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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)

Magdy El-Salhy [1,2,3,*], Trygve Hausken [2,3] and Jan Gunnar Hatlebakk [2,3]

1 Section for Gastroenterology, Department of Medicine, Stord Hospital, Box 4000, 54 09 Stord, Norway
2 Department of Clinical Medicine, University of Bergen, 5007 Bergen, Norway; trygve.hausken@helse-bergen.no (T.H.); jan.gunnar.hatlebakk@helse-bergen.no (J.G.H.)
3 National Centre for Functional Gastrointestinal Disorders, 5021 Bergen, Norway
* Correspondence: magdy.el-salhy@helse-fonna.no; Tel.: +47-53491000; Fax: +47-53491001

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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).

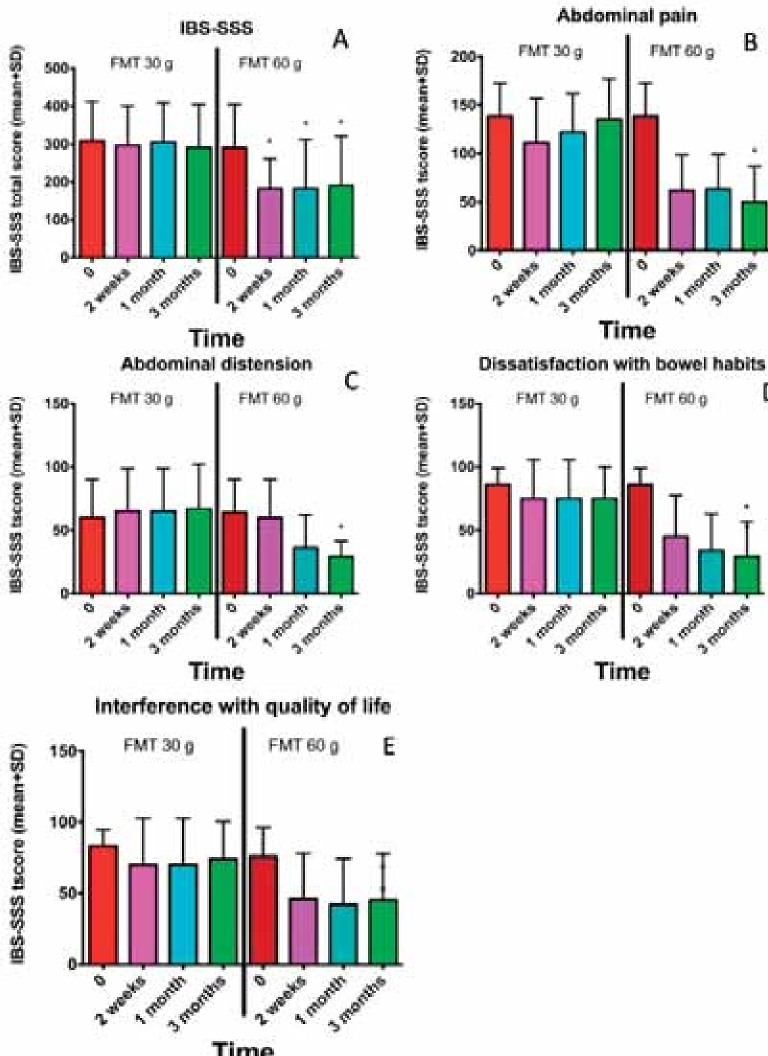

Figure 1. The Irritable Bowel Syndrome Symptom Severity Score (IBS-SSS) at different intervals after receiving 30-g and 60-g transplants for the total score (**A**), abdominal pain (**B**), abdominal distension (**C**), dissatisfaction with bowel habits (**D**) and interference with quality of life (**E**). * $p < 0.05$ compared to baseline. FMT: faecal microbiome transplantation.

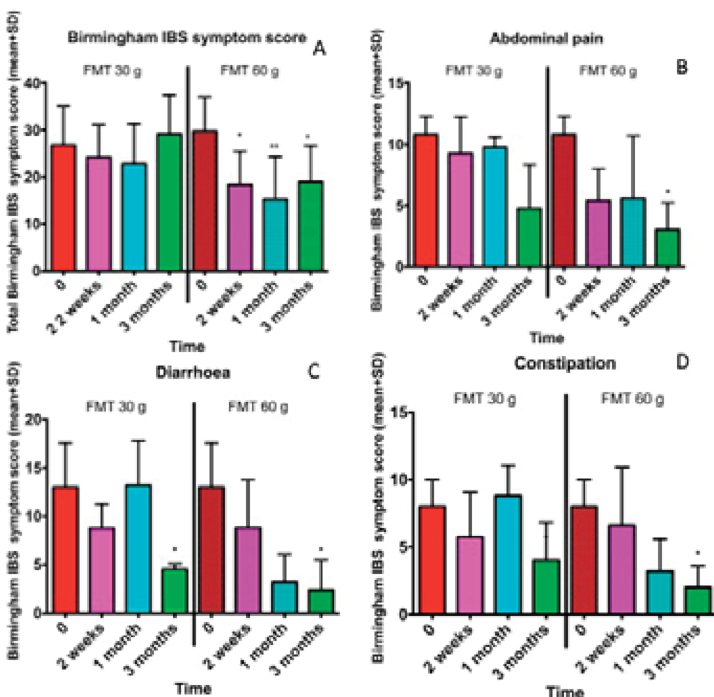

Figure 2. The total Birmingham IBS symptom score and its three items in IBS patients who initially received a 30-g transplant that was subsequently followed by a 60-g transplant for the total score (**A**), abdominal pain (**B**), diarrhoea (**C**) and constipation (**D**). * $p < 0.05$; ** $p < 0.01$ compared to baseline.

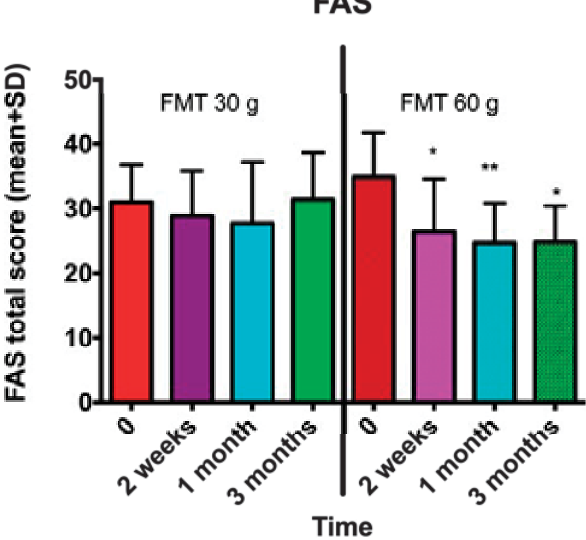

Figure 3. Fatigue as measured by the Fatigue Assessment Scale (FAS) questionnaire was reduced in IBS patients after they received a 60-g transplant but not when they received a 30-g transplant. * $p < 0.05$; ** $p < 0.01$ compared to baseline.

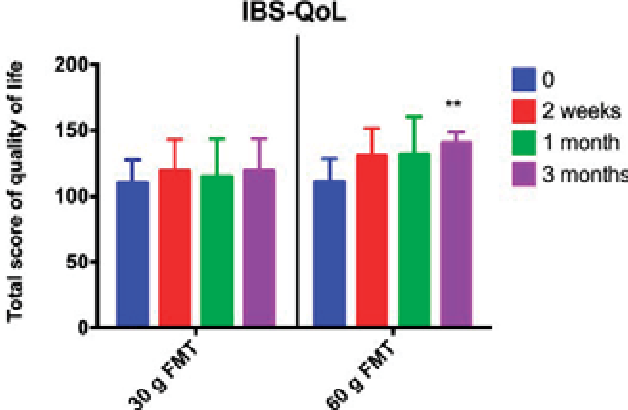

Figure 4. The quality of life as assessed by the IBS Quality of Life (IBS-QoL) questionnaire after FMT with 30-g and 60-g transplants. ** $p < 0.01$ compared to baseline.

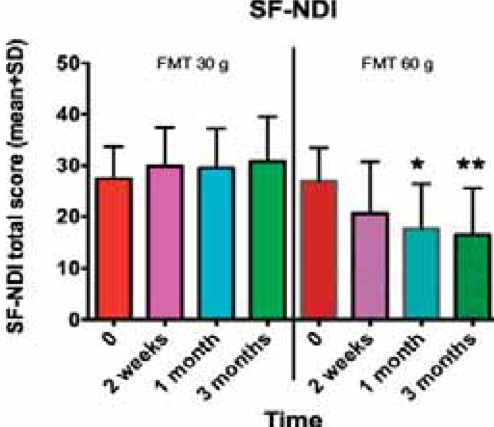

Figure 5. The change in the quality of life as measured by the Short-Form Dyspepsia Index (SF-NDI) questionnaire in IBS patients following FMT with 30-g and 60-g transplants. * $p < 0.05$; ** $p < 0.01$ compared to baseline.

3.2. Dysbiosis Index

The DI decreased significantly following FMT with a 60-g transplant but not with a 30-g transplant (Figure 6).

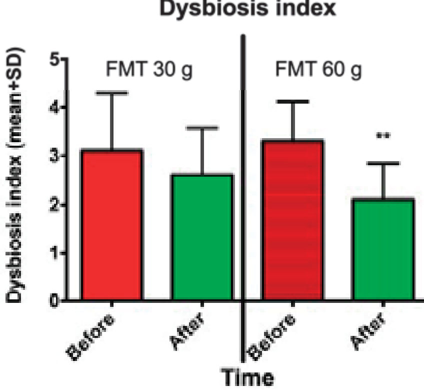

Figure 6. The dysbiosis index (DI) in IBS patients at baseline and 1 month after FMT with 30-g and 60-g transplants. ** $p < 0.01$ compared to baseline.

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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Conflicts of Interest: The authors declare no conflict of interest.

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Research Article

Are Nonnutritive Sweeteners Obesogenic? Associations between Diet, Faecal Microbiota, and Short-Chain Fatty Acids in Morbidly Obese Subjects

Per G. Farup[1,2] **Stian Lydersen**[3] **and Jørgen Valeur**[4]

[1]*Department of Research, Innlandet Hospital Trust, PB 104, N-2381 Brumunddal, Norway*
[2]*Unit for Applied Clinical Research, Department of Clinical and Molecular Medicine, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology, Box 8905, N-7491 Trondheim, Norway*
[3]*Regional Centre for Child and Youth Mental Health and Child Welfare, Department of Mental Health, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology, Box 8905, N-7491 Trondheim, Norway*
[4]*Unger-Vetlesen Institute, Lovisenberg Diaconal Hospital, N-0440 Oslo, Norway*

Correspondence should be addressed to Per G. Farup; per.farup@ntnu.no

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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.

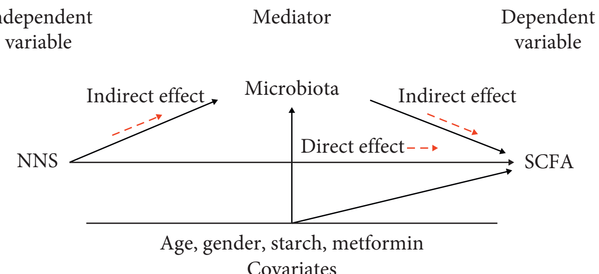

Figure 1: A directed acyclic graph of the direct and indirect effects (mediated via the microbiota) of nonnutritive sweeteners (NNSs) on short-chain fatty acids (SCFA) adjusted for age, gender, and intake of starch and metformin.

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

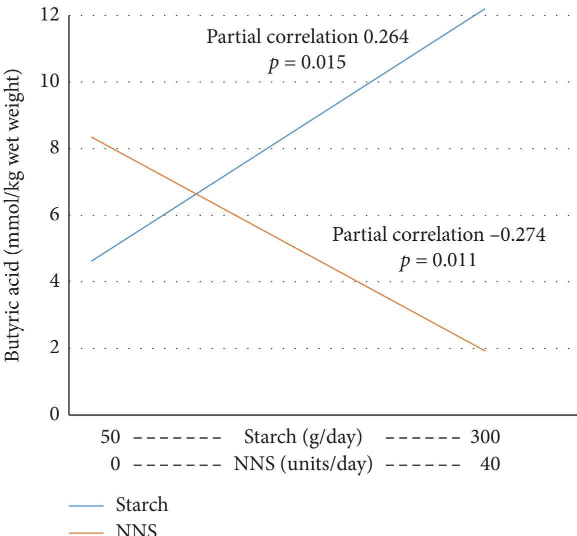

Figure 2: Regression lines for the associations between starch and NNSs (on the x-axis) and butyric acid (on the y-axis) calculated at the mean of the covariates age, gender, use of metformin, and/or NNSs/starch.

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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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

Eva Klingberg[1,2*], Maria K. Magnusson[3], Hans Strid[4,5], Anna Deminger[1], Arne Ståhl[3], Johanna Sundin[3,5], Magnus Simrén[3,6], Hans Carlsten[1], Lena Öhman[3] and Helena Forsblad-d'Elia[1,7]

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

* Correspondence: eva.klingberg@vgregion.se
[1]Department of Rheumatology and Inflammation Research, Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden
[2]Department of Rheumatology, Sahlgrenska University Hospital, Gröna stråket 14, SE-41345 Gothenburg, Sweden
Full list of author information is available at the end of the article

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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 $R2$, 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 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

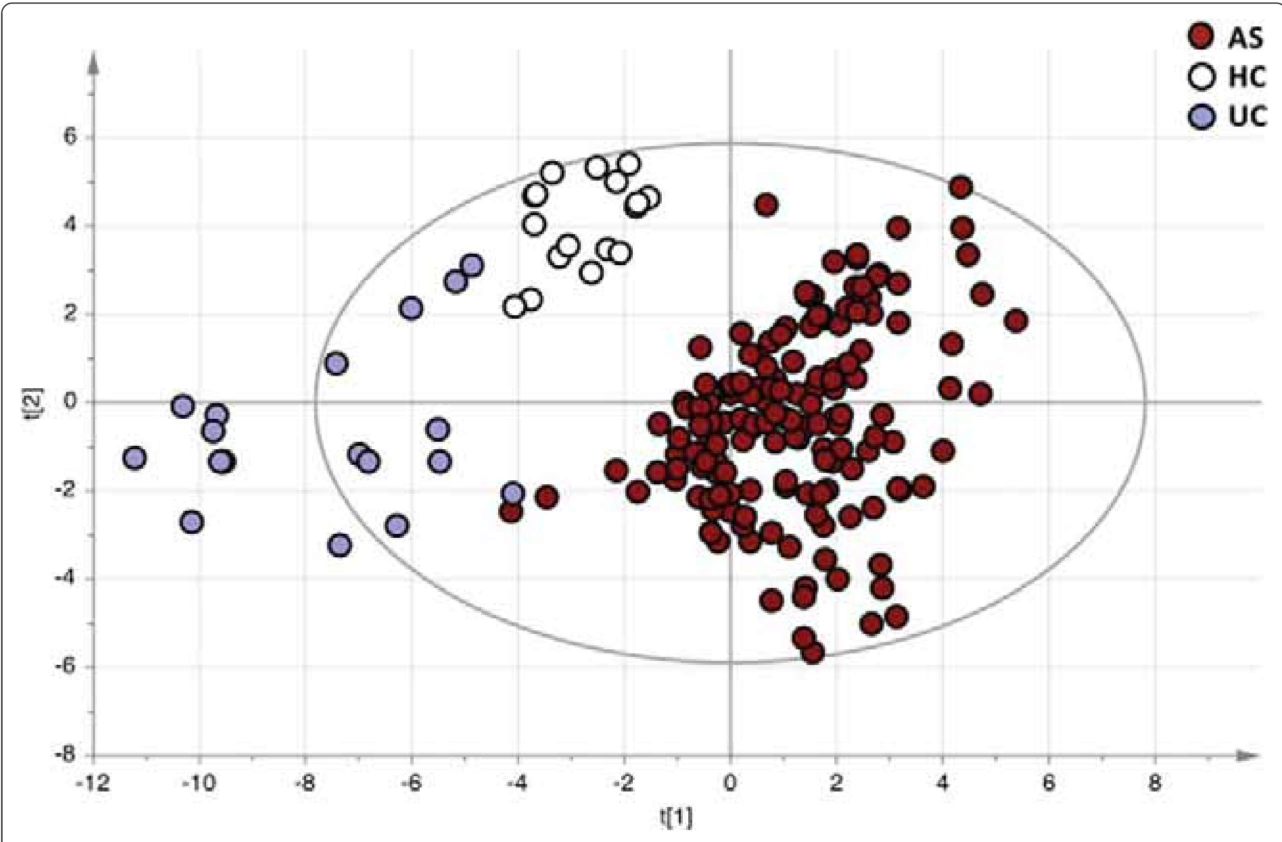

Fig. 1 Fecal microbiota composition in patients with ankylosing spondylitis (AS, n=150), patients with ulcerative colitis (UC, *n* = 18), and healthy controls (HC, *n* = 17) analyzed by the GA-map™ Dysbiosis Test. Total variance of the analyzed 54 bacterial targets are shown in the principal component 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

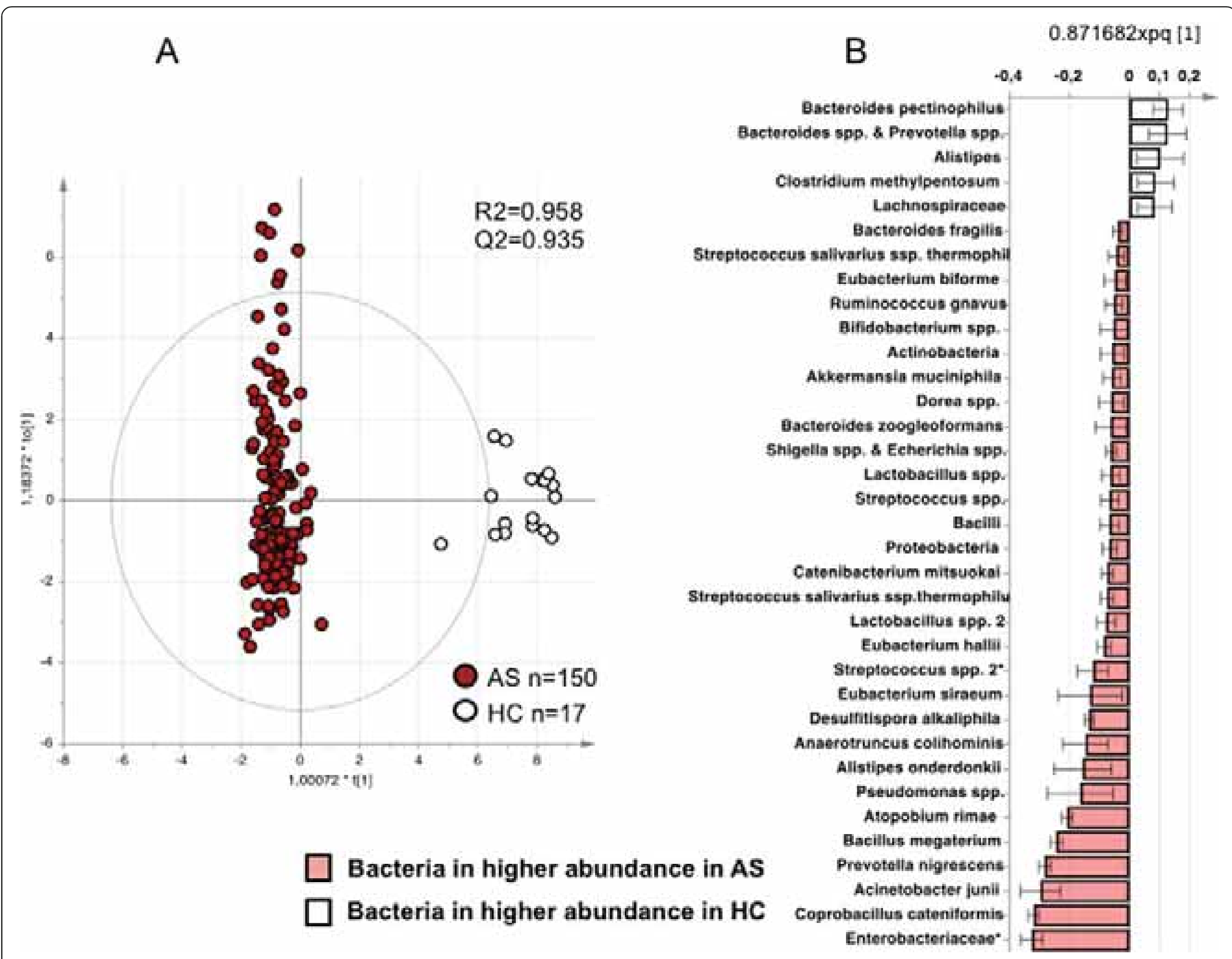

Fig. 2 Comparison of fecal microbiota composition in patients with ankylosing spondylitis (AS) and healthy controls (HC). Fecal samples were analyzed by the GA-map™ Dysbiosis Test and evaluated using orthogonal partial least squares discriminant analysis (OPLS-DA). AS (n = 150) and HC (n = 17). **a** 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

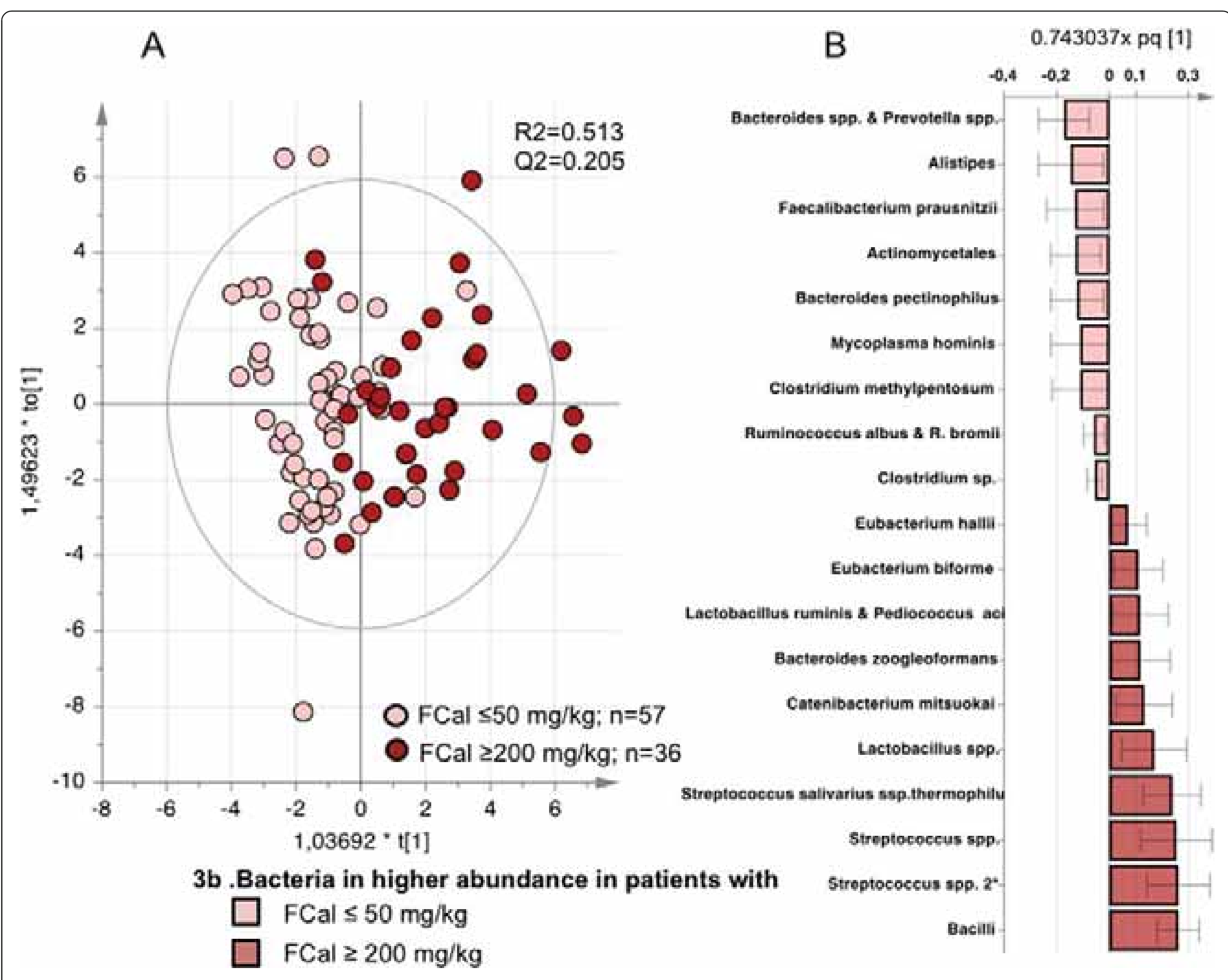

Fig. 3 Fecal microbiota composition in relation to levels of fecal calprotectin. Comparison of the fecal microbiota composition in ankylosing spondylitis (AS) patients with normal (≤ 50 mg/kg, $n = 57$) vs. increased (≥ 200 mg/kg, $n = 36$) fecal calprotectin (Fcal). Orthogonal partial least squares 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*

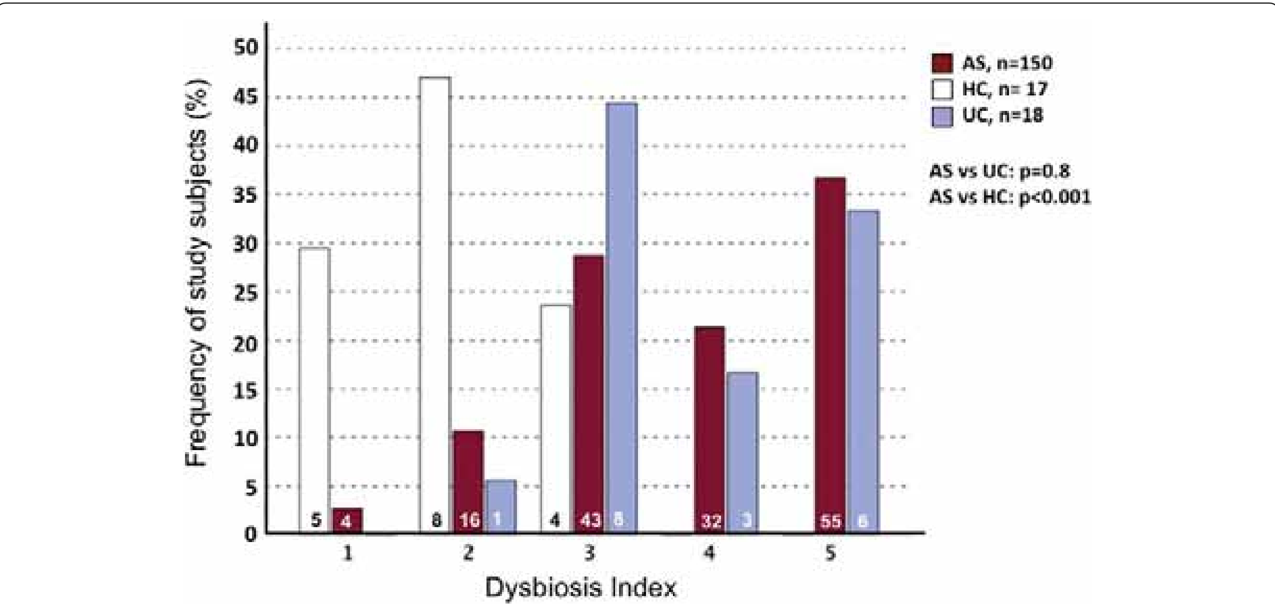

Fig. 4 The distribution of the Dysbiosis Index (DI) score among patients with ankylosing spondylitis (AS, n = 150), patients with ulcerative colitis (UC, n = 18), and healthy controls (HC, n = 17). Fecal samples were analyzed by the GA-map™ Dysbiosis Test. DI is scored between 1 and 5, where a score 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

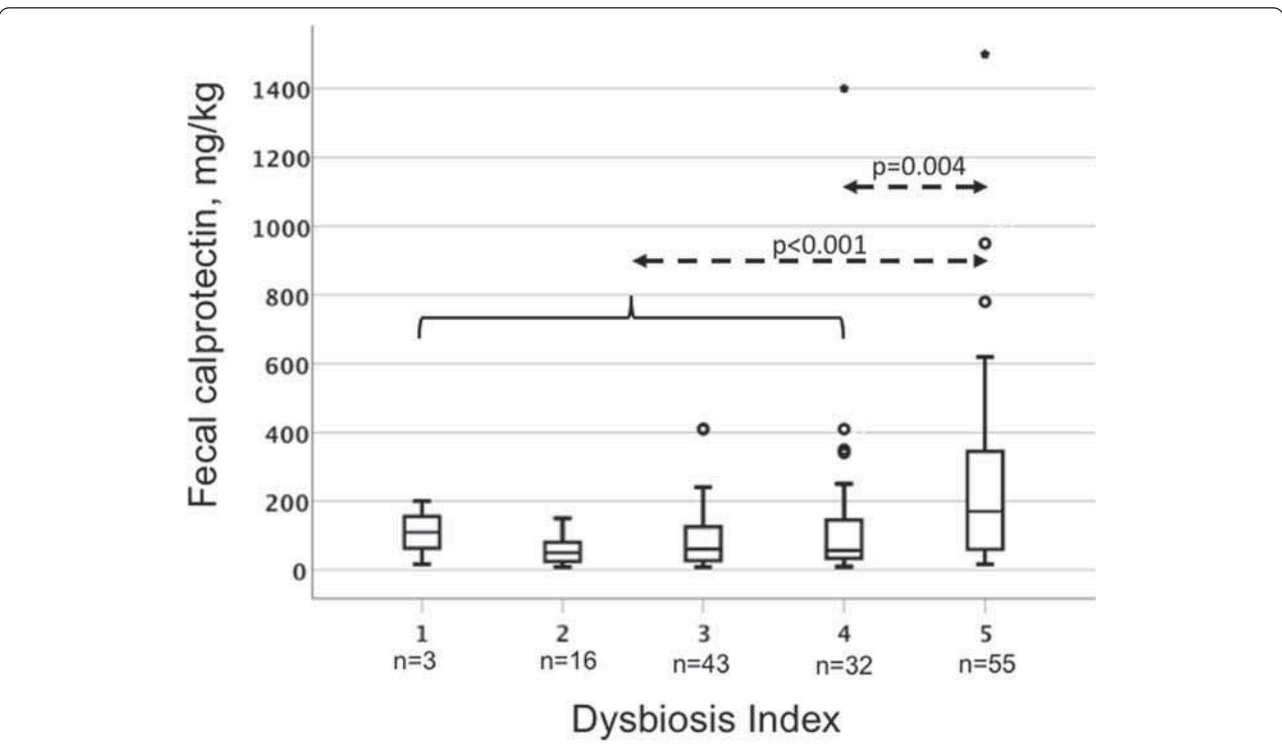

Fig. 5 Fecal calprotectin (Fcal) in relation to dysbiosis index (DI) in patients with ankylosing spondylitis (AS). Boxplots represent the Fcal concentration within each DI score. Values are the medians (horizontal line), interquartile range (box), and range (whiskers). Outliers: circles show cases with 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.

Author details

[1]Department of Rheumatology and Inflammation Research, Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden. [2]Department of Rheumatology, Sahlgrenska University Hospital, Gröna stråket 14, SE-41345 Gothenburg, Sweden. [3]Department of Microbiology and Immunology, Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden. [4]Department of Internal Medicine, Södra Älvsborgs sjukhus, Borås, Sweden. [5]Department of Internal Medicine and Clinical Nutrition, Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden. [6]Center for Functional Gastrointestinal and Motility Disorders, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA. [7]Department of Public Health and Clinical Medicine, Rheumatology, Umeå University, Umeå, Sweden.

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ORIGINAL RESEARCH

Efficacy of faecal microbiota transplantation for patients with irritable bowel syndrome in a randomised, double-blind, placebo-controlled study

Magdy El-Salhy ,[1,2] Jan Gunnar Hatlebakk,[2] Odd Helge Gilja,[2] Anja Bråthen Kristoffersen,[3] Trygve Hausken[2]

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[1]Stord Hospital, Stord, Norway
[2]Department of Clinical Medicine, University of Bergen Faculty of Medicine and Dentistry, Bergen, Norway
[3]Genetic Analysis As, Oslo, Norway

Correspondence to
Professor Magdy El-Salhy, Stord Hospital, Stord, Norway; magdy.elsalhy@sklbb.no

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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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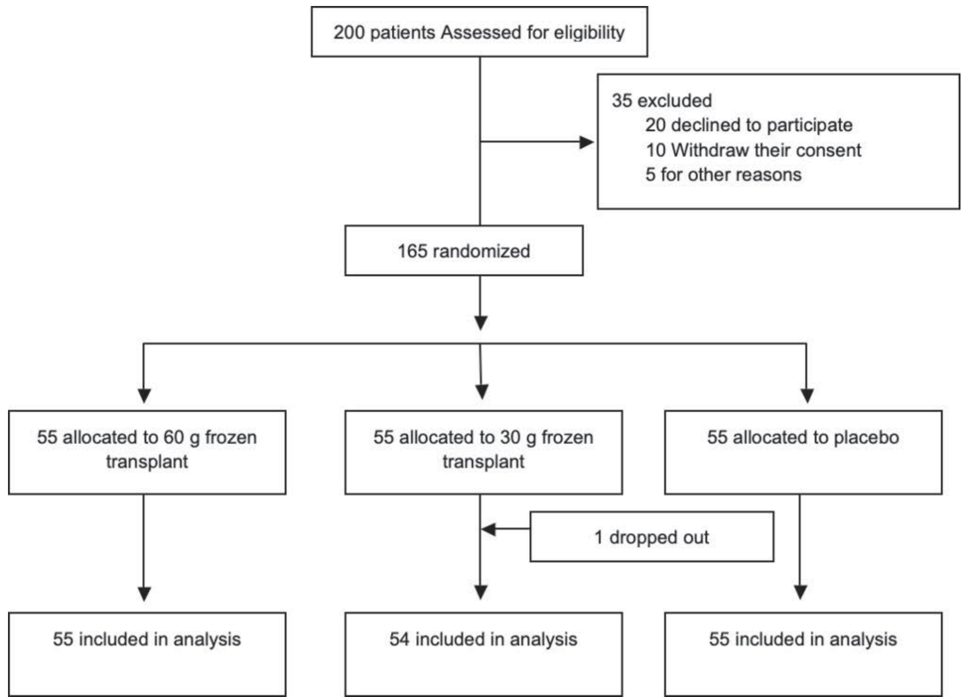

Figure 1 Consolidated standards of reporting trials) diagram showing the enrolment and randomisation of the patients.

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 (30g of their own faeces), 30g FMT and 60g 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.

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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

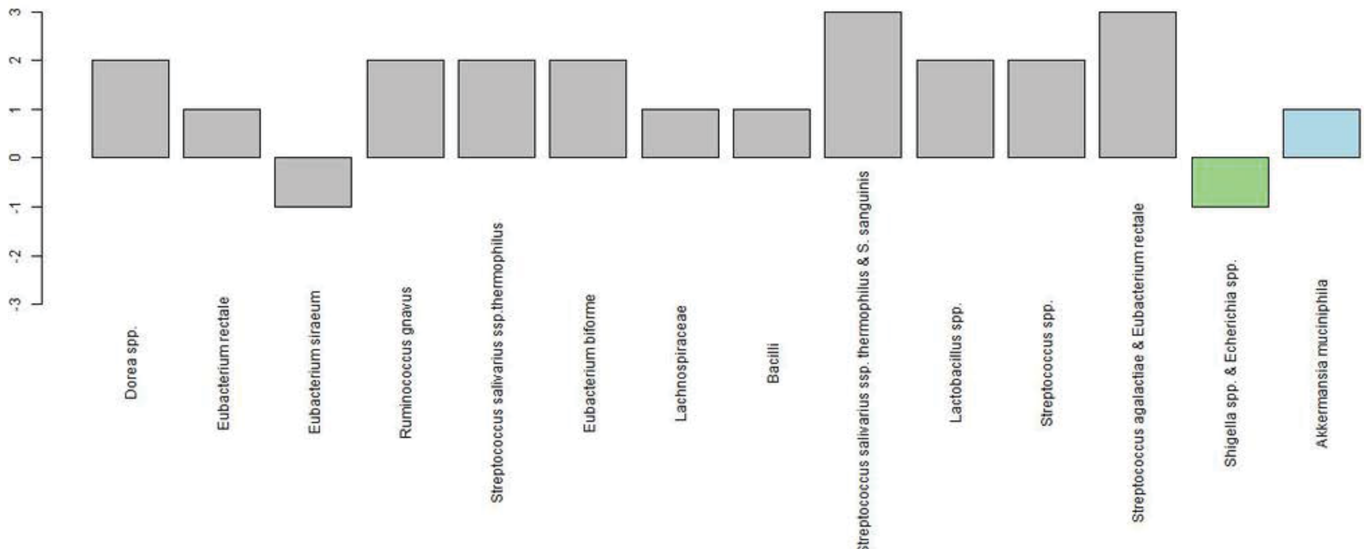

Figure 2 Although the superdonor was normobiotic, his bacterial profile deviated from the expected normal abundance in 14 of the 39 bacteria markers. The deviating bacteria belong to the typical commensal bacteria species that do not contribute to dysbiosis. In all, 12 of these bacteria belong to the phylum Firmicutes (grey), one to the phylum Proteobacteria (light green) and one to the phylum Verrucomicrobia (light blue).

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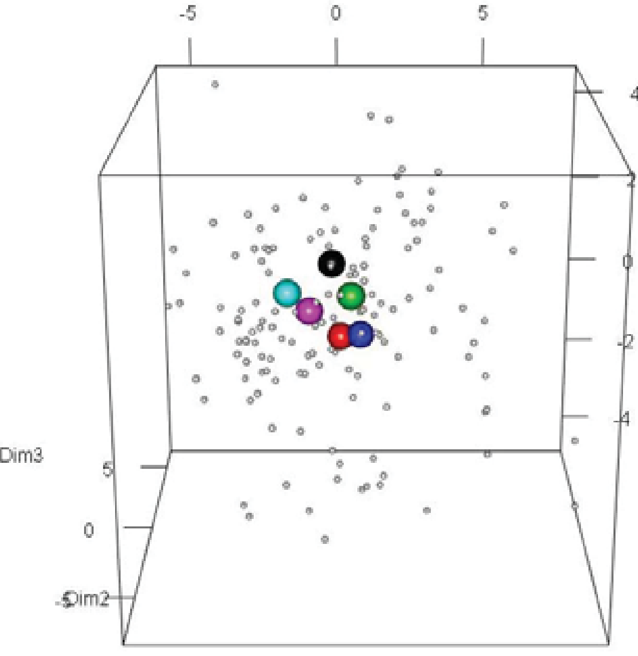

Figure 3 Scaled principal component analysis plot of faecal samples from the superdonor and patients before transplantation. The patient samples are indicated by small grey circles. The superdonor samples are indicated by the larger circles of different colours that indicate the sampling times: Black, 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.

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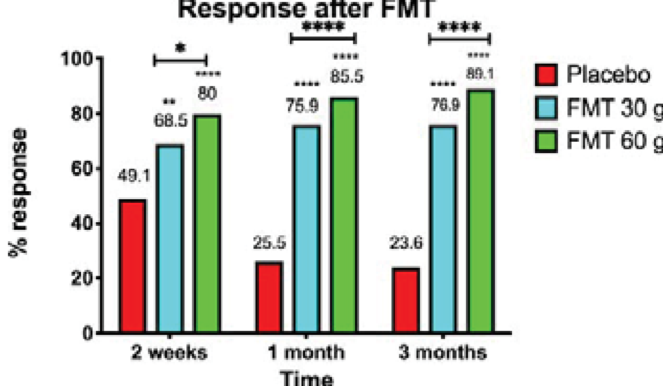

Figure 4 Responses of patients with IBS to placebo, 30 g FMT and 60 g FMT at different intervals after transplantation. **, p<0.001; ****, p<0.0001 compared with placebo. *, p<0.001; ***, p<0.0001 for 30 g FMT compared with 60 g FMT. IBS, irritable bowel syndrome; FMT, faecal microbiota transplantation.

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 (α=0.05, 1–β=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 dropouts.[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.

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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

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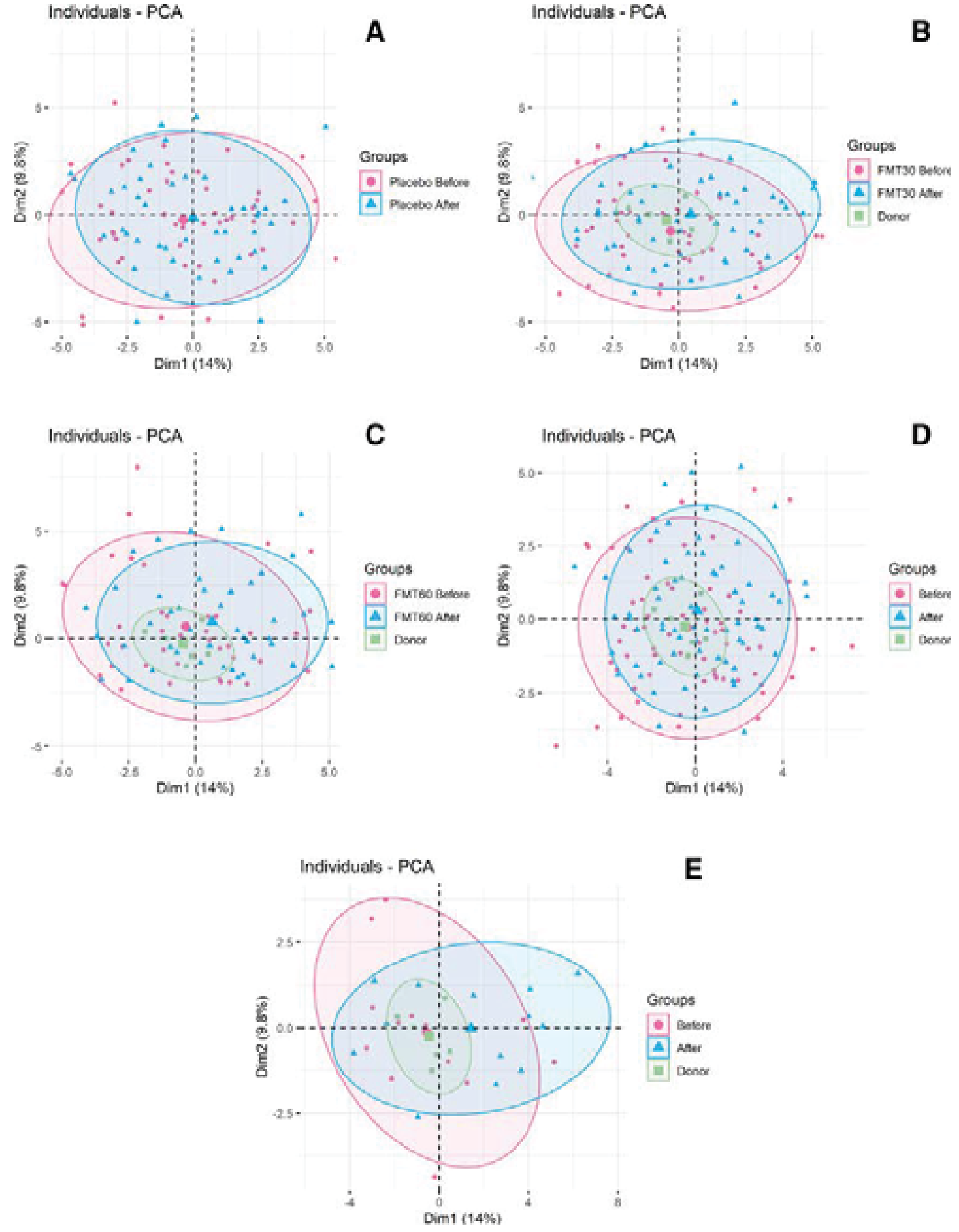

Figure 5 Scaled PCA plot of faecal samples before and after transplantation for placebo (A), 30 g FMT (B), 60 g FMT (C), responders (D) and non-responders (E). Faecal samples before and after transplantation are indicated by pink circles and blue triangles, respectively. The ellipses cover 80% of the 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.

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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 nonsmoker, 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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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval The study was performed in accordance with the Declaration of Helsinki and was approved by the Regional Committee for Medical and Health Research Ethics West, Bergen, Norway (2017/1197/REK vest).

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Data availability statement Data are available upon reasonable request. Data are stored at Helse Vest server and anonymous data are available upon reasonable request.

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ORCID iD

Magdy El-Salhy http://orcid.org/0000-0003-3398-3288

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ORIGINAL RESEARCH

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Fecal microbiota profiles in treatment-naïve pediatric inflammatory bowel disease – associations with disease phenotype, treatment, and outcome

Christine Olbjørn,[1,2] Milada Cvancarova Småstuen,[3] Espen Thiis-Evensen,[4] Britt Nakstad,[1,2] Morten Harald Vatn,[5] Jørgen Jahnsen,[2,6] Petr Ricanek,[2,6] Simen Vatn,[2,6] Aina EF Moen,[5] Tone M Tannæs,[5] Jonas C Lindstrøm,[7] Johan D Söderholm,[8] Jonas Halfvarson,[9] Fernando Gomollón,[10] Christina Casén,[11] Magdalena K Karlsson,[11] Rahul Kalla,[12] Alex T Adams,[12,13] Jack Satsangi,[12,13] Gøri Perminow[14]

[1]Department of Pediatric and Adolescent Medicine, Akershus University Hospital, Lørenskog, Norway; [2]Institute of Clinical Medicine, Campus Ahus, University of Oslo, Oslo, Norway; [3]Faculty of Health Sciences, Oslo Metropolitan University, Oslo, Norway; [4]Department of Gastroenterology, Oslo University Hospital, Rikshospitalet, Oslo, Norway; [5]Department of Clinical Molecular Biology (EpiGen), Division of Medicine, Akershus University Hospital, Lørenskog, and University of Oslo, Oslo, Norway; [6]Department of Gastroenterology, Akerhus University Hospital, Lørenskog, Norway; [7]Institute of Clinical Medicine, University of Oslo, Health Services Research Unit, Akershus University Hospital, Lørenskog, Norway; [8]Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Linköping, Sweden; [9]Department of Clinical and Experimental Medicine, Faculty of Medicine and Health Sciences, Linköping University, Linköping, Sweden; [10]Digestive Diseases Unit, IIS Aragón, Zaragoza, Spain; [11]Genetic-Analysis AS, Oslo, Norway; [12]Gastrointestinal Unit, Centre for Genomics and Molecular Medicine, University of Edinburgh, Edinburgh, UK; [13]Translational Gastroenterology Unit, Experimental Medicine Division, University of Oxford, Oxford, UK; [14]Department of Pediatrics, Oslo University Hospital, Ullevål, Oslo, Norway

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.

Correspondence: Christine Olbjørn
Department of Pediatric and Adolescent Medicine, Akershus University Hospital, Sykehusveien 25, 1474 Lørenskog, Norway
Tel +47 9 302 1216
Email chrisolb@gmail.com

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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.

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.

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.

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.

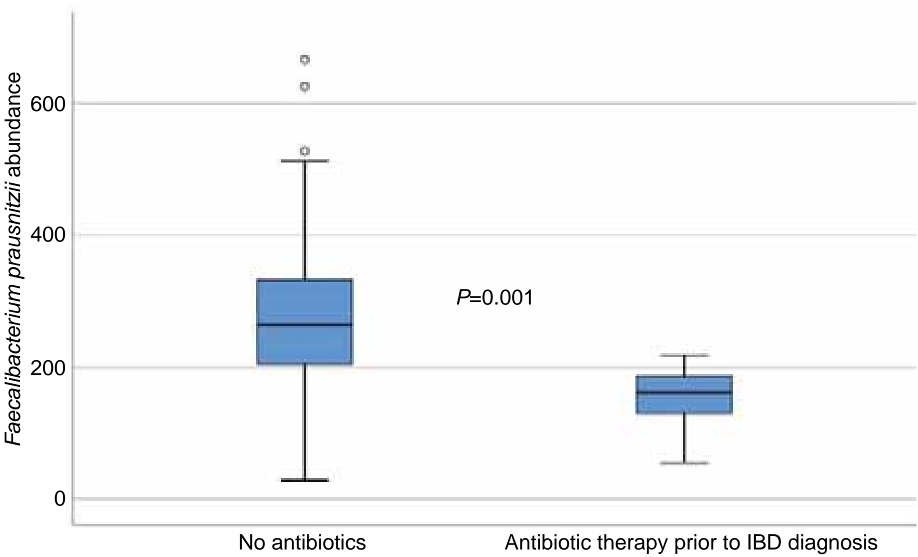

Figure 1 *Faecalibacterium prausnitzii* abundance in IBD patients according to whether they had received antibiotics prior to the diagnosis (measured in fluorescence signal strength in 1,000 units).
Abbreviation: IBD, inflammatory bowel disease.

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 ileo-colonoscopy) was not achieved in 40 (36%) of the patients

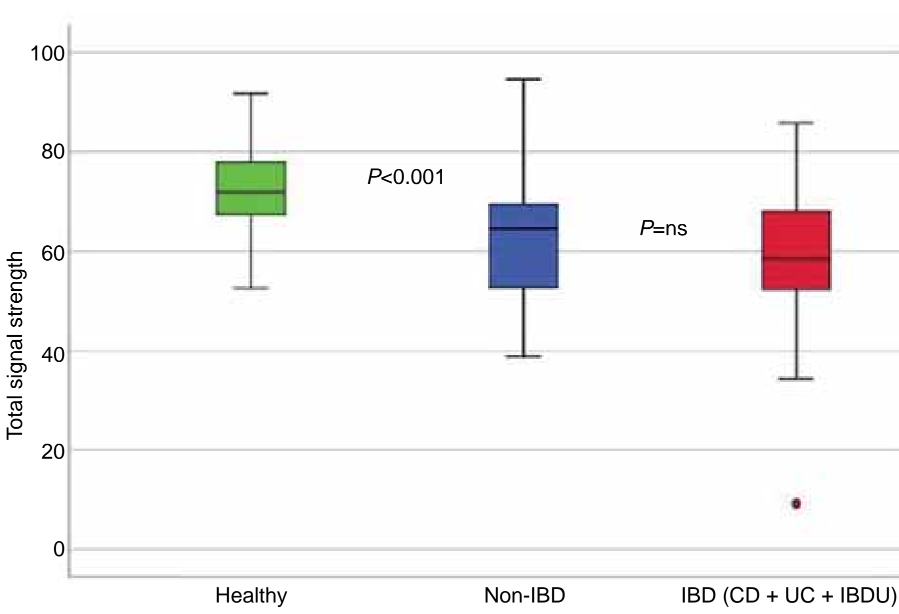

Figure 2 Boxplot illustrating the differences in the total fluorescence signal strength measured in 1,000 units between IBD, non-IBD symptomatic patients, and healthy controls.

Abbreviations: IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; IBDU, inflammatory bowel disease unclassified; ns, not significant.

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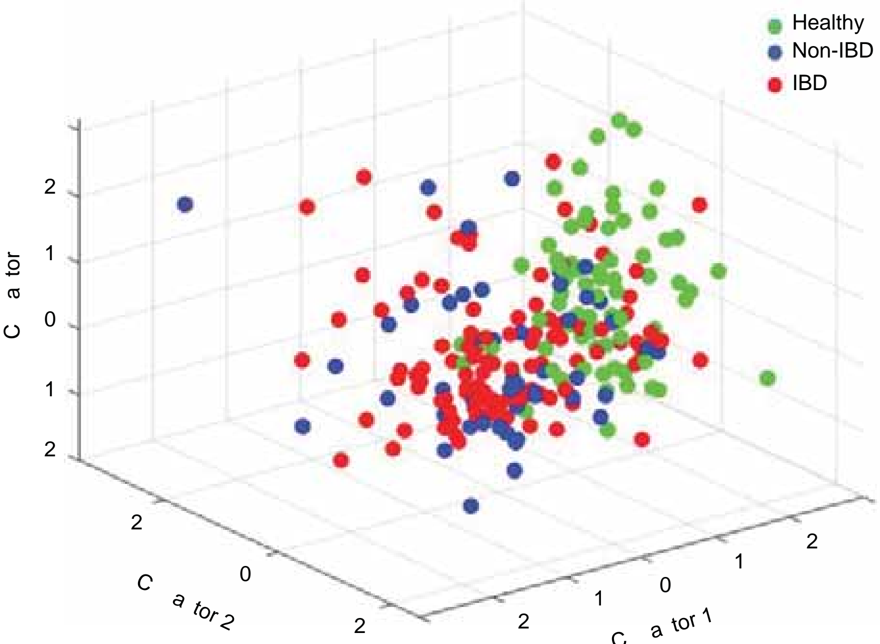
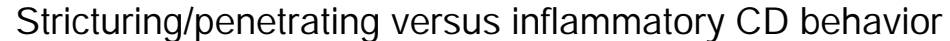


Figure 3 Principal component analysis, illustrating the difference in microbiota abundance of all 54 bacterial probes between IBD, non-IBD symptomatic patients, and healthy controls.
Notes: Each dot represents one individual. The units represent the total item loadings on each of the extracted factors.
Abbreviations: IBD, inflammatory bowel disease; PCA, principal component analysis.



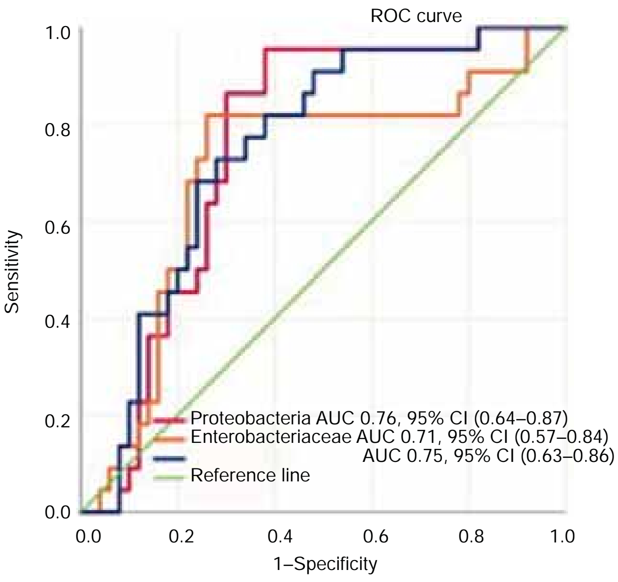


Figure 4 Sensitivity and specificity of Proteobacteria, *Enterobacteriaceae,* and *Shigella/Escherichia* abundance in differentiating Crohn's disease phenotypes (stricturing/penetrating vs inflammatory disease behavior) using the area under the receiver operating characteristics curve analysis.
Abbreviations: 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,

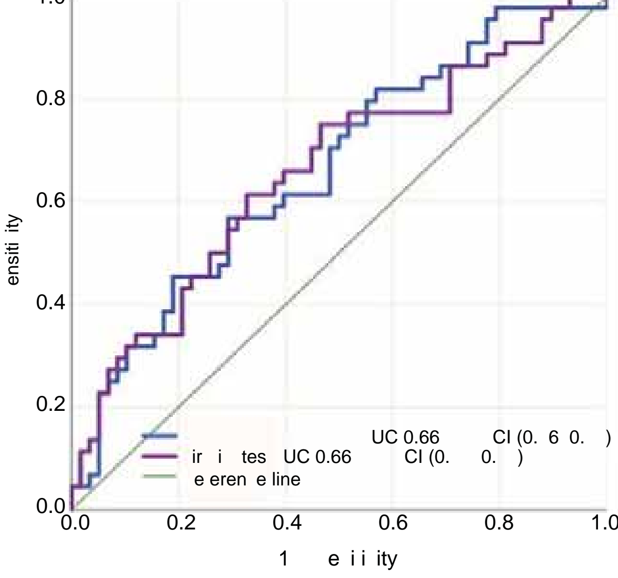


Figure 5 Sensitivity and specificity of Firmicutes and *Mycoplasma hominis* abundance in differentiating conventional- vs biologic therapy-treated IBD patients using the area under the receiver operating characteristics curve analysis.
Abbreviations: IBD, inflammatory bowel disease; CD, Crohn's disease; AUC, area under the curve.

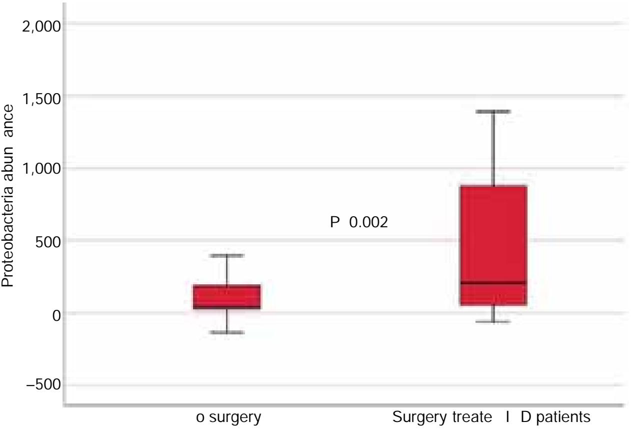


Figure 6 Proteobacteria abundance in IBD patients according to whether they needed surgery or not (measured in fluorescence signal strength in 1,000 units).
Abbreviation: IBD, inflammatory bowel disease.

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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Temporal Development of the Infant Gut Microbiota in Immunoglobulin E-Sensitized and Nonsensitized Children Determined by the GA-Map Infant Array▽†

Heidi C. Vebø,[1] Monika Sekelja,[1,2] Ragnhild Nestestog,[1] Ola Storrø,[4] Roar Johnsen,[4] Torbjørn Øien,[4] and Knut Rudi[1,2,3]*

Genetic Analysis AS, Frederik A Dahlsvei 20, N-1432, Ås, Norway[1]; Norwegian University for Life Sciences, Ås, Norway[2]; Hedemark University College, Hamar, Norway[3]; and Department of Public Health and General Practice, Norwegian University of Science and Technology, Trondheim, Norway[4]

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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).

* Corresponding author. Mailing address: Norwegian University for Life Sciences, IKBM, Postboks 5003, N-1432, Ås, Norway. Phone: 47 64 96 58 73. Fax: 47 64 96 59 01. E-mail: knut.rudi@hihm.no.

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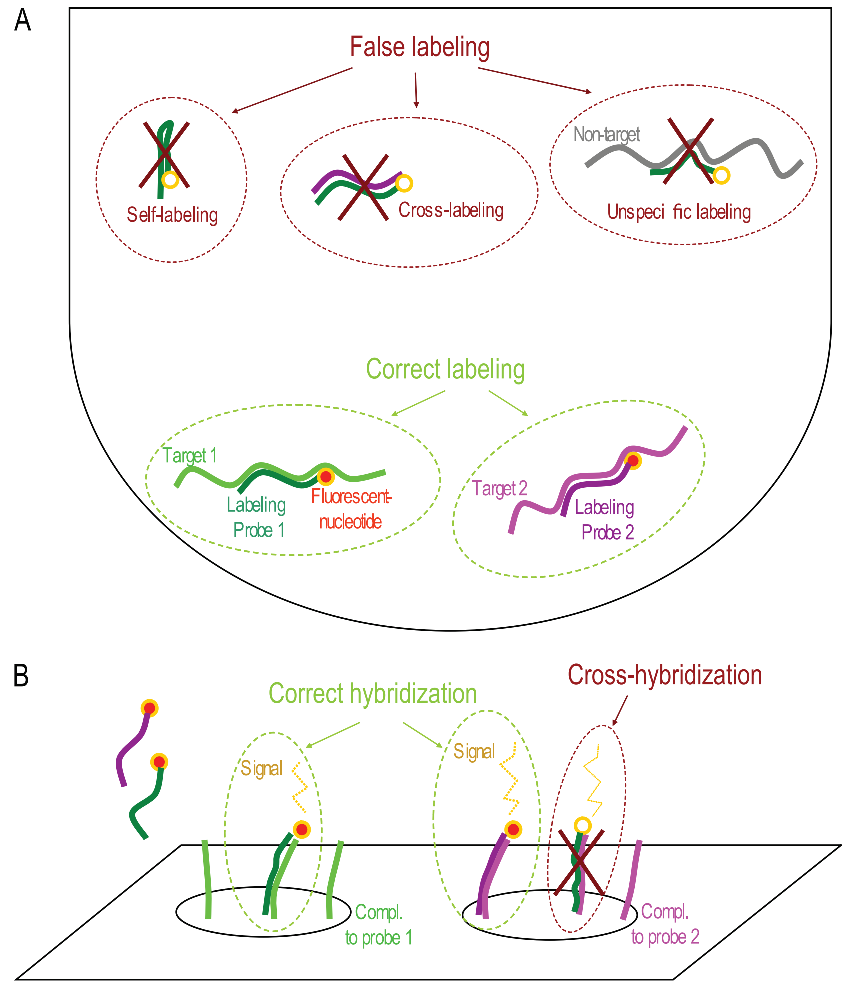

FIG. 1. Illustration of the principle and challenges associated with the GA-map array technology. In the labeling step, there are several ways probes can be nonspecifically labeled through self-labeling (internal regions of the probe are used as targets), cross-labeling (a region internal to another 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 ($\geq$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.

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

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 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

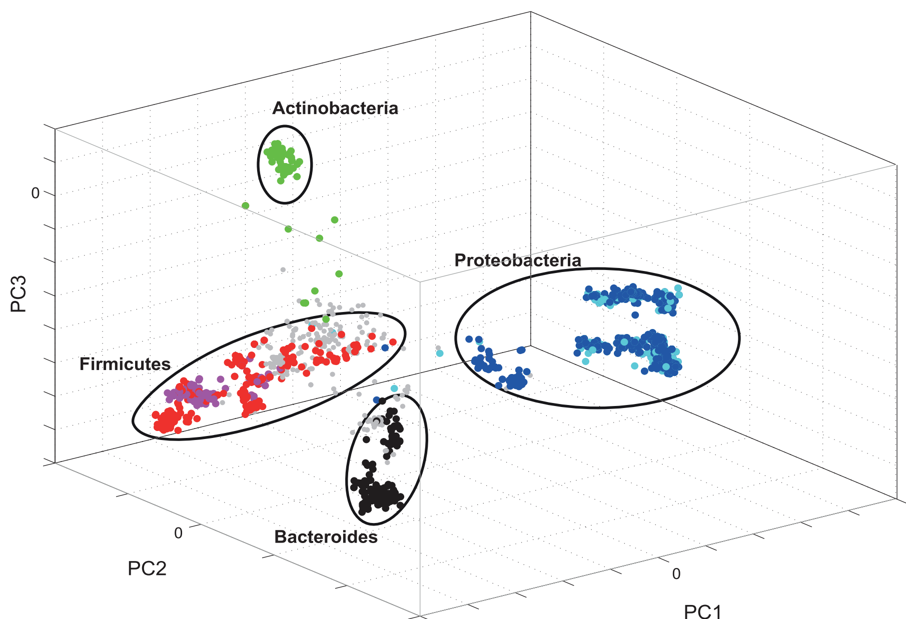

FIG. 2. Score plot of bacteria isolated from infant feces. Each object represents one bacterial clone. The objects are clustered based on 16S rRNA gene phylogeny. The colors indicate theoretical phylum probe specificities: 6_2, green; 5_1, red; 4_1, magenta; 2_1_min1b, cyan; 3_2, blue; and 1_1, black.The 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-

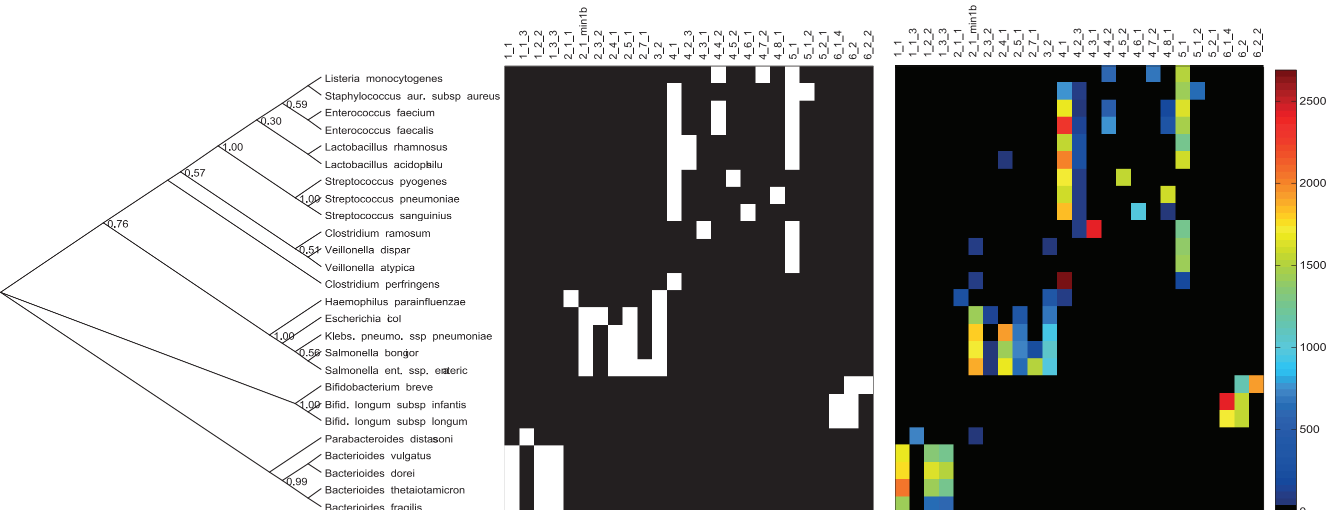

FIG. 3. Strain classification by GA-map infant array. (Left) Phylogenetic tree of all bacterial strains used to investigate probe accuracy and sensitivity. The numbers at the nodes indicate bootstrap support. (Middle) The theoretical result for the array experiment, where the classification as nontarget 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).

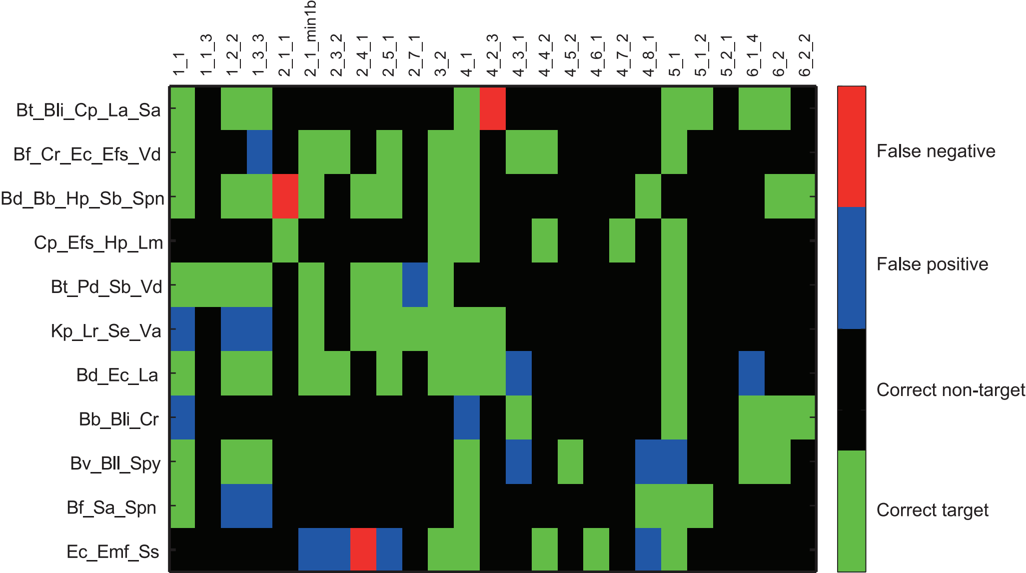

FIG. 4. Classification of mixed samples by the GA-map infant array. Signals from defined one-to-one mixtures of bacteria were evaluated. The following abbreviations were used for the bacterial species in the sample mixtures: Bd, *Bacteroides dorei*; Bf, *Bacteroides fragilis*; Bt, *Bacteroides thetaiotaomicron*; 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^2[d]
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).

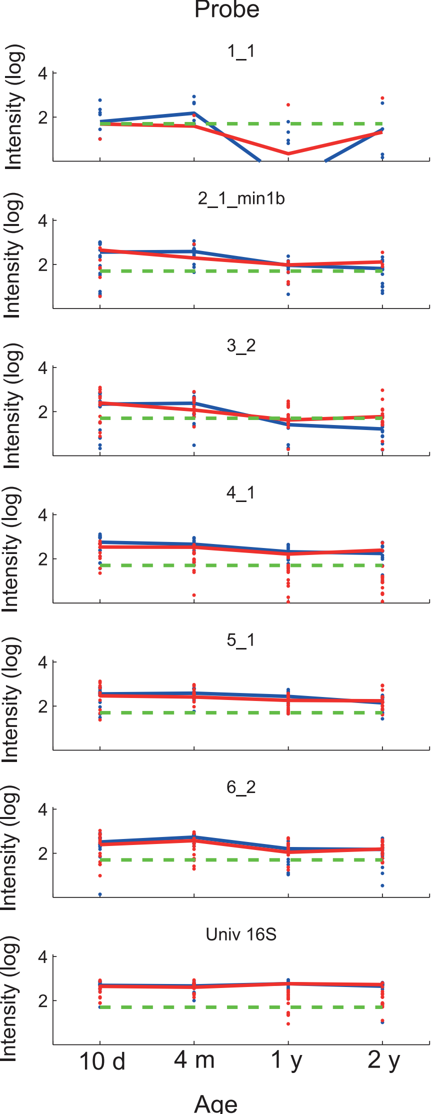

FIG. 5. Temporal development of bacterial phyla in sensitized and nonsensitized infants. Each panel shows the temporal development of probe signals within the study population for the respective probe. The log average signal for each probe is shown as a line, while the log signals of all time points 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.

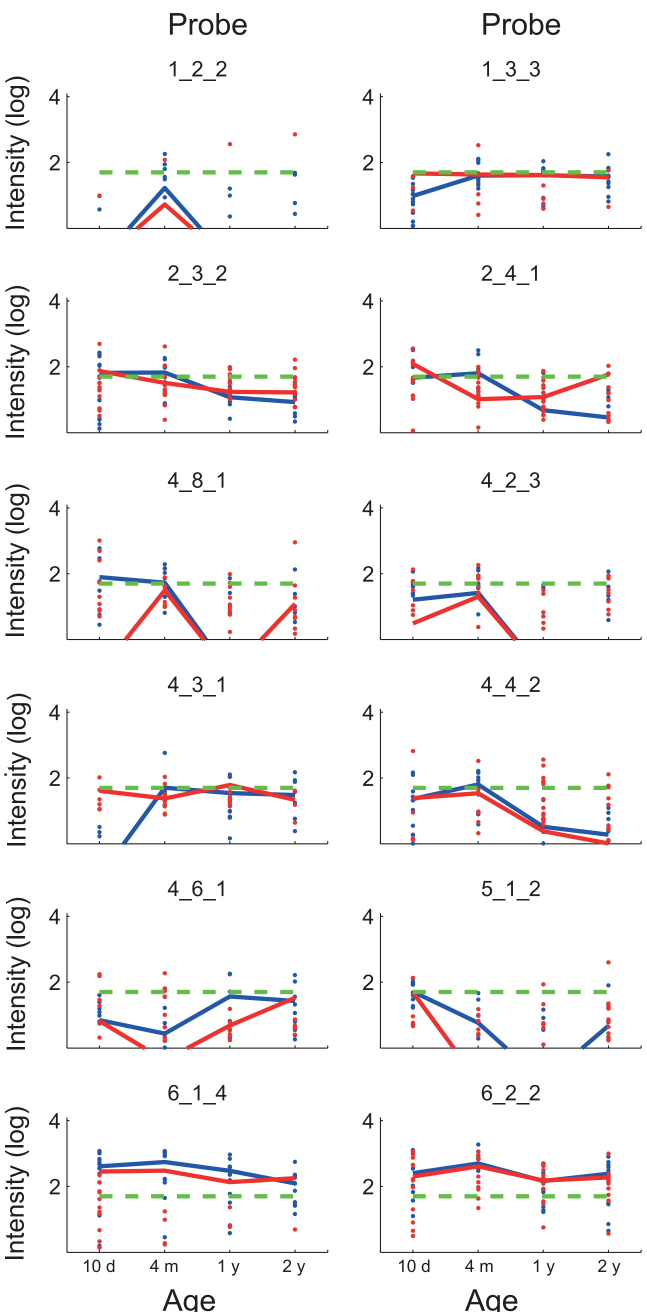

FIG. 6. Temporal development of bacterial genera/species in sensitized and nonsensitized infants. Each panel shows the temporal development of probe signals within the study population for the respective probe. The log average signal for each probe is shown as a line, while the log signals of all time 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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Research Article

Dominant Fecal Microbiota in Newly Diagnosed Untreated Inflammatory Bowel Disease Patients

Lill Therese Thorkildsen,[1] Felix Chinweije Nwosu,[1] Ekaterina Avershina,[2] Petr Ricanek,[3,4] Gøri Perminow,[5] Stephan Brackmann,[4] Morten H. Vatn,[4,6] and Knut Rudi[1,2]

[1] *Hedmark University College, 2306 Hamar, Norway*
[2] *Department of Chemistry, Biotechnology and Food Science, Norwegian University for Life Sciences, 1432 Ås, Norway*
[3] *Department of Gastroenterology, Akershus University Hospital, 1478 Lørenskog, Norway*
[4] *Department of Clinical Molecular Biology and Laboratory Sciences (EpiGen), Division of Medicine, Akershus University Hospital, University of Oslo, 0316 Oslo, Norway*
[5] *Pediatric Department, Oslo University Hospital, Ullevål, 0450 Oslo, Norway*
[6] *Medical Clinic, Oslo University Hospital, Rikshospitalet, 0372 Oslo, Norway*

Correspondence should be addressed to Knut Rudi; knut.rudi@umb.no

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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.

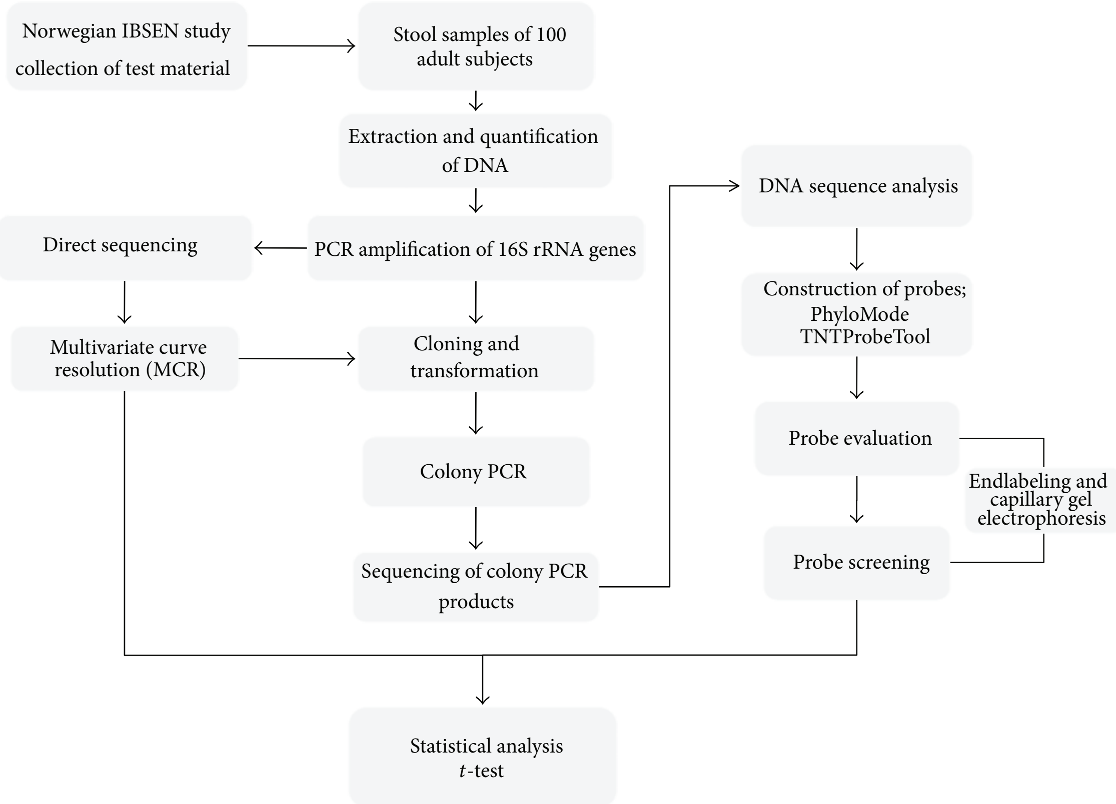

Figure 1: Schematic outline of the methodology.

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

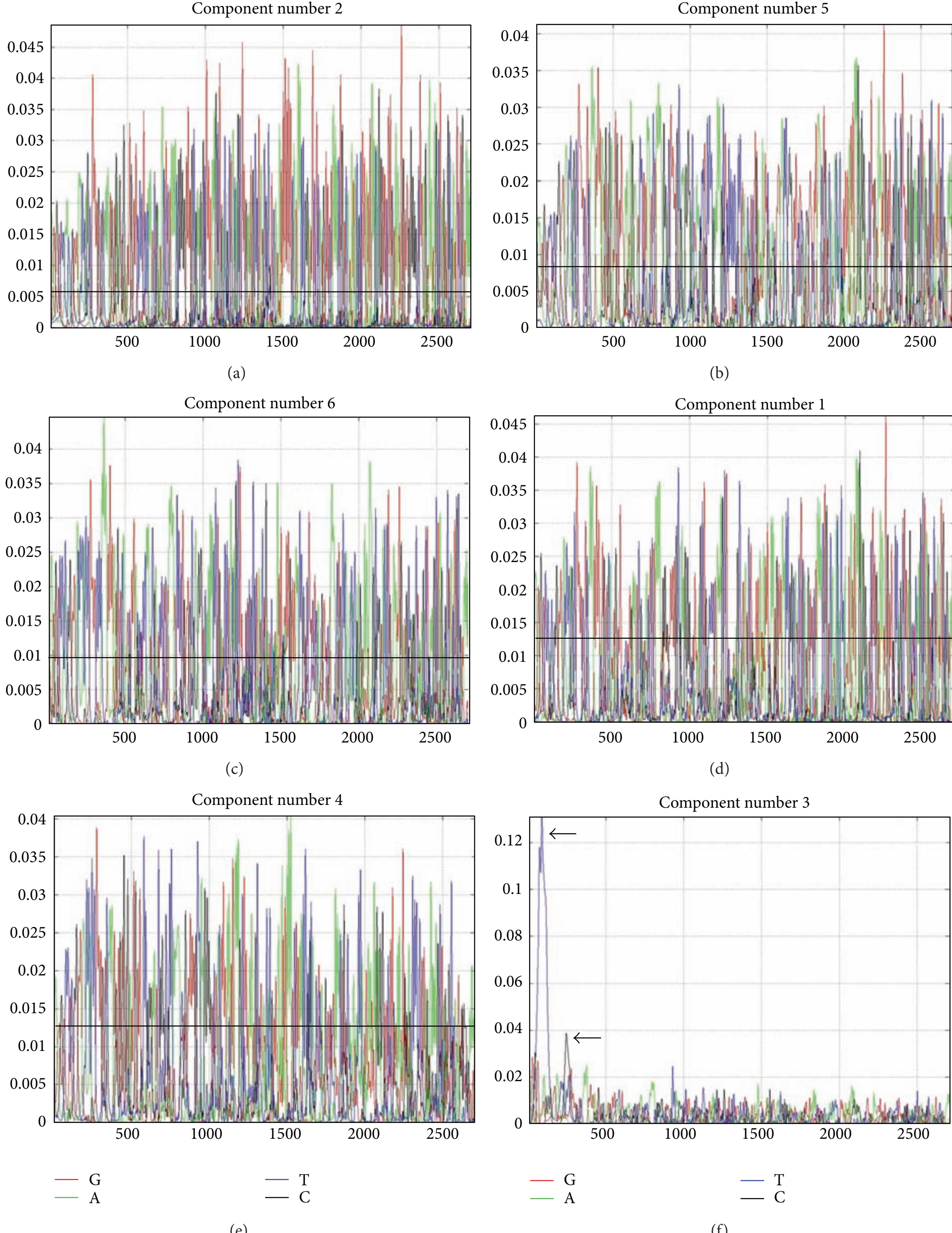

Figure 2: Spectra of the six components resolved by MCR analysis. Visual examination reveal components 2, 5, and 6 ((a)–(c)) to have well resolved spectra with low background sequences. Components 1 and 4 ((d) and (e)) also have well resolved spectra, although with somewhat higher background sequences than components 2, 5, and 6. Component 3 (f) has two high peaks (black arrows) and a poorly resolved spectrum.

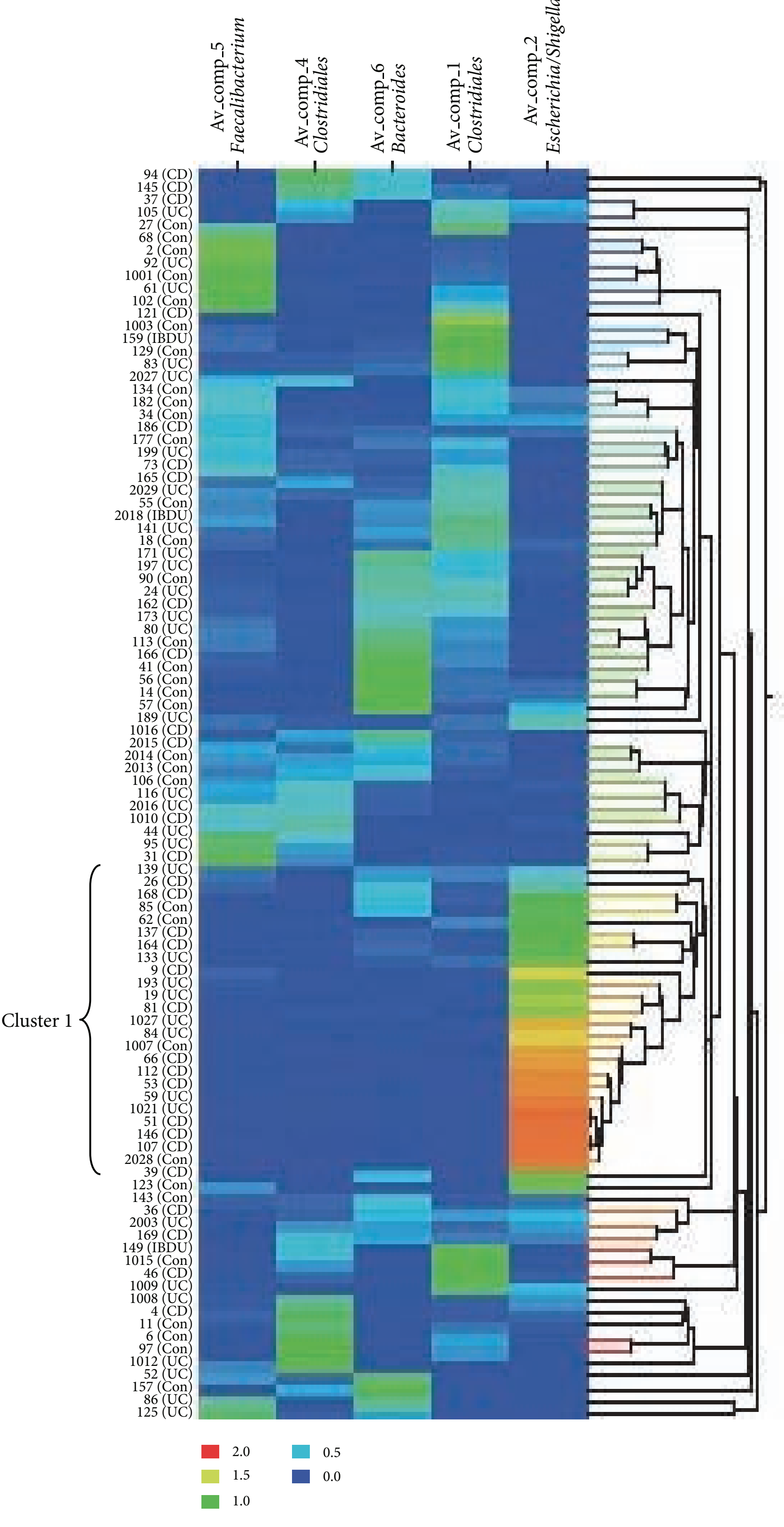

Figure 3: Data matrix of the amount of components 1, 2, 4, 5, and 6 in the stool samples. The graded colors indicate the abundance of a particular component in a sample. Red color indicates a high amount of the component, and blue color indicates a low amount of the component. To the left of the matrix, 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

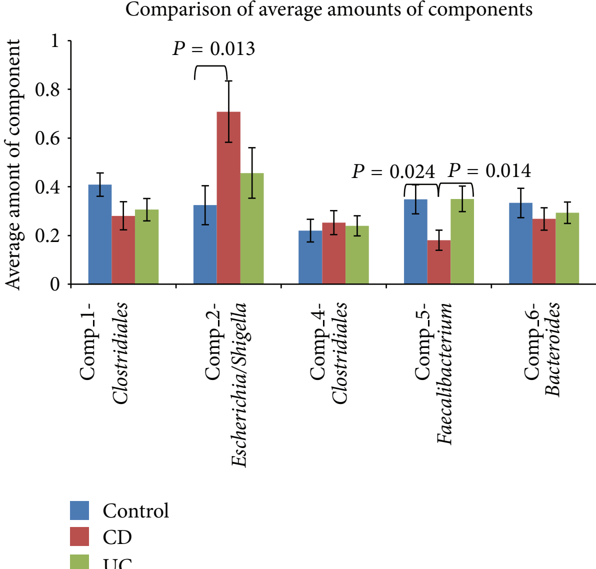

Figure 4: Comparison of the average amount of each component in the three disease states. The averages are calculated based on MCR analysis of stool samples from 33 subjects in the control group, 30 subjects diagnosed with CD (Crohn's Disease), and 33 subjects diagnosed with UC (Ulcerative Colitis). 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.

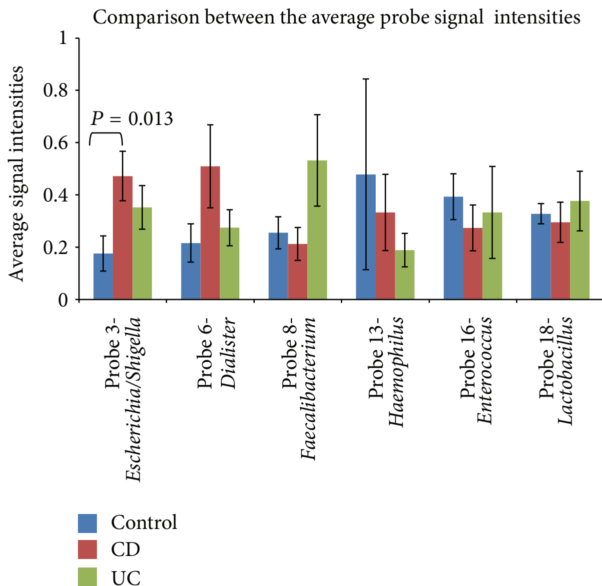

Figure 5: Comparison between the average amount of specific bacteria groups in the different disease states. The averages are calculated based on the height of the probe signals normalized by the signal height of the universal bacterial probe. Further, the averages are normalized to one because of Probe 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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AP&T Alimentary Pharmacology and Therapeutics

Deviations in human gut microbiota: a novel diagnostic test for determining dysbiosis in patients with IBS or IBD

C. Casén*, H. C. Vebø*, M. Sekelja*, F. T. Hegge*, M. K. Karlsson*, E. Ciemniejewska*, S. Dzankovic*, C. Frøyland*, R. Nestestog*, L. Engstrand[†], P. Munkholm[‡], O. H. Nielsen[§], G. Rogler[¶], M. Simrén**, L. Öhman**[,††], M. H. Vatn[‡‡,§§] & K. Rudi[¶¶]

*Genetic Analysis AS, Oslo, Norway.
[†]Karolinska Institute, Stockholm, Sweden.
[‡]Department of Gastroenterology, Northzealand Hospital, University of Copenhagen, Copenhagen, Denmark.
[§]Department of Gastroenterology, Herlev Hospital, University of Copenhagen, Copenhagen, Denmark.
[¶]Clinic for Gastroenterology and Hepatology, University of Zürich, Zürich, Switzerland.
**Department of Internal Medicine and Clinical Nutrition, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden.
[††]Department of Microbiology and Immunology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden.
[‡‡]EpiGen Institute, Campus Ahus, Institute of Clinical Medicine, University of Oslo, Lørenskog, Norway.
[§§]Section of Gastroenterology, Oslo University Hospital, Rikshospitalet, Oslo, Norway.
[¶¶]Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Aas, Norway.

Correspondence to:
C. Casén, Genetic Analysis AS, Nycoveien 2 0485 Oslo, Norway.
E-mail: cc@genetic-analysis.com

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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.

Aliment Pharmacol Ther

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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'.

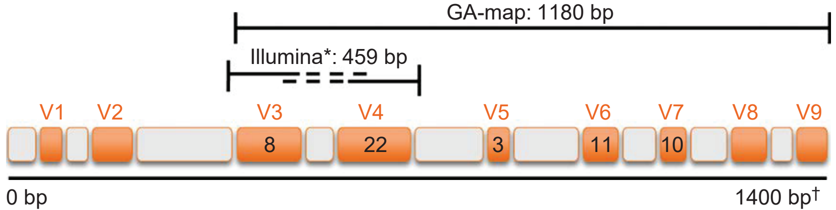

Figure 1 | Target regions for the GA primer (1180 bp) and the Illumina primer (459 bp) showing variable (orange V1-V9) and conserved (grey) regions in the bacterial 16S rRNA gene (1400 bp) utilised by the two methods. The numbers in V3 to V7 denote the number of GA probes targeting each variable region, 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	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

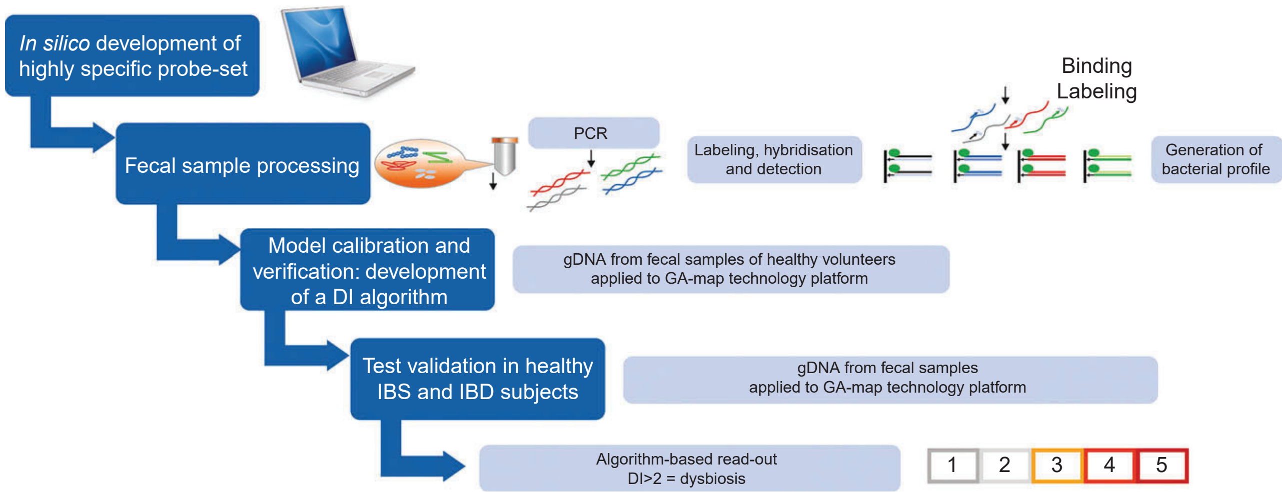

Figure 2 | Flow chart illustrating the GA-map Dysbiosis Test development, starting with *in silico* development of bacterial probe set, standardisation of laboratory analysis process, model calibration and verification in healthy individuals (normobiotic reference collection), and validation in healthy, 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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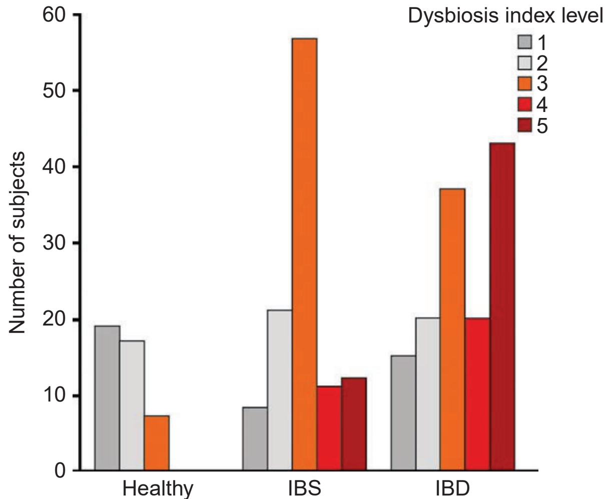

Figure 3 | Distribution of DI scores 1–5 for the validation cohort as determined by GA-map Dysbiosis Test, showing the increase in DI from healthy individuals through IBS patients and finally in IBD patients.

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

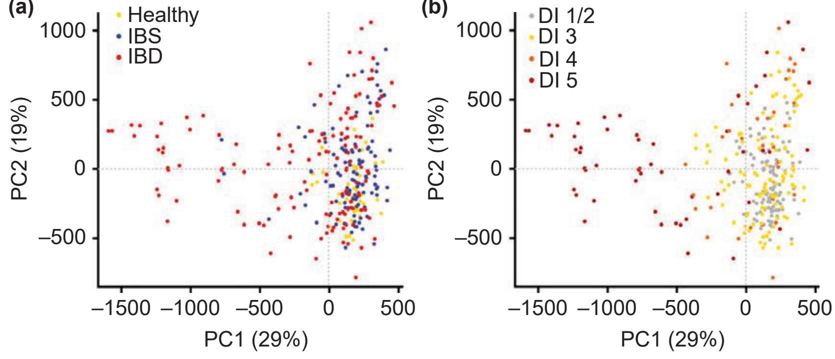

Figure 4 | PCA scores for the first two principal components for validation cohort ($n = 287$) based on 54 probes. The two PCs account for 48% of the variation, and points are coloured according to (a) cohort: yellow – healthy, blue – IBS, and red – IBD; and (B) DI: grey = 1–2, orange = 3, red = 4, dark red = 5.

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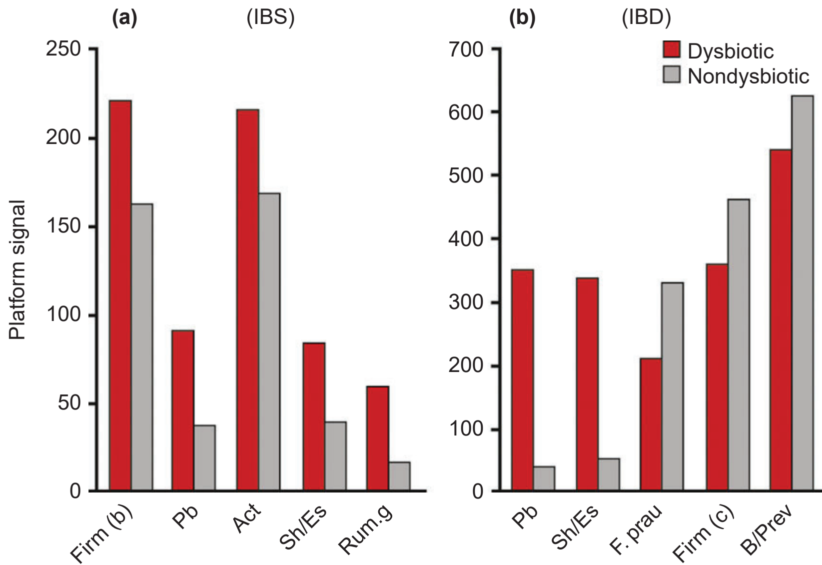

Figure 5 | Mean normalised signal for top five probes sorted by absolute relative difference between dysbiotic (red) and nondysbiotic (grey) as determined by the GA-map Dysbiosis Test for (a) IBS patients (n = 109), and (b) IBD patients (n = 135). Act, Actinobacteria; B/Prev, Bacteroides/Prevotella; 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.

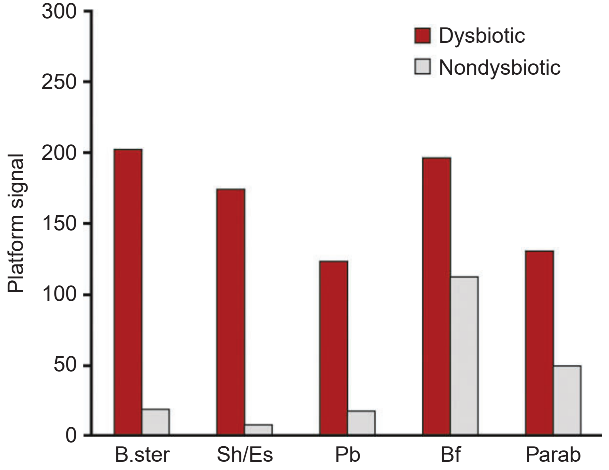

Figure 6 | Mean normalised signal for probes sorted by absolute relative difference between dysbiotic (red) and nondysbiotic (grey) as determined by the GA-map Dysbiosis Test for Spanish cohort (n = 24). Bf; Bifidobacterium, B. ster; *Bacteroides stercoris*, Parab; Parabacteroides, Pb; Proteobacteria, Sh/Es; *Shigella/Escherichia*.

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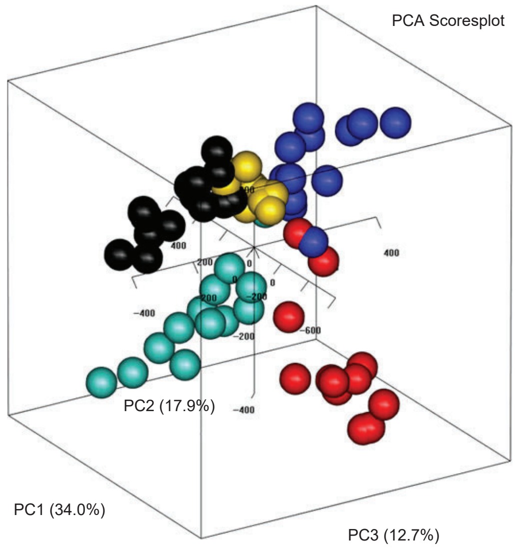

Figure 7 | Scores for the first three principal components from PCA of normalised data from five healthy subjects collected weekly for up to 14 weeks ($n = 64$). One point is one sample for donor x taken at time point y. The first three PCs account for 65% of the variation, and points are coloured according 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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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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Note

Bead-beating artefacts in the *Bacteroidetes* to *Firmicutes* ratio of the human stool metagenome

Heidi C. Vebø [a,1], Magdalena Kauczynska Karlsson [a], Ekaterina Avershina [b], Lene Finnby [a], Knut Rudi [b,*]

[a] Genetic Analysis AS, Oslo, Norway
[b] Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway

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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.

* Corresponding author.
E-mail address: knut.rudi@nmbu.no (K. Rudi).
[1] Current address: Gentian AS, Moss, Norway.

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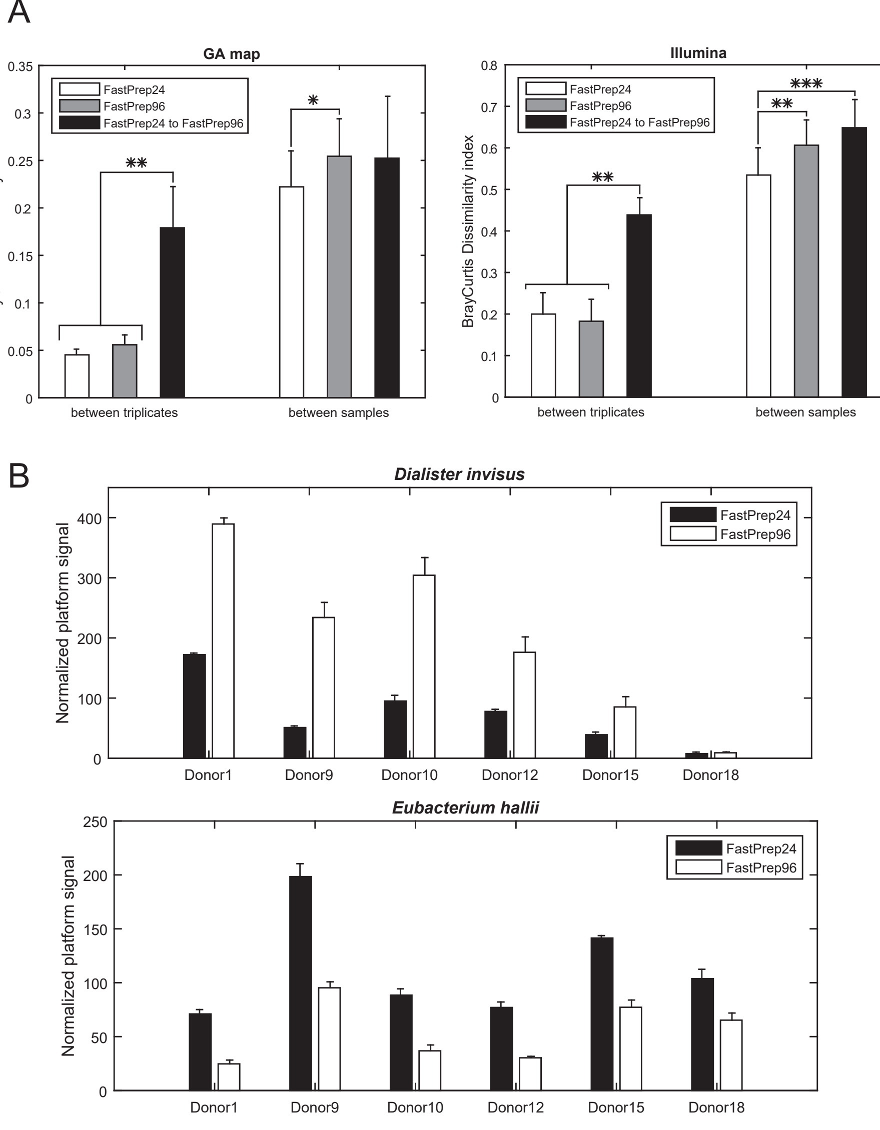

Fig. 1. Effect of bead-beating on (A) microbiota beta diversity and (B) species composition. (A) Mean Bray-Curtis beta diversity within and between sample categories are shown. Error bars represent standard error of the mean. The significance levels are designated by asterisks: $^{*}p < 0.05$, $^{**}p < 0.01$ 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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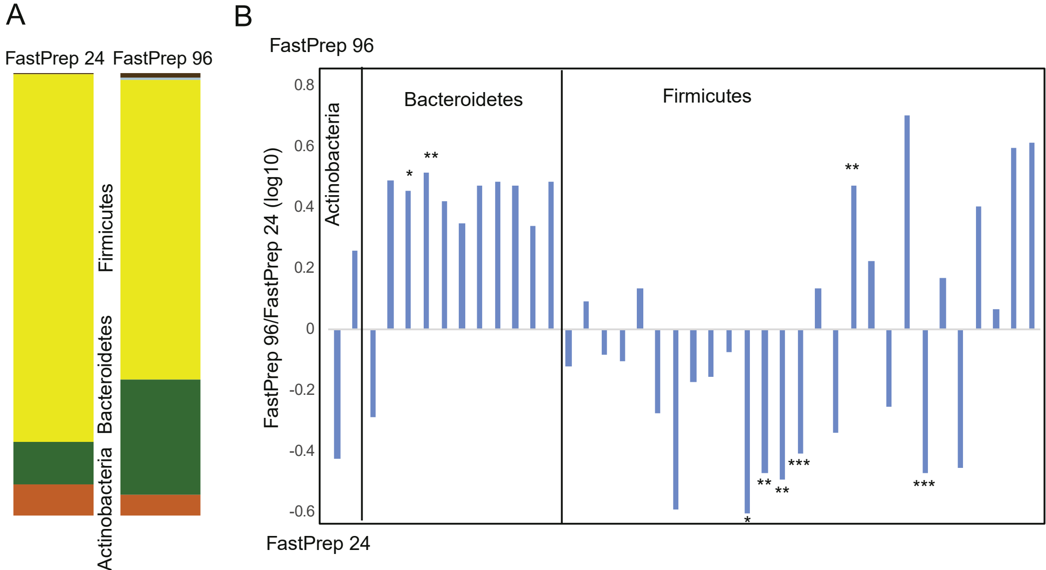

Fig. 2. Effect of bead-beating on microbiota taxonomic composition as determined by Illumina sequencing. (A) Effect on phylum level and (B) on OTU level (for OTU's that have an abundance of >1% on average in the dataset) as determined by Illumina sequencing. The ordinate represents the average number 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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Arthritis Research & Therapy

RESEARCH ARTICLE
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Intestinal dysbiosis is common in systemic sclerosis and associated with gastrointestinal and extraintestinal features of disease

Kristofer Andréasson[1*], Zaid Alrawi[1], Anita Persson[1], Göran Jönsson[2] and Jan Marsal[3,4,5]

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

* Correspondence: kristofer.andreasson@med.lu.se
[1]Section of Rheumatology, Department of Clinical Sciences, Lund University, Lund, Sweden
Full list of author information is available at the end of the article

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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 χ2 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

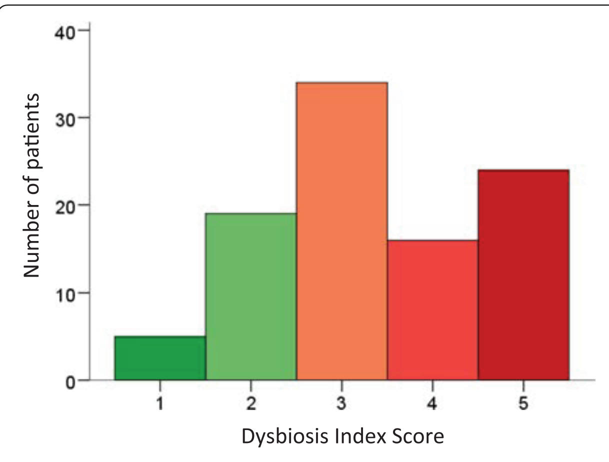

Fig. 1 Dysbiosis is common in patients with systemic sclerosis.A majority of the study population suffers from dysbiosis, as defined by the GA-map™ Dysbiosis Test, with 25% exhibiting pronounced dysbiosis

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

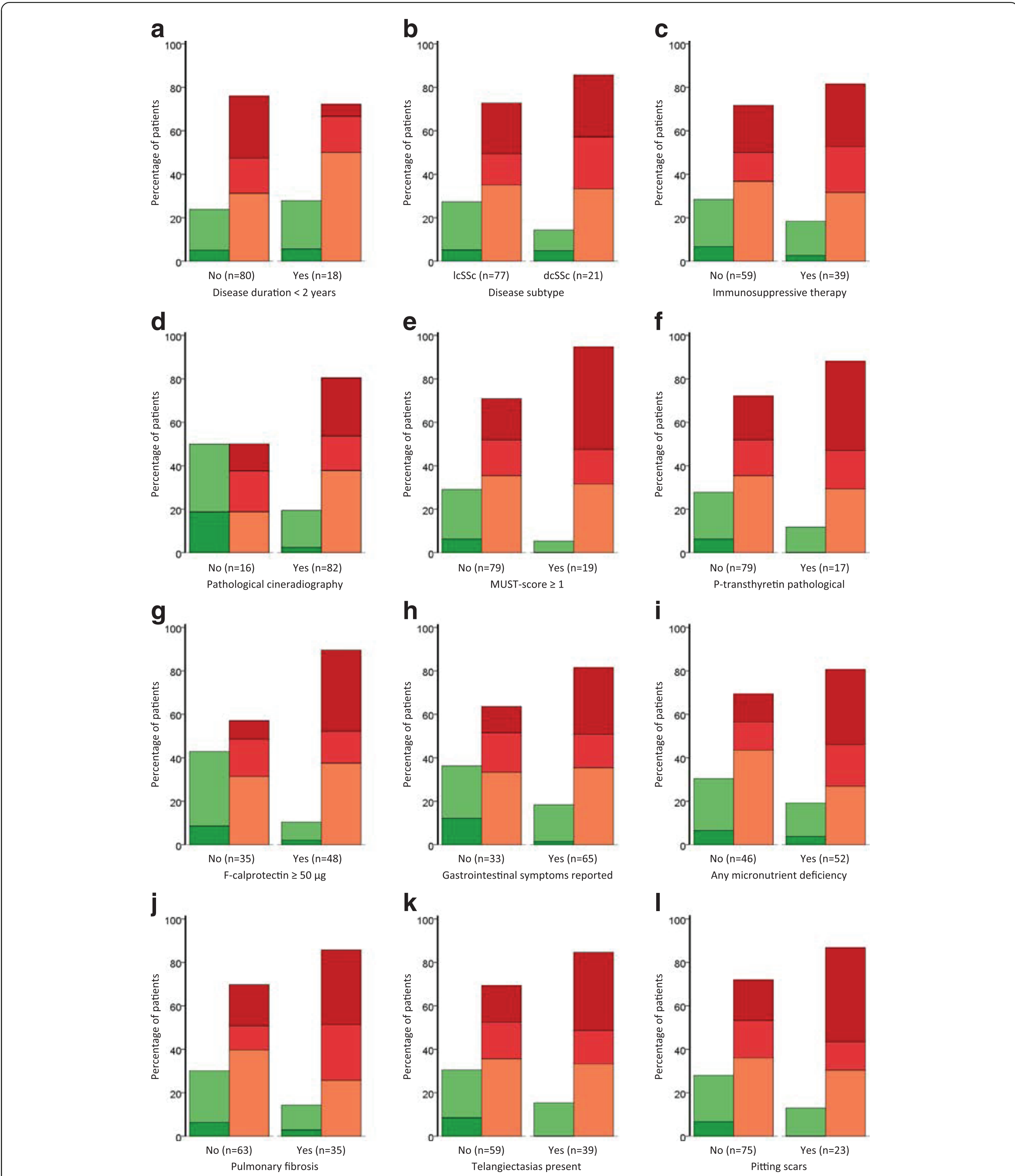

Fig. 2 Dysbiosis correlates with gastrointestinal and some extraintestinal manifestations of SSc, but not disease subtype or immunosuppressive therapy. Dysbiosis was prevalent in patients with both short and long disease duration (**a**), lcSSc and dcSSc (**b**) as well as in patients with and without 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.

Author details

[1]Section of Rheumatology, Department of Clinical Sciences, Lund University, Lund, Sweden. [2]Section of Infectious Diseases, Department of Clinical Sciences, Lund University, Lund, Sweden. [3]Department of Clinical Sciences, Lund University, Lund, Sweden. [4]Immunology Section, Lund University, Lund, Sweden. [5]Department of Gastroenterology, Skåne University Hospital, Lund, Sweden.

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Evaluation of a faecal dysbiosis test for irritable bowel syndrome in subjects with and without obesity

Martin Aasbrenn, Jørgen Valeur & Per G. Farup

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ORIGINAL ARTICLE

Evaluation of a faecal dysbiosis test for irritable bowel syndrome in subjects with and without obesity

Martin Aasbrenn[a,b], Jørgen Valeur[c] and Per G. Farup[b,d]

[a]Department of Surgery, Innlandet Hospital Trust, Gjøvik, Norway; [b]Department of Clinical and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway; [c]Unger-Vetlesen Institute, Lovisenberg Diaconal Hospital, Oslo, Norway; [d]Department of Research, Innlandet Hospital Trust, Brumunddal, Norway

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

CONTACT Martin Aasbrenn Martin.Aasbrenn@gmail.com Department of Surgery, Innlandet Hospital Trust, Kyrre Greppsgt. 11, N-2819 Gjøvik, Norway
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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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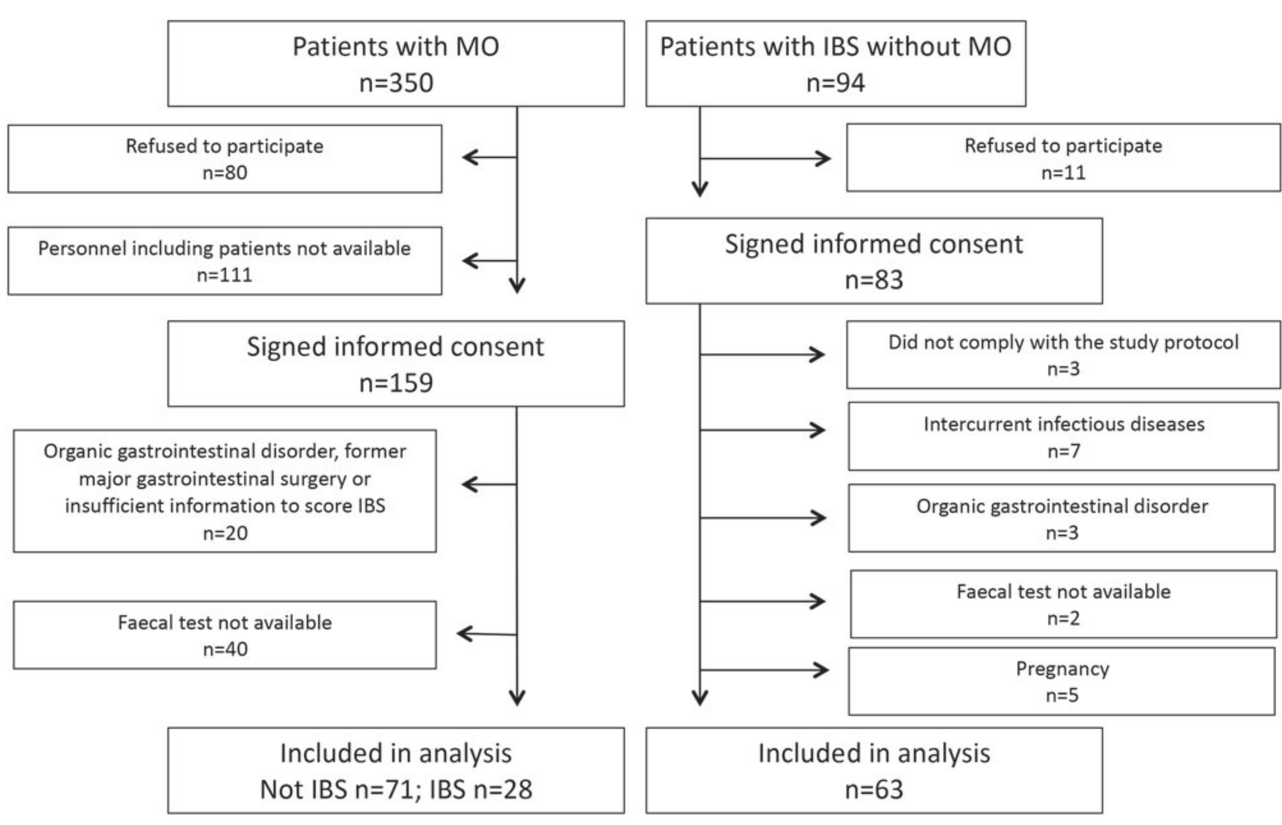

Figure 1. Flow chart depicting inclusion of subjects.

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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), $p = .047$).

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.

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 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.

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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

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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ORCID

Martin Aasbrenn http://orcid.org/0000-0003-3637-5763

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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

Maria K. Magnusson, PhD,[*,†] *Hans Strid, MD, PhD,*[‡] *Stefan Isaksson, BSc,*[*,†] *Magnus Simrén, MD, PhD,*[†] *and Lena Öhman, PhD*[*,†,§]

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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From the *Department of Microbiology and Immunology, Institute for Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden; †Department of Internal Medicine and Clinical Nutrition, Institute for Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden; ‡Department of Internal Medicine, Södra Älvsborg Hospital, Borås, Sweden; and §School of Health and Education, University of Skövde, Skövde, Sweden.

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Address correspondence to: Maria K. Magnusson, PhD, Department of Microbiology and Immunology, University of Gothenburg, Box 435, 405 30 Gothenburg, Sweden (e-mail: maria.magnusson@microbio.gu.se).

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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 non-viable 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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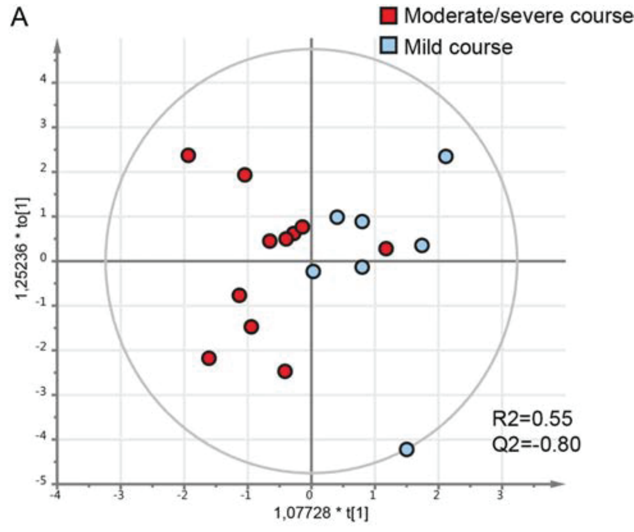
TABLE 2. Health Care Use and Medical Treatment During the 3 Years Follow-up

	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.

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.



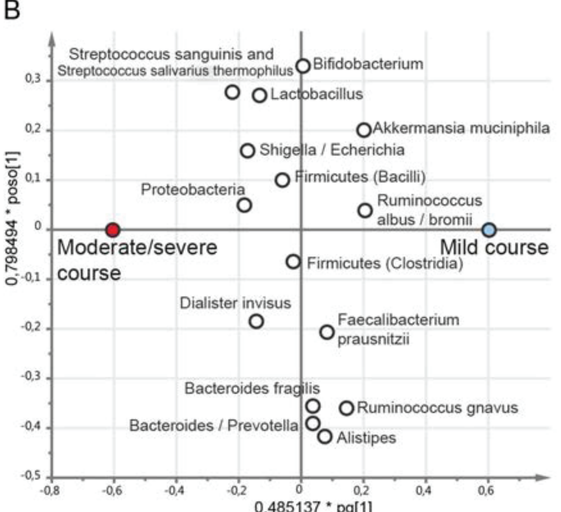


FIGURE 1. Patients with a mild and a moderate/severe disease course differ at diagnosis of disease with respect to their fecal microbiota composition. Fecal samples obtained from patients with newly diagnosed UC were analyzed by the GA-map Dysbiosis test (mild disease n = 7, moderate/severe disease n = 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.

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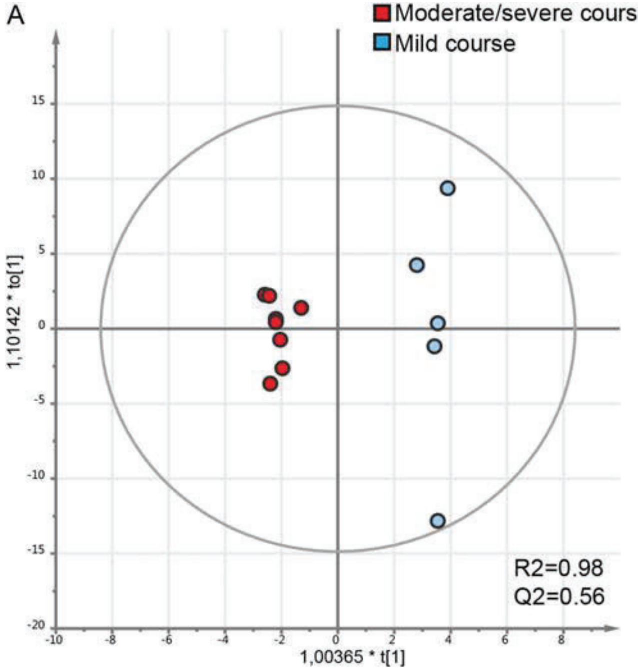

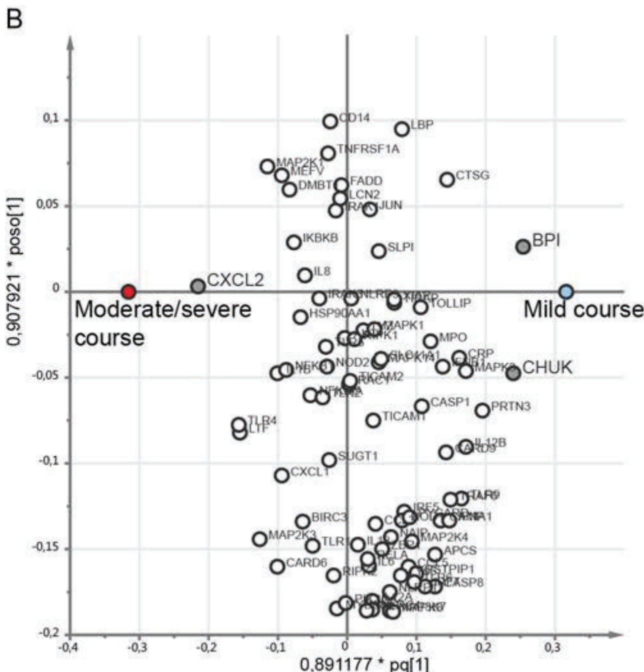

FIGURE 2. Patients with a mild and a moderate/severe disease course display different mucosal antibacterial response profiles at diagnosis of disease. Rectal mucosal biopsies obtained from patients with newly diagnosed UC were analyzed using a PCR array for 84 genes involved in antibacterial response (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 ($\geq$9) versus moderate ($\leq$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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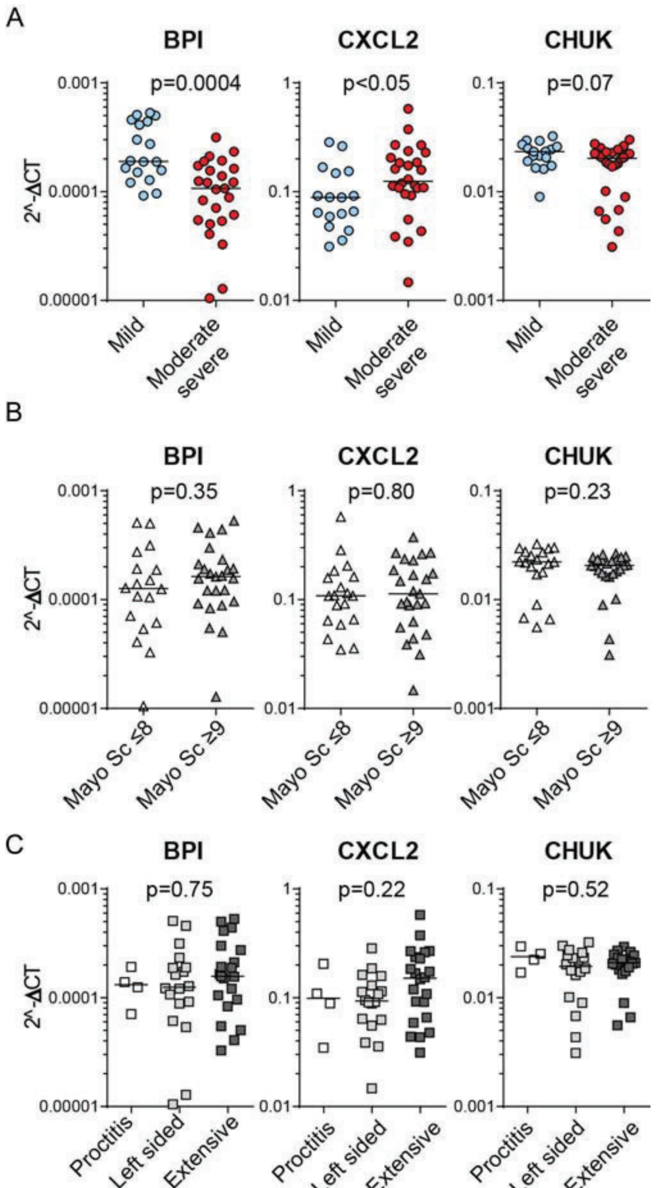

FIGURE 3. Mucosal expression of BPI, CHUK, and CXCL2 in patients with newly diagnosed UC is associated with disease course. Rectal mucosal biopsies obtained were analyzed for mRNA expression of *BPI*, *CXCL2*, and *CHUK* using RT-PCR. For patients with newly diagnosed UC, gene expression was compared 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.

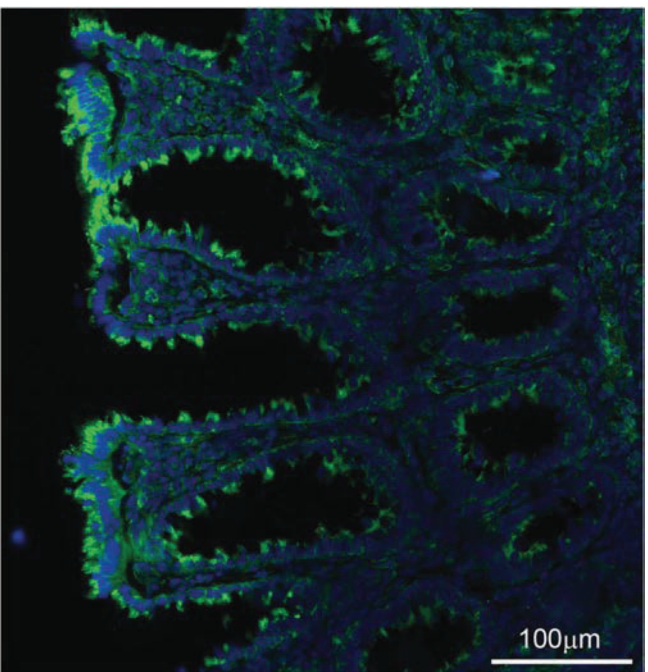

FIGURE 4. Localization of BPI in the epithelial cell layer and crypts. Biopsy specimens from rectum stained with polyclonal rabbit-anti-BPI-IgG followed by goat-anti-rabbit-AlexaFluor488. Sections were mounted with 4′, 6-Diamidino-2-Phenylindole Dihydrochloride (DAPI) (chromosome stain) and visualized 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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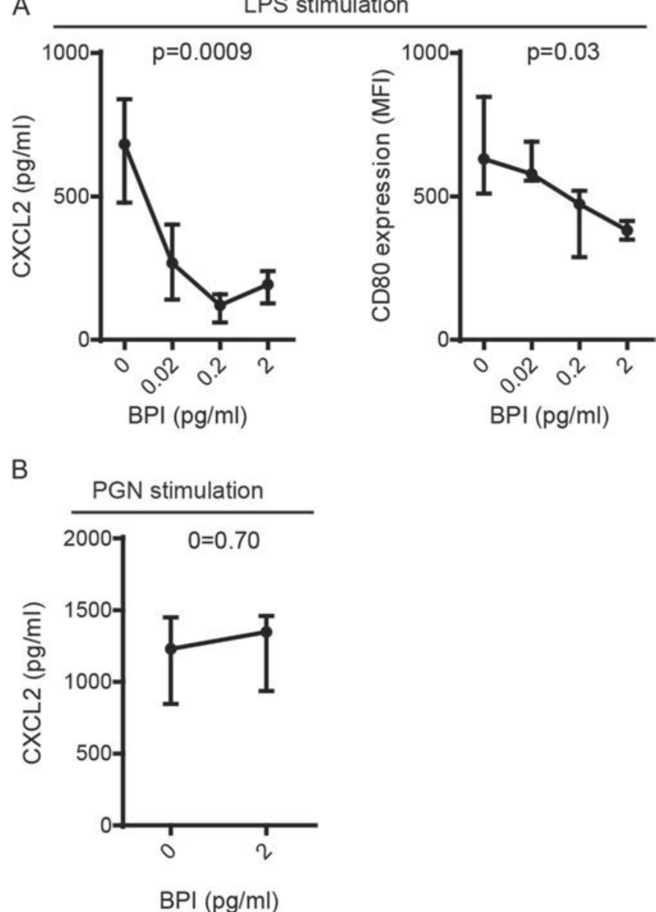
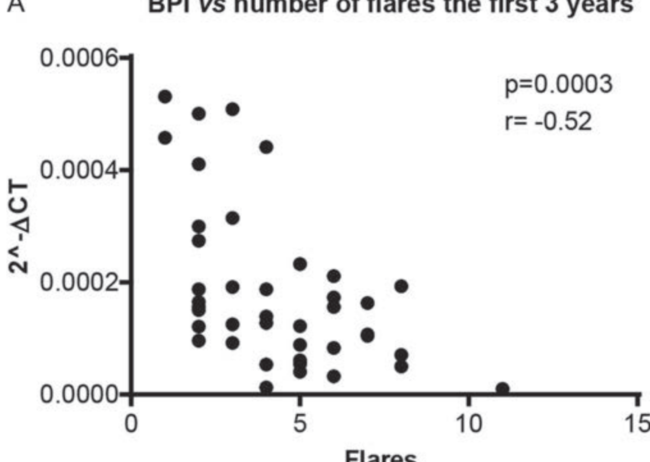


FIGURE 5. BPI reduces CXCL2 secretion from monocytes during LPS stimulation. PBMCs from healthy controls were stimulated with (A) LPS or (B) peptidoglycan (PGN) without and with increasing concentrations of BPI for 18 hours. Secretion of CXCL2 into the media was determined by enzyme-linked immunosorbent 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).



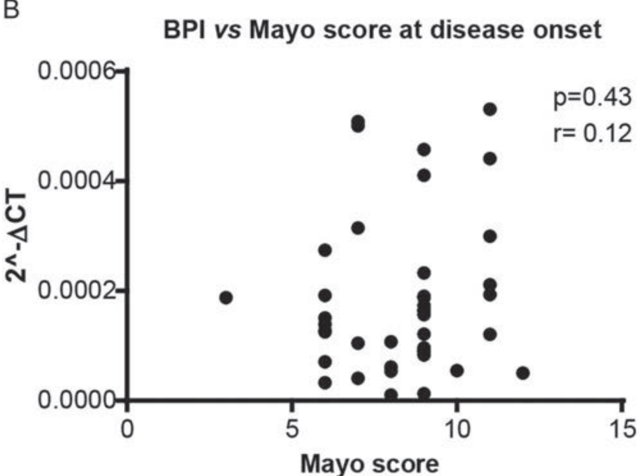


FIGURE 6. Mucosal gene expression of BPI in patients with newly diagnosed UC correlates to the number of flares during the 3 years follow-up. Rectal mucosal biopsies obtained from patients with newly diagnosed UC (n = 44) were analyzed for mRNA expression of *BPI* using RT-PCR. Gene expression was correlated 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 (≤2) or many flares (≥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

Exploring Gut Microbiota Composition as an Indicator of Clinical Response to Dietary FODMAP Restriction in Patients with Irritable Bowel Syndrome

Jørgen Valeur[1] · Milada Cvancarova Småstuen[2] · Torunn Knudsen[3] · Gülen Arslan Lied[4,5] · Arne Gustav Røseth[6]

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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

✉ Jørgen Valeur
jorgen.valeur@lds.no

Milada Cvancarova Småstuen
miladacv@medisin.uio.no

Torunn Knudsen
torunn.knudsen@lds.no

Gülen Arslan Lied
gulen.arslan@uib.no

Arne Gustav Røseth
arneroseth@gmail.com

[1] Unger-Vetlesen Institute, Lovisenberg Diaconal Hospital, Lovisenberggata 21, 0440 Oslo, Norway

[2] Faculty of Health Sciences, Oslo and Akershus University College of Applied Sciences, Oslo, Norway

[3] Department of Clinical Nutrition, Lovisenberg Diaconal Hospital, Oslo, Norway

[4] Department of Clinical Medicine, Center for Nutrition, University of Bergen, Bergen, Norway

[5] Department of Medicine, Section of Gastroenterology, Haukeland University Hospital, Bergen, Norway

[6] Department of Medicine, Lovisenberg Diaconal Hospital, Oslo, Norway

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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

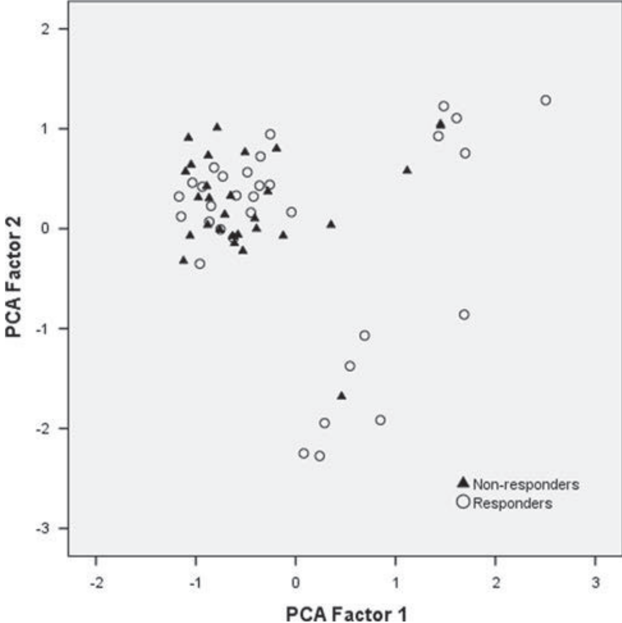

Fig. 1 Principal component analysis (PCA) showing a decomposition of gut microbiota markers using two-factor solution, as assessed by the GA-map™ Dysbiosis Test. The PCA confirmed that it is possible to distinguish between patients with IBS classified as responders ($n = 32$, depicted with open circles) 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

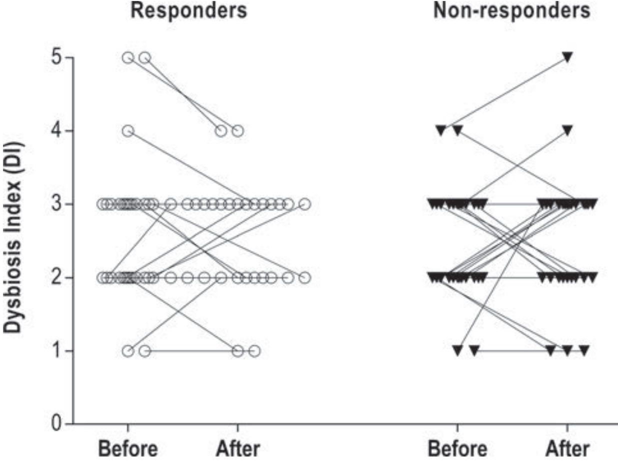

Fig. 2 Dysbiosis Index (DI), as provided by the producer of the GA-map™ Dysbiosis Test, assessed before and after a 4-week FODMAP-restricted diet in IBS patients defined as responders ($n = 32$) and non-responders ($n = 29$). Each line represents one individual

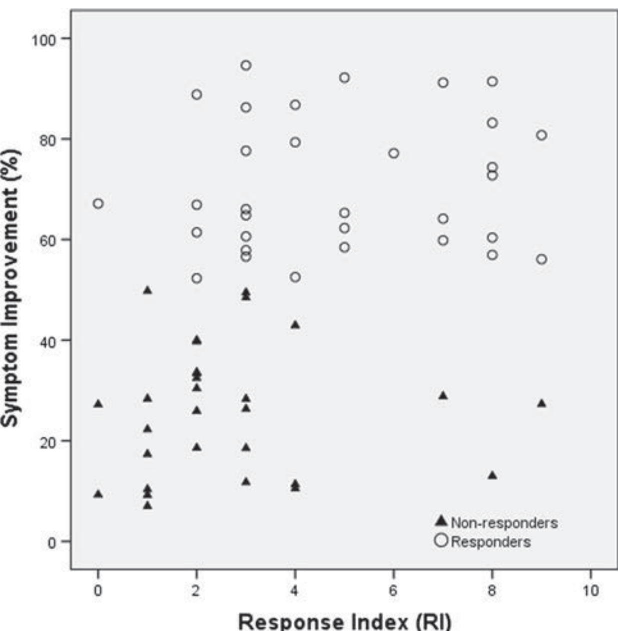

Fig. 3 Association between Response Index (RI), based on gut microbiota composition results, and symptom response, as assessed as reduction in IBS-SSS in percent from baseline values. There was a statistically significant, however, low to moderate correlation between the variables (Spearman's rho = 0.44, $p < 0.001$)

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

Multivariate modelling of faecal bacterial profiles of patients with IBS predicts responsiveness to a diet low in FODMAPs

Sean M P Bennet,[1,2] Lena Böhn,[1,3] Stine Störsrud,[1,3] Therese Liljebo,[4] Lena Collin,[5] Perjohan Lindfors,[1,5,6] Hans Törnblom,[1,3] Lena Öhman,[1,2,7] Magnus Simrén[1,3]

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For numbered affiliations see end of article.

Correspondence to
Professor Magnus Simrén, Department of Internal Medicine and Clinical Nutrition, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg 405 30, Sweden; magnus.simren@medicine.gu.se

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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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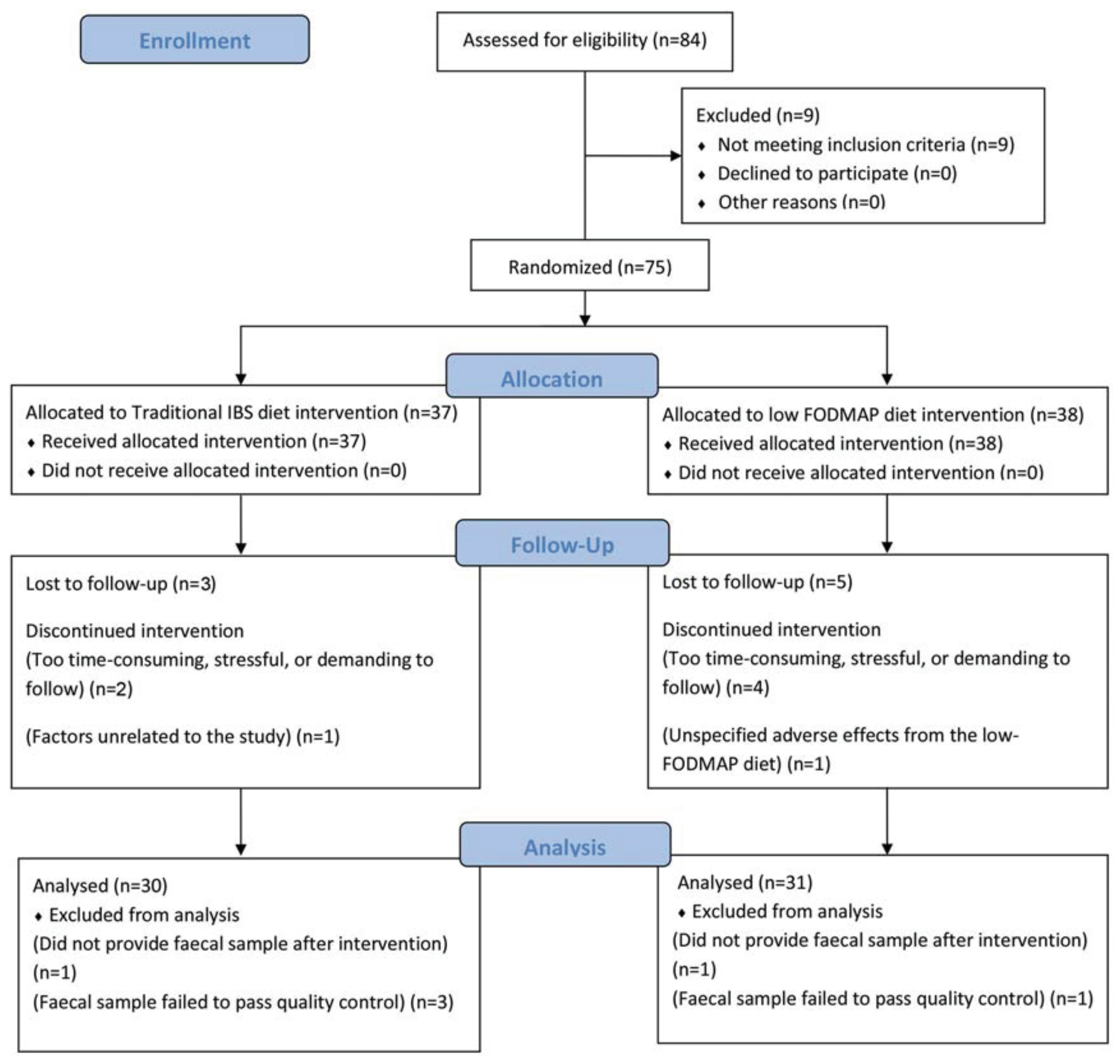

Figure 1 CONSORT flow diagram. Flow chart depicting patient numbers during the different phases of the study. FODMAP, fermentable oligosaccharides, disaccharides, monosaccharides and polyols.

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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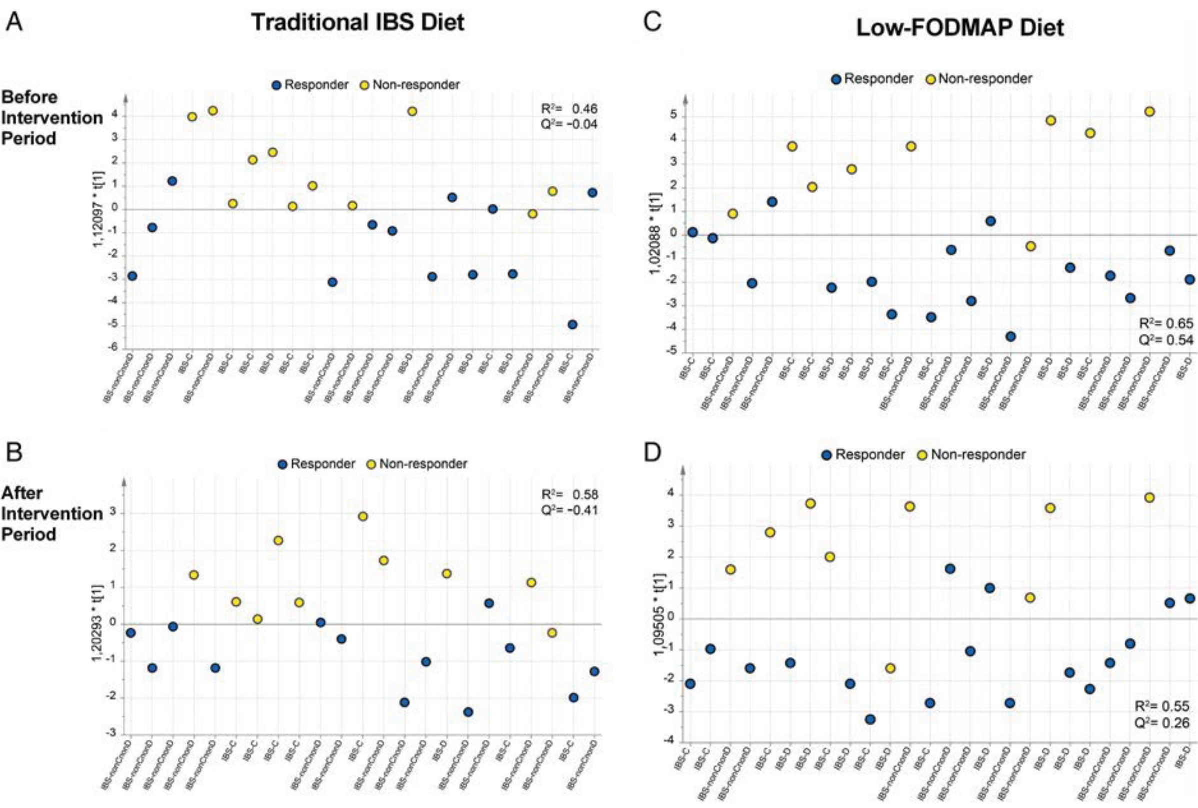

Figure 2 Bacterial profile analysis of non-responders and responders to dietary intervention. The GA-map Dysbiosis Test[22] analysing signals from 54 probes targeting ≥300 bacteria on different taxonomic levels was used to create bacterial profiles for patients undergoing either traditional IBS diet (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 (n=30)	*Phascolarctobacterium**	25 (0–207)	0 (0–20)	0.03	0.8
	Firmicutes (*Bacilli* and *Clostridia*)*	109 (87–173)	168 (132–221)	0.03	0.8
Low FODMAP diet (n=31)	*Acinetobacter*	201.8 (199.7–203)	204 (202–206)	0.005	0.07
	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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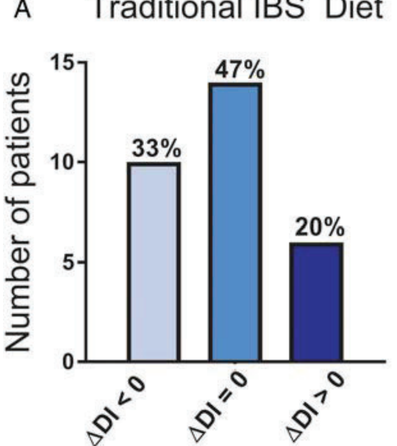

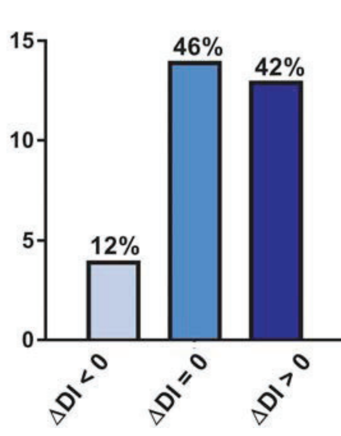

Figure 3 Change in Dysbiosis Index scores from before to after the traditional IBS diet or low fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAP) intervention period. The composition of faecal bacteria was determined by the GA-map Dysbiosis Test[22] in patients following 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

Distribution of patients		Frequency of patients who had a change in Dysbiosis Index (%) 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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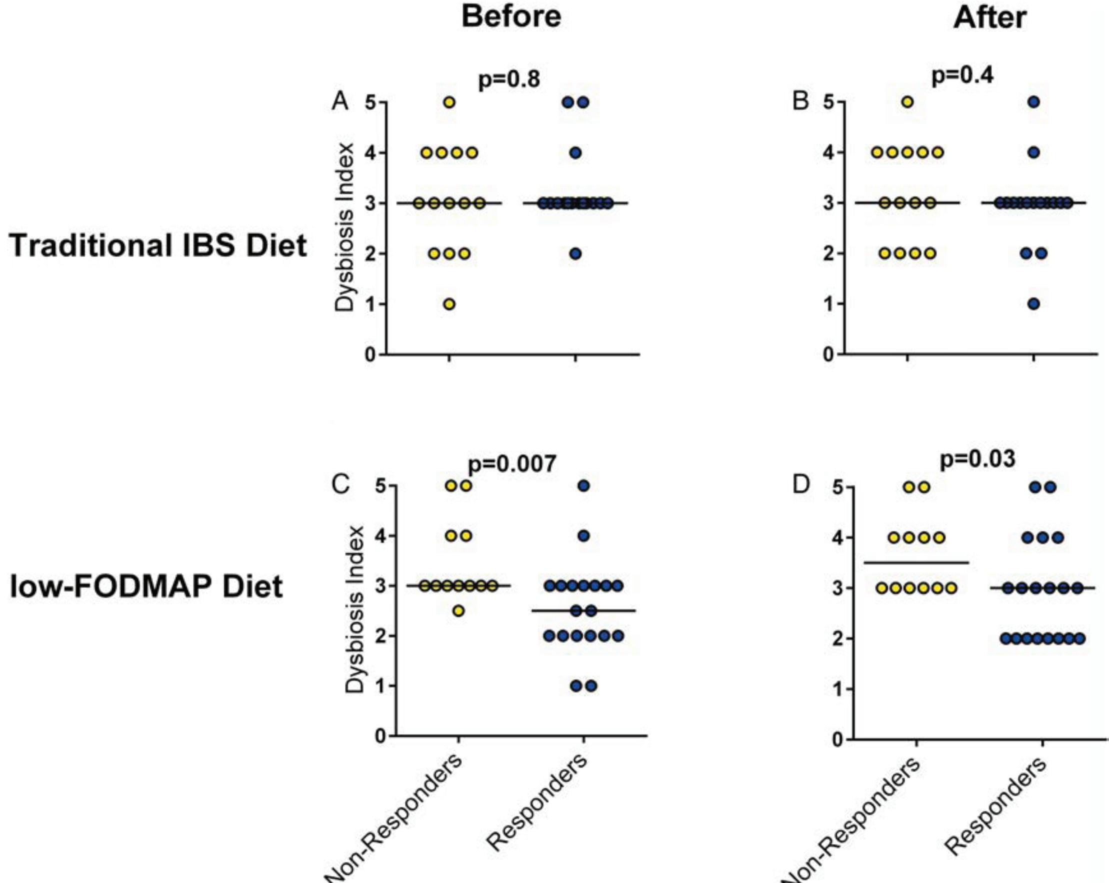

Figure 4 Comparison of Dysbiosis Index between non-responders and responders both before and after the respective intervention diets. The composition of faecal bacteria was determined by the GA-map Dysbiosis Test[22] in patients following traditional IBS dietary advice (n=30) or low fermentable oligosaccharides, 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.

Author affiliations
[1]Department of Internal Medicine and Clinical Nutrition, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden
[2]Department of Microbiology and Immunology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden
[3]Centre for Person-Centred Care (GPCC), Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden
[4]Department of Nutrition, Karolinska University Hospital, Stockholm, Sweden
[5]Department of Gastroenterology, Sabbatsbergs Hospital, Stockholm, Sweden
[6]Department of Medicine, Huddinge, Karolinska Institutet, Stockholm, Sweden
[7]School of Health and Education, University of Skövde, Skövde, Sweden

Contributors SB: data acquisition, assemblage of database, interpretation of data and drafting and finalising of manuscript. Has approved the final draft submitted. LB: assemblage of database. Has approved the final draft submitted. HT: collection of study subject materials, interpretation of data and finalising of manuscript. Has

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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.

* Correspondence: per.farup@ntnu.no
[1]Department of Research, Innlandet Hospital Trust, N-2381 Brumunddal, Norway
[2]Unit for Applied Clinical Research, Department of Clinical and Molecular Medicine, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology, N-7491 Trondheim, Norway
Full list of author information is available at the end of the article

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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.

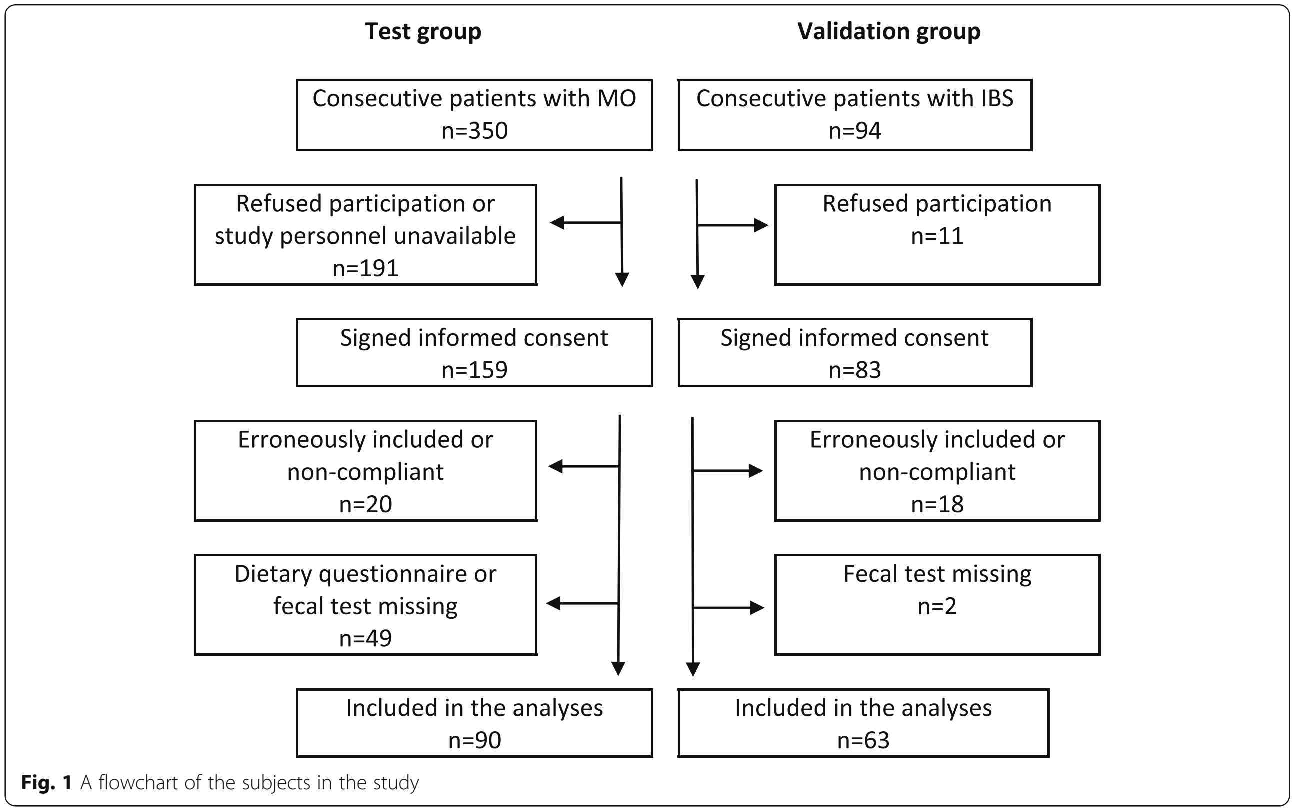

Fig. 1 A flowchart of the subjects in the study

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

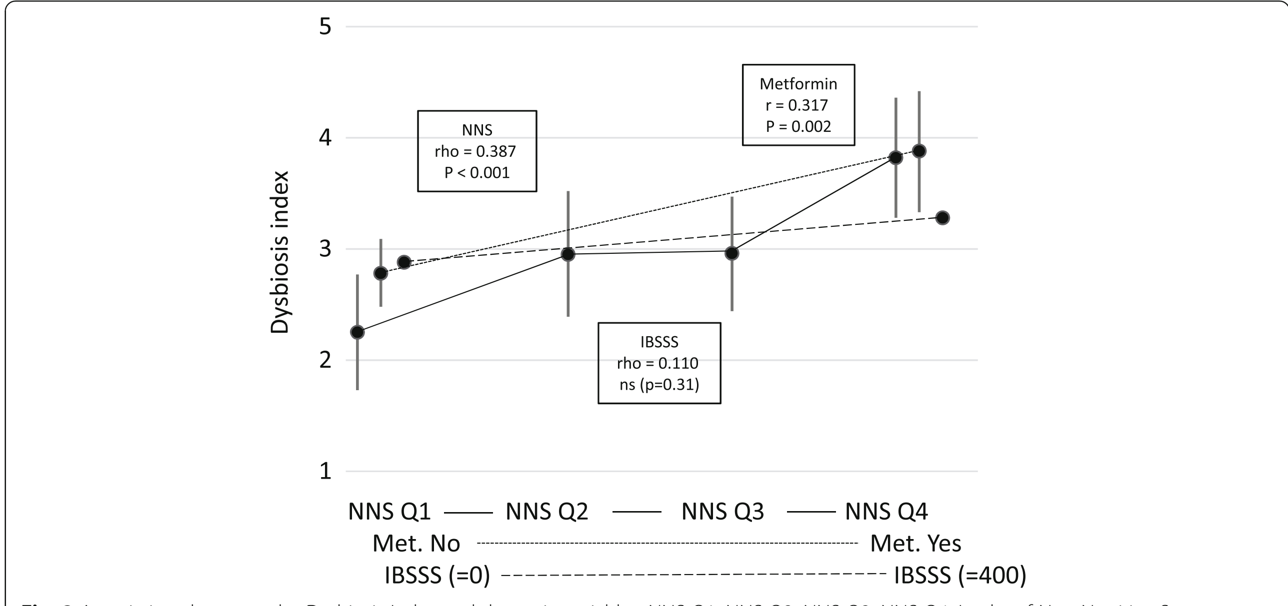

Fig. 2 Associations between the Dysbiosis Index and the main variables. NNS Q1, NNS Q2, NNS Q3, NNS Q4: Intake of Non-Nutritive Sweeteners divided into quartiles. Met: Metformin. IBSSS: Irritable Bowel Severity Scoring System. The results for NNS and Met are given as mean with 95% CI. The associations 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

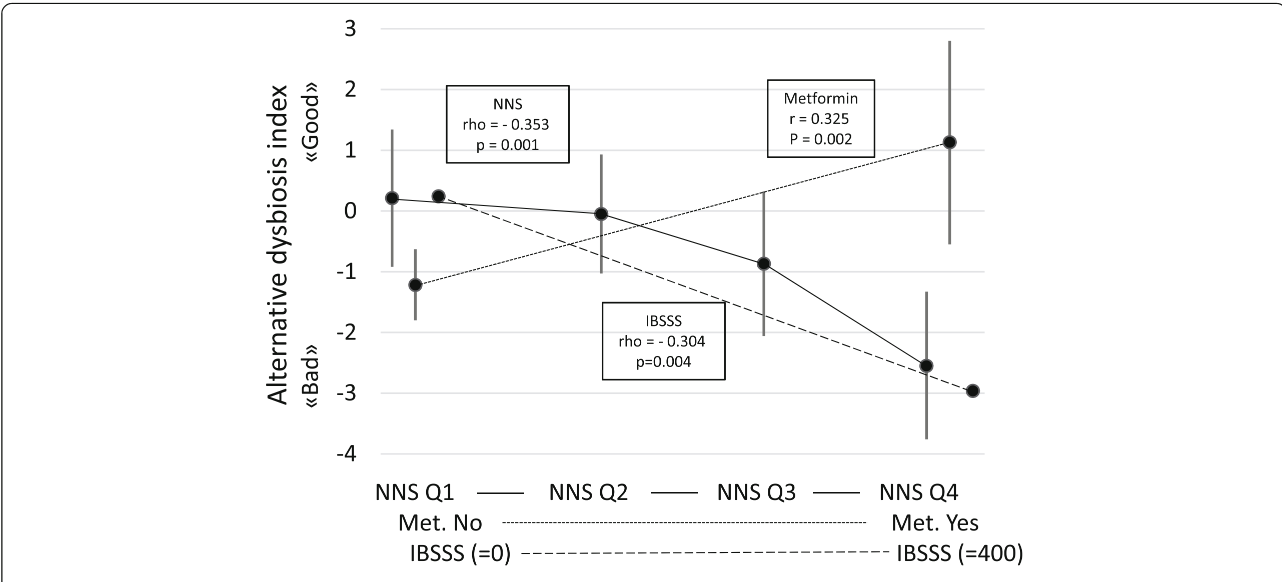

Fig. 3 Associations between the Alternative Dysbiosis Index and the main variables. NNS Q1, NNS Q2, NNS Q3, NNS Q4: Intake of Non-Nutritive Sweeteners divided into quartiles. Met: Metformin. IBSSS: Irritable Bowel Severity Scoring System. The results for NNS and Met are given as mean with 95% CI. The 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 Test group (IBS no/yes: no 63/25)	ADI Validation group (IBS yes: 63)	Statistics *p*-value
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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Author details
[1]Department of Research, Innlandet Hospital Trust, N-2381 Brumunddal, Norway. [2]Unit for Applied Clinical Research, Department of Clinical and Molecular Medicine, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology, N-7491 Trondheim, Norway. [3]Department of Surgery, Innlandet Hospital Trust, N-2819 Gjøvik, Norway. [4]Novo Nordisk Foundation Center for Basic Metabolic Research, Section of Metabolic Genetics, Faculty of Health and Medical Sciences, University of Copenhagen, DK-2200 Copenhagen, Denmark. [5]Unger-Vetlesen Institute, Lovisenberg Diaconal Hospital, N-0440 Oslo, Norway.

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Article

Faecal Microbial Markers and Psychobiological Disorders in Subjects with Morbid Obesity. A Cross-Sectional Study

Per G Farup [1,2,*] and Jørgen Valeur [3]

1 Department of Research, Innlandet Hospital Trust, PB 104, N-2381 Brumunddal, Norway
2 Unit for Applied Clinical Research, Department of Clinical and Molecular Medicine, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology, N-7491 Trondheim, Norway
3 Unger-Vetlesen Institute, Lovisenberg Diaconal Hospital, N-0440 Oslo, Norway; Jorgen.Valeur@lds.no
* Correspondence: per.farup@ntnu.no; Tel.: +47-94-818-603; Fax: +47-61-157-437

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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 ≤ 50 indicate low mood and scores ≤ 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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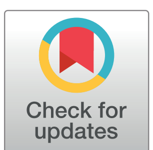
RESEARCH ARTICLE

The kinetics of gut microbial community composition in patients with irritable bowel syndrome following fecal microbiota transplantation

Tarek Mazzawi[1,2,3]*, **Gülen Arslan Lied**[1,2,3], **Dag André Sangnes**[1], **Magdy El-Salhy**[2,3,4], **Johannes R. Hov**[5], **Odd Helge Gilja**[1,2,3], **Jan Gunnar Hatlebakk**[1,2,3], **Trygve Hausken**[1,2,3]

1 Section of Gastroenterology, Department of Medicine, Haukeland University Hospital, Bergen, Norway, **2** Norwegian Competence Centre for Functional Gastro-Intestinal Disorders, Section of Gastroenterology, Department of Medicine, Haukeland University Hospital, Bergen, Norway, **3** Center for Nutrition, Department of Clinical Medicine, University of Bergen, Bergen, Norway, **4** Section of Gastroenterology, Department of Medicine, Stord Hospital, Helse-Fonna, Stord, Norway, **5** Norwegian PSC Research Center, Section of Gastroenterology and Research Institute of Internal Medicine, Division of Surgery, Inflammatory Diseases and Transplantation, Oslo University Hospital and University of Oslo, Oslo, Norway

* tarek.mazzawi@gmail.com



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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

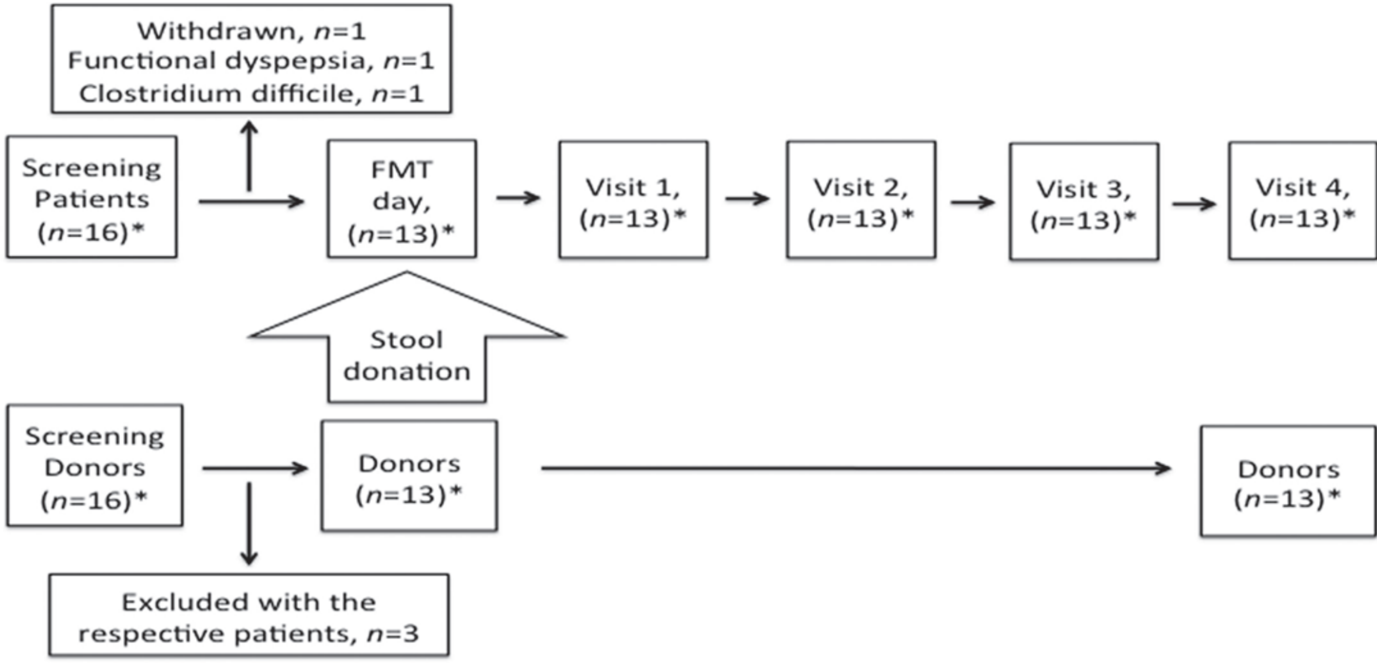

Fig 1. Study flow chart.

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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

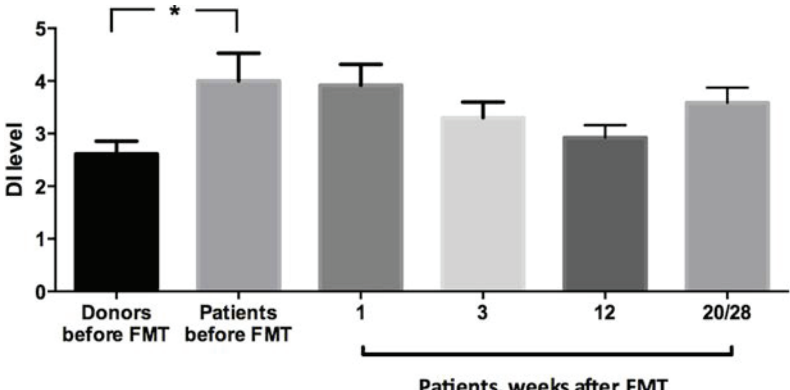

Fig 2. Dysbiosis index of the donors before fecal microbiota transplantation (FMT) and for the patients before FMT (day 0) and following FMT (weeks 1, 3, 12 and 20/28).

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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.

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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

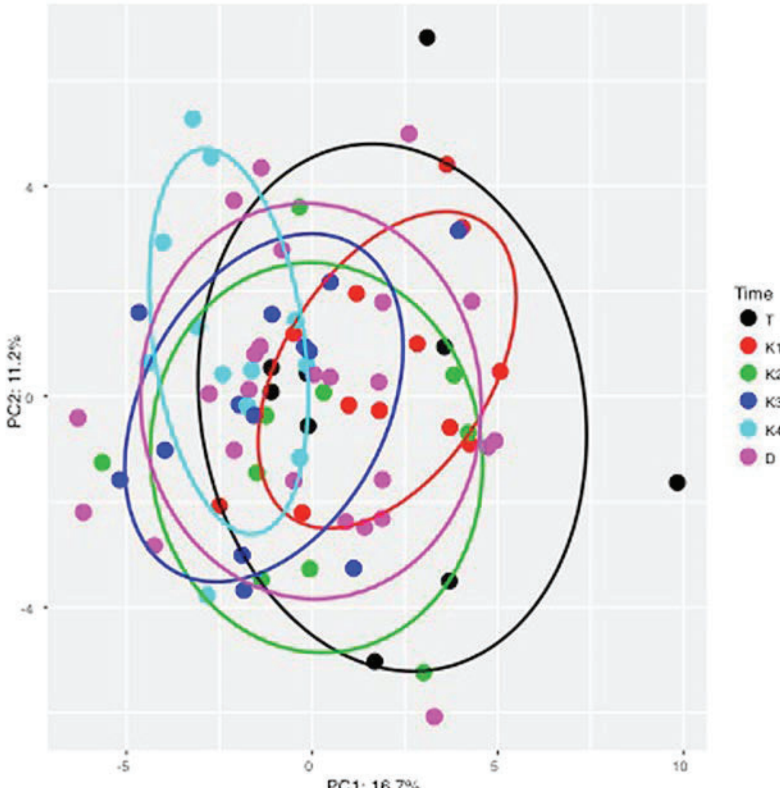

Fig 3. Scores for the first two principal component analysis (PCA) of fecal microbiota in donors at the beginning of the study and patients with irritable bowel syndrome after fecal microbiota transplantation (FMT, *n* = 13). Data have been centered within each donor series to remove donor differences 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.

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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.

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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.

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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

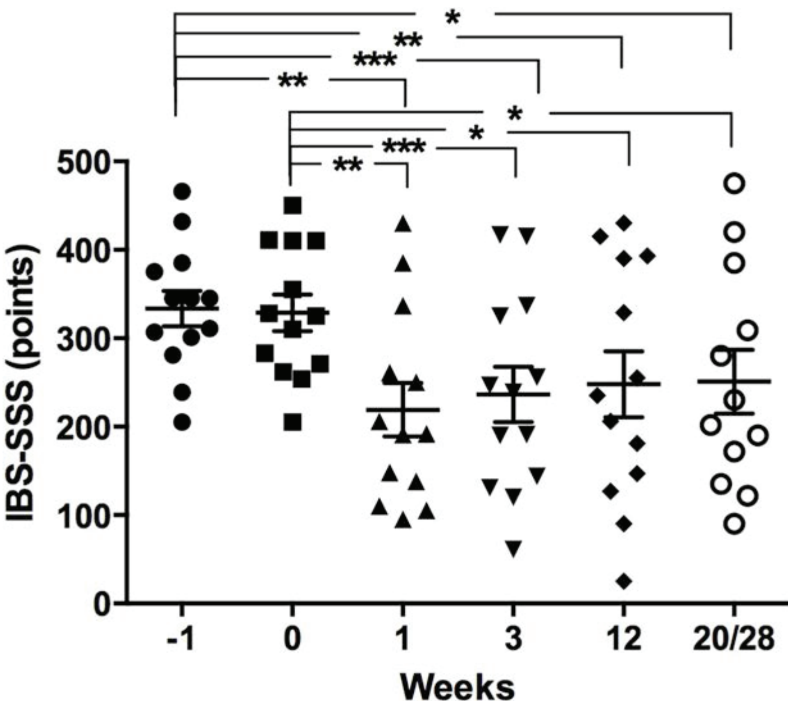

Fig 5. IBS-SSS scores of the patients one week before fecal microbiota transplantation (FMT, week -1), at FMT day (0), and following FMT (weeks 1, 3, 12 and 20/28).

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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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Author Contributions

Data curation: Gülen Arslan Lied, Johannes R. Hov.

Formal analysis: Tarek Mazzawi.

Funding acquisition: Johannes R. Hov, Trygve Hausken.

Investigation: Tarek Mazzawi, Gülen Arslan Lied, Trygve Hausken.

Methodology: Tarek Mazzawi, Gülen Arslan Lied, Dag André Sangnes, Jan Gunnar Hatlebakk.

Project administration: Tarek Mazzawi, Gülen Arslan Lied, Dag André Sangnes, Magdy El-Salhy, Odd Helge Gilja, Jan Gunnar Hatlebakk, Trygve Hausken.

Supervision: Tarek Mazzawi, Magdy El-Salhy, Odd Helge Gilja, Jan Gunnar Hatlebakk, Trygve Hausken.

Writing – original draft: Tarek Mazzawi.

Writing – review & editing: Tarek Mazzawi, Gülen Arslan Lied, Dag André Sangnes, Magdy El-Salhy, Johannes R. Hov, Odd Helge Gilja, Jan Gunnar Hatlebakk, Trygve Hausken.

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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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Christine Olbjørn,[1,2] Milada Cvancarova Småstuen,[3] Espen Thiis-Evensen,[4] Britt Nakstad,[1,2] Morten Harald Vatn,[5] Jørgen Jahnsen,[2,6] Petr Ricanek,[2,6] Simen Vatn,[2,6] Aina EF Moen,[5] Tone M Tannæs,[5] Jonas C Lindstrøm,[7] Johan D Söderholm,[8] Jonas Halfvarson,[9] Fernando Gomollón,[10] Christina Casén,[11] Magdalena K Karlsson,[11] Rahul Kalla,[12] Alex T Adams,[12,13] Jack Satsangi,[12,13] Gøri Perminow[14]

[1]Department of Pediatric and Adolescent Medicine, Akershus University Hospital, Lørenskog, Norway; [2]Institute of Clinical Medicine, Campus Ahus, University of Oslo, Oslo, Norway; [3]Faculty of Health Sciences, Oslo Metropolitan University, Oslo, Norway; [4]Department of Gastroenterology, Oslo University Hospital, Rikshospitalet, Oslo, Norway; [5]Department of Clinical Molecular Biology (EpiGen), Division of Medicine, Akershus University Hospital, Lørenskog, and University of Oslo, Oslo, Norway; [6]Department of Gastroenterology, Akerhus University Hospital, Lørenskog, Norway; [7]Institute of Clinical Medicine, University of Oslo, Health Services Research Unit, Akershus University Hospital, Lørenskog, Norway; [8]Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Linköping, Sweden; [9]Department of Clinical and Experimental Medicine, Faculty of Medicine and Health Sciences, Linköping University, Linköping, Sweden; [10]Digestive Diseases Unit, IIS Aragón, Zaragoza, Spain; [11]Genetic-Analysis AS, Oslo, Norway; [12]Gastrointestinal Unit, Centre for Genomics and Molecular Medicine, University of Edinburgh, Edinburgh, UK; [13]Translational Gastroenterology Unit, Experimental Medicine Division, University of Oxford, Oxford, UK; [14]Department of Pediatrics, Oslo University Hospital, Ullevål, Oslo, Norway

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.

Correspondence: Christine Olbjørn
Department of Pediatric and Adolescent Medicine, Akershus University Hospital, Sykehusveien 25, 1474 Lørenskog, Norway
Tel +47 9 302 1216
Email chrisolb@gmail.com

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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

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.

Wilcoxon signed-rank tests (before and after treatment) for continuous variables and chi-squared tests for categorical data.

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.

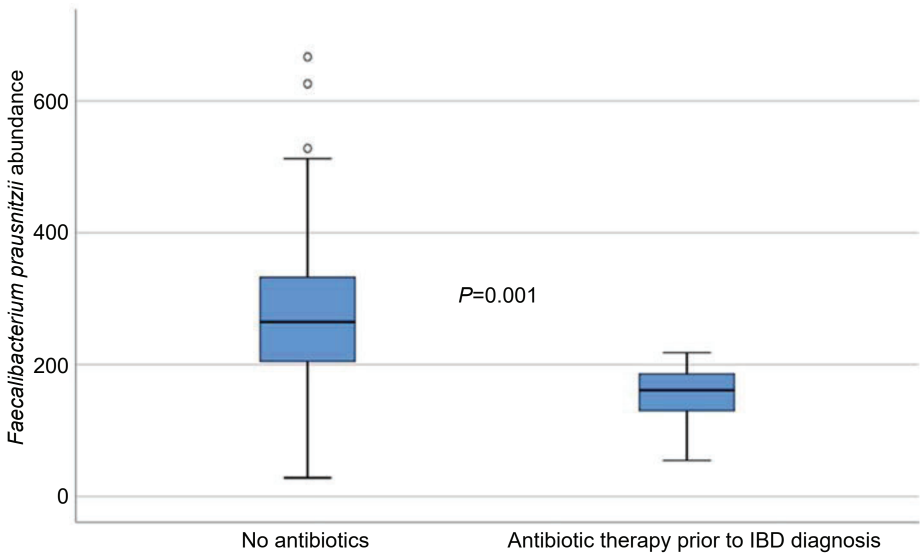

Figure 1 *Faecalibacterium prausnitzii* abundance in IBD patients according to whether they had received antibiotics prior to the diagnosis (measured in fluorescence signal strength in 1,000 units).
Abbreviation: IBD, inflammatory bowel disease.

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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

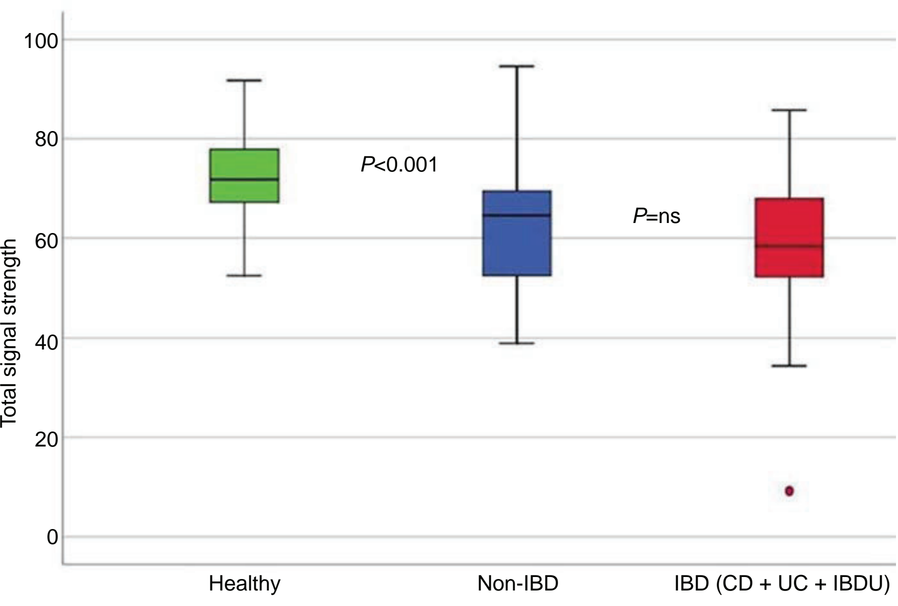

Figure 2 Boxplot illustrating the differences in the total fluorescence signal strength measured in 1,000 units between IBD, non-IBD symptomatic patients, and healthy controls.

Abbreviations: IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; IBDU, inflammatory bowel disease unclassified; ns, not significant.

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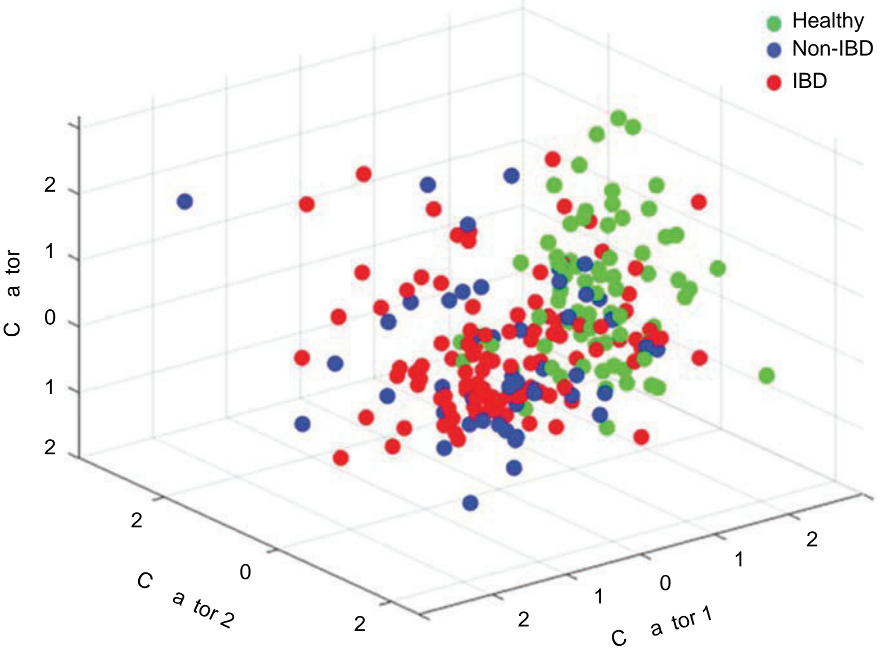

Figure 3 Principal component analysis, illustrating the difference in microbiota abundance of all 54 bacterial probes between IBD, non-IBD symptomatic patients, and healthy controls.
Notes: Each dot represents one individual. The units represent the total item loadings on each of the extracted factors.
Abbreviations: IBD, inflammatory bowel disease; PCA, principal component analysis.

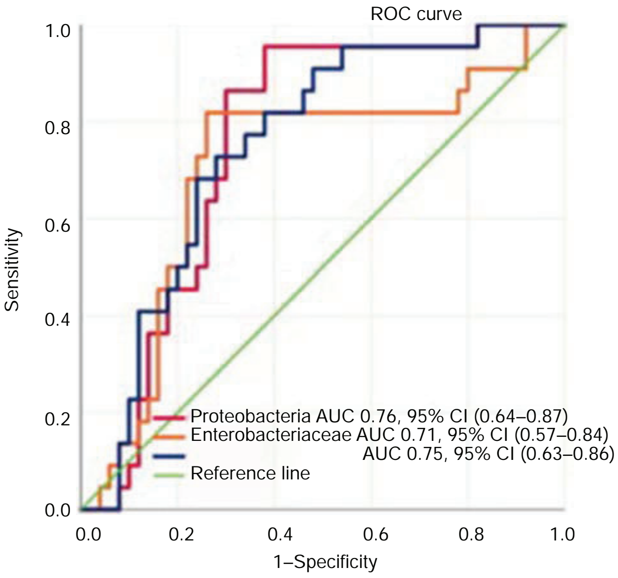

Figure 4 Sensitivity and specificity of Proteobacteria, *Enterobacteriaceae,* and *Shigella/Escherichia* abundance in differentiating Crohn's disease phenotypes (stricturing/penetrating vs inflammatory disease behavior) using the area under the receiver operating characteristics curve analysis.
Abbreviations: 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,

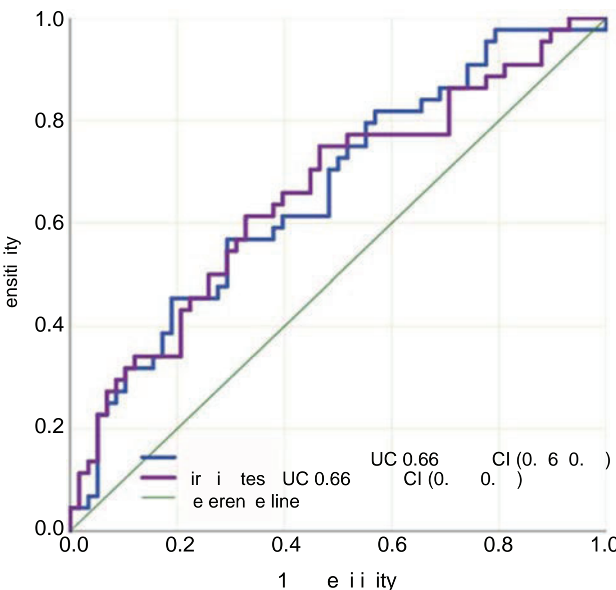

Figure 5 Sensitivity and specificity of Firmicutes and *Mycoplasma hominis* abundance in differentiating conventional- vs biologic therapy-treated IBD patients using the area under the receiver operating characteristics curve analysis.
Abbreviations: IBD, inflammatory bowel disease; CD, Crohn's disease; AUC, area under the curve.

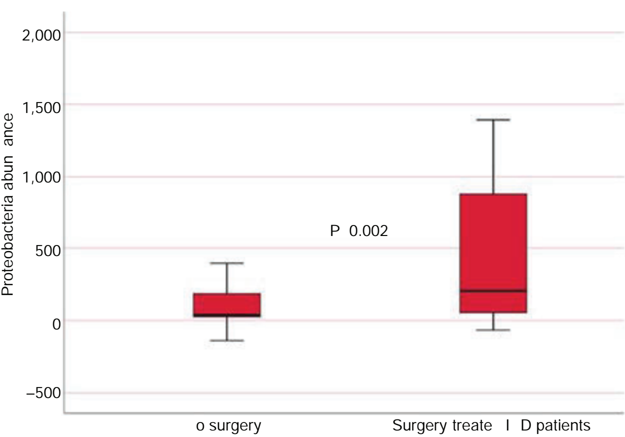

Figure 6 Proteobacteria abundance in IBD patients according to whether they needed surgery or not (measured in fluorescence signal strength in 1,000 units).
Abbreviation: IBD, inflammatory bowel disease.

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

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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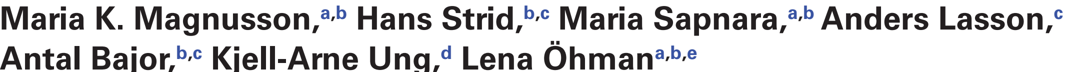
Anti-TNF Therapy Response in Patients with Ulcerative Colitis Is Associated with Colonic Antimicrobial Peptide Expression and Microbiota Composition

Maria K. Magnusson,[a,b] Hans Strid,[b,c] Maria Sapnara,[a,b] Anders Lasson,[c] Antal Bajor,[b,c] Kjell-Arne Ung,[d] Lena Öhman[a,b,e]



[a]Department of Microbiology and Immunology, University of Gothenburg, Institute for Biomedicine, Gothenburg, Sweden [b]Department of Internal Medicine and Clinical Nutrition, University of Gothenburg, Institute for Medicine, Gothenburg, Sweden [c]Södra Älvsborg Hospital, Department of Internal Medicine, Borås, Sweden [d]Kärnsjukhuset, Department of Internal Medicine, Skövde, Sweden [e]School of Health and Education, University of Skövde, Skövde, Sweden

Corresponding author: Maria K. Magnusson, PhD, Department of Microbiology and Immunology, University of Gothenburg, Box 435, 405 30 Gothenburg, Sweden. Tel: +46 31 786 6220; email: maria.magnusson@microbio.gu.se

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

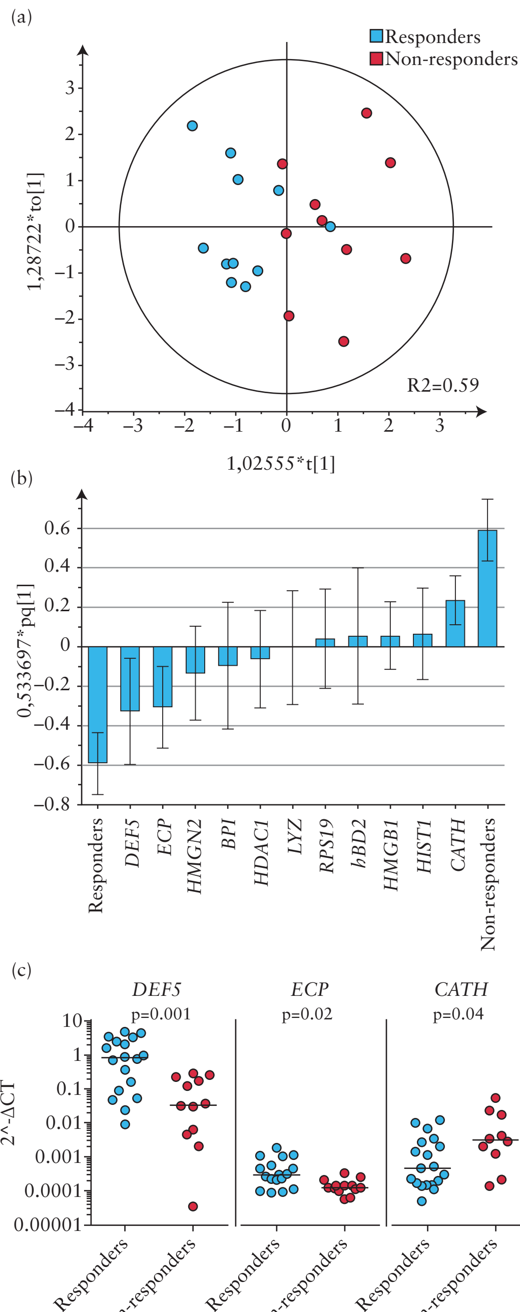

Figure 1. Anti-TNF therapy responders and non-responders show different mucosal AMP expression before start of treatment. Biopsy samples were obtained at baseline and mucosal mRNA expression of *DEF5*, *ECP*, *HMGN2*, *BPI*, *HDAC1*, *LYZ*, *RPS19*, *hBD2*, *HMGB1*, *HIST1*, and *CATH* was analysed with 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].

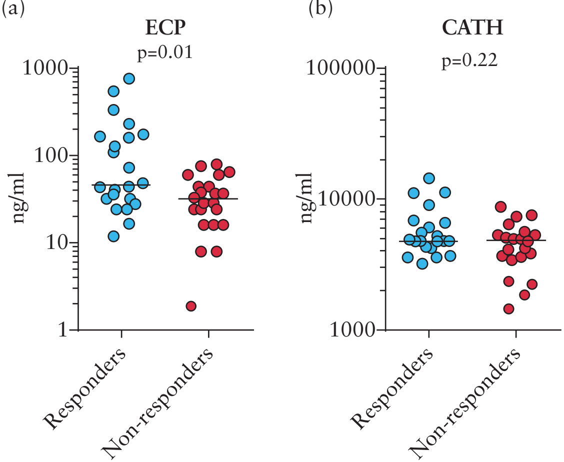

Figure 2. Serum levels of ECP and CATH in anti-TNF therapy responders and non-responders. Serum samples were obtained at baseline [responders $n = 23$ and non-responders $n = 21$], and levels of ECP [A] and CATH [B] were analysed by ELISA. Each symbol represents one individual and horizontal lines indicates 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

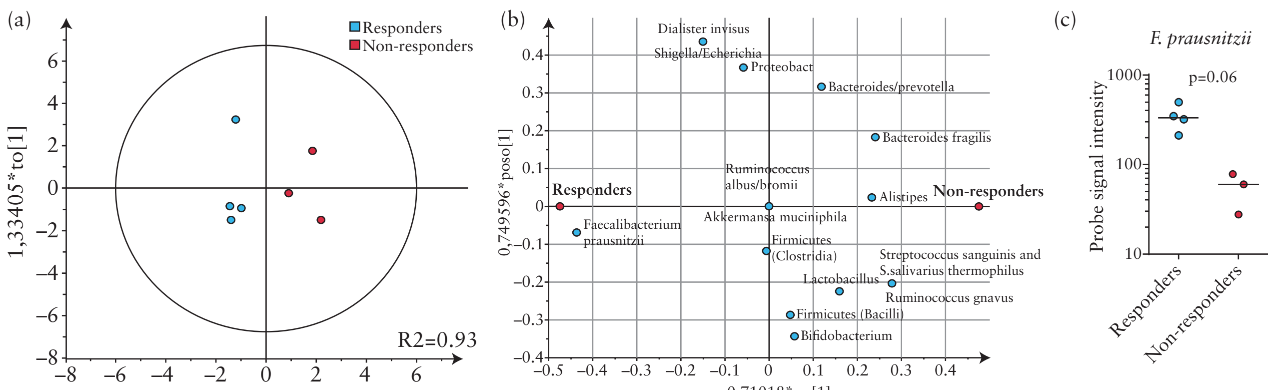

Figure 3. Anti-TNF therapy responders and non-responders differ at baseline with respect to their fecal microbiota composition. Fecal samples were obtained at baseline [responders $n = 4$ and non-responders $n = 3$] and analysed by the GA-map™ Dysbiosis Test. OPLS-DA scatter plot [A] and loading scatter 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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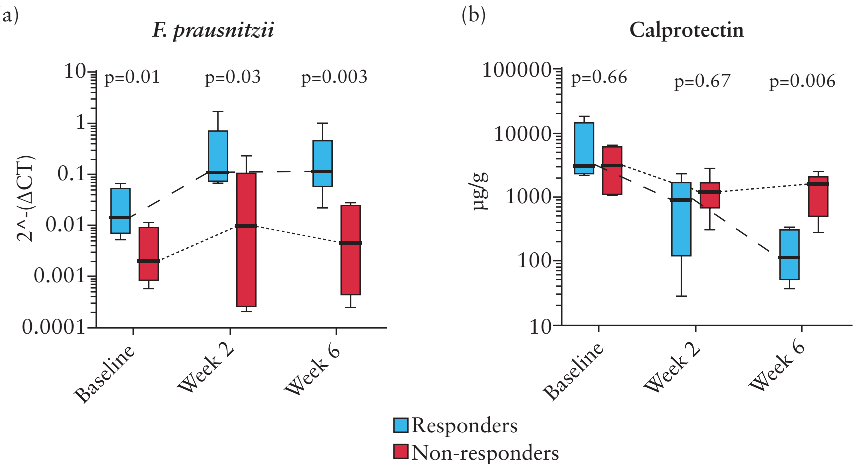

Figure 4. Anti-TNF therapy responders show higher abundance of *F. prausnitzii* compared with non-responders. Fecal samples were obtained at baseline [responders $n = 4$ and non-responders $n = 3$], Week 2 [responders $n = 8$ and non-responders $n = 7$], and Week 6 [responders $n = 8$ and non-responders $n = 5$]. [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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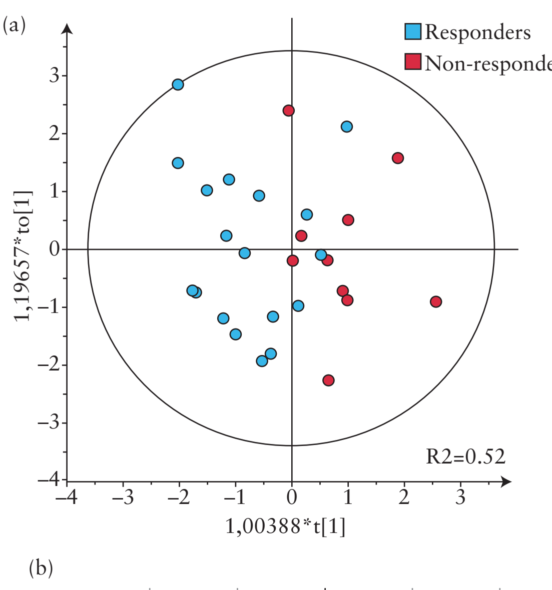

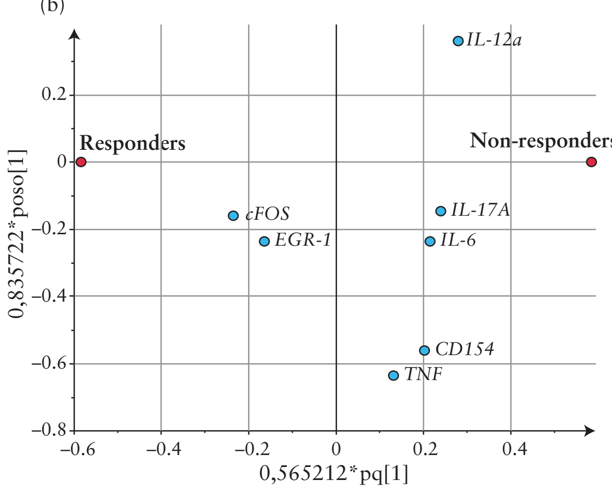

Figure 5. Multivariate factor analysis depicting associations between anti-TNF therapy response and genes involved in mucosal inflammation and regulation. Biopsy samples were obtained at baseline [responders n = 18 and non-responders n = 12] and mucosal mRNA expression *IL-6*, *IL-12a*, *IL17A*, *TNF*, *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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