqman Gene Expression assays [Applied Biosystems] according to the manufacturer's protocol. Expression of defensin 5 [DEF5, gene name = *DEFA5*], bactericidal/permeability-increasing protein [BPI], histone H1.5 [HIST1, gene name = *HIST1H1B*], ribosomal protein S19 [*RPS19*], eosinophil cationic protein [ECP, gene name = *RNASE3*], high mobility group nucleosomal binding domain 2 [*HMGN2*], high mobility group box 1 [*HMGB1*], histone deacetylase 1 [*HDAC1*], lysozyme [LYZ], cathelicidin antimicrobial peptide [CATH, gene name = *CAMP*], human β-defensin 2 [hBD2, gene name = *DEFB4A*], interleukin 6 [*IL-6*], *IL-12a*, *IL-17A*, *TNF*, and also CD154 [gene name = *CD40LG*], *cFOS,* and *EGR-1,* were determined. Amplification was carried out using a 7500 RT-PCR system [Applied Biosystems] and all samples were run in triplicate. The results were normalised to the expression level of *GAPDH* and *HPRT* and expressed as $2^{-[\text{Target-Housekeeping}]}$.

2.5. Analysis of serum samples

Serum samples were collected for enzyme-linked immunosorbent assay [ELISA] analysis. Serum levels of ECP [Diagnostics Development, Uppsala, Sweden] and CATH [CSB-EL004476HU, Cusabio, Wuhan, China] were analysed according to the manufacturers' instructions.

2.6. Microbiota analysis

Microbiota analysis was performed using the GA-map™ Dysbiosis Test [Genetic Analysis AS, Oslo, Norway]. The GA-test is based on regular molecular biology techniques, comprising human fecal

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sample homogenisation and mechanical bacterial cell disruption; automated total bacterial gDNA extraction using magnetic beads; 16S rRNA PCR DNA amplification covering V3–V9; probe labelling by single nucleotide extension; hybridisation to complementary probes coupled to magnetic beads; and signal detection using BioCode 1000A 128-Plex Analyzer [Applied BioCode, Santa Fe Springs, CA, USA].[48] The GA-test consists of 54 DNA probes targeting ≥ 300 bacteria on different taxonomic levels. The probes were selected based on the ability to distinguish between healthy controls, irritable bowel syndrome [IBS], and IBD patients. The model algorithmically assesses fecal bacterial abundance and profile and potential clinically relevant deviation in the microbiome from normobiosis. The dysbiosis model output is a bacterial profile and a Dysbiosis Index score. Dysbiosis Indexes above 2 [maximum 5] indicate a microbiota that differs from the reference group.

2.7. Fecal DNA extraction and PCR

DNA was extracted from 100-200 mg of feces using the QIAamp Fast DNA Stool Mini Kit [Qiagen] and stored at -20°C.

Specific oligonucleotide primers and probes targeted to the 16srRNA gene [rDNA] were used to amplify the sequences of *F. prausnitzii* [primer/probe set: *Fprau*] and conserved rDNA present in all bacteria [universal primer/probe set: *Uni*]. Primers for Fprau generated a 203 bp amplicon using the forward primer FprauF [5' GGA GGA TTG ACC CCT TCA GT 3', T_m 59.4°C] and reverse primer FprauR [5' CTG GTC CCG AAG AAA CAC AT 3', T_m 57.3°C].[49] The probe *FprauP* [6-FAM 5' CTT GAC ATC CTG CGA CGC GC 3' TAMRA, T_m 68.5°C] was designed in house. Primers for *Uni* generated a 466-bp amplicon using the forward primer UniF [5' TCC TAC GGG AGG CAG CAG T 3', T_m 59.4°C] and the reverse primer UniR [5' GGA CTA CCA GGG TAT CTA ATC CTG TT 3', T_m 58.1°C]; the probe used was UniP [6-FAM 5' CGT ATT ACC GCG GCT GCT GGC AC 3' TAMRA, T_m 69.9°C].[50]

PCR amplification was performed in a final volume of 20 µl using Taqman Universal PCR Master Mix [Applied Biosystems] containing 100 nM of each primer and 250 nM probe. Reaction conditions were 2 min at 50°C, 10 min at 95°C, and 45 cycles of 15 s at 95°C and 60 s at 60°C. Amplification was carried out using a 7500 real-time PCR system [Applied Biosystems] and all samples were run in triplicate. The CT values of *Fprau* were normalised to the CT values of *Uni* and expressed as $2^{-[Fprau-Uni]}$.

2.8. Statistical analysis

To examine the relation between anti-TNF therapy response and non-response [Y-variables] and various AMP, cytokine, transcription factor, and bacterial factors [X-variables], multivariate factor analysis [SIMCA-P+ software; Umetrics, Umeå, Sweden] was used. Orthogonal partial least squares discriminant analyses [OPLS-DA] was implemented to correlate a selected Y-variable and X-variables with each other in linear multivariate models. The quality of the OPLS-DA was based on the parameter R2, that is, the goodness of the fit of the model [best possible fit, R2 = 1]. In the OPLS-DA loading column plots, the importance of each X-variable [cytokine] to Y [therapy outcome] is represented by column bars. The variables positioned in the same direction as the bar representing therapy response are positively associated, whereas variables in the opposite direction are inversely related to therapy response. The larger the bar and smaller the error bar, the stronger and more reliable is the contribution to the model. The whiskers show the range [95% confidence intervals] of the measured X- and Y-variables.

Mann-Whitney U test was used to evaluate differences between two groups, and Kruskal-Wallis test followed by Dunn's multiple comparisons test were used to evaluate differences between three groups. All analyses were performed using GraphPad Prism 6.0 [GraphPad Software, La Jolla, USA]; *p*-values < 0.05 were considered as statistically significant.

2.9. Ethical considerations

The study was performed after receiving written informed consent from all subjects and the protocol was approved by the Regional Ethical Review Board at the University of Gothenburg. Evaluations of treatment response and laboratory analyses were blinded to each other.

3. Results

3.1. The baseline expression patterns of mucosal antimicrobial peptides differ in anti-TNF therapy responders and non-responders

To investigate proteins associated with anti-TNF therapy response, proteomic analysis of biopsies obtained at baseline from six patients, three responders, and three non-responders, was performed. Among the identified proteins for responders [*n* = 1505] and non-responders [*n* = 1822], only responders showed detectable expression of DEF5, BPI, ECP, HIST1, RPS19, HMGB1, and HMGN2, which are proteins with antimicrobial functions or which can affect expression of AMPs [Table 2]. In contrast, only non-responders showed detectable

Table 2. Protein identification in biopsies at baseline from anti-TNF therapy responders and non-responders.

Protein name [abbreviation, Swiss prot Accession no]	Function	Responders [no. of peptides[a]]	Non-responders [no. of peptides]
Defensin-5 [*DEF5, Q01523*]	Antimicrobial	Yes [1]	No [0]
Bactericidal permeability-increasing protein [*BPI, P17213*]	Antimicrobial	Yes [1]	No [0]
Eosinophil cationic protein [*ECP, P12724*]	Antimicrobial	Yes [3]	No [0]
Histone H1.5 [*HIST1, P16401*]	DNA binding	Yes [10]	No [0]
40S ribosomal protein S19 [*RPS19, P39019*]	RNA processing and maturation	Yes [5]	No [0]
High-mobility group protein B1 [*HMGB1, P09429*]	DNA binding	Yes [5]	No [0]
Non-histone chromosomal protein HMG-17 [*HMGN2, P05204*]	DNA binding	Yes [2]	No [0]
Histone deacetylase 1 [*HDAC1, Q13547*]	Transcriptional regulation	No [0]	Yes [2]
Lysozyme C [*LYZ, P61626*]	Antimicrobial	Yes [2]	Yes [1]

TNF, tumour necrosis factor.

[a]Number of tryptically digested peptides that match the identified protein. At least one matching peptide for each identified protein must fulfil significance criteria.

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expression of HDAC1, which inhibits AMP expression, whereas the AMP LYZ was found among both responders and non-responders [Table 2].

To confirm these findings in a quantitative manner, gene expression of the proteins presented in Table 2 together with the classical AMPs hBD2 and CATH was then analysed by RT-PCR in mucosal biopsies obtained at baseline. Multivariate factor component analysis by OPLS-DA revealed different expression patterns for responders and non-responders with regard to the AMP and AMP-related genes, with a model fit of R2 = 0.59 [Figure 1A]. The variables contributing most to the model were identified in the OPLS-DA loading column plot and showed that high levels of *DEFA5* and *ECP* characterised response, whereas high levels of *CATH* characterised non-response [Figure 1B]. These results were further confirmed by univariate analysis comparing expression of *DEFA5*, *ECP,* and *CATH*, respectively. Thus, responders had higher expression of *DEFA5* and *ECP* but lower expression of *CATH* as compared with non-responders [Figure 1C]. Furthermore, serum analysis at baseline showed that responders had higher levels of circulating *ECP* whereas no differences were detected for *CATH* [Figure 2A and B, respectively]. Serum levels of *DEFA5* were below the detection limit [data not shown]. Altogether, this indicates that the outcome of anti-TNF therapy is linked to the expression pattern of the antimicrobial peptide response before treatment start.

3.2. The fecal microbiota differs between treatment responders and non-responders at baseline

As differences in the antimicrobial response may be an indication of deviations in the gut microbiota, the fecal bacterial composition and the grade of dysbiosis were analysed by the GA-map™ Dysbiosis Test. Fecal samples obtained at baseline from four responders and three non-responders were analysed and showed that responders tended to have lower dysbiosis indexes as compared with non-responders [2, 3, 3, and 5 vs 4, 5 and 5, one-tailed p = 0.097]. In addition, multivariate factor discriminant analysis of the bacteria showed that treatment responders and non-responders clustered into different groups with a model fit of R2 = 0.93 [Figure 3A]. In this model, the bacteria were gathered into 15 different groups [shown in Figure 3B] and the OPLS-DA loading scatter plot identified *F. prausnitzii* as the most important factor associated with treatment response [Figure 3B]. The probe intensity of *F. prausnitzii* tended to be higher among responders as compared with non-responders [Figure 3C].

3.3. Anti-TNF therapy responders show high abundance of *F. prausnitzii* that increase during induction therapy

To further investigate *F. prausnitzii*, PCR analyses of fecal samples obtained at baseline, Week 2 and Week 6, were performed where *F. prausnitzii* was related to total bacteria by detection of 16srRNA genes. Responders had higher abundance of *F. prausnitzii* as compared with non-responders at Weeks 2 and 6, and a tendency towards higher levels already at baseline [Figure 4A]. Also, the abundance of *F. prausnitzii* increased during induction therapy for anti-TNF responders [Figure 4A, medians linked by striped line, p = 0.01] whereas no differences were detected for non-responders over time [Figure 4A, medians linked by dotted line, p = 0.83]. The differences in abundance of *F. prausnitzii* followed a different pattern from calprotectin which was similar in the two groups at baseline and Week 2 and only decreased in responders at Week 6 [Figure 4B]. As

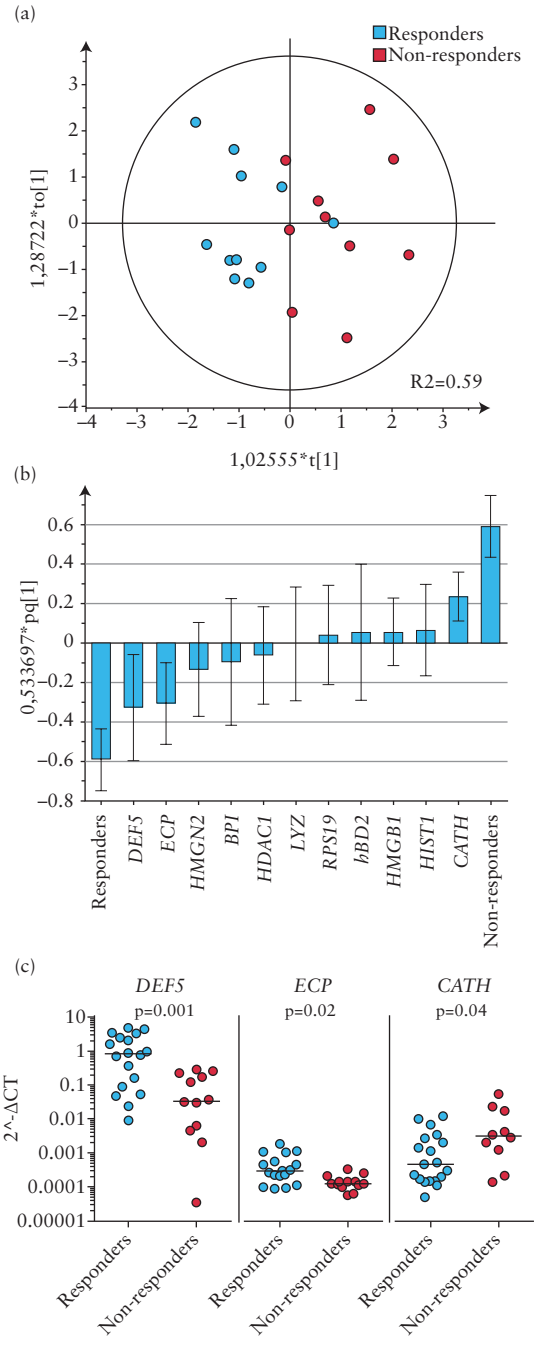

Figure 1. Anti-TNF therapy responders and non-responders show different mucosal AMP expression before start of treatment. Biopsy samples were obtained at baseline and mucosal mRNA expression of *DEF5, ECP, HMGN2, BPI, HDAC1, LYZ, RPS19, hBD2, HMGB1, HIST1,* and *CATH* was analysed with RT-PCR. [A] Multivariate discriminant analysis [OPLS-DA] scatter plot showing the separation between responders [n = 11] and non-responders [n = 10] based on the analyzsd X-variables [n = 11]. [B] OPLS-DA column loading plots depicting the association between treatment outcome and analysed variables. [C] Univariate analysis of mucosal *DEF5, ECP,* and *CATH* mRNA expression comparing treatment responders and non-responders [responders n = 17–19 and non-responders n = 10–12]. Results are displayed as the normalised ratios between the relative expression of the gene of interest and the housekeeping genes *GAPDH* and *HPRT*. Each symbol represents one individual and horizontal lines indicates median of the group. TNF, tumour necrosis factor; AMP, antimicrobial peptide; RT-PCR, real-time polymerase chain reaction; OPLS-DA, orthogonal partial least squares discriminant analyses.

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expected, the calprotectin levels decreased during induction therapy for responders [Figure 4B, medians linked by striped line, $p = 0.005$] whereas no differences were detected for non-responders [Figure 4B, medians linked by dotted line, $p = 0.50$]. Taken together this shows that a favourable treatment outcome is linked to high abundance of *F. prausnitzii*.

3.4. The local inflammatory milieu differs between treatment responders and non-responders

For further insight into the local milieu, we examined mucosal gene expression of proteins known to be influenced by the microbiota: *cFOS*, *EGR-1*, *IL-6*, *IL-12a*, *IL-17A*, *TNF*, and *CD154*. When taking all seven genes into account, responders and non-responders formed different clusters with a model fit of R2 = 0.52 [Figure 5A]. The loading scatter plot showed that response was associated with higher levels of *cFOS* and *EGR-1*, whereas non-response was defined by higher levels of *IL-6*, *IL-12a*, *IL-17A*, *TNF*, and *CD154* [Figure 5B]. When performing univariate analyses, *IL-6*, *IL-17A*, and *TNF* expression was higher among non-responders [Table 3].

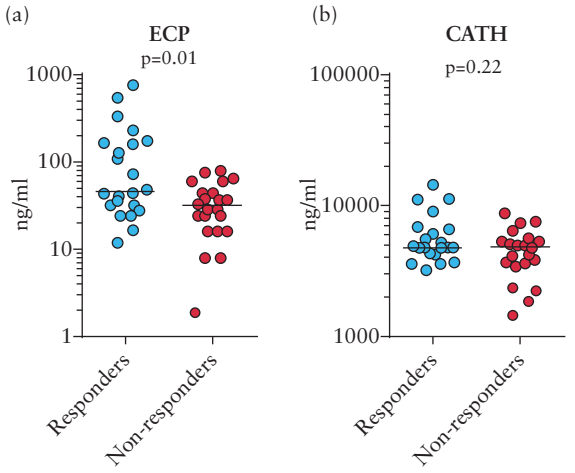

Figure 2. Serum levels of ECP and CATH in anti-TNF therapy responders and non-responders. Serum samples were obtained at baseline [responders $n = 23$ and non-responders $n = 21$], and levels of ECP [A] and CATH [B] were analysed by ELISA. Each symbol represents one individual and horizontal lines indicates median of the group. TNF, tumour necrosis factor; ELISA, enzyme-linked immunosorbent assay.

4. Discussion

The interplay between anti-TNF therapy and the antimicrobial/microbial milieu in the gut is so far an area poorly explored but may in fact be of importance for treatment outcome. In this study we show that response to anti-TNF therapy is related to specific AMP, microbiota, and cytokine profiles of the gut. When comparing the two groups, treatment responders have high levels of *DEF5*, *ECP*, and *F. prausnitzii*, whereas non-responders have high levels of *CATH*, *IL-12*, *IL-17A*, and *TNF* before treatment start.

Ulcerative colitis is a multifactorial disease and it appears reasonable that the underlying cause for treatment outcome would also be complex. Thus, multivariate factor analysis is becoming an important tool to study systems rather than investigating single factors. In the primary phase of this study we used proteomic analysis to identify proteins of interest which differed between the groups. The aim here was not to perform quantification but to identify possible targets to validate in a larger cohort. By doing this we found that AMP expression patterns differed before anti-TNF treatment start for responders and non-responders. A reason for this may be genetic predisposal for aberrant AMP expression, but so far no convincing data concerning genomic predictors for AMP expression, or for anti-TNF therapy response, in UC have been identified. In fact, it has been shown that dysregulation of many AMPs is a consequence of inflammation.[36] In line with this, it was recently shown in mice that bacterial dysbiosis had a causal role in the development of chronic ileal inflammation with failure of Paneth cell function as a secondary effect.[51] Thus, as an alternative, the AMP expression patterns could be related to the composition of the gut microbiota, which in this study was analysed by the GA-map™ Dysbiosis Test and via specific PCR for *F. prausnitzii*. The GA-technology, in contrast to high-throughput sequencing, detects a panel of pre-defined bacteria and is mainly focused on revealing patterns which differ from normal, with the breadth of microbial knowledge gained from microbiome projects.[48]

Despite low numbers of fecal samples at baseline, we revealed discrimination between the patient groups based on the fecal microbiota

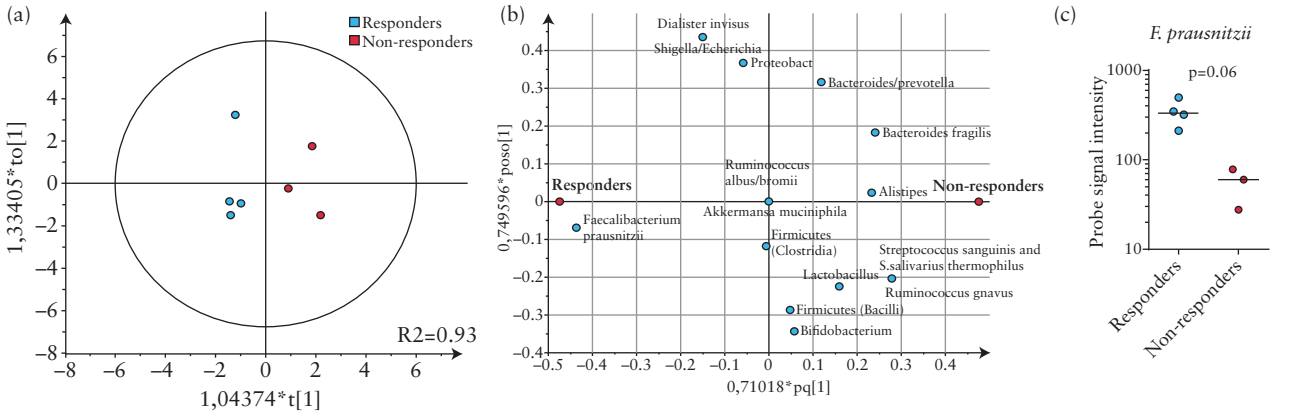

Figure 3. Anti-TNF therapy responders and non-responders differ at baseline with respect to their fecal microbiota composition. Fecal samples were obtained at baseline [responders $n = 4$ and non-responders $n = 3$] and analysed by the GA-map™ Dysbiosis Test. OPLS-DA scatter plot [A] and loading scatter plot [B] depicting associations between treatment response [Y-variables] and fecal bacterial groups [X-variables, $n = 15$]. [C] Probe signal intensities for *F. prausnitzii* for anti-TNF therapy responders and non-responders. TNF, tumour necrosis factor; OPLS-DA, orthogonal partial least squares discriminant analyses.

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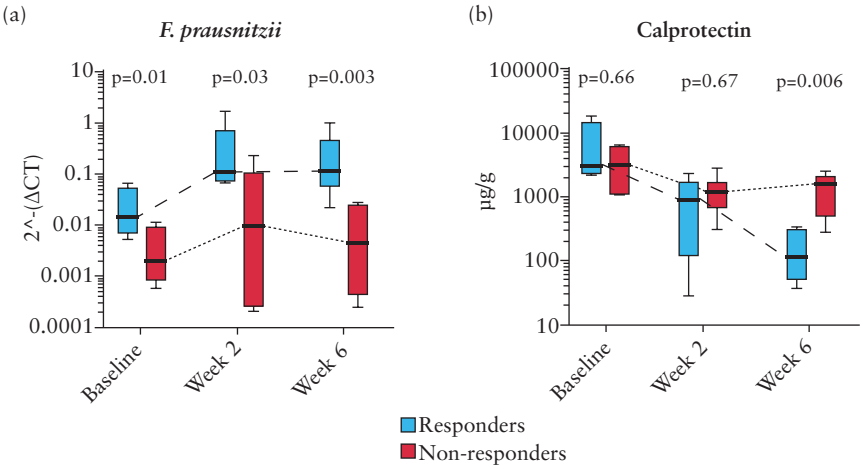

Figure 4. Anti-TNF therapy responders show higher abundance of *F. prausnitzii* compared with non-responders. Fecal samples were obtained at baseline [responders $n = 4$ and non-responders $n = 3$], Week 2 [responders $n = 8$ and non-responders $n = 7$], and Week 6 [responders $n = 8$ and non-responders $n = 5$]. [A] The abundance of *F. prausnitzii* in relation to total bacteria was analysed by PCR. [B] Fecal calprotectin was analysed by ELISA. The boxes range from the first to the third quartile, the median being shown as the thick horizontal line. The whiskers show the range. The lines link the medians in each group for each time point [striped lines; responders, and dotted lines; non-responders]. TNF, tumour necrosis factor; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.

composition and a tendency towards differences in dysbiosis indexes. Importantly, the abundance of *F. prausnitzii* tended to be higher in treatment responders than non-responders at baseline. Further analysis of *F. prausnitzii* during induction therapy revealed an increasing abundance of this species in treatment responders. Interestingly, *F. prausnitzii* has been suggested as a sensor of intestinal health,[52] and the recovery of *F. prausnitzii* in patients with UC after relapse is associated with maintained remission.[17] In murine models of colitis, intra-gastric administration of *F. prausnitzii* has been shown to ameliorate inflammation.[19,53] Despite these data, it seems unlikely that one bacterial species would be the only nominator for gut balance and, indeed, reduction of other bacteria has been linked to IBD and/or anti-TNF therapy; *Roseburia hominis*, *Eubacterium rectale*, and *Bifidobacterium spp.*[10,18] A common function for these bacteria, including *F. prausnitzii*, is that they are all producers of SCFAs, and the total production of microbiota metabolites is probably more important than presence of certain species. Unfortunately, no samples were collected to measure SCFA levels in the gut mucosa in this study and the metabolic function of the gut bacteria warrants further investigation. However, factors influenced by SCFA were studied in the mucosa and revealed different patterns between the groups, indicating higher levels of SCFA in responders. Few of the cytokines and transcription factors reached significant differences between the groups by themselves, but again the global pattern of expression is likely to be of more importance. Clear deviations as seen in *in vitro* studies, like the reduction of IL6 and IL12a expression upon addition of HDAC inhibitors to macrophages,[23,24] would be difficult to detect *in vivo*.

The fact that the fecal microbiota is related to inflammation and also anti-TNF therapy, was recently shown in a study of paediatric patients with IBD. Similar to results in our study, they showed that the intestinal microbiota can predict therapeutic responses and might also be a potential biomarker for inflammation.[10] In this study, we extended the analyses to include inflammatory responses of the host, to strengthen the concept of cross-talk between the microbiota and the host. The studies of fecal calprotectin also underline the importance of microbiota-host interaction, since the microbial change precedes decreased fecal calprotectin levels. This also indicates that microbial fecal analyses may be a faster way of detecting treatment response than calprotectin analysis.

There are limitations in this study, mainly concerning the numbers of fecal and biopsy samples obtained before treatment start. Many of the patients were included on the day of their first anti-TNF injection, and thus no biopsy or stool samples at baseline could be obtained. Also, some patients were unwilling to give stool samples and this was not an exclusion criterion to join the study. Retrospectively, it would have been desirable to collect samples from all patients and also for metabolomic studies. New patient cohorts need to be recruited in order to confirm the present data. Also, if *F. prausnitzii* has a future as a biomarker for treatment response, this needs to be validated in a separate patient cohort. It would also be interesting to investigate if AMP levels in fecal samples may be used as biomarkers for treatment response. However, mucosal levels of *DEF5* and *ECP* showed large individual variance and may be more of importance in a multivariate factor analysis approach. Importantly, the aim of this study was not to develop biomarkers but to investigate mucosal interactions in relation to treatment response. In contrast, a study employing a whole human genome array analysis identified five predictive genes for anti-TNF therapy response: osteoprotegerin, stanniocalcin-1, prostaglandin-endoperoxide synthase 2, interleukin 13 receptor alpha 2, and interleukin 11, all involved in the adaptive immune response. [54] Whether the expression of these genes is related to the antimicrobial defence described in this study warrants further investigation.

Despite the data presented in this study, the question still remains why some patients have an antimicrobial/microbial milieu which seems to favour anti-TNF response and others do not. Also, is there anything we can do to change it? It is known that many factors influence the ecosystem in our gut, eg diet, stress, medications, genetics, health, and lifestyle, and most of these factors are beyond reach of this study. However, we do show that there are no differences in age, Mayo score [total and endoscopic], medications, or smoking habits between the study groups at baseline. Questionnaires concerning diet, stress, and lifestyle before baseline may shed light on this issue and remain to be elucidated. In addition to this, microbial changes during the years

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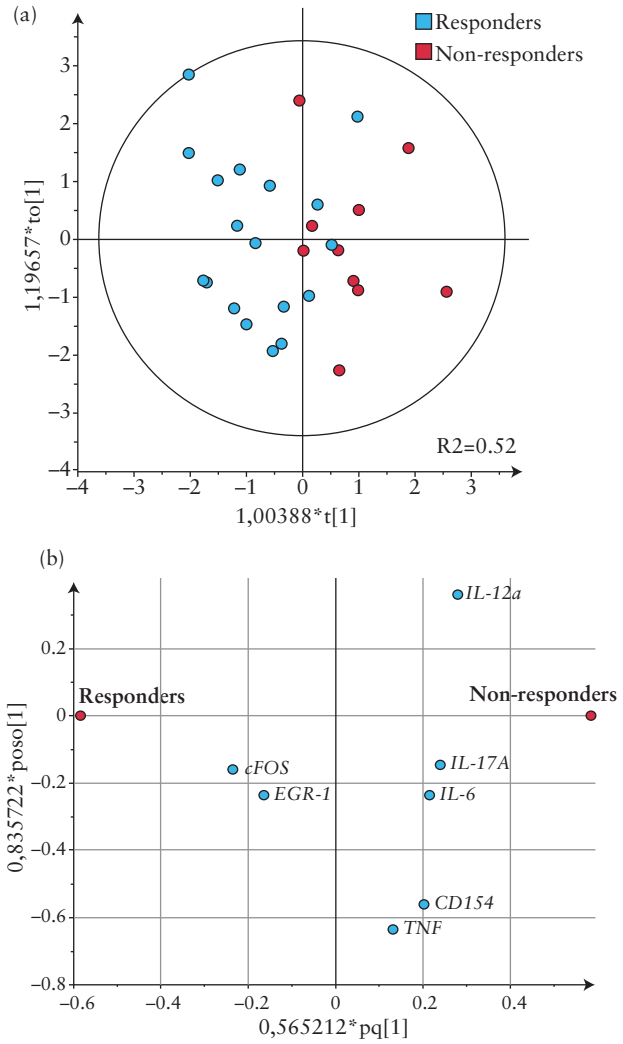

Figure 5. Multivariate factor analysis depicting associations between anti-TNF therapy response and genes involved in mucosal inflammation and regulation. Biopsy samples were obtained at baseline [responders *n* = 18 and non-responders *n* = 12] and mucosal mRNA expression *IL-6, IL-12a, IL17A, TNF, CD154, cFOS,* and *EGR-1* were analysed with RT-PCR. [A] OPLS-DA scatter plot showing the separation between responders and non-responders based on the analysed X-variables [*n* = 7]. [B] OPLS-DA loading scatter plot depicting the association between treatment outcome and analysed variables. TNF, tumour necrosis factor; OPLS-DA, orthogonal partial least squares discriminant analyses; RT-PCR, real-time polymerase chain reaction.

of disease would be interesting to investigate. Such studies could provide clues how to counteract a non-permissive gut profile for anti-TNF therapy outcome. Indeed, we have learned from multiple mouse studies that deliberate alteration of the microbiota can induce diseases or even change behaviour.[55,56] The most compelling evidence for this in humans with gastrointestinal diseases is fecal transplantation for treatment of patients with severe *Clostridium difficile* infection.[57]

Finally, there may be multiple other reasons for success or failure of anti-TNF therapy, such as differences in drug turn-over time, drug secretion, unknown genetic factors, and differences in disease progression. With this study we would like to highlight the challenge anti-TNF therapy faces in relation to the gut ecology. We have shown that the AMP, cytokine, and microbial expression patterns differ substantially in anti-TNF therapy responders and non-responders before treatment start. At this time point we are unable to reveal the cause of the differences in gut ecology; but we provide an insight into the system and suggest that the antimicrobial/microbial milieu in the gut can be of importance for anti-TNF therapy outcome.

Table 3. Mucosal gene expression in biopsies at baseline for anti-TNF therapy responders and non-responders.

Gene	Responders [x 10^{-3}][a] *n* = 18	Non-responders [x 10^{-3}][a] *n* = 12	*p*-Value[b]
cFOS	1.51 [0.72–5.90][c]	0.86 [0.22–2.42]	0.14
EGR-1	21.30 [9.92–49.49]	11.22 [8.19–19.24]	0.10
IL-6	0.77 [0.24–1.46]	1.67 [1.09–2.34]	0.06
IL-12a	0.53 [0.29–0.83]	0.91 [0.53–2.81]	0.03
IL-17A	0.92 [0.46–1.57]	1.65 [1.42–2.27]	0.03
TNF	0.26 [0.18–1.01]	0.65 [0.47–0.81]	< 0.05
CD154	2.91 [1.68–4.91]	4.12 [3.12–5.66]	0.10

TNF, tumour necrosis factor; *cFOS*, proto-oncogene cFOS; *EGR-1*, early growth response protein 1; *IL-6*, interleukin 6; *IL-12a*, interleukin 6 subunit alpha; *IL-17A*, interleukin 17A.

[a]The results were normalised to the expression level of GAPDH and HPRT and expressed as $2^{-[\text{Target-Housekeeping}]}$. Data in the column should be multiplied by 10^{-3}.

[b]Mann-Whitney test.

[c]Data are shown as median [25–75th percentile].

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Conflict of Interest

None.

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Author Contributions

MKM and LÖ contributed to the concept and design of the study. MKM and MS performed the experiments. HS, AB, AL, and KAU recruited and enrolled patients in the study. All the authors contributed to the analysis and interpretation of data. MKM and LÖ wrote the manuscript and HS, MS, AB, AL, and KAU critically reviewed it and approved the final draft.

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AP&T Alimentary Pharmacology and Therapeutics

Deviations in human gut microbiota: a novel diagnostic test for determining dysbiosis in patients with IBS or IBD

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SUMMARY

Background
Dysbiosis is associated with many diseases, including irritable bowel syndrome (IBS), inflammatory bowel diseases (IBD), obesity and diabetes. Potential clinical impact of imbalance in the intestinal microbiota suggests need for new standardised diagnostic methods to facilitate microbiome profiling.

Aim
To develop and validate a novel diagnostic test using faecal samples to profile the intestinal microbiota and identify and characterise dysbiosis.

Methods
Fifty-four DNA probes targeting ≥300 bacteria on different taxonomic levels were selected based on ability to distinguish between healthy controls and IBS patients in faecal samples. Overall, 165 healthy controls (normobiotic reference collection) were used to develop a dysbiosis model with a bacterial profile and Dysbiosis Index score output. The model algorithmically assesses faecal bacterial abundance and profile, and potential clinically relevant deviation in the microbiome from normobiosis. This model was tested in different samples from healthy volunteers and IBS and IBD patients (n = 330) to determine the ability to detect dysbiosis.

Results
Validation confirms dysbiosis was detected in 73% of IBS patients, 70% of treatment-naïve IBD patients and 80% of IBD patients in remission, vs. 16% of healthy individuals. Comparison of deep sequencing and the GA-map Dysbiosis Test, (Genetic Analysis AS, Oslo, Norway) illustrated good agreement in bacterial capture; the latter showing higher resolution by targeting pre-determined highly relevant bacteria.

Conclusions
The GA-map Dysbiosis Test identifies and characterises dysbiosis in IBS and IBD patients, and provides insight into a patient's intestinal microbiota. Evaluating microbiota as a diagnostic strategy may allow monitoring of prescribed treatment regimens and improvement in new therapeutic approaches.

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INTRODUCTION

Intestinal microbiota is generally comparable for individuals comprising the general adult population, with recent evidence supporting the gut microbiota as representing a healthy state defined as normobiosis.[1–3] Notably, deviations from normobiosis can result in a transient or permanent microbiotic imbalance known as dysbiosis, which has been linked to several disorders, including Crohn's disease (CD), ulcerative colitis (UC), irritable bowel syndrome (IBS), obesity, nonalcoholic steatohepatitis, and type I and type II diabetes.[4–8]

Traditionally, evaluation of intestinal microbiota composition has been based on breath-testing methods, small-bowel culture techniques and culture-independent techniques such as high-throughput next-generation sequencing.[9–11] The use of these methods has significantly increased our understanding of the role of gut microbiota in health and disease[10]; for example, small intestinal bacterial overgrowth[12] and altered intestinal microbiota[13] are implicated in subgroups of patients with functional bowel disorders. Firm evidence for a causal role of microbiota composition on disease pathogenesis has, however, remained elusive due to inherent limitations in the diagnostic methods used. For instance, breath-testing and culture techniques have not been validated, the majority of species cannot be cultured with standard methods, and the effect of potentially confounding polypharmacy has not been thoroughly evaluated.[11, 14] Nevertheless, increasing awareness of the potential clinical impact of imbalance in the intestinal microbiota has led to a call for new standardised diagnostic methods, such as high-throughput DNA sequencing, that facilitate profiling of the microbiome and possible differentiation between normobiosis and dysbiosis.[15]

Analysis of faecal samples from individuals with dysbiosis is anticipated to enable characterisation of the bacterial profile associated with different pathological conditions, thus aiding clinical diagnosis of pathological conditions and improving therapeutic regimens. Furthermore, detailed sequential profiling of intestinal microbiota over the course of a therapeutic regimen may allow for monitoring of inflammatory bowel disease (IBD) progression[16] and the prediction of relapse, for example in CD.[17] The ability to characterise the bacterial profiles both of normobiotic and dysbiotic patients may also help in evaluating the efficacy and further development of therapeutic approaches such as faecal microbiota transplantation (FMT), special diets and use of probiotics.[17–21]

In the present publication, a novel diagnostic test (GA-map Dysbiosis Test, Genetic Analysis AS, Oslo, Norway) is evaluated that allows mapping of the intestinal microbiota profile for a selected set of bacteria, and used to identify and characterise dysbiosis in a clinical setting. The GA-map Dysbiosis Test (GA-test) is based on advances in DNA profiling using probes targeting variable regions (V3 to V7) of the bacterial 16S rRNA gene to characterise and identify bacteria present (Figure 1). The probes comprise a highly selective and specific bacterial probe set that is used with a unique algorithm to facilitate determination of dysbiosis level. The method provides a rapid, high-throughput analysis of a large number of individual faecal samples. The breadth of knowledge gained from microbiome projects was used to develop a test aimed at characterising dysbiosis by deviation from a normobiotic state for use in a clinical diagnostic setting. For this purpose, the test was technically documented in accordance with EU requirements for an *in vitro* diagnostic test comprising the following intended use claim: 'The GA-test is intended to be used as a gut microbiota DNA analysis tool to identify and characterise dysbiosis'.

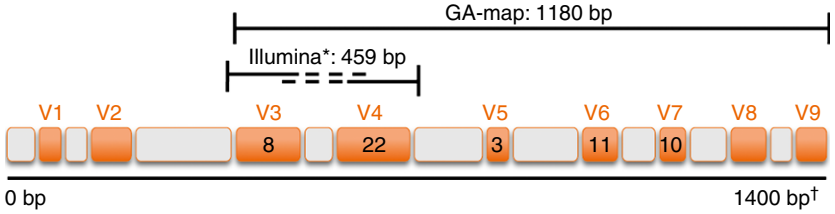

Figure 1 | Target regions for the GA primer (1180 bp) and the Illumina primer (459 bp) showing variable (orange V1-V9) and conserved (grey) regions in the bacterial 16S rRNA gene (1400 bp) utilised by the two methods. The numbers in V3 to V7 denote the number of GA probes targeting each variable region, in total 54 probes. *Illumina application note; http://res.illumina.com/documents/products/appnotes/appnote_16s_sequencing.pdf. †Position in *E. coli* (number of base pairs)[40]

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MATERIALS AND METHODS

Human samples

Faecal samples were collected from 668 adults (aged 17–76; 69% women), including controls from healthy volunteers (n = 297) and patients with IBS (n = 236) and IBD (n = 135) (Table 1). Faecal samples were collected from hospitals in Norway, Sweden, Denmark and Spain (72%), as well as from workplaces in Oslo, Norway (28%), in an effort to achieve heterogeneity. The healthy donors had no clinical signs, symptoms or history of IBD, IBS or other organic gastrointestinal-related disorders (e.g. colon cancer). Additional demographics are shown in Table 1, and sample inclusion and exclusion criteria are summarised in Data S1. The IBS samples were collected as part of prospective studies that used Rome II and III diagnostic criteria (depending on collection site) to identify IBS. The distribution of IBS subtypes was 44% IBS-diarrhoea, 22% IBS-alternating, 17% IBS-constipation, 11% IBS-unsubtyped and 4% IBS-mixed. The diagnosis of IBD was based on clinical presentation confirmed by colonoscopy. Of the 135 IBD samples, 80 (59%) were treatment-naïve patients and 55 (41%) were IBD patients in remission. The distribution of IBD types was 62% UC and 38% CD for the treatment-naïve group, and 67% UC and 33% CD for the IBD in remission group. Informed consent was obtained for all samples along with approval from local scientific ethics committees. Samples were collected at home, office or hospital, and frozen within 3–5 days (for faecal sample collection, storage and processing, see Data S2).

Table 1 | Demographic information

Categories	Total	Females (%)	Age (years)* Mean	Age (years)* Range
Healthy controls	297	63	41	21–70
Nordic	254	64	42	21–70
Danish	19	63	42	23–61
Spanish	24	50	35	22–56
IBS†	236	78	40	17–76
IBS-D	102	79	40	18–70
IBS-C	41	85	42	22–73
IBS-M	10	80	37	19–55
IBS-U	25	88	41	19–68
IBS-A	51	67	39	20–62
IBD treatment-naïve	80	56	34	18–61
CD	30	50	33	19–53
UC	50	63	35	18–61
IBD remission‡	55	76	42	20–69
CD	18	72	38	20–59
UC	36	78	44	24–69

A, alternating; C, constipation; CD, Cohn's disease; D, diarrhoea; IBS, irritable bowel syndrome; IBD, inflammatory bowel disease; M, mixed; U, unsubtyped; UC, ulcerative colitis.

* Precise ages were known for 99% of the total samples used.

† IBStype known for 97% of the total IBS samples used.

‡ CD/UC diagnosis known for 99% of the total IBD samples used.

Probe identification, selection, *in silico* and *in vitro* testing

To establish and optimise the most applicable bacterial probeset, data from previous IBD and IBS intestinal microbiota research was compiled based on pre-defined search criteria (Data S3) to provide >500 bacterial observations associated with the occurrence of IBD and IBS. From a combined dataset of 496 16S rRNA gene sequences (consensus sequence[s] for each species, chosen from all available long 16S rRNA sequences and purified to avoid sequences errors) from 269 bacterial species, probes were designed to cover major bacterial observations made from the literature. All probes were designed according to Vebø et al.[22] with a minimum melting temperature (T_m) of 60 °C by the nearest-neighbour method[23] for the target group where the nucleotide 3′ end of the probe is a cytosine; nontarget group probe requirements were a T_m of 30 °C or absence of a cytosine as the nucleotide adjacent to the 3′ end of the probe. Each probe was designed to target a bacterial species or group, i.e. *Faecalibacterium prausnitzii* (species), Lactobacillus (genus), Clostridia (class) and Proteobacteria (phylum), based on their 16S rRNA sequence (V3–V9). Probes that satisfied target detection and nontarget exclusion *in silico* were evaluated for cross-labelling, self-labelling and cross hybridisation before final validation was performed against bacterial strains *in vitro*.

After *in vitro* testing, a panel of 124 optimal probes was further selected using variable selection methods: variable importance in projection, selectivity ratio and interval partial least squares using data from a selection of healthy and IBS samples (data not shown). The variables (probes) were selected based on their ability to distinguish between samples isolated from healthy individuals and IBS patients. A final panel of 54 probes was selected covering the sites across V3 to V7 on the 16S rRNA sequence (Figure 1). Bacterial target specificity, tested with the 54-probe set against 368 available single bacterial strains (Data S4), was performed to define the target bacteria for each probe. The probes detect bacteria within the six phyla; Firmicutes, Proteobacteria,

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Bacteroidetes, Actinobacteria, Tenericutes and Verrucomicrobia, covering 10 taxonomic bacterial classes and 36 genera (for more details on the bacterial targets for the 54 probes see Data S5).

Sample preparation and detection

The GA-test is based on regular molecular biology techniques, comprising human faecal sample homogenisation and mechanical bacterial cell disruption; automated total bacterial gDNA extraction using magnetic beads; 16S rRNA PCR DNA amplification covering V3–V9; probe labelling by single nucleotide extension; hybridisation to complementary probes coupled to magnetic beads; and signal detection using BioCode 1000A 128-Plex Analyzer (Applied BioCode, Santa Fe Springs, CA, USA). The method is described in detail in Data S2, and an overview to the whole process from sample preparation to result is shown in Figure 2.

Data pre-processing

To ensure high quality assurance, several quality control criteria were applied to the detection data for each sample: (i) a bead count >2 for each probe; (ii) the hybridisation control (HYC) median signal >13 000; (iii) a median background signal <500 and (iv) a universal control median signal >4500. Normalisation was applied by first dividing the signal intensity of each probe in each sample by the signal intensity for HYC for that sample, and multiplying by 1000. This was done to adjust for sample differences due to pipetting or hybridisation. Subsequently, normalisation to adjust for run differences was applied by dividing the HYC-normalised signal of each probe in each sample by the median HYC-normalised signal of each probe for replicates of a synthetic DNA control (Data S2; Table S1), and multiplying by 1000. Prior to normobiotic microbiota profile calibration, normalised signal intensities below 15 were set to 0 to remove for low background noise and data was mean centred. Test and validation samples were normalised, and normalised signals below 15 were set to 0 before data were mean centred using mean probe signals from the normobiotic reference cohort.

Dysbiosis test development and validation

Principal component analysis (PCA)[24] was used to build a normobiotic microbiota profile (model). The boundary between nondysbiotic and dysbiotic was determined by calculating confidence regions for the values of Hotelling's T-square and Q statistics given by PCA scores in the model. Geometrically this corresponds to a rectangle with one corner located at the origin which classifies samples located within the rectangle as nondysbiotic and samples located outside as dysbiotic. Analysis of T-square and Q statistics scaled by the confidence limit showed that the Euclidian distance from the origin had a log-normal like distribution (data not shown). Euclidian

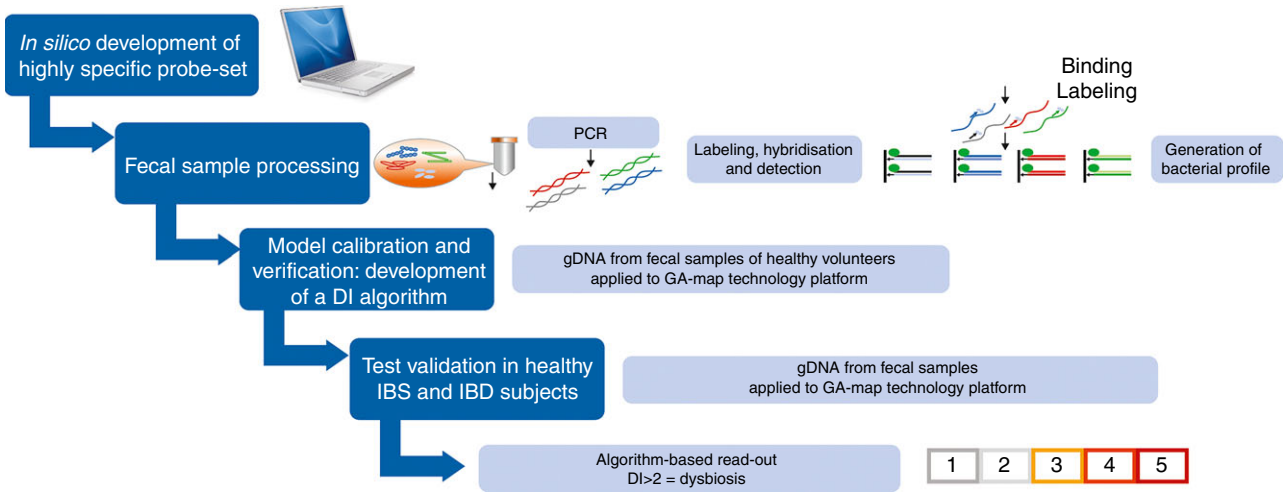

Figure 2 | Flow chart illustrating the GA-map Dysbiosis Test development, starting with *in silico* development of bacterial probe set, standardisation of laboratory analysis process, model calibration and verification in healthy individuals (normobiotic reference collection), and validation in healthy, IBS and IBD individuals. Derivation of a DI based on bacterial 16S rRNA DNA analysis in faecal samples demonstrates that a DI score >2 confirms microbiota profile deviations from the normobiotic reference collection.

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distance from the origin was used to merge the two dimensions, and weighting was performed to capture the effect of *T*-squared and *Q* statistics as appropriate. A single numeric representation of the degree of dysbiosis, defined as the Dysbiosis Index (DI), was derived from a log-normal distribution by assigning estimated portions of the distribution to different values on a scale set from 0 to 5. A DI value of 2 was defined as class separation represented by the identified confidence limits; a DI of 2 or lower being the nondysbiotic region and a DI of 3 or higher being the dysbiotic region. The higher the DI above 2, the more the sample is considered to deviate from normobiosis, e.g. sample A with DI = 4 is farther away from the normobiotic reference cohort in the Euclidian space than sample B with DI = 3, thus A is more dysbiotic than B. The scale was optimised with emphasis on reducing technical variation between replicates, meaning that the integer part of the numeric output is decided by pre-determined levels of the Euclidian distance.

To create the GA-test, 211 healthy individuals were selected and randomly split into a training set (n = 165) designed to build models and a test set (n = 46) designed to tune parameters. Duplicate samples were run, and mean normalised signal was used for training and testing. Sample demographics for the two groups were similar (Table 2). In addition, a set of IBS patients were included in the test set (n = 127). A number of models were developed and evaluated, and the frequency of dysbiosis in the test set was used as measure of model performance. For the final PCA model, 15 principal components were used, and a 98% confidence limit was determined for *T*-squared and *Q* statistics to define class separation. When the model is used to score other samples, values outside these limits are defined as dysbiotic.

External validation using an independent test set comprising healthy, IBS and IBD subjects (n = 287) was used to assess the clinical diagnostic performance of the model (Table 3). The validation set subjects were all from unique donors who had not been included in the healthy reference collection used for normobiotic profile calibration or in parameter tuning. Each sample was processed using the finalised algorithm which converts data for each sample into a single integer, i.e. the DI, which represents the degree of dysbiosis based on bacterial abundance and profile within a sample relative to the established normobiotic profile. A DI > 2 represents a potentially clinically relevant deviation in microbiotic profile from that of the normobiotic reference collection. Finally, the dysbiosis frequency was calculated. In addition, PCA was performed on the validation set to investigate differences in microbiota profile between the three subject groups.

Table 2 | Sample sets used for GA-map Dysbiosis Test development and validation

Cohort	Samples, *n*	Age, mean	Female (%)	Sample type, *n*		
				Healthy	IBS	IBD
Training	165	42	64	165	–	–
Test	173	40	73	46	127	–
Validation	287	39	71	43	109	135
Full cohort	625	40	70	254	236	135

Table 3 | Percentage dysbiosis and mean DI score in validation cohort

Cohort	Total	Dysbiotic, % (95% CI)	DI, mean
Healthy controls	43	16 (±11)	1.72
IBS	109	73 (±8)	2.98
IBS-D	34	76 (±14)	3.03
IBS-C	26	73 (±17)	3.00
IBS-M	3	67	3.33
IBS-U	25	72 (±18)	3.04
IBS-A	20	70 (±20)	2.85
IBD treatment-naïve	80	70 (±10)	3.31
CD	30	80 (±14)	3.60
UC	50	64 (±13)	3.14
IBD remission	55	80 (±11)	3.15
CD	18	89 (±14)	3.65
UC	36	75 (±14)	2.92

A, alternating; C, constipation; CD, Crohn's disease; D, diarrhoea; DI, Dysbiosis Index; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; M, mixed; U, un-subtyped; UC, ulcerative colitis.

Technical performance

The EU directive for *in vitro* diagnostic tests was followed to ensure compliance with a CE-marked test.[25] The main technical parameters evaluated were precision and quantitative range of the test; both at probe signal level and at final output level (i.e. DI). At probe level, precision of signals [coefficient of variation [CV], %) varied with raw signal intensity. Signals below 500 IU were regarded as background noise; therefore, measurement of variance was not applicable. For signals above 500 IU precision was estimated to be 8.4%, using repeated runs for six donors over six faecal extractions per donor over 2 days (n = 328). A CV below 10% was

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set as a criterion in development of the DI algorithm. Based on repetitive measurements of 139 dysbiotic samples, 94% of the samples showed CVs below 10%. In addition, several in-process test steps were evaluated (data not shown).

Faecal microbiota variation over time

Variation in microbiota over time was investigated both for normalised data across the selected probe set, and for the test result (DI). Faecal samples were collected from five donors (aged 24–38; 80% women) at a 1-week interval for up to 14 weeks. PCA of normalised data was performed, and statistical assessment of variation in the signals for donor and sampling time (weekly) was conducted using R package *ffmanova*, an implementation of 50–50 multivariate analysis of variance.[26]

Comparison to Illumina deep sequencing

To compare the performance of deep sequencing and the GA-test data for the gut microbiota, a total of 188 samples from 162 subjects (89 healthy subjects and 73 IBS subjects; from the training and test cohorts described in Table 2) were randomly selected. Sequencing was performed using the paired-end 250 bp sequencing on the Illumina MiSeq platform[27] at the Norwegian High Throughput Sequencing Center (UiO, Oslo, Norway). Demultiplexed Illumina readings were clustered into Operational Taxonomic Units (OTUs) using Qiime pipeline (v.1.7), StarCluster (http://star.mit.edu/cluster) and Amazon Web Services (https://aws.amazon.com, virtual machine identifier ami-9bc9a7f2) at 97% sequence similarity. Standard tools and parameters for Qiime downstream analysis were used, such as *uclust* for OTU picking and Ribosomal Database Classifier for taxonomy assignment. A pre-defined taxonomy map of reference sequence OTU to taxonomy was used rather than open-reference picking and assignment, as the reference database of 16S rRNA sequences found in human gut is comprehensive. Thereafter, one representative read for each OTU group was extracted and aligned to create a phylogenetic tree and an OTU Biological Observation Matrix table was constructed (data not shown). The OTU table was rarefied to 5000 sequences to remove sample heterogeneity. Four samples which had less read count than the set threshold were excluded from further analysis.[28]

To compare the MiSeq sequence reads to the GA-map Technology (GA-Technology, Genetic Analysis AS, Oslo, Norway) probe signals, we identified probes that were specific for a maximum of two species or genera, and compared the normalised signals from the probes for each sample to the number of sequences of the corresponding sample and closest matching taxonomic bins found by MiSeq sequencing. If a genus found by sequencing corresponded to several probes, the sum of the probes was used in the comparison, and if a probe represented two genera the sum of sequences from both genera was used. Finally, correlation between deep sequencing data and GA-technology data was calculated using Pearson correlation.

Furthermore, we applied the approach for defining the healthy reference collection, as described in the Dysbiosis test development section, to the Illumina sequence data set. In order to compare the results from this model with GA-technology data, we constructed a new model using GA-technology data limited to the same 188 samples. The samples were classified either as dysbiotic or non-dysbiotic using both models, and the results compared by counting the number of samples that were classified equally across the models.

Statistical analysis

All data were analysed at GA (Genetic Analysis AS). Categorical data were expressed as the number of subjects (and percentage) with a specified condition or clinical variable, and the mean as appropriate. A test for association between the two technologies were performed using an independent *t*-test based on Pearson's product moment correlation coefficient. The Mann–Whitney *U*-test was used for testing DI values. All tests were two-sided, and the chosen level of significance was $P < 0.05$. Analysis was done using the statistical computing language R version 3.0.2[29] and MATLAB 2011b (The MathWorks, Inc., Natick, MA, USA).

RESULTS

Frequency of dysbiosis in healthy, IBS and IBD subjects

Validation of the developed GA-test was performed by comparing frequency of dysbiosis in a set of 287 samples, including healthy individuals previously not included in the normobiotic profile calibration ($n = 43$) and patients with IBS ($n = 109$) and IBD ($n = 135$) (Table 2). The results in the validation cohort are given in Table 3. Of the 43 samples from healthy volunteers included in the validation cohort, seven (16%) were determined as being dysbiotic, with the distribution of DI scores for validation cohort shown in Figure 3. Among the IBS patients, 80 of 109 (73%) were determined as being dysbiotic. In the

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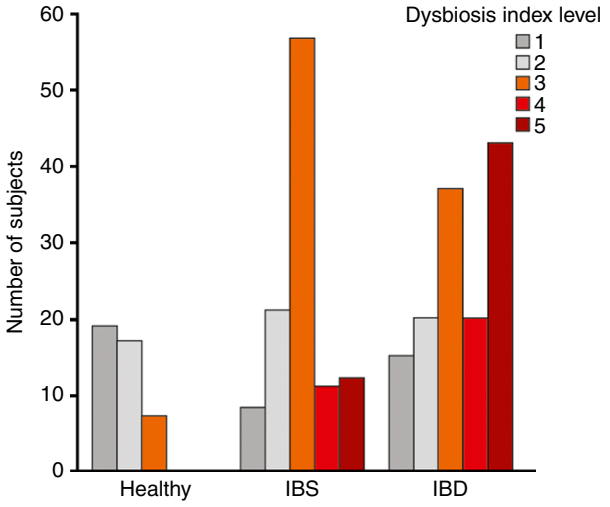

Figure 3 | Distribution of DI scores 1–5 for the validation cohort as determined by GA-map Dysbiosis Test, showing the increase in DI from healthy individuals through IBS patients and finally in IBD patients.

IBD cohort, 100 of 135 (74%) were determined as being dysbiotic, including 56 of 80 (70%) treatment-naïve IBD patients, and 44 of 55 (80%) IBD patients in clinical remission. The distribution of DI between IBS and IBD patients was significantly different ($P < 0.01$) and more IBD patients than IBS patients had a DI >4 (Figure 3). Furthermore, both in treatment-naïve IBD patients and in IBD patients in remission, the frequency of dysbiosis was higher in CD (80% and 89% respectively) than UC (64% and 75%), with significantly higher DI values in CD than UC ($P = 0.03$).

The test was also applied to a set of 43 available samples from healthy volunteers from Denmark ($n = 19$; aged 23–61; 63% women) and Spain ($n = 24$; aged 22–56; 50% women). Seven of the 19 Danish samples were determined as being dysbiotic with mean DI of 2.16, resulting in 37% dysbiotic (95% CI, ±22%) healthy volunteers in this cohort. Among the Spanish samples, 10 of 24 were determined as being dysbiotic with mean DI of 2.58, resulting in 42% dysbiotic (95% CI, ±20%). While the result for the Danish healthy cohort was not significantly different from the healthy validation samples ($P > 0.05$), we observed that 50% (5/10) of the dysbiotic samples in the Spanish samples showed a DI above 3.

Bacterial profile in dysbiosis

Applying PCA to the validation cohort using normalised data for all 54 probes demonstrated a relative clustering of samples by disease cohorts. The scores for the first two principal components (PC), accounting for 48% of the variance in the data, showed a tighter cluster for healthy subjects in the bottom right corner compared with a more diverse spread for subjects with IBD and IBS (Figure 4a). The sample distribution in the scores plot was found to be linked to the degree of dysbiosis, with a central cluster of nondysbiotic samples surrounded by samples with weak dysbiosis (DI = 3), and the samples with the most severe dysbiosis (DI = 5) scattered outside this cluster (Figure 4b). Both the first and second PC each separate the samples from healthy volunteers from IBS and IBD samples to a certain degree. The scatter of DI values implies that different bacteria dominate dysbiosis for different samples. To further investigate which bacterial groups were the main contributors to dysbiosis in IBD and IBS, differences in overall mean normalised signal between dysbiotic and nondysbiotic status for each of the 54 probes were

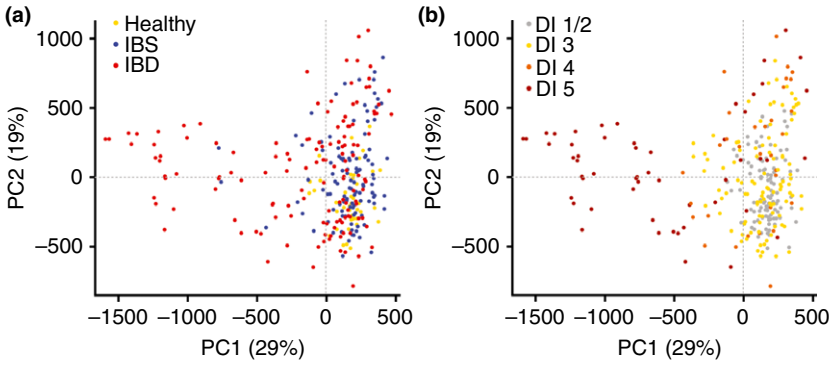

Figure 4 | PCA scores for the first two principal components for validation cohort ($n = 287$) based on 54 probes. The two PCs account for 48% of the variation, and points are coloured according to (a) cohort: yellow – healthy, blue – IBS, and red – IBD; and (B) DI: grey = 1–2, orange = 3, red = 4, dark red = 5.

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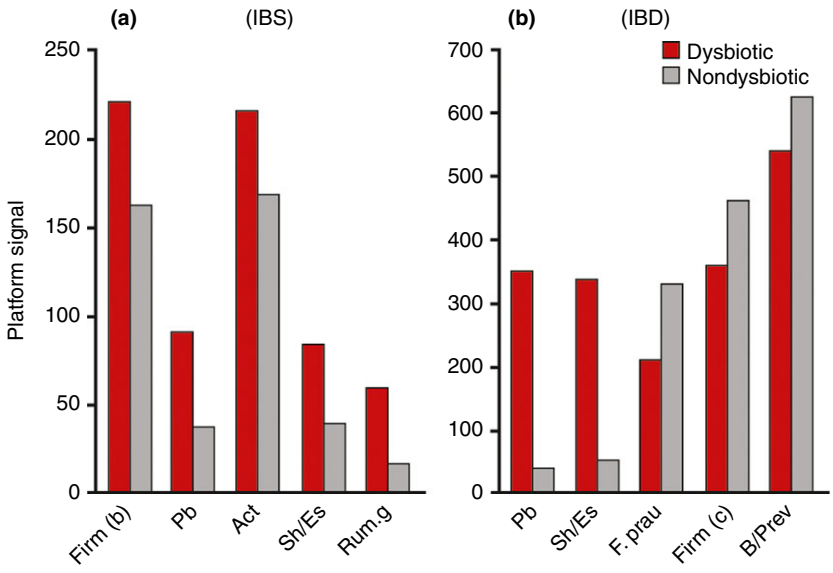

Figure 5 | Mean normalised signal for top five probes sorted by absolute relative difference between dysbiotic (red) and nondysbiotic (grey) as determined by the GA-map Dysbiosis Test for (a) IBS patients (n = 109), and (b) IBD patients (n = 135). Act, Actinobacteria; B/Prev, Bacteroides/Prevotella; Firm(b), Firmicutes (Bacilli); Firm (c), Firmicutes (Clostridia); F. prau, *Faecalibacterium prausnitzii*; Pb, Proteobacteria; Rum.g, *Ruminococcus gnavus*; Sh/Es, *Shigella/Escherichia*.

calculated. The pre-dominant bacteria contributing to dysbiosis within the IBS cohort were Firmicutes (Bacilli), Proteobacteria (*Shigella/Escherichia*), Actinobacteria and *Ruminococcus gnavus* (Figure 5a). Similarly, the pre-dominant bacteria within the IBD cohort were Proteobacteria (*Shigella/Escherichia*), Firmicutes, specifically *F. prausnitzii*, and Bacteroidetes (Bacteroides and Prevotella) (Figure 5b). Interestingly, Proteobacteria (*Shigella/Escherichia*) was among the top five dysbiosis-contributing bacterial groups for both IBS and IBD, implying similarities in dysbiosis between IBS and IBD. However, all bacterial groups that contributed most to dysbiosis in the IBS cohort showed increased probe signal intensity compared to nondysbiotic patients, while for the IBD cohort, both reduced (*F. prausnitzii*) and increased probe signal intensities were the main contributors to dysbiosis.

We found a single probe with a differential signal between samples from the Spanish and Scandinavian cohorts ($P < 0.01$; Benjamini–Hochberg correction). The probe targets Firmicutes (*Streptococcus*), and this signal was found to be elevated in the Spanish samples compared to the Scandinavian cohort. Figure 6 shows the pre-dominant bacteria contributing to dysbiosis within the Spanish samples. As expected, Proteobacteria (*Shigella/Escherichia*) is again found to be a contributing bacteria in dysbiosis. In addition, *Bacteroides stercoris* and *Bifidobacterium* contribute to dysbiosis, which potentially could be linked to differences, in e.g. diet between Scandinavian countries and the Mediterranean region.

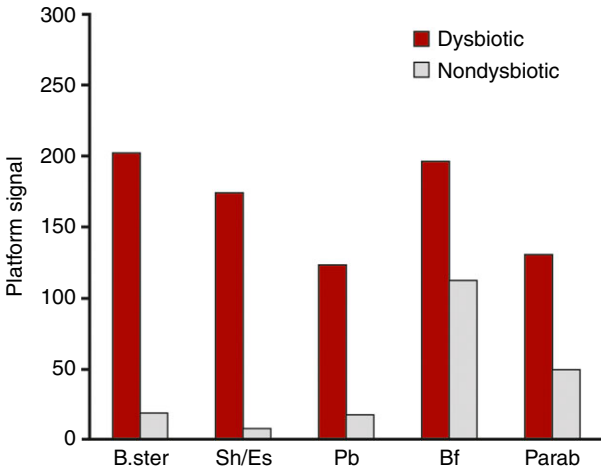

Figure 6 | Mean normalised signal for probes sorted by absolute relative difference between dysbiotic (red) and nondysbiotic (grey) as determined by the GA-map Dysbiosis Test for Spanish cohort (n = 24). Bf; Bifidobacterium, B. ster; *Bacteroides stercoris*, Parab; Parabacteroides, Pb; Proteobacteria, Sh/Es; *Shigella/Escherichia*.

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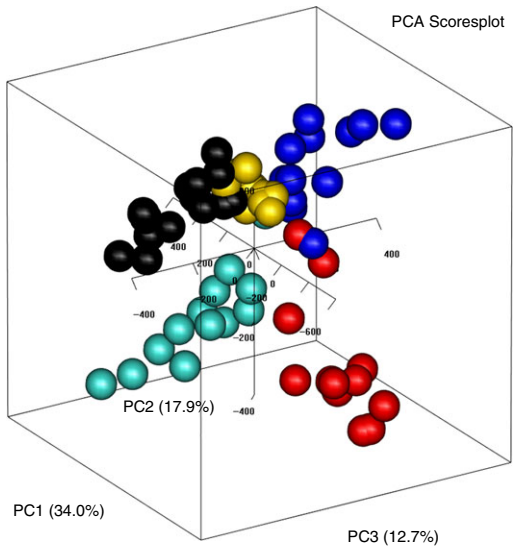

Figure 7 | Scores for the first three principal components from PCA of normalised data from five healthy subjects collected weekly for up to 14 weeks (n = 64). One point is one sample for donor x taken at time point y. The first three PCs account for 65% of the variation, and points are coloured according to donor.

Faecal microbiota variation over time

Faecal samples were collected from five individuals at 1-week intervals for up to 14 weeks. PCA of the normalised data (n = 64) revealed that most variability in the longitudinal faecal microbiota analysis was related to inter-individual variability; donors could clearly be distinguished by the three-first and most important PCs in the score plot (Figure 7). The samples were clustered according to faecal donor independently of sample collection time. The three first PCs described 65% of the total variability in the faecal microbiota data.

The significance of the PCs was analysed by *ffmanova* and performed using normalised data with only the main effects of donor and sampling time (weekly) included in the model. The results show that the average amount of variation between donors was greater than that within a donor ($P < 0.001$) with explained variances based on sums of squares of 0.48. The variation between sampling time points was not significant ($P = 0.26$), with explained variances based on sums of squares of 0.11. The low level of variation within one individual over time is crucial in utilising the test for monitoring changes during treatment for altering the microbiota profile.

Comparison to deep sequencing

The randomly selected set of 188 samples was sequenced using MiSeq Illumina to investigate similarities with GA-technology profiles. Any reads that did not match a reference sequence at greater than or equal to 97% sequence identity were discarded according to a closed-reference OTU-picking protocol. A total of 7 564 142 reads were binned into 254 OTUs at higher taxonomic levels and 165 of these were identified at genus level. Of the 165 genera, 77 were found in more than 10% of samples.

After identifying the genera in the samples with MiSeq sequencing, a comparison was performed with the closest matching taxonomic bins detected by GA-technology. In general, we found strong correlations between the GA-technology signals and Qiime taxonomically assigned MiSeq reads (Table 4), where *Alistipes, Bifidobacterium, Dialister, Lactobacillus/Pediococcus, R. gnavus* and *Shigella/Escherichia* all had a Pearson correlation of $r > 0.85$. For some species the correlation was moderate, e.g. *B. fragilis* ($r = 0.38$), *Ruminococcus albus/bromii* ($r = 0.31$) and *Streptococcus sanguinis/thermophilus* ($r = 0.49$). However, since MiSeq sequencing did not

Table 4 | Correlation between normalised GA-map signal data and MiSeq Illumina sequence data (97% sequence identity) for 188 healthy and IBS samples

Taxonomic group	Correlation coefficient (r)*	P-value
Mycoplasma hominis†	−0.05	0.50
Ruminococcus albus/bromii‡	0.31	<0.001
Bacteroides fragilis‡	0.38	<0.001
Streptococcus sanguinis/thermophilus‡	0.49	<0.001
Phascolarctobacterium	0.72	<0.001
Faecalibacterium prausnitzii	0.75	<0.001
Streptococcus thermophilus	0.78	<0.001
Akkermansia	0.79	<0.001
Eubacterium	0.79	<0.001
Megashera/dialister	0.83	<0.001
Ruminococcus gnavus	0.86	<0.001
Dialister	0.88	<0.001
Alistipes	0.90	<0.001
Bifidobacterium	0.90	<0.001
Shigella/Escherichia	0.93	<0.001
Lactobacillus/Pediococcus	0.94	<0.001

* Correlation coefficients were determined for the closest matching taxonomic bins.

† Mycoplasma only identified in one sample by MiSeq Illumina sequencing.

‡ Illumina sequencing did not enable selective detection of species.

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allow for detection at species level, a direct comparison to the specific probe signals can be complicated if the specific species is not the dominating species in a genera. Interestingly, no correlation was found between the two methods for the species *Mycoplasma hominis* ($r = -0.05$). Using MiSeq sequencing, *Mycoplasma* genus was only detected in one sample, implying that MiSeq sequencing does not allow for the selective detection of this genus at all. In contrast, *M. hominis* was detected in a majority of the 188 samples with the GA-technology. The highly specific GA-technology probe detecting *M. hominis* binds to V6 on the 16S rRNA gene, a variable region not covered by MiSeq Illumina sequencing (Figure 1).

In addition, two new models were built using Illumina sequencing data and GA-technology probe intensity data with eight and nine PCs, respectively. The number of PCs was selected by optimising the frequency of dysbiosis in test samples (at the most 20% of healthy individuals and 60% of IBS patients were determined as dysbiotic). The training data set consisted of 100 samples from healthy volunteers, and the test set included 15 healthy and 73 IBS samples. The results were compared across the two models and yielded 80% concordance.

DISCUSSION

In this article, we demonstrate the performance of a novel gut microbiota test, aiming to identify and characterise dysbiosis by determining deviation from normobiosis. Such a diagnostic approach contrasts to direct diagnosis of a particular disease. Characteristic sets of bacteria are required in a healthy normobiotic gut microbiota, and deviation will represent a dysbiotic state. Quantitative measurement of deviation in bacterial microbiota makes it possible to characterise dysbiosis in samples from IBS and IBD patients based on a single diagnostic algorithm targeting normobiosis.

Ideal enabling technologies will be those that can profile the microbiome as a whole and, at the same time, reliably target deviations (and their degree) from normobiosis. Notably, gut microbiota also harbour a range of transient colonisers with no diagnostic value that have the potential to generate obscure diagnostic results. Furthermore, recent evidence suggests that species-level information is important in gut microbiota diagnostics.[30] Techniques with a low error rate which target a wide range of variable positions in the 16S rRNA gene would therefore be preferable for discriminating between normobiosis and dysbiosis.

The present test is a broad-spectrum, reproducible, precise, high throughput, easy to use method of quantifying the extent of dysbiosis that is especially suitable for clinical use. This test gives an algorithmically derived DI based on bacterial abundance and profile within a sample. This DI is an indicator of the degree to which an individual's microbiome deviates from that of a healthy reference collection and could potentially be highly relevant in clinical diagnosis and monitoring of the progression of conditions such as IBD and IBS. The stability of the human gut microbiota is another important feature if microbial characterisation is to play a role in diagnosis, treatment and prevention of disease. Faith et al.[31] showed that, in an individual's microbiota, 60% of the bacterial strains persisted over the course of 5 years. Our data also suggest that there is little variation in an individual's gut microbiota over time, since we found only a low within individual variation in weekly sampling over 14 weeks.

High-throughput sequencing is an excellent tool for exploratory analyses of the gut microbiota, and is widely used. A limitation to this technology is the relatively short read-lengths used for sequencing, only allowing for a limited region of the 16S rRNA gene to be exploited (usually V3 and/or V4) (Figure 1); thus, less than 50% of obtained sequences can be annotated at genus level.[32] The lack of detection of *Mycoplasma* with MiSeq Illumina sequencing, detected in a majority of the samples using a probe targeting V6 with the GA-map test, further illustrates the limitations of using only limited variable regions of the 16S rRNA gene. Moreover, since MiSeq sequencing does not allow for detection at species level, a direct comparison to the specific probe signals can be challenging if the specific species is not the dominating species in a genera. Even so, it is possible to gain important insights to an individuals' gut microbiota using high-throughput sequencing. Compared to the GA-technology, this technology is superior towards exploring novel bacterial biomarkers, and gaining in-depth information regarding all bacteria present in a sample. However, in terms of the human gut microbiota, the main patterns have been explored,[32] and GA-technology has consolidated on this information in designing 54 highly specific DNA probes exploiting a broad range of gene variability (V3–V7). These 54 probes have further been converted into a diagnostic test but without the laborious data-analysis required following high-throughput sequencing. Therefore, the GA-technology provides a unique opportunity to study changes in gut microbiota profiles potentially associated with

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gastrointestinal-related disorders. Our results show agreement between the two technologies regarding determining dysbiosis, as well as strong correlations in detecting several bacteria. However, results also show weak correlations for some specific species, possibly due to lack of selective species detection by MiSeq Illumina sequencing.

The GA-test identifies a high frequency of dysbiosis in IBS and IBD patients and low frequency in healthy individuals. Both IBD patients in remission and treatment-naïve IBD patients reported DI scores well above the threshold of two with a dysbiosis frequency of 80% and 70%, respectively. IBS patients, defined according to Rome II and III-criteria (depending on collection site), showed a dysbiosis frequency of 73%, confirming previous observations,[30, 33, 34] while the frequency of dysbiosis in healthy individuals was 16%. The normobiotic reference collection comprised healthy Scandinavian individuals, which may be a potential limitation of the test. We found slightly increased DI in healthy controls from Denmark (DI $\geq$ 3 in 33%, n = 19) and Spain (DI $\geq$ 3 in 42%, n = 24); however, the sample size is too small to allow any definitive conclusions to be drawn regarding differences in frequency of dysbiosis or microbiota between the populations. Further investigation is needed with increased sample numbers from across Europe to firmly establish the broad clinical utility of the test.

The intestinal microbiome is a dynamic environment in which the relative balance of the composition of pro- and anti-inflammatory bacterial species is known to be highly relevant.[35] For example, the microbial signature of Firmicutes species present in the intestinal tract in patients with UC differs significantly from that in CD patients.[36] Compared with CD patients in long-term remission, patients with relapsing CD have lower levels of all Firmicutes species, and a bacterial profile significantly predictive of relapse for up to 1 year before infliximab withdrawal.[17]

Dysbiosis is associated with many diseases, including IBS, IBD, obesity and diabetes,[4–8] and has also been implicated in depression and autism.[37, 38] In recent years, new treatment options have emerged with respect to restoring the balance of the microbiota in dysbiotic patients. FMT is now regarded as the most effective treatment in relapsing *Clostridium difficile* colitis,[18, 39] and is currently being studied in phase I to IV clinical trials in many of the aforementioned conditions (CD, phase II/III NCT01793831; UC, phase I NCT01947101, phase II NCT01896635, phase II/III NCT01790061; IBD including CD and UC, phase IV NCT02033408). A key barrier in the interpretation of FMT data has been the variability in bacterial composition of donor microbiota, not only related to pathogenic organisms but also to the composition of the normally occurring microbiota, further highlighting the importance of identifying a method to sufficiently characterise both pathogenic and nonpathogenic microbes. The ability to characterise an individual's microbiome and monitor alterations may allow for the prediction of therapeutic outcome or even relapse in such conditions.[17] It may also help to explain why a patient is refractory to particular therapeutic regimens and aid adaptation of the regimen accordingly. Furthermore, rapid and reproducible detailed bacterial profiles from normobiotic and dysbiotic individuals may aid the continuation of innovative therapeutic approaches such as FMT.[18] Thus, use of the test could prove clinically useful in determining dysbiosis, not only in IBS and IBD patients, but also in other conditions where knowledge about the microbiota profile might prove clinically useful, in the subsequent monitoring of prescribed treatment regimens, and in the evolution of new therapeutic approaches.

In conclusion, this is the first clinical test, aiming to identify and characterise dysbiosis based on faecal specimens. The diagnostic applicability of the test will have to await further clinical experience, also from international studies, as one might expect geographical deviations related to microbial patterns. Nevertheless, the present standardised and reproducible method represents, in particular, a step forward as a combined practical, ready to use, clinical and research tool. The method will allow us to gain more knowledge on the microbial component of intestinal disorders, and in general, provide the possibility of increasing our understanding of the part played by the microbiome in the disease process.

AUTHORSHIP

Guarantor of the article: Professor Knut Rudi, Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Science, Aas, Norway.

Author contributions: C Casén, HC Vebø, FT Hegge and K Rudi conceived and designed the technical and clinical studies. M Sekelja and MK Karlsson analysed the data. C Casén and S Dzankovic administered sample collection and clinical data recording. E Ciemniejewska, S Dzankovic, C Frøyland and R Nestestog performed the laboratory work. L Engstrand, P Munkholm, OH Nielsen, G Rogler, M Simrén, L Öhman, MH Vatn and K Rudi contributed to strategic development decisions and clinical supervision. All authors contributed to writing the manuscript and approved the final submitted version.

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Declaration of personal interests: GR has been a consultant for Abbot, Abbvie, Augurix, Boehringer, Calypso, FALK, Genentech, Genetic Analysis, Essex, MSD, Novartis, Pfizer, Roche, UCB, Takeda, Tillots and Vifor; has received speaker honoraria from AstraZeneca, Abbott, Abbvie, FALK, MSD, Phadia, Tillots, UCB, and Vifor; and has received educational and research grants from Abbot, Abbvie, Ardeypharm, Essex/MSD, FALK, Flamentera, Novartis, Roche, Tillots, UCB and Zeller. PM has been a consultant for AstraZeneca, MSD, Tillots and Calpro. MHV has been an advisor for Genetic Analysis and organizer of the International Advisory Board of Genetic Analysis, a member of the Advisory Board of Tillots, and has received speaker honoraria from AstraZeneca, Abbott, MSD and Falk. OHN has been a member of the International Advisory Board of Genetic Analysis. KR is a board member of Genetic Analysis. LE has been an adviser for Genetic Analysis. LÖ has received speaker honoraria from Abbvie, UCB and Takeda, and is a member of the Advisory Board of Genetic Analysis, and has received an unrestricted research grant from AstraZeneca. MS has received unrestricted research grants from Danone, and served as a Consultant/Advisory Board member for Danone, Nestlé, Chr Hansen, Almirall, Albireo, AstraZeneca, Sucampo, and Shire. C Casén, HC Vebø, M Sekelja, FT Hegge, MK Karlsson, E Ciemniejewska, S Dzankovic, C Frøyland and R Nestestog are employees of Genetic Analysis AS. C Casén, HC Vebø, M Sekelja, FT Hegge, S Dzankovic, R Nestestog and K Rudi owns stocks and shares in Genetic Analysis AS. Genetic Analysis AS owns patent invented by HC Vebø and K Rudi: Methods of amplifying a target sequence of a 16S rRNA or 16S rDNA in a prokaryotic species, US patent No. 8889358, the 16S oligonucleotide primers used in this work. Genetic Analysis AS owns patents invented by M Sekelja, K Rudi and HC Vebø: Oligonucleotide probe set and methods of microbiota profiling, US application No 13/919056 describing a set of oligonucleotide probes used for profiling the microbiotia in the GI tract used in this work. Genetic Analysis AS owns patent invented by K Rudi: Nucleic acid detection method with US patent No. 6617138, describing the method of selective binding, labeling and hybridization of a oligonucelotide probe used in this work.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Data S1. Sample inclusion and exclusion criteria.

Data S2. Faecal sample collection, storage and processing and data processing.

Data S3. NCBI MeSH search terms used to compile initial IBD and IBS intestinal microbiota bacterial observations.

Data S4. Probe set (54 probes): 368 strains used to test bacterial target specificity.

Data S5. Probes and bacterial target validation.

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Research Article

Dominant Fecal Microbiota in Newly Diagnosed Untreated Inflammatory Bowel Disease Patients

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Our knowledge about the microbiota associated with the onset of IBD is limited. The aim of our study was to investigate the correlation between IBD and the fecal microbiota for early diagnosed untreated patients. The fecal samples used were a part of the Inflammatory Bowel South-Eastern Norway II (IBSEN II) study and were collected from CD patients ($n = 30$), UC patients ($n = 33$), unclassified IBD (IBDU) patients ($n = 3$), and from a control group ($n = 34$). The bacteria associated with the fecal samples were analyzed using a direct 16S rRNA gene-sequencing approach combined with a multivariate curve resolution (MCR) analysis. In addition, a 16S rRNA gene clone library was prepared for the construction of bacteria-specific gene-targeted single nucleotide primer extension (SNuPE) probes. The MCR analysis resulted in the recovery of five pure components of the dominant bacteria present: *Escherichia/Shigella*, *Faecalibacterium*, *Bacteroides*, and two components of unclassified Clostridiales. *Escherichia/Shigella* was found to be significantly increased in CD patients compared to control subjects, and *Faecalibacterium* was found to be significantly reduced in CD patients compared to both UC patients and control subjects. Furthermore, a SNuPE probe specific for *Escherichia/Shigella* showed a significant overrepresentation of *Escherichia/Shigella* in CD patients compared to control subjects. In conclusion, samples from CD patients exhibited an increase in *Escherichia/Shigella* and a decrease in *Faecalibacterium* indicating that the onset of the disease is associated with an increase in proinflammatory and a decrease in anti-inflammatory bacteria.

1. Introduction

The gut microbiota has the potential to exert both pro- and anti-inflammatory responses [1–3]. The gut microbiota is also supposed to be an epigenetic factor modifying the pathogenesis of extraintestinal disorders, including type I diabetes [4], obesity [5], atopic disorders such as asthma and eczema [6], and a contributing factor in the pathogenesis of inflammatory bowels disease (IBD) [7]. Knowledge of the composition of the intestinal microbiota, therefore, is vital to our understanding of which groups of bacteria are of importance in maintaining gut health or promoting disease.

The two major forms of IBD are ulcerative colitis (UC) and Crohn's disease (CD) [8, 9]. The etiology of IBD is complex and the causes are not yet fully understood. The pathogenesis of IBD involves interactions between the intestinal microbiota, the immune system, and epithelial cells. In addition, genetic and environmental factors modify this interplay towards or away from disease [10]. While these results are not conclusive, environmental factors do seem to influence the development of IBD.

Intestinal microorganisms have been implicated in the pathogenesis of IBD, with abnormal interactions between the host and either pathogens or commensal bacteria. Altered

Table 1: Patient characteristics.

	CD	UC	IBDU	IBD total	Non-IBD
Total number	30	33	3	66	33
Median age	32.9	33.8	41.2	33.7	32.3
Min.–max.	20.1–52.7	16.1–60.1	34.9–52.2	16.1–60.1	19.1–55.1
Male	10	17	1	28	14
Female	20	16	2	38	19

microbial composition and function result in increased immune stimulation, epithelial dysfunction, or enhanced mucosal permeability [11]. Studies have revealed that experimental colitis does not develop in animals when they are kept in a germ-free environment, suggesting that normal mucosal microbiota is required to initiate or maintain an inflammatory process [12]. The link between enteric bacteria and mucosal inflammation is also strengthened by the role of the CD susceptibility gene, NOD2/CARD15, in bacterial peptidoglycan recognition [13]. Moreover, IBD especially occurs in the colon and distal ileum, which contain the highest intestinal bacterial concentrations. Furthermore, antibiotics can reduce inflammation [14] while diversion of the fecal stream can prevent recurrence in CD [15].

In most previous studies, where samples from IBD patients have been under study, the samples have often been from long-term patients who have already received treatment for their medical conditions. Such treatment can lead to modifications of the fecal microbiota that subsequently influence the analytical outcome. It has been proposed that analysis of gastrointestinal microbiota in established IBD more accurately reflects changes associated with chronic disease, and as such should not be extrapolated to the onset of disease [16]. In the current study, however, fecal samples were collected from newly diagnosed IBD patients that had not yet received treatment for their disease. Hence, the sample set used in this study is unique as it describes the fecal microbiota at the onset of disease in untreated IBD patients.

The aim of the current study was to determine any correlation of fecal microbiota composition to IBD patients (both CD and UC) by comparing fecal samples of IBD patients to non-IBD control subjects, in an attempt to study the relationship between microbiota and established inflammation. In order to achieve this aim, we used direct sequencing of 16S rRNA gene sequences amplified from bacterial DNA extracted from the fecal samples [17, 18], in addition to a validation of our findings using a targeted probe approach [19].

2. Materials and Methods

A schematic outline of the methodology used in this work is given in Figure 1.

2.1. Subjects and Study Design. The stool samples used in the current study were from patients with newly diagnosed untreated IBD, and non-IBD patients were used as controls (Table 1). These samples were part of the Inflammatory Bowel South-Eastern Norway II study (the IBSEN II study) and were provided by Akershus University Hospital (Ahus) during 2005–2007. The subjects included were patients suspected to have IBD on the basis of a set of predefined symptoms, including abdominal pain, diarrhea, and/or blood in the stools for more than 10 days. An IBD diagnosis was based upon endoscopic and histologic findings. The IBD diagnosed patients were classified as CD, UC, or IBDU (IBD unclassified) based on ileocolonoscopy with addition of histology for each segment of the bowel, according to the Lennard-Jones criteria [20] and the Vienna classification [21]. Patients with IBD that could not be attributed to CD or UC were classified as IBD unclassified (IBDU).

Subjects who did not meet the diagnostic criteria for IBD and who displayed no evidence of infection or other pathology in the gut were included as a symptomatic non-IBD control group. Subjects with infection of pathogenic gut bacteria, microscopic colitis, or cancer were excluded from the IBSEN II study, both for cases and controls [22].

Of the 30 CD patients, four (13%) showed ileal disease (L1), 17 (57%) colonic disease (L2), and 9 (30%) ileocolonic disease (L3). A fistula was found in two (7%) and a stenosis in in four (13%) CD patients. Twenty-four had a nonstricturing/nonpenetrating behaviour. Most of these patients had a mild clinical disease with a median Harvey Bradshaw Index of five (range 0 to 29).

Among the 33 patients with UC, 17 (52%) had total or extensive colitis, four (12%) had left sided, and 12 (36%) proctitis. Also in the group of UC patients, the clinical disease was relatively mild with a median Simple Clinical Colitis Activity Index of four (range 0 to 14).

In total, ninety-nine patients from the IBSEN II study were included in this present study, ages ranging from 16 to 60 years. Out of the hundred patient samples, 33 were diagnosed with UC, while 30 were diagnosed with CD. In addition, 3 patients were diagnosed with unclassified IBD (IBDU). Samples of 34 subjects were in the non-IBD control group. Extraintestinal manifestations were found in three (10%), three (9%), and two (67%) of the patients with CD, UC and IBDU, respectively.

All CD, UC, and IBDU patients were included in the primary stage of treatment naive active disease.

Among the included patients, four (6%) IBD patients had been using antibiotics within one week and five patients (8%) within one month prior to stool sampling. Among the non-IBD controls, none had used antibiotics within one week, but three (9%) within one month prior to stool sampling.

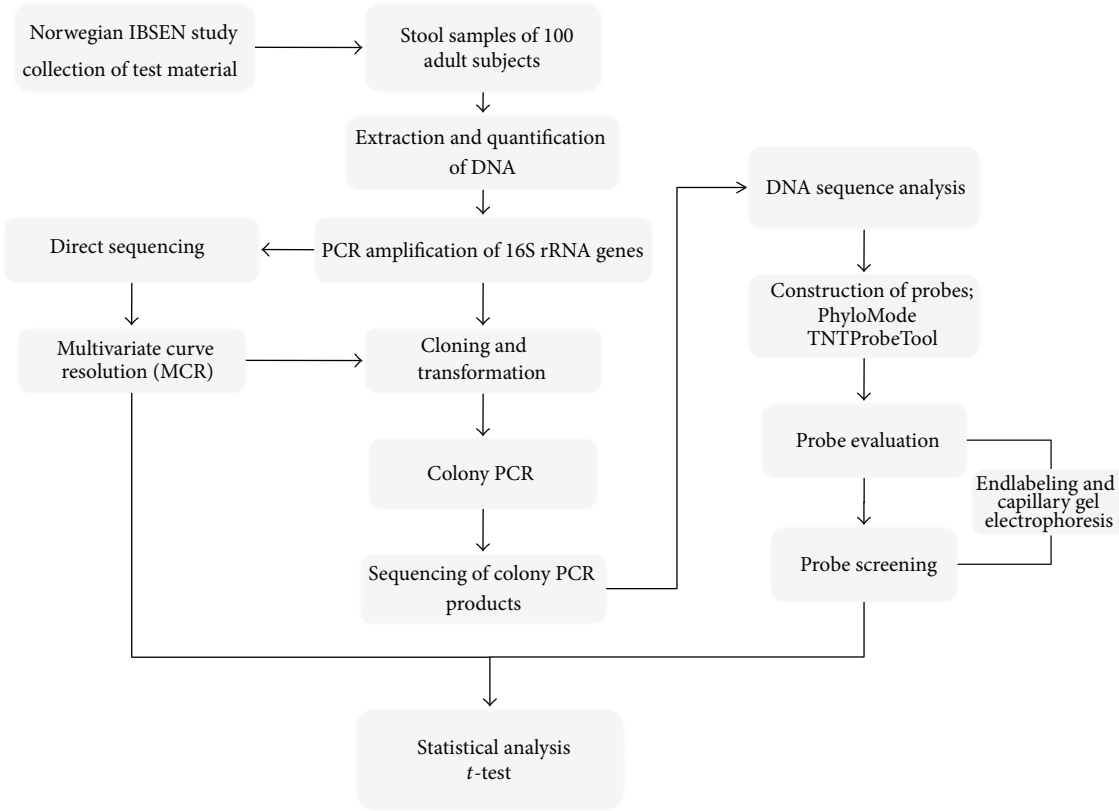

Figure 1: Schematic outline of the methodology.

2.2. Stool Samples. Patients were informed to collect stool before cleansing and received equipment for collection. Samples were kept cooled by the patients in a refrigerator and delivered at the day of the endoscopic examination. The samples were then deep frozen at −80°C the same day. Only a few patients failed to deliver a stool sample at inclusion.

2.3. DNA Extraction. DNA was extracted using the QIAGEN QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). The purification of the DNA from the stool samples was done according to the manufacturer's instructions. The stool samples were stored at −80°C before approximately 200 mg of the samples was used for the DNA extraction. The samples were lysed in 1.6 mL ASL buffer (Qiagen) with a bead-beating step of 2 minutes at 20 Hz in order to ensure maximum yield. The samples were then heated at 95°C for 5 minutes for further lysis. After cooling in room temperature the samples were vortexed before being centrifuged at 17 g for 1 minute to pellet stool particles. One InhibitEXtablet was added to 1.4 mL of the supernatant. The samples were incubated for one minute in room temperature to allow inhibitors to adsorb the InhibitEX matrix. The samples were then centrifuged at 17 g for 3 minutes to pellet stool particles and inhibitors bound to the InhibitEX matrix. Finally, 600 μL of the supernatant was placed in the QiaCube purifier (Qiagen) for automated purification of the DNA. The QiaCube purifier was preloaded with proteinase K, AL buffer, ethanol, AW1 and AW2 buffers, and AE elution buffer.

2.4. Polymerase Chain Reaction. Polymerase chain reaction (PCR) was performed in order to amplify the 16S rRNA genes. Each PCR reaction was performed in a total volume of 25 μL, and the PCR conditions were as follows: HotFirePol 1.25 U (Solis Biodyne, Tartu, Estonia), B2 buffer 1x (SolisBiodyne), $MgCl_2$ 2.5 mM (Solis Biodyne), dNTP 200 μM (Termo Fisher scientific, Surrey, USA), forward primer 0.2 μM, reverse primer 0.2 μM. The amount of DNA template used was 5 ng. Amplicons were checked with 1.5% Agarose gel (80 V; 60 min).

The 16S rRNA genes were PCR amplified from each DNA extract using the GA universal cover-all 16S rRNA primers (Genetic Analysis, Oslo, Norway), providing a PCR product of approximately 1200 bp [19].

PCR amplification was carried out with an initial denaturation step at 95°C for 15 min, followed by 30 cycles consisting of denaturation for 30 sec at 95°C, annealing for 30 sec at 55°C, and elongation for 1 min 20 sec at 72°C. The reaction was completed with a final primer elongation step at 72°C for 7 min.

2.5. Mixed Sequencing. 16S rRNA genes form the stool samples were sequenced using the universally conserved primer U515FC30 [17]. Direct sequencing was performed for all the samples in order to obtain an overview of the bacteria composition and check for any indication that any of the dominant bacteria correlated with IBD. This operation was performed in replicates where both the PCR and the direct

sequencing were repeated. In addition, 10 random samples were sequenced twice to function as technical replicates.

Different dilution factors of the ExoI and SAP treated PCR products were used for the different samples. In order to decide the dilution factor, dilution series were performed based on the band strength of the agarose gel. This was done in order to obtain good sequencing signals, where the raw signals strength should be under 8000 relative fluorescent unit (rfu) (not saturated) and over 1000 rfu [18].

A multivariate curve resolution analysis (MCR) was carried out to resolve the mixed DNA sequence spectra into pure components and their relative amounts in each of the mixed DNA samples. This analysis included principal component analysis (PCA) in order to predict the number of components to be present in the dataset, followed by the MCR analysis to finally resolve the predicted number of components. This gives two outputs (i) the relative amount of each of the components in every sample of the dataset and (ii) the spectral information of each of the components. The spectral information was base called, and the components were aligned against entries in the Ribosomal Database Project II in order to classify them.

2.6. Sequencing and Analyses of Clones. A total of 15 samples were selected for cloning. The cloning reaction and the transformation were performed using TOPO TA-cloning kit (Invitrogen) in accordance with the manufacturer recommendations for electrocompetent *E. coli*.

Low quality sequences (poor signals and short sequences) were filtered out manually, and the forward and reverse sequencing reads that were of high quality were assembled using assemble sequences (default settings) in CLC Main Workbench v6.0.1. The assembled sequences that contained a high level of conflicting information were also filtered out. All the assembled sequences were aligned in CLC using default settings with *E. coli* U0096 being used as a reference.

The sequences were further examined for chimeric artifacts using the chimeric sequence removal with chimera slayer in mothur (http://www.mothur.org/). The input in the chimera slayer was a fasta file of the filtered sequences in addition to a template file, and the outputs were potentially chimeric sequences based on the chimera slayer algorithm. The template reference set was obtained from Haas et al. [23].

The Ribosomal Database Project II Sequence Match and Classifier were used to classify the sequences to a taxonomical hierarchy.

A phylogenetic tree was constructed based on the sequences from the clone libraries. The DNA sequences were aligned using the MUCSLE algorithm in CLC (default settings) before being imported as a fasta file into the online tool BioNJ which is a part of the online service Phylogeny.fr (http://www.phylogeny.fr/). The phylogenetic tree was constructed using the Kimura 2 parameters as substitution model and 1000 as bootstrap number. The tree was subsequently imported into the computer program Dendroscope (http://ab.inf.uni-tuebingen.de/software/dendroscope/) for editing.

2.7. Probe Analyses. The 16S rRNA clone libraries were used to construct probes targeting the main clusters of bacteria. The DNA sequences in the clone libraries were first used to create a principal component (PC) plot by using the GA in-house-developed computer program PhyloMode (http://www.nofimamat.no/phylomode). Principal components analysis (PCA) is a method used for extracting a set of components that explain as much of the variability of a dataset as possible. The PhyloMode computer program is based on alignment-independent bilinear multivariate modeling (AIBIMM) [24]. The first step was to transform DNA sequence data into DNA n-mer frequencies. The n-mer frequency data was obtained by sliding a window of size n. A given pair of multimers can either be equal due to a common ancestor (homology) or equal due to mutational events (equal multimers with different evolutionary origin). A window size of $n = 5$ multimer was chosen as a trade-off between detecting phylogenetic signals (homologous multimer equalities) and avoiding base composition biases arising from nonhomologous multimer equalities [24]. The frequencies of the pentamers were counted and stored in a table. The multimer frequency data was normalized before being compressed into principal components (PCs) as previously described for the AIBIMM approach. The PCA model was exported as a "pcam" file for further use in TNTProbeTool.

Before importing the sequences into PhyloMode as a file in FASTA format, all the sequences (with chimeras removed) were aligned in CLC. The sequences were cut at conserved regions at the beginning and end, giving them the same starting and ending point.

The probe construction software TNTProbeTool was used for construction of the probes. TNTProbeTool is a GA in-house developed software for the design of single nucleotide primer extension (SNuPE) probes for analysis of microbial communities [19]. The TNTProbeTool has been developed to be able to find specific areas within the 16S rRNA gene and identify these as unique probes that can be used to identify a specific phyla, genera, family, or individual strains. The first step in the probe construction process was to define a set of multiple target and nontarget microbial DNA sequences in the PCA plot imported from the PhyloMode program. A matching region of eight nucleotides was chosen, and the labeling nucleotide was set as C. The next step was identification of probes that satisfied the criteria for target detection and nontarget exclusion, based on the combined criteria of hybridization and labeling. All probes were designed with minimum melting temperature (T_m) of 60°C by the nearest-neighbor method for the target group, while the maximum T_m between probes and nontarget sequences was set at 30°C [19]. Finally, found probes were checked against nontarget sequences, and the probes that were not good enough were filtered out. The constructed probes were exported as a "fastagr" file.

The bacterial strain-specific probe was end-labeled with fluorescence dye TAMRA bound to a ddCTP (5-propagylamino-ddCTP-5/6 TAMARA) for detection using capillary electrophoresis. The designed probes were bound to the complementary 16S rRNA sequence of that particular bacterium or groups of bacteria, and ddCTP-TAMRA was then bound as a single nucleotide to the 3′ end of the probe.

Table 2: Sequence of the probes used in the project.

Probe	Sequence
Probe 3-*Escherichia/Shigella*	GCCTCAAGGGCACAAC
Probe 6-*Dialister*	AAGAACTCCGCATTTCTGC
Probe 8-*Faecalibacterium*	CGTAGTTAGCCGTCACTTC
Probe 13-*Haemophilus*	TCGCTTCCCTCTGTATACG
Probe 16-*Enterococcus*	CCCTCCAACACTTAGCA
Probe 18-*Lactobacillus*	CCTGTTTGCTACCCATACTTT
Universal probe	CGTATTACCGCGGCTGCTGGCA

This reaction was done in a cyclic manner by thermocycling, and gave rise to free labeled probes in the solution. In a total volume of 10 μL HOT Termipol DNA polymerase 2.5 U (Solis Biodyne), HOT Termipol buffer C 1x, $MgCl_2$ 4 mM, ddCTP Tamra 0.4 μM, designed probe 0.1 μM, and 10x diluted ExoI and SAP treated template (2 μL). The labeling reaction was carried out with an initial denaturation step at 95°C for 15 min, followed by five cycles consisting of denaturation for 20 sec at 96°C, and combined annealing and extension for 35 sec at 60°C.

Before performing probe screening on all the samples, all the constructed probes were evaluated experimentally by cloned target sequences and nontarget sequences (both close to the target sequences and random sequences). Finally, suitable probes that satisfied the criteria for target sequences detection and exclusion of nontarget sequences were included in the screening.

All samples were hybridized with six probes in separate reactions. A universal 16S rRNA gene probe was also included to measure the total abundance of bacterial DNA in the samples (Table 2). After labeling, the samples were treated with 8 U SAP and incubated at 37°C for 1 hour and inactivated at 80°C for 15 min. Then 1 μL of the SAP-treated and labeled probes were mixed with 9 μL of Hi-Di formamide and 0.5 μL GeneScan 120 Liz Size Standard (Applied Biosystems). The samples were incubated at 95°C for 5 min before being placed on ice. The samples were then loaded onto a 36 cm 3130xl capillary array in the ABI Genetic Analyzer 3130xl sequencer (Applied Biosystems), containing the performance optimized polymer 7 (POP-7, Applied Biosystems). Injection time was 16–22 s and the electrophoretic conditions were run time 180 s at 15000 V, run current 100 μA, and 60°C run temperature. Data analysis was performed using the GeneMapper 4.0 software (Applied Biosystems).

3. Results

3.1. Resolving Mixed Sequences into Pure Components. The mixed sequences were resolved into six main components using MCR analysis. The spectra of the six components are presented in Figure 2. One of the components (component 3) was regarded as noise and excluded as it exhibited two high peaks and a poorly resolved spectrum. The other five components showed well-resolved spectra with nearly the same signal heights. There were, however, some variance in the signal height of the background sequences compared to the components and hence also in the purity of the components. A visual examination indicated that components 2, 5, and 6 had lower background sequences and better resolved spectra than did components 1 and 4.

The base-called sequences of the five components with well-resolved spectra are shown in Table 3.

The components were classified using the Ribosomal Database Project II (RDP) Classifier (Table 4), which estimates the classification reliability using bootstrapping. Components 2, 5, and 6 were classified with relatively high bootstrap confidence estimates (above 85%) at the genus level, whereas for components 1 and 4, classification at the genus level gave low bootstrap confidence estimates (10% and 9% resp.). The confidence threshold for short sequences was set at 50%. And as a result, the components were classified as Clostridiales (Comp_1), *Escherichia/Shigella* (Comp_2), Clostridiales (Comp_4), *Faecalibacterium* (Comp_5), and *Bacteroides* (Comp_6).

The technical quality of the resolved components were evaluated both by analyses of sample replicates and comparison with the results from cloning and sequencing. Taken together, these results support a high technical quality and reliability. Details for the analyses and comparisons are shown in the Supplementary Material available online at http://dx.doi.org/10.1155/2013/636785.

3.2. MCR Clusters. The data matrix in Figure 3 summarizes the amount of each component in each sample.

One cluster of twenty-five samples (Cluster 1, Figure 3) has a very low diversity flora. There is mainly one component present; there are high amounts of Comp_2-*Escherichia/Shigella*, while the amounts of other components are low, or other components are not present at all. This cluster of samples consists of all disease states, though there is an overrepresentation of CD (52%) and UC (32%) compared to controls (16%). Most of the other samples show an overall mix of several components, and the data matrix does not reveal any apparent clustering of the different disease states.

3.3. Comparison of the Average Amount of Components in the Different Disease States. The average amount of each component was calculated for each of the three disease states in order to facilitate comparison between them. The averages are presented in Figure 4, and the most striking difference is for Comp_2-*Escherichia/Shigella*, where the CD average is high compared to both control and UC. Another considerable difference is the amount of Comp_5-*Faecalibacterium* present in the control and UC group, compared to the CD group. In addition, there is a slightly higher amount of Comp_1-Clostridiales in the control group compared to both CD and UC.

In order to investigate whether the observed differences are statistically significant, a two-tail t-test for independent data was conducted. The amount of Comp_2-*Escherichia/Shigella* in CD patients was found to be statistically significantly higher ($P = 0.013$) than in controls, while the amount of Comp_5-*Faecalibacterium* was found to be significantly lower in CD patients than in both control subjects

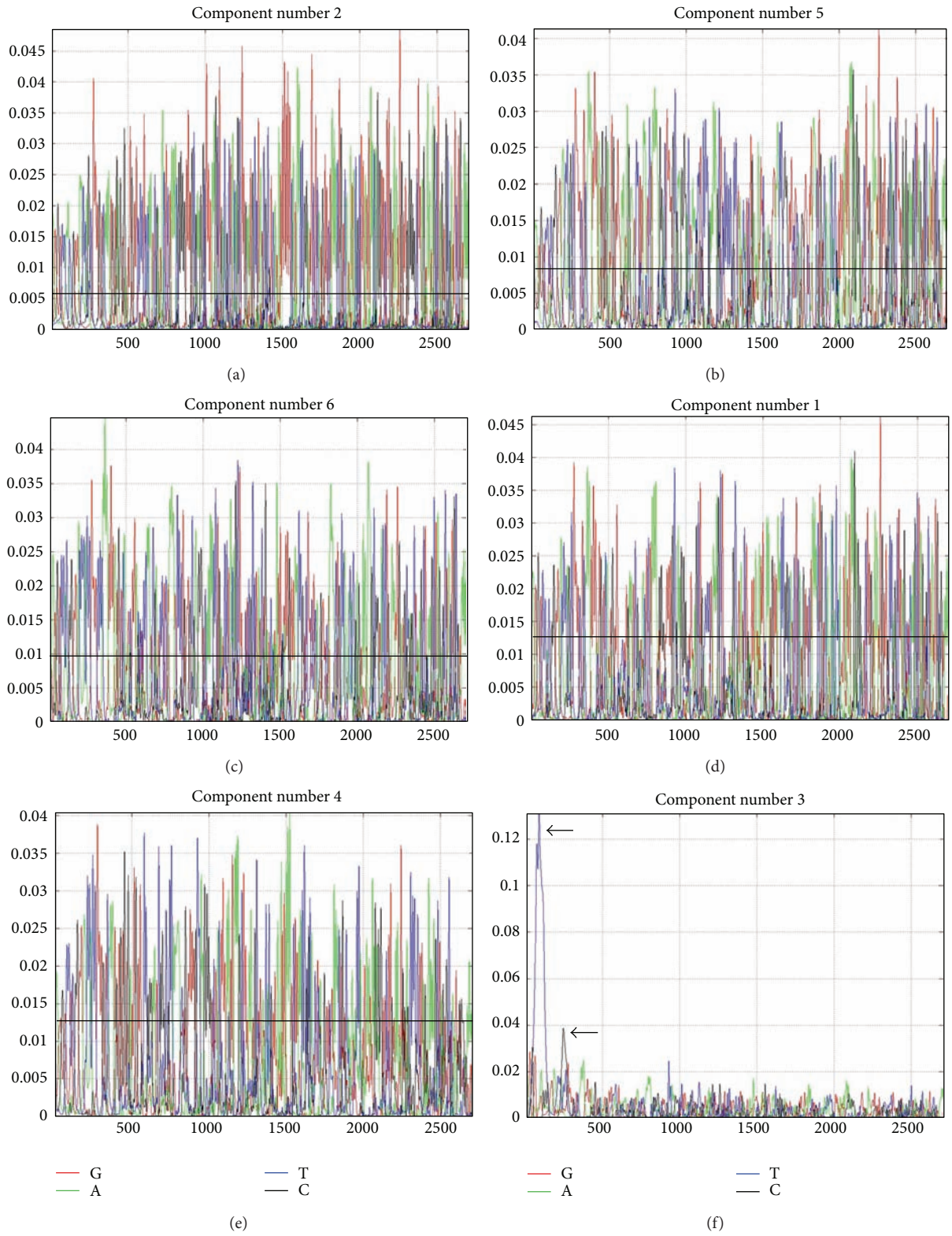

Figure 2: Spectra of the six components resolved by MCR analysis. Visual examination reveal components 2, 5, and 6 ((a)–(c)) to have well resolved spectra with low background sequences. Components 1 and 4 ((d) and (e)) also have well resolved spectra, although with somewhat higher background sequences than components 2, 5, and 6. Component 3 (f) has two high peaks (black arrows) and a poorly resolved spectrum.

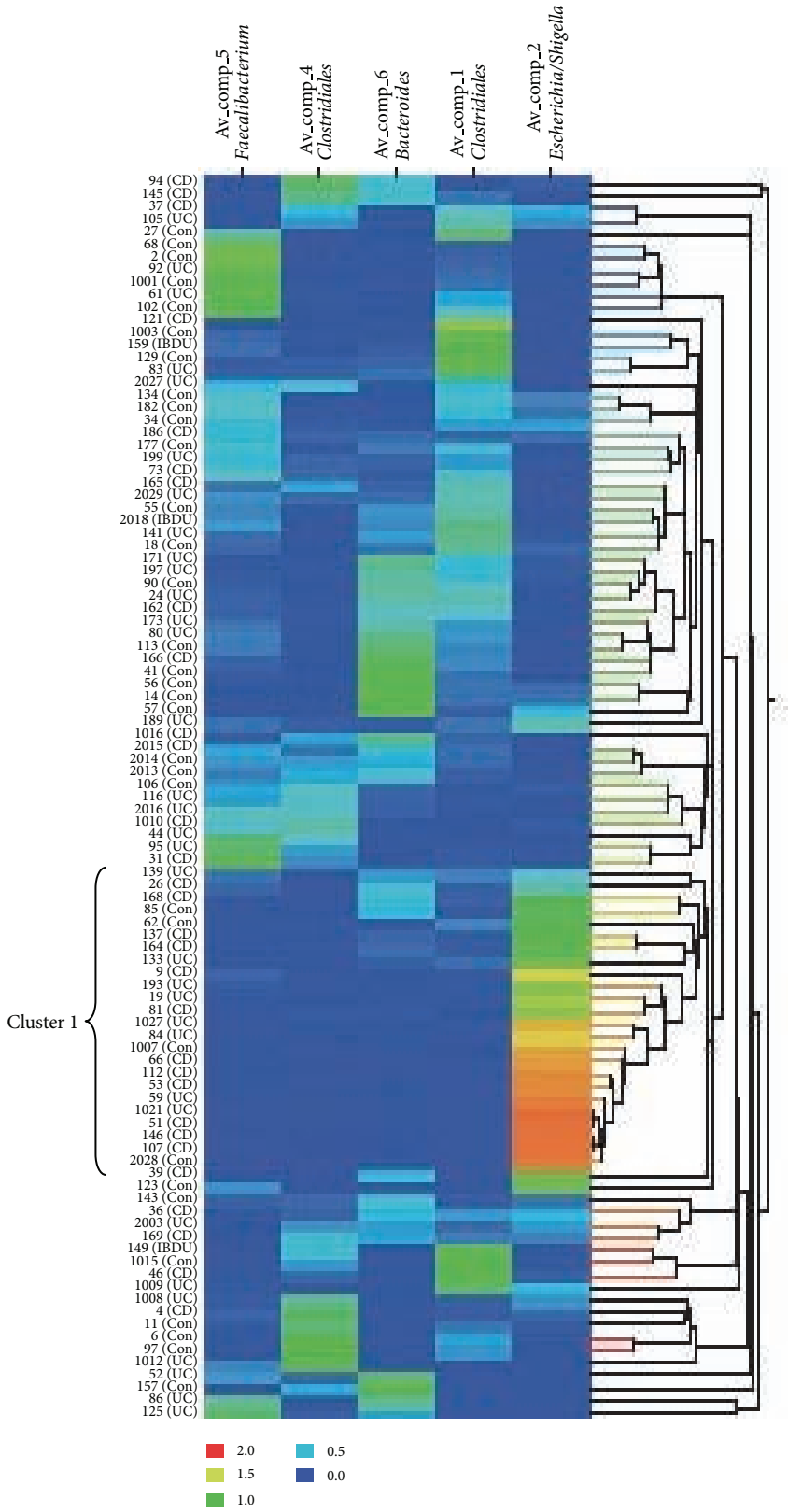

Figure 3: Data matrix of the amount of components 1, 2, 4, 5, and 6 in the stool samples. The graded colors indicate the abundance of a particular component in a sample. Red color indicates a high amount of the component, and blue color indicates a low amount of the component. To the left of the matrix, sample numbers are shown together with the diagnosis UC (Ulcerative colitis), CD (Crohn's disease), and Con (control).

Table 3: Base-called sequences of the five components that showed well resolved spectra obtained from the MCR analysis.

Component	Sequence
1 (Clostridiales)[1]	AGCGTTAGTCCGGATTTACTGGGTGTAAAGGGWGCGTAGGACGGWTGTGCAAGTCATG GAWGTGAAAGSCCCGGGGCTRAACCCCTGGYACTGCWTTTGGAAACTGTGAGACTAGG AGTGACWCGGAGYGGCTAASCGGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGG AGGAACACCAGTGGCGAAGGCGGCTTAGCTGGACTTGTAACTGACGRTGAGGCATCGAAA
2 (*Escherichia/Shigella*)[1]	AGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTG AAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGA GGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTG GCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAA
4 (Clostridiales)[1]	AGGCGTTGTCCGGAATTATTGGGCGTAAAGSGCGCGCAGGCGGTTCCCTAAGTCCCTCTT AAAGTGGCGGGGCTTAACCCCGTGGATGGGAWGGAAACTGTGGAAGCTMGAGATTATC GGAAAGGAAAGTGGAATTCTCTATGTTYCGGTGGAAATGCGTAAAGAATTAGGAAGAAC AKCGGTTGGCGGAAGAGSCGACTTTCTGGAGCAAAACTGTAGCGCTCGTAGAGCSCCCAAA
5 (*Faecalibacterium*)[1]	AAGCGTTGTCCGATTACTGGGTGTAAGGGAGCGCAGGCGGAAGACAGTTGGAAGTGAAAC CATGGGCTCAACCCATGAATCTTGCTTTCAAAACRGMTTTTCTTGAYTWGTGCAAAGG GTAGAGTGGGAATTCCGGTTGTACCGTGGAATGCGTAATATCGGGAGGAACACCAGTGGC GAAGGCGGCRTACTGGGCACCAACTGACGCTGAGGCTCGAAA
6 (*Bacteroides*)[1]	AGCGTTATCCGGATTTATTGGGTTTAAAGGGAGCGTAGACTGGACTMTGTTAAGTCAGT TGTGAAAGTTTGCGGCTCAACCGTAAAATTGCAGTTGAWACTGGTGTCTTGAGTYCAGTW GAAGGCTYGGCGGAATTCGTGGTGTACGGTGAAATGCTTAATATCACGAAGAACRCCGAT TGCAAGGCAGCRTAGCTGAACTGAACTGACARTGATGCTCGAAA

[1] Classification of the components is done according to Table 3.

Table 4: The five well-resolved components from the MCR analysis of the results of the direct sequencing classified using the Classifier in Ribosomal Database Project II. Classification at the phylum, class, order, family, and genus levels are shown with the corresponding bootstrap confidence estimate.

	Phylum	Class	Order	Family	Genus
Comp_1	Firmicutes 84%	Clostridia 84%	Clostridiales* 84%	Lactinospiraceae 22%	*Lactinofactor* 10%
Comp_2	Proteobacteria 100%	Gammaproteobacteria 100%	Enterobacteriales 100%	Enterobacteriaceae 100%	*Escherichia/Shigella** 85%
Comp_4	Frimicutes 77%	Clostridia 65%	Clostridiales* 64%	Incertae Sedis XI 15%	*Parvimonas* 9%
Comp_5	Firmicutes 98%	Clostridia 98%	Clostridiales 98%	Ruminococcaceae 97%	*Faecalibacterium** 94%
Comp_6	Bacteroidetes 100%	Bacteroidia 99%	Bacteroidales 99%	Bacteroidaceae 91%	*Bacteroides** 91%

* The cut-off value of the bootstrap confidence threshold was set at 50%. Comp_1 and Comp_4 were classified at the order level whereas the other components were classified at the genus level.

and UC patients ($P = 0.024$ and 0.014, resp.). The difference between the average amounts of Comp_1-Clostridiales in the control group and in CD and UC patients was not statistically significant at a 5% level (control versus CD; $P = 0.097$ and control versus UC; $P = 0.129$).

3.4. Comparison of the Average Signal Strength of the Probes in the Different Disease States. The probe identification and evaluation are presented in the Supplementary Information. Only one of the constructed probes did not satisfy the criteria of target detection and nontarget exclusion.

In order to make a comparison between the disease states, the average peak height for all the probes in the three disease states was calculated. The probe signals were normalized using signals from the universal probe, and the average of the ratios are presented in Figure 5. Because of considerable differences in absolute signal strength, all the signals were normalized to one. The most obvious difference is the signals of Probe 3-*Escherichia/Shigella* for both CD and UC compared to control. There is also a marked difference ($P = 0.142$ and 0.093, resp.) between UC and both CD and control for the signals of Probe 8-*Faecalibacterium*. In addition, CD has higher signals than both control and UC ($P = 0.100$ and 0.182, resp.) for Probe 6-*Dialister*. The average signal strength values were compared using a t-test and the only statistically significant ($P = 0.013$) difference was the higher amount of Probe 3-*Escherichia/Shigella* compared to controls.

4. Discussion

The presence of *Escherichia/Shigella* was found to be significantly increased in CD patients compared to controls. The

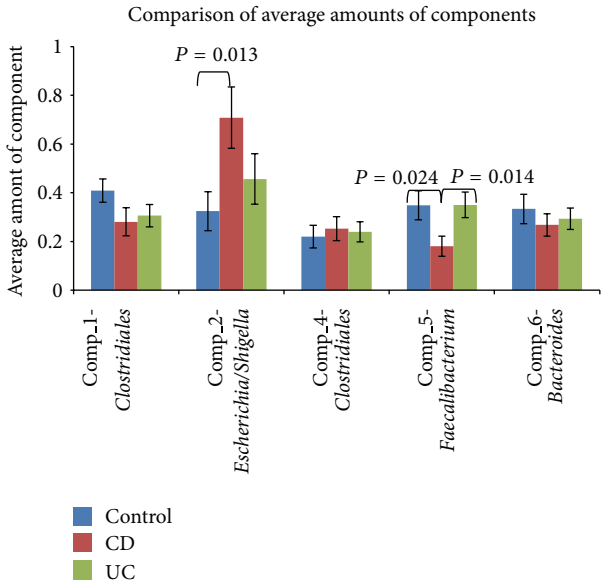

Figure 4: Comparison of the average amount of each component in the three disease states. The averages are calculated based on MCR analysis of stool samples from 33 subjects in the control group, 30 subjects diagnosed with CD (Crohn's Disease), and 33 subjects diagnosed with UC (Ulcerative Colitis). Standard error of arithmetic mean is shown. The significance of the differences between the averages of the three groups, control, CD, and UC, was tested using the t-test where the statistical significance was accepted at $P < 0.05$. Only the statistically significant P values are shown in the figure.

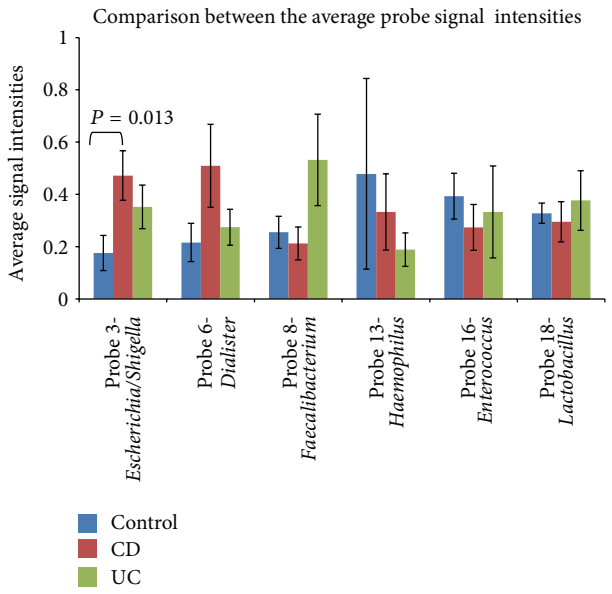

Figure 5: Comparison between the average amount of specific bacteria groups in the different disease states. The averages are calculated based on the height of the probe signals normalized by the signal height of the universal bacterial probe. Further, the averages are normalized to one because of Probe 18-*Lactobacillus* in particular, which gave much higher signals. There are 34 subjects in the control group, and the CD (Crohn's Disease) and UC (Ulcerative colitis) group have 30 and 33 subjects, respectively. Standard error of arithmetic mean is shown. The significance of the differences between the averages were tested using a t-test where the statistical significance was accepted at $P < 0.05$. Only the statistically significant P-value is shown in the figure.

fact that both the MCR data and the probe screening data reveal a correlation of higher amounts of *Escherichia/Shigella* in patients with CD strongly supports this fact. The result, that is, increased numbers of *Escherichia/Shigella* in fecal samples from CD patients compared to control subjects is supported by several previous studies. Using a semiquantitative microbiological method, Giaffer et al. [25] found that patients with active CD had significantly higher total scores of *E. coli* compared to patients with quiescent disease, patients with UC, and healthy controls. Seksik et al. [26] further reported that enterobacteria were observed significantly more frequently in patients suffering from CD than in healthy subjects using dot blot hybridization. Using qRT-PCR and microarray approaches, Mondot et al. [27] revealed that *E. coli* is more represented in CD patients compared to controls.

The predominant mucosa-associated bacterial communities in the colon differ significantly from those in feces [28, 29], and this is an important fact to recognize when studying the role of the endogenous microbiota in IBD [28]. An increased level of Proteobacteria (with *E. coli* being the most common phylotype) in CD patients compared to UC and controls was found in a study of tissue-associated intestinal microflora [30]. Also, an increased amount of Enterobacteriaceae has been found in CD mucosal biopsies [31]. Baumgart et al. [32] reported that the ileal mucosa of patients with CD involving the ileum were enriched in sequences of a novel group of invasive and potentially pathogenic *E. coli*, and that the number of *E. coli in situ* correlated with the severity of the disease. These mucosa-associated pathogenic *E. coli* are invasive and highly adherent to intestinal cells and are designated adherent-invasive *E. coli* (AIEC) [33, 34]. Darfeuille-Michaud et al. [35] found a high prevalence of AIEC in the ileal mucosa of patients suffering from CD. AIEC strains were found more frequently in early recurrent lesions after surgery, leading to the suggestion that AIEC could be involved in the initiation of the inflammatory process and not only secondary invaders. Sepehri et al. [36] characterized AIEC from IBD patients at first diagnosis, which suggests that they may have a role in the early stages of disease onset. The fact that AIEC is also detected in healthy mucosa [35], may indicate that the presence of these strains is in itself insufficient to cause disease. It has been suggested that AIEC may be opportunistic pathogens that have the ability to exploit the mucosal environment of a CD susceptible individual. Alternatively, the proliferation of these microorganisms may be a consequence of depletion of the normal flora [32]. AIEC may have the ability to exploit host defects in bacterial clearance and autophagy for survival and replication [37]. Furthermore, AIEC is able to initiate an inflammatory process by the induction of the first stages of cell aggregation leading to the formation of granulomatous structures [38] which is a histological characteristic of CD. Such granulomas are also associated with several infectious

diseases involving among others *Salmonella* spp and *Shigella* spp (reviewed in Rolhion and Darfeuille-Michaud, [34]).

In this study, *Faecalibacterium* were significantly less abundant in individuals with CD compared to both controls and individuals with UC when investigating the average of the MCR data. However, the probe for *Faecalibacterium* did not show significantly lower signals for the CD group compared to the control group or UC group. Although the average of the probe signals for the UC group showed a sizable difference compared to both the control group and the CD group, and the difference between UC and CD had a low P value; this was not statistically significant at the 5% level. There are, thus, some inconsistent results concerning *Faecalibacterium*. The abundance of *Faecalibacterium* seems, all the same, to be reduced in the CD group compared to both controls and the UC group. The Firmicutes phylum has previously been reported underrepresented in IBD, and in CD particularly. Manichanh et al. [39] reported a reduced diversity of Firmicutes, and the *Clostridium leptum* phylogenetic group in particular was reported to be less abundant in fecal samples from CD patients compared to those of healthy individuals. The *C. leptum* group contains numerous butyrate-producing bacteria. Butyrate is a major source of energy for colonic epithelial cells and inhibits inflammatory responses by decreasing proinflammatory cytokine expression via inhibition of NF-κB activation in immune cells [40, 41]. Decreased butyrate levels could, thus, be implicated in the increased inflammatory state that occurs in IBD (reviewed in Fava and Danese, [42]). *Faecalibacterium prausnitzii* is a predominant species of the *C. leptum* group [43], and analysis has revealed that *F. prausnitzii* exhibit an anti-inflammatory effect and thus is important for the gut homeostasis [44]. A reduction of *Faecalibacterium* in fecal samples of patients with CD and an underrepresentation of the phylum Firmicutes, particularly *F. prausnitzii* in both active UC and CD patients as well as in infectious colitis patients has been reported [40, 45, 46]. Mondot et al. [27] also revealed that *F. prausnitzii* was more represented in fecal samples from healthy subjects compared to those of CD patients. One study, on the other hand, revealed a significant increase of *F. prausnitzii* at the time of diagnosis in pediatric CD suggesting a possibly more complex role for *F. prausnitzii* in CD pathogenesis. However, there may be important distinctions between adult and pediatric IBD [16].

The genus *Dialister* showed a higher abundance in CD patients in our study when comparing average probe signal strength although this was not significant at a 5% level when comparing the averages using t-test. In contradiction with this finding Joossens et al. [45] reported a decrease in *Dialister invisus* in patients with CD. This species is typically isolated from the oral cavity but has also been detected in samples of the normal gastrointestinal microbiota [47]. However, in the present study the probe for *Dialister* does not target the species *D. invisus* specifically which may be a possible explanation for the discrepancy.

For the Bacteroides group we were not able to identify any significant correlations related to IBD. In the literature, however, there are conflicting evidence for Bacteroides. For a mouse model, it has been shown that Bacteroides species can induce colitis [48], while both significant [49, 50] and not significant [39] correlations have been identified for human cohorts.

At a higher taxonomic level, the MCR analysis revealed one cluster of twenty-five samples consisting of all three disease states, although with an overrepresentation of CD and UC. These clusters showed low diversity flora with only one dominant component, Comp_2-*Escherichia/Shigella*. The low diversity flora was not expected, and in healthy individuals the abundance of Proteobacteria (including *E. coli*) is expected to be low [51]. However, the control group in this study are patients hospitalized with GI symptoms, and it can be disputed whether these patients can be characterized as healthy controls. Subjects in the control group may for instance be suffering from irritable bowel syndrome (IBS) which is a common intestinal disorder. The fecal microbiota has also been shown to be altered in patients suffering from IBS [52, 53], and discriminating IBS from IBD is a common clinical challenge [54]. The control patients in the present study all had symptoms without inflammation, probably also including IBS in several cases. Consequently, one strength of the study is its potential to differentiate between the characteristic of microbiota in inflammatory compared to noninflammatory states.

It is difficult to establish whether the altered microbiota composition observed in IBD patients is a cause or a consequence of the inflamed mucosa. The altered composition of microbiota may result from colonization by an enteric pathogen, from host-mediated inflammatory responses, or from both (reviewed in [55]). Infecting mice with *Salmonella enterica* serovar Typhimurium shows that this intestinal pathogen overcomes colonization resistance by inducing the host's inflammatory immune response and exploiting it for its own purpose and for promoting its own growth. An inflammatory response induced by *S. enterica* also alters the composition of the resident microflora. Other closely related proteobacteria, such as *E. coli,* is also believed to benefit from inflammation. The altered microbiota composition in IBD patients might, thus, not be the cause, but rather one of the many symptoms, of intestinal inflammation in IBD patients [56]. In a mouse model of gut infection, Lupp et al. [57] demonstrated that host-mediated inflammation in response to an infecting agent or genetic predisposition markedly alters the colonic microbial community. The resident colonic bacteria become significantly reduced whereas such an inflammation supports the growth of potentially pathogenic bacteria, particularly Enterobacteriaceae. These findings may suggest that the onset of an inflammatory response by the host could be the initiating factor in the dysregulation of the intestinal microbiota balance and cause of the persistent inflammatory state of IBD. An increased risk of developing IBD after an episode of acute gastroenteritis has also been indicated [58], which may lead to speculation that a bacterial infection-driven dysbiosis could lead to IBD in a predisposed individual [40]. Shifts in microbial populations are also associated with particular CD risk alleles, indicating that dysbiosis is not only a consequence of chronic disease [59]. Gophna et al. [30] found no significant difference in the flora between the ulcerated and nonulcerated tissues within

the same individual suffering from CD and suggested that it is unlikely that inflammation is directly caused by a mucosa-associated pathogen. This is in agreement with another study reporting no qualitative difference between ulcerated and nonulcerated mucosa in CD patients [60]. In contradiction with this, Walker et al. [31] found differences in microbial community structure between inflamed and noninflamed mucosal sites. In UC patients, Zhang et al. [61] found a localized dysbiosis where lactobacilli and the Clostridium leptum subgroup were significantly different between the ulcerated and the nonulcerated regions of the mucosa-associated intestinal flora and that this may be related to UC.

In conclusion, the evaluation of the fecal microbiota in newly diagnosed, untreated IBD patients and control subjects revealed significant changes in the fecal microbiota, whether causative of or responsive to disease.

Conflict of Interests

None of the coauthors has any conflict of interest related to the data presented in the current work.

Acknowledgment

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Temporal Development of the Infant Gut Microbiota in Immunoglobulin E-Sensitized and Nonsensitized Children Determined by the GA-Map Infant Array▽†

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At birth, the human infant gut is sterile, but it becomes fully colonized within a few days. This initial colonization process has a major impact on immune development. Our knowledge about the correlations between aberrant colonization patterns and immunological diseases, however, is limited. The aim of the present work was to develop the GA-map (Genetic Analysis microbiota array platform) infant array and to use this array to compare the temporal development of the gut microbiota in IgE-sensitized and nonsensitized children during the first 2 years of life. The GA-map infant array is composed of highly specific 16S rRNA gene-targeted single nucleotide primer extension (SNuPE) probes, which were designed based on extensive infant 16S rRNA gene sequence libraries. For the clinical screening, we analyzed 216 fecal samples collected from a cohort of 47 infants (16 sensitized and 31 nonsensitized) from 1 day to 2 years of age. The results showed that at a high taxonomic level, *Actinobacteria* was significantly overrepresented at 4 months while *Firmicutes* was significantly overrepresented at 1 year for the sensitized children. At a lower taxonomic level, for the sensitized group, we found that *Bifidobacterium longum* was significantly overrepresented at the age of 1 year and *Enterococcus* at the age of 4 months. For most phyla, however, there were consistent differences in composition between age groups, irrespective of the sensitization state. The main age patterns were a rapid decrease in staphylococci from 10 days to 4 months and a peak of bifidobacteria and bacteroides at 4 months. In conclusion, our analyses showed consistent microbiota colonization and IgE sensitization patterns that can be important for understanding both normal and diseased immunological development in infants.

The colonization of the human infant gut is a remarkable process in which the gut goes from sterile to fully colonized with no further increase in bacterial concentration within just a few days (19). During this colonization, there is an intimate interaction between the microbiota and the host, including training of the immune system with respect to the responses to microorganisms (24). Early aberrant colonization may lead to a situation in which the immune system does not respond properly later in life. More than 20 years ago, the hygiene hypothesis stated that the clean Western lifestyle is the main underlying cause of the current increase in allergic disorders (3, 30). However, discussion about the validity of the hygiene hypothesis is ongoing (1, 4, 7).

The KOALA study is currently one of the largest culture-independent studies of infant gut bacterial composition and atopy development (21). In this study, five bacterial phylogroups were investigated, and the composition was determined at 1 month after birth by real-time PCR. Limitations of the KOALA study, however, were that the temporal development of the microbiota was not investigated and a relatively limited number of bacteria were tested. In the IM-PACT study, therefore, we have investigated the effects of the temporal development of 12 selected bacteria on allergy development. We found that specific IgE antibodies to mites (*Dermatophagoides pteronyssinus*); mold (*Cladosporium herbarum*); cat and dog dander; birch, timothy (grass), and mugwort pollens; cow's milk; hen's egg white; codfish; hazelnut; and peanut gave the best correlation with bacterial profiles, while we found relatively low correlation with the other measured atopic markers (O. Storrø, T. Øien, Ø. Langsrud, K. Rudi, O. K. Dotterud, and R. Johnsen, unpublished results). Atopy is an allergic disease mediated through elevated IgE antibody levels.

Still, a challenge in understanding the effect of the microbiota on atopy development is the complexity of the microbiota (24). Only recent technological advances in 16S rRNA gene deep-sequencing (22) and array technologies (20, 23) have enabled large-scale analyses of the dominant microbiota in infants. The most extensive analysis until now is the detailed description of the colonization of 14 children up to the age of 1 year using a 16S rRNA gene array approach (19). These analyses revealed a highly complex colonization pattern at the genus level, while the pattern was more deterministic and predictable at the phylum level (34).

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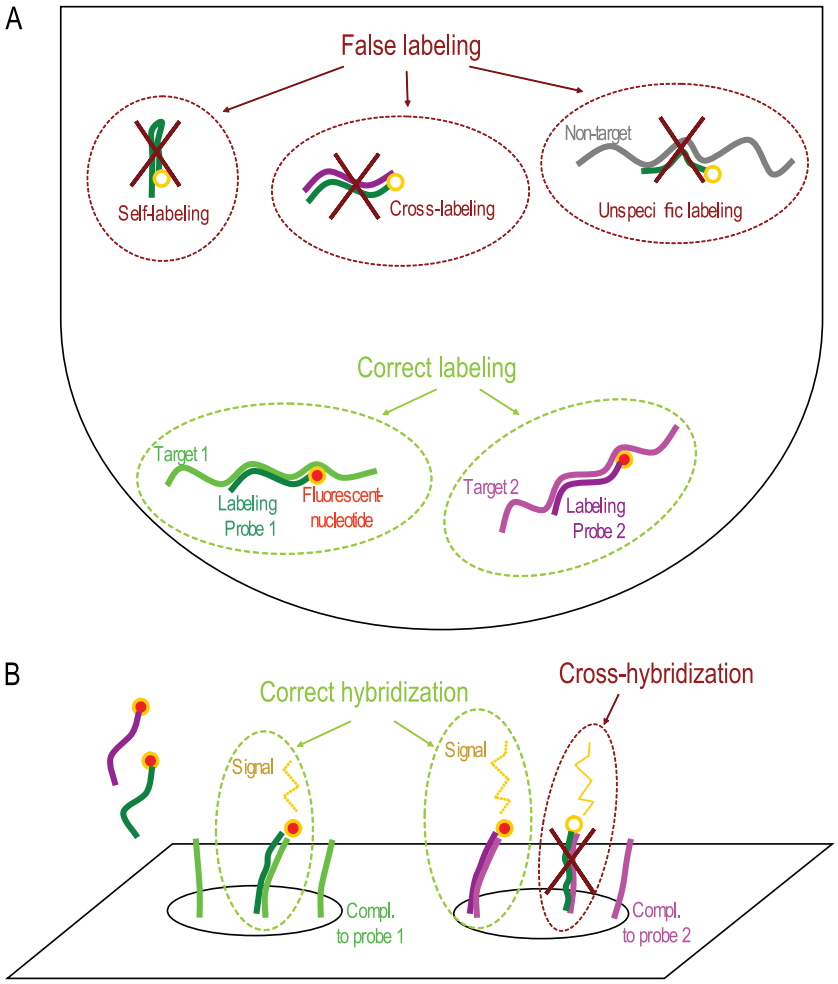

FIG. 1. Illustration of the principle and challenges associated with the GA-map array technology. In the labeling step, there are several ways probes can be nonspecifically labeled through self-labeling (internal regions of the probe are used as targets), cross-labeling (a region internal to another probe is used as a target), and nonspecific labeling (a probe is labeled based on a nontarget template) (A), while in the hybridization step, the probes may bind to the wrong position in the array in the process of cross-hybridization (B). A nontemplate control reaction was included to detect potential self-labeling and cross-labeling. The nonspecific labeling was evaluated by control experiments on samples with defined compositions, while the hybridization step was controlled by the inclusion of both positive and negative hybridization controls.

To our knowledge, no studies have yet correlated the temporal development of a comprehensive set of the dominant microbiota with atopic disease. The aim of the present work was therefore to prospectively compare the development of the dominant microbiota in IgE-sensitized children and nonsensitized children during the first 2 years of life. In order to accomplish this, a tool to rapidly screen for the complexity and composition of the bacteria in stool samples was needed. We therefore developed an infant high-throughput 16S rRNA gene microarray, called the GA-map (Genetic Analysis microbiota array platform) infant assay, that is applicable to any infant gut microbiota-related task. The microarray analyses were performed on a selected subset of the IM-PACT cohort. Specific IgE was chosen as an atopy marker, since we have previously shown that this marker is correlated with gut bacteria (Storrø et al., unpublished).

The main difference between the GA-map infant array and alternative 16S rRNA gene array approaches (19, 23) is the use of highly specific single nucleotide primer extension (SNuPE) probes for target/nontarget discrimination (17, 27). The high specificity of the SNuPE assay is obtained by the combined fidelity provided by DNA polymerase-based incorporation of a fluorescently labeled dideoxynucleotide and target hybridization (16, 31). The SNuPE probes are constructed so that they hybridize adjacent to discriminative gene positions. If the target bacterium is present, then a labeled dideoxynucleotide is incorporated by the polymerase. To reduce complexity and to increase throughput, the GA-map infant assay was targeted to bacteria expected to colonize the infant gut (19, 26). The probes were selected based on the criterion of the minimum number of probes covering the expected diversity of bacteria in the infant gut. A schematic outline of the GA-map assay is shown in Fig. 1.

We present results showing that there were significant phylum and genus level differences between the sensitized and nonsensitized children. We also identified surprisingly consistent age-specific colonization patterns independent of the sensitization state.

MATERIALS AND METHODS

Cohort. The Prevention of Allergy Among Children in Trondheim (PACT) study is a large population-based intervention study in Norway focused on childhood allergy (18). The sample included here is a subset of the PACT study in which we undertook immunology and microbiology measurements. For the substudy, family doctors and midwives in Trondheim participated in recruiting an unselected population of women during ordinary early pregnancy checkups until 720 had been approved to participate. The women filled in questionnaires on risk factors during pregnancy, at 6 weeks after delivery, and 1 and 2 years after giving birth. The questions were on allergy in the family, housing conditions, diet, and lifestyle and, after birth, on breastfeeding, food supplements, diet, infections, vaccines, antibiotics, stays in day care centers, and nicotine exposure. When the infants turned 2 years old, another questionnaire on health and disease was submitted. Atopic sensitization was assessed as elevated specific IgE (≥0.35 kU/ml) in serum using an assay for a range of allergens (Immulite 2000 Allergen-Specific IgE system; Siemens Medical Solutions Diagnostics). The cohort was initially analyzed for 12 specific bacteria by quantitative PCR (qPCR) (Storrø et al., unpublished). Here, we selected a range of infants for in-depth GA-map infant array testing based on the number of samples and the sensitization state. A total of 16 sensitized and 31 nonsensitized children were selected, representing a total of 216 fecal samples. We were blinded to the information about the other factors in this selection.

Samples for validation of reproducibility and specificity. Forty-three samples were randomly picked to examine the reproducibility of the GA-map infant assay. These 43 samples were processed twice, starting from the labeling reaction. From one fecal shedding, we did three independent samplings and analyses. This was done to evaluate if a single sample would give representative results for the fecal microbiota. The classification accuracy was evaluated by mixtures of 50 ng/μl PCR products from 2 (1:1) to 5 (1:1:1:1:1) pure bacterial strains (see Table 3). Subsequently, 2 μl (100 ng) of the mixed PCR product was used as input in the labeling reaction. As a test of the quantitative range of the assay, PCR products from pure cultures of 5 different species (see Table 3) were diluted from 10^0 to 10^{-4} and included in the labeling reaction and downstream array analysis. Finally, we tested the relative quantification of mixed samples using PCR products (50 ng/μl) following the experimental design illustrated in Table S4 in the supplemental material and using 2 μl (100 ng) as a template in the end-labeling reaction.

Sample preparation and PCR amplification. Feces were collected from the diaper and transferred to Carry Blair transport medium by the parents and stored immediately at −18°C at home before being transported to permanent storage at −80°C until further analysis. Mechanical lysis was used for cell disruption, and an automated magnetic-bead-based method was used for DNA purification. The approach was previously described by Skånseng et al. (29).

We combined the use of a forward primer targeting the conserved region between V2 and V3 (15) with a reverse primer targeting the 3′ end of the 16S rRNA gene (35). We used 1.5 U HotFirePol (Solis Biodyne, Tartu, Estonia), 1× B2 buffer (Solis Biodyne), 2.5 mM $MgCl_2$ (Solis Biodyne), 200 μM deoxynucleoside triphosphate (dNTP) (Thermo Fisher Scientific, Waltham, MA), 0.2 μM each forward and reverse primer, and approximately 10 to 50 ng template in a total volume of 25 μl. One of the samples was amplified three times to examine the reproducibility of the PCR (described in further detail below [see Capillary electrophoresis]) (see Fig. S2 in the supplemental material). The amplification protocol included a 15-min activation stage at 95°C, followed by 30 cycles with 30 s denaturation at 95°C, 30 s annealing at 55°C, and 90 s extension at 72°C. A final elongation for 7 min at 72°C was included for completion of all the PCR products. For the initial tests of the array, 16S rRNA gene PCR was performed on bacterial DNA from pure cultures of 26 strains listed in Table 1, and the PCR products were tested in the downstream GA-map infant assay. The strains were sequenced to confirm their identities and possible mutations (the sequence accession numbers are listed in Table 1). A positive control consisting of a mixture of DNAs from pure cultures of 8 relevant bacterial strains, as well as a negative control consisting of H_2O, was included during the 16S rRNA gene PCR and the downstream GA-map infant assay. The positive controls were used as a quality control of the labeling reaction and hybridization of the arrays (results not shown).

Design of the GA-map infant assay. The GA-map assay is based on the SNuPE in combination with microarray hybridization (25). An overview of the GA-map principle and considerations in assay design is shown in Fig. 1.

The bacterial strains shown in Table 1 were used for probe validation. For probe construction, we used a combined data set consisting of a total of 3,580 16S rRNA gene sequences (19, 26), in addition to a set of known pathogens.

We used a four-step process in designing the probes. (i) First, we defined a set of target and nontarget groups based on a coordinate classification system (see Fig. S1A in the supplemental material). (ii) The next step was to identify probes that satisfied the criteria of target detection and nontarget exclusion. This was based on combined criteria of hybridization and labeling. All probes were designed with a minimum melting temperature (T_m) of 60°C by the nearest-neighbor method for the target group, while the nontarget group should have a T_m of <30°C or absence of a cytosine as the nucleotide adjacent to the 3′ end of the probe. All probes satisfying the criteria were identified (see Fig. S1B in the supplemental material). (iii) Then, the potential cross-labeling or self-labeling probes were evaluated, in addition to potential cross hybridization on the array (see Fig. S1C in the supplemental material). (iv) Finally, by combining the knowledge about target/nontarget groups and compatibility for each of the probes, final arrays were designed using a hierarchical approach.

The strategy for searching for the most appropriate probe sets is described in detail in the supplemental material.

A universal 16S rRNA gene probe (UNI01) (13) was included in the probe sets to measure the total abundance of bacterial DNA in the sample. One additional probe was added in the hybridization step: a 1:4 mixture of prelabeled and unlabeled hybridization control probe (HYC01). HYC01 is used to measure the efficiency of the hybridization step on the slide and to normalize the probe signals between slides. The microarrays used in the GA-map infant assay were superaldehyde slides produced by ArrayIt (Sunnyvale, CA) spotted as described on the company's homepage. One glass slide contains 24 separate identical microarrays, and the probes (complementary to the probes listed in Table 2) were spotted in triplicate on each array. Furthermore, the arrays also included two nonbinding control probes (NBC01 and NBC02) (28). An overview of the control probes found on the array and their sequences is shown in Table S3 in the supplemental material.

GA-map infant assay. Before the labeling reaction, the 16S rRNA gene PCR products (amplified as described above) were treated with 3 U exonuclease I (New England BioLabs, Ipswich, MA) and 8 U shrimp alkaline phosphatase (USB, Cleveland, OH) at 37°C for 2 h and inactivated at 80°C for 15 min. The exonuclease I-shrimp alkaline phosphatase (ExoSAP)-treated PCR products were then quantified using Kodak molecular imaging software (version 4.0) based on pictures from gel electrophoresis. A 1-kb DNA ladder (N3232; New

TABLE 1. Bacterial strains used for probe evaluation

Class	Species	Strain	Accession no.
Actinobacteria	*Bifidobacterium breve*	DSM20213	HQ012023
	Bifidobacterium longum subsp. *infantis*	DSM20088	HQ012021
	Bifidobacterium longum subsp. *longum*	DSM20219	HQ012022
Bacteroidetes	*Bacteroides dorei*	DSM17855	HQ012025
	Bacteroides fragilis	DSM2151	HQ012027
	Bacteroides thetaiotaomicron	DSM2079	HQ012026
	Bacteroides vulgatus	DSM1447	HQ012024
	Parabacteroides distasonis	DSM 20701	NA
Firmicutes	*Clostridium perfringens*	DSM756	HQ012013
	Clostridium ramosum	DSM1402	HQ012012
	Enterococcus faecalis	DSM20478	HQ012029
	Enterococcus faecium	DSM20477	HQ012007
	Lactobacillus acidophilus	DSM20079	HQ012028
	Lactobacillus rhamnosus	DSM20021	HQ012008
	Listeria monocytogenes	DSM20600	HQ012006
	Staphylococcus aureus subsp. *aureus*	DSM20231	HQ012011
	Streptococcus pneumoniae	DSM20566	HQ012009
	Streptococcus pyogenes	DSM20565	HQ012030
	Streptococcus sanguinis	DSM20567	HQ012010
	Veillonella atypical	DSM20739	HQ012015
	Veillonella dispar	DSM20735	HQ012014
Proteobacteria	*Escherichia coli*	DSM30083	HQ012019
	Haemophilus parainfluenzae	DSM8978	HQ012020
	Klebsiella pneumoniae subsp. *pneumoniae*	DSM30104	HQ012018
	Salmonella bongori	DSM13772	HQ012016
	Salmonella enterica subsp. *enterica*	DSM17058	HQ012017

TABLE 2. Probes included in probe set 3

Probe identifier	Taxonomic group(s) detected	Probe sequence	% False positive/% false negative[a]	Mean correct signal[a]	Standard deviation correct signal[a]
1_1	*Bacteroides*	TTGCGGCTCAACCGTAAAATTG	0/0	1,723.54	245.51
1_1_3	*Parabacteroides*	CGCCTGCCTCAAACATA	0/0	733.62	NA
1_2_2	*Bacteroides* (*dorei*, *fragilis*, *thetaiotaomicron*, *vulgatus*)	GCACTCAAGACATCCAGTATCA ACTG	0/0	1,261.71	435.04
1_3_3	*Bacteroides* (*dorei*, *fragilis*, *thetaiotaomicron*, *vulgatus*)	AGGGCAGTCATCCTTCACG	0/0	1,157.96	391.09
2_1_min1b	*Gamma-proteobacteria*	CAGGTGTAGCGGTGAAATGCGTA GAGAT	14/0	1,711.24	201.24
2_1_1	*Haemophilus*	ACGCTCGCACC	0/0	270.16	NA
2_3_2	Gamma-proteobacteria subgroup	CGGGGATTTCACATCTGA	8/0	141.42	NA
2_4_1	Gamma-proteobacteria subgroup	TGCCAGTTTCGAATGCAGTT	4/0	1,677.81	251.28
2_5_1	Gamma-proteobacteria subgroup	GTGCTTCTTCTGCGGGTAA	0/0	611.51	155.12
2_7_1	*Salmonella*	TGTTGTGGTTAATAACCGCAGCAA TTGA	4/0	1,527.71	NA
3_2	*Proteobacteria*	ACGCTTGCACCCT	5/0	809.64	278.90
4_1	*Firmicutes* (*Lactobacillales*, *Clostridium perfringens*, *Staphylococcus*)	CGATCCGAAAACCTTCTTCACT	6/0	1,799.51	538.14
4_2_3	*Lactobacillus* subgroup	GCTACACATGGAGTTCCA	29/0	278.64	14.67
4_3_1	*Clostridium ramosum*	CCGTCACTCGGCTACCATTTC	0/0	2,429.10	NA
4_4_2	*Enterococcus*, *Listeria*	TCCAATGACCCTCCC	0/0	640.06	125.05
4_5_2	*Streptococcus pyogenes*	GATTTTCCACTCCCACCAT	0/0	1,556.65	NA
4_6_1	*Streptococcus sanguinis*	CACTCTCACACCCGTT	0/0	978.28	NA
4_7_2	*Listeria*	CCGTCAAGGGACAAG	0/0	678.60	NA
4_8_1	*Streptococcus pneumoniae*, *Enterococcus*	GTTGCTCGGTCAGACTT	12/0	1,593.28	NA
5_1	*Firmicutes* (*Clostridia*, *Bacillales*, *Enterococcus*, *Lactobacillus*)	GGACAACGCTTGCCAC	6/0	1,315.09	417.36
5_1_2	*Staphylococcus*	CGTGGCTTTCTGATTAGGTA	0/0	654.06	NA
5_2_1	*Clostridium neonatale*	CGTAGTTAGCCGTGG	0/0	0.00	0.00
6_1_4	*Bifidobacterium longum*	TGCTTATTCAACGGGTAAACT	0/0	2,071.50	492.05
6_2	*Actinobacteria*	CGTAGGCGGTTCGTCGCGT	0/0	1,417.55	243.38
6_2_2	*Bifidobacterium breve*	CGGTGCTTATTCGAAAGGTACACT	0/0	1,928.16	NA
UNI01	16S Universal	CGTATTACCGCGGCTGCTGGCA	NA	NA	NA
HYC01	Hybridization control	GTAGCATTCGATTCGGGCAA	NA	NA	NA

[a] NA, not applicable because the probe has only one control target bacterium.

England BioLabs) with specified concentrations was included on all gels. Based on the quantification from the gel images, the PCR products were diluted to equal concentrations of 50 ng/μl/sample, and approximately 100 ng template was used in the following labeling reaction mixture: in a total reaction volume of 10 μl, 2.5 U Hot TermiPol (Solis Biodyne), 1× buffer C (Solis Biodyne), 4 mM $MgCl_2$ (Solis Biodyne), 0.4 μM ddCTP-TAMRA (6-carboxytetramethylrhodamine) (Jena Bioscience, Jena, Germany) and 2.9 μM probe set 3 (Table 2). The labeling protocol included a 12-min activation stage at 95°C, followed by 10 cycles with 20 s denaturation at 96°C and 35 s combined annealing and extension at 60°C. The number of cycles used was a tradeoff between sensitivity and saturation for high-concentration targets.

The arrays were prehybridized to prevent background signal by soaking the glass slides in BlockIt (ArrayIt) at room temperature. After 2 h, the slides were washed for 2 min in a wash buffer containing 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate; Sigma-Aldrich, St. Louis, MO) plus 0.1% Sarkosyl (room temperature [RT]; VWR International, Ltd., Poole, United Kingdom) and then for 2 min in 2× SSC (Sigma-Aldrich). The slides were then placed in a beaker with ultrapure H_2O (100°C) for 2 min and immediately transferred to a beaker containing 100% ethanol (−20°C) for 20 s before they were dried by centrifugation at 91 × *g* in a Multifuge 3 S-R centrifuge (Heraeus, Buckinghamshire, United Kingdom) for 12 min and used within an hour.

Immediately prior to the actual array hybridization, 60 μl hybridization buffer containing 7.2% polyethylene glycol 8000 (Sigma-Aldrich), 1.2× SSC (Sigma-Aldrich), and 0.17 μM hybridization control probe HYC01 mixture (a 1:4 mixture of TAMRA-labeled HYC01 and unlabeled HYC01) were added to the samples. The samples were denatured at 95°C for 2 min and then left at 45°C for 2 min. The glass slides were placed in a 96-well hybridization chamber (ArrayIt) before the samples were loaded onto the arrays. Two arrays per slide were used for the positive- and negative-control samples. The hybridization chamber was placed in a humid chamber and hybridized for 16 h in an Innova 4000 incubator shaker (New Brunswick Scientific, Champaign, IL) at 45°C and 60 rpm.

After hybridization, the arrays were washed for 5 min in the wash buffer containing 2× SSC (Sigma-Aldrich) and 0.1% Sarkosyl (VWR International, Ltd.), then for 5 min in 2× SSC (Sigma-Aldrich), and finally for 10 s in 0.2× SSC (Sigma-Aldrich) before they were dried by centrifugation at 91 × *g* for 12 min in a Multifuge 3 S-R centrifuge (Heraeus). The hybridized arrays were scanned at a wavelength of 532 nm with a Tecan LS reloaded scanner (Tecan, Männedorf, Austria). Fluorescence intensities and spot morphologies were analyzed using Axon GenePix Pro 6.0. Pictures of two example arrays can be seen in Fig. S3 in the supplemental material.

Capillary electrophoresis. The GA-map labeling step was evaluated by capillary electrophoresis. To test the labeling, single probes were tested against their target bacteria (DNA from pure cultures and a complementary synthetic template with five additional nucleotides in both the 5′ and 3′ ends if a pure culture was lacking) by performing 16S rRNA gene PCR amplification for the pure DNA and labeling reactions as described above (with 1 μM single probes instead of probe set 3, which was used in the final assay), and the performances of the probes were evaluated using capillary electrophoresis. The compatibility of different sets of functioning probes (see Table S2 in the supplemental material) was also evaluated using capillary electrophoresis with water as the template and different probes sets (see Table S2 in the supplemental material) instead of probe set 3 in the labeling reaction described above. Furthermore, the reproducibility of the 16S rRNA gene PCR was examined on one of the samples (amplified in three separate PCRs) using capillary electrophoresis. Two probes (6_1_4 and 5_1_2) were chosen to examine the signal for each of the three PCR products, and a triplicate run on a pool of the three PCR products was also examined using

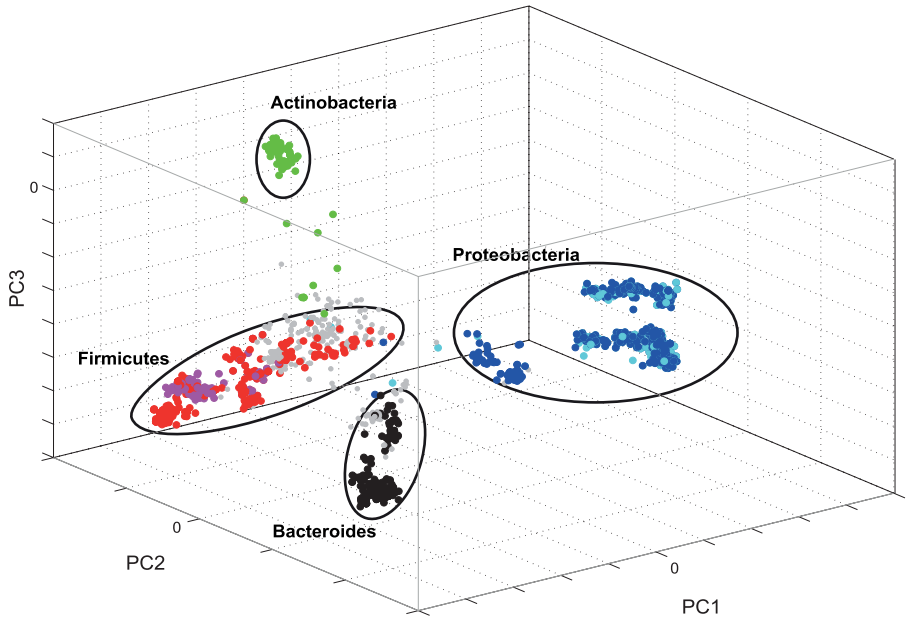

FIG. 2. Score plot of bacteria isolated from infant feces. Each object represents one bacterial clone. The objects are clustered based on 16S rRNA gene phylogeny. The colors indicate theoretical phylum probe specificities: 6_2, green; 5_1, red; 4_1, magenta; 2_1_min1b, cyan; 3_2, blue; and 1_1, black.The gray objects are not detected by any probes.

the same probes (see Fig. S2 in the supplemental material). After labeling, the samples were treated with 8 U SAP (USB), incubated at 37°C for 1 h, and inactivated at 80°C for 15 min. Then, 1 μl of the SAP-treated and labeled probes was mixed with 9 μl of Hi-Di formamide (Applied Biosystems, Warrington, United Kingdom) and 0.5 μl GeneScan 120 Liz Size Standard (Applied Biosystems), and the samples were incubated at 95°C for 5 min and immediately put on ice. The samples were then loaded onto a 50-cm 3130xl capillary array (Applied Biosystems) in the ABI Genetic Analyzer 3130xl sequencer (Applied Biosystems) containing the performance-optimized polymer 7 (POP-7; Applied Biosystems). The injection time was 16 to 22 s, and the electrophoretic conditions were as follows: run time, 1,500 s at 15,000 V; run current, 100 μA; run temperature, 60°C. GeneMapper 4.0 software was used to analyze the results.

DNA sequence analysis. The 16S rRNA gene PCR products from the 26 bacterial strains used to evaluate the probes were sequenced to confirm their identities and to examine if there were any mutations in their gene sequences compared to the sequences used to design the probes. The ExoSAP-treated PCR products were diluted 10-fold, and 1 μl was used in the sequencing reaction using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). The same forward and reverse primers used for the 16S rRNA PCR described above (0.32 μM) were used in two separate sequencing reactions. A BigDye XTerminator Purification Kit (Applied Biosystems. Warrington, United Kingdom) was used according to the manufacturer's recommendations to clean up the sequencing reactions. The samples were analyzed on a 36-cm 3130xl capillary array (Applied Biosystems) in the ABI Genetic Analyzer 3130xl sequencer (Ap-

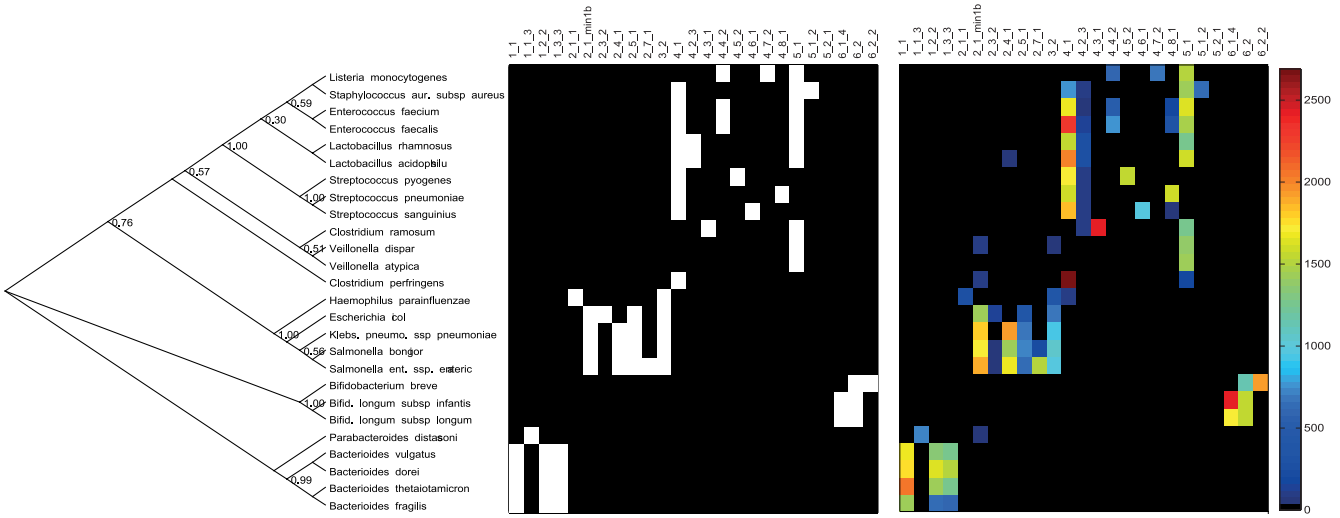

FIG. 3. Strain classification by GA-map infant array. (Left) Phylogenetic tree of all bacterial strains used to investigate probe accuracy and sensitivity. The numbers at the nodes indicate bootstrap support. (Middle) The theoretical result for the array experiment, where the classification as nontarget and target is given as a color code from black to white. (Right) Experimental results with signal intensities color coded as shown in the color bar at the right of the image. The numbering of probes is based on an in-house classification system (Table 2).

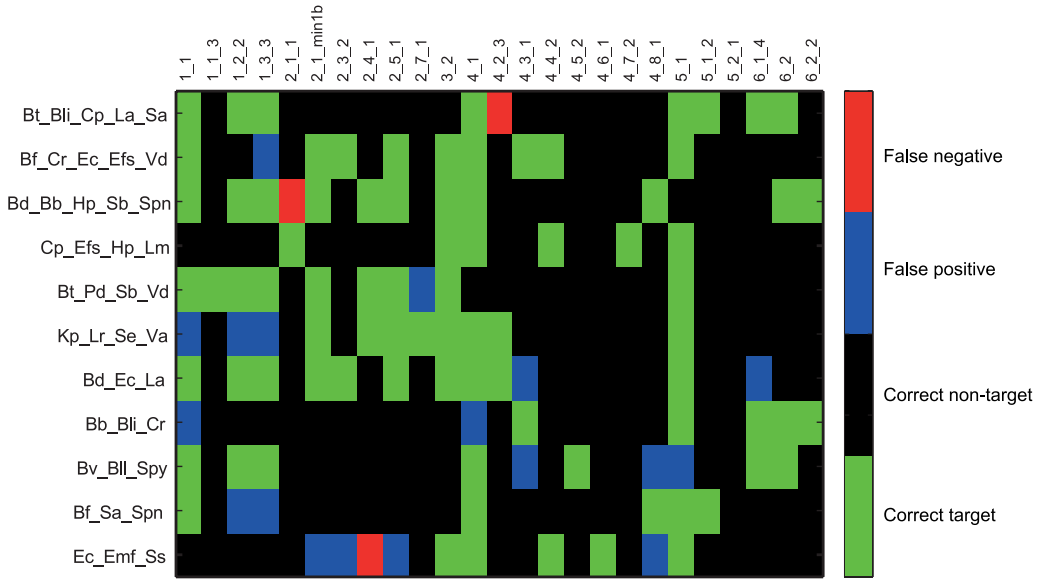

FIG. 4. Classification of mixed samples by the GA-map infant array. Signals from defined one-to-one mixtures of bacteria were evaluated. The following abbreviations were used for the bacterial species in the sample mixtures: Bd, *Bacteroides dorei*; Bf, *Bacteroides fragilis*; Bt, *Bacteroides thetaiotaomicron*; Bv, *Bacteroides vulgatus*; Bb, *Bifidobacterium breve*; Bli, *Bifidobacterium longum* subsp. *infantis*; Bll, *Bifidobacterium longum* subsp. *longum*; Cp, *Clostridium perfringens*; Cr, *Clostridium ramosum*; Efs, *Enterococcus faecalis*; Efm, *Enterococcus faecium*; Ec, *Escherichia coli*; Hp, *Haemophilus parainfluenzae*; Kp, *Klebsiella pneumoniae* subsp. *pneumoniae*; La, *Lactobacillus acidophilus*; Lr, *Lactobacillus rhamnosus*; Lm, *Listeria monocytogenes*; Pd, *Parabacteroides distasonis*; Sb, *Salmonella bongori*; Se, *Salmonella enterica* subsp. *enterica*; Sa, *Staphylococcus aureus* subsp. *aureus*; Spn, *Streptococcus pneumoniae*; Spy, *Streptococcus pyogenes*; Ss, *Streptococcus sanguinis*; Va, *Veillonella atypica*; Vd, *Veillonella dispar.*

plied Biosystems) containing the performance-optimized POP-7 (Applied Biosystems). The injection time was 3 s, and the electrophoretic conditions were as follows: run time, 2,780 s at 8,500 V; run current, 5.0 μA; run temperature, 60°C. The sequences were base called by Sequence Scanner Software v1.0 (Applied Biosystems).

The sequences were aligned, and a bootstrapped neighbor-joining tree of all 26 bacterial strains used to evaluate the probes was constructed using the program Mega 4 with default settings (32).

Data preprocessing and analysis. The probe signals were corrected for undesired hybridization variations that are observed from slide to slide. In each experiment, a probe that is already labeled (HYC01) is added to the probe mixture to evaluate the hybridization step and to normalize differences in hybridization efficiencies. To correct for varying hybridization between slides, we divide all sample signals by the average signal of all replicas from the probe. In addition, self-labeling and/or cross-labeling from each individual probe was removed by subtracting the average signal from a nontemplate control sample included on all slides used in the experiment. Finally, the nonbinding control probes NBC01 and NBC02 were used to evaluate cross-hybridization.

Statistical analyses. The probe specificity was evaluated by comparing the theoretical target/nontarget values with the experimental results on single strains, using an empirically determined background signal threshold value of 50.

Microarray data usually contain both threshold and saturation values and are therefore very seldom normally distributed. Thus, in order to test the significance of microarray data, it is common to use permutation-based approaches instead of standard statistical tests, such as analysis of variance (ANOVA) and *t* tests, which require normal distribution. Permutation testing is an exact statistical test, even for data with a complex distribution structure (6). Hence, the *P* values for group differences within each age category were calculated by permutation testing (14), using 50 as the background threshold value.

Nucleotide sequence accession numbers. The sequences for the bacterial strains have been deposited in GenBank, and the strains' respective accession numbers are listed in Table 1.

RESULTS

Probe construction and evaluation. A set of 88 probes was constructed based on the criteria described in Materials and Methods. Six probes for the main phyla covered 88% of the clones in our evaluated data set, as illustrated in Fig. 2, indicating that the majority of the bacteria expected in the human gut can be covered by broad-range probes. Single-probe evaluations of the 88 probes using capillary gel electrophoresis and the strains in Table 1 (in addition to a synthetic oligonucleotide for probe 5_2_1) as templates showed that 76% of the probes satisfy the criterion of target detection (see Materials and Methods), indicating a relatively high success rate for the probes constructed based on the criteria described in the supplemental material. We identified 10 probe sets among the probes that satisfied the labeling criterion (see Table S2 in the

TABLE 3. Quantification in a mixed species background

Probe identifier[a]	Species[b]	Detection limit[c]	R^{2d}
1_1	*Bacteroides fragillis*	0.01	0.94
2_1_min1b	*Escherichia coli*	0.02	0.93
2_5_1	*Escherichia coli*	0.02	0.95
3_2	*Escherichia coli*	0.01	0.98
4_3_1	*Clostridium ramosum*	0.01	0.96
4_4_2	*Enterococcus faecalis*	0.01	0.84
4_5_2	*Streptococcus pyogenes*	0.01	0.96
5_1_2	*Staphylococcus aureus* subsp. *aureus*	0.01	0.98
6_1_4	*Bifidobacterium longum* subsp. *infantis*	0.01	0.97
6_2_2	*Bifidobacterium breve*	0.01	0.95

[a] Only probes that uniquely detect the respective bacteria are shown.
[b] Bacterial PCR products were subjected to dilution series following the experimental scheme shown in Table S4 in the supplemental material.
[c] The detection limits represent the relative amounts of the respective bacterial PCR products for which two sample *t* tests between two consecutive dilutions showed significance ($P < 0.05$).
[d] R^2, the squared regression coefficient.

TABLE 4. Phylum level differences between sensitized and nonsensitized children

Probe	Taxonomic group	Difference at age (days)[a]:			
		10	120	360	720
1_1	*Bacteroides*	0.640	0.868	1.00	0.903
2_1_min1b	*Gammaproteobacteria*	0.760	0.220	0.801	0.542
3_2	*Proteobacteria*	0.922	0.3126	0.126	0.465
4_1	*Firmicutes* (*Lactobacillales*, *Clostridium perfringens*, *Staphylococcus*)	0.164	0.190	0.360	0.599
5_1	*Firmicutes* (*Clostridium*, *Bacillales*, *Enterococcus*, *Lactobacillus*)	0.486	0.127	**0.049**	0.556
6_2	*Actinobacteria*	0.152	**0.042**	0.196	0.989
UNI01	16S universal	0.450	0.867	0.917	0.216

[a] The significances of differences were determined by permutation testing. Significant differences ($P < 0.05$) are in boldface.

supplemental material) based on a set of bioinformatics criteria (see the supplemental material). Each probe set consisted of 25 probes that were selected based on their *in silico* compatibility with each other. The compatibility estimations were based on melting temperature calculations and the thermodynamics of the probe: self-hybridization and hybridization to other probes in the probe set or their target bacteria as described the supplemental material. Experimental validation by capillary gel electrophoresis showed that probe set 3 gave the lowest cross-labeling, as determined by labeling without template (results not shown). This probe set was therefore selected for array construction (Table 2).

Specificity, reproducibility, and quantitative range of the GA-map infant array. The first evaluation of the array was on pure cultures. The evaluation was based on comparing *in silico*-determined targets/nontargets with experimental signals (Fig. 3).This analysis showed good concordance between the theoretical and experimental probe specificities. Using a signal cutoff value of 50, we found that there were no false negatives, while the numbers of false positives were more variable (Table 2). Probe 4_2_3 showed the highest level, with 29% false-positive signals, while the rest of the probes showed <15% false-positive signals. Unfortunately, we did not have a target bacterium for probe 5_2_1, but what this evaluation shows is that the probe at least does not cross-react with the nontarget bacteria.

The next step in the evaluation was to determine the classification accuracy of mixed samples. This was done by analyzing a set of defined one-to-one mixtures of PCR products from pure bacterial strains. The evaluation of these data showed that the majority of the probes accurately identified their target bacteria (Fig. 4). In total, there were 9.0% false positives and 1.6% false negatives given a background signal threshold of 50. The quantitative range of selected probes was subsequently evaluated by template dilutions in a mixed strain background (see Table S4 in the supplemental material for the experimental setup). These analyses showed quantitative responses for all the probes evaluated (Table 3; see Fig. S4 in the supplemental material). In addition, we evaluated the effect of the total amount of template in the labeling reaction. This evaluation showed that given more than 10 ng of target, the linearity between the template concentration and the signal is lost. We also showed that the smallest amount of template that could be detected was between 0.1 and 0.01 ng (see Table S5 in the supplemental material).

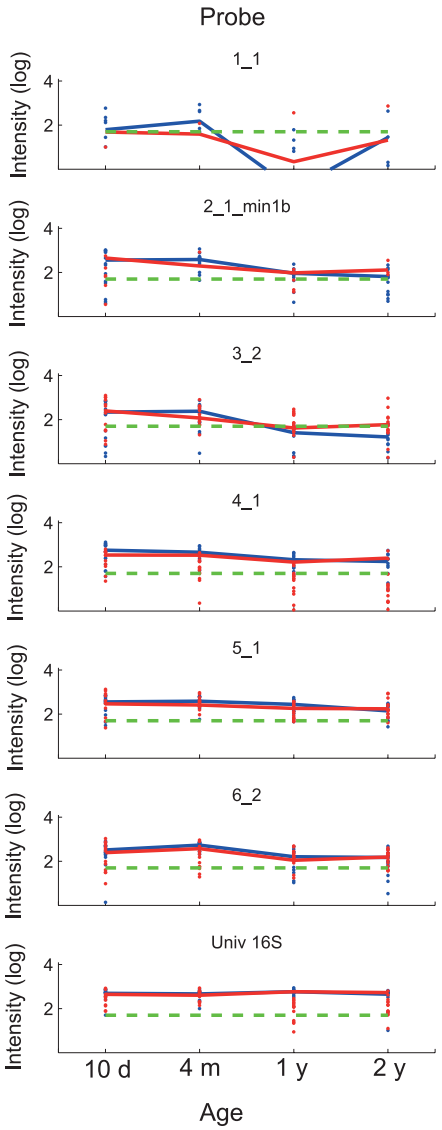

FIG. 5. Temporal development of bacterial phyla in sensitized and nonsensitized infants. Each panel shows the temporal development of probe signals within the study population for the respective probe. The log average signal for each probe is shown as a line, while the log signals of all time points measured are shown as dots (levels above a signal threshold of 50, denoted by dashed green lines). The blue lines and dots represent sensitized children ($n = 16$), while the red lines and dots represent nonsensitized children ($n = 16$). Values of <0 were set to 0.001 before log transformation. d, day; m, month; y, year.

TABLE 5. Genus/species differences between sensitized and nonsensitized children

Probe	Taxonomic group	Difference at age (days)[a]:			
		10	120	360	720
1_1_3	*Parabacteroides*	1	0.866	1.000	1.000
1_2_2	*Bacteroides* (*B. dorei*, *B. fragilis*, *B. thetaiotaomicron*, *B. vulgatus*)	1	0.884	1.000	1.000
1_3_3	*Bacteroides* (*B. dorei*, *B. fragilis*, *B. thetaiotaomicron*, *B. vulgatus*)	0.756	0.488	0.206	0.741
2_1_1	*Haemophilus*	0.783	1.000	1.000	1.000
2_3_2	*Gammaproteobacteria* subgroup	0.668	0.347	1.000	0.494
2_4_1	*Gammaproteobacteria* subgroup	0.182	0.622	1.000	1.000
2_5_1	*Gammaproteobacteria* subgroup	0.695	0.913	0.870	0.949
2_7_1	*Salmonella*	0.754	1.000	1.000	1.000
4_2_3	*Lactobacillus* subgroup	0.938	0.909	1.000	0.405
4_3_1	*Clostridium ramosum*	0.786	0.765	0.828	0.537
4_4_2	*Enterococcus*, *Listeria*	0.9736	**0.020**	1.000	1.000
4_6_1	*Streptococcus sanguinis*	1.000	1.000	**0.038**	0.689
4_8_1	*Streptococcus pneumoniae*, *Enterococcus*	*0.084*	0.169	1.000	0.935
5_1_2	*Staphylococcus*	0.847	1.000	1.000	0.399
6_1_4	*Bifidobacterium longum*	*0.097*	*0.066*	**0.016**	0.837
6_2_2	*Bifidobacterium breve*	0.711	0.679	0.844	0.784

[a] The significances of differences were determined by permutation testing. Significant differences ($P < 0.05$) are in boldface, while differences in the range $0.05 < P < 0.1$ are italicized.

The reproducibility of the assay was evaluated by duplicate analyses of 43 samples.

The mean percent variation and R^2 for each probe were evaluated individually (see Table S1 in the supplemental material). These results confirmed the reproducibility of the assay with relatively high R^2 values and low mean percent variation. Furthermore, the repeated analyses from the same fecal shedding showed R^2 values of >0.93 for all pairwise comparisons of probe signal intensities. This indicates that the microbiota is homogeneous among the different samples and that the sample preparation does not introduce a large amount of variance.

Finally, we compared GA-map infant array data for *Bifidobacterium breve* (probe 6_2_2) and *Bifidobacterium longum* (probe 6_1_4) to previously generated qPCR results (Storrø et al., unpublished). There was relatively high correlation for all age groups for the *B. longum* subsp. *longum*/*B. longum* subsp. *infantis* group ($R^2 = 0.42$; $n = 159$), while for *B. breve*, the correlation between qPCR and the array was age dependent. For the 10-day age category, the correlation was relatively high ($R^2 = 0.45$; $n = 30$), while it was lower for the 4-month-old group ($R^2 = 0.33$; $n = 27$); for the 1-year-old group, it was even lower ($R^2 = 0.20$; $n = 28$), and for the 2-year-old group, there was nearly no correlation ($R^2 = 0.08$; $n = 32$).

Phylum level development of the gut microbiota. We found that *Actinobacteria* (probe 6_2) and *Firmicutes* (probe 5_1) were significantly overrepresented at 4 months and 1 year, respectively, in the IgE-sensitized children (Table 4 and Fig. 5). There was also an overall consistent age-specific colonization pattern at the phylum level, irrespective of the sensitization state. The general pattern was an initial dominance of *Firmicutes* and *Proteobacteria* at 10 days. At 4 months, the *Proteobacteria*/*Firmicutes* dominance was replaced with *Bacteroides*/*Actinobacteria*, while after 1 and 2 years, the initially colonizing phyla were apparently becoming low in abundance.

Genus and species level development of the gut microbiota. The main difference between the sensitized and nonsensitized groups was that *B. longum* (probe 6_1_4) was significantly overrepresented in the sensitized group compared to the nonsensitized group at 1 year. We also found that *Enterococcus* (probe 4_4_2) was significantly overrepresented at 4 months. It also seems that streptococci are associated with sensitization, with *Streptococcus sanguinis* (probe 4_6_1) being significantly overrepresented at 1 year and *Streptococcus pneumoniae* (probe 4_8_1) at the border of significance at 10 days (Table 5 and Fig. 6).

The bacterial groups with the most consistent colonization patterns correlating with age were *Staphylococcus* (probe 5_1_2) and *B. breve* (probe 6_2_2). *Staphylococcus* dominated initially, while *B. breve* had a dominance peak at 4 months.

DISCUSSION

Major challenges with traditional 16S rRNA gene microarrays are probe specificity and cross-reactivity between closely related species. For microarrays, these challenges have recently been addressed by tiling probes covering the variable region of the 16S rRNA gene (23). The principle of tilling is that a large number of overlapping probes cover the region of interest, with the combined probe signals providing a relatively good signal-to-noise ratio. However, to our knowledge, no other array approaches have yet demonstrated quantitative differentiation of the microbiota based on point mutations.

With the SNuPE-based GA-map assay, we obtained high specificity and sensitivity with only a few single-nucleotide differences targeting probes. The obvious benefit of this is that the assay enables high-throughput applications due to reduced complexity. Few well-defined polymorphic sites also allow easier validation of target and nontarget bacteria. A requirement of SNuPE arrays, however, is that the polymorphic sites targeted must be very well characterized to cover the phylogenetic groups of interest. A further challenge with SNuPE arrays is that all factors affecting labeling are not yet completely known. This is illustrated with probe 4_2_3, which cross-reacted with a range of theoretical nontarget bacteria.

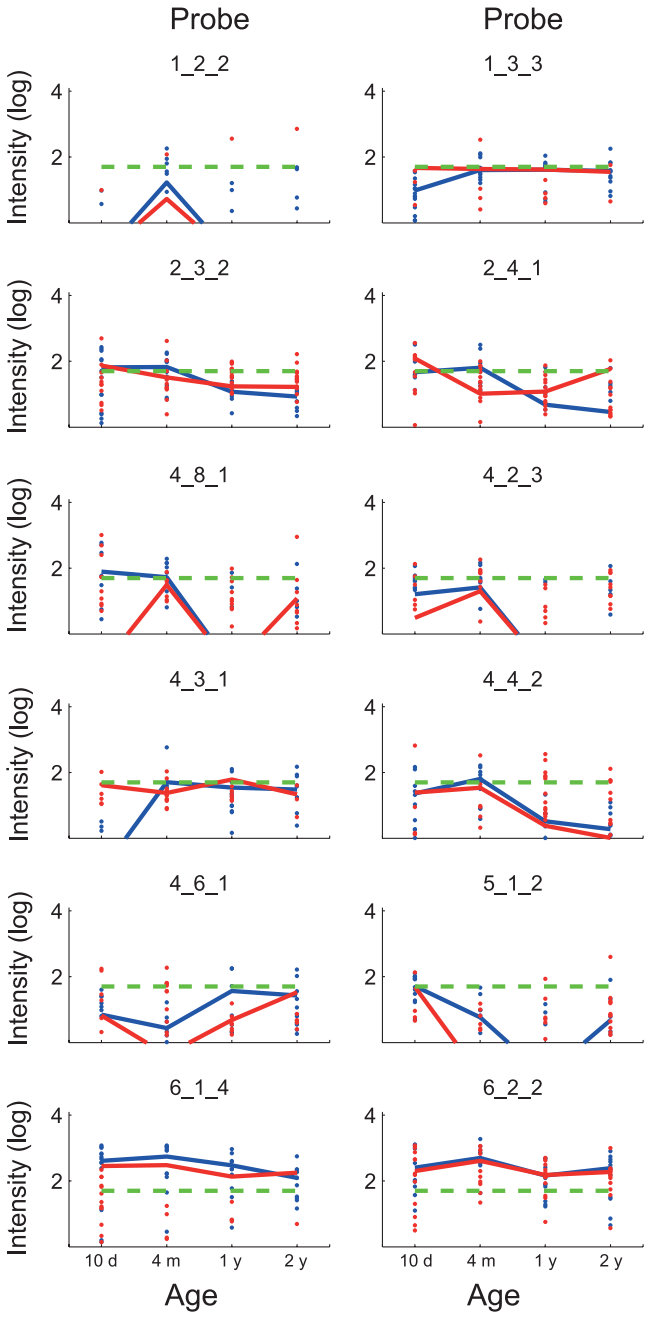

FIG. 6. Temporal development of bacterial genera/species in sensitized and nonsensitized infants. Each panel shows the temporal development of probe signals within the study population for the respective probe. The log average signal for each probe is shown as a line, while the log signals of all time points measured are shown as dots (levels above a signal threshold of 50, denoted by dashed green lines). The blue lines and dots represent sensitized children ($n = 16$), while the red lines and dots represent nonsensitized children ($n = 16$). Values of <0 were set to 0.001 before log transformation. Probes for which the signals for all the samples were below the threshold are not shown.

Not only is the specificity of the assays for microbiota characterization important, but also the quantitative properties. Since SNuPE assays include linear amplification, the quantitative range is limited by label saturation for highly abundant phylogroups, while the detection of low-abundance phylotypes is limited by the sensitivity of the assay. We designed our SNuPE assay to quantify bacteria in the range down to 1% of the total microbiota. This choice was a trade-off between sensitivity and the ability to quantify the dominant species. In the linear range, we found the quantitative properties of our assay were very good ($R^2 > 0.9$). We also found a relatively good correlation with that of qPCR. These comparisons, however, are challenging, due to differences in both the phylogenetic widths and the quantitative ranges of the assays. E.g., the age-dependent reduction in correlation for *B. breve* between qPCR and the SNuPE array suggests that the phylogenetic widths are different in the two assays. Although our assay does not have a linear dose response for high-abundance taxa, the reproducibility between parallel samples was very high, suggesting that the main quantitative information is captured in the GA-map assay. Finally, as for most 16S rRNA gene microarray approaches, the broad-range PCR amplification can introduce quantification biases (8).

The most surprising biological finding in our data was that *B. longum* was significantly overrepresented in the IgE-sensitized group at 360 days, in addition to low *P* values for 10 days and 120 days. This finding has also been independently confirmed by qPCR for the IM-PACT data (Storrø et al., unpublished). Taken together, the multiple independent observations support the validity of the correlations. The surprise was because most previous work has actually suggested that *B. longum* is protective with respect to sensitization (9, 11, 33). Experiments with mouse models, however, have shown that the time and order of bifidobacterial colonization are important for the immunomodulatory effects (10). This may explain the differences in effects between different studies.

We also found that the *Firmicutes* subgroup containing streptococci and enterococci was significantly overrepresented in the IgE-sensitized group. These correlations, however, need to be verified further due to the possibility of type I errors. Furthermore, relatively little has been described about these bacterial groups with respect to sensitization. However, it has been suggested that *S. pneumoniae* infections can be correlated with increased IgE levels in chronic bronchitis (12). Thus, there could be common underlying mechanisms for the infant and bronchitis sensitizations.

The generally lower levels of most phyla in the nonsensitized group compared to the sensitized group suggests that there are phyla missing in the GA-map infant assay that are negatively correlated with sensitization. There are probably phyla missing in the GA-map assay for the high-age groups. Although the assay was constructed to detect the major phylogroups in a relatively large data set (19, 26), this data set may not completely represent the phylogroups in the IM-PACT cohort. A requirement in order to use targeted microarrays is that the human gut microbiota consists of a limited number of taxa. Recent deep sequencing suggests that this is in fact the case (2). Therefore, it should be possible to develop future assays including all phylogroups expected to colonize the infant gut. Recent extensive in-depth sequencing may help to identify these phylogroups (5).

Since we analyzed the fecal microbiota, our observations may not reflect the bacteria directly interacting with the immune system in the intestine. Neither can we determine from

our data if our observations are a cause or a consequence of the sensitization state. Further experimental documentation is therefore needed to determine the mechanistic nature of the correlations detected. What we have shown, however, is that there is a difference in the fecal microbiota between sensitized and nonsensitized children in the IM-PACT cohort. Furthermore, we have also shown an age-specific colonization pattern, irrespective of the sensitization state.

This study demonstrates the usefulness of the GA-map infant assay in determining variations in the composition of the infant gut microbiota, and we believe that with both future temporal and interactional results from large-scale screenings, several of the apparently controversial issues in the current literature can be resolved and a better understanding of the interaction of the complex gut microbiota can be obtained. Such understanding could lead to early diagnosis of disease and better prophylactic or therapeutic treatments of various gut-related diseases.

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