Publications

2019

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]

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.
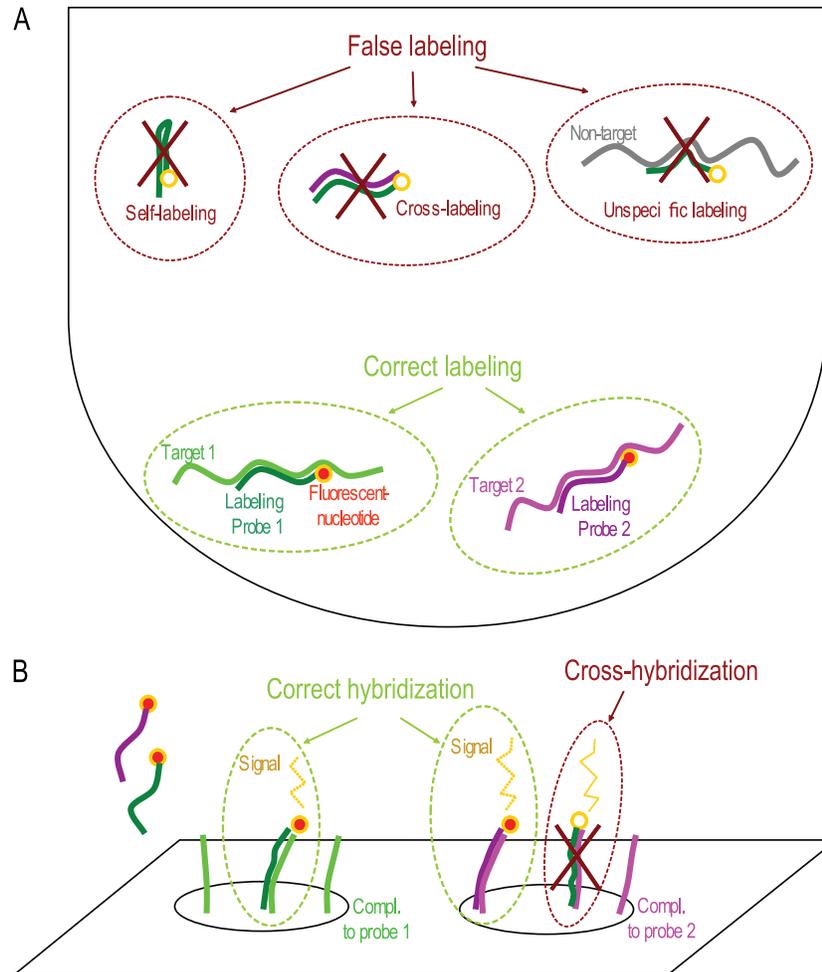
FIG. 1. Illustration of the principle and challenges associated with the GA-map array technology. In the labeling step, there are several ways probes can be nonspecifically labeled through self-labeling (internal regions of the probe are used as targets), cross-labeling (a region internal to another 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$_2$O, 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

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

of target and nontarget groups based on a coordinate classification system (see Fig. S1A in the supplemental material). (ii) The next step was to identify probes that satisfied the criteria of target detection and nontarget exclusion. This was based on combined criteria of hybridization and labeling. All probes were designed with a minimum melting temperature (T_m) of 60°C by the nearest-neighbor method for the target group, while the nontarget group should have a T_m of <30°C or absence of a cytosine as the nucleotide adjacent to the 3′ end of the probe. All probes satisfying the criteria were identified (see Fig. S1B in the supplemental material). (iii) Then, the potential cross-labeling or self-labeling probes were evaluated, in addition to potential cross hybridization on the array (see Fig. S1C in the supplemental material). (iv) Finally, by combining the knowledge about target/nontarget groups and compatibility for each of the probes, final arrays were designed using a hierarchical approach.

The strategy for searching for the most appropriate probe sets is described in detail in the supplemental material.

A universal 16S rRNA gene probe (UNI01) (13) was included in the probe sets to measure the total abundance of bacterial DNA in the sample. One additional probe was added in the hybridization step: a 1:4 mixture of prelabeled and unlabeled hybridization control probe (HYC01). HYC01 is used to measure the efficiency of the hybridization step on the slide and to normalize the probe signals between slides. The microarrays used in the GA-map infant assay were superaldehyde slides produced by ArrayIt (Sunnyvale, CA) spotted as described on the company's homepage. One glass slide contains 24 separate identical microarrays, and the probes (complementary to the probes listed in Table 2) were spotted in triplicate on each array. Furthermore, the arrays also included two nonbinding control probes (NBC01 and NBC02) (28). An overview of the control probes found on the array and their sequences is shown in Table S3 in the supplemental material.

GA-map infant assay. Before the labeling reaction, the 16S rRNA gene PCR products (amplified as described above) were treated with 3 U exonuclease I (New England BioLabs, Ipswich, MA) and 8 U shrimp alkaline phosphatase (USB, Cleveland, OH) at 37°C for 2 h and inactivated at 80°C for 15 min. The exonuclease I-shrimp alkaline phosphatase (ExoSAP)-treated PCR products were then quantified using Kodak molecular imaging software (version 4.0) based on pictures from gel electrophoresis. A 1-kb DNA ladder (N3232; New

TABLE 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*)	GCACTCAAGACATCCAGTATCAACTG	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*	CAGGTGTAGCGGTGAAATGCGTAGAGAT	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*	TGTTGTGGTTAATAACCGCAGCAATTGA	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$_2$O (100°C) for 2 min and immediately transferred to a beaker containing 100% ethanol (−20°C) for 20 s before they were dried by centrifugation at 91 × g in a Multifuge 3 S-R centrifuge (Heraeus, Buckinghamshire, United Kingdom) for 12 min and used within an hour.

Immediately prior to the actual array hybridization, 60 μl hybridization buffer containing 7.2% polyethylene glycol 8000 (Sigma-Aldrich), 1.2× SSC (Sigma-Aldrich), and 0.17 μM hybridization control probe HYC01 mixture (a 1:4 mixture of TAMRA-labeled HYC01 and unlabeled HYC01) were added to the samples. The samples were denatured at 95°C for 2 min and then left at 45°C for 2 min. The glass slides were placed in a 96-well hybridization chamber (ArrayIt) before the samples were loaded onto the arrays. Two arrays per slide were used

for the positive- and negative-control samples. The hybridization chamber was placed in a humid chamber and hybridized for 16 h in an Innova 4000 incubator shaker (New Brunswick Scientific, Champaign, IL) at 45°C and 60 rpm.

After hybridization, the arrays were washed for 5 min in the wash buffer containing 2× SSC (Sigma-Aldrich) and 0.1% Sarkosyl (VWR International, Ltd.), then for 5 min in 2× SSC (Sigma-Aldrich), and finally for 10 s in 0.2× SSC (Sigma-Aldrich) before they were dried by centrifugation at 91 × g for 12 min in a Multifuge 3 S-R centrifuge (Heraeus). The hybridized arrays were scanned at a wavelength of 532 nm with a Tecan LS reloaded scanner (Tecan, Männedorf, Austria). Fluorescence intensities and spot morphologies were analyzed using Axon GenePix Pro 6.0. Pictures of two example arrays can be seen in Fig. S3 in the supplemental material.

Capillary electrophoresis. The GA-map labeling step was evaluated by capillary electrophoresis. To test the labeling, single probes were tested against their target bacteria (DNA from pure cultures and a complementary synthetic template with five additional nucleotides in both the 5′ and 3′ ends if a pure culture was lacking) by performing 16S rRNA gene PCR amplification for the pure DNA and labeling reactions as described above (with 1 μM single probes instead of probe set 3, which was used in the final assay), and the performances of the probes were evaluated using capillary electrophoresis. The compatibility of different sets of functioning probes (see Table S2 in the supplemental material) was also evaluated using capillary electrophoresis with water as the template and different probes sets (see Table S2 in the supplemental material) instead of probe set 3 in the labeling reaction described above. Furthermore, the reproducibility of the 16S rRNA gene PCR was examined on one of the samples (amplified in three separate PCRs) using capillary electrophoresis. Two probes (6_1_4 and 5_1_2) were chosen to examine the signal for each of the three PCR products, and a triplicate run on a pool of the three PCR products was also examined using
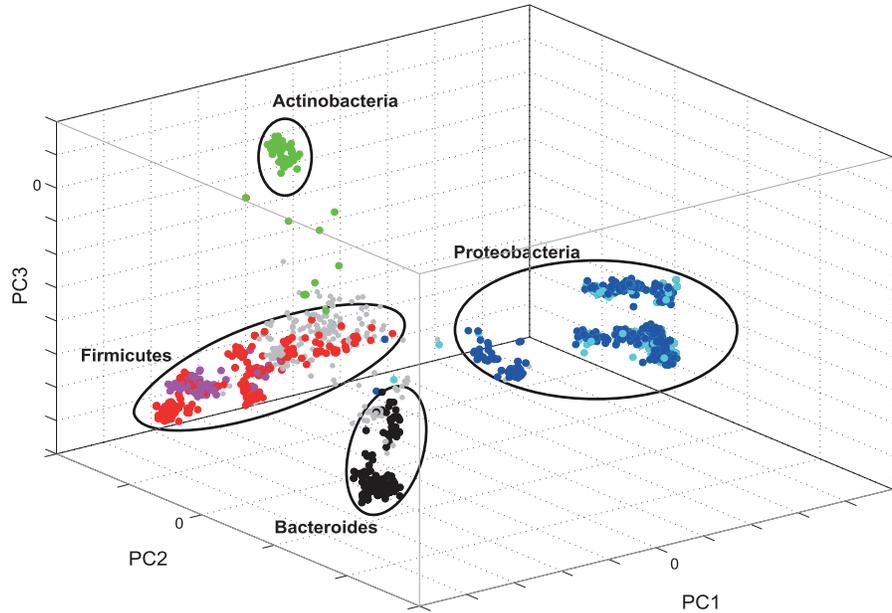
FIG. 2. Score plot of bacteria isolated from infant feces. Each object represents one bacterial clone. The objects are clustered based on 16S rRNA gene phylogeny. The colors indicate theoretical phylum probe specificities: 6_2, green; 5_1, red; 4_1, magenta; 2_1_min1b, cyan; 3_2, blue; and 1_1, black.The gray objects are not detected by any probes.

the same probes (see Fig. S2 in the supplemental material). After labeling, the samples were treated with 8 U SAP (USB), incubated at 37°C for 1 h, and inactivated at 80°C for 15 min. Then, 1 μl of the SAP-treated and labeled probes was mixed with 9 μl of Hi-Di formamide (Applied Biosystems, Warrington, United Kingdom) and 0.5 μl GeneScan 120 Liz Size Standard (Applied Biosystems), and the samples were incubated at 95°C for 5 min and immediately put on ice. The samples were then loaded onto a 50-cm 3130xl capillary array (Applied Biosystems) in the ABI Genetic Analyzer 3130xl sequencer (Applied Biosystems) containing the performance-optimized polymer 7 (POP-7; Applied Biosystems). The injection time was 16 to 22 s, and the electrophoretic conditions were as follows: run time, 1,500 s at 15,000 V; run current, 100 μA; run temperature, 60°C. GeneMapper 4.0 software was used to analyze the results.

DNA sequence analysis. The 16S rRNA gene PCR products from the 26 bacterial strains used to evaluate the probes were sequenced to confirm their identities and to examine if there were any mutations in their gene sequences compared to the sequences used to design the probes. The ExoSAP-treated PCR products were diluted 10-fold, and 1 μl was used in the sequencing reaction using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). The same forward and reverse primers used for the 16S rRNA PCR described above (0.32 μM) were used in two separate sequencing reactions. A BigDye XTerminator Purification Kit (Applied Biosystems. Warrington, United Kingdom) was used according to the manufacturer's recommendations to clean up the sequencing reactions. The samples were analyzed on a 36-cm 3130xl capillary array (Applied Biosystems) in the ABI Genetic Analyzer 3130xl sequencer (Ap-
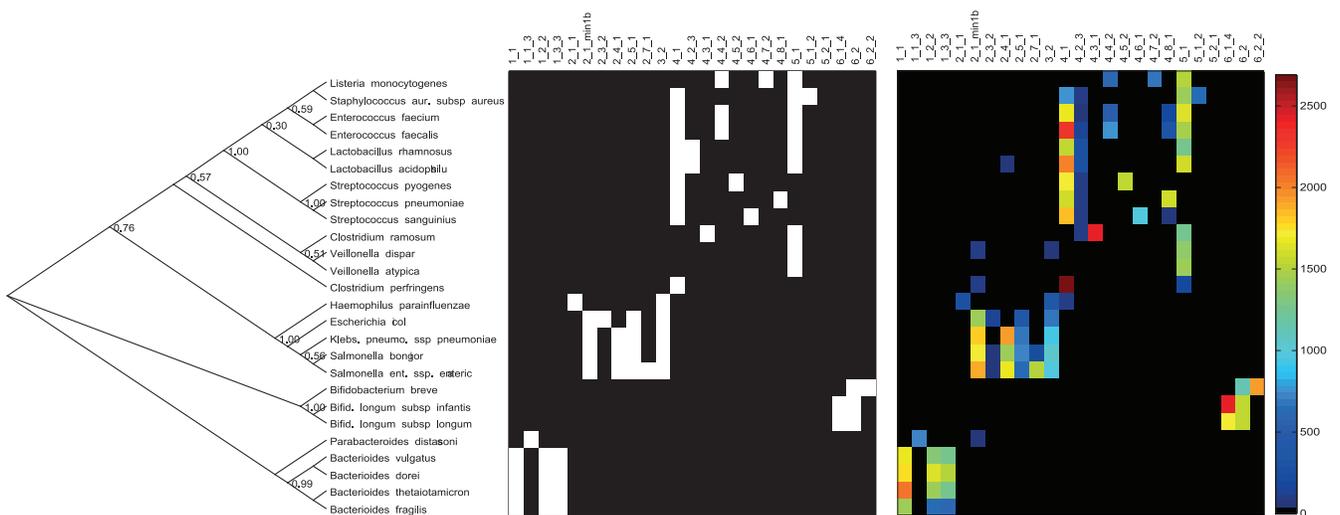

FIG. 3. Strain classification by GA-map infant array. (Left) Phylogenetic tree of all bacterial strains used to investigate probe accuracy and sensitivity. The numbers at the nodes indicate bootstrap support. (Middle) The theoretical result for the array experiment, where the classification as nontarget and target is given as a color code from black to white. (Right) Experimental results with signal intensities color coded as shown in the color bar at the right of the image. The numbering of probes is based on an in-house classification system (Table 2).
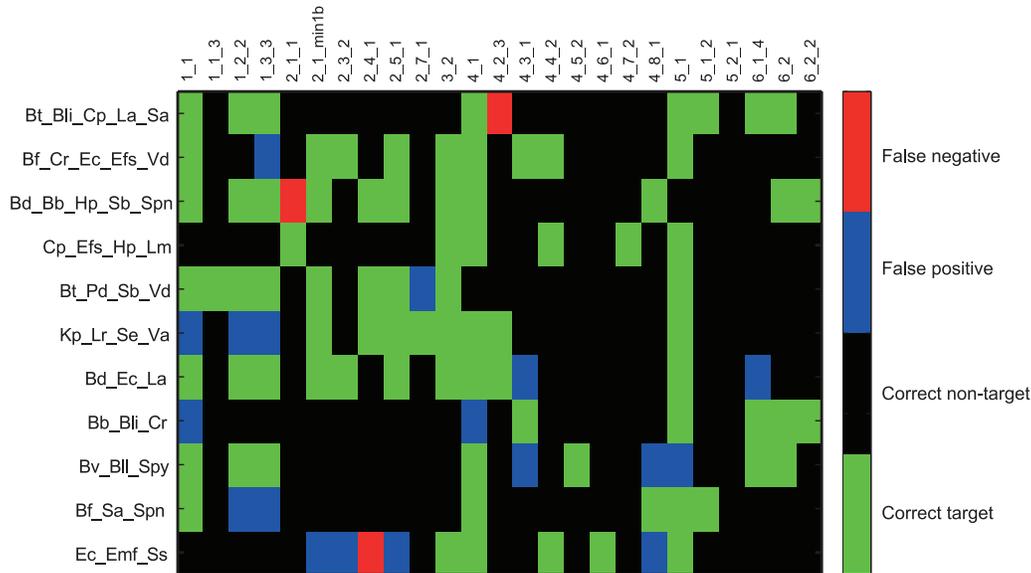
FIG. 4. Classification of mixed samples by the GA-map infant array. Signals from defined one-to-one mixtures of bacteria were evaluated. The following abbreviations were used for the bacterial species in the sample mixtures: Bd, *Bacteroides dorei*; Bf, *Bacteroides fragilis*; Bt, *Bacteroides thetaiotaomicron*; Bv, *Bacteroides vulgatus*; Bb, *Bifidobacterium breve*; Bli, *Bifidobacterium longum* subsp. *infantis*; Bll, *Bifidobacterium longum* subsp. *longum*; Cp, *Clostridium perfringens*; Cr, *Clostridium ramosum*; Efs, *Enterococcus faecalis*; Efm, *Enterococcus faecium*; Ec, *Escherichia coli*; Hp, *Haemophilus parainfluenzae*; Kp, *Klebsiella pneumoniae* subsp. *pneumoniae*; La, *Lactobacillus acidophilus*; Lr, *Lactobacillus rhamnosus*; Lm, *Listeria monocytogenes*; Pd, *Parabacteroides distasonis*; Sb, *Salmonella bongori*; Se, *Salmonella enterica* subsp. *enterica*; Sa, *Staphylococcus aureus* subsp. *aureus*; Spn, *Streptococcus pneumoniae*; Spy, *Streptococcus pyogenes*; Ss, *Streptococcus sanguinis*; Va, *Veillonella atypica*; Vd, *Veillonella dispar*.

plied Biosystems) containing the performance-optimized POP-7 (Applied Biosystems). The injection time was 3 s, and the electrophoretic conditions were as follows: run time, 2,780 s at 8,500 V; run current, 5.0 μA; run temperature, 60°C. The sequences were base called by Sequence Scanner Software v1.0 (Applied Biosystems).

The sequences were aligned, and a bootstrapped neighbor-joining tree of all 26 bacterial strains used to evaluate the probes was constructed using the program Mega 4 with default settings (32).

Data preprocessing and analysis. The probe signals were corrected for undesired hybridization variations that are observed from slide to slide. In each experiment, a probe that is already labeled (HYC01) is added to the probe mixture to evaluate the hybridization step and to normalize differences in hybridization efficiencies. To correct for varying hybridization between slides, we divide all sample signals by the average signal of all replicas from the probe. In addition, self-labeling and/or cross-labeling from each individual probe was removed by subtracting the average signal from a nontemplate control sample included on all slides used in the experiment. Finally, the nonbinding control probes NBC01 and NBC02 were used to evaluate cross-hybridization.

Statistical analyses. The probe specificity was evaluated by comparing the theoretical target/nontarget values with the experimental results on single strains, using an empirically determined background signal threshold value of 50.

Microarray data usually contain both threshold and saturation values and are therefore very seldom normally distributed. Thus, in order to test the significance of microarray data, it is common to use permutation-based approaches instead of standard statistical tests, such as analysis of variance (ANOVA) and *t* tests, which require normal distribution. Permutation testing is an exact statistical test, even for data with a complex distribution structure (6). Hence, the *P* values for group differences within each age category were calculated by permutation testing (14), using 50 as the background threshold value.

Nucleotide sequence accession numbers. The sequences for the bacterial strains have been deposited in GenBank, and the strains' respective accession numbers are listed in Table 1.

RESULTS

Probe construction and evaluation. A set of 88 probes was constructed based on the criteria described in Materials and Methods. Six probes for the main phyla covered 88% of the

clones in our evaluated data set, as illustrated in Fig. 2, indicating that the majority of the bacteria expected in the human gut can be covered by broad-range probes. Single-probe evaluations of the 88 probes using capillary gel electrophoresis and the strains in Table 1 (in addition to a synthetic oligonucleotide for probe 5_2_1) as templates showed that 76% of the probes satisfy the criterion of target detection (see Materials and Methods), indicating a relatively high success rate for the probes constructed based on the criteria described in the supplemental material. We identified 10 probe sets among the probes that satisfied the labeling criterion (see Table S2 in the

TABLE 3. Quantification in a mixed species background

Probe identifier[a]	Species[b]	Detection limit[c]	R^{2d}
1_1	*Bacteroides fragillis*	0.01	0.94
2_1_min1b	*Escherichia coli*	0.02	0.93
2_5_1	*Escherichia coli*	0.02	0.95
3_2	*Escherichia coli*	0.01	0.98
4_3_1	*Clostridium ramosum*	0.01	0.96
4_4_2	*Enterococcus faecalis*	0.01	0.84
4_5_2	*Streptococcus pyogenes*	0.01	0.96
5_1_2	*Staphylococcus aureus* subsp. *aureus*	0.01	0.98
6_1_4	*Bifidobacterium longum* subsp. *infantis*	0.01	0.97
6_2_2	*Bifidobacterium breve*	0.01	0.95

[a] Only probes that uniquely detect the respective bacteria are shown.
[b] Bacterial PCR products were subjected to dilution series following the experimental scheme shown in Table S4 in the supplemental material.
[c] The detection limits represent the relative amounts of the respective bacterial PCR products for which two sample *t* tests between two consecutive dilutions showed significance (*P* < 0.05).
[d] R^2, the squared regression coefficient.

TABLE 4. Phylum level differences between sensitized and nonsensitized children

Probe	Taxonomic group	Difference at age (days)[a]:			
		10	120	360	720
1_1	*Bacteroides*	0.640	0.868	1.00	0.903
2_1_min1b	*Gammaproteobacteria*	0.760	0.220	0.801	0.542
3_2	*Proteobacteria*	0.922	0.3126	0.126	0.465
4_1	*Firmicutes* (*Lactobacillales, Clostridium perfringens, Staphylococcus*)	0.164	0.190	0.360	0.599
5_1	*Firmicutes* (*Clostridium, Bacillales, Enterococcus, Lactobacillus*)	0.486	0.127	**0.049**	0.556
6_2	*Actinobacteria*	0.152	**0.042**	0.196	0.989
UNI01	16S universal	0.450	0.867	0.917	0.216

[a] The significances of differences were determined by permutation testing. Significant differences ($P < 0.05$) are in boldface.

supplemental material) based on a set of bioinformatics criteria (see the supplemental material). Each probe set consisted of 25 probes that were selected based on their *in silico* compatibility with each other. The compatibility estimations were based on melting temperature calculations and the thermodynamics of the probe: self-hybridization and hybridization to other probes in the probe set or their target bacteria as described the supplemental material. Experimental validation by capillary gel electrophoresis showed that probe set 3 gave the lowest cross-labeling, as determined by labeling without template (results not shown). This probe set was therefore selected for array construction (Table 2).

Specificity, reproducibility, and quantitative range of the GA-map infant array. The first evaluation of the array was on pure cultures. The evaluation was based on comparing *in silico*-determined targets/nontargets with experimental signals (Fig. 3).This analysis showed good concordance between the theoretical and experimental probe specificities. Using a signal cutoff value of 50, we found that there were no false negatives, while the numbers of false positives were more variable (Table 2). Probe 4_2_3 showed the highest level, with 29% false-positive signals, while the rest of the probes showed <15% false-positive signals. Unfortunately, we did not have a target bacterium for probe 5_2_1, but what this evaluation shows is that the probe at least does not cross-react with the nontarget bacteria.

The next step in the evaluation was to determine the classification accuracy of mixed samples. This was done by analyzing a set of defined one-to-one mixtures of PCR products from pure bacterial strains. The evaluation of these data showed that the majority of the probes accurately identified their target bacteria (Fig. 4). In total, there were 9.0% false positives and 1.6% false negatives given a background signal threshold of 50. The quantitative range of selected probes was subsequently evaluated by template dilutions in a mixed strain background (see Table S4 in the supplemental material for the experimental setup). These analyses showed quantitative responses for all the probes evaluated (Table 3; see Fig. S4 in the supplemental material). In addition, we evaluated the effect of the total amount of template in the labeling reaction. This evaluation showed that given more than 10 ng of target, the linearity between the template concentration and the signal is lost. We also showed that the smallest amount of template that could be detected was between 0.1 and 0.01 ng (see Table S5 in the supplemental material).
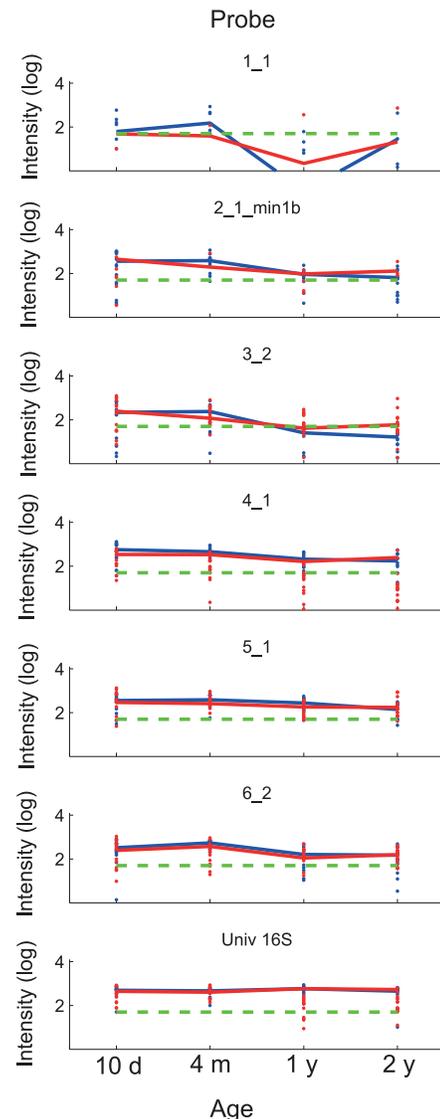

FIG. 5. Temporal development of bacterial phyla in sensitized and nonsensitized infants. Each panel shows the temporal development of probe signals within the study population for the respective probe. The log average signal for each probe is shown as a line, while the log signals of all time points measured are shown as dots (levels above a signal threshold of 50, denoted by dashed green lines). The blue lines and dots represent sensitized children ($n = 16$), while the red lines and dots represent nonsensitized children ($n = 16$). Values of <0 were set to 0.001 before log transformation. d, day; m, month; y, year.

TABLE 5. Genus/species differences between sensitized and nonsensitized children

Probe	Taxonomic group	Difference at age (days)[a]:			
		10	120	360	720
1_1_3	*Parabacteroides*	1	0.866	1.000	1.000
1_2_2	*Bacteroides* (*B. dorei, B. fragilis, B. thetaiotaomicron, B. vulgatus*)	1	0.884	1.000	1.000
1_3_3	*Bacteroides* (*B. dorei, B. fragilis, B. thetaiotaomicron, B. vulgatus*)	0.756	0.488	0.206	0.741
2_1_1	*Haemophilus*	0.783	1.000	1.000	1.000
2_3_2	*Gammaproteobacteria* subgroup	0.668	0.347	1.000	0.494
2_4_1	*Gammaproteobacteria* subgroup	0.182	0.622	1.000	1.000
2_5_1	*Gammaproteobacteria* subgroup	0.695	0.913	0.870	0.949
2_7_1	*Salmonella*	0.754	1.000	1.000	1.000
4_2_3	*Lactobacillus* subgroup	0.938	0.909	1.000	0.405
4_3_1	*Clostridium ramosum*	0.786	0.765	0.828	0.537
4_4_2	*Enterococcus, Listeria*	0.9736	**0.020**	1.000	1.000
4_6_1	*Streptococcus sanguinis*	1.000	1.000	**0.038**	0.689
4_8_1	*Streptococcus pneumoniae, Enterococcus*	*0.084*	0.169	1.000	0.935
5_1_2	*Staphylococcus*	0.847	1.000	1.000	0.399
6_1_4	*Bifidobacterium longum*	*0.097*	*0.066*	**0.016**	0.837
6_2_2	*Bifidobacterium breve*	0.711	0.679	0.844	0.784

[a] The significances of differences were determined by permutation testing. Significant differences ($P < 0.05$) are in boldface, while differences in the range $0.05 < P < 0.1$ are italicized.

The reproducibility of the assay was evaluated by duplicate analyses of 43 samples.

The mean percent variation and R^2 for each probe were evaluated individually (see Table S1 in the supplemental material). These results confirmed the reproducibility of the assay with relatively high R^2 values and low mean percent variation. Furthermore, the repeated analyses from the same fecal shedding showed R^2 values of >0.93 for all pairwise comparisons of probe signal intensities. This indicates that the microbiota is homogeneous among the different samples and that the sample preparation does not introduce a large amount of variance.

Finally, we compared GA-map infant array data for *Bifidobacterium breve* (probe 6_2_2) and *Bifidobacterium longum* (probe 6_1_4) to previously generated qPCR results (Storrø et al., unpublished). There was relatively high correlation for all age groups for the *B. longum* subsp. *longum*/*B. longum* subsp. *infantis* group ($R^2 = 0.42$; $n = 159$), while for *B. breve*, the correlation between qPCR and the array was age dependent. For the 10-day age category, the correlation was relatively high ($R^2 = 0.45$; $n = 30$), while it was lower for the 4-month-old group ($R^2 = 0.33$; $n = 27$); for the 1-year-old group, it was even lower ($R^2 = 0.20$; $n = 28$), and for the 2-year-old group, there was nearly no correlation ($R^2 = 0.08$; $n = 32$).

Phylum level development of the gut microbiota. We found that *Actinobacteria* (probe 6_2) and *Firmicutes* (probe 5_1) were significantly overrepresented at 4 months and 1 year, respectively, in the IgE-sensitized children (Table 4 and Fig. 5). There was also an overall consistent age-specific colonization pattern at the phylum level, irrespective of the sensitization state. The general pattern was an initial dominance of *Firmicutes* and *Proteobacteria* at 10 days. At 4 months, the *Proteobacteria*/*Firmicutes* dominance was replaced with *Bacteroides*/*Actinobacteria*, while after 1 and 2 years, the initially colonizing phyla were apparently becoming low in abundance.

Genus and species level development of the gut microbiota. The main difference between the sensitized and nonsensitized groups was that *B. longum* (probe 6_1_4) was significantly overrepresented in the sensitized group compared to the nonsensitized group at 1 year. We also found that *Enterococcus* (probe 4_4_2) was significantly overrepresented at 4 months. It also seems that streptococci are associated with sensitization, with *Streptococcus sanguinis* (probe 4_6_1) being significantly overrepresented at 1 year and *Streptococcus pneumoniae* (probe 4_8_1) at the border of significance at 10 days (Table 5 and Fig. 6).

The bacterial groups with the most consistent colonization patterns correlating with age were *Staphylococcus* (probe 5_1_2) and *B. breve* (probe 6_2_2). *Staphylococcus* dominated initially, while *B. breve* had a dominance peak at 4 months.

DISCUSSION

Major challenges with traditional 16S rRNA gene microarrays are probe specificity and cross-reactivity between closely related species. For microarrays, these challenges have recently been addressed by tiling probes covering the variable region of the 16S rRNA gene (23). The principle of tilling is that a large number of overlapping probes cover the region of interest, with the combined probe signals providing a relatively good signal-to-noise ratio. However, to our knowledge, no other array approaches have yet demonstrated quantitative differentiation of the microbiota based on point mutations.

With the SNuPE-based GA-map assay, we obtained high specificity and sensitivity with only a few single-nucleotide differences targeting probes. The obvious benefit of this is that the assay enables high-throughput applications due to reduced complexity. Few well-defined polymorphic sites also allow easier validation of target and nontarget bacteria. A requirement of SNuPE arrays, however, is that the polymorphic sites targeted must be very well characterized to cover the phylogenetic groups of interest. A further challenge with SNuPE arrays is that all factors affecting labeling are not yet completely known. This is illustrated with probe 4_2_3, which cross-reacted with a range of theoretical nontarget bacteria.
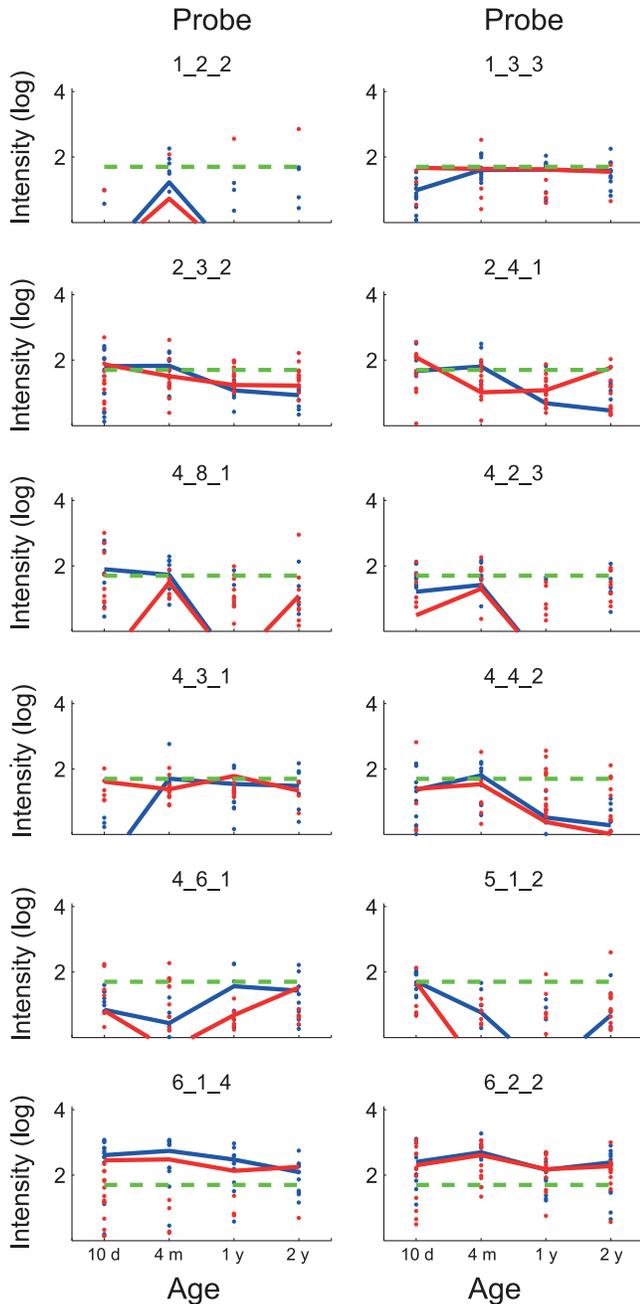
FIG. 6. Temporal development of bacterial genera/species in sensitized and nonsensitized infants. Each panel shows the temporal development of probe signals within the study population for the respective probe. The log average signal for each probe is shown as a line, while the log signals of all time points measured are shown as dots (levels above a signal threshold of 50, denoted by dashed green lines). The blue lines and dots represent sensitized children ($n = 16$), while the red lines and dots represent nonsensitized children ($n = 16$). Values of <0 were set to 0.001 before log transformation. Probes for which the signals for all the samples were below the threshold are not shown.

Not only is the specificity of the assays for microbiota characterization important, but also the quantitative properties. Since SNuPE assays include linear amplification, the quantitative range is limited by label saturation for highly abundant

phylogroups, while the detection of low-abundance phylotypes is limited by the sensitivity of the assay. We designed our SNuPE assay to quantify bacteria in the range down to 1% of the total microbiota. This choice was a trade-off between sensitivity and the ability to quantify the dominant species. In the linear range, we found the quantitative properties of our assay were very good ($R^2 > 0.9$). We also found a relatively good correlation with that of qPCR. These comparisons, however, are challenging, due to differences in both the phylogenetic widths and the quantitative ranges of the assays. E.g., the age-dependent reduction in correlation for *B. breve* between qPCR and the SNuPE array suggests that the phylogenetic widths are different in the two assays. Although our assay does not have a linear dose response for high-abundance taxa, the reproducibility between parallel samples was very high, suggesting that the main quantitative information is captured in the GA-map assay. Finally, as for most 16S rRNA gene microarray approaches, the broad-range PCR amplification can introduce quantification biases (8).

The most surprising biological finding in our data was that *B. longum* was significantly overrepresented in the IgE-sensitized group at 360 days, in addition to low *P* values for 10 days and 120 days. This finding has also been independently confirmed by qPCR for the IM-PACT data (Storrø et al., unpublished). Taken together, the multiple independent observations support the validity of the correlations. The surprise was because most previous work has actually suggested that *B. longum* is protective with respect to sensitization (9, 11, 33). Experiments with mouse models, however, have shown that the time and order of bifidobacterial colonization are important for the immunomodulatory effects (10). This may explain the differences in effects between different studies.

We also found that the *Firmicutes* subgroup containing streptococci and enterococci was significantly overrepresented in the IgE-sensitized group. These correlations, however, need to be verified further due to the possibility of type I errors. Furthermore, relatively little has been described about these bacterial groups with respect to sensitization. However, it has been suggested that *S. pneumoniae* infections can be correlated with increased IgE levels in chronic bronchitis (12). Thus, there could be common underlying mechanisms for the infant and bronchitis sensitizations.

The generally lower levels of most phyla in the nonsensitized group compared to the sensitized group suggests that there are phyla missing in the GA-map infant assay that are negatively correlated with sensitization. There are probably phyla missing in the GA-map assay for the high-age groups. Although the assay was constructed to detect the major phylogroups in a relatively large data set (19, 26), this data set may not completely represent the phylogroups in the IM-PACT cohort. A requirement in order to use targeted microarrays is that the human gut microbiota consists of a limited number of taxa. Recent deep sequencing suggests that this is in fact the case (2). Therefore, it should be possible to develop future assays including all phylogroups expected to colonize the infant gut. Recent extensive in-depth sequencing may help to identify these phylogroups (5).

Since we analyzed the fecal microbiota, our observations may not reflect the bacteria directly interacting with the immune system in the intestine. Neither can we determine from

our data if our observations are a cause or a consequence of the sensitization state. Further experimental documentation is therefore needed to determine the mechanistic nature of the correlations detected. What we have shown, however, is that there is a difference in the fecal microbiota between sensitized and nonsensitized children in the IM-PACT cohort. Furthermore, we have also shown an age-specific colonization pattern, irrespective of the sensitization state.

This study demonstrates the usefulness of the GA-map infant assay in determining variations in the composition of the infant gut microbiota, and we believe that with both future temporal and interactional results from large-scale screenings, several of the apparently controversial issues in the current literature can be resolved and a better understanding of the interaction of the complex gut microbiota can be obtained. Such understanding could lead to early diagnosis of disease and better prophylactic or therapeutic treatments of various gut-related diseases.

ACKNOWLEDGMENTS

REFERENCES

1. **Adlerberth, I., et al.** 2007. Gut microbiota and development of atopic eczema in 3 European birth cohorts. J. Allergy Clin. Immunol. **120:**343–350.
2. **Arumugam, M., et al.** 2011. Enterotypes of the human gut microbiome. Nature **473:**174–180.
3. **Bjorksten, B., P. Naaber, E. Sepp, and M. Mikelsaar.** 1999. The intestinal microflora in allergic Estonian and Swedish 2-year-old children. Clin. Exp. Allergy **29:**342–346.
4. **Bjorksten, B., E. Sepp, K. Julge, T. Voor, and M. Mikelsaar.** 2001. Allergy development and the intestinal microflora during the first year of life. J. Allergy Clin. Immunol. **108:**516–520.
5. **Dominguez-Bello, M. G., et al.** 2010. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc. Natl. Acad. Sci. **107:**11971–11975.
6. **Edgington, E. S.** 1995. Randomization tests, 3rd ed. Marcel Dekker, New York, NY.
7. **Forno, E., et al.** 2008. Diversity of the gut microbiota and eczema in early life. Clin. Mol. Allergy **6:**11.
8. **Hong, S., J. Bunge, C. Leslin, S. Jeon, and S. S. Epstein.** 2009. PCR primers miss half of rRNA microbial diversity. ISME J. **3:**1365–1373.
9. **Inoue, Y., N. Iwabuchi, J. Z. Xiao, T. Yaeshima, and K. Iwatsuki.** 2009. Suppressive effects of bifidobacterium breve strain M-16V on T-helper type 2 immune responses in a murine model. Biol. Pharm. Bull. **32:**760–763.
10. **Kim, H., S. Y. Lee, and G. E. Ji.** 2005. Timing of bifidobacterium administration influences the development of allergy to ovalbumin in mice. Biotechnol. Lett. **27:**1361–1367.
11. **Kirjavainen, P. V., T. Arvola, S. J. Salminen, and E. Isolauri.** 2002. Aberrant composition of gut microbiota of allergic infants: a target of bifidobacterial therapy at weaning? Gut **51:**51–55.
12. **Kjaergard, L. L., et al.** 1996. Basophil-bound IgE and serum IgE directed against Haemophilus influenzae and Streptococcus pneumoniae in patients with chronic bronchitis during acute exacerbations. APMIS **104:**61–67.
13. **Lane, D. J.** 1991. Nucleic acid techniques in bacterial systematics John Wiley and Sons, New York, NY.
14. **Langsrud, Ø.** 2002. 50-50 multivariate analysis of variance for collinear responses. J. R. Stat. Soc. D **51:**305–317.
15. **Nadkarni, M. A., F. E. Martin, N. A. Jacques, and N. Hunter.** 2002. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology **148:**257–266.
16. **Nikolausz, M., A. Chatzinotas, A. Tancsics, G. Imfeld, and M. Kastner.** 2009. Evaluation of single-nucleotide primer extension for detection and typing of phylogenetic markers used for investigation of microbial communities. Appl Environ. Microbiol. **75:**2850–2860.
17. **Nikolausz, M., A. Chatzinotas, A. Tancsics, G. Imfeld, and M. Kastner.** 2009. The single-nucleotide primer extension (SNuPE) method for the multiplex detection of various DNA sequences: from detection of point mutations to microbial ecology. Biochem. Soc. Trans. **37:**454–459.
18. **Øien, T., O. Storrø, and R. Johnsen.** 2006. Intestinal microbiota and its effect on the immune system—a nested case-cohort study on prevention of atopy among small children in Trondheim: the IMPACT study. Contemp. Clin. Trials **27:**389–395.
19. **Palmer, C., E. M. Bik, D. B. DiGiulio, D. A. Relman, and P. O. Brown.** 2007. Development of the human infant intestinal microbiota. PLoS Biol. **5:**e177.
20. **Palmer, C., et al.** 2006. Rapid quantitative profiling of complex microbial populations. Nucleic Acids Res. **34:**e5.
21. **Penders, J., et al.** 2007. Gut microbiota composition and development of atopic manifestations in infancy: the KOALA Birth Cohort Study. Gut **56:**661–667.
22. **Quince, C., et al.** 2009. Accurate determination of microbial diversity from 454 pyrosequencing data. Nat. Methods. **6:**639–641.
23. **Rajilic-Stojanovic, M., et al.** 2009. Development and application of the human intestinal tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. Environ. Microbiol. **11:**1736–1751.
24. **Round, J. L., and S. K. Mazmanian.** 2009. The gut microbiota shapes intestinal immune responses during health and disease. Nat. Rev. Immunol. **9:**313–323.
25. **Rudi, K., O. M. Skulberg, F. Larsen, and K. S. Jakobsen.** 1998. Quantification of toxic cyanobacteria in water by use of competitive PCR followed by sequence-specific labeling of oligonucleotide probes. Appl. Environ. Microbiol. **64:**2639–2643.
26. **Rudi, K., et al.** 2007. Alignment-independent comparisons of human gastrointestinal tract microbial communities in a multidimensional 16S rRNA gene evolutionary space. Appl. Environ. Microbiol. **73:**2727–2734.
27. **Rudi, K., M. Zimonja, P. Trosvik, and T. Naes.** 2007. Use of multivariate statistics for 16S rRNA gene analysis of microbial communities. Int. J. Food Microbiol. **120:**95–99.
28. **Sanguin, H., et al.** 2006. Development and validation of a prototype 16S rRNA-based taxonomic microarray for *Alphaproteobacteria*. Environ. Microbiol. **8:**289–307.
29. **Skånseng, B., M. Kaldhusdal, and K. Rudi.** 2006. Comparison of chicken gut colonisation by the pathogens Campylobacter jejuni and Clostridium perfringens by real-time quantitative PCR. Mol. Cell. Probes **20:**269–279.
30. **Strachan, D. P.** 1989. Hay fever, hygiene, and household size. BMJ **299:**1259–1260.
31. **Syvanen, A. C., K. Aalto-Setala, L. Harju, K. Kontula, and H. Soderlund.** 1990. A primer-guided nucleotide incorporation assay in the genotyping of apolipoprotein E. Genomics **8:**684–692.
32. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24:**1596–1599.
33. **Tanaka, K., and H. Ishikawa.** 2004. Role of intestinal bacterial flora in oral tolerance induction. Histol. Histopathol. **19:**907–914.
34. **Trosvik, P., N. C. Stenseth, and K. Rudi.** 2010. Convergent temporal dynamics of the human infant gut microbiota. ISME J. **4:**151–158.
35. **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane.** 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173:**697–703.

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

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.
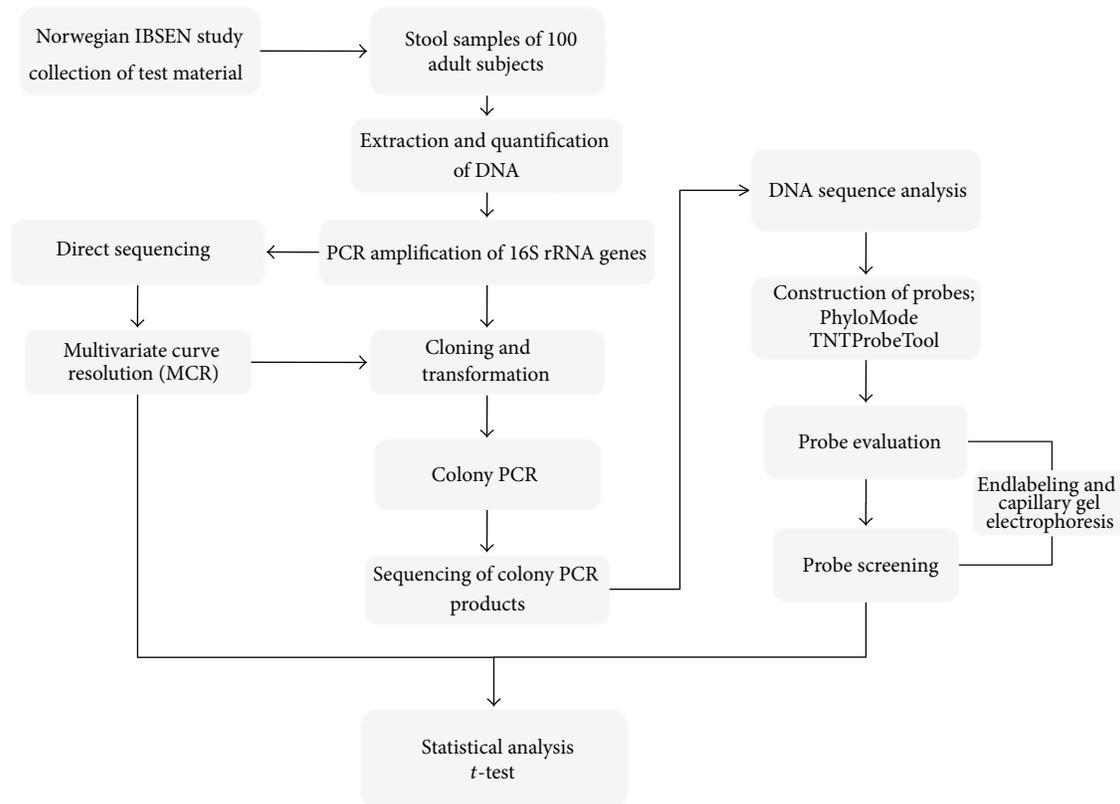
FIGURE 1: Schematic outline of the methodology.

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
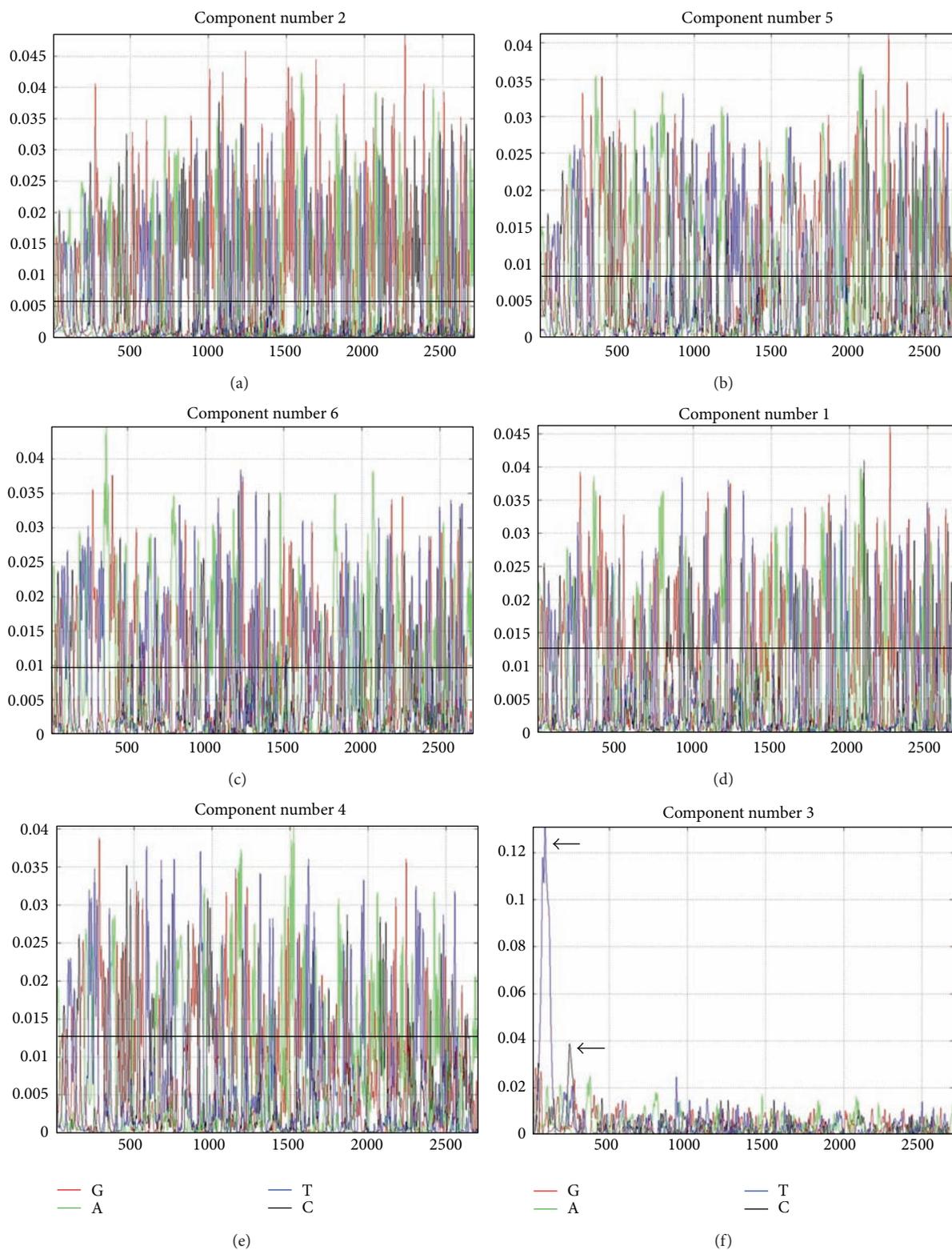
Figure 2: Spectra of the six components resolved by MCR analysis. Visual examination reveal components 2, 5, and 6 ((a)–(c)) to have well resolved spectra with low background sequences. Components 1 and 4 ((d) and (e)) also have well resolved spectra, although with somewhat higher background sequences than components 2, 5, and 6. Component 3 (f) has two high peaks (black arrows) and a poorly resolved spectrum.
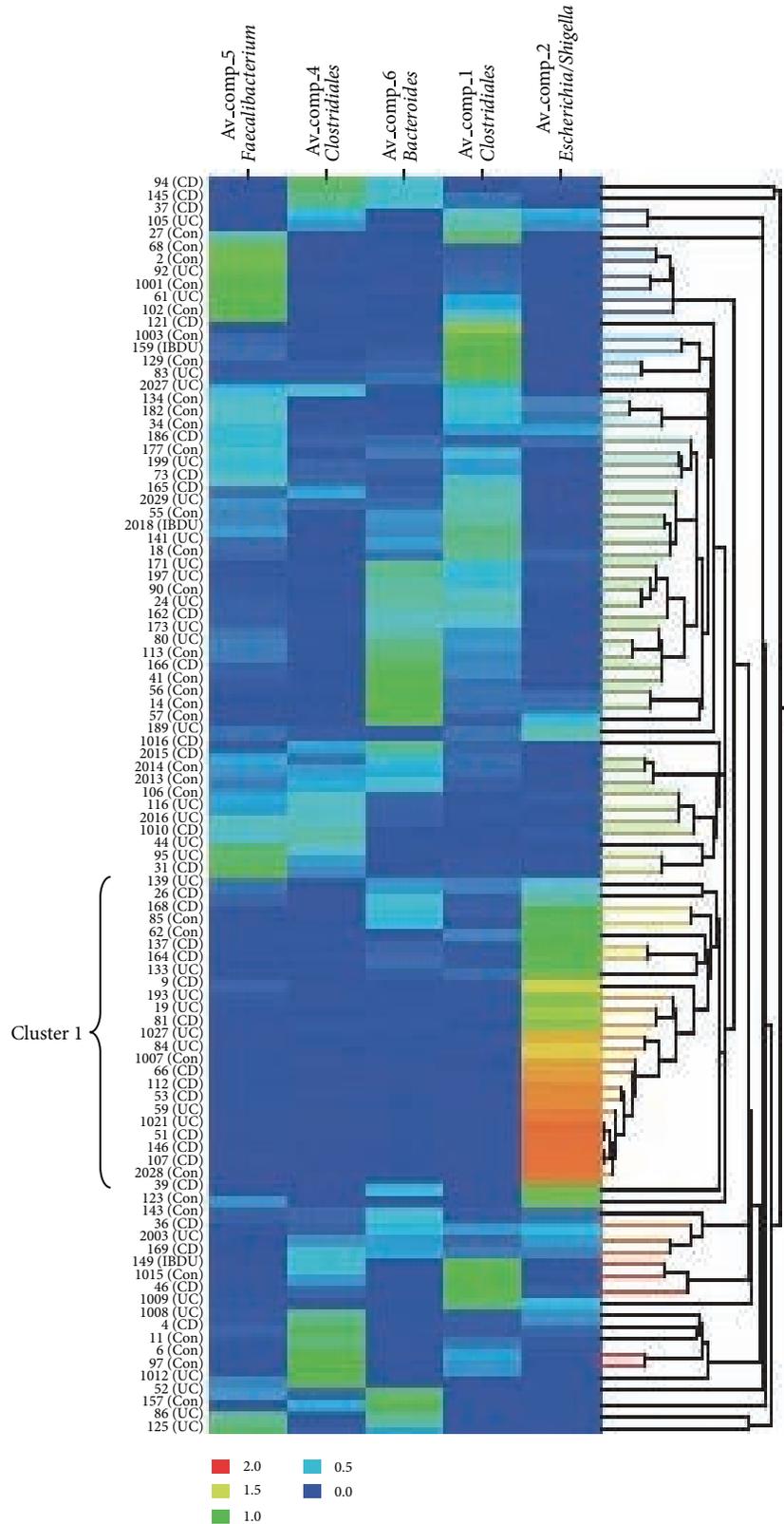
FIGURE 3: Data matrix of the amount of components 1, 2, 4, 5, and 6 in the stool samples. The graded colors indicate the abundance of a particular component in a sample. Red color indicates a high amount of the component, and blue color indicates a low amount of the component. To the left of the matrix, 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
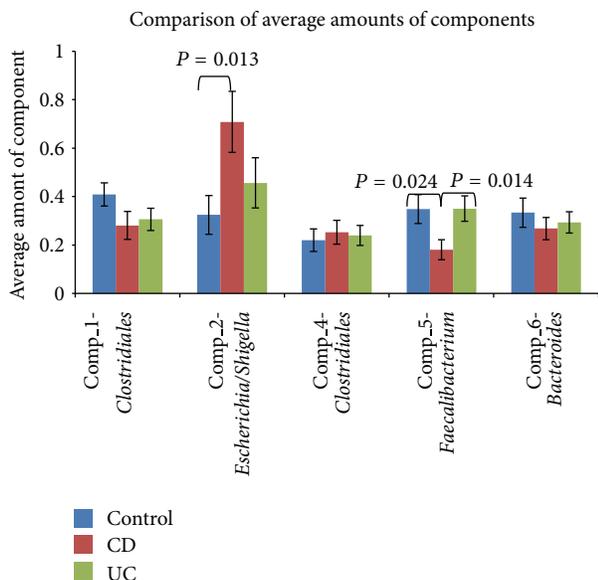
FIGURE 4: Comparison of the average amount of each component in the three disease states. The averages are calculated based on MCR analysis of stool samples from 33 subjects in the control group, 30 subjects diagnosed with CD (Crohn's Disease), and 33 subjects diagnosed with UC (Ulcerative Colitis). 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.
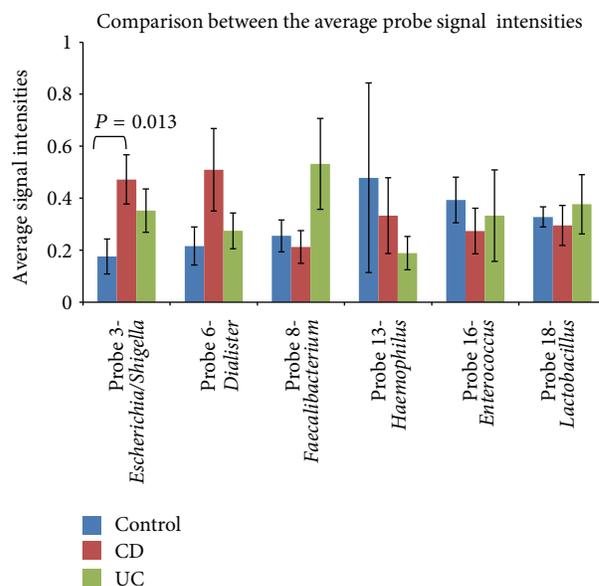

FIGURE 5: Comparison between the average amount of specific bacteria groups in the different disease states. The averages are calculated based on the height of the probe signals normalized by the signal height of the universal bacterial probe. Further, the averages are normalized to one because of Probe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

References

[1] L. V. Hooper, D. R. Littman, and A. J. Macpherson, "Interactions between the microbiota and the immune system," *Science*, vol. 336, pp. 1268–1273, 2012.

[2] J. L. Round and S. K. Mazmanian, "The gut microbiota shapes intestinal immune responses during health and disease," *Nature Reviews Immunology*, vol. 9, no. 5, pp. 313–323, 2009.

[3] F. Shanahan and C. N. Bernstein, "The evolving epidemiology of inflammatory bowel disease," *Current Opinion in Gastroenterology*, vol. 25, no. 4, pp. 301–305, 2009.

[4] A. Giongo, K. A. Gano, D. B. Crabb et al., "Toward defining the autoimmune microbiome for type 1 diabetes," *ISME Journal*, vol. 5, no. 1, pp. 82–91, 2011.

[5] R. E. Ley, P. J. Turnbaugh, S. Klein, and J. I. Gordon, "Microbial ecology: human gut microbes associated with obesity," *Nature*, vol. 444, no. 7122, pp. 1022–1023, 2006.

[6] J. Penders, C. Thijs, P. A. Van Den Brandt et al., "Gut microbiota composition and development of atopic manifestations in infancy: the KOALA birth cohort study," *Gut*, vol. 56, no. 5, pp. 661–667, 2007.

[7] D. N. Frank, A. L. St. Amand, R. A. Feldman, E. C. Boedeker, N. Harpaz, and N. R. Pace, "Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 34, pp. 13780–13785, 2007.

[8] R. J. Xavier and D. K. Podolsky, "Unravelling the pathogenesis of inflammatory bowel disease," *Nature*, vol. 448, no. 7152, pp. 427–434, 2007.

[9] R. B. Sartor, "Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis," *Nature Clinical Practice Gastroenterology and Hepatology*, vol. 3, no. 7, pp. 390–407, 2006.

[10] J. Braun and B. Wei, "Body traffic: ecology, genetics, and immunity in inflammatory bowel disease," *Annual Review of Pathology*, vol. 2, pp. 401–429, 2007.

[11] R. B. Sartor, "Key questions to guide a better understanding of host-commensal microbiota interactions in intestinal inflammation," *Mucosal Immunology*, vol. 4, no. 2, pp. 127–132, 2011.

[12] G. Bouma and W. Strober, "The immunological and genetic basis of inflammatory bowel disease," *Nature Reviews Immunology*, vol. 3, no. 7, pp. 521–533, 2003.

[13] C. P. Tamboli, C. Neut, P. Desreumaux, and J. F. Colombel, "Dysbiosis in inflammatory bowel disease," *Gut*, vol. 53, no. 1, pp. 1–4, 2004.

[14] J. B. Ewaschuk, Q. Z. Tejpar, I. Soo, K. L. Madsen, and R. N. Fedorak, "The role of antibiotic and probiotic therapies in current and future management of inflammatory bowel disease," *Current Gastroenterology Reports*, vol. 8, no. 6, pp. 486–498, 2006.

[15] P. Rutgeerts, K. Goboes, M. Peeters et al., "Effect of faecal stream diversion on recurrence of Crohn's disease in the neoterminal ileum," *Lancet*, vol. 338, no. 8770, pp. 771–774, 1991.

[16] R. Hansen, R. K. Russell, C. Reiff et al., "Microbiota of De-Novo Pediatric IBD: increased *Faecalibacterium prausnitzii* and reduced bacterial diversity in Crohn's but not in ulcerative colitis," *American Journal of Gastroenterology*, vol. 107, pp. 1913–1922, 2012.

[17] M. Sekelja, I. Rud, S. H. Knutsen et al., "Abrupt temporal fluctuations in chicken fecal microbiota explained by gastrointestinal origin," *Applied and Environmental Microbiology*, 2012.

[18] M. Zimonja, K. Rudi, P. Trosvik, and T. Næs, "Multivariate curve resolution of mixed bacterial DNA sequence spectra: identification and quantification of bacteria in undefined mixture samples," *Journal of Chemometrics*, vol. 22, no. 5, pp. 309–322, 2008.

[19] H. C. Vebø, M. Sekelja, R. Nestestog et al., "Temporal development of the infant gut microbiota in immunoglobulin E-sensitized and nonsensitized children determined by the GA-map infant array," *Clinical and Vaccine Immunology*, vol. 18, no. 8, pp. 1326–1335, 2011.

[20] J. E. Lennard-Jones, "Classification of inflammatory bowel disease," *Scandinavian Journal of Gastroenterology*, vol. 24, no. 170, pp. 2–6, 1989.

[21] C. Gasche, J. Scholmerich, J. Brynskov et al., "A simple classification of Crohn's disease: report of the working party for the world congresses of gastroenterology, Vienna 1998," *Inflammatory Bowel Diseases*, vol. 6, no. 1, pp. 8–15, 2000.

[22] P. Ricanek, S. Brackmann, G. Perminow et al., "Evaluation of disease activity in IBD at the time of diagnosis by the use of clinical, biochemical, and fecal markers," *Scandinavian Journal of Gastroenterology*, vol. 46, no. 9, pp. 1081–1091, 2011.

[23] B. J. Haas, D. Gevers, A. M. Earl et al., "Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons," *Genome Research*, vol. 21, no. 3, pp. 494–504, 2011.

[24] K. Rudi, M. Zimonja, and T. Næs, "Alignment-independent bilinear multivariate modelling (AIBIMM) for global analyses of 16S rRNA gene phylogeny," *International Journal of Systematic and Evolutionary Microbiology*, vol. 56, no. 7, pp. 1565–1575, 2006.

[25] M. H. Giaffer, C. D. Holdsworth, and B. I. Duerden, "The assessment of faecal flora in patients with inflammatory bowel disease by a simplified bacteriological technique," *Journal of Medical Microbiology*, vol. 35, no. 4, pp. 238–243, 1991.

[26] P. Seksik, L. Rigottier-Gois, G. Gramet et al., "Alterations of the dominant faecal bacterial groups in patients with Crohn's disease of the colon," *Gut*, vol. 52, no. 2, pp. 237–242, 2003.

[27] S. Mondot, S. Kang, J. P. Furet et al., "Highlighting new phylogenetic specificities of Crohn's disease microbiota," *Inflammatory Bowel Diseases*, vol. 17, no. 1, pp. 185–192, 2011.

[28] P. Lepage, P. Seksik, M. Sutren et al., "Biodiversity of the mucosa-associated microbiota is stable along the distal digestive tract in healthy individuals and patients with IBD," *Inflammatory Bowel Diseases*, vol. 11, no. 5, pp. 473–480, 2005.

[29] E. G. Zoetendal, A. Von Wright, T. Vilpponen-Salmela, K. Ben-Amor, A. D. L. Akkermans, and W. M. De Vos, "Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces," *Applied and Environmental Microbiology*, vol. 68, no. 7, pp. 3401–3407, 2002.

[30] U. Gophna, K. Sommerfeld, S. Gophna, W. F. Doolittle, and S. J. O. Veldhuyzen Van Zanten, "Differences between tissue-associated intestinal microfloras of patients with Crohn's disease and ulcerative colitis," *Journal of Clinical Microbiology*, vol. 44, no. 11, pp. 4136–4141, 2006.

[31] A. W. Walker, J. D. Sanderson, C. Churcher et al., "High-throughput clone library analysis of the mucosa-associated microbiota reveals dysbiosis and differences between inflamed and non-inflamed regions of the intestine in inflammatory bowel disease," *BMC Microbiology*, vol. 11, article 7, 2011.

[32] M. Baumgart, B. Dogan, M. Rishniw et al., "Culture independent analysis of ileal mucosa reveals a selective increase in invasive *Escherichia coli* of novel phylogeny relative to depletion of Clostridiales in Crohn's disease involving the ileum," *ISME Journal*, vol. 1, no. 5, pp. 403–418, 2007.

[33] J. Boudeau, A.-L. Glasser, E. Masseret, B. Joly, and A. Darfeuille-Michaud, "Invasive ability of an *Escherichia coli* strain isolated from the ileal mucosa of a patient with Crohn's disease," *Infection and Immunity*, vol. 67, no. 9, pp. 4499–4509, 1999.

[34] N. Rolhion and A. Darfeuille-Michaud, "Adherent-invasive *Escherichia coli* in inflammatory bowel disease," *Inflammatory Bowel Diseases*, vol. 13, no. 10, pp. 1277–1283, 2007.

[35] A. Darfeuille-Michaud, J. Boudeau, P. Bulois et al., "High prevalence of adherent-invasive *Escherichia coli* associated with ileal mucosa in Crohn's disease," *Gastroenterology*, vol. 127, no. 2, pp. 412–421, 2004.

[36] S. Sepehri, E. Khafipour, C. N. Bernstein et al., "Characterization of *Escherichia coli* isolated from gut biopsies of newly diagnosed patients with inflammatory bowel disease," *Inflammatory Bowel Diseases*, vol. 17, no. 7, pp. 1451–1463, 2011.

[37] P. Lapaquette, A.-L. Glasser, A. Huett, R. J. Xavier, and A. Darfeuille-Michaud, "Crohn's disease-associated adherent-invasive E. coli are selectively favoured by impaired autophagy to replicate intracellularly," *Cellular Microbiology*, vol. 12, no. 1, pp. 99–113, 2010.

[38] S. Meconi, A. Vercellone, F. Levillain et al., "Adherent-invasive *Escherichia coli* isolated from Crohn's disease patients induce granulomas in vitro," *Cellular Microbiology*, vol. 9, no. 5, pp. 1252–1261, 2007.

[39] C. Manichanh, L. Rigottier-Gois, E. Bonnaud et al., "Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach," *Gut*, vol. 55, no. 2, pp. 205–211, 2006.

[40] H. Sokol, P. Seksik, J. P. Furet et al., "Low counts of *Faecalibacterium prausnitzii* in colitis microbiota," *Inflammatory Bowel Diseases*, vol. 15, no. 8, pp. 1183–1189, 2009.

[41] J.-P. Segain, J.-P. Galmiche, D. Raingeard De La Blétière et al., "Butyrate inhibits inflammatory responses through NFκB inhibition: implications for Crohn's disease," *Gut*, vol. 47, no. 3, pp. 397–403, 2000.

[42] F. Fava and S. Danese, "Intestinal microbiota in inflammatory bowel disease: friend of foe?" *World Journal of Gastroenterology*, vol. 17, no. 5, pp. 557–566, 2011.

[43] S. H. Duncan, P. Louis, and H. J. Flint, "Cultivable bacterial diversity from the human colon," *Letters in Applied Microbiology*, vol. 44, no. 4, pp. 343–350, 2007.

[44] H. Sokol, B. Pigneur, L. Watterlot et al., "*Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 43, pp. 16731–16736, 2008.

[45] M. Joossens, G. Huys, M. Cnockaert et al., "Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives," *Gut*, vol. 60, no. 5, pp. 631–637, 2011.

[46] B. P. Willing, J. Dicksved, J. Halfvarson et al., "A pyrosequencing study in twins shows that gastrointestinal microbial profiles vary with inflammatory bowel disease phenotypes," *Gastroenterology*, vol. 139, no. 6, pp. 1844–1854, 2010.

[47] M. Rajilić-Stojanović, H. Smidt, and W. M. De Vos, "Diversity of the human gastrointestinal tract microbiota revisited," *Environmental Microbiology*, vol. 9, no. 9, pp. 2125–2136, 2007.

[48] S. M. Bloom, V. N. Bijanki, G. M. Nava et al., "Commensal Bacteroides species induce colitis in host-genotype-specific fashion in a mouse model of inflammatory bowel disease," *Cell Host and Microbe*, vol. 9, no. 5, pp. 390–403, 2011.

[49] A. Andoh, H. Kuzuoka, T. Tsujikawa et al., "Multicenter analysis of fecal microbiota profiles in Japanese patients with Crohn's disease," *Journal of Gastroenterology*, vol. 47, pp. 1298–1307, 2012.

[50] A. Andoh, H. Imaeda, T. Aomatsu et al., "Comparison of the fecal microbiota profiles between ulcerative colitis and Crohn's disease using terminal restriction fragment length polymorphism analysis," *Journal of Gastroenterology*, vol. 46, no. 4, pp. 479–486, 2011.

[51] P. B. Eckburg, E. M. Bik, C. N. Bernstein et al., "Microbiology: diversity of the human intestinal microbial flora," *Science*, vol. 308, no. 5728, pp. 1635–1638, 2005.

[52] G. C. Parkes, N. B. Rayment, B. N. Hudspith et al., "Distinct microbial populations exist in the mucosa-associated microbiota of sub-groups of irritable bowel syndrome," *Neurogastroenterology and Motility*, vol. 24, no. 1, pp. 31–39, 2012.

[53] C. Tana, Y. Umesaki, A. Imaoka, T. Handa, M. Kanazawa, and S. Fukudo, "Altered profiles of intestinal microbiota and organic acids may be the origin of symptoms in irritable bowel syndrome," *Neurogastroenterology and Motility*, vol. 22, no. 5, p. 512-e115, 2010.

[54] A. M. Schoepfer, M. Trummler, P. Seeholzer, B. Seibold-Schmid, and F. Seibold, "Discriminating IBD from IBS: comparison of the test performance of fecal markers, blood leukocytes, CRP, and IBD antibodies," *Inflammatory Bowel Diseases*, vol. 14, no. 1, pp. 32–39, 2008.

[55] B. Chassaing and A. Darfeuillemichaud, "The commensal microbiota and enteropathogens in the pathogenesis of inflammatory bowel diseases," *Gastroenterology*, vol. 140, no. 6, pp. 1720–1728, 2011.

[56] B. Stecher, R. Robbiani, A. W. Walker et al., "Salmonella enterica serovar typhimurium exploits inflammation to compete with the intestinal microbiota," *PLoS Biology*, vol. 5, no. 10, pp. 2177–2189, 2007.

[57] C. Lupp, M. L. Robertson, M. E. Wickham et al., "Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae," *Cell Host and Microbe*, vol. 2, no. 2, pp. 119–129, 2007.

[58] L. A. G. Rodríguez, A. Ruigómez, and J. Panés, "Acute gastroenteritis is followed by an increased risk of inflammatory bowel disease," *Gastroenterology*, vol. 130, no. 6, pp. 1588–1594, 2006.

[59] D. N. Frank, C. E. Robertson, C. M. Hamm et al., "Disease phenotype and genotype are associated with shifts in intestinal-associated microbiota in inflammatory bowel diseases," *Inflammatory Bowel Diseases*, vol. 17, no. 1, pp. 179–184, 2011.

[60] P. Seksik, P. Lepage, M.-F. De La Cochetière et al., "Search for localized dysbiosis in Crohn's disease ulcerations by temporal temperature gradient gel electrophoresis of 16S rRNA," *Journal of Clinical Microbiology*, vol. 43, no. 9, pp. 4654–4658, 2005.

[61] M. Zhang, B. Liu, Y. Zhang, H. Wei, Y. Lei, and L. Zhao, "Structural shifts of mucosa-associated lactobacilli and *Clostridium leptum* subgroup in patients with ulcerative colitis," *Journal of Clinical Microbiology*, vol. 45, no. 2, pp. 496–500, 2007.

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

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.

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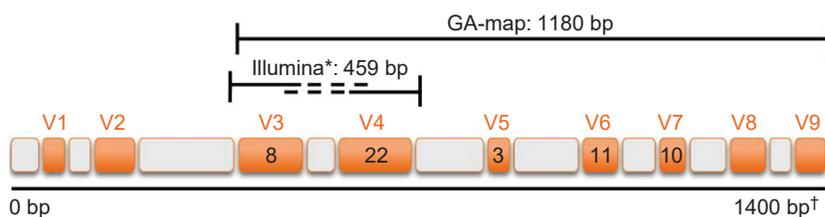
Figure 1 | Target regions for the GA primer (1180 bp) and the Illumina primer (459 bp) showing variable (orange V1-V9) and conserved (grey) regions in the bacterial 16S rRNA gene (1400 bp) utilised by the two methods. The numbers in V3 to V7 denote the number of GA probes targeting each variable region,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

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).

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,

Table 1 | Demographic information

Categories	Total	Females (%)	Age (years)* Mean	Age (years)* Range
Healthy controls	297	63	41	21–70
Nordic	254	64	42	21–70
Danish	19	63	42	23–61
Spanish	24	50	35	22–56
IBS†	236	78	40	17–76
IBS-D	102	79	40	18–70
IBS-C	41	85	42	22–73
IBS-M	10	80	37	19–55
IBS-U	25	88	41	19–68
IBS-A	51	67	39	20–62
IBD treatment-naïve	80	56	34	18–61
CD	30	50	33	19–53
UC	50	63	35	18–61
IBD remission‡	55	76	42	20–69
CD	18	72	38	20–59
UC	36	78	44	24–69

A, alternating; C, constipation; CD, Cohn's disease; D, diarrhoea; IBS, irritable bowel syndrome; IBD, inflammatory bowel disease; M, mixed; U, unsubtyped; UC, ulcerative colitis.

* Precise ages were known for 99% of the total samples used.

† IBStype known for 97% of the total IBS samples used.

‡ CD/UC diagnosis known for 99% of the total IBD samples used.

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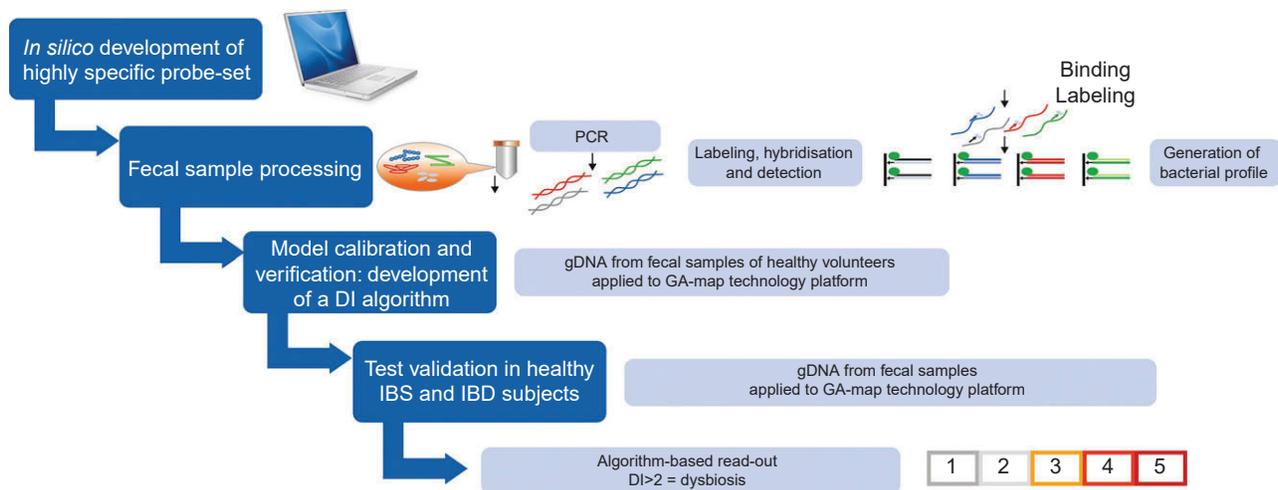
Figure 2 | Flow chart illustrating the GA-map Dysbiosis Test development, starting with *in silico* development of bacterial probe set, standardisation of laboratory analysis process, model calibration and verification in healthy individuals (normobiotic reference collection), and validation in healthy,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

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

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 ($\pm$11)	1.72
IBS	109	73 ($\pm$8)	2.98
IBS-D	34	76 ($\pm$14)	3.03
IBS-C	26	73 ($\pm$17)	3.00
IBS-M	3	67	3.33
IBS-U	25	72 ($\pm$18)	3.04
IBS-A	20	70 ($\pm$20)	2.85
IBD treatment-naïve	80	70 ($\pm$10)	3.31
CD	30	80 ($\pm$14)	3.60
UC	50	64 ($\pm$13)	3.14
IBD remission	55	80 ($\pm$11)	3.15
CD	18	89 ($\pm$14)	3.65
UC	36	75 ($\pm$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.

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.

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

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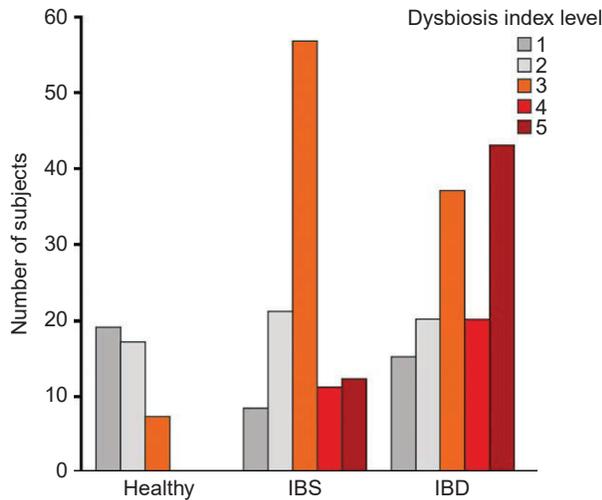
Figure 3 | Distribution of DI scores 1–5 for the validation cohort as determined by GA-map Dysbiosis Test, showing the increase in DI from healthy individuals through IBS patients and finally in IBD patients.

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
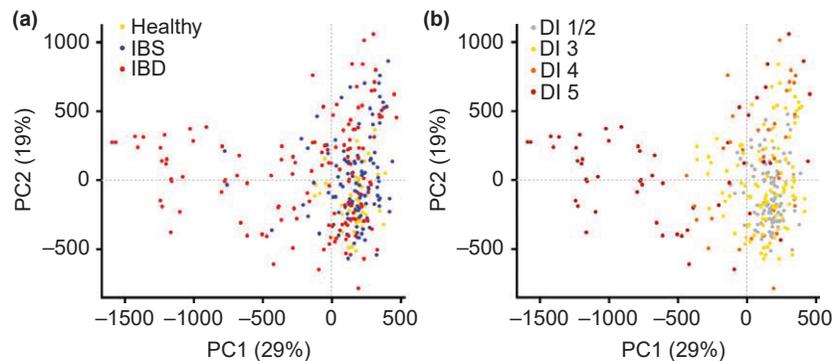

Figure 4 | PCA scores for the first two principal components for validation cohort ($n = 287$) based on 54 probes. The two PCs account for 48% of the variation, and points are coloured according to (a) cohort: yellow – healthy, blue – IBS, and red – IBD; and (B) DI: grey = 1–2, orange = 3, red = 4, dark red = 5.
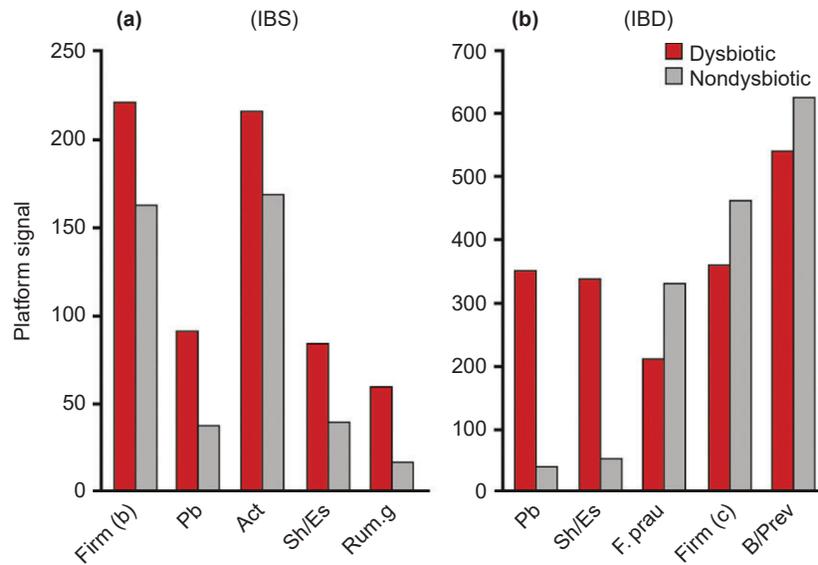
Figure 5 | Mean normalised signal for top five probes sorted by absolute relative difference between dysbiotic (red) and nondysbiotic (grey) as determined by the GA-map Dysbiosis Test for (a) IBS patients (*n* = 109), and (b) IBD patients (*n* = 135). Act, Actinobacteria; B/Prev, Bacteroides/Prevotella; 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*
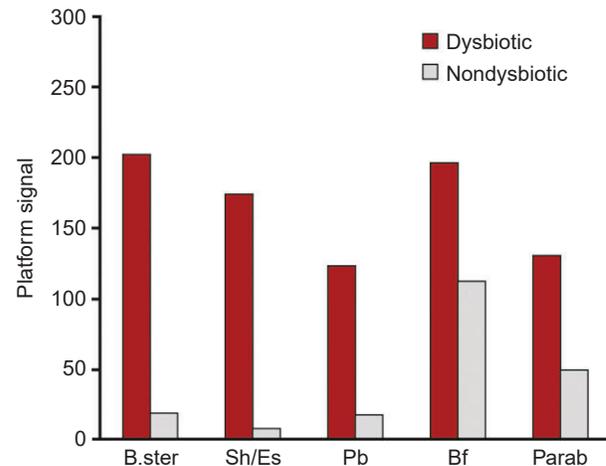
Figure 6 | Mean normalised signal for probes sorted by absolute relative difference between dysbiotic (red) and nondysbiotic (grey) as determined by the GA-map Dysbiosis Test for Spanish cohort (*n* = 24). Bf; Bifidobacterium, B. ster; *Bacteroides stercoris*, Parab; Parabacteroides, Pb; Proteobacteria, Sh/Es; *Shigella/Escherichia*.

and *Bifidobacterium* contribute to dysbiosis, which potentially could be linked to differences, in e.g. diet between Scandinavian countries and the Mediterranean region.
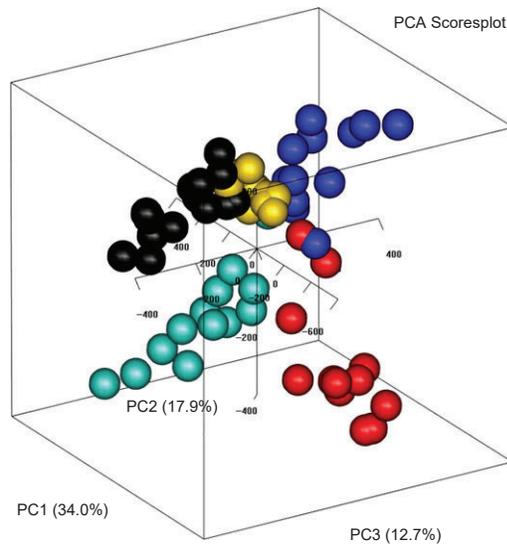
Figure 7 | Scores for the first three principal components from PCA of normalised data from five healthy subjects collected weekly for up to 14 weeks ($n = 64$). One point is one sample for donor x taken at time point y. The first three PCs account for 65% of the variation, and points are coloured according to donor.

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

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.

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.

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

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

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.

REFERENCES

1. Arumugam M, Raes J, Pelletier E, *et al.* Enterotypes of the human gut microbiome. *Nature* 2011; **473**: 174–80.
2. Sekelja M, Berget I, Naes T, Rudi K. Unveiling an abundant core microbiota in the human adult colon by a phylogroup-independent searching approach. *ISME J* 2011; **5**: 519–31.
3. Ding T, Schloss PD. Dynamics and associations of microbial community types across the human body. *Nature* 2014; **509**: 357–60.
4. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. *Nature* 2006; **444**: 1022–3.
5. Manichanh C, Rigottier-Gois L, Bonnaud E, *et al.* Reduced diversity of faecal microbiota in Cohn's disease revealed by a metagenomic approach. *Gut* 2006; **55**: 205–11.
6. Larsen N, Vogensen FK, van den Berg FW, *et al.* Gut microbiota in human adults with type 2 diabetes differs from

non-diabetic adults. *PLoS ONE* 2010; **5**: e9085.

7. Kostic AD, Xavier RJ, Gevers D. The microbiome in inflammatory bowel disease: current status and the future ahead. *Gastroenterology* 2014; **146**: 1489–99.

8. Hong SN, Rhee PL. Unraveling the ties between irritable bowel syndrome and intestinal microbiota. *World J Gastroenterol* 2014; **20**: 2470–81.

9. Gasbarrini A, Corazza GR, Gasbarrini G, *et al.* Methodology and indications of H2-breath testing in gastrointestinal diseases: the Rome Consensus Conference. *Aliment Pharmacol Ther* 2009; **29**(Suppl 1): 1–49.

10. Sekirov I, Russell SL, Antunes LC, Finlay BB. Gut microbiota in health and disease. *Physiol Rev* 2010; **90**: 859–904.

11. Simren M, Barbara G, Flint HJ, *et al.* Intestinal microbiota in functional bowel disorders: a Rome foundation report. *Gut* 2013; **62**: 159–76.

12. Ford AC, Spiegel BM, Talley NJ, Moayyedi P. Small intestinal bacterial overgrowth in irritable bowel syndrome: systematic review and meta-analysis. *Clin Gastroenterol Hepatol* 2009; **7**: 1279–86.

13. Salonen A, de Vos WM, Palva A. Gastrointestinal microbiota in irritable bowel syndrome: present state and perspectives. *Microbiology* 2010; **156**: 3205–15.

14. Vanner S. The small intestinal bacterial overgrowth. Irritable bowel syndrome hypothesis: implications for treatment. *Gut* 2008; **57**: 1315–21.

15. Budding AE, Grasman ME, Lin F, *et al.* IS-pro: high-throughput molecular fingerprinting of the intestinal microbiota. *FASEB J* 2010; **24**: 4556–64.

16. Thorkildsen LT, Nwosu FC, Avershina E, *et al.* Dominant fecal microbiota in newly diagnosed untreated inflammatory bowel disease patients. *Gastroenterol Res Pract* 2013; **2013**: 636785.

17. Rajca S, Grondin V, Louis E, *et al.* Alterations in the intestinal microbiome (dysbiosis) as a predictor of relapse after infliximab withdrawal in Cohn's disease. *Inflamm Bowel Dis* 2014; **20**: 978–86.

18. Kelly CP. Fecal microbiota transplantation – an old therapy comes of age. *N Engl J Med* 2013; **368**: 474–5.

19. Pedersen N, Vegh Z, Burisch J, *et al.* Ehealth monitoring in irritable bowel syndrome patients treated with low fermentable oligo-, di-, mono-saccharides and polyols diet. *World J Gastroenterol* 2014; **20**: 6680–4.

20. Pedersen N, Vinding K, Vegh Z, *et al.* Gut microbiota in IBD patients with IBS before and after 6 weeks of low FODMAP diet. *Gastroenterology* 2014; **146**: S-241.

21. Macfarlane S, Cleary S, Bahrami B, Reynolds N, Macfarlane GT. Synbiotic consumption changes the metabolism and composition of the gut microbiota in older people and modifies inflammatory processes: a randomised, double-blind, placebo-controlled crossover study. *Aliment Pharmacol Ther* 2013; **38**: 804–16.

22. Vebø HC, Sekelja M, Nestestog R, *et al.* Temporal development of the infant gut microbiota in immunoglobulin E-sensitized and nonsensitized children determined by the GA-map infant array. *Clin Vaccine Immunol* 2011; **18**: 1326–35.

23. Sugimoto N, Katoh M, Nakano S, Ohmichi T, Sasaki M. RNA/DNA hybrid duplexes with identical nearest-neighbor base-pairs have identical stability. *FEBS Lett* 1994; **354**: 74–8.

24. Mardia KV, Kent JT, Bibby JM. *Multivariate Analysis*. London: Academic Press, 1979.

25. Directive 98/79/EC of the European Parliament and of the Council of 27 October 1998 on in vitro diagnostic medical devices. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:1998L0079:20090807:en:PDF.

26. Langsrud Ø. 50-50 multivariate analysis of variance for collinear responses. *J R Stat Soc Ser D* 2002; **51**: 305–17.

27. Naseribafrouei A, Hestad K, Avershina E, *et al.* Correlation between the human fecal microbiota and depression. *Neurogastroenterol Motil* 2014; **26**: 1155–62.

28. Lozupone CA, Knight R. Species divergence and the measurement of microbial diversity. *FEMS Microbiol Rev* 2008; **32**: 557–78.

29. R Development Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: the R Foundation for Statistical Computing. Available at: http://www.R-project.org/.

30. Jeffery IB, O'Toole PW, Ohman L, *et al.* An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. *Gut* 2012; **61**: 997–1006.

31. Faith JJ, Guruge JL, Charbonneau M, *et al.* The long-term stability of the human gut microbiota. *Science* 2013; **341**: 1237439.

32. Aravindraja C, Viszwapriya D, Karutha PS. Ultradeep 16S rRNA sequencing analysis of geographically similar but diverse unexplored marine samples reveal varied bacterial community composition. *PLoS ONE* 2013; **8**: e76724.

33. Collins SM. A role for the gut microbiota in IBS. *Nat Rev Gastroenterol Hepatol* 2014; **11**: 497–505.

34. Karantanos T, Markoutsaki T, Gazouli M, Anagnou NP, Karamanolis DG. Current insights in to the pathophysiology of irritable bowel syndrome. *Gut Pathog* 2010; **2**: 3.

35. Dupont HL. Review article: evidence for the role of gut microbiota in irritable bowel syndrome and its potential influence on therapeutic targets. *Aliment Pharmacol Ther* 2014; **39**: 1033–42.

36. Machiels K, Joossens M, Sabino J, *et al.* A decrease of the butyrate-producing species *Roseburia hominis* and *Faecalibacterium prausnitzii* defines dysbiosis in patients with ulcerative colitis. *Gut* 2014; **63**: 1275–83.

37. Dinan TG, Cryan JF. Melancholic microbes: a link between gut microbiota and depression? *Neurogastroenterol Motil* 2013; **25**: 713–9.

38. Finegold SM, Dowd SE, Gontcharova V, *et al.* Pyrosequencing study of fecal microflora of autistic and control children. *Anaerobe* 2010; **16**: 444–53.

39. Sha S, Liang J, Chen M, *et al.* Systematic review: faecal microbiota transplantation therapy for digestive and nondigestive disorders in adults and children. *Aliment Pharmacol Ther* 2014; **39**: 1003–32.

40. Mizrahi-Man O, Davenport ER, Gilad Y. Taxonomic classification of bacterial 16S rRNA genes using short sequencing reads: evaluation of effective study designs. *PLoS ONE* 2013; **8**: e53608.

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*

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.

© 2016 Published by Elsevier B.V.

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.
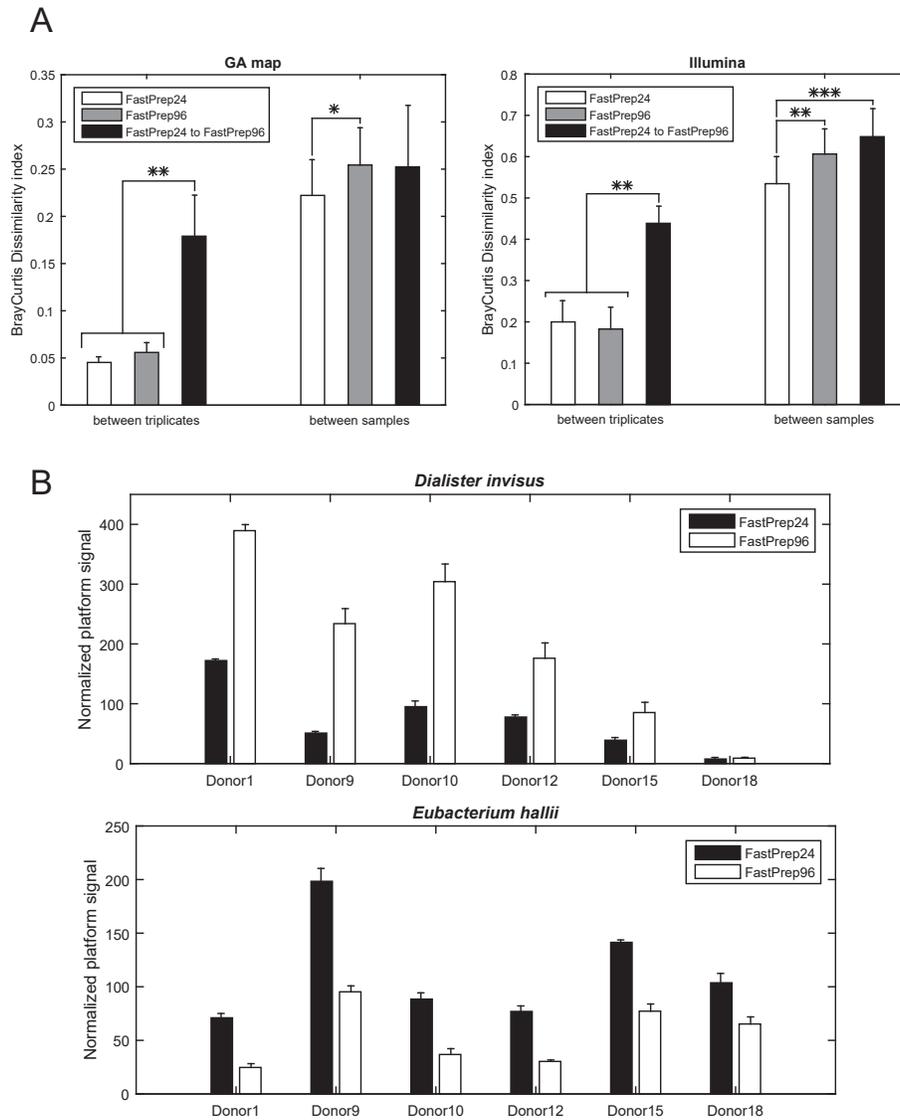
Fig. 1. Effect of bead-beating on (A) microbiota beta diversity and (B) species composition. (A) Mean Bray-Curtis beta diversity within and between sample categories are shown. Error bars represent standard error of the mean. The significance levels are designated by asterisks: *$p < 0.05$, **$p < 0.01$ 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.
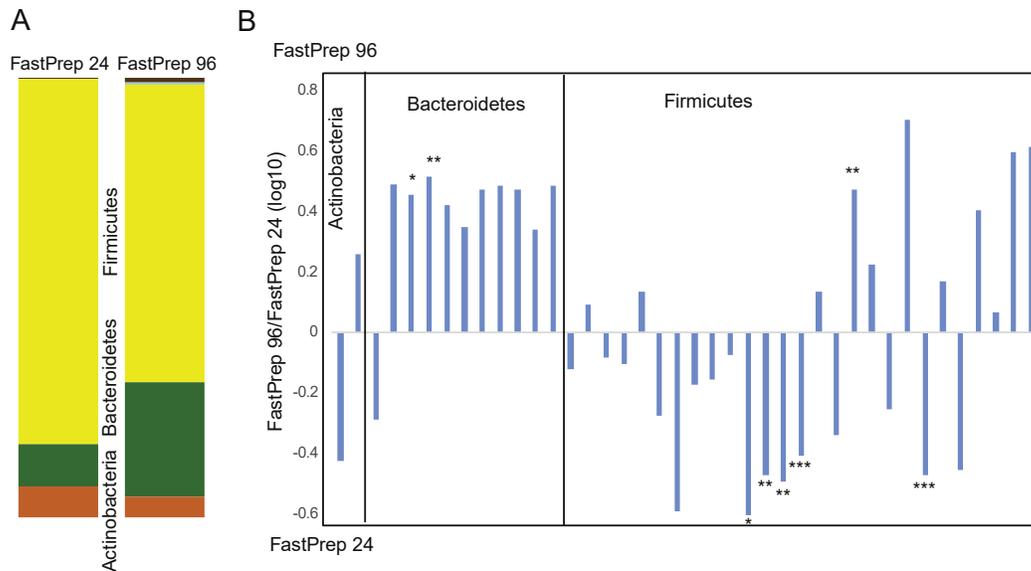
Fig. 2. Effect of bead-beating on microbiota taxonomic composition as determined by Illumina sequencing. (A) Effect on phylum level and (B) on OTU level (for OTU's that have an abundance of >1% on average in the dataset) as determined by Illumina sequencing. The ordinate represents the average number 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.

References

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D.R., Fernandes, G.R., Tap, J., Bruls, T., Batto, J.M., Bertalan, M., Borruel, N., Casellas, F., Fernandez, L., Gautier, L., Hansen, T., Hattori, M., Hayashi, T., Kleerebezem, M., Kurokawa, K., Leclerc, M., Levenez, F., Manichanh, C., Nielsen, H.B., Nielsen, T., Pons, N., Poulain, J., Qin, J., Sicheritz-Ponten, T., Tims, S., Torrents, D., Ugarte, E., Zoetendal, E.G., Wang, J., Guarner, F., Pedersen, O., de Vos, W.M., Brunak, S., Dore, J., Antolin, M., Artiguenave, F., Blottiere, H.M., Almeida, M., Brechot, C., Cara, C., Chervaux, C., Cultrone, A., Delorme, C., Denariaz, G., Dervyn, R., Foerstner, K.U., Friss, C., van de Guchte, M., Guedon, E., Haimet, F., Huber, W., van Hylckama-Vlieg, J., Jamet, A., Juste, C., Kaci, G., Knol, J., Lakhdari, O., Layec, S., Le Roux, K., Maguin, E., Merieux, A., Melo Minardi, R., M'Rini, C., Muller, J., Oozeer, R., Parkhill, J., Renault, P., Rescigno, M., Sanchez, N., Sunagawa, S., Torrejon, A., Turner, K., Vandemeulebrouck, G., Varela, E., Winogradsky, Y., Zeller, G., Weissenbach, J., Ehrlich, S.D., Bork, P., 2011. Enterotypes of the human gut microbiome. Nature 473, 174–180.

Bahl, M.I., Bergstrom, A., Licht, T.R., 2012. Freezing fecal samples prior to DNA extraction affects the Firmicutes to Bacteroidetes ratio determined by downstream quantitative PCR analysis. FEMS Microbiol. Lett. 329, 193–197.

Casen, C., Vebo, H.C., Sekelja, M., Hegge, F.T., Karlsson, M.K., Ciemniejewska, E., Dzankovic, S., Froyland, C., Nestestog, R., Engstrand, L., Munkholm, P., Nielsen, O.H., Rogler, G., Simren, M., Ohman, L, Vatn, M.H., Rudi, K., 2015. Deviations in human gut microbiota: a novel diagnostic test for determining dysbiosis in patients with IBS or IBD. Aliment. Pharmacol. Ther. 42, 71–83.

Greenhill, C., 2015. Gut microbiota: Firmicutes and Bacteroidetes involved in insulin resistance by mediating levels of glucagon-like peptide 1. Nat. Rev. Endocrinol. 11, 254.

Hanage, W.P., 2014. Microbiology: microbiome science needs a healthy dose of scepticism. Nature 512, 247–248.

Ley, R.E., Turnbaugh, P.J., Klein, S., Gordon, J.I., 2006. Microbial ecology: human gut microbes associated with obesity. Nature 444, 1022–1023.

Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R., Gordon, J.I., 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444, 1027–1031.

Walker, A.W., Martin, J.C., Scott, P., Parkhill, J., Flint, H.J., Scott, K.P., 2015. 16S rRNA gene-based profiling of the human infant gut microbiota is strongly influenced by sample processing and PCR primer choice. Microbiome 3, 26.

Arthritis Research & Therapy

Open Access

CrossMark

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

Andréasson *et al. Arthritis Research & Therapy* (2016) 18:278

Page 2 of 8

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.

Andréasson *et al. Arthritis Research & Therapy* (2016) 18:278

Page 3 of 8

Assessment of intestinal symptoms

All patients were systematically questioned regarding the following GI symptoms: heartburn (dyspepsia), dysphagia, diarrhea, and/or constipation. These were recorded as present or not.

Laboratory examinations

Blood tests included measurements of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), haptoglobin, orosomucoid, α_1-antitrypsin, immunoglobulin (Ig)G, IgM, IgA, vitamin B_{12}, folic acid, ferritin, iron, transferrin iron-binding capacity (TIBC) and transthyretin. Subjects with an iron/TIBC ratio < 0.16 were considered to be iron deficient [25]. F-calprotectin was measured using a commercially available enzyme-linked immunosorbent assay (ELISA, Calpro, Lysaker, Norway). The lower limit of the ELISA was 30 µg/g and values below this cutoff were estimated as 20 µg/g. In accordance with published data and recommendations from the manufacturer, we considered F-calprotectin levels < 50 µg/g to be within normal range [26].

Assessment of gut dysbiosis

The GA-map™ Dysbiosis Test (Genetic Analysis, Oslo, Norway) has been developed and validated in relation to a Scandinavian control population to identify dysbiosis in adults by genetic analysis of a stool sample. The test makes use of 54 bacterial ribosomal RNA probes specific for various intestinal bacterial species or clades to generate genomic data on the intestinal microbiota composition. Using a defined algorithm, these data are subsequently translated into a Dysbiosis Index Score ranging from 1 to 5 (grades 1–2 are defined as eubiosis and 3–5 as dysbiosis). The test has been compared with MiSeq Illumina sequencing-based protocols and proven successful in identifying dysbiosis [6, 27]. In a healthy control population, 84% exhibited eubiosis and 16% dysbiosis [27]. In the current study, gut microbiota eubiosis and dysbiosis were delineated as per the standardised GA-map™ Dysbiosis Test results.

Statistical analyses

The Mann-Whitney U test was used to compare the degree of dysbiosis and the $\chi 2$ test to compare the frequency of dysbiosis in patients with and without various manifestations of SSc. Spearman correlation coefficient (r_s) was used to correlate the Dysbiosis Index Score with other continuous variables.

Results

Study population characteristics and levels of dysbiosis

Systemic sclerosis patients (n = 98) were examined for an array of characteristics and assessed for intestinal dysbiosis analysing their stools using the GA-map™ Dysbiosis

Test. Patient characteristics are presented in Table 1. A majority (75.5%) of the patients exhibited dysbiosis to some degree (score 3–5), and a significant proportion (24.9%) suffered from severe dysbiosis (score 5, Fig. 1).

Dysbiosis was associated with gastrointestinal manifestations of systemic sclerosis

A majority of the patients (84%) exhibited esophageal dysfunction, and dysbiosis was significantly more common in this group ($p = 0.013$; Fig. 2). The degree of dysmotility correlated with intestinal dysbiosis (Table 2). Malnutrition was frequent; 53% of the patients exhibited

Table 1 Patient characteristics

	n	(%)
Systemic sclerosis subtype		
limited cutaneous SSc	77	(78)
diffuse cutaneous SSc	21	(22)
Autoantibodies		
ANA-positive	87	(89)
ACA-positive	33	(34)
ARA-positive	10	(10)
ATA-positive	11	(11)
Smoking		
smoker	11	(11)
ex-smoker	43	(44)
non-smoker	44	(45)
Telangiectasias	39	(40)
Pulmonary arterial hypertension[a]	13	(13)
Pitting scars, current	23	(23)
Lung fibrosis[b]	35	(36)
Pathological cineradiography	82	(84)
Regular PPI usage	78	(80)
Immunosuppressive therapy		
mycophenolate mofetil	23	(23)
methotrexate	5	(5)
azathioprine	10	(10)
no immunosuppressive therapy	60	(61)
	median	interquartile range
Modified Rodnan skin score		
limited cutaneous	2	(0, 4)
diffuse cutaneous	10	(4, 22)
Disease duration, years[c]	6	(2, 16)
Prednisolone, daily intake (mg)	0	(0, 4)

ANA anti-nuclear antibodies, *ACA* anti-centrome antibodies, *ARA* anti-RNA polymerase III antibodies, *ATA* anti-topoisomerase1 antibodies, *PPI* proton pump inhibitor
[a]As determined by right heart catheterisation
[b]As determined on high-resolution computed tomography
[c]Years since first non-Raynaud's phenomena symptom

Andréasson *et al. Arthritis Research & Therapy* (2016) 18:278

Page 4 of 8

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
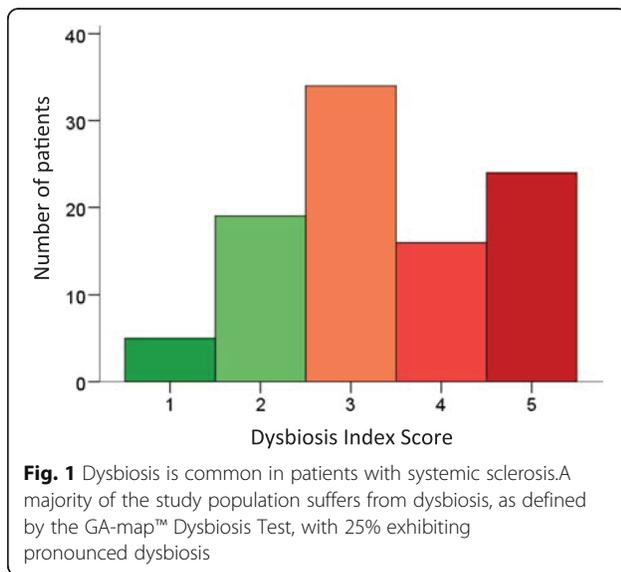
deficiency of folic acid, vitamin B12, and/or iron. Nineteen patients had a MUST score of ≥ 1, of which 18 exhibited dysbiosis, and 17 patients had pathological levels of P-transthyretin of which 15 exhibited dysbiosis. Patients with these malnutrition-associated characteristics (any deficiency, MUST ≥ 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 $\pm$ 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

Andréasson *et al. Arthritis Research & Therapy* (2016) 18:278

Page 5 of 8
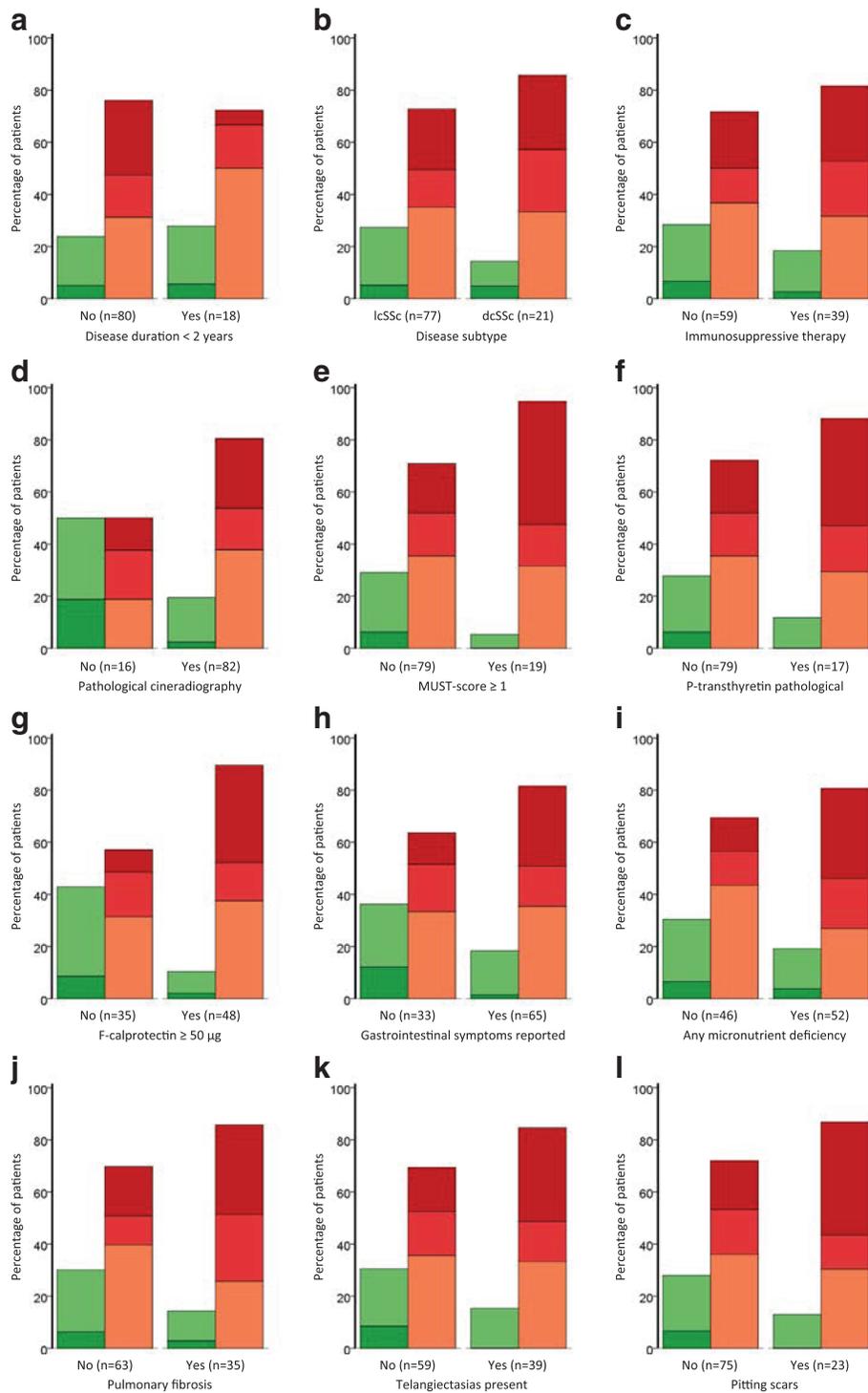
Fig. 2 Dysbiosis correlates with gastrointestinal and some extraintestinal manifestations of SSc, but not disease subtype or immunosuppressive therapy. Dysbiosis was prevalent in patients with both short and long disease duration (**a**), lcSSc and dcSSc (**b**) as well as in patients with and without 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 Univeersal Screening Tool

Andréasson *et al. Arthritis Research & Therapy* (2016) 18:278

Page 6 of 8

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

Andréasson *et al. Arthritis Research & Therapy* (2016) 18:278

Page 7 of 8

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

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.

References

1. Gabrielli A, Avvedimento EV, Krieg T. Scleroderma. N Engl J Med. 2009;360(19):1989–2003.
2. Qin J, Li R, Raes J, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464(7285):59–65.
3. Honda K, Littman DR. The microbiota in adaptive immune homeostasis and disease. Nature. 2016;535(7610):75–84.
4. Forbes JD, Van Domselaar G, Bernstein CN. The gut microbiota in immune-mediated inflammatory diseases. Front Microbiol. 2016;7:1081.
5. Andreasson K, Marsal J, Mansson B, Saxne T, Wollheim FA. Diet-induced arthritis in pigs: comment on the article by Scher et al. Arthritis Rheumatol. 2016;68(6):1568–9.
6. Iebba V, Totino V, Gagliardi A, et al. Eubiosis and dysbiosis: the two sides of the microbiota. New Microbiol. 2016;39(1):1–12.
7. Scher JU, Littman DR, Abramson SB. Microbiome in inflammatory arthritis and human rheumatic diseases. Arthritis Rheumatol. 2016;68(1):35–45.
8. Hevia A, Milani C, Lopez P, et al. Intestinal dysbiosis associated with systemic lupus erythematosus. MBio. 2014;5(5):e01548–14.
9. Rosser EC, Mauri C. A clinical update on the significance of the gut microbiota in systemic autoimmunity. J Autoimmun. 2016;74:85–93.
10. Costello ME, Ciccia F, Willner D, et al. Intestinal dysbiosis in ankylosing spondylitis. Arthritis Rheumatol. 2014. doi: 10.1002/art.38967. [Epub ahead of print].
11. de Paiva CS, Jones DB, Stern ME, et al. Altered mucosal microbiome diversity and disease severity in Sjogren syndrome. Sci Rep. 2016;6:23561.
12. Alipour B, Homayouni-Rad A, Vaghef-Mehrabany E, et al. Effects of Lactobacillus casei supplementation on disease activity and inflammatory cytokines in rheumatoid arthritis patients: a randomized double-blind clinical trial. Int J Rheum Dis. 2014;17(5):519–27.
13. Pineda Mde L, Thompson SF, Summers K, de Leon F, Pope J, Reid G. A randomized, double-blinded, placebo-controlled pilot study of probiotics in active rheumatoid arthritis. Med Sci Monit. 2011;17(6):CR347–54.
14. Marie I, Ducrotte P, Denis P, Menard JF, Levesque H. Small intestinal bacterial overgrowth in systemic sclerosis. Rheumatology (Oxford). 2009;48(10):1314–9.

Andréasson *et al. Arthritis Research & Therapy* (2016) 18:278

Page 8 of 8

15. Marie I, Leroi AM, Menard JF, Levesque H, Quillard M, Ducrotte P. Fecal calprotectin in systemic sclerosis and review of the literature. Autoimmun Rev. 2015;14(6):547–54.
16. Volkmann ER, Chang YL, Barroso N, et al. Association of systemic sclerosis with a unique colonic microbial consortium. Arthritis Rheumatol. 2016;68(6):1483–92.
17. Clements PJ, Becvar R, Drosos AA, Ghattas L, Gabrielli A. Assessment of gastrointestinal involvement. Clin Exp Rheumatol. 2003;21(3 Suppl 29):S15–8.
18. Andreasson K, Scheja A, Saxne T, Ohlsson B, Hesselstrand R. Faecal calprotectin: a biomarker of gastrointestinal disease in systemic sclerosis. J Intern Med. 2011;270(1):50–7.
19. Codullo V, Cereda E, Klersy C, et al. Serum prealbumin is an independent predictor of mortality in systemic sclerosis outpatients. Rheumatology (Oxford). 2016;55(2):315–9.
20. Baron M, Hudson M, Steele R. Canadian Scleroderma Research G. Malnutrition is common in systemic sclerosis: results from the Canadian scleroderma research group database. J. Rheumatol. 2009;36(12):2737–43.
21. Ingenbleek Y, Bernstein LH. Plasma transthyretin as a biomarker of lean body mass and catabolic states. Adv Nutr. 2015;6(5):572–80.
22. LeRoy EC, Black C, Fleischmajer R, et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol. 1988;15(2):202–5.
23. Summerton SL. Radiographic evaluation of esophageal function. Gastrointest Endosc Clin N Am. 2005;15(2):231–42.
24. Clements P, Lachenbruch P, Siebold J, et al. Inter and intraobserver variability of total skin thickness score (modified Rodnan TSS) in systemic sclerosis. J Rheumatol. 1995;22(7):1281–5.
25. Moreno Chulilla JA, Romero Colas MS, Gutierrez MM. Classification of anemia for gastroenterologists. World J Gastroenterol. 2009;15(37):4627–37.
26. Manz M, Burri E, Rothen C, et al. Value of fecal calprotectin in the evaluation of patients with abdominal discomfort: an observational study. BMC Gastroenterol. 2012;12:5.
27. Casen C, Vebo HC, Sekelja M, et al. Deviations in human gut microbiota: a novel diagnostic test for determining dysbiosis in patients with IBS or IBD. Aliment Pharmacol Ther. 2015;42(1):71–83.
28. Miyoshi J, Chang EB. The gut microbiota and inflammatory bowel diseases. Transl Res. 2016;S1931–5244(16):30095.
29. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. Cell. 2014;157(1):121–41.
30. Dolan KT, Chang EB. Diet, gut microbes, and the pathogenesis of inflammatory bowel diseases. Mol Nutr Food Res. 2016. doi: 10.1002/mnfr. 201600129. [Epub ahead of print].
31. Manetti M, Neumann E, Milia AF, et al. Severe fibrosis and increased expression of fibrogenic cytokines in the gastric wall of systemic sclerosis patients. Arthritis Rheum. 2007;56(10):3442–7.
32. Manetti M, Neumann E, Muller A, et al. Endothelial/lymphocyte activation leads to prominent CD4+ T cell infiltration in the gastric mucosa of patients with systemic sclerosis. Arthritis Rheum. 2008;58(9):2866–73.
33. Shaw KA, Bertha M, Hofmekler T, et al. Dysbiosis, inflammation, and response to treatment: a longitudinal study of pediatric subjects with newly diagnosed inflammatory bowel disease. Genome Med. 2016;8(1):75.
34. Sokol H, Seksik P, Furet JP, et al. Low counts of Faecalibacterium prausnitzii in colitis microbiota. Inflamm Bowel Dis. 2009;15(8):1183–9.
35. Bishop V, Harrison E, Lal S, Herrick AL. Evidence for a clinical association between body mass index and malabsorption in patients with systemic sclerosis. Scand J Rheumatol. 2015;44(4):341–3.
36. Krause L, Becker MO, Brueckner CS, et al. Nutritional status as marker for disease activity and severity predicting mortality in patients with systemic sclerosis. Ann Rheum Dis. 2010;69(11):1951–7.
37. Tsuda A, Suda W, Morita H, et al. Influence of proton-pump inhibitors on the luminal microbiota in the gastrointestinal tract. Clin Transl Gastroenterol. 2015;6:e89.
38. Khanna D, Hays RD, Maranian P, et al. Reliability and validity of the University of California, Los Angeles Scleroderma Clinical Trial Consortium Gastrointestinal Tract Instrument. Arthritis Rheum. 2009;61(9):1257–63.
39. Abdollahi-Roodsaz S, Abramson SB, Scher JU. The metabolic role of the gut microbiota in health and rheumatic disease: mechanisms and interventions. Nat Rev Rheumatol. 2016;12(8):446–55.

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

Martin Aasbrenn, Jørgen Valeur & Per G. Farup

Published online: 22 Dec 2017.

Submit your article to this journal

Article views: 19

View related articles

View Crossmark data

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Check for updates

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

Martin Aasbrenn[a,b] (iD), 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^{TM} 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.

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

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^{TM} 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

Figure 1. Flow chart depicting inclusion of subjects.
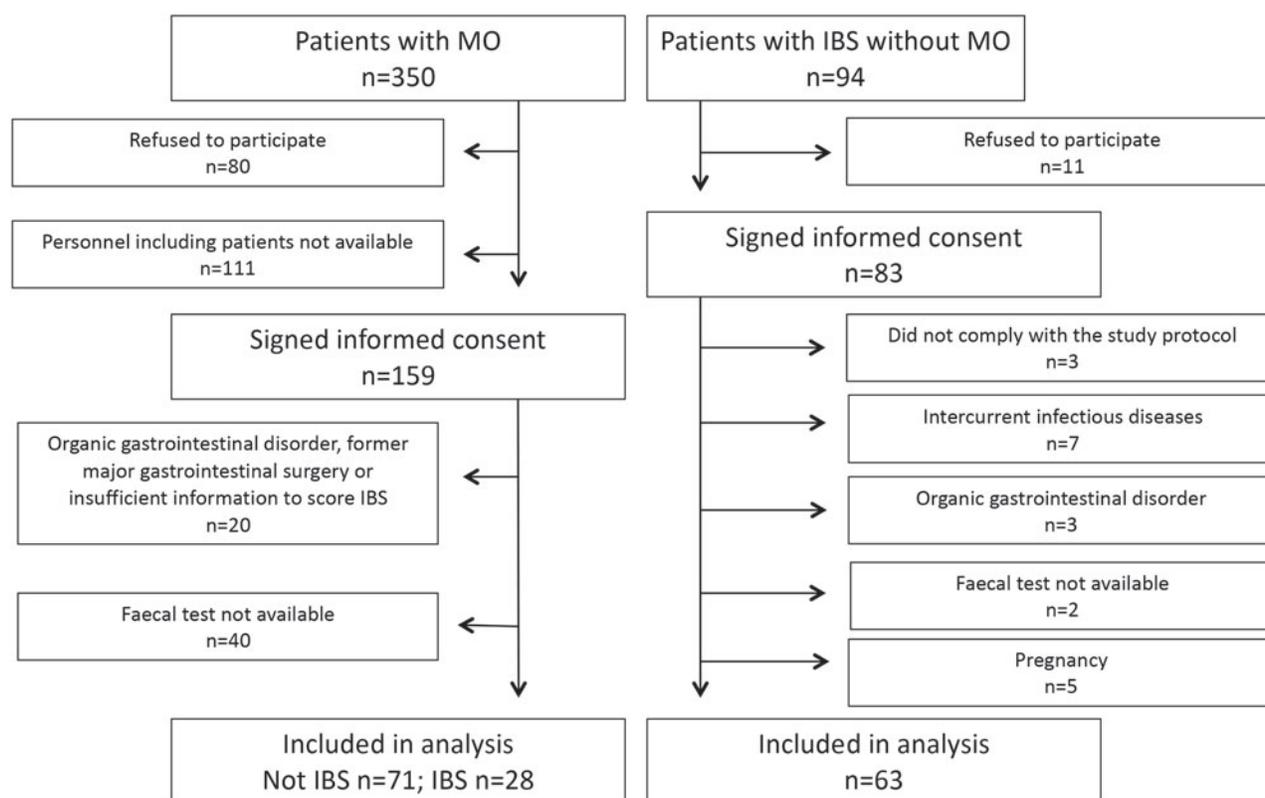
Abbreviations: MO = Morbid obesity, IBS = Irritable bowel syndrome

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).

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.

obesity than in healthy volunteers (Table 3). There were no significant differences in the prevalence of dysbiosis between the groups with and without IBS stratified for morbid obesity (Table 3). Among the subjects with IBS, the prevalence of dysbiosis was higher in the subgroup IBS-D than in subjects without IBS-D (odds ratio 2.48 (95% confidence interval 1.01–6.09), p = .047).

In subjects with morbid obesity, the sensitivity, specificity, LR+ and LR− of the dysbiosis test for the diagnosis of IBS were 64%, 37%, 1.01 and 0.98, respectively (Table 4). Data on the DI were available in three groups. In these groups, there were no significant differences in the DI (Table 2). In subjects with morbid obesity, the area under the ROC curve for the DI was 0.54 (95% CI 0.42–0.67, p = .50). In subjects without morbid obesity, the sensitivity, specificity, LR+, and LR− were 49%, 58%, 1.18 and 0.87 respectively (Table 4).

Table 4. Diagnostic properties of the dysbiosis test for the diagnosis of irritable bowel syndrome.

	Sensitivity	Specificity	PPV	NPV	Accuracy	DOR	LR+	LR−
Morbid obesity	64% (44%–81%)	37% (26%–49%)	29% (18%–41%)	72% (55%–86%)	44% (34%–54%)	1.04 (0.41–2.59)	1.01 (0.73–1.41)	0.98 (0.54–1.75)
Without morbid obesity	49% (36%–62%)	58% (47%–69%)	n.a	n.a	n.a	1.35 (0.71–2.58)	1.18 (0.83–1.67)	0.87 (0.65–1.18)

DOR: diagnostic odds ratio; LR+: positive likelihood ratio; LR−: negative likelihood ratio; NPV: negative predictive value; PPV: positive predictive values. All values are given with 95% confidence intervals.

Discussion

The prevalence of dysbiosis was above 40% in all four groups of subjects examined in this study. The subjects with IBS had a lower prevalence and the healthy volunteers had a higher prevalence of dysbiosis than previously reported [11,18]. The association between dysbiosis and obesity with this dysbiosis test was in accordance with studies using other methods [7,13].

IBS and dysbiosis were not significantly associated, either among subjects with or without morbid obesity. This was unexpected, as the dysbiosis test was created by selecting the probes that best could separate subjects with and without IBS [11]. The high prevalence of dysbiosis in subjects with IBS-D could be explained by the association between stool consistency and the microbiota [19].

The dysbiosis test was unsuitable as a diagnostic test for IBS. The upper and lower limits of the 95% confidence intervals for the LR + and LR- were below 2 and above 0.5 respectively, which have been described as the limits for unimportant effects [20]. Other cut-off values on the dysbiosis index did not improve the diagnostic usefulness.

Standard DNA-based methods for the study of microbiota produce huge data sets and are expensive [4]. The faecal dysbiosis test evaluated in this paper uses 54 probes and analyses only a small proportion of the genetic material. The test, therefore, gives limited knowledge about the entire faecal microbiota, though high overlap between the dysbiosis test and metagenomic sequencing has been reported for some bacteria [11,21]. For the use in clinical practice, knowledge about tests that give a summary of the genetic material is nevertheless very important, as such tests are easily available and less expensive.

Prevalence rates of dysbiosis as measured by the dysbiosis test appear to vary widely between different groups of healthy volunteers as well as between groups of subjects with the same disorder. A large number of confounding factors are relevant, including but not restricted to diet, geography and stool consistency [19]. This study reminds us that biomarkers based on a limited number of subjects and promoted by the producer demand validation in daily practice and in larger and different groups of subjects before taken into regular use.

The imperfect properties of the test for the diagnosis of IBS do not exclude other areas of application. The symptom-based diagnosis of IBS defined by the Rome III criteria may represent several disorders with similar symptoms. Subjects with and without dysbiosis or with different degrees of dysbiosis might be subgroups of IBS with different aetiology, pathophysiology, severity or prognosis and profit from different treatment alternatives. For instance, the effect of fermentable oligosaccharides, disaccharides and monosaccharides and polyols (FODMAP) reduced diet has been shown to be best in subjects without dysbiosis [12]. Such areas of application of the test deserve further evaluation. The test has also been related to the prognosis of inflammatory bowel disease [22].

The microbiota may be a modifiable cause of obesity-related metabolic comorbidity [23]. Subjects with obesity and subjects with a metabolic risk profile have been shown to be identifiable by less than ten bacterial species from faecal samples [7]. If the dysbiosis test or other tests based on the same technology can characterise obesity-related changes in the microbiota, they may become useful for the management of subgroups of subjects with obesity and metabolic disorders.

Strengths and limitations

To achieve high quality, the manufacturer's laboratory performed all the faecal analyses. In the two groups of patients with morbid obesity, the laboratory was blinded to the diagnosis of IBS; and for all subjects, the laboratory was blinded to symptom severity. The diagnosis of IBS was based on a validated Norwegian translation of the Rome III criteria [16]. The study size resulted in narrow confidence intervals that excluded the test as a diagnostic tool for IBS. All the faecal samples were handled according to the producer's recommendations. The small difference in preanalytical handling at the two centres was judged as unimportant. A limitation of the study was that the data in the healthy volunteers were based on previously published data and not our own data.

Selection bias is unlikely. The main reason for the inadequate inclusion of subjects with morbid obesity was the absence of a study nurse 2–3 days a week (Figure 1). In the group with IBS without morbid obesity, consecutive subjects were included. As expected, symptoms were more severe in the group with IBS without obesity than in the group with obesity, because subjects referred to a gastroenterological outpatient clinic for abdominal complaints are likely to have more severe symptoms than subjects referred for obesity and asked about abdominal complaints (Table 1).

Conclusions

The dysbiosis test was inappropriate as a diagnostic test of IBS. Dysbiosis was more common in subjects with morbid

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.

Acknowledgments

Disclosure statement

Funding

ORCID

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

References

[1] Ford AC, Lacy BE, Talley NJ. Irritable bowel syndrome. N Engl J Med. 2017;376:2566–2578.

[2] Camilleri M. Review article: biomarkers and personalised therapy in functional lower gastrointestinal disorders. Aliment Pharmacol Ther. 2015;42:818–828.

[3] Sood R, Gracie DJ, Law GR, et al. Systematic review with meta-analysis: the accuracy of diagnosing irritable bowel syndrome with symptoms, biomarkers and/or psychological markers. Aliment Pharmacol Ther. 2015;42:491–503.

[4] Lynch SV, Pedersen O. The human intestinal microbiome in health and disease. N Engl J Med. 2016;375:2369–2379.

[5] GA-map ™ Dysbiosis Test [Internet]. Oslo: Genetic Analysis; 2016 [cited 2017 Nov 11]. Available from: http://www.genetic-analysis.com/ga-map-dysbiosis-test

[6] Major G, Spiller R. Irritable bowel syndrome, inflammatory bowel disease and the microbiome. Curr Opin Endocrinol Diabetes Obes. 2014;21:15–21.

[7] Le Chatelier E, Nielsen T, Qin J, et al. Richness of human gut microbiome correlates with metabolic markers. Nature. 2013; 500:541–546.

[8] Qin J, Li Y, Cai Z, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature. 2012;490:55–60.

[9] Palleja A, Kashani A, Allin KH, et al. Roux-en-Y gastric bypass surgery of morbidly obese patients induces swift and persistent changes of the individual gut microbiota. Genome Med. 2016;8:67.

[10] Zhuang X, Xiong L, Li L, et al. Alterations of gut microbiota in patients with irritable bowel syndrome: A systematic review and meta-analysis. J Gastroenterol Hepatol. 2017;32:28–38.

[11] Casen C, Vebø HC, Sekelja M, et al. Deviations in human gut microbiota: a novel diagnostic test for determining dysbiosis in patients with IBS or IBD. Aliment Pharmacol Ther. 2015;42: 71–83.

[12] Bennet SMP, Böhn L, Störsrud S, et al. Multivariate modelling of faecal bacterial profilse of patients with IBS predicts responsiveness to a diet low in FODMAPs. Gut. 2017. doi: 10.1136/gutjnl-2016-313128.

[13] Schwiertz A, Taras D, Schäfer K, et al. Microbiota and SCFA in lean and overweight healthy subjects. Obesity (Silver Spring). 2009;18:190–195.

[14] NIH conference. Gastrointestinal surgery for severe obesity. Consensus Development Conference Panel. Ann Intern Med. 1991;115:956–961.

[15] Ricanek PV, Vatn S, Kalla R, et al. The Ibd-Character Consortium. Microbiota Alterations in Treatment Naïve IBD and Non-IBD Patients - the EU IBD-Character Project. United European Gastroenterol J. 2016;4:A95.

[16] Longstreth GF, Thompson WG, Chey WD, et al. Functional bowel disorders. Gastroenterology. 2006;130:1480–1491.

[17] Francis CY, Morris J, Whorwell PJ. The irritable bowel severity scoring system: a simple method of monitoring irritable bowel syndrome and its progress. Alimentary Pharmacol Ther. 1997; 11:395–402.

[18] Sauar J, Halvorsen F, Corwin C, Olafsson S. Dysbiosis and stability over two years in patients with irritable bowel syndrome. United European Gastroenterology J. 2016;4(Supplement 1):A524.

[19] Falony G, Joossens M, Vieira-Silva S, et al. Population-level analysis of gut microbiome variation. Science. 2016;352: 560–564.

[20] Portney LG, Watkins MP. Foundations of Clinical Research Applications to Practice. Upper Saddle River (NJ): Pearson International Edition 2009. Statistical measures of validity, p. 619–658.

[21] Vebø HC, Sekelja M, Nestestog R, et al. Temporal development of the infant gut microbiota in immunoglobulin E-sensitized and nonsensitized children determined by the GA-map infant array. Clin Vaccine Immunol. 2011;18:1326–1335.

[22] Magnusson MK, Strid H, Sapnara M, et al. Anti-TNF therapy response in patients with ulcerative colitis is associated with colonic antimicrobial peptide expression and microbiota composition. ECCOJC. 2016;10:943–952.

[23] Hansen TH, Gøbel RJ, Hansen T, et al. The gut microbiome in cardio-metabolic health. Genome Med. 2015;7:33.

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 RT² 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.

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

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

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.

RT² Profiler PCR Array

Complementary DNA was prepared using the RT² First Strand Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The RT² 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 RT² qPCR SYBR Green MasterMix (Qiagen). Data were analyzed in the CFX Manager software (BioRad) and the RT² Profiler PCR Array Data Analysis version 3.5 (Qiagen). All samples except two passed the quality checks for PCR Array reproducibility, RT efficiency, and genomic DNA contamination. *GAPDH* and *HPRT1* were chosen as housekeeping genes. A complete list of the genes in the array is shown in Table 1, Supplemental Digital Content 1, http://links.lww.com/IBD/B501.

Quantitative RT-PCR Analysis

Complementary DNA was prepared using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. Complementary DNA was then used for RT-PCR using Taqman Universal PCR MasterMix (Applied Biosystems, Foster City, CA) and Taqman Gene Expression assays (Applied Biosystems) according to the manufacturer's protocol. Expression of *BPI* (Hs01552756_m1), conserved helix-loop-helix ubiquitous kinase (*CHUK*, Hs00989502_m1), and chemokine (C-X-C motif) ligand 2 (*CXCL2*, Hs00601975_m1) was determined. Amplification was carried out using a 7500 RT-PCR system (Applied Biosystems), and all samples were run in triplicate. The results were normalized to the expression level of *GAPDH* (Hs03929097_g1) and *HPRT* (Hs02800695_m1) and expressed as $2^{-(\text{Target-Housekeeping})}$.

PBMC Cultivation Assay and Flow Cytometry Analysis

PBMCs were isolated from heparinized venous blood from healthy volunteers by density gradient centrifugation using Ficoll-Paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Cells were cultured in Iscove's medium supplemented with 100 μg/mL gentamicin, 3 μg/mL L-glutamine (all from Sigma-Aldrich, St Louis, MO), and 10% fetal bovine serum (Gibco by Thermo Fisher Scientific, Waltham, MA). Cell cultures from each subject were stimulated with a final concentration of 1 ng/mL LPS (Sigma-Aldrich) or 10 μg/mL peptidoglycan (tlrl-pgnb2; Invivogen, San Diego, CA) with and without addition of 0.02, 0.2, or 2 μg/mL BPI (SRP6307; Sigma-Aldrich) in flat-bottomed 96-well plates (Nunc, Roskilde, Denmark). Before addition to the cells, LPS or peptidoglycan were preincubated with or without BPI at 37°C for 15 minutes. The preincubation step was performed at 10× concentration in a total volume of 20 μL 0.1 M citric acid buffer (pH 5.5). Subsequently, 180 μL supplemented media was added to generate a 1× solution, the mixture was added to the cells, and the plates were incubated for 18 hours at 37°C. Cell culture supernatants were collected and stored at −80°C at the end of the cell culture. Cultured cells were stained for flow cytometry analysis using the following antibodies: anti-HLADR-Alexa700, anti-CD14-PeCy7, anti-CD80-APC, lineage markers (anti-CD3-PE-CF594, anti-CD19-PE-CF594, anti-CD56-PE-CF594) (all from BD Biosciences, San Jose, CA). 7-Aminoactinomycin D (BD Biosciences) was used according to the manufacturer's protocol to exclude nonviable cells. Flow cytometry analysis was performed using an LSR II flow cytometer (BD Pharmingen, San Jose, CA). The data were analyzed using Flow Jo software (Treestar Inc., Ashland, OR).

Analysis of CXCL2 Protein and Anti-BPI antibodies

Serum samples were analyzed by enzyme-linked immunosorbent assay for levels of CXCL2 protein (Human MIP2 Enzyme-Linked Immunosorbent Assay Kit; Abcam, Cambridge, MA) and anti-BPI-IgG antibodies (Demeditec Diagnostics GmbH, Kiel, Germany) according to the manufacturers' instructions. In vitro PBMC cultivation supernatants were analyzed for levels of CXCL2 protein (Abcam).

Immunohistochemistry

Frozen sections were prepared (7 μm) on microslides with a cryostat (Leitz, Wetzlar, Germany), fixed in 100% ice-cold acetone, blocked in phosphate-buffered saline with 1% bovine serum albumin and 5% goat serum, and stained with polyclonal rabbit-anti-BPI-IgG (PA5-26069, diluted 1:200; Thermo Fisher Scientific) followed by goat-anti-rabbit-AlexaFluor488 (Molecular Probes A11008, diluted 1:50; Invitrogen). Mounting was performed with 4′, 6-Diamidino-2-Phenylindole Dihydrochloride (DAPI)-UltraCruz mounting medium (sc-24941; Santa Cruz Biotech,

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.

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

	Mild Disease Course (n = 19)	Moderate/Severe Disease Course (n = 29)
Health care seeking during the year[a], n (%)		
Year 1 (≥4 times/1–3 times)	1 (5)/5 (26)	10 (34)/10 (34)
Year 2 (≥4 times/1–3 times)	0 (0)/2 (11)	3 (10)/14 (48)
Year 3 (≥4 times/1–3 times)	0 (0)/5 (26)	2 (7)/14 (48)
Hospitalized during the year[a], n (%)		
Year 1	2 (11)	9 (31)
Year 2	0 (0)	1 (3)
Year 3	0 (0)	2 (7)
5-ASA use during the year[b], n (%)		
Year 1	14 (74)	24 (83)
Year 2	12 (63)	25 (86)
Year 3	11 (59)	24 (83)
Thiopurine use during the year, n (%)		
Year 1	0 (0)	4 (14)
Year 2	0 (0)	5 (17)
Year 3	0 (0)	11 (38)
Corticosteroid use during the year[b], n (%)		
Year 1	1 (5)	7 (24)
Year 2	1 (5)	3 (10)
Year 3	2 (11)	1 (3)

[a]For gastrointestinal symptoms.
[b]Excluding treatment during the first flare.
5-ASA, 5-aminosalicylic acid.

FIGURE 1. Patients with a mild and a moderate/severe disease course differ at diagnosis of disease with respect to their fecal microbiota composition. Fecal samples obtained from patients with newly diagnosed UC were analyzed by the GA-map Dysbiosis test (mild disease n = 7, moderate/severe disease n = 11). A, Multivariate discriminant analysis (OPLS-DA) scatter plot showing separation between patients with mild disease course (blue circles) and moderate/severe disease course (red circles). R2 defines the goodness of fit and Q2 the goodness of prediction. B, OPLS-DA loading scatter plot showing associations between fecal bacterial groups and disease outcome. The multivariate analysis was performed with bacterial groups as X variables (n = 15) and disease course (mild disease and moderate/severe disease) as Y variables.

obtained from 18 patients with newly diagnosed UC was analyzed using the GA-map Dysbiosis test. Stool samples obtained at the time of UC diagnosis were provided by 7 patients who had a mild disease course and 11 patients with a moderate/severe disease course during the 3 years follow-up. All patients, except one, had dysbiosis (Dysbiosis index > 2), but no differences in Dysbiosis index were found between patients with a mild and a moderate/severe disease (median [range], 3 [3–5] versus 5 [2–5], *P* = 0.30). Multivariate analysis revealed that fecal microbiota composition at the time of UC diagnosis differed between patients who had a mild versus a moderate/severe disease course, including the total set of bacteria (Fig. 1A). The model showed a good R2 value (0.55), defining a good separation between the groups, but a low Q2 value (−0.80), showing low predictive ability.

The OPLS-DA loading scatter plot showed that no single bacteria at the diagnosis of disease defined either patients with a mild disease course or patients with a moderate/severe disease course because all X variables (bacteria) were distributed relatively close to the mid-vertical line (Fig. 1B). Nevertheless, a bacterial composition with higher abundance of *Proteobacteria*

and *Streptococcus* was seen in patients with a moderate/severe disease course, whereas a composition including higher abundance of *Ruminococcus* and *Akkermansia* was demonstrated in patients with a mild disease course (Fig. 1B). Together, these data suggest that there are differences in the fecal microbial composition, but without strong prognostic value, between patients with newly diagnosed UC who will have a mild or a moderate/severe disease course over a 3-year follow-up period.

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

Mucosal Antibacterial Response Profile in Patients with Newly Diagnosed UC Is Associated with Disease Course

The composition of the gut microbial milieu develops in symbiosis with the host's innate immune response. Thus, an exploratory mRNA array was performed to study the mucosal antibacterial response profile in biopsies obtained from the patients at diagnosis. Samples from the patients with gut microbiota profiling were intended for inclusion in these analyses, but 2 samples did not pass quality control for the array (mild n = 1, moderate/severe n = 1) and 3 samples had insufficient RNA quantity (mild n = 1, moderate/severe n = 2), which resulted in 13 patients for these analyses. The overall mucosal antibacterial response profiles at the time of UC diagnosis showed excellent discrimination and high predictive ability when comparing patients with a mild and a moderate/severe disease course, respectively (Fig. 2A, R2 = 0.98, Q2 = 0.56). The most important nominators for the discrimination between the groups were higher expression of *CXCL2* for patients with a moderate/severe disease course, whereas patients with a mild disease course had higher expression of *BPI* and *CHUK* (Fig. 2B). Among the 84 genes, 25 had a variable influence on projection larger than 1 and were thus the most important genes for the explanation of the different mucosal antibacterial response profiles between the 2 patient groups (see Fig. 1, Supplemental Digital Content 2, http://links.lww.com/IBD/B502). A multivariate factor analysis combining data from fecal microbiota and mucosal antibacterial response (X variables, n = 99) and disease course (Y variable, mild n = 5, moderate/severe n = 8) did not result in an improved discrimination between the groups (R2 = 0.89, Q2 = 0.16, data not shown).

Next, mucosal gene expression of *BPI*, *CXCL2*, and *CHUK* at the time of UC diagnosis was evaluated by RT-PCR in the full patient cohort (n = 44), and it was confirmed that patients with a mild disease course had higher levels of *BPI* and lower levels of *CXCL2* compared with patients with a moderate/severe disease course (Fig. 3A). In contrast, no differences in gene expression were detected when patients were subgrouped according to high (≥9) versus moderate (≤8) Mayo score (Fig. 3B) or according to disease extent at the time of UC diagnosis (Fig. 3C). *BPI* expression was also analyzed in noninflamed control patients (n = 7, median age 42 yrs; 4 males/3 females) who showed higher *BPI* gene expression compared with patients with newly diagnosed UC (n = 44) (median [interquartile range], 0.00024 [0.00020–0.00067] versus 0.00015 [0.00009–0.00021], P = 0.01). Protein expression of BPI was confirmed by immunohistochemistry, where BPI expression was detected in the epithelial cell layer and crypts (Fig. 4). However, no quantifiable differences in BPI protein expression could be detected by immunohistochemistry between noninflamed controls and UC patients with a mild or a moderate/severe disease course (data not shown).

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*.

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.
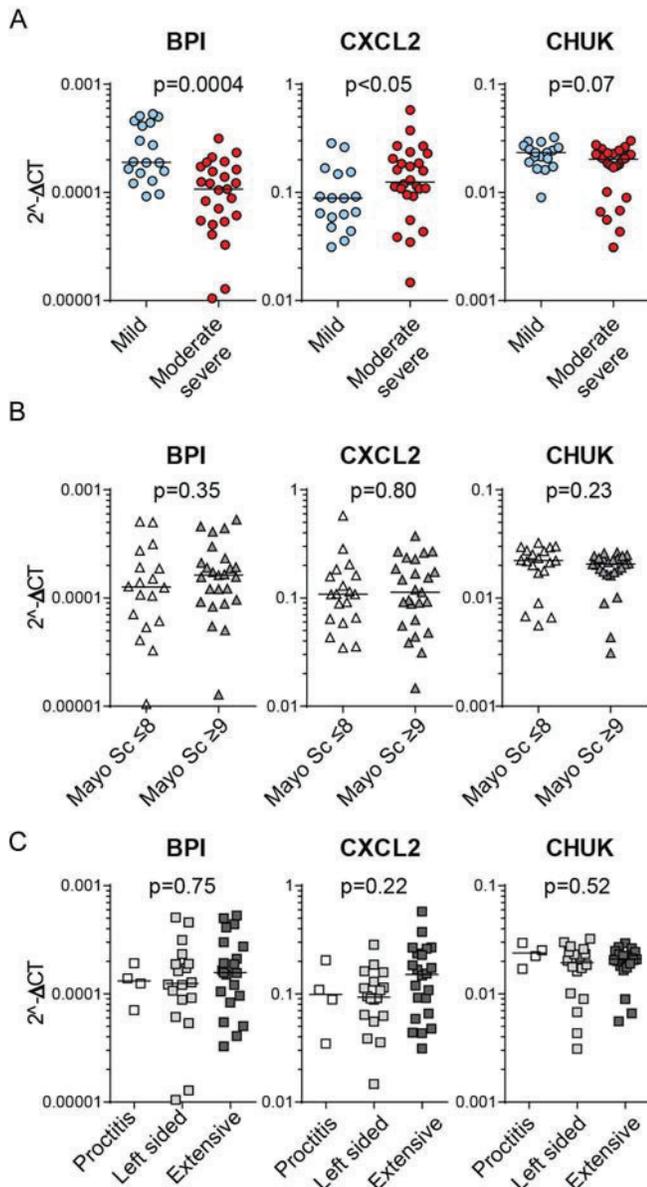

FIGURE 3. Mucosal expression of BPI, CHUK, and CXCL2 in patients with newly diagnosed UC is associated with disease course. Rectal mucosal biopsies obtained were analyzed for mRNA expression of *BPI*, *CXCL2*, and *CHUK* using RT-PCR. For patients with newly diagnosed UC, gene expression was compared 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], $P = 0.19$)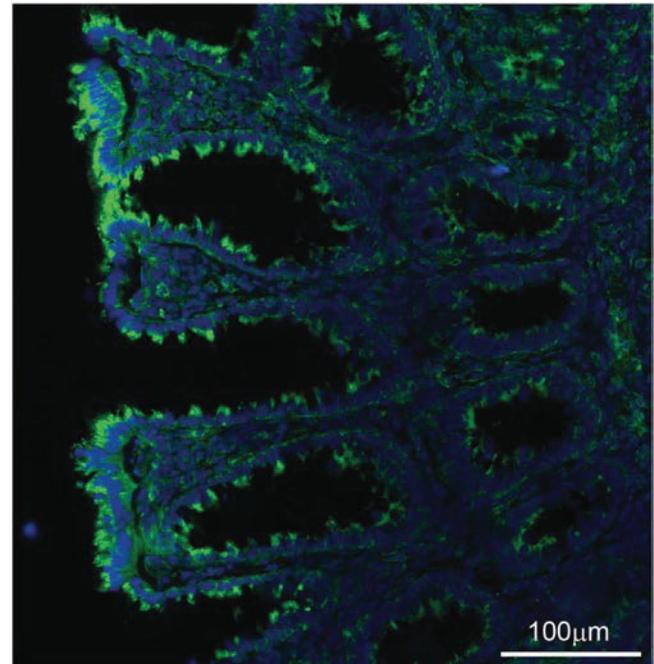 or for BPI autoantibodies (median [range], 39 U/mL [20–510] versus 34 U/mL [23–182], $P = 0.53$) between patients with a mild and a moderate/severe disease course.

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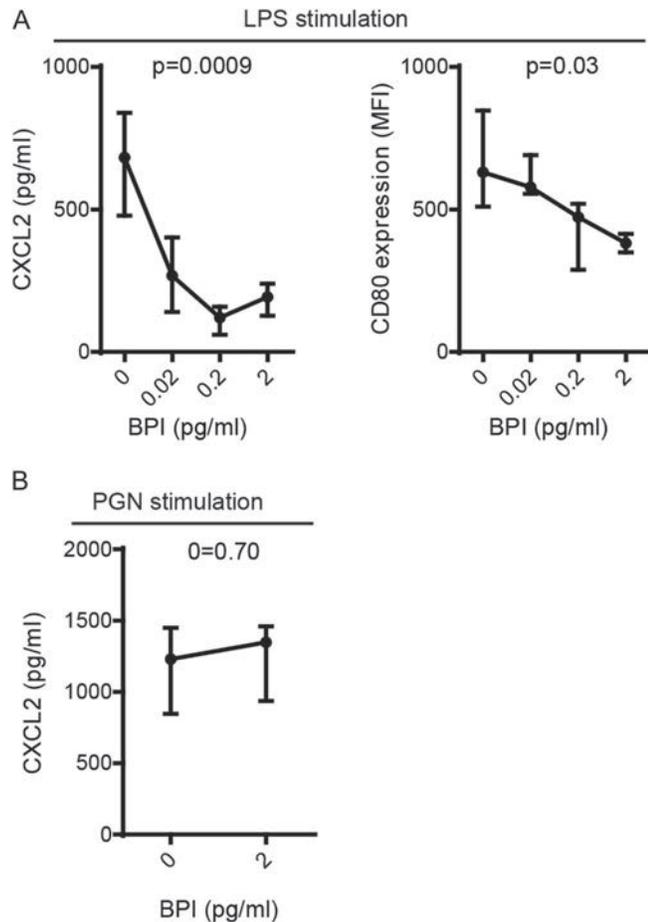
FIGURE 5. BPI reduces CXCL2 secretion from monocytes during LPS stimulation. PBMCs from healthy controls were stimulated with (A) LPS or (B) peptidoglycan (PGN) without and with increasing concentrations of BPI for 18 hours. Secretion of CXCL2 into the media was determined by enzyme-linked immunosorbent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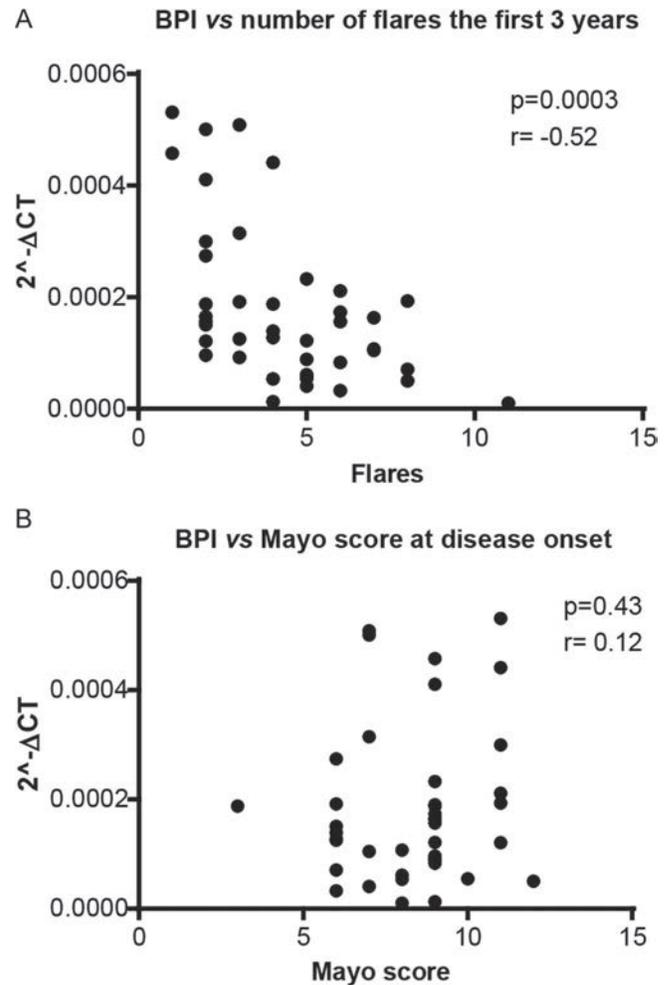

FIGURE 6. Mucosal gene expression of BPI in patients with newly diagnosed UC correlates to the number of flares during the 3 years follow-up. Rectal mucosal biopsies obtained from patients with newly diagnosed UC (n = 44) were analyzed for mRNA expression of *BPI* using RT-PCR. Gene expression was correlated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

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

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.

REFERENCES

1. Langholz E, Munkholm P, Davidsen M, et al. Course of ulcerative colitis: analysis of changes in disease activity over years. *Gastroenterology*. 1994;107:3–11.
2. Solberg IC, Lygren I, Jahnsen J, et al. Clinical course during the first 10 years of ulcerative colitis: results from a population-based inception cohort (IBSEN Study). *Scand J Gastroenterol*. 2009;44:431–440.
3. Roth LS, Chande N, Ponich T, et al. Predictors of disease severity in ulcerative colitis patients from Southwestern Ontario. *World J Gastroenterol*. 2010;16:232–236.
4. Waterman M, Knight J, Dinani A, et al. Predictors of outcome in ulcerative colitis. *Inflamm Bowel Dis*. 2015;21:2097–2105.
5. Romberg-Camps MJ, Dagnelie PC, Kester AD, et al. Influence of phenotype at diagnosis and of other potential prognostic factors on the course of inflammatory bowel disease. *Am J Gastroenterol*. 2009;104:371–383.
6. Ohman L, Dahlen R, Isaksson S, et al. Serum IL-17A in newly diagnosed treatment-naive patients with ulcerative colitis reflects clinical disease severity and predicts the course of disease. *Inflamm Bowel Dis*. 2013;19:2433–2439.
7. Yarur AJ, Strobel SG, Deshpande AR, et al. Predictors of aggressive inflammatory bowel disease. *Gastroenterol Hepatol (N Y)*. 2011;7:652–659.
8. Ott SJ, Musfeldt M, Wenderoth DF, et al. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. *Gut*. 2004;53:685–693.
9. Frank DN, St Amand AL, Feldman RA, et al. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A*. 2007;104:13780–13785.
10. Casen C, Vebo HC, Sekelja M, et al. Deviations in human gut microbiota: a novel diagnostic test for determining dysbiosis in patients with IBS or IBD. *Aliment Pharmacol Ther*. 2015;42:71–83.
11. Canny G, Levy O, Furuta GT, et al. Lipid mediator-induced expression of bactericidal/permeability-increasing protein (BPI) in human mucosal epithelia. *Proc Natl Acad Sci U S A*. 2002;99:3902–3907.
12. Weiss J, Elsbach P, Olsson I, et al. Purification and characterization of a potent bactericidal and membrane active protein from the granules of human polymorphonuclear leukocytes. *J Biol Chem*. 1978;253:2664–2672.
13. Gazzano-Santoro H, Parent JB, Grinna L, et al. High-affinity binding of the bactericidal/permeability-increasing protein and a recombinant amino-terminal fragment to the lipid A region of lipopolysaccharide. *Infect Immun*. 1992;60:4754–4761.
14. Heumann D, Gallay P, Betz-Corradin S, et al. Competition between bactericidal/permeability-increasing protein and lipopolysaccharide-binding protein for lipopolysaccharide binding to monocytes. *J Infect Dis*. 1993;167:1351–1357.
15. Marra MN, Wilde CG, Griffith JE, et al. Bactericidal/permeability-increasing protein has endotoxin-neutralizing activity. *J Immunol*. 1990;144:662–666.
16. Monajemi H, Meenan J, Lamping R, et al. Inflammatory bowel disease is associated with increased mucosal levels of bactericidal/permeability-increasing protein. *Gastroenterology*. 1996;110:733–739.
17. Haapamaki MM, Haggblom JO, Gronroos JM, et al. Bactericidal/permeability-increasing protein in colonic mucosa in ulcerative colitis. *Hepatogastroenterology*. 1999;46:2273–2277.
18. Walmsley RS, Zhao MH, Hamilton MI, et al. Antineutrophil cytoplasm autoantibodies against bactericidal/permeability-increasing protein in inflammatory bowel disease. *Gut*. 1997;40:105–109.
19. Schinke S, Fellermann K, Herlyn K, et al. Autoantibodies against the bactericidal/permeability-increasing protein from inflammatory bowel disease patients can impair the antibiotic activity of bactericidal/permeability-increasing protein. *Inflamm Bowel Dis*. 2004;10:763–770.
20. Schroeder KW, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. *N Engl J Med*. 1987;317:1625–1629.
21. Satsangi J, Silverberg MS, Vermeire S, et al. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. *Gut*. 2006;55:749–753.
22. Geuking MB, Koller Y, Rupp S, et al. The interplay between the gut microbiota and the immune system. *Gut Microbes*. 2014;5:411–418.
23. Thaiss CA, Zmora N, Levy M, et al. The microbiome and innate immunity. *Nature*. 2016;535:65–74.
24. Sartor RB, Wu GD. Roles for intestinal bacteria, Viruses, and Fungi in pathogenesis of inflammatory bowel diseases and Therapeutic Approaches. *Gastroenterology*. 2017;152:327–339.
25. Thorkildsen LT, Nwosu FC, Avershina E, et al. Dominant fecal microbiota in newly diagnosed untreated inflammatory bowel disease patients. *Gastroenterol Res Pract*. 2013;2013:636785.

26. Shah R, Cope JL, Nagy-Szakal D, et al. Composition and function of the pediatric colonic mucosal microbiome in untreated patients with ulcerative colitis. *Gut Microbes.* 2016;7:384–396.

27. Gevers D, Kugathasan S, Denson LA, et al. The treatment-naive microbiome in new-onset Crohn's disease. *Cell Host Microbe.* 2014;15: 382–392.

28. Haberman Y, Tickle TL, Dexheimer PJ, et al. Pediatric Crohn disease patients exhibit specific ileal transcriptome and microbiome signature. *J Clin Invest.* 2014;124:3617–3633.

29. Hacker H, Karin M. Regulation and function of IKK and IKK-related kinases. *Sci STKE.* 2006;2006:re13.

30. Ling L, Cao Z, Goeddel DV. NF-kappaB-inducing kinase activates IKK-alpha by phosphorylation of Ser-176. *Proc Natl Acad Sci U S A.* 1998;95: 3792–3797.

31. Iovine N, Eastvold J, Elsbach P, et al. The carboxyl-terminal domain of closely related endotoxin-binding proteins determines the target of protein-lipopolysaccharide complexes. *J Biol Chem.* 2002;277: 7970–7978.

32. Tobias PS, Soldau K, Iovine NM, et al. Lipopolysaccharide (LPS)-binding proteins BPI and LBP form different types of complexes with LPS. *J Biol Chem.* 1997;272:18682–18685.

33. Peeters M, Joossens S, Vermeire S, et al. Diagnostic value of anti-Saccharomyces cerevisiae and antineutrophil cytoplasmic autoantibodies in inflammatory bowel disease. *Am J Gastroenterol.* 2001;96:730–734.

34. Stoffel MP, Csernok E, Herzberg C, et al. Anti-neutrophil cytoplasmic antibodies (ANCA) directed against bactericidal/permeability increasing protein (BPI): a new seromarker for inflammatory bowel disease and associated disorders. *Clin Exp Immunol.* 1996;104:54–59.

35. Lasson A, Simren M, Stotzer PO, et al. Fecal calprotectin levels predict the clinical course in patients with new onset of ulcerative colitis. *Inflamm Bowel Dis.* 2013;19:576–581.

ORIGINAL ARTICLE

CrossMark

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]

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

✌ Springer

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 Bio-Code 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/m², 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
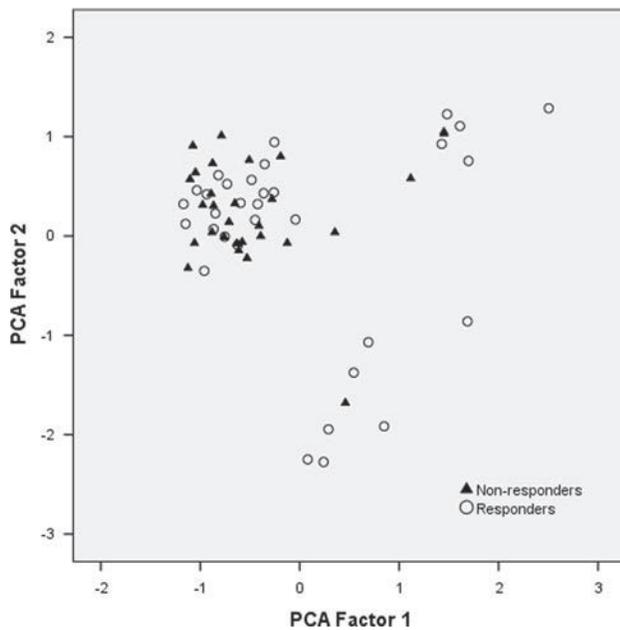
Fig. 1 Principal component analysis (PCA) showing a decomposition of gut microbiota markers using two-factor solution, as assessed by the GA-map™ Dysbiosis Test. The PCA confirmed that it is possible to distinguish between patients with IBS classified as responders ($n = 32$, depicted with open circles) 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
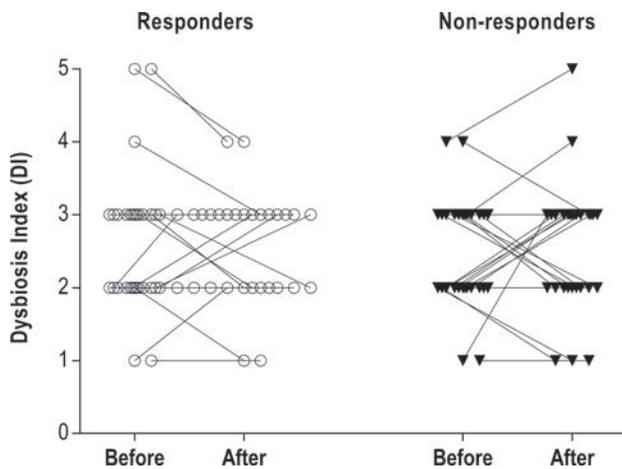
Fig. 2 Dysbiosis Index (DI), as provided by the producer of the GA-map™ Dysbiosis Test, assessed before and after a 4-week FODMAP-restricted diet in IBS patients defined as responders ($n = 32$) and non-responders ($n = 29$). Each line represents one individual
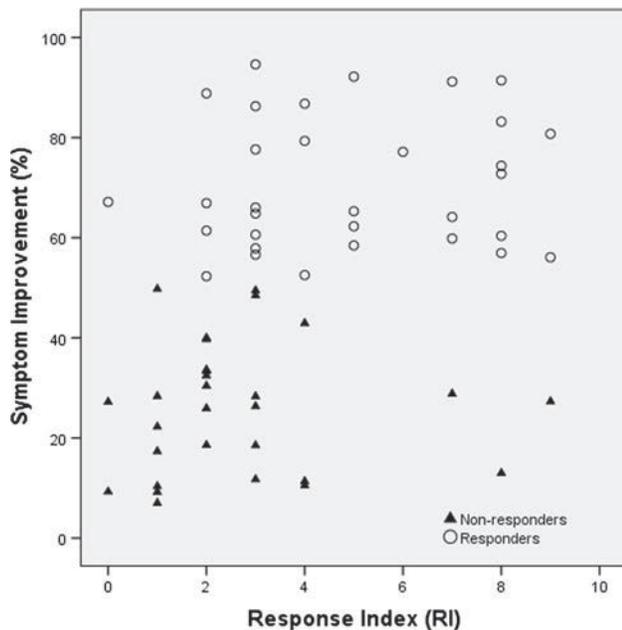
Fig. 3 Association between Response Index (RI), based on gut microbiota composition results, and symptom response, as assessed as reduction in IBS-SSS in percent from baseline values. There was a statistically significant, however, low to moderate correlation between the variables (Spearman's rho $= 0.44$, $p < 0.001$)

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.

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

References

1. Sperber AD, Dumitrascu D, Fukudo S, et al. The global prevalence of IBS in adults remains elusive due to the heterogeneity of studies: a Rome Foundation working team literature review. Gut 2016.

2. Enck P, Aziz Q, Barbara G, et al. Irritable bowel syndrome. *Nat Rev Dis Primers*. 2016;2:16014.

3. Rajilic-Stojanovic M, Jonkers DM, Salonen A, et al. Intestinal microbiota and diet in IBS: causes, consequences, or epiphenomena? *Am J Gastroenterol*. 2015;110:278–287.

4. Nanayakkara WS, Skidmore PM, O'Brien L, Wilkinson TJ, Gearry RB. Efficacy of the low FODMAP diet for treating irritable bowel syndrome: the evidence to date. *Clin Exp Gastroenterol*. 2016;9:131–142.

5. Halmos EP, Christophersen CT, Bird AR, Shepherd SJ, Gibson PR, Muir JG. Diets that differ in their FODMAP content alter the colonic luminal microenvironment. *Gut*. 2015;64:93–100.

6. Valeur J, Roseth AG, Knudsen T, et al. Fecal fermentation in irritable bowel syndrome: influence of dietary restriction of fermentable oligosaccharides, disaccharides, monosaccharides and polyols. *Digestion*. 2016;94:50–56.

7. Yao CK, Muir JG, Gibson PR. Review article: insights into colonic protein fermentation, its modulation and potential health implications. *Aliment Pharmacol Ther*. 2016;43:181–196.

8. Daien CI, Pinget GV, Tan JK, Macia L. Detrimental impact of microbiota-accessible carbohydrate-deprived diet on gut and immune homeostasis: an overview. *Front Immunol*. 2017;8:548.

9. Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. *Gastroenterology*. 2006;130:1480–1491.

10. Tveito K, Brunborg C, Bratlie J, et al. Intestinal malabsorption of D-xylose: comparison of test modalities in patients with celiac disease. *Scand J Gastroenterol*. 2010;45:1289–1294.

11. Gibson PR, Shepherd SJ. Evidence-based dietary management of functional gastrointestinal symptoms: the FODMAP approach. *J Gastroenterol Hepatol*. 2010;25:252–258.

12. Francis CY, Morris J, Whorwell PJ. The irritable bowel severity scoring system: a simple method of monitoring irritable bowel syndrome and its progress. *Aliment Pharmacol Ther*. 1997;11:395–402.

13. Irvine EJ, Whitehead WE, Chey WD, et al. Design of treatment trials for functional gastrointestinal disorders. *Gastroenterology*. 2006;130:1538–1551.

14. Zigmond AS, Snaith RP. The hospital anxiety and depression scale. *Acta Psychiatr Scand*. 1983;67:361–370.

15. Lind R, Berstad A, Hatlebakk J, Valeur J. Chronic fatigue in patients with unexplained self-reported food hypersensitivity and irritable bowel syndrome: validation of a Norwegian translation of the Fatigue Impact Scale. *Clin Exp Gastroenterol*. 2013;6:101–107.

16. Casen C, Vebo HC, Sekelja M, et al. Deviations in human gut microbiota: a novel diagnostic test for determining dysbiosis in patients with IBS or IBD. *Aliment Pharmacol Ther*. 2015;42:71–83.

17. Altman DG, Machin D, Bryant TN, Gardner MJ. *Statistics with confidence*. 2nd ed. New York: BMJ Books; 2000.

18. James G, Witten D, Hastie T, Tibshirani R. *An introduction to statistical learning*. New York: Springer; 2013.

19. Collins SM. A role for the gut microbiota in IBS. *Nat Rev Gastroenterol Hepatol*. 2014;11:497–505.

20. Parkes GC, Brostoff J, Whelan K, Sanderson JD. Gastrointestinal microbiota in irritable bowel syndrome: their role in its pathogenesis and treatment. *Am J Gastroenterol*. 2008;103:1557–1567.

21. Carroll IM, Ringel-Kulka T, Keku TO, et al. Molecular analysis of the luminal- and mucosal-associated intestinal microbiota in diarrhea-predominant irritable bowel syndrome. *Am J Physiol Gastrointest Liver Physiol*. 2011;301:G799–G807.

22. Jalanka-Tuovinen J, Salojarvi J, Salonen A, et al. Faecal microbiota composition and host-microbe cross-talk following gastroenteritis and in postinfectious irritable bowel syndrome. *Gut*. 2014;63:1737–1745.

23. Hustoft TN, Hausken T, Ystad SO, et al. Effects of varying dietary content of fermentable short-chain carbohydrates on symptoms, fecal microenvironment, and cytokine profiles in patients with irritable bowel syndrome. Neurogastroenterol Motil 2016.

24. Chumpitazi BP, Cope JL, Hollister EB, et al. Randomised clinical trial: gut microbiome biomarkers are associated with clinical response to a low FODMAP diet in children with the irritable bowel syndrome. *Aliment Pharmacol Ther*. 2015;42:418–427.

25. Vieira-Silva S, Falony G, Darzi Y, et al. Species-function relationships shape ecological properties of the human gut microbiome. *Nat Microbiol*. 2016;1:16088.

26. Khanna S, Montassier E, Schmidt B, et al. Gut microbiome predictors of treatment response and recurrence in primary Clostridium difficile infection. *Aliment Pharmacol Ther*. 2016;44:715–727.

27. Magnusson MK, Strid H, Sapnara M, et al. Anti-TNF therapy response in patients with ulcerative colitis is associated with colonic antimicrobial peptide expression and microbiota composition. *J Crohns Colitis*. 2016;10:943–952.

28. Bennet SMP, Bohn L, Storsrud S, et al. Multivariate modelling of faecal bacterial profiles of patients with IBS predicts responsiveness to a diet low in FODMAPs. Gut 2017.

29. David LA, Maurice CF, Carmody RN, et al. Diet rapidly and reproducibly alters the human gut microbiome. *Nature*. 2014;505:559–563.

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]

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

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

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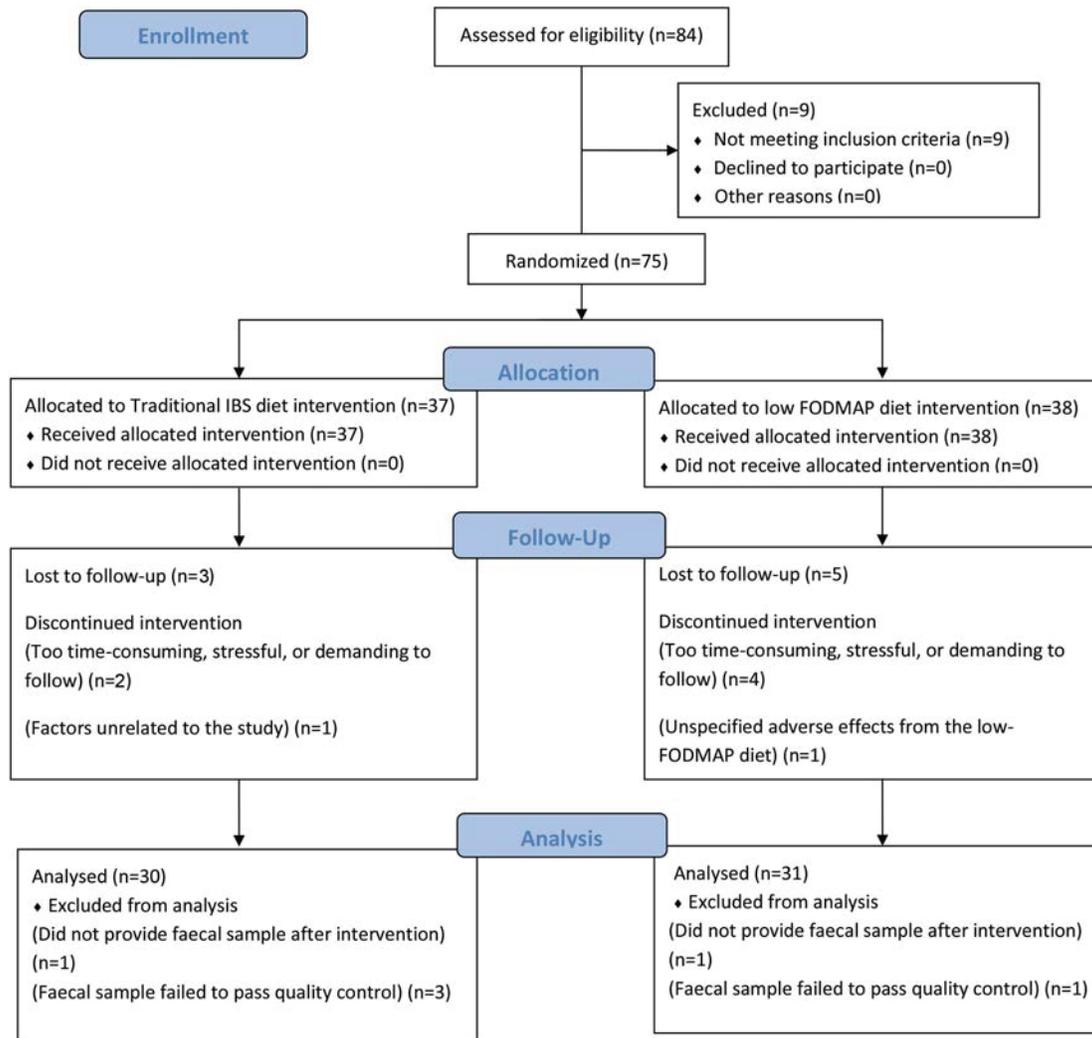
Figure 1 CONSORT flow diagram. Flow chart depicting patient numbers during the different phases of the study. FODMAP, fermentable oligosaccharides, disaccharides, monosaccharides and polyols.

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/m²*	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
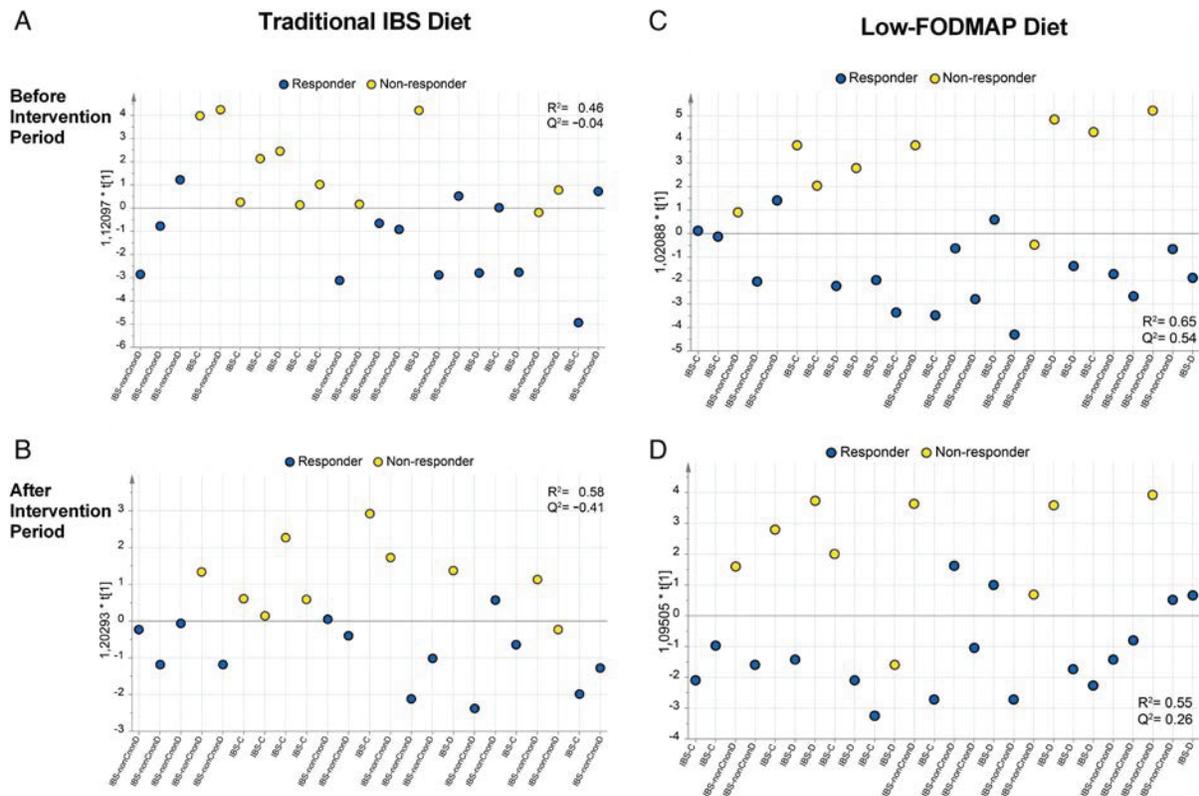
Figure 2 Bacterial profile analysis of non-responders and responders to dietary intervention. The GA-map Dysbiosis Test[22] analysing signals from 54 probes targeting ≥300 bacteria on different taxonomic levels was used to create bacterial profiles for patients undergoing either traditional IBS diet (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

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

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.

A. Traditional IBS Diet

B. low-FODMAP Diet
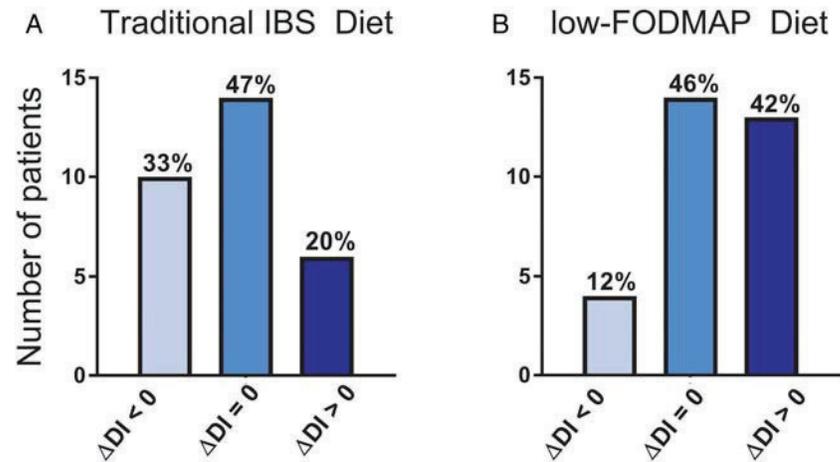
Table 5 Overview of responder and non-responder change in dysbiosis during intervention

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

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

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

REFERENCES

1 Lovell RM, Ford AC. Global prevalence of and risk factors for irritable bowel syndrome: a meta-analysis. *Clin Gastroenterol Hepatol* 2012;10:712–21.e4.
2 Longstreth GF, Thompson WG, Chey WD, et al. Functional Bowel Disorders. *Gastroenterology* 2006;130:1480–91.
3 Bennet SM, Ohman L, Simren M. Gut microbiota as potential orchestrators of irritable bowel syndrome. *Gut Liver* 2015;9:318–31.
4 Metchnikoff E, Mitchell PC. *The prolongation of life: optimistic studies*. Putnam, 1908.
5 Simrén M, Barbara G, Flint HJ, et al., Rome Foundation Committee. Intestinal microbiota in functional bowel disorders: a Rome foundation report. *Gut* 2013;62:159–76.
6 Jeffery IB, O'Toole PW, Öhman L, et al. An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. *Gut* 2012;61:997–1006.
7 Chassard C, Dapoigny M, Scott KP, et al. Functional dysbiosis within the gut microbiota of patients with constipated-irritable bowel syndrome. *Aliment Pharmacol Ther* 2012;35:828–38.
8 Collins S, Verdu E, Denou E, et al. The role of pathogenic microbes and commensal bacteria in irritable bowel syndrome. *Dig Dis* 2009;27(Suppl 1):85–9.
9 Jernberg C, Löfmark S, Edlund C, et al. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *ISME J* 2007;1:56–66.
10 De Filippo C, Cavalieri D, Di Paola M, et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Natl Acad Sci USA* 2010;107:14691–6.
11 McKenzie YA, Alder A, Anderson W, et al. British Dietetic Association evidence-based guidelines for the dietary management of irritable bowel syndrome in adults. *J Hum Nutr Diet* 2012;25:260–74.
12 Blanchard-Smith J, Bullock I, Dalrymple J, et al. NICE Guidelines: irritable bowel syndrome in adults: diagnosis and management of irritable bowel syndrome in primary care. Published February 2008. Available at: https://www.nice.org.uk/guidance/cg61. (accessed May 16 2016).
13 Dalrymple J, Bullock I. Diagnosis and management of irritable bowel syndrome in adults in primary care: summary of NICE guidance. *BMJ* 2008;336:556–8.
14 Böhn L, Störsrud S, Liljebo T, et al. Diet low in FODMAPs reduces symptoms of irritable bowel syndrome as well as traditional dietary advice: a randomized controlled trial. *Gastroenterology* 2015;149:1399–1407.e2.
15 Marciani L, Cox EF, Hoad CL, et al. Postprandial changes in small bowel water content in healthy subjects and patients with irritable bowel syndrome. *Gastroenterology* 2010;138:469–77, 77.e1.
16 Barrett JS, Gearry RB, Muir JG, et al. Dietary poorly absorbed, short-chain carbohydrates increase delivery of water and fermentable substrates to the proximal colon. *Aliment Pharmacol Ther* 2010;31:874–82.
17 Ong DK, Mitchell SB, Barrett JS, et al. Manipulation of dietary short chain carbohydrates alters the pattern of gas production and genesis of symptoms in irritable bowel syndrome. *J Gastroenterol Hepatol* 2010;25:1366–73.
18 Halmos EP, Power VA, Shepherd SJ, et al. A diet low in FODMAPs reduces symptoms of irritable bowel syndrome. *Gastroenterology* 2014;146:67–75.e5.
19 Halmos EP, Christophersen CT, Bird AR, et al. Diets that differ in their FODMAP content alter the colonic luminal microenvironment. *Gut* 2015;64:93–100.
20 Staudacher HM, Lomer MC, Anderson JL, et al. Fermentable carbohydrate restriction reduces luminal bifidobacteria and gastrointestinal symptoms in patients with irritable bowel syndrome. *J Nutr* 2012;142:1510–18.
21 Francis CY, Morris J, Whorwell PJ. The irritable bowel severity scoring system: a simple method of monitoring irritable bowel syndrome and its progress. *Aliment Pharmacol Ther* 1997;11:395–402.
22 Casén C, Vebø HC, Sekelja M, et al. Deviations in human gut microbiota: a novel diagnostic test for determining dysbiosis in patients with IBS or IBD. *Aliment Pharmacol Ther* 2015;42:71–83.
23 Umetrics. SIMCA-P and Multivariate Analysis FAQ. Version 1.01. http://umetrics.com/sites/default/files/kb/multivariate_faq.pdf (accessed 26 Sep 2016).
24 Eriksson L, Johansson E, Kettaneh-Wold N, et al. Multi- and megavariate data analysis: part I: basic principles and applications. Umeå: Umetrics, 2006.
25 Rajilić-Stojanović M, Biagi E, Heilig HG, et al. Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. *Gastroenterology* 2011;141:1792–801.
26 Chumpitazi BP, Cope JL, Hollister EB, et al. Randomised clinical trial: gut microbiome biomarkers are associated with clinical response to a low FODMAP diet in children with the irritable bowel syndrome. *Aliment Pharmacol Ther* 2015;42:418–27.
27 Wu GD, Chen J, Hoffmann C, et al. Linking long-term dietary patterns with gut microbial enterotypes. *Science* 2011;334:105–8.
28 Lappi J, Salojärvi J, Kolehmainen M, et al. Intake of whole-grain and fiber-rich rye bread versus refined wheat bread does not differentiate intestinal microbiota composition in Finnish adults with metabolic syndrome. *J Nutr* 2013;143:648–55.
29 Cotillard A, Kennedy SP, Kong LC, et al. Dietary intervention impact on gut microbial gene richness. *Nature* 2013;500:585–8.
30 David LA, Maurice CF, Carmody RN, et al. Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 2014;505:559–63.
31 Salonen A, Lahti L, Salojärvi J, et al. Impact of diet and individual variation on intestinal microbiota composition and fermentation products in obese men. *ISME J* 2014;8:2218–30.
32 McIntosh K, Reed DE, Schneider T, et al. FODMAPs alter symptoms and the metabolome of patients with IBS: a randomised controlled trial. *Gut* 2016. doi: 10.1136/gutjnl-2015-311339. [Epub ahead of print 14 Mar 2016].
33 O'Mahony L, McCarthy J, Kelly P, et al. Lactobacillus and Bifidobacterium in irritable bowel syndrome: Symptom responses and relationship to cytokine profiles. *Gastroenterology* 2005;128:541–51.
34 Whorwell PJ, Altringer L, Morel J, et al. Efficacy of an encapsulated probiotic Bifidobacterium infantis 35624 in women with irritable bowel syndrome. *Am J Gastroenterol* 2006;101:1581–90.
35 Guyonnet D, Chassany O, Ducrotte P, et al. Effect of a fermented milk containing Bifidobacterium animalis DN-173 010 on the health-related quality of life and symptoms in irritable bowel syndrome in adults in primary care: a multicentre, randomized, double-blind, controlled trial. *Aliment Pharmacol Ther* 2007;26:475–86.
36 Fischbach MA, Sonnenburg JL. Eating for two: How metabolism establishes interspecies interactions in the gut. *Cell Host Microbe* 2011;10:336–47.
37 Duncan SH, Belenguer A, Holtrop G, et al. Reduced dietary intake of carbohydrates by obese subjects results in decreased concentrations of butyrate and butyrate-producing bacteria in feces. *Appl Environ Microbiol* 2007;73:1073–8.
38 Walker AW, Ince J, Duncan SH, et al. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *ISME J* 2011;5:220–30.
39 Parche S, Beleut M, Rezzonico E, et al. Lactose-over-glucose preference in Bifidobacterium longum NCC2705: glcP, encoding a glucose transporter, is subject to lactose repression. *J Bacteriol* 2006;188:1260–5.

BMC Obesity

CrossMark

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

Farup *et al. BMC Obesity* (2018) 5:30

Page 2 of 10

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

Farup *et al. BMC Obesity* (2018) 5:30

Page 3 of 10

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.

Farup *et al. BMC Obesity* (2018) 5:30

Page 4 of 10

Fig. 1 A flowchart of the subjects in the study
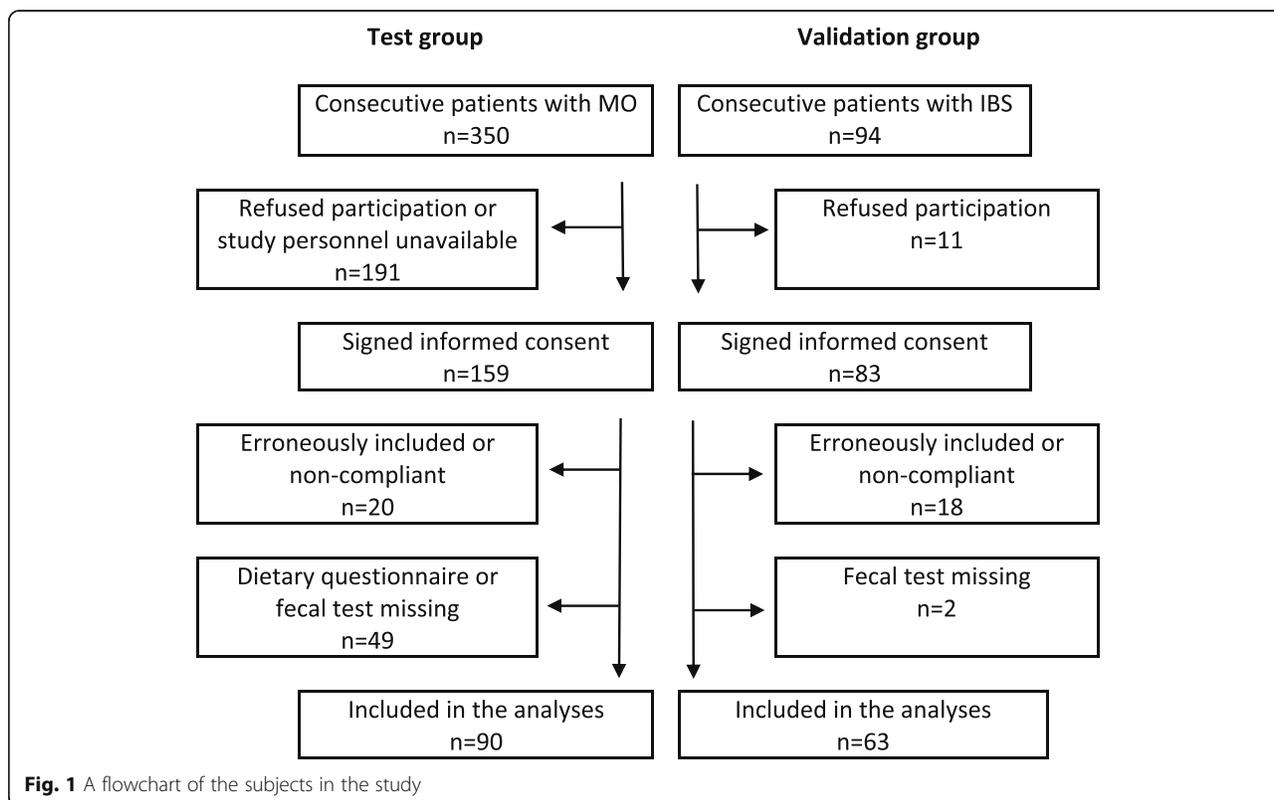
The results of the multivariable analyses with all variables in the equation and the stepwise forward analyses are given in Table 2. The main findings were the positive associations between DI and use of metformin and NNS, the positive association between ADI and metformin (the "good" dysbiosis), and 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 Yes (no 59)	Dysbiosis No (31)	Dysbiosis p-values	Association with DI [b]	Association with ADI [b]
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

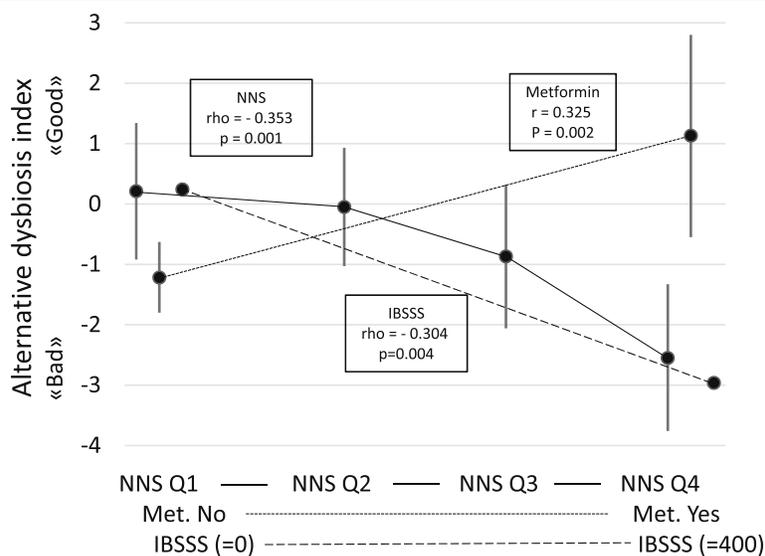Farup *et al. BMC Obesity* (2018) 5:30

Page 6 of 10
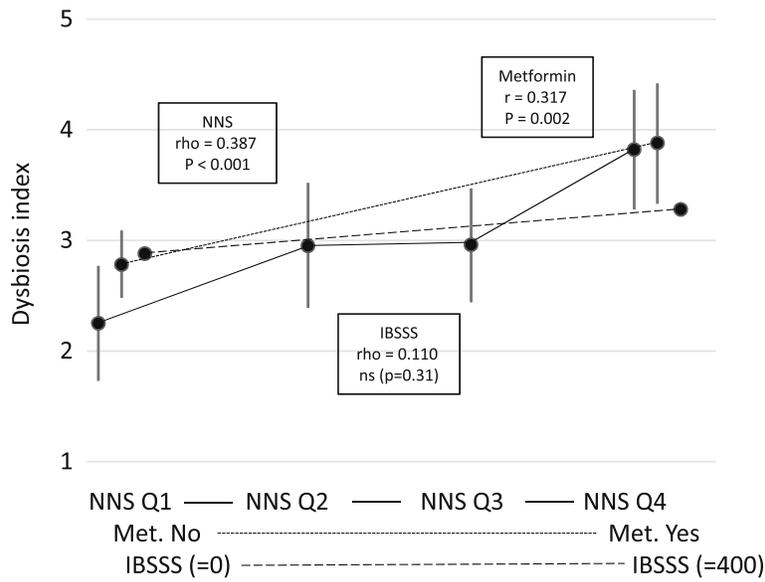
Fig. 2 Associations between the Dysbiosis Index and the main variables. NNS Q1, NNS Q2, NNS Q3, NNS Q4: Intake of Non-Nutritive Sweeteners divided into quartiles. Met: Metformin. IBSSS: Irritable Bowel Severity Scoring System. The results for NNS and Met are given as mean with 95% CI. The associations 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)

Farup *et al. BMC Obesity* (2018) 5:30

Page 7 of 10

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

Farup *et al. BMC Obesity* (2018) 5:30

Page 8 of 10

Table 3 ADI scores and associations with IBS and IBSSS with comparisons between the groups

Variables	ADI	ADI	Statistics
	Test group	Validation group	*p*-value
	(IBS no/yes: no 63/25)	(IBS yes: 63)	
IBS (no / yes)	−0.41 (2.75) / −1.64 (2.77) (*p* = 0.11)†	−1.68 (2.26)†	0.013*
IBSSS	rho = −0.304 (*p* = 0.004)	rho = −0.249 (*p* = 0.049)	0.86 #

ADI Alternative Dysbiosis Index, *IBS* Irritable Bowel Syndrome, *IBSSS* Irritable Bowel Severity Score System
*One-Way ANOVA with comparisons between the three groups
† Post hoc comparisons (Tukey) between the validation group and subjects with and without IBS in the test group were *p* = 1.00 and 0.016 respectively
# Univariate analysis of variance with ADI as the dependent variable and IBSSS and group as independent variables. The *p*-value is the interaction between IBSSS and group and indicates no significant difference between the correlations

[14, 32–35]. The dysbiosis caused by NNS induces glucose intolerance and has been linked to obesity by the obesity-associated metabolic changes [15–17]. Therefore, the NNS associated dysbiosis is probably "bad" for subjects with morbid obesity.

IBS is one among many disorders that has been associated with alterations in the gut microbiota [18–21]. The dysbiosis in subjects with IBS is a "bad" dysbiosis since faecal microbiota transplantation may normalise the microbiota and improve symptoms [36]. In all, there are several types of dysbiosis that might be separated into "good" and "bad".

The producer's test response does not differentiate between types of dysbiosis. The ADI based on simple explorative analyses of available results in the producer's report could easily separate the "good" metformin-type dysbiosis from the "bad" NNS-type dysbiosis. The ADI-score was adjusted so that "good" and "bad" dysbiosis had positive and negative scores respectively. If the results are reproducible, and the dysbiosis test allows construction of other clinically relevant dysbiosis indexes, the potential usefulness of the test increases markedly.

The ADI was not constructed to explore dysbiosis associated with IBS and gastrointestinal complaints. The negative correlations between ADI and IBS and gastrointestinal symptoms were therefore new and interesting findings, which were confirmed with unadjusted and adjusted analyses in the validation group. The findings are in accordance with other reports indicating associations between IBS and dysbiosis [19–21]. The ADI could be a test for detection of "bad" dysbiosis in subjects with IBS and gastrointestinal complaints and replace complex, resource demanding and costly 16S gene sequencing.

Further research, aiming at enlarging the producer's test response with the specification of the type of dysbiosis related to dietary factors, drugs, disorders and diseases (e.g. metformin- or NNS-like, or "good" or "bad") is desirable. Specified results might predict response to treatment, e.g. antibiotics and other drugs, probiotics, prebiotics, diet, and faecal microbiota transplant. Treatment aiming at prevention or normalising of a "bad" dysbiosis or induction of a "good" dysbiosis could change the treatment of a range of disorders [37].

Strengths and limitation
The test group and the validation group were consecutive subjects representative of subjects referred to outpatient clinics for morbid obesity and gastrointestinal complaints respectively. Because the ADI was constructed to detect differences between metformin and NNS, the significant differences between the ADI scores for metformin and NNS were expected. It was nevertheless pleasing that the ADI could be constructed so easily. The most impressive findings were the associations between IBS and IBSSS and the negative ADI score. The ADI was not constructed to find these differences, and they were not detected with the producer's result report. It was a strength that these findings were confirmed in the validation group, which substantiates that a negative ADI indicates a "bad" NNS- or IBS-like dysbiosis.

The external validity could be questioned since the ADI was based on results from subjects with morbid obesity who might have a high prevalence of dysbiosis also without having gastrointestinal comorbidity and use of metformin and NNS. The exclusion of subjects using antibiotics the last month might have been a too short period.

Conclusions
A commercially available test for faecal dysbiosis showed a high prevalence of dysbiosis in subjects with morbid obesity, particularly in users of metformin and NNS, but no association with gastrointestinal complaints. An ADI based on explorative analyses of the results from the test could differentiate between the "good" dysbiosis associated with metformin and the "bad" dysbiosis associated with NNS. The "bad" dysbiosis was also associated with gastrointestinal symptom severity. The associations between IBS and gastrointestinal symptom severity were confirmed in an independent validation group, indicating that ADI might be a valid diagnostic tool for the diagnosis of IBS-associated dysbiosis. Rather than merely reporting dysbiosis and degrees of dysbiosis, diagnostic tests for faecal dysbiosis should separate between types of dysbiosis.

Farup *et al. BMC Obesity* (2018) 5:30

Page 9 of 10

Bowel Syndrome; IBSSS: Irritable Bowel Severity Scoring System; NNS: Non-Nutritive Sweeteners; pc: partial correlation; SD: Standard Deviation

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.

Publisher's Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

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.

References
1. Bouter KE, van Raalte DH, Groen AK, Nieuwdorp M. Role of the gut microbiome in the pathogenesis of obesity and obesity-related metabolic dysfunction. Gastroenterology. 2017;152(7):1671–8.
2. Gao R, Zhu C, Li H, Yin M, Pan C, Huang L, Kong C, Wang X, Zhang Y, Qu S, et al. Dysbiosis signatures of gut microbiota along the sequence from healthy, young patients to those with overweight and obesity. Obesity (Silver Spring). 2018;26(2):351–61.
3. Zhang X, Shen D, Fang Z, Jie Z, Qiu X, Zhang C, Chen Y, Ji L. Human gut microbiota changes reveal the progression of glucose intolerance. PLoS One. 2013;8(8):e71108.
4. Montandon SA, Jornayvaz FR. Effects of Antidiabetic Drugs on Gut Microbiota Composition. Genes (Basel). 2017;8(10). https://doi.org/10.3390/genes8100250.
5. Le Bastard Q, Al-Ghalith GA, Gregoire M, Chapelet G, Javaudin F, Dailly E, Batard E, Knights D, Montassier E. Systematic review: human gut dysbiosis induced by non-antibiotic prescription medications. Aliment Pharmacol Ther. 2018;47(3):332–45.
6. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014;505(7484):559–63.
7. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R, et al. Linking long-term dietary patterns with gut microbial enterotypes. Science. 2011;334(6052):105–8.
8. Ferrannini E. The target of metformin in type 2 diabetes. N Engl J Med. 2014;371(16):1547–8.
9. Desilets AR, Dhakal-Karki S, Dunican KC. Role of metformin for weight management in patients without type 2 diabetes. Ann Pharmacother. 2008; 42(6):817–26.
10. Abdelgadir E, Ali R, Rashid F, Bashier A. Effect of metformin on different non-diabetes related conditions, a special focus on malignant conditions: review of literature. J Clin Med Res. 2017;9(5):388–95.
11. Wu H, Esteve E, Tremaroli V, Khan MT, Caesar R, Manneras-Holm L, Stahlman M, Olsson LM, Serino M, Planas-Felix M, et al. Metformin alters the gut microbiome of individuals with treatment-naive type 2 diabetes, contributing to the therapeutic effects of the drug. Nat Med. 2017;23(7): 850–8.
12. Maniar K, Moideen A, Bhattacharyya R, Banerjee D. Metformin exerts anti-obesity effect via gut microbiome modulation in prediabetics: a hypothesis. Med Hypotheses. 2017;104:117–20.
13. Forslund K, Hildebrand F, Nielsen T, Falony G, Le Chatelier E, Sunagawa S, Prifti E, Vieira-Silva S, Gudmundsdottir V, Pedersen HK, et al. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. Nature. 2015;528(7581):262–6.
14. Winther R, Aasbrenn M, Farup PG. Intake of non-nutritive sweeteners is associated with an unhealthy lifestyle: a cross-sectional study in subjects with morbid obesity. BMC Obes. 2017;4:41.
15. Suez J, Korem T, Zeevi D, Zilberman-Schapira G, Thaiss CA, Maza O, Israeli D, Zmora N, Gilad S, Weinberger A, et al. Artificial sweeteners induce glucose intolerance by altering the gut microbiota. Nature. 2014;514(7521):181–6.
16. Feehley T, Nagler CR. Health: the weighty costs of non-caloric sweeteners. Nature. 2014;514(7521):176–7.
17. Abbott A. Sugar substitutes linked to obesity. Nature. 2014;513(7518):290.
18. Aasbrenn M, Valeur J, Farup PG. Evaluation of a faecal dysbiosis test for irritable bowel syndrome in subjects with and without obesity. Scand J Clin Lab Invest. 2018;76(1–2):109–13.
19. Zhuang X, Xiong L, Li L, Li M, Chen M. Alterations of gut microbiota in patients with irritable bowel syndrome: a systematic review and meta-analysis. J Gastroenterol Hepatol. 2017;32(1):28–38.
20. Collins SM. A role for the gut microbiota in IBS. Nat Rev Gastroenterol Hepatol. 2014;11(8):497–505.
21. Bhattarai Y, Muniz Pedrogo DA, Kashyap PC. Irritable bowel syndrome: a gut microbiota-related disorder? Am J Physiol Gastrointest Liver Physiol. 2017; 312(1):G52–62.
22. Zaneveld JR, McMinds R, Vega Thurber R. Stress and stability: applying the Anna Karenina principle to animal microbiomes. Nat Microbiol. 2017;2: 17121.
23. Casen C, Vebo HC, Sekelja M, Hegge FT, Karlsson MK, Ciemniejewska E, Dzankovic S, Froyland C, Nestestog R, Engstrand L, et al. Deviations in human gut microbiota: a novel diagnostic test for determining dysbiosis in patients with IBS or IBD. Aliment Pharmacol Ther. 2015;42(1):71–83.
24. Genetic Analysis AS. GAMap TM Dysbiosis Test [http://www.genetic-analysis.com/patent] Accessed 31 July 2018.
25. Francis CY, Morris J, Whorwell PJ. The irritable bowel severity scoring system: a simple method of monitoring irritable bowel syndrome and its progress. Aliment Pharmacol Ther. 1997;11(2):395–402.
26. Norwegian Food Safety Authority. Food Database [http://www.matvaretabellen.no/?language=en]. Accessed 31 July 2018.

Farup *et al. BMC Obesity* (2018) 5:30

Page 10 of 10

27. Suez J, Korem T, Zilberman-Schapira G, Segal E, Elinav E. Non-caloric artificial sweeteners and the microbiome: findings and challenges. Gut Microbes. 2015;6(2):149–55.

28. Sze MA, Schloss PD. Looking for a Signal in the Noise: Revisiting Obesity and the Microbiome. MBio. 2016:7(4). https://doi.org/10.1128/mBio.01018-16.

29. Madiraju AK, Erion DM, Rahimi Y, Zhang XM, Braddock DT, Albright RA, Prigaro BJ, Wood JL, Bhanot S, MacDonald MJ, et al. Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. Nature. 2014;510(7506):542–6.

30. Bonora E, Cigolini M, Bosello O, Zancanaro C, Capretti L, Zavaroni I, Coscelli C, Butturini U. Lack of effect of intravenous metformin on plasma concentrations of glucose, insulin, C-peptide, glucagon and growth hormone in non-diabetic subjects. Curr Med Res Opin. 1984;9(1):47–51.

31. Sum CF, Webster JM, Johnson AB, Catalano C, Cooper BG, Taylor R. The effect of intravenous metformin on glucose metabolism during hyperglycaemia in type 2 diabetes. Diabet Med. 1992;9(1):61–5.

32. Mandrioli D, Kearns CE, Bero LA. Relationship between research outcomes and risk of bias, study sponsorship, and author financial conflicts of interest in reviews of the effects of artificially sweetened beverages on weight outcomes: a systematic review of reviews. PLoS One. 2016;11(9):e0162198.

33. Pepino MY. Metabolic effects of non-nutritive sweeteners. Physiol Behav. 2015;152(Pt B):450–5.

34. Lohner S, Toews I, Meerpohl JJ. Health outcomes of non-nutritive sweeteners: analysis of the research landscape. Nutr J. 2017;16(1):55.

35. Shearer J, Swithers SE. Artificial sweeteners and metabolic dysregulation: lessons learned from agriculture and the laboratory. Rev Endocr Metab Disord. 2016;17(2):179–86.

36. Johnsen PH, Hilpusch F, Cavanagh JP, Leikanger IS, Kolstad C, Valle PC, Goll R. Faecal microbiota transplantation versus placebo for moderate-to-severe irritable bowel syndrome: a double-blind, randomised, placebo-controlled, parallel-group, single-Centre trial. Lancet Gastroenterol Hepatol. 2018;3(1): 17–24.

37. Valeur J, Smastuen MC, Knudsen T, Lied GA, Roseth AG. Exploring gut microbiota composition as an indicator of clinical response to dietary FODMAP restriction in patients with irritable bowel syndrome. Dig Dis Sci. 2018;63(2):429–36.

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

Abstract: Morbidly obese subjects have a high prevalence of comorbidity and gut microbial dysbiosis, and are thus suitable for the study of gut-brain interactions. The aim was to study the associations between the faecal microbiota's composition and function and psychobiological comorbidity in subjects with BMI > 40 kg/m^2 or >35 kg/m^2 with obesity-related complications. The faecal microbiota was assessed with GA-Map dysbiosis test ™ (Genetic Analysis, Oslo Norway) and reported as dysbiosis (yes/no) and degree of dysbiosis, and the relative abundance of 39 bacteria. The microbiota's function was assessed by measuring the absolute and relative amount of faecal short chain fatty acids. Associations were made with well-being, mental distress, fatigue, food intolerance, musculoskeletal pain, irritable bowel syndrome, and degree of abdominal complaints. One hundred and two subjects were included. The results confirmed the high prevalence of comorbidity and dysbiosis (62/102; 61%) and showed a high prevalence of significant associations (41/427; 10%) between the microbiota's composition and function and the psychobiological disorders. The abundant, but in part divergent, associations supported the close gut-brain interaction but revealed no clear-cut and straightforward communication pathways. On the contrary, the study illustrates the complexity of gut-brain interactions.

Keywords: gut-brain axis; faecal microbiota; faecal short chain fatty acids; morbid obesity; psychobiological disorders; well-being; mental distress; irritable bowel syndrome

1. Introduction

Obesity is a worldwide health problem that has nearly tripled since 1975 and it affects 13% of the adult population. It is associated with a wide range of comorbidities, such as cardiovascular diseases, diabetes, musculoskeletal disorders, cancer, and psychobiological disorders, and an increased risk of death [1].

Faecal dysbiosis has been defined as an imbalance in the faecal microbiota. It is common in subjects with morbid obesity (MO) and it has been mentioned as a causal factor for obesity and the comorbidities such as insulin resistance, glucose intolerance and diabetes type II, as well as psychiatric and functional disorders [2–6].

The gut-brain axis is a bidirectional link between the gut and the brain and of importance for various psychobiological disorders, such as anxiety, depression, fatigue, stress reactions, pain syndromes, and functional gastrointestinal disorders [7–9]. The absolute or relative amounts of the gut microbes per se and the microbes' metabolic products are possible mediators of the gut-brain

effects. Faecal short chain fatty acids (SCFA), which are products of bacterial fermentation, have been proposed as mediators of the health-related effects [10,11].

The primary aim of this study in subjects with morbid obesity was to explore associations between the faecal microbiota's composition and metabolic products and a selection of psychobiological disorders. The secondary aims were to compare the subjects' microbiota with that of healthy reference populations. The high prevalence of psychobiological disorders and faecal dysbiosis in subjects with morbid obesity makes this group of special interest for the study of gut-brain interactions.

2. Materials and Methods

2.1. Study Design

A cross-sectional study in subjects with morbid obesity. The microbial composition was analysed with a commercially available test, and the results were compared with the test producer's reference population [12]. The faecal SCFA were compared with the faecal samples from healthy volunteers, as previously published [13].

2.2. Participants

Consecutive subjects aged 18–65 years with MO (defined as BMI > 40 or >35 kg/m^2 with morbidity related comorbidity), referred to the Unit for Obesity at Innlandet Hospital Trust–Gjøvik, Norway in the period from December 2012 to September 2014 were eligible for the study. Subjects with organic gastrointestinal disorders, major psychiatric disorders, severe not obesity-related somatic disorders, alcohol or drug addiction, and previous obesity surgery or other major abdominal surgery were excluded.

The healthy volunteers were healthcare workers and students from Haukeland University Hospital, Bergen, Norway who considered themselves healthy.

2.3. Accomplishment

In all morbidly obese subjects, a medical history was taken, a physical examination was performed, and blood and faecal samples were collected. The information was collected on paper-based questionnaires that were filled in by the doctors, the study nurse, and the participants. Other examinations were performed at the doctors' discretion. Except for some demographic data, no information was available about the healthy volunteers.

2.4. Variables

2.4.1. Participants' Characteristics

- Gender, age (years), height (m), weight (kg), BMI (kg/m^2), coffee (cups/day), smoking (daily, previously, never), and previous and present diseases.
- Physical activity was the sum of two questions: Easy activity (not sweaty/breathless): None; <1 h; 1–2 h; >3 h/week (score 0–3). Strenuous activity (sweaty/breathless): none; <1 h; 1–2 h; >3 h/week (score 0, 3, 4, 5). Sum score physical activity 0–8.
- Use of Metformin and other drugs (Yes/No)
- Use of Non-Nutritive Sweeteners (NNS). One unit of NNS was defined as 100 mL NNS-containing beverage or two NNS tablets/teaspoons for use in tea or coffee. A validated food frequency questionnaire that is based on the official Norwegian food composition table was used for the calculation [14].

2.4.2. Psychobiological Disorders

- WHO-5 Well-being index (score 0–100; scores $\leq$ 50 indicate low mood and scores $\leq$ 28 indicate likely depression) [15]

- Hopkins symptom checklist 10, (score 1–4; scores $\geq$ 1.85 indicate mental distress) [16]
- Fatigue (Score 9–63; scores $\geq$ 36 indicate further evaluation). The diagnose was based on a validated Norwegian translation of the Fatigue Severity Scale [17].
- Musculoskeletal pain from six parts of the body (score 0–12).
- Food intolerance (yes/no) as reported by the participants.
- Irritable Bowel Syndrome (IBS) (yes/no) was diagnosed with a validated Norwegian translation of the Rome III criteria [18].
- Abdominal complaints were scored with IBS Severity Score system (IBS-SSS) (score 0–500) [19]. All of the subjects with abdominal complaints, and not only those with IBS, filled in the questionnaire.

2.4.3. Faecal Microbiota

The CE marked GA-map™ Dysbiosis Test (Genetic Analysis AS, Oslo, Norway) was used for the analyses of the faecal microbiota [12]. The test has a US (Patent No. 9243297) and a European patent (Patent No. 2652145) for its technology governing the oligonucleotide probe set and methods of microbiota profiling [20]. It uses 54 oligonucleotide probes targeting the 16S rRNA gene at different bacterial taxonomic levels.

The overall result is given as the Dysbiosis Index (DI) with scores 1 to 5; values above 2 indicate a microbiota profile that differs from the producer's reference population (i.e., dysbiosis). The results are also given as the relative abundance compared to a reference population (score -3 to 3) of 39 bacteria at different taxonomic levels (Actinobacteria, Actinomycetales, *Bifidobacterium* spp., Alistipes, *Alistipes onderdonkii*, *Bacteroides fragilis*, *Bacteroides* spp. & *Prevotella* spp., *Bacteroides stercoris*, *Bacteroides zoogleoformans*, *Parabacteroides johnsonii*, *Parabacteroides* spp., Firmicutes, Bacilli, *Catenibacterium mitsuoka*, Clostridi a, *Clostridium* sp., *Dialister invisus*, *Dialister invisus* & *Megasphaera micronuciformis*, *Dorea* spp., *Eubacterium biforme*, *Eubacterium hallii*, *Eubacterium rectale*, *Eubacterium siraeum*, *Faecalibacterium prausnitzii*, Lachnospiraceae, *Lactobacillus ruminis* & *Pediococcus acidilactic*, *Lactobacillus* spp., *Phascolarctobacterium* sp., *Ruminococcus albus* & *R. bromii*, *Ruminococcus gnavus*, *Streptococcus agalactiae* & *Eubacterium rectale*, *Streptococcus salivarius* ssp. *thermophiles* & *S. sanguinis*, *Streptococcus salivarius* ssp. *Thermophilus*, *Streptococcus* spp., *Veillonella* spp., Proteobacteria, *Shigella* spp. & *Escherichia* spp., *Mycoplasma hominis*, and *Akkermanasia muciniphilia*). The test is a commercial and patented product—hence the dysbiosis scores are the producer's secret.

In addition, an Alternative Dysbiosis Index (ADI) that is based on the relative abundance of the bacteria Alistipes, Proteobacteria and *Shigella* spp. & *Escherichia* spp., and the relative scarcity of *Bacteroides fragilis*, *Ruminococcus gnavus*, *Bacteroides* spp. & *Prevotella* spp., and *Dialister invisus* was calculated. The ADI has been claimed to separate the favourable dysbiosis (positive scores) from the unfavourable one (negative scores) [21].

2.4.4. Faecal Short Chain Fatty Acids

The subjects with morbid obesity collected the faecal material at home in kits that were provided by the producer of the microbial test and stored it at room temperature for maximum five days before freezing at minus 70 °C [12].

Distilled water containing 3 mmol/L of 2-ethylbutyric acid (as internal standard) and 0.5 mmol/L of H_2SO_4 was added to 0.5 g of the faecal content and homogenized. According to the method of Zijlstra et al. as modified by Høverstad et al. 2.5 mL of the homogenate was vacuum distilled [22,23]. The distillate was analysed with gas chromatography (Agilent 7890 A; Agilent, CA, USA) using a capillary column (serial no. USE400345H, Agilent J&W GC columns; Agilent, CA, USA) and quantified while using internal standardisation. Flame ionisation detection was employed. The total amount of SCFA and the total and relative amount of acetic, propionic, n-butyric, i-butyric, n-valeric, i-valeric, n-caproic, and i-caproic acids expressed in mmol/kg wet weight and proportion (percentage) were measured and reported.

The following variables were also calculated:

- Index A (saccharolytic fermentation), which was the concentration of acetic minus propionate minus butyrate divided by the total amount of SCFAs [24].
- Index B (proteolytic fermentation), which was the sum of concentrations of isobutyrate and isovalerate [24].
- The ratio "Propionic acid/Butyric acid". A high ratio has been proposed as unfavourable [25].

In principle, the analyses of SCFA were performed with identical methods in the subjects with morbid obesity and the healthy volunteers. However, since the analyses were performed in different laboratories and with slightly different preanalytical handling of the samples, only the relative amounts of the SCFA were compared between the groups to avoid bias in the measuring of the total amounts of SCFA.

2.5. Statistics

Student *t*-test was used for comparisons between groups, Wilcoxon sign-rank test for comparisons with a reference standard, and linear and logistic regression analyses for the study of associations. In each analysis, all of the cases with data on the relevant variables were included ("available case analysis"). *p*-values < 0.05 were judged as being statistically significant. The analyses were performed with IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY, USA: IBM Corp.

2.6. Ethics

The study was approved by the Norwegian Regional Committees for Medical and Health Research Ethics, (reference numbers 2012/966 and 030.08) and was performed in accordance with the Declaration of Helsinki. All the participants gave written informed consent before inclusion.

3. Results

3.1. Subject Characteristics

Out of 350 consecutive subjects with morbid obesity, 111 were excluded because the study nurse was unavailable, and 80 refused participation. Out of 159 subjects included in the study, 17 were erroneously included or non-compliant and 40 did not provide faecal samples. Table 1 gives the characteristics of the 102 subjects that were included in this study.

Table 1. Subject characteristics.

Subject Characteristics	Number (%) Mean and/or Median	SD and/or Range
Gender (male/female)	15 (14.7%)/87 (85.3)	
Age (years)	44.2	8.6
Height (cm)	170	7.8
Weight (kg)	120.8	16.1
BMI (kg/m^2)	41.8	3.6
Coffee (cups/day)	3.2	2.5
Smoking (daily/previously/never)	14 (13.7%)/46 (45.1%)/42 (41.2%)	
Physical activity (0–8)	4.5	2.3
Diabetes (yes/no)	23 (23.2%)/76 (76.8%)	
Metformin use (yes/no)	16 (18.0%)/73 (82.0%)	
Non-nutritive sweeteners (units *)	7.5 (median 3.3)	10.1 (0–43)
WHO-5 (0–100)	60.4 (median 60)	16 (12–92)
HSCL-10 (1–4)	1.58 (median 1.4)	0.54 (1.0–3.2)
HSCL-10 Mental distress (yes/no)	26 (26.5%)/72 (73.5%)	
Fatigue (6–63)	35.9	14.8
Musculoskeletal pain (0–12)	4.4	2.9
Food intolerance (Yes/No)	55 (55.6%)/44 (44.4%)	
IBS (Yes/No)	27 (27%)/73 (73%)	
IBS Severity scoring system (0–500)	103	0–389

* One unit = 100 mL beverage with non-nutritive sweeteners or 2 tablets/teaspoons for coffee of tea. WHO-5: WHO Well-being index. HSCL-10: Hopkin Symptom Check List 10. IBS: Irritable Bowel Syndrome.

The healthy volunteers that were used for comparisons of the SCFA were four men and eleven women with a mean age of 32.1 years (range 22–68) and BMI 23.7 (range 20.1–27.8 kg/m^2).

3.2. Dysbiosis Test

Dysbiosis [Dysbiosis Index (DI) > 2] was present in 62/102 (61%). The mean DI and Alternative Dysbiosis Index (ADI) scores were 2.8 (1.3) and −0.4 (2.6), respectively. When compared with producer's reference population [12], the relative amount of 22 bacteria were significantly elevated (*p*-values < 0.05, of which 12 bacteria with *p* < 0.001) and the relative amount of 10 bacteria was significantly reduced (*p*-values < 0.05, of which 5 bacteria with *p* < 0.001) The most marked deviations from the reference population were: *Bacteroides* spp. & *Prevotella* spp.: Score 1.59 (1.27) (*p* < 0.001). Bacteroides fragilis: Score 0.54 (0.89) (*p* < 0.001). Bacteroides stercoris: Score 0.44 (0.74) (*p* < 0.001). Eubacterium hallii: Score −0.54 (0.54) (*p* < 0.001). Faecalibacterium prausnitzii: −0.49 (0.71) (*p* < 0.001).

3.3. Short Chain Fatty Acids

Total amount of SCFA was 35.99 (SD 21.24) mmol/kg wet weight. Table 2 gives the results in the subjects with morbid obesity and the healthy volunteers with comparisons between the relative amounts of SCFA in the two groups.

Table 2. Short chain fatty acids (SCFA) in subjects with morbid obesity and healthy volunteers. The results are given as mean (SD).

SCFA	Subjects with Morbid Obesity		Healthy Volunteers	MO vs. HV Relative Amounts
	mmol/kg Wet Weight	Relative Amount (%)	Relative Amount (%)	*p*-Value
SCFA total	35.99 (21.24)			
Acetic acid	19.57 (10.72)	55.1 (6.4)	76.9 (9.6)	<0.001
Propionic acid	6.25 (4.16)	17.3 (4.4)	8.5 (3.7)	<0.001
Iso-butyric acid	0.72 (0.61)	2.1 (0.9)	1.4 (0.7)	0.006
Butyric acid	7.13 (5.28)	19.2 (5.3)	9.5 (4.6)	<0.001
Iso-valeric acid	1.05 (0.93)	3.0 (1.5)	2.0 (1.2)	0.017
Valeric acid	0.96 (0.84)	2.6 (1.2)	1.3 (0.8)	<0.001
Iso-capronic acid	0.00 (0.01)	0.0 (0.0)	0.0 (0.0)	0.163
Capronic acid	0.29 (0.51)	0.7 (1.0)	0.4 (0.5)	0.187
Index A	0.19 (0.11)			
Index B	1.77 (1.53)			
Pro/But ratio	1.01 (0.53)	1.01 (0.53)	1.0 (0.4)	0.864

SCFA: Short chain fatty acids. MO: Subjects with morbid obesity. HV: Healthy volunteers. Pro/But ratio: The ratio Propionic acid/Butyric acid.

3.4. Associations between the Psychobiological Disorders and the Microbial Markers

Out of 427 analysed associations between the faecal microbiota and the psychobiological disorders, 41 (10%) were statistically significant. Table 3 gives all of the statistically significant associations between the psychological disorders and the microbiota, and Table 4 provides all of the statistically significant associations between the functional somatic disorders and the microbiota.

Table 3. The significant associations between the psychological disorders and the faecal microbiota and SCFA. Regression analyses with the psychological variables as dependent variables.

Microbiota	WHO-5		HSC-10		Fatigue	
	B; *p*-Value *	B; *p*-Value †	B; *p*-Value *	B; *p*-Value †	B; *p*-Value *	B; *p*-Value †
Dysbiosis Index	−2.86; 0.024					
ADI			−0.056; 0.011		−1.98; 0.001	−1.81; 0.002
Alistipes					−5.42; 0.022	
Bacteroides spp. & *Prevotella* spp.	−3.43; 0.010				2.84; 0.021	
Bacteroides stercoris			0.174; 0.019	0.159; 0.028		
Bacilli	4.86; 0.039					
Dorea spp.	12.18; 0.014	11.44;0.016				
Faecalibacterium prausnitzii	6.37; 0.007	5.65; 0.013	−0.205; 0.011	−0.191; 0.015		
Phascolarctobacterium sp.					−6.77; 0.005	−5.94; 0.009
SCFA total	−0.179; 0.019					
Acetic acid	−0.342; 0.024					
Propionic acid	−0.890; 0.022					
Butyric acid	−0.681; 0.026	−0.675; 0.020				

* Linear regression analyses with the psychological variable as dependent variable and one-by-one of the microbiota variables adjusted for gender, age and BMI; † Stepwise forward linear regression analyses. All the significant variables in the one-by-one analyses were included adjusted for gender, age and BMI.

Table 4. The significant associations between the functional somatic disorders and the faecal microbiota and SCFA. Regression analyses with the functional somatic disorders as dependent variables.

Microbiota	Food Intolerance		Musculoskeletal Pain		IBS		IBS-SSS	
	OR; *p*-Value *	OR; *p*-Value †	B; *p*-Value *	B; *p*-Value †	OR; *p*-Value *	OR; *p*-Value †	B; *p*-Value *	B; *p*-Value †
ADI							−10.86; 0.010	−10.86; 0.010
Actinomycetales			1.34; 0.034					
Bifidobacterium spp.			1.22; 0.012	0.94; 0.039				
Alistipes	0.34; 0.019	0.34; 0.019					−40.3; 0.012	
Alistipes onderdonkii	0.52; 0.041							
Bacteroides stercoris			1.25; 0.001	1.07; 0.004				
Bacteroides zoogleoformans					4.64; 0.026	15.55; 0.009		
Parabacteroides johnsonii								
Parabacteroides spp.					2.10; 0.037	3.31; 0.007		
Firmicutes					2.30; 0.037			
Dialister 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.

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

References

1. WHO Fact Sheet N 311, Obesity and Overweight. Updated June 2016. Available online: http://www.who.int/mediacentre/factsheets/fs311/en/ (accessed on 10 September 2018).

2. Bouter, K.E.; van Raalte, D.H.; Groen, A.K.; Nieuwdorp, M. Role of the Gut Microbiome in the Pathogenesis of Obesity and Obesity-Related Metabolic Dysfunction. *Gastroenterology* **2017**, *152*, 1671–1678. [CrossRef] [PubMed]

3. Gao, R.; Zhu, C.; Li, H.; Yin, M.; Pan, C.; Huang, L.; Kong, C.; Wang, X.; Zhang, Y.; Qu, S.; et al. Dysbiosis Signatures of Gut Microbiota Along the Sequence from Healthy, Young Patients to Those with Overweight and Obesity. *Obesity (Silver Spring)* **2018**, *26*, 351–361. [CrossRef] [PubMed]

4. Zhang, X.; Shen, D.; Fang, Z.; Jie, Z.; Qiu, X.; Zhang, C.; Chen, Y.; Ji, L. Human gut microbiota changes reveal the progression of glucose intolerance. *PLoS ONE* **2013**, *8*, e71108. [CrossRef] [PubMed]

5. Zhuang, X.; Xiong, L.; Li, L.; Li, M.; Chen, M. Alterations of gut microbiota in patients with irritable bowel syndrome: A systematic review and meta-analysis. *J. Gastroenterol. Hepatol.* **2017**, *32*, 28–38. [CrossRef] [PubMed]

6. Bhattarai, Y.; Muniz Pedrogo, D.A.; Kashyap, P.C. Irritable bowel syndrome: A gut microbiota-related disorder? *Am. J. Physiol. Gastrointest. Liver Physiol.* **2017**, *312*, G52–G62. [CrossRef] [PubMed]

7. Moser, G.; Fournier, C.; Peter, J. Intestinal microbiome-gut-brain axis and irritable bowel syndrome. *Wien. Med. Wochenschr.* **2018**, *168*, 62–66. [CrossRef] [PubMed]

8. Naseribafrouei, A.; Hestad, K.; Avershina, E.; Sekelja, M.; Linlokken, A.; Wilson, R.; Rudi, K. Correlation between the human fecal microbiota and depression. *Neurogastroenterol. Motil.* **2014**, *26*, 1155–1162. [CrossRef]

9. Cenit, M.C.; Sanz, Y.; Codoner-Franch, P. Influence of gut microbiota on neuropsychiatric disorders. *World J. Gastroenterol.* **2017**, *23*, 5486–5498. [CrossRef]

10. Gill, P.A.; van Zelm, M.C.; Muir, J.G.; Gibson, P.R. Review article: Short chain fatty acids as potential therapeutic agents in human gastrointestinal and inflammatory disorders. *Aliment. Pharmacol. Ther.* **2018**, *48*, 15–34. [CrossRef]

11. Szczesniak, O.; Hestad, K.; Hanssen, J.F.; Rudi, K. Isovaleric acid in stool correlates with human depression. *Nutr. Neurosci.* **2015**, *19*, 279–283. [CrossRef]

12. Casen, C.; Vebo, H.C.; Sekelja, M.; Hegge, F.T.; Karlsson, M.K.; Ciemniejewska, E.; Dzankovic, S.; Froyland, C.; Nestestog, R.; Engstrand, L.; et al. Deviations in human gut microbiota: A novel diagnostic test for determining dysbiosis in patients with IBS or IBD. *Aliment. Pharmacol. Ther.* **2015**, *42*, 71–83. [CrossRef] [PubMed]

13. Valeur, J.; Morken, M.H.; Norin, E.; Midtvedt, T.; Berstad, A. Intestinal fermentation in patients with self-reported food hypersensitivity: Painful, but protective? *Clin. Exp. Gastroenterol.* **2010**, *3*, 65–70. [CrossRef] [PubMed]

14. The Norwegian Food Composition Table. Available online: http://www.matvaretabellen.no/?language=en (accessed on 10 September 2018).

15. Topp, C.W.; Ostergaard, S.D.; Sondergaard, S.; Bech, P. The WHO-5 Well-Being Index: A systematic review of the literature. *Psychother. Psychosom.* **2015**, *84*, 167–176. [CrossRef] [PubMed]

16. Søgaard, A.J.; Bjelland, I.; Tell, G.S.; Røysamb, E. A comparison of the CONOR Mental Health Index to the HSCL-10 and HADS. *Nor. Epidemiol.* **2003**, *13*, 279–284.

17. Lerdal, A.; Wahl, A.; Rustoen, T.; Hanestad, B.R.; Moum, T. Fatigue in the general population: A translation and test of the psychometric properties of the Norwegian version of the fatigue severity scale. *Scand. J. Public Health* **2005**, *33*, 123–130. [CrossRef] [PubMed]

18. Longstreth, G.F.; Thompson, W.G.; Chey, W.D.; Houghton, L.A.; Mearin, F.; Spiller, R.C. Functional bowel disorders. *Gastroenterology* **2006**, *130*, 1480–1491. [CrossRef] [PubMed]

19. Francis, C.Y.; Morris, J.; Whorwell, P.J. The irritable bowel severity scoring system: A simple method of monitoring irritable bowel syndrome and its progress. *Aliment. Pharmacol. Ther.* **1997**, *11*, 395–402. [CrossRef] [PubMed]

20. Genetic Analysis AS. GAMap TM Dysbiosis Test. Available online: http://www.genetic-analysis.com/patent (accessed on 10 September 2018).

21. Farup, P.G.; Kvehaugen, A.S.; Aasbrenn, M. Could a test discriminate between the "good" and the "bad" fecal dysbiosis? A study in subjects with morbid obesity. *Obes. Facts* **2018**, *11*, 112–113.

22. Zijlstra, J.B.; Beukema, J.; Wolthers, B.G.; Byrne, B.M.; Groen, A.; Dankert, J. Pretreatment methods prior to gaschromatographic analysis of volatile fatty acids from faecal samples. *Clin. Chim. Acta* **1977**, *78*, 243–250. [CrossRef]

23. Hoverstad, T.; Bjorneklett, A.; Midtvedt, T.; Fausa, O.; Bohmer, T. Short-chain fatty acids in the proximal gastrointestinal tract of healthy subjects. *Scand. J. Gastroenterol.* **1984**, *19*, 1053–1058. [PubMed]

24. Tjellstrom, B.; Hogberg, L.; Stenhammar, L.; Magnusson, K.E.; Midtvedt, T.; Norin, E.; Sundqvist, T. Effect of exclusive enteral nutrition on gut microflora function in children with Crohn's disease. *Scand. J. Gastroenterol.* **2012**, *47*, 1454–1459. [CrossRef] [PubMed]

25. Farup, P.G.; Rudi, K.; Hestad, K. Faecal short-chain fatty acids—A diagnostic biomarker for irritable bowel syndrome? *BMC Gastroenterol.* **2016**, *16*, 51. [CrossRef] [PubMed]

26. Liu, Y.; Zhang, L.; Wang, X.; Wang, Z.; Zhang, J.; Jiang, R.; Wang, X.; Wang, K.; Liu, Z.; Xia, Z.; et al. Similar Fecal Microbiota Signatures in Patients With Diarrhea-Predominant Irritable Bowel Syndrome and Patients With Depression. *Clin. Gastroenterol. Hepatol.* **2016**, *14*, 1602–1611. [CrossRef] [PubMed]

27. Sundin, J.; Rangel, I.; Fuentes, S.; Heikamp-de Jong, I.; Hultgren-Hornquist, E.; de Vos, W.M.; Brummer, R.J. Altered faecal and mucosal microbial composition in post-infectious irritable bowel syndrome patients correlates with mucosal lymphocyte phenotypes and psychological distress. *Aliment. Pharmacol. Ther.* **2015**, *41*, 342–351. [CrossRef] [PubMed]

28. Shin, N.R.; Whon, T.W.; Bae, J.W. Proteobacteria: Microbial signature of dysbiosis in gut microbiota. *Trends Biotechnol.* **2015**, *33*, 496–503. [CrossRef] [PubMed]

29. Litvak, Y.; Byndloss, M.X.; Tsolis, R.M.; Baumler, A.J. Dysbiotic Proteobacteria expansion: A microbial signature of epithelial dysfunction. *Curr. Opin. Microbiol.* **2017**, *39*, 1–6. [CrossRef] [PubMed]

30. Feng, J.; Tang, H.; Li, M.; Pang, X.; Wang, L.; Zhang, M.; Zhao, Y.; Zhang, X.; Shen, J. The abundance of fecal Faecalibacterium prausnitzii in relation to obesity and gender in Chinese adults. *Arch. Microbiol.* **2014**, *196*, 73–77. [CrossRef] [PubMed]

31. Ferreira-Halder, C.V.; Faria, A.V.S.; Andrade, S.S. Action and function of Faecalibacterium prausnitzii in health and disease. *Best Pract. Res. Clin. Gastroenterol.* **2017**, *31*, 643–648. [CrossRef] [PubMed]

32. Tana, C.; Umesaki, Y.; Imaoka, A.; Handa, T.; Kanazawa, M.; Fukudo, S. Altered profiles of intestinal microbiota and organic acids may be the origin of symptoms in irritable bowel syndrome. *Neurogastroenterol. Motil.* **2010**, *22*, 512–515. [CrossRef] [PubMed]

33. Stilling, R.M.; van de Wouw, M.; Clarke, G.; Stanton, C.; Dinan, T.G.; Cryan, J.F. The neuropharmacology of butyrate: The bread and butter of the microbiota-gut-brain axis? *Neurochem. Int.* **2016**, *99*, 110–132. [CrossRef] [PubMed]

34. Schwiertz, A.; Taras, D.; Schafer, K.; Beijer, S.; Bos, N.A.; Donus, C.; Hardt, P.D. Microbiota and SCFA in lean and overweight healthy subjects. *Obesity (Silver Spring)* **2010**, *18*, 190–195. [CrossRef] [PubMed]

35. Rahat-Rozenbloom, S.; Fernandes, J.; Gloor, G.B.; Wolever, T.M. Evidence for greater production of colonic short-chain fatty acids in overweight than lean humans. *Int. J. Obes. (Lond.)* **2014**, *38*, 1525–1531. [CrossRef] [PubMed]

36. Dugas, L.R.; Lie, L.; Plange-Rhule, J.; Bedu-Addo, K.; Bovet, P.; Lambert, E.V.; Forrester, T.E.; Luke, A.; Gilbert, J.A.; Layden, B.T. Gut microbiota, short chain fatty acids, and obesity across the epidemiologic transition: The METS-Microbiome study protocol. *BMC Public Health* **2018**, *18*, 978. [CrossRef] [PubMed]

37. Vanhoutvin, S.A.; Troost, F.J.; Kilkens, T.O.; Lindsey, P.J.; Hamer, H.M.; Jonkers, D.M.; Venema, K.; Brummer, R.J. The effects of butyrate enemas on visceral perception in healthy volunteers. *Neurogastroenterol. Motil.* **2009**, *21*, 952-e76. [CrossRef] [PubMed]

38. Montandon, S.A.; Jornayvaz, F.R. Effects of Antidiabetic Drugs on Gut Microbiota Composition. *Genes (Basel)* **2017**, *8*, 250. [CrossRef]

39. Wu, H.; Esteve, E.; Tremaroli, V.; Khan, M.T.; Caesar, R.; Manneras-Holm, L.; Stahlman, M.; Olsson, L.M.; Serino, M.; Planas-Felix, M.; et al. Metformin alters the gut microbiome of individuals with treatment-naive type 2 diabetes, contributing to the therapeutic effects of the drug. *Nat. Med.* **2017**, *23*, 850–858. [CrossRef] [PubMed]

40. Suez, J.; Korem, T.; Zilberman-Schapira, G.; Segal, E.; Elinav, E. Non-caloric artificial sweeteners and the microbiome: Findings and challenges. *Gut Microbes* **2015**, *6*, 149–155. [CrossRef] [PubMed]

41. Sohail, M.U.; Althani, A.; Anwar, H.; Rizzi, R.; Marei, H.E. Role of the Gastrointestinal Tract Microbiome in the Pathophysiology of Diabetes Mellitus. *J. Diabetes Res.* **2017**, *2017*, 9631435. [CrossRef] [PubMed]

42. Kannampalli, P.; Shaker, R.; Sengupta, J.N. Colonic butyrate-algesic or analgesic? *Neurogastroenterol. Motil.* **2011**, *23*, 975–979. [CrossRef] [PubMed]

43. Li, Z.; Yi, C.X.; Katiraei, S.; Kooijman, S.; Zhou, E.; Chung, C.K.; Gao, Y.; van den Heuvel, J.K.; Meijer, O.C.; Berbee, J.F.P.; et al. Butyrate reduces appetite and activates brown adipose tissue via the gut-brain neural circuit. *Gut* **2018**, *67*, 1269–1279. [CrossRef] [PubMed]

44. Bouter, K.; Bakker, G.J.; Levin, E.; Hartstra, A.V.; Kootte, R.S.; Udayappan, S.D.; Katiraei, S.; Bahler, L.; Gilijamse, P.W.; Tremaroli, V.; et al. Differential metabolic effects of oral butyrate treatment in lean versus metabolic syndrome subjects. *Clin. Transl. Gastroenterol.* **2018**, *9*, 155. [CrossRef] [PubMed]

45. Duncan, S.H.; Belenguer, A.; Holtrop, G.; Johnstone, A.M.; Flint, H.J.; Lobley, G.E. Reduced dietary intake of carbohydrates by obese subjects results in decreased concentrations of butyrate and butyrate-producing bacteria in feces. *Appl. Environ. Microbiol.* **2007**, *73*, 1073–1078. [CrossRef] [PubMed]

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

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 pseudo-membranous 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.

https://doi.org/10.1371/journal.pone.0194904.t001

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 Bio-Code, 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
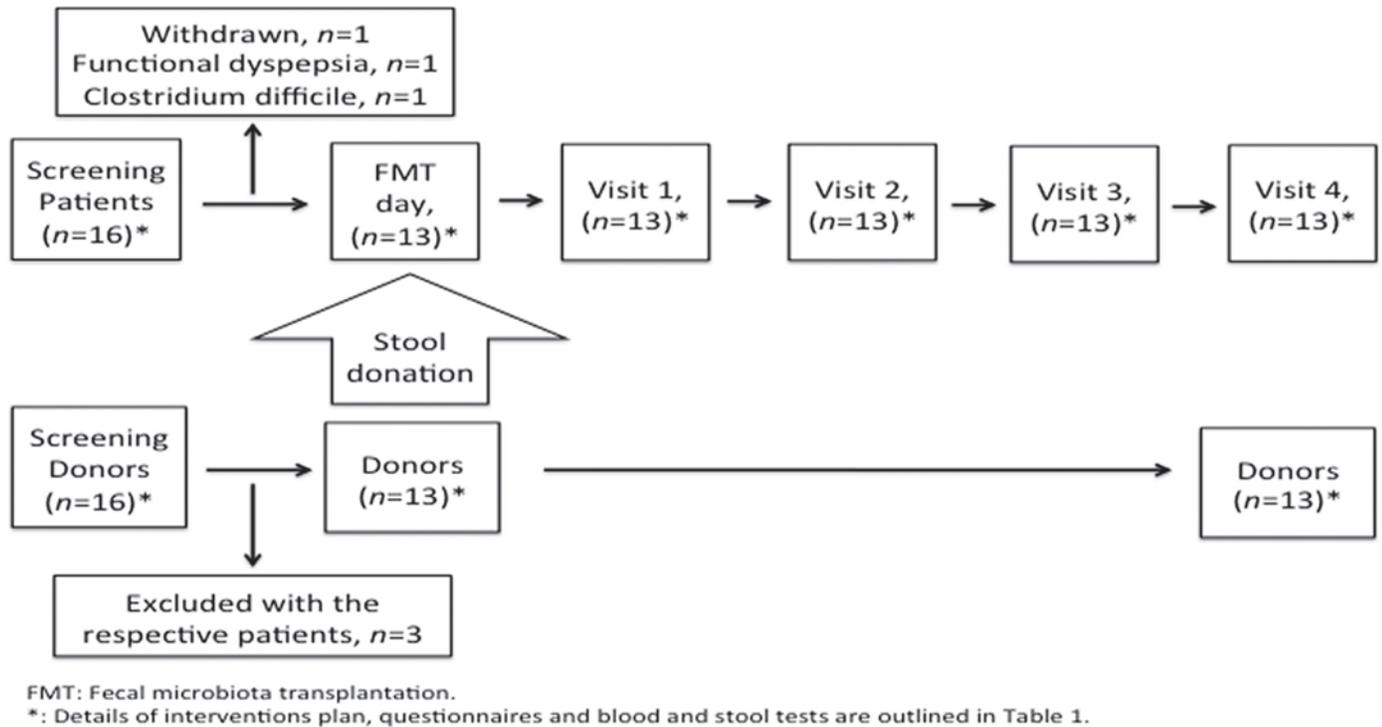
FMT: Fecal microbiota transplantation.
*: Details of interventions plan, questionnaires and blood and stool tests are outlined in Table 1.

Fig 1. Study flow chart.

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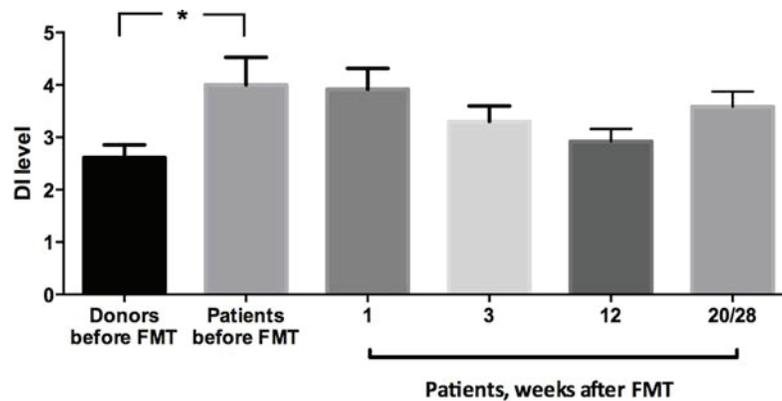
Fig 2. Dysbiosis index of the donors before fecal microbiota transplantation (FMT) and for the patients before FMT (day 0) and following FMT (weeks 1, 3, 12 and 20/28).

https://doi.org/10.1371/journal.pone.0194904.g002

plot of the gut microbiota profiles corrected for sample differences showed a gradual shift of the gut microbiota profile over time (Fig 3).

Comparing the microbiota between the groups, responders ($n = 8$) and non-responders ($n = 5$), which were defined based on achieving >50 or <50 IBS-SSS points, respectively, at week 20/28 compared to baseline, showed significant differences (adjusted P-values) in the *Bacteroides* signals between the donors at the beginning and the end of the study ($P<0.0001$ and 0.23, respectively) and between the patients at weeks 0, 1, 3, 12 and 20/28 ($P<0.0001$, <0.0001, 0.23, 0.08 and 0.08, respectively), Fig 4A. The respective values for *Desulfitispora* signals between the donors at the beginning and the end of the study ($P = 0.0005$ and 0.98, respectively) and between the patients at weeks 0, 1, 3, 12 and 20/28 ($P = 0.0002$, <0.0001, 0.0005, 0.0025 and <0.0001, respectively), Fig 4B, and for *Megasphaera/Dialister* signals between the donors at the beginning and the end of the study ($P = 0.54$ and 0.53, respectively) and between the patients at weeks 0, 1, 3, 12 and 20/28 ($P = 0.13$, 0.029, 0.24, 0.013 and 0.029, respectively), Fig 4C. As for *Bifidobacteria*, differences in the bacterial signals between responders and non-responders were noted, however, they were statistically not significant, Fig 4D.

Questionnaires

The score of IBS-SSS (mean±SEM) for the donors was 18±8.9 and for IBS-SQ is 0.7±0.3, which indicated asymptomatic status. The score of IBS-SSS of 11 patients were considered severe (IBS-SSS score >300) and only two patients had moderate severity (IBS-SSS score = 176–300) before FMT. The score of IBS-SSS (mean±SEM) for the patients at screening day was 333.6±20. IBS-SSS scores at FMT-day and at weeks 1, 3, 12 and 20/28 after FMT and comparisons between FMT-day and at each week are presented in Table 3. Using paired *t* test showed no significant difference in the aforementioned IBS-SSS scores for the patients between screening and FMT-day ($P = 0.45$), however, a significant reduction was noted in IBS-SSS scores of the patients between screening and weeks 1, 3, 12 and 20/28 ($P = 0.003$, 0.0004, 0.0095 and 0.012, respectively). No significant differences were observed by comparing the scores in weeks 1, 3, 12 and 20/28 interchangeably between each other, Fig 5. Four out of the 13 patients did not achieve >50 points reduction in IBS-SSS scores from baseline at week 1. However, two out of these four patients were late responders and achieved >50 points reduction in IBS-SSS scores from baseline at week 3. Therefore, a total of 9, 11, 9 and 8 out of the 13 patients achieved >50 points reduction in IBS-SSS scores from baseline at weeks 1, 3, 12 and 20/28 following FMT, respectively, and were considered as responders.

Table 2. Characteristics of the fecal bacterial signals between the donors at the beginning and end of the study, and patients in weeks 0, 1, 3, 12 and 20/28. The left part of the table shows the bacterial signals given for donors and recipients at different time points before and after FMT and the directionality towards or away from that of the donors at baseline. The right part of the table shows the *P*-values when comparing the bacterial signals for the recipients at different time points to that for the donors at baseline.

Bacteria strain	Donors, Beginning of study, n = 13	Patients					Donors, End of study, n = 10	P[a] Before FMT	P[b] After 1 week	P[c] After 3 weeks	P[d] After 12 weeks	P[e] After 20/28 weeks	P[f] Donors
		FMT day, n = 9	After 1 week, n = 12	After 3 weeks, n = 9	After 12 weeks, n = 13	After 20/28 weeks, n = 12							
Ruminococcus gnavus	4.6±1.1	40±15.6	8±2.3	15.3±9	25±17	8.1±1.8	8.8±2	**0.015**	>0.9	0.15	0.44	>0.9	0.19
Bacteroides	27.2±4.1	38.9 ±8.3	47.1 ±16.6	30.9±3.7	55.4±12.7	49.3±11	42±5.2	>0.9	>0.9	>0.9	**0.02**	0.1	0.097
Bacteroides/ Prevotella	483±51.4	634 ±28.5	599 ±25.8	551±63.9	783±49.7	731±52	788±58	0.7	>0.9	>0.9	**0.005**	**0.02**	**0.009**
Alistipes	100.5±16.8	100.5 ±23	119 ±17.4	140±26.8	186±11.9	208±9.6	188±15.5	>0.9	>0.9	0.9	**0.011**	**0.0006**	**0.03**
Parabacteroides	7.6±1.8	7.9±2.9	8.3±2	14.6±5.4	11.8±1.8	15.7±3.5	19.5±3.8	>0.9	>0.9	>0.9	0.4	0.3	**0.03**
Actinobacteria	287±45	66.6±13	95±23	197±54	138±29	92±23	204±57	**0.0010**	**0.007**	0.7	0.2	**0.003**	**0.018**
Bifidobacteria	324±57	65±13	97±25	205±57	150±34	92.5±25	241±72	**0.0011**	**0.008**	0.6	0.2	**0.004**	**0.017**
Proteobacteria	27±7.8	195 ±121.6	565±162	63.8 ±28.5	105±70	56.5±40	17±1.9	>0.9	**0.03**	>0.9	0.9	>0.9	0.5
Shigella/ Escherichia	62±23	260 ±124	578±128	116±45	188±77	90.9±65	41±14	>0.9	**0.002**	0.8	>0.9	>0.9	0.8

Data are presented as the mean±SEM. Comparison: Kruskal-Wallis multiple comparisons test with Dunn's post test

[a] Donors at the beginning of the study vs. patients on FMT day before fecal installation.

[b] Donors at the beginning of the study vs. patients 1 week after FMT.

[c] Donors at the beginning of the study vs. patients 3 weeks after FMT.

[d] Donors at the beginning of the study vs. patients 12 weeks after FMT.

[e] Donors at the beginning of the study vs. patients 20/28 weeks after FMT.

Paired *t* test

[f] Donors at the beginning vs. end of the study.

FMT: fecal microbiota transplantation.

https://doi.org/10.1371/journal.pone.0194904.t002

The scores for the following questionnaires are presented in Table 3: Bristol stool form scale, total SF-NDI scores, EPQ-N-12 and HAD. Bristol stool form scale showed significant changes in the stool form from diarrhea type before FMT to normal following FMT (weeks 1 and 3, *P* = 0.07 and 0.04, respectively), however, no significant differences were noted between weeks 1, 3, 12 and 20/28 when comparing them interchangeably with each other. The total SF-NDI scores showed a significant improvement in the QoL following FMT that lasted to the end of the study and no significant difference was found between weeks 3 and 20/28. The scores for EPQ-N-12 and HAD showed only significant improvement in HAD scores (anxiety and depression) 3 weeks after FMT (*P* = 0.016 and 0.038, respectively). The scores for the different domains of IBS-SQ during the first 3 weeks (20 days) showed significant improvements (except for anorexia) after receiving FMT, as presented in Table 4.

Post-FMT complications

No complications were reported during and following FMT until the end of the study.

Discussion

In this study of the kinetics of gut microbial community composition after FMT in IBS patients, the gut microbiota profile of the patients, which differed significantly from the donors
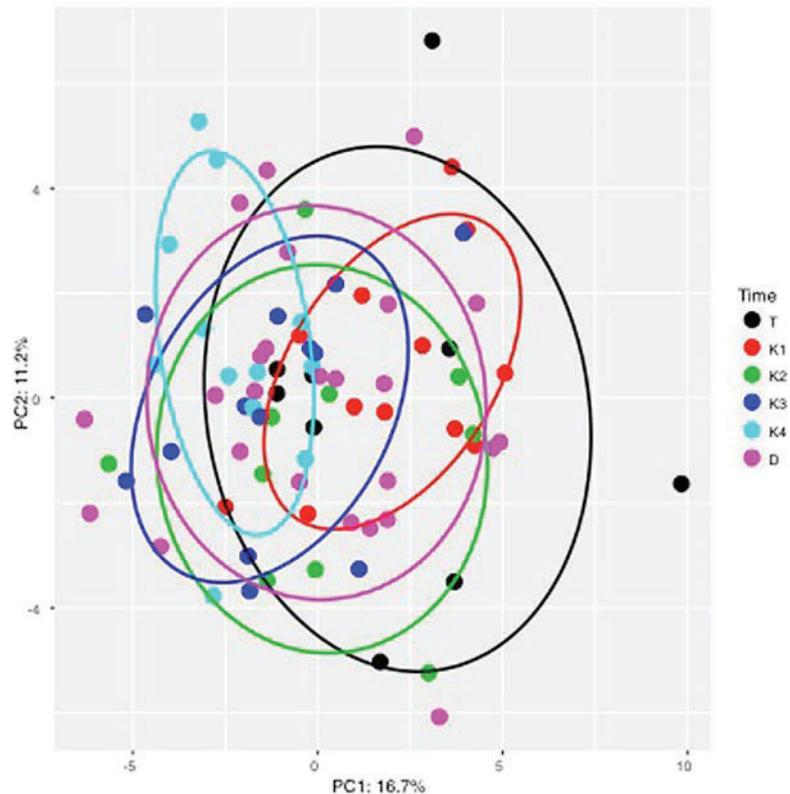
Fig 3. Scores for the first two principal component analysis (PCA) of fecal microbiota in donors at the beginning of the study and patients with irritable bowel syndrome after fecal microbiota transplantation (FMT, *n* = 13). Data have been centered within each donor series to remove donor differences for this analysis. Data are shown as donor (D: pink dots), FMT day (T: black dots), visit at week 1 (K1: red dots), visit at week 3 (K2: green dots), visit at week 12 (K3: dark blue dots) and visit at week 20/28 (K4: light blue dots). Each dot represents data from one patient for one visit. The first two PCs account for 27.9% (sum of PC1 and PC2) of the variation. The colored ellipses demonstrate the 68% confidence interval for PC1 and PC2. The PCA scores show that FMT seem to have an effect on the gut microbiota as systematic change is going forward.

https://doi.org/10.1371/journal.pone.0194904.g003

before FMT, have shown some dynamic changes during the 28 weeks of follow up period post FMT. In addition, the patients complained of severe IBS symptoms and low QoL before FMT. The change in the gut microbiota in IBS patients during the course of the study parallels a rapid improvement in the patients' symptoms and QoL that lasts up to 28 weeks.

The DI of the patients changed from severe dysbiosis before FMT to moderate dysbiosis after 12 weeks following FMT and maintained its new status throughout the course of the study. This showed that administrating the FMT via gastroscope in to the duodenum did not cause/worsen dysbiosis, on the contrary, it helped change the gut microbiota towards normobiosis. In general, dysbiosis in IBS is characterized by a decrease in *Actinobacteria*, *Bifidobacteria* and *Lactobacillus* [41], and an increase in *Bacteroidetes*, *Firmicutes* and *Proteobacteria* in the feces [41, 42]. Increased *Proteobacteria* in diarrhea-predominant IBS (IBS-D) including *E. coli* is associated with increased inflammation [41]. *Bifidobacteria* count is either decreased [41, 43] or increased in IBS patients [44]. In the current study, the bacterial signals for *Actinobacteria* in general and especially in *Bifidobacteria* in the total IBS group and the subgroups of IBS patients are significantly reduced compared to the donors' group at the beginning of the study.
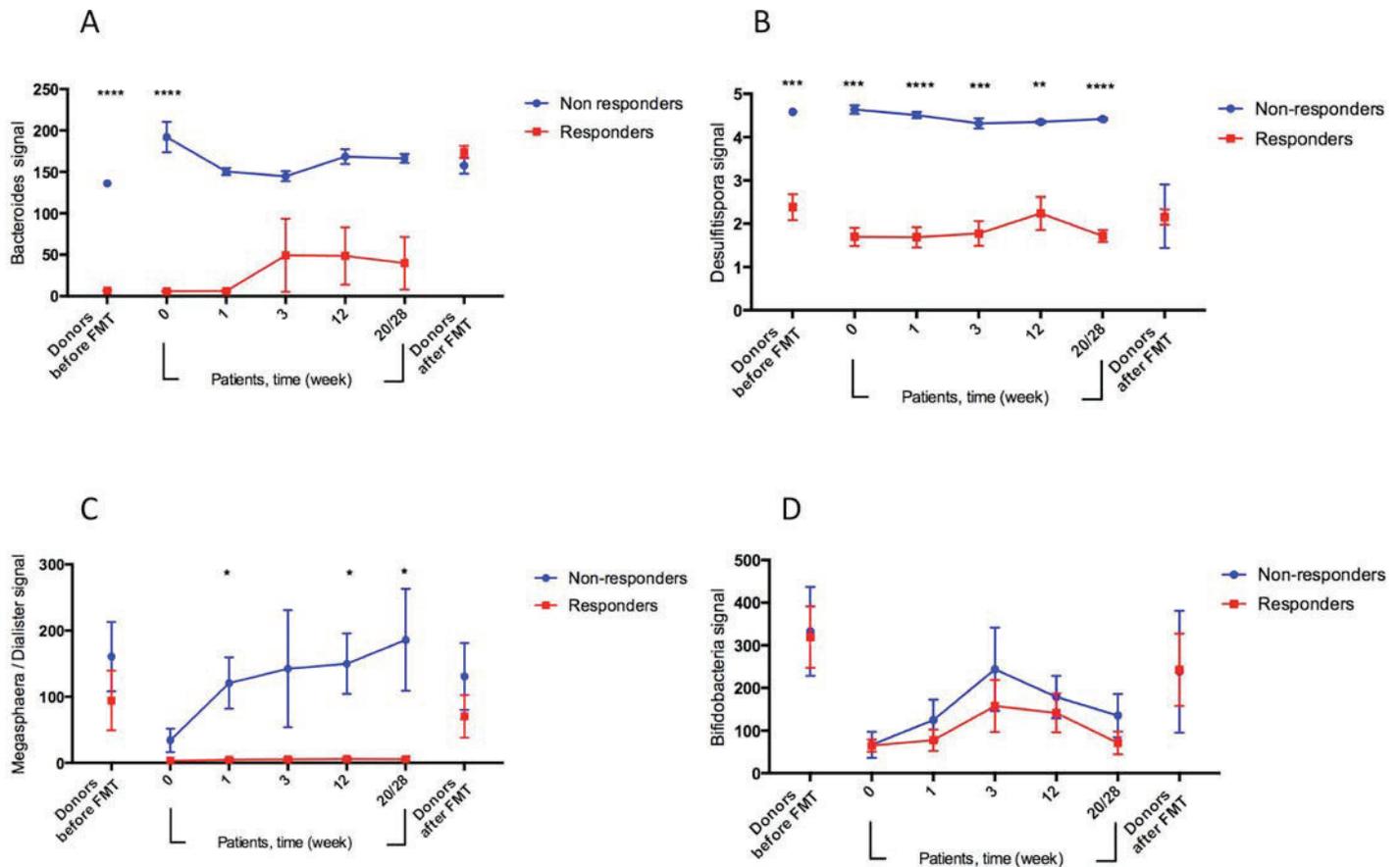
Fig 4. The characteristic differences of gut microbiota between responders and non-responders groups before and after FMT. The bacterial signals of (A) *Bacteroides*, (B) *Desulfitispora*, (C) *Megasphaera/Dialister* and (D) *Bifidobacteria*, in the responders and non-responders groups between the donors at the beginning of the study (before FMT), and between the patients in weeks 0, 1, 3, 12 and 20/28.

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.

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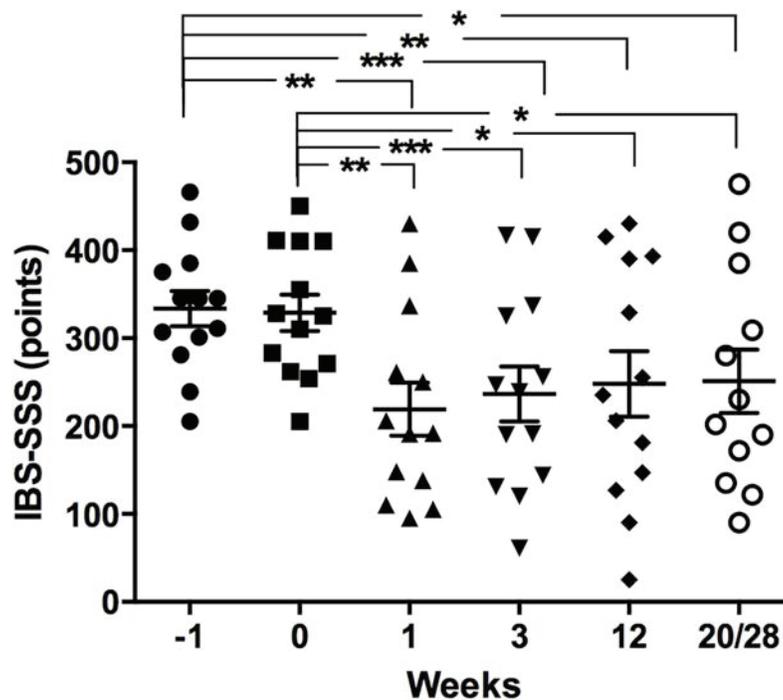
Fig 5. IBS-SSS scores of the patients one week before fecal microbiota transplantation (FMT, week -1), at FMT day (0), and following FMT (weeks 1, 3, 12 and 20/28).

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.

https://doi.org/10.1371/journal.pone.0194904.t004

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)

Acknowledgments

The authors thank all study patients and family members for participating in the study, and Genetic Analysis AS (Oslo, Norway) for performing the GA-map Dysbiosis test.

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.

References

1. Hungin AP, Whorwell PJ, Tack J, Mearin F. The prevalence, patterns and impact of irritable bowel syndrome: an international survey of 40,000 subjects. Alimentary pharmacology & therapeutics. 2003; 17 (5):643–50. Epub 2003/03/19. PMID: 12641512.

2. Hong SN, Rhee PL. Unraveling the ties between irritable bowel syndrome and intestinal microbiota. World journal of gastroenterology: WJG. 2014; 20(10):2470–81. Epub 2014/03/15. https://doi.org/10.3748/wjg.v20.i10.2470 PMID: 24627584; PubMed Central PMCID: PMCPmc3949257.

3. Tana C, Umesaki Y, Imaoka A, Handa T, Kanazawa M, Fukudo S. Altered profiles of intestinal microbiota and organic acids may be the origin of symptoms in irritable bowel syndrome. Neurogastroenterology and motility: the official journal of the European Gastrointestinal Motility Society. 2010; 22(5):512–9, e114-5. Epub 2009/11/12. https://doi.org/10.1111/j.1365-2982.2009.01427.x PMID: 19903265.

4. El-Salhy M, Gundersen D, Gilja OH, Hatlebakk JG, Hausken T. Is irritable bowel syndrome an organic disorder? World journal of gastroenterology: WJG. 2014; 20(2):384–400. https://doi.org/10.3748/wjg.v20.i2.384 PMID: 24574708

5. Grover M, Camilleri M, Smith K, Linden DR, Farrugia G. On the fiftieth anniversary. Postinfectious irritable bowel syndrome: mechanisms related to pathogens. Neurogastroenterology and motility: the official journal of the European Gastrointestinal Motility Society. 2014; 26(2):156–67. Epub 2014/01/21. https://doi.org/10.1111/nmo.12304 PMID: 24438587.

6. Ishihara S, Tada Y, Fukuba N, Oka A, Kusunoki R, Mishima Y, et al. Pathogenesis of irritable bowel syndrome—review regarding associated infection and immune activation. Digestion. 2013; 87(3):204–11. Epub 2013/05/29. https://doi.org/10.1159/000350054 PMID: 23712295.

7. Saito YA. The role of genetics in IBS. Gastroenterology clinics of North America. 2011; 40(1):45–67. Epub 2011/02/22. https://doi.org/10.1016/j.gtc.2010.12.011 PMID: 21333900; PubMed Central PMCID: PMCPmc3056499.

8. Simren M, Mansson A, Langkilde AM, Svedlund J, Abrahamsson H, Bengtsson U, et al. Food-related gastrointestinal symptoms in the irritable bowel syndrome. Digestion. 2001; 63(2):108–15. Epub 2001/03/13. 51878. https://doi.org/10.1159/000051878 PMID: 11244249.

9. Monsbakken KW, Vandvik PO, Farup PG. Perceived food intolerance in subjects with irritable bowel syndrome—etiology, prevalence and consequences. European journal of clinical nutrition. 2006; 60 (5):667–72. Epub 2006/01/05. https://doi.org/10.1038/sj.ejcn.1602367 PMID: 16391571.

10. Aroniadis OC, Brandt LJ. Fecal microbiota transplantation: past, present and future. Current opinion in gastroenterology. 2013; 29(1):79–84. Epub 2012/10/09. https://doi.org/10.1097/MOG.0b013e32835a4b3e PMID: 23041678.

11. Ringel Y, Maharshak N. Intestinal microbiota and immune function in the pathogenesis of irritable bowel syndrome. American journal of physiology Gastrointestinal and liver physiology. 2013; 305(8):G529–41. Epub 2013/07/28. https://doi.org/10.1152/ajpgi.00207.2012 PMID: 23886861; PubMed Central PMCID: PMCPmc3798736.

12. Spiller R, Garsed K. Postinfectious irritable bowel syndrome. Gastroenterology. 2009; 136(6):1979–88. Epub 2009/05/22. https://doi.org/10.1053/j.gastro.2009.02.074 PMID: 19457422.

13. Karlsson FH, Fak F, Nookaew I, Tremaroli V, Fagerberg B, Petranovic D, et al. Symptomatic athero-sclerosis is associated with an altered gut metagenome. Nature communications. 2012; 3:1245. Epub 2012/12/06. https://doi.org/10.1038/ncomms2266 PMID: 23212374; PubMed Central PMCID: PMCPMC3538954.

14. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature. 2006; 444(7122):1022–3. Epub 2006/12/22. https://doi.org/10.1038/4441022a PMID: 17183309.

15. Vrieze A, Van Nood E, Holleman F, Salojarvi J, Kootte RS, Bartelsman JF, et al. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. Gastro-enterology. 2012; 143(4):913–6.e7. Epub 2012/06/26. https://doi.org/10.1053/j.gastro.2012.06.031 PMID: 22728514.

16. Sisson G, Ayis S, Sherwood RA, Bjarnason I. Randomised clinical trial: A liquid multi-strain probiotic vs. placebo in the irritable bowel syndrome—a 12 week double-blind study. Alimentary pharmacology & therapeutics. 2014; 40(1):51–62. Epub 2014/05/13. https://doi.org/10.1111/apt.12787 PMID: 24815298.

17. Menees SB, Maneerattannaporn M, Kim HM, Chey WD. The efficacy and safety of rifaximin for the irri-table bowel syndrome: a systematic review and meta-analysis. The American journal of gastroenterol-ogy. 2012; 107(1):28–35; quiz 6. Epub 2011/11/03. https://doi.org/10.1038/ajg.2011.355 PMID: 22045120.

18. Brandt LJ. American Journal of Gastroenterology Lecture: Intestinal microbiota and the role of fecal microbiota transplant (FMT) in treatment of C. difficile infection. The American journal of gastroenterol-ogy. 2013; 108(2):177–85. Epub 2013/01/16. https://doi.org/10.1038/ajg.2012.450 PMID: 23318479.

19. Jeffery IB, O'Toole PW, Ohman L, Claesson MJ, Deane J, Quigley EM, et al. An irritable bowel syn-drome subtype defined by species-specific alterations in faecal microbiota. Gut. 2012; 61(7):997–1006. Epub 2011/12/20. https://doi.org/10.1136/gutjnl-2011-301501 PMID: 22180058.

20. Eiseman B, Silen W, Bascom GS, Kauvar AJ. Fecal enema as an adjunct in the treatment of pseudo-membranous enterocolitis. Surgery. 1958; 44(5):854–9. Epub 1958/11/01. PMID: 13592638.

21. Schwan A, Sjolin S, Trottestam U, Aronsson B. Relapsing clostridium difficile enterocolitis cured by rec-tal infusion of homologous faeces. Lancet. 1983; 2(8354):845. Epub 1983/10/08. PMID: 6137662.

22. van Nood E, Vrieze A, Nieuwdorp M, Fuentes S, Zoetendal EG, de Vos WM, et al. Duodenal infusion of donor feces for recurrent Clostridium difficile. The New England journal of medicine. 2013; 368(5):407–15. Epub 2013/01/18. https://doi.org/10.1056/NEJMoa1205037 PMID: 23323867.

23. Pinn DM, Aroniadis OC, Brandt LJ. Is fecal microbiota transplantation the answer for irritable bowel syn-drome? A single-center experience. The American journal of gastroenterology. 2014; 109(11):1831–2. Epub 2014/11/07. https://doi.org/10.1038/ajg.2014.295 PMID: 25373585.

24. Holvoet T, Joossens M, Wang J, Boelens J, Verhasselt B, Laukens D, et al. Assessment of faecal microbial transfer in irritable bowel syndrome with severe bloating. Gut. 2016. Epub 2016/08/12. https://doi.org/10.1136/gutjnl-2016-312513 PMID: 27511198.

25. Zoller V, Laguna AL, Prazeres Da Costa O, Buch T, Goke B, Storr M. [Fecal microbiota transfer (FMT) in a patient with refractory irritable bowel syndrome]. Deutsche medizinische Wochenschrift (1946). 2015; 140(16):1232–6. Epub 2015/08/12. https://doi.org/10.1055/s-0041-103798 PMID: 26261935.

26. Bennet JD, Brinkman M. Treatment of ulcerative colitis by implantation of normal colonic flora. Lancet. 1989; 1(8630):164. Epub 1989/01/21. PMID: 2563083.

27. Borody TJ, Warren EF, Leis S, Surace R, Ashman O. Treatment of ulcerative colitis using fecal bacter-iotherapy. Journal of clinical gastroenterology. 2003; 37(1):42–7. Epub 2003/06/18. PMID: 12811208.

28. Finegold SM, Molitoris D, Song Y, Liu C, Vaisanen ML, Bolte E, et al. Gastrointestinal microflora studies in late-onset autism. Clinical infectious diseases: an official publication of the Infectious Diseases

Society of America. 2002; 35(Suppl 1):S6–s16. Epub 2002/08/13. https://doi.org/10.1086/341914 PMID: 12173102.

29. Francis CY, Morris J, Whorwell PJ. The irritable bowel severity scoring system: a simple method of monitoring irritable bowel syndrome and its progress. Alimentary pharmacology & therapeutics. 1997; 11(2):395–402. Epub 1997/04/01. PMID: 9146781.

30. Association WM. Declaration of Helsinki. Ethical Principles for Medical Research Involving Human Subjects. Jahrbuch für Wissenschaft Und Ethik. 2009; 14(1):233–8.

31. Cammarota G, Ianiro G, Gasbarrini A. Fecal microbiota transplantation for the treatment of Clostridium difficile infection: a systematic review. Journal of clinical gastroenterology. 2014; 48(8):693–702. Epub 2014/01/21. https://doi.org/10.1097/MCG.0000000000000046 PMID: 24440934.

32. Casen C, Vebo HC, Sekelja M, Hegge FT, Karlsson MK, Ciemniejewska E, et al. Deviations in human gut microbiota: a novel diagnostic test for determining dysbiosis in patients with IBS or IBD. Alimentary pharmacology & therapeutics. 2015; 42(1):71–83. Epub 2015/05/15. https://doi.org/10.1111/apt.13236 PMID: 25973666.

33. Mathias JR, Clench MH, Reeves-Darby VG, Fox LM, Hsu PH, Roberts PH, et al. Effect of leuprolide acetate in patients with moderate to severe functional bowel disease. Double-blind, placebo-controlled study. Digestive diseases and sciences. 1994; 39(6):1155–62. Epub 1994/06/01. PMID: 8200247.

34. Kane SV, Sandborn WJ, Rufo PA, Zholudev A, Boone J, Lyerly D, et al. Fecal lactoferrin is a sensitive and specific marker in identifying intestinal inflammation. The American journal of gastroenterology. 2003; 98(6):1309–14. Epub 2003/06/24. https://doi.org/10.1111/j.1572-0241.2003.07458.x PMID: 12818275.

35. Lewis SJ, Heaton KW. Stool form scale as a useful guide to intestinal transit time. Scandinavian journal of gastroenterology. 1997; 32(9):920–4. Epub 1997/09/23. https://doi.org/10.3109/00365529709011203 PMID: 9299672.

36. Arslan G, Lind R, Olafsson S, Florvaag E, Berstad A. Quality of life in patients with subjective food hypersensitivity: applicability of the 10-item short form of the Nepean Dyspepsia Index. Digestive diseases and sciences. 2004; 49(4):680–7. Epub 2004/06/10. PMID: 15185878.

37. Eysenck HJ. The structure of human personality: London: Methuen; 1953.

38. Botega NJ, Bio MR, Zomignani MA, Garcia C Jr., Pereira WA. [Mood disorders among inpatients in ambulatory and validation of the anxiety and depression scale HAD]. Revista de saude publica. 1995; 29(5):355–63. Epub 1995/10/01. PMID: 8731275.

39. Zigmond AS, Snaith RP. The hospital anxiety and depression scale. Acta psychiatrica Scandinavica. 1983; 67(6):361–70. Epub 1983/06/01. PMID: 6880820.

40. Hanevik K, Dizdar V, Langeland N, Hausken T. Development of functional gastrointestinal disorders after Giardia lamblia infection. BMC gastroenterology. 2009; 9:27. Epub 2009/04/23. https://doi.org/10.1186/1471-230X-9-27 PMID: 19383162; PubMed Central PMCID: PMCPMC2676300.

41. Bennet SM, Ohman L, Simren M. Gut microbiota as potential orchestrators of irritable bowel syndrome. Gut and liver. 2015; 9(3):318–31. Epub 2015/04/29. https://doi.org/10.5009/gnl14344 PMID: 25918261; PubMed Central PMCID: PMCPMC4413965.

42. Chang C, Lin H. Dysbiosis in gastrointestinal disorders. Best practice & research Clinical gastroenterology. 2016; 30(1):3–15. Epub 2016/04/07. https://doi.org/10.1016/j.bpg.2016.02.001 PMID: 27048892.

43. Duboc H, Rainteau D, Rajca S, Humbert L, Farabos D, Maubert M, et al. Increase in fecal primary bile acids and dysbiosis in patients with diarrhea-predominant irritable bowel syndrome. Neurogastroenterology and motility: the official journal of the European Gastrointestinal Motility Society. 2012; 24(6):513–20, e246-7. Epub 2012/02/24. https://doi.org/10.1111/j.1365-2982.2012.01893.x PMID: 22356587.

44. Kassinen A, Krogius-Kurikka L, Makivuokko H, Rinttila T, Paulin L, Corander J, et al. The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects. Gastroenterology. 2007; 133(1):24–33. Epub 2007/07/17. https://doi.org/10.1053/j.gastro.2007.04.005 PMID: 17631127.

45. Jalanka-Tuovinen J, Salojarvi J, Salonen A, Immonen O, Garsed K, Kelly FM, et al. Faecal microbiota composition and host-microbe cross-talk following gastroenteritis and in postinfectious irritable bowel syndrome. Gut. 2014; 63(11):1737–45. Epub 2013/12/07. https://doi.org/10.1136/gutjnl-2013-305994 PMID: 24310267.

46. Hustoft TN, Hausken T, Ystad SO, Valeur J, Brokstad K, Hatlebakk JG, et al. Effects of varying dietary content of fermentable short-chain carbohydrates on symptoms, fecal microenvironment, and cytokine profiles in patients with irritable bowel syndrome. Neurogastroenterology and motility: the official journal of the European Gastrointestinal Motility Society. 2017; 29(4). Epub 2016/10/18. https://doi.org/10.1111/nmo.12969 PMID: 27747984.

47. Staley C, Vaughn BP, Graiziger CT, Singroy S, Hamilton MJ, Yao D, et al. Community dynamics drive punctuated engraftment of the fecal microbiome following transplantation using freeze-dried, encapsulated fecal microbiota. Gut microbes. 2017:1–13. Epub 2017/03/11. https://doi.org/10.1080/19490976.2017.1299310 PMID: 28282270.

48. Nanayakkara WS, Skidmore PM, O'Brien L, Wilkinson TJ, Gearry RB. Efficacy of the low FODMAP diet for treating irritable bowel syndrome: the evidence to date. Clinical and experimental gastroenterology. 2016; 9:131–42. Epub 2016/07/07. https://doi.org/10.2147/CEG.S86798 PMID: 27382323; PubMed Central PMCID: PMCPMC4918736.

49. Mazzawi T, El-Salhy M. Effect of diet and individual dietary guidance on gastrointestinal endocrine cells in patients with irritable bowel syndrome (Review). International journal of molecular medicine. 2017; 40 (4):943–52. Epub 2017/08/30. https://doi.org/10.3892/ijmm.2017.3096 PMID: 28849091.

50. Mazzawi T, Hausken T, Gundersen D, El-Salhy M. Effects of dietary guidance on the symptoms, quality of life and habitual dietary intake of patients with irritable bowel syndrome. Molecular medicine reports. 2013; 8(3):845–52. Epub 2013/07/04. https://doi.org/10.3892/mmr.2013.1565 PMID: 23820783.

51. Niec AM, Frankum B, Talley NJ. Are adverse food reactions linked to irritable bowel syndrome? The American journal of gastroenterology. 1998; 93(11):2184–90. Epub 1998/11/20. https://doi.org/10.1111/j.1572-0241.1998.00531.x PMID: 9820394.

52. Gough E, Shaikh H, Manges AR. Systematic review of intestinal microbiota transplantation (fecal bacteriotherapy) for recurrent Clostridium difficile infection. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America. 2011; 53(10):994–1002. Epub 2011/10/18. https://doi.org/10.1093/cid/cir632 PMID: 22002980.

53. Ghoshal UC, Srivastava D. Irritable bowel syndrome and small intestinal bacterial overgrowth: meaningful association or unnecessary hype. World journal of gastroenterology: WJG. 2014; 20(10):2482–91. Epub 2014/03/15. https://doi.org/10.3748/wjg.v20.i10.2482 PMID: 24627585; PubMed Central PMCID: PMCPMC3949258.

54. Gupta S, Allen-Vercoe E, Petrof EO. Fecal microbiota transplantation: in perspective. Therapeutic advances in gastroenterology. 2016; 9(2):229–39. Epub 2016/03/02. https://doi.org/10.1177/1756283X15607414 PMID: 26929784; PubMed Central PMCID: PMCPMC4749851.

55. Brandt LJ, Aroniadis OC, Mellow M, Kanatzar A, Kelly C, Park T, et al. Long-term follow-up of colonoscopic fecal microbiota transplant for recurrent Clostridium difficile infection. The American journal of gastroenterology. 2012; 107(7):1079–87. Epub 2012/03/28. https://doi.org/10.1038/ajg.2012.60 PMID: 22450732.

ORIGINAL RESEARCH

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, Akershus 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

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

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.

- 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

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.

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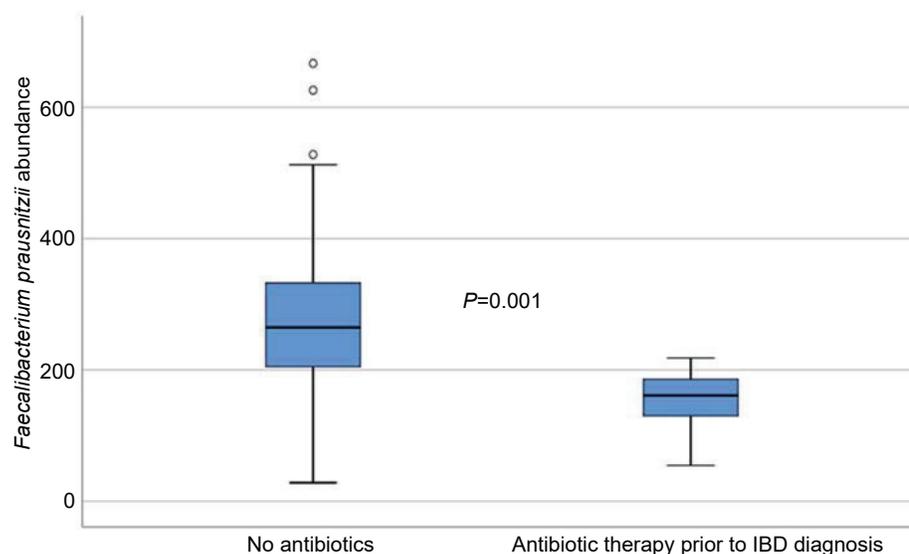
Figure 1 *Faecalibacterium prausnitzii* abundance in IBD patients according to whether they had received antibiotics prior to the diagnosis (measured in fluorescence signal strength in 1,000 units).
Abbreviation: IBD, inflammatory bowel disease.

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 ileocolonoscopy) was not achieved in 40 (36%) of the patients
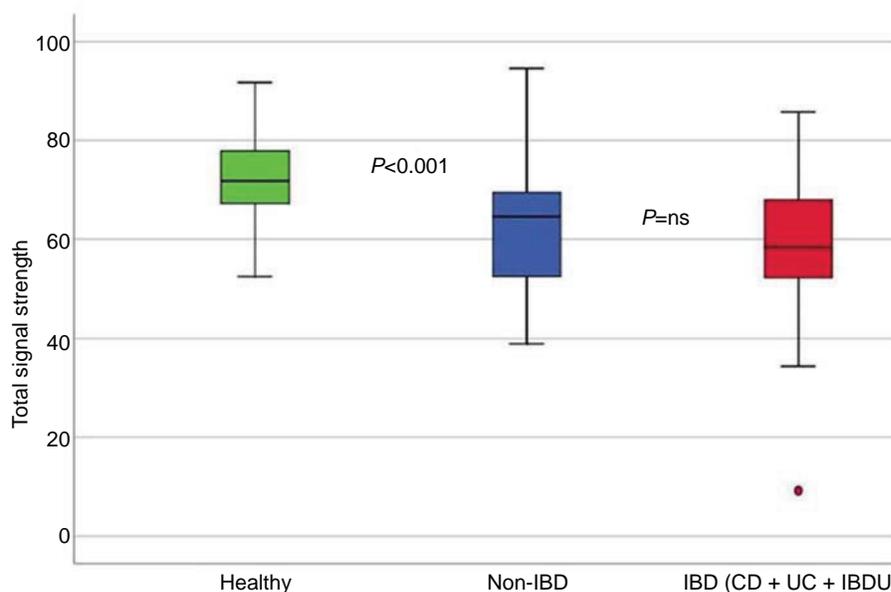
Figure 2 Boxplot illustrating the differences in the total fluorescence signal strength measured in 1,000 units between IBD, non-IBD symptomatic patients, and healthy controls.
**Abbreviations:** IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; IBDU, inflammatory bowel disease unclassified; ns, not significant.
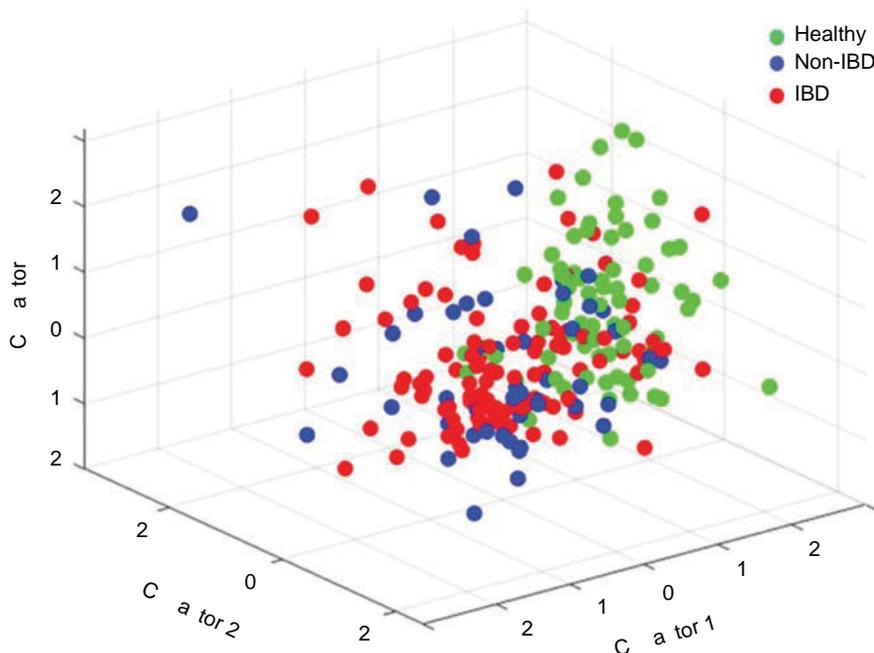
Figure 3 Principal component analysis, illustrating the difference in microbiota abundance of all 54 bacterial probes between IBD, non-IBD symptomatic patients, and healthy controls.
Notes: Each dot represents one individual. The units represent the total item loadings on each of the extracted factors.
**Abbreviations:** IBD, inflammatory bowel disease; PCA, principal component analysis.
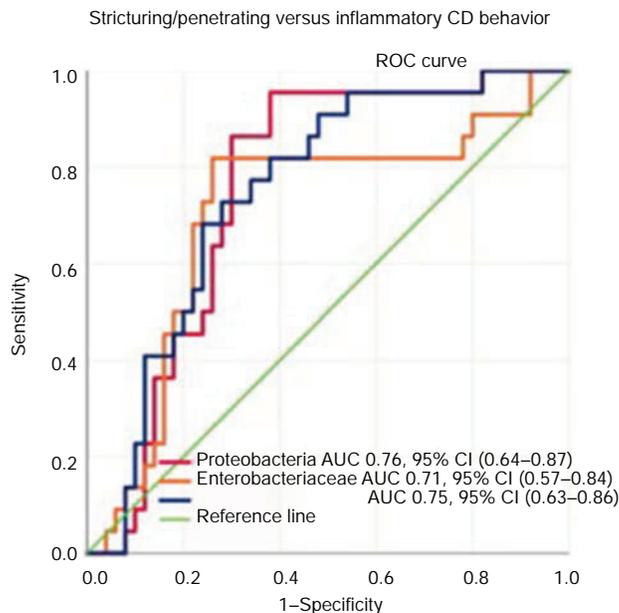
Figure 4 Sensitivity and specificity of Proteobacteria, *Enterobacteriaceae,* and *Shigella/Escherichia* abundance in differentiating Crohn's disease phenotypes (stricturing/penetrating vs inflammatory disease behavior) using the area under the receiver operating characteristics curve analysis.
Abbreviations: 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,
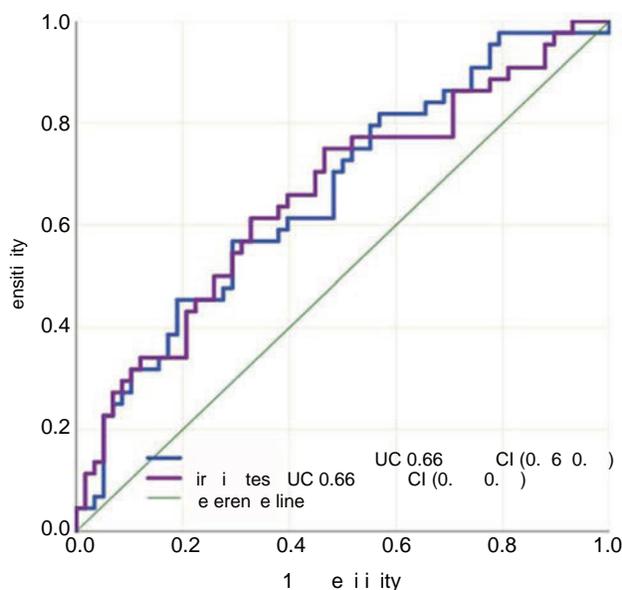
Figure 5 Sensitivity and specificity of Firmicutes and *Mycoplasma hominis* abundance in differentiating conventional- vs biologic therapy-treated IBD patients using the area under the receiver operating characteristics curve analysis.
Abbreviations: IBD, inflammatory bowel disease; CD, Crohn's disease; AUC, area under the curve.
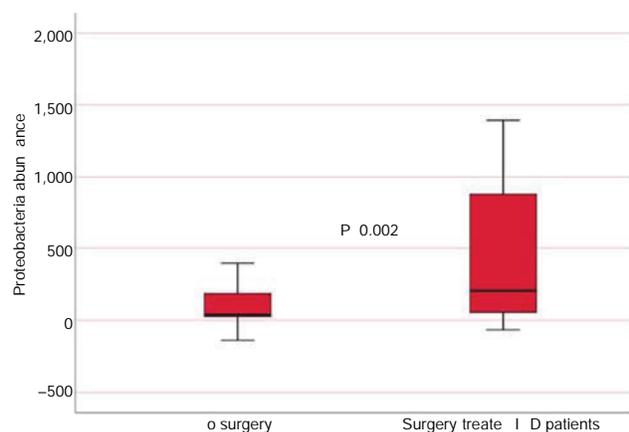
Figure 6 Proteobacteria abundance in IBD patients according to whether they needed surgery or not (measured in fluorescence signal strength in 1,000 units).
Abbreviation: IBD, inflammatory bowel disease.

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

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

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.

Acknowledgments

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.

References

1. Chu H, Khosravi A, Kusumawardhani IP, et al. Gene-microbiota interactions contribute to the pathogenesis of inflammatory bowel disease. *Science*. 2016;352(6289):1116–1120.
2. Ng SC, Shi HY, Hamidi N, et al. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet*. 2018;390(10114):2769–2778.
3. Kaplan GG, Ng SC. Understanding and preventing the global increase of inflammatory bowel disease. *Gastroenterology*. 2017;152(2):313–321.
4. Lin L, Zhang J. Role of intestinal microbiota and metabolites on gut homeostasis and human diseases. *BMC Immunol*. 2017;18(1):2.
5. Spiller R, Major G. IBS and IBD – separate entities or on a spectrum? *Nat Rev Gastroenterol Hepatol*. 2016;13(10):613–621.
6. Sartor RB, Wu GD. Roles for intestinal bacteria, viruses, and fungi in pathogenesis of inflammatory bowel diseases and therapeutic approaches. *Gastroenterology*. 2017;152(2):327–339.
7. Chang C, Lin H. Dysbiosis in gastrointestinal disorders. *Best Pract Res Clin Gastroenterol*. 2016;30(1):3–15.
8. Sundin J, Ohman L, Simren M. Understanding the gut microbiota in inflammatory and functional gastrointestinal diseases. *Psychosom Med*. 2017.
9. Miyoshi J, Chang EB. The gut microbiota and inflammatory bowel diseases. *Transl Res*. 2017;179:38–48.
10. Hold GL, Smith M, Grange C, Watt ER, El-Omar EM, Mukhopadhya I. Role of the gut microbiota in inflammatory bowel disease pathogenesis: what have we learnt in the past 10 years? *World J Gastroenterol*. 2014;20(5):1192–1210.
11. Perminow G, Brackmann S, Lyckander LG, et al. A characterization in childhood inflammatory bowel disease, a new population-based inception cohort from South-Eastern Norway, 2005-07, showing increased incidence in Crohn's disease. *Scand J Gastroenterol*. 2009;44(4):446–456.
12. Olbjørn C, Cvancarova Småstuen M, Thiis-Evensen E, Nakstad B, Vatn MH, Perminow G. Serological markers in diagnosis of pediatric inflammatory bowel disease and as predictors for early tumor necrosis factor blocker therapy. *Scand J Gastroenterol*. 2017;52(4):414–419.
13. Ricanek P, Vatn S, Kalla R. Microbiota alterations in treatment naïve IBD and non-IBD patients – the EU IBD character project. *United Eur Gastroenterol J*. 2016;4(5 suppl):A721–A754.
14. IBD Working Group of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition. Inflammatory bowel disease in children and adolescents: recommendations for diagnosis – the Porto criteria. *J Pediatr Gastroenterol Nutr*. 2005;41(1):1–7.
15. Fagerberg UL, Lööf L, Merzoug RD, Hansson LO, Finkel Y. Fecal calprotectin levels in healthy children studied with an improved assay. *J Pediatr Gastroenterol Nutr*. 2003;37(4):468–472.
16. Olbjørn C, Nakstad B, Småstuen MC, Thiis-Evensen E, Vatn MH, Perminow G. Early anti-TNF treatment in pediatric Crohn's disease. Predictors of clinical outcome in a population-based cohort of newly diagnosed patients. *Scand J Gastroenterol*. 2014;49(12):1425–1431.
17. Levine A, Griffiths A, Markowitz J, et al. Pediatric modification of the Montreal classification for inflammatory bowel disease: the Paris classification. *Inflamm Bowel Dis*. 2011;17(6):1314–1321.
18. Casén C, Vebø HC, Sekelja M, et al. Deviations in human gut microbiota: a novel diagnostic test for determining dysbiosis in patients with IBS or IBD. *Aliment Pharmacol Ther*. 2015;42(1):71–83.
19. Lopetuso LR, Petito V, Graziani C, et al. Gut microbiota in health, diverticular disease, irritable bowel syndrome, and inflammatory bowel diseases: time for microbial marker of gastrointestinal disorders. *Dig Dis*. 2018;36(1):56–65.
20. Bennet SM, Ohman L, Simren M. Gut microbiota as potential orchestrators of irritable bowel syndrome. *Gut Liver*. 2015;9(3):318–331.
21. Maukonen J, Kolho KL, Paasela M, et al. Altered fecal microbiota in paediatric inflammatory bowel disease. *J Crohns Colitis*. 2015;9(12):1088–1095.
22. Gevers D, Kugathasan S, Denson LA, et al. The treatment-naive microbiome in new-onset Crohn's disease. *Cell Host Microbe*. 2014;15(3):382–392.
23. Papa E, Docktor M, Smillie C, et al. Non-invasive mapping of the gastrointestinal microbiota identifies children with inflammatory bowel disease. *PLoS ONE*. 2012;7(6):e39242.
24. Satokari R. Contentious host–microbiota relationship in inflammatory bowel disease – can foes become friends again? *Scand J Gastroenterol*. 2015;50(1):34–42.
25. Smith PM, Howitt MR, Panikov N, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science*. 2013;341(6145):569–573.
26. Arrieta M-C, Stiemsma LT, Amenyogbe N, Brown EM, Finlay B. The intestinal microbiome in early life: health and disease. *Front Immunol*. 2014;5(Suppl 1):427.
27. Forbes JD, van Domselaar G, Bernstein CN. The gut microbiota in immune-mediated inflammatory diseases. *Front Microbiol*. 2016;7(19032):1081.
28. Mottawea W, Chiang CK, Mühlbauer M, et al. Altered intestinal microbiota-host mitochondria crosstalk in new onset Crohn's disease. *Nat Commun*. 2016;7(1):13419.
29. Joossens M, Huys G, Cnockaert M, et al. Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. *Gut*. 2011;60(5):631–637.
30. Berry D, Reinisch W. Intestinal microbiota: a source of novel biomarkers in inflammatory bowel diseases? *Best Pract Res Clin Gastroenterol*. 2013;27(1):47–58.

31. Frank DN, St. Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci USA*. 2007;104(34):13780–13785.

32. Mukhopadhya I, Hansen R, El-Omar EM, Hold GL. IBD – what role do Proteobacteria play? *Nat Rev Gastroenterol Hepatol*. 2012;9(4): 219–230.

33. Kaakoush NO, Day AS, Huinao KD, et al. Microbial dysbiosis in pediatric patients with Crohn's disease. *J Clin Microbiol*. 2012;50(10):3258–3266.

34. Dunn KA, Moore-Connors J, Macintyre B, et al. Early changes in microbial community structure are associated with sustained remission after nutritional treatment of pediatric Crohn's disease. *Inflamm Bowel Dis*. 2016;22(12):2853–2862.

35. Thorkildsen LT, Nwosu FC, Avershina E, et al. Dominant fecal microbiota in newly diagnosed untreated inflammatory bowel disease patients. *Gastroenterol Res Pract.* 2013;2013(170):1–13.

36. Kolho KL, Korpela K, Jaakkola T, et al. Fecal microbiota in pediatric inflammatory bowel disease and its relation to inflammation. *Am J Gastroenterol*. 2015;110(6):921–930.

37. Mccarville JL, Caminero A, Verdu EF. Novel perspectives on therapeutic modulation of the gut microbiota. *Therap Adv Gastroenterol*. 2016;9(4):580–593.

38. Burman S, Hoedt EC, Pottenger S, Mohd-Najman NS, Ó Cuív P, Morrison M. An (anti)-inflammatory microbiota: defining the role in inflammatory bowel disease? *Dig Dis*. 2016;34(1–2):64–71.

39. Magnusson MK, Strid H, Sapnara M, et al. Anti-TNF therapy response in patients with ulcerative colitis is associated with colonic antimicrobial peptide expression and microbiota composition. *J Crohns Colitis*. 2016;10(8):943–952.

40. Rajca S, Grondin V, Louis E, et al. Alterations in the intestinal microbiome (dysbiosis) as a predictor of relapse after infliximab withdrawal in Crohn's disease. *Inflamm Bowel Dis*. 2014;20(6):978–986.

41. Shaw KA, Bertha M, Hofmekler T, et al. Dysbiosis, inflammation, and response to treatment: a longitudinal study of pediatric subjects with newly diagnosed inflammatory bowel disease. *Genome Med*. 2016;8(1):75.

42. Lewis JD, Chen EZ, Baldassano RN, et al. Inflammation, antibiotics, and diet as environmental stressors of the gut microbiome in pediatric Crohn's disease. *Cell Host Microbe*. 2015;18(4):489–500.

43. Gerasimidis K, Bertz M, Hanske L, et al. Decline in presumptively protective gut bacterial species and metabolites are paradoxically associated with disease improvement in pediatric Crohn's disease during enteral nutrition. *Inflamm Bowel Dis*. 2014;20(5):861–871.

44. Maclellan A, Connors J, Grant S, Cahill L, Langille M, van Limbergen J. The Impact of Exclusive Enteral Nutrition (EEN) on the gut microbiome in Crohn's disease: a review. *Nutrients*. 2017;9(5):0447.

45. Qiao YQ, Cai CW, Ran ZH. Therapeutic modulation of gut microbiota in inflammatory bowel disease: more questions to be answered. *J Dig Dis*. 2016;17(12):800–810.

46. Gaufin T, Tobin NH, Aldrovandi GM. The importance of the microbiome in pediatrics and pediatric infectious diseases. *Curr Opin Pediatr*. 2018;30(1):117–124.

47. Lane ER, Zisman TL, Suskind DL. The microbiota in inflammatory bowel disease: current and therapeutic insights. *J Inflamm Res*. 2017;10:63–73.

48. Sokol H, Leducq V, Aschard H, et al. Fungal microbiota dysbiosis in IBD. *Gut*. 2017;66(6):1039–1048.

49. Norman JM, Handley SA, Baldridge MT, et al. Disease-specific alterations in the enteric virome in inflammatory bowel disease. *Cell*. 2015;160(3):447–460.

50. Ni J, Wu GD, Albenberg L, Tomov VT. Gut microbiota and IBD: causation or correlation? *Nat Rev Gastroenterol Hepatol*. 2017;14(10):573–584.

51. Moen AE, Tannæs TM, Vatn S, et al. Simultaneous purification of DNA and RNA from microbiota in a single colonic mucosal biopsy. *BMC Res Notes*. 2016;9(1):328.

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*

OXFORD

Original Article

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

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.

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 $2^{-[Target-Housekeeping]}$.

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

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.

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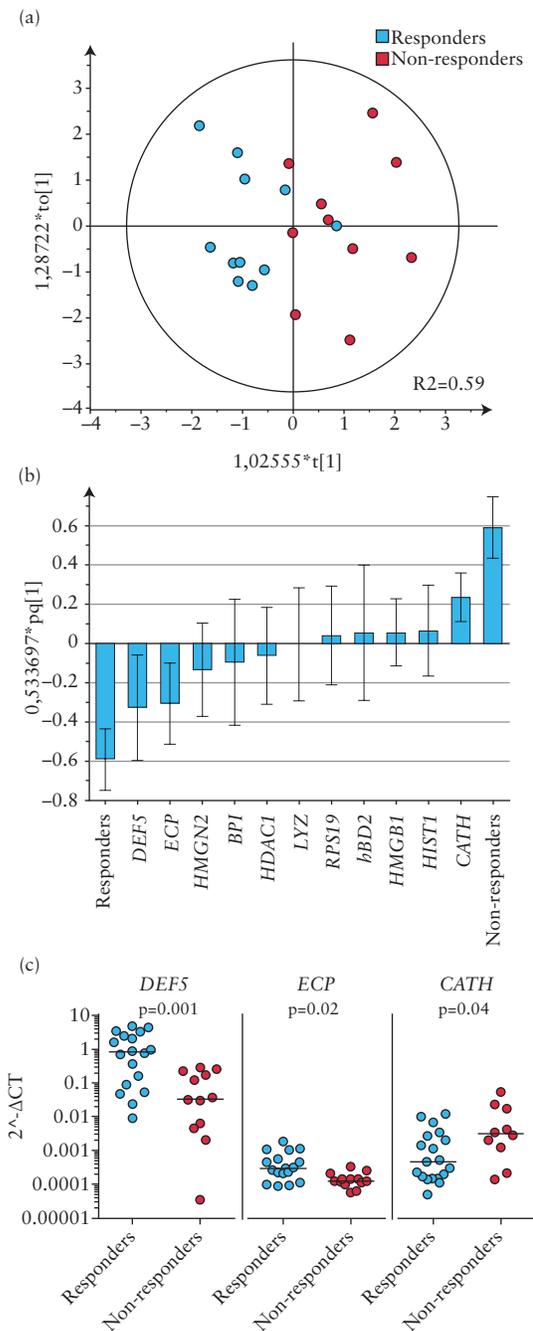
Figure 1. Anti-TNF therapy responders and non-responders show different mucosal AMP expression before start of treatment. Biopsy samples were obtained at baseline and mucosal mRNA expression of *DEF5, ECP, HMGN2, BPI, HDAC1, LYZ, RPS19, hBD2, HMGB1, HIST1,* and *CATH* was analysed with RT-PCR. [A] Multivariate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

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].

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
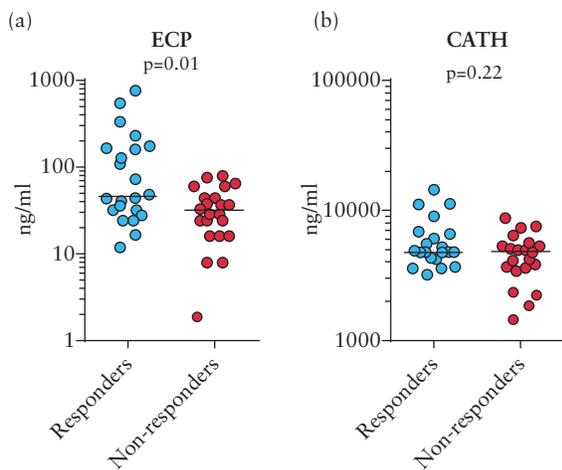
Figure 2. Serum levels of ECP and CATH in anti-TNF therapy responders and non-responders. Serum samples were obtained at baseline [responders *n* = 23 and non-responders *n* = 21], and levels of ECP [A] and CATH [B] were analysed by ELISA. Each symbol represents one individual and horizontal lines indicates median of the group. TNF, tumour necrosis factor; ELISA, enzyme-linked immunosorbent assay.
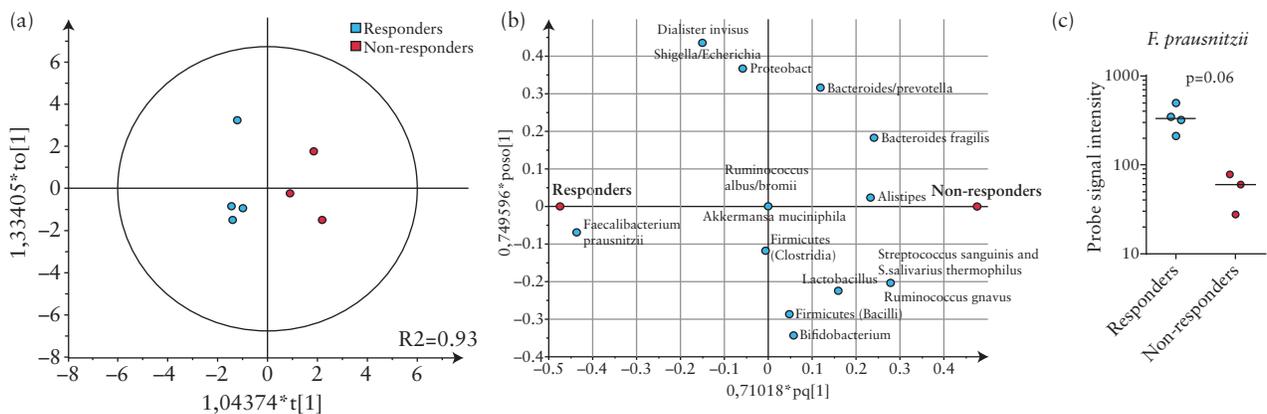
Figure 3. Anti-TNF therapy responders and non-responders differ at baseline with respect to their fecal microbiota composition. Fecal samples were obtained at baseline [responders *n* = 4 and non-responders *n* = 3] and analysed by the GA-map™ Dysbiosis Test. OPLS-DA scatter plot [A] and loading scatter 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.
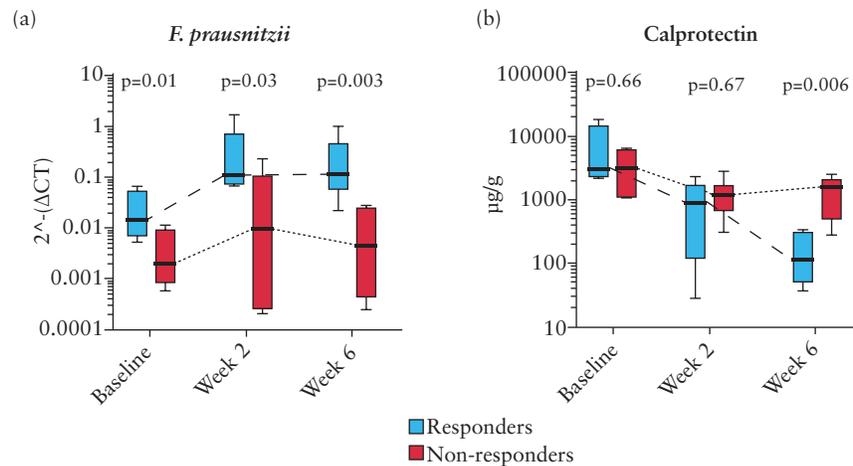
Figure 4. Anti-TNF therapy responders show higher abundance of *F. prausnitzii* compared with non-responders. Fecal samples were obtained at baseline [responders *n* = 4 and non-responders *n* = 3], Week 2 [responders *n* = 8 and non-responders *n* = 7], and Week 6 [responders *n* = 8 and non-responders *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

(a)

(b)
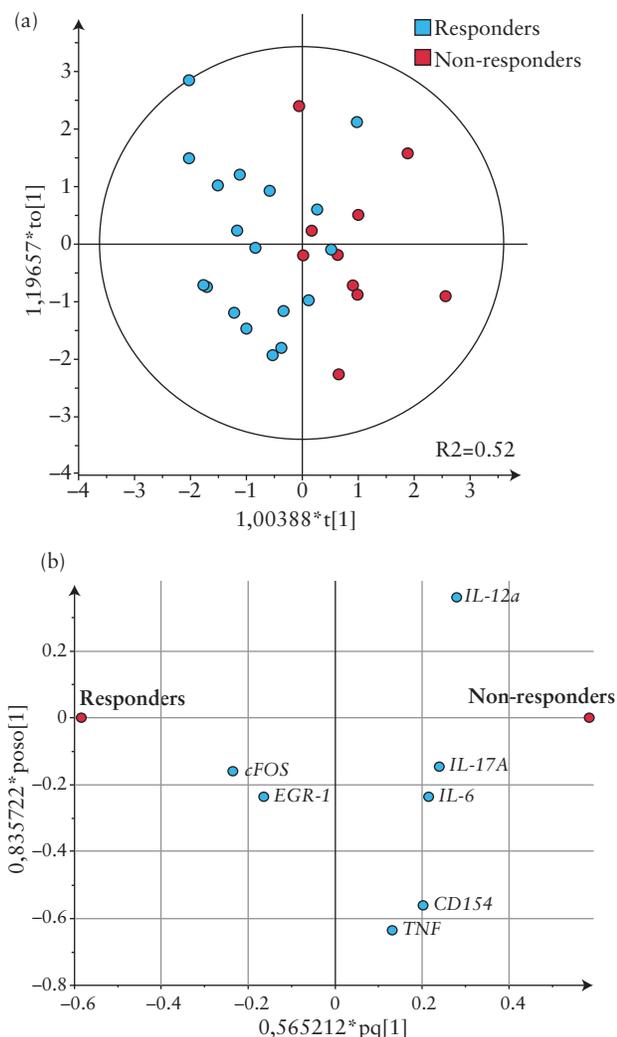
Figure 5. Multivariate factor analysis depicting associations between anti-TNF therapy response and genes involved in mucosal inflammation and regulation. Biopsy samples were obtained at baseline [responders n = 18 and non-responders n = 12] and mucosal mRNA expression *IL-6, IL-12a, IL17A, TNF, CD154, 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.

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 $2^{-[Target-Housekeeping]}$. Data in the column should be multiplied by 10^{-3}.

[b]Mann-Whitney test.

[c]Data are shown as median [25–75th percentile].

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.

Conflict of Interest

None.

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.

References

1. Rutgeerts P, Goboes K, Peeters M, *et al*. Effect of faecal stream diversion on recurrence of Crohn's disease in the neoterminal ileum. *Lancet* 1991;**338**:771–4.
2. Sartor RB. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology* 2004;**126**:1620–33.

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

3. Casellas F, Borruel N, Papo M, *et al*. Antiinflammatory effects of enterically coated amoxicillin-clavulanic acid in active ulcerative colitis. *Inflamm Bowel Dis* 1998;**4**:1–5.

4. Sellon RK, Tonkonogy S, Schultz M, *et al*. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infect Immun* 1998;**66**:5224–31.

5. Dianda L, Hanby AM, Wright NA, *et al*. T cell receptor-alpha beta-deficient mice fail to develop colitis in the absence of a microbial environment. *Am J Pathol* 1997;**150**:91–7.

6. Frank DN, St Amand AL, Feldman RA, *et al*. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A* 2007;**104**:13780–5.

7. Manichanh C, Rigottier-Gois L, Bonnaud E, *et al*. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut* 2006;**55**:205–11.

8. Swidsinski A, Ladhoff A, Pernthaler A, *et al*. Mucosal flora in inflammatory bowel disease. *Gastroenterology* 2002;**122**:44–54.

9. Dicksved J, Halfvarson J, Rosenquist M, *et al*. Molecular analysis of the gut microbiota of identical twins with Crohn's disease. *ISME J* 2008;**2**:716–27.

10. Kolho KL, Korpela K, Jaakkola T, *et al*. Fecal microbiota in pediatric inflammatory bowel disease and its relation to inflammation. *Am J Gastroenterol* 2015;**110**:921–30.

11. Lepage P, Hasler R, Spehlmann ME, *et al*. Twin study indicates loss of interaction between microbiota and mucosa of patients with ulcerative colitis. *Gastroenterology* 2011;**141**:227–36.

12. Lupp C, Robertson ML, Wickham ME, *et al*. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. *Cell Host Microbe* 2007;**2**:204.

13. Ohkusa T, Sato N, Ogihara T, *et al*. Fusobacterium varium localized in the colonic mucosa of patients with ulcerative colitis stimulates species-specific antibody. *J Gastroenterol Hepatol* 2002;**17**:849–53.

14. Ohkusa T, Yoshida T, Sato N, *et al*. Commensal bacteria can enter colonic epithelial cells and induce proinflammatory cytokine secretion: a possible pathogenic mechanism of ulcerative colitis. *J Med Microbiol* 2009;**58**:535–45.

15. Sokol H, Lepage P, Seksik P, *et al*. Temperature gradient gel electrophoresis of fecal 16S rRNA reveals active Escherichia coli in the microbiota of patients with ulcerative colitis. *J Clin Microbiol* 2006;**44**:3172–7.

16. Willing B, Halfvarson J, Dicksved J, *et al*. Twin studies reveal specific imbalances in the mucosa-associated microbiota of patients with ileal Crohn's disease. *Inflamm Bowel Dis* 2009;**15**:653–60.

17. Varela E, Manichanh C, Gallart M, *et al*. Colonisation by Faecalibacterium prausnitzii and maintenance of clinical remission in patients with ulcerative colitis. *Aliment Pharmacol Ther* 2013;**38**:151–61.

18. Machiels K, Joossens M, Sabino J, *et al*. A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. *Gut* 2014;**63**:1275–83.

19. Sokol H, Pigneur B, Watterlot L, *et al*. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci U S A* 2008;**105**:16731–6.

20. Atarashi K, Tanoue T, Oshima K, *et al*. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* 2013;**500**:232–6.

21. Wilson AJ, Chueh AC, Togel L, *et al*. Apoptotic sensitivity of colon cancer cells to histone deacetylase inhibitors is mediated by an Sp1/Sp3-activated transcriptional program involving immediate-early gene induction. *Cancer Res* 2010;**70**:609–20.

22. Nepelska M, Cultrone A, Beguet-Crespel F, *et al*. Butyrate produced by commensal bacteria potentiates phorbol esters induced AP-1 response in human intestinal epithelial cells. *PLoS One* 2012;**7**:e52869.

23. Chang PV, Hao L, Offermanns S, *et al*. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proc Natl Acad Sci U S A* 2014;**111**:2247–52.

24. Roger T, Lugrin J, Le Roy D, *et al*. Histone deacetylase inhibitors impair innate immune responses to Toll-like receptor agonists and to infection. *Blood* 2011;**117**:1205–17.

25. Masui R, Sasaki M, Funaki Y, *et al*. G protein-coupled receptor 43 moderates gut inflammation through cytokine regulation from mononuclear cells. *Inflamm Bowel Dis* 2013;**19**:2848–56.

26. Skov S, Rieneck K, Bovin LF, *et al*. Histone deacetylase inhibitors: a new class of immunosuppressors targeting a novel signal pathway essential for CD154 expression. *Blood* 2003;**101**:1430–8.

27. Mishra N, Brown DR, Olorenshaw IM, *et al*. Trichostatin A reverses skewed expression of CD154, interleukin-10, and interferon-gamma gene and protein expression in lupus T cells. *Proc Natl Acad Sci U S A* 2001;**98**:2628–33.

28. Marchesi JR, Holmes E, Khan F, *et al*. Rapid and noninvasive metabonomic characterization of inflammatory bowel disease. *J Proteome Res* 2007;**6**:546–51.

29. Le Gall G, Noor SO, Ridgway K, *et al*. Metabolomics of fecal extracts detects altered metabolic activity of gut microbiota in ulcerative colitis and irritable bowel syndrome. *J Proteome Res* 2011;**10**:4208–18.

30. De Preter V, Machiels K, Joossens M, *et al*. Faecal metabolite profiling identifies medium-chain fatty acids as discriminating compounds in IBD. *Gut* 2015;**64**:447–58.

31. Ho S, Pothoulakis C, Koon HW. Antimicrobial peptides and colitis. *Curr Pharm Des* 2013;**19**:40–7.

32. Jager S, Stange EF, Wehkamp J. Antimicrobial peptides in gastrointestinal inflammation. *Int J Inflam* 2010;**2010**:910283.

33. Nuding S, Fellermann K, Wehkamp J, *et al*. Reduced mucosal antimicrobial activity in Crohn's disease of the colon. *Gut* 2007;**56**:1240–7.

34. Wehkamp J, Salzman NH, Porter E, *et al*. Reduced Paneth cell alpha-defensins in ileal Crohn's disease. *Proc Natl Acad Sci U S A* 2005;**102**:18129–34.

35. Schauber J, Rieger D, Weiler F, *et al*. Heterogeneous expression of human cathelicidin hCAP18/LL-37 in inflammatory bowel diseases. *Eur J Gastroenterol Hepatol* 2006;**18**:615–21.

36. Arijs I, De Hertogh G, Lemaire K, *et al*. Mucosal gene expression of antimicrobial peptides in inflammatory bowel disease before and after first infliximab treatment. *PLoS One* 2009;**4**:e7984.

37. Rutgeerts P, Sandborn WJ, Feagan BG, *et al*. Infliximab for induction and maintenance therapy for ulcerative colitis. *N Engl J Med* 2005;**353**:2462–76.

38. Jarnerot G, Hertervig E, Friis-Liby I, *et al*. Infliximab as rescue therapy in severe to moderately severe ulcerative colitis: a randomized, placebo-controlled study. *Gastroenterology* 2005;**128**:1805–11.

39. Sandborn WJ, Colombel JF, D'Haens G, *et al*. One-year maintenance outcomes among patients with moderately-to-severely active ulcerative colitis who responded to induction therapy with adalimumab: subgroup analyses from ULTRA 2. *Aliment Pharmacol Ther* 2013;**37**:204–13.

40. Ferrante M, Vermeire S, Katsanos KH, *et al*. Predictors of early response to infliximab in patients with ulcerative colitis. *Inflamm Bowel Dis* 2007;**13**:123–8.

41. Taxonera C, Olivares D, Mendoza JL, *et al*. Need for infliximab dose intensification in Crohn's disease and ulcerative colitis. *World J Gastroenterol* 2014;**20**:9170–7.

42. Wolf D, D'Haens G, Sandborn WJ, *et al*. Escalation to weekly dosing recaptures response in adalimumab-treated patients with moderately to severely active ulcerative colitis. *Aliment Pharmacol Ther* 2014;**40**:486–97.

43. Olsen T, Goll R, Cui G, *et al*. TNF-alpha gene expression in colorectal mucosa as a predictor of remission after induction therapy with infliximab in ulcerative colitis. *Cytokine* 2009;**46**:222–7.

44. Dahlen R, Magnusson MK, Bajor A, *et al*. Global mucosal and serum cytokine profile in patients with ulcerative colitis undergoing anti-TNF therapy. *Scand J Gastroenterol* 2015;**50**:1–9.

45. Schroeder KW, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. *N Engl J Med* 1987;**317**:1625–9.

46. Magnusson MK, Strid H, Isaksson S, *et al*. Cultured blood T-cell responses predict anti-TNF therapy response in patients with ulcerative colitis. *Aliment Pharmacol Ther* 2015;**41**:1149–61.

47. Magnusson MK, Strid H, Isaksson S, *et al*. Response to infliximab therapy in ulcerative colitis is associated with decreased monocyte activation, reduced CCL2 expression and downregulation of Tenascin C. *J Crohns Colitis* 2014;**9**:56-65.

48. Casen C, Vebo HC, Sekelja M, *et al*. Deviations in human gut microbiota: a novel diagnostic test for determining dysbiosis in patients with IBS or IBD. *Aliment Pharmacol Ther* 2015;**42**:71–83.

49. Balamurugan R, Janardhan HP, George S, *et al*. Molecular studies of fecal anaerobic commensal bacteria in acute diarrhea in children. *J Pediatr Gastroenterol Nutr* 2008;**46**:514–9.

50. Nadkarni MA, Martin FE, Jacques NA, *et al*. Determination of bacterial load by real-time PCR using a broad-range [universal] probe and primers set. *Microbiology* 2002;**148**:257–66.

51. Schaubeck M, Clavel T, Calasan J, *et al*. Dysbiotic gut microbiota causes transmissible Crohn's disease-like ileitis independent of failure in antimicrobial defence. *Gut* 2016;**65**:225–37.

52. Miquel S, Martin R, Rossi O, *et al*. Faecalibacterium prausnitzii and human intestinal health. *Curr Opin Microbiol* 2013;**16**:255–61.

53. Martin R, Chain F, Miquel S, *et al*. The commensal bacterium Faecalibacterium prausnitzii is protective in DNBS-induced chronic moderate and severe colitis models. *Inflamm Bowel Dis* 2014;**20**:417–30.

54. Arijs I, Li K, Toedter G, *et al*. Mucosal gene signatures to predict response to infliximab in patients with ulcerative colitis. *Gut* 2009;**58**:1612–9.

55. Bercik P, Denou E, Collins J, *et al*. The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. *Gastroenterology* 2011;**141**:599–609.

56. Janssen AW, Kersten S. The role of the gut microbiota in metabolic health. *FASEB J* 2015;**29**:3111–23.

57. Cammarota G, Ianiro G, Gasbarrini A. Fecal microbiota transplantation for the treatment of Clostridium difficile infection: a systematic review. *J Clin Gastroenterol* 2014;**48**:693–702.