ORIGINAL ARTICLE

Diarrhoea-predominant irritable bowel syndrome distinguishable by 16S rRNA gene phylotype quantification

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Abstract

AIM: To study whether selected bacterial 16S ribosomal RNA (rRNA) gene phylotypes are capable of distinguishing irritable bowel syndrome (IBS).

METHODS: The faecal microbiota of twenty volunteers with IBS, subdivided into eight diarrhoea-predominant (IBS-D), eight constipation-predominant (IBS-C) and four mixed symptom-subtype (IBS-M) IBS patients, and fifteen control subjects, were analysed at three time-points with a set of fourteen quantitative real-time polymerase chain reaction assays. All assays targeted 16S rRNA gene phylotypes putatively associated with IBS, based on 16S rRNA gene library sequence analysis. The target phylotypes were affiliated with *Actinobacteria*, *Bacteroidetes* and *Firmicutes*. Eight of the target phylotypes had less than 95% similarity to cultured bacterial species according to their 16S rRNA gene sequence. The data analyses were made with repeated-measures ANCOVA-type modelling of the data and principle component analysis (PCA) with linear mixed-effects models applied to the principal component scores.

RESULTS: Bacterial phylotypes *Clostridium cocleatum* 88%, *Clostridium thermosuccinogenes* 85%, *Coprobacillus catenaformis* 91%, *Ruminococcus bromii*-like, *Ruminococcus torques* 91%, and *R. torques* 93% were detected from all samples analysed. A multivariate analysis of the relative quantities of all 14 bacterial 16S rRNA gene phylotypes suggested that the intestinal microbiota of the IBS-D patients differed from other sample groups. The PCA on the first principal component (PC1), explaining 30.36% of the observed variation in the IBS-D patient group, was significantly altered from all other sample groups (IBS-D *vs* control, *P* = 0.01; IBS-D *vs* IBS-M, *P* = 0.00; IBS-D *vs* IBS-C, *P* = 0.05). Significant differences were also observed in the levels of distinct phylotypes using relative values in proportion to the total amount of bacteria. A phylotype with 85% similarity to *C. thermosuccinogenes* was quantified in significantly different quantities among the IBS-D and control subjects (-4.08 ± 0.90 *vs* -3.33 ± 1.16, *P* = 0.04) and IBS-D and IBS-M subjects (-4.08 ± 0.90 *vs* -3.08 ± 1.38, *P* = 0.05). Furthermore, a phylotype with 94% similarity to *R. torques* was more prevalent in IBS-D patients' intestinal microbiota than in that of control subjects (-2.43 ± 1.49 *vs* -4.02 ± 1.63, *P* = 0.01). A phylotype with 93% similarity to *R. torques* was associated with control samples when compared with IBS-M (-2.41 ± 0.53 *vs* -2.92 ± 0.56, *P* = 0.00). Additionally, a *R. bromii*-like phylotype was associated with IBS-C patients in comparison to control subjects (-1.61 ± 1.83 *vs* -3.69 ± 2.42, *P* = 0.01). All of the above mentioned phylotype specific alterations were independent of the effect of time.

CONCLUSION: Significant phylotype level alterations

in the intestinal microbiotas of IBS patients were observed, further emphasizing the possible contribution of the gastrointestinal microbiota in IBS.

INTRODUCTION

Irritable bowel syndrome (IBS) is a functional gastrointestinal (GI) disorder with a worldwide prevalence of 10%-20%[1]. The main symptoms include abdominal pain or discomfort, diarrhoea, constipation, abdominal bloating, and flatulence. The symptoms are associated with changes in the frequency and form of stool, improved by defecation, and they typically fluctuate with time. Although IBS does not predispose to malignancies, it essentially lowers the patients' quality of life. Multiple interacting mechanisms lie behind IBS aetiology[2,3]. These include psychological stress and disturbances, physiological features, such as altered GI motility and visceral hypersensitivity, low-grade inflammation, and bacterial gastroenteritis.

The possible role of the GI microbiota in IBS aetiology (for review, see Parkes *et al*[4]) is supported by low-grade mucosal inflammation in the GI tract of IBS patients[5,6], onset of GI symptoms after a gastroenteritis (generating a subset of patients diagnosed with post-infectious IBS[7,8]), and observations suggesting the presence of altered GI microbiota in IBS[9-12]. Recently, Gecse *et al*[13] associated the elevated level of non-endogenous colonic serine protease in diarrhoea-predominant IBS patients with increased mucosal permeability and subsequent visceral hypersensitivity. The detected increase in the level of colonic serine protease was suggested to originate from intestinal bacteria. In addition, antibodies to bacterial flagellins A4-Fla2 and Fla-X associated with the *Clostridium* cluster XIVa are elevated in IBS compared to healthy controls[14]. The potential role of GI microbiota in IBS is further supported by studies where probiotics have alleviated IBS symptoms (for a review, see Spiller *et al*[15]). In the recent study of Kajander *et al*[16], a multispecies probiotic was also shown to stabilize the gut microbiota, but the microbial alterations were not specified.

16S ribosomal acid (rRNA) gene based methods have identified almost 900 bacterial phylotypes in the human GI tract with, of which only 18% represent cultured species[17]. Richness estimates within an individual's colon extend to 300 phylotypes[18], while a vast variation is introduced by disparities in the phylotype composition between individuals[18-20]. The main phyla found in 16S rRNA gene sequencing based studies are *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria*, and *Verrucomicrobia*[18,21-23].

Using culture-based techniques, the GI microbiota of IBS patients has been characterized to have less lactobacilli and bifidobacteria and an elevated amount of aerobes relative to anaerobes[24-26]. Specific divergences have been observed with quantitative real-time polymerase chain reaction (qPCR) assays targeting *Lactobacillus* spp, *Veillonella* spp, *Bifidobacterium* spp, *Clostridium coccoides*, and *Bifidobacterium catenulatum*[10], and with 16S rRNA cloned sequence-based assays targeting phylotypes within the genera *Coprococcus, Collinsella,* and *Coprobacillus*[11]. With a 16S rRNA gene-based phylogenetic microarray analysis targeting over a 1000 human intestinal phylotypes, the faecal microbiota of IBS patients and control subjects could be distinguished by hierarchical cluster analysis and stronger variation in the composition of the microbiota was seen in the IBS patients' profiles[12]. Furthermore, a higher degree of temporal instability among IBS patients has been detected with ribosomal RNA-based denaturing gradient gel electrophoresis[9]. Mucosal bacteria have also been found to be more abundant in IBS patients than in healthy controls[27].

In this study, we applied a set of eight novel and six previously published[11] qPCR assays to the analysis of faecal samples obtained from IBS patients and healthy controls to detect possible aberrations in the GI microbiotas of IBS patients. The design of the novel qPCR assays was based on comparing the 16S rRNA clone libraries of IBS patients and healthy controls, but in this study three time-points per subject during a 6-mo survey were analysed instead of one[11].

MATERIALS AND METHODS

Subjects and study design

Faecal samples were collected from 20 IBS patients and 15 healthy control subjects (Table 1) at time-points 0, 3 and 6 mo of a 6-mo follow-up period.

The IBS patients were recruited by experienced physicians and fulfilled the Rome II criteria[28], except for three subjects who reported slightly less than 12 wk of abdominal pain during the preceding year[29]. All patients had undergone clinical investigation and endoscopy or barium enema of the GI tract less than a year prior to the study. Exclusion criteria included pregnancy, lactation, organic intestinal disease, other severe systematic disease, antimicrobial medication during the previous 2 mo, previous major or complicated abdominal surgery, severe endometriosis and dementia or otherwise inadequate cooperation capability. Patients with lactose intolerance

Table 1 Characteristics of IBS patients and control subjects

	IBS-D	IBS-C	IBS-M	Controls
n	8	8	4	15
Age (yr): mean (range)	43.6 (26-60)	48.6 (24-64)	50.8 (31-62)	47 (25-64)
Gender (female/male)	4/4	9/1	3/1	10/5
Predominant bowel habit	Diarrhoea	Constipation	Mixed-type	-

IBS: Irritable bowel syndrome; IBS-D: Diarrhoea-predominant IBS; IBS-C: Constipation-predominant IBS; IBS-M: Mixed symptom-subtype IBS.

were included if they were reported to follow a low-lactose or lactose-free diet. All IBS patients were advised not to make any changes to their medication, including ongoing IBS medication (mainly commercial fibre analogues, laxatives, or antidiarrhoeals). The IBS patients formed the placebo group of a 6-mo probiotic intervention study[29]. They received daily a placebo capsule consisting of microcrystalline cellulose, magnesium stearate, and gelatine as the encapsulating material. Consumption of probiotic products was not allowed during the study.

Control subjects devoid of GI symptoms were also recruited and originally age- and gender-matched with the IBS patients as a whole[26]. Volunteers with regular intestinal disturbances, lactose intolerance, celiac disease, or antibiotic therapy during the preceding 2 mo of the study were excluded. The faecal samples of the controls and IBS subjects[9-11,26,29,30] have been studied previously. The novelty in the present study resides in the eight previously unpublished 16S rRNA phylotype targeting assays, the analysis of several time-points during the 6-mo survey, and in the in-depth statistical analysis of the results.

Ethics

All participants gave their written informed consent and were told that they could withdraw from the study at any time. The Human Ethics Committee of the Joint Authority for the Hospital District of Helsinki and Uusimaa (HUS) approved the study protocol for the IBS patients. The ethical committee of the Technical Research Centre of Finland (VTT) approved the study protocol for the healthy controls.

Extraction and purification of DNA from faecal samples

Faecal samples were preserved anaerobically immediately after defecation, stirred and aliquoted, and stored at -70℃ within 4 h of delivery. For qPCR analysis, total DNA was isolated from 1 g of faecal material according to Apajalahti *et al*[31], which included removing the undigested particles from the faecal material by three rounds of low-speed (200 × *g*) centrifugation and collection of the bacterial cells with high-speed centrifugation (30 000 × *g*) at 15℃ for 15 min using a Beckman Avanti™ centrifuge (Fullerton, CA, USA) with the rotor JA 25.50 or JLA 16.250 rotor, respectively. The bacterial cells were lysed after centrifugation with a combination of freeze-thaw

cycles (freezing for 1 h at -70℃ and thawing for 15 min in a 37℃ water bath), lysozyme and vortexing with glass beads. DNA concentrations were determined with a NanoDrop ND-1000 Spectrophotometer (NanoDrop products, Wilmington, DE, USA).

Design of qPCR assays

Divergences detected by comparing the sequence data of 16S rRNA gene clone libraries of healthy controls and symptomatically sub-grouped IBS patients (diarrhoea-predominant IBS, IBS-D; constipation-predominant IBS, IBS-C; and mixed symptom-subtype IBS, IBS-M) were used as the basis for selection of qPCR targets[11]. Prior to cloning and sequencing, the faecal microbial genomes had been profiled and fractioned on the basis of genomic guanine-plus-cytosine content[11]. Partial 16S rRNA gene sequences encompassing the variable regions V1 and V2 combined from all four sample types were aligned using either the version Beta 2003-08-22 of ARB[11,32] or ClustalW 1.83[33]. For the ARB alignment, an aligned sequence database (ssu_jan04_corr_opt.arb) was downloaded from the ARB home page (http://www.arb-home.de) and the in-house sequences[11] were aligned using the ARB-EDIT FastAlign function, followed by manual correction of the alignments with special attention to the ends of the sequences. Finally, the sequences were imported into an existing tree file of the database (Tree-Bacteria) by filtering the data against a sequence of similar length as the imported partial 16S rRNA gene sequences. Regions of the tree where sequences derived from one subject group (healthy *vs* IBS or healthy *vs* IBS subtypes) dominated over the other groups were considered as potentially interesting. In addition, a ClustalW 1.83 alignment (FAST DNA pairwise alignment algorithm option, gap penalty 3, word size 4, number of top diagonals 1 and window size 1) was constructed covering approximately 450 bp from the 5' end of the 16S rRNA gene and visually inspected and cut from the *Escherichia coli* position 430 (universally conserved GTAAA) with BioEdit version 7.0.5.3[34]. Distance matrices were calculated from the ClustalW alignment with Phylip 3.66 Dnadist[35] using Jukes-Cantor correction. The distribution of sequences into operative taxonomic units (OTUs) was determined using DOTUR[36] by applying the furthest neighbour rule option and 98% cut-off for sequence similarity. Uneven distribution of sequences originating from the different sample types within an OTU was used as criteria for qPCR target selection.

Potential primer target sites for specific quantitative analyses were assessed manually from ClustalW 1.83 alignments. Primer 3 online interface[37] and mfold 3.3 DNA-folding servers[38] were used for optimizing the final primer sequences and secondary structure analyses. The primer specificity against publicly available prokaryotic 16S rRNA sequences was checked with FASTA[39] provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/) and against in-house 16S rRNA clone library sequences of human faecal origin, using the blastall option of Parallel BLAST[40] with Corona hardware

(http://corona.csc.fi) maintained by the Finnish IT Center for Science (CSC - Scientific Computing Ltd., Finland). The qPCR primers were synthesized commercially by Oligomer Oy (Helsinki, Finland). The clone sequences used to generate the standard curve in each qPCR assay were classified using The Ribosomal Database Project Ⅱ Classifier[41]. The assays were named according to the most similar 16S rRNA gene sequence of a cultured bacterial species with the similarity percentages below 98% indicated.

qPCR optimization and conditions

For each assay, the optimal annealing temperature and MgCl₂ concentration were defined using the iCycler iQ Real-Time Detection System (Bio-Rad, Hercules, CA, USA) associated with the iCycler Optical System Interface software (version 2.3; Bio-Rad). Actual samples were run as triplicates with optimized reaction conditions using SYBR Green I chemistry and 25 ng (specific phylotype targeting assays) or 0.5 ng (universal 16S rRNA gene assay) of faecal bacterial DNA. For all assays, the samples were run with different sample groups randomly mixed in the individual runs to minimize the effect of technical deviation between runs. Amplified clonal 16S rRNA genes were used as standards, ranging from 10^2 to 10^7 gene copies per reaction. The reaction mixtures consisted of a 1:75 000 dilution of SYBR Green I (Lonza biosciences, Basel, Switzerland), 10 mmol/L Tris-HCl (pH 8.8), 50 mmol/L KCl, 0.1 % Triton X-100, 2-5 mmol/L MgCl₂, 100 μmol/L each dNTP, 0.5 μmol/L each primer, 0.024 U Dynazyme Ⅱ polymerase (Finnzymes, Espoo, Finland) and 5 μL of either template or water. The amplification involved one cycle at 95℃ for 5 min for initial denaturation, followed by 40 cycles of denaturation at 95℃ for 20 s, primer annealing at the defined optimal temperatures for 20 s, extension at 72℃ for 30 s and a fluorescence detection step at 80-89℃ for 30 s. The specificity of the qPCR assays was checked with a reassociation curve analysis after amplification by slow cooling from 95℃ to 60℃, with fluorescence collection at 0.3℃ intervals for 10 s at each decrement. The qPCR efficiencies were calculated from the standard curves using the equation $E = (10^{1/k})-1$, where E and k stand for efficiency and slope, respectively.

Statistical analysis

In the raw data from qPCR assays, microbe groups with low abundance were occasionally undetected (below qPCR detection limit). These values may not be truly zero or missing values, but are caused by limitations in the technical accuracy of the qPCR equipment. Therefore, for data analysis, zeros and missing values were imputed with the mean values obtained from the qPCR runs with the same primer pair applied to molecular grade water. If those too were undetected, the minimum of all the detected water runs was used.

After imputing the undetected values, the raw data was transformed to log₁₀ ratios of relative amount of 16S rRNA gene copies detected *vs* the amount of bacterial

16S rRNA gene copies detected with the universal qPCR assay. Using the ratio will, to some extent, control the sample specific variation due to lab procedures and sample handling affecting the overall bacterial concentration. All the statistical analyses were carried out with these values.

Statistical analyses were made with standard mixed-effect linear models having fixed effects for the time, and the IBS subtype, and a random effect for individual (taking into account the repeated measures from the same subject). In summary, this set up results in a repeated-measures ANOVA-type modelling of the data.

The model selection between whether to use the full model with interaction term between time and group and the age term, or the simpler model without interaction and the age was based on F-tests. The inference from the estimated models was based on the standard F-tests and t-tests.

For multivariate analysis of the data, principal component analysis (PCA) was used to visualize the data sets. Linear mixed-effects models were also applied to the first four principal component scores to quantify potential multivariate effects present in the data.

All the analyses were made with statistical programming language R 2.6.2[42] utilizing the package *lme* for mixed-effects linear models[43] and contrast for computing the contrasts.

RESULTS

Design and optimization of qPCR assays

A total of 14 qPCR assays were designed and optimized (Table 2) for analyzing alterations in the faecal microbiotas of IBS patients sub-grouped according to symptom subtype and healthy controls. The optimized annealing and detection temperatures ranged from 60℃ to 67℃ and 80℃ to 89℃, respectively. For the universal assay, an annealing temperature of 50℃ was used. The PCR efficiencies for the optimized qPCR reactions were above 80% with the exception of *Collinsella aerofaciens*-like, *Coprococcus eutactus* 97% and *Spiroplasma chinense* 84% assays.

Non-specific product peaks with a lower melting temperature than the desired product were observed for some of the faecal DNA samples in the reassociation analyses of several assays (*Bacteroides intestinalis*-like, *Butyrivibrio crossotus*-like, *Clostridium cocleatum* 88%, *C. eutactus* 97%, *S. chinense* 84%, *Ruminococcus torques* 91%, *R. torques* 94% and *Slackia faecicanis* 91%). The fluorescence detection temperatures in these assays were set above the melting point of the unspecific products to avoid detecting them.

Analysis of faecal samples

The log₁₀ number of bacterial 16S rRNA gene copies detected ranged from 11.71 to 11.93 per gram of faeces (wet weight) and the average relative log₁₀ numbers of 16S rRNA gene copies detected with phylotype targeting assays in proportion to the universal bacterial assay ranged from -7.34 to -0.72 (Table 3; time-point specific averages are presented in Supplementary Table 1). Target

Table 2 Phylum level classification, primers and assay conditions of qPCR assays

qPCR assay (Phylum)	Primers (5'→3')	Standard	Target size (bp)	MgCl2 (mmol/L)	Annealing T (℃)	Detection T (℃)	Average PCR efficiency ± SD
Bacteroides intestinalis-like (Bacteroidetes)	F: AGCATGACCTAGCAATAGGTT R: CCTTCTCGTTATACTATCCGGTAT	AM277809	124	3	63	83	87 ± 6
Bifidobacterium catenulatum/ Bifidobacterium pseudocatenulatum-like[11] (Actinobacteria)	F: ACTCCTCGCATGGGGTGTC R: CCGAAGGCTTGCTCCCGAT	AM277149	275	3	68	87	90 ± 8
Butyrivibrio crossotus-like (Firmicutes)	F: TGCTAATACCGCATAAAACAGCAGA R: CGCTGGATCAGGCTTTCG	AM275497	232	4	63	85	82 ± 5
Clostridium cocleatum 88%[11] (Firmicutes)	F: AATACATAAGTAACCTGGCRTC R: CGTAGCACTTTTCATATAGAGTT	AM276544	104	4	60	80	88 ± 10
Clostridium thermosuccinogenes 85% (Firmicutes)	F: ACATGCAAGTCGAACGGAAGTC R: TGCGTCAGAGTTTCCTCCATTG	AM275406	373	2	62	81	88 ± 8
Collinsella aerofacien-like[11] (Actinobacteria)	F: CCCGACGGGAGGGGAT R: CTTCTGCAGGTACAGTCTTGAC	AM276090	260	4	67	89	75 ± 3
Coprobacillus catenaformis 91% (Firmicutes)	F: CGGACGCGATGCTTCT(A/G)GC R: AACATATCTCCCATGCGGTTG	AM275478	133	4	62	82	93 ± 8
Coprococcus eutactus 97%[11] (Firmicutes)	F: AGCTTGCTCCGGCYGATTTA R: CGGTTTTACCAGTCGTTTCCAA	AM275825	97	2	63	83	73 ± 5
Ruminococcus bromii-like (Firmicutes)	F: CGAACGGAACTGTTTTGAAAGA R: CAAAACCATGTGGTTCCGATAT	AM275413	156	4	62	81	97 ± 6
Ruminococcus torques 91%[11] (Firmicutes)	F: TGCTTAACTGATCTTCTTCGGA R: CGGTATTAGCAGTCATTTCTG	AM276624	119	5	62	82	88 ± 5
R. torques 93%[1] (Firmicutes)	F: GACTGCTTTTGAAACTGTCA R: AGGTCCGGTTAAGGA	AM275798	396	4	61	83	85 ± 4
R. torques 94%[11] (Firmicutes)	F: AATCTTCGGAGGAAGAGGACA R: ACACTACACCATGCGGTCCT	AM275522	137	2	65	85	81 ± 6
Slackia faecicanis 91% (Actinobacteria)	F: GAGTAACGCGTGACCGACCTT R: CCCGGAGTACCCGGTATCA	AM276086	75	4	64	86	90 ± 5
Spiroplasma chinense 84% (Firmicutes)	F: ATGGCCCAGTGAAGGTTG R: CCCAACGAAAAGGTAGGTCA	AM275518	101	4	66	83	79 ± 4
Universal[61]	F: TCCTACGGGAGGCAGCAGT R: GGACTACCAGGGTATCTAATCCTGTT	*B. longum*[2]	466	3	50	80	92 ± 6

[1]For the *R. torques* 93% assay the sequence AY305319[62] was used for primer design; [2]The *Bifidobacterium longum* DSM 20219T 16S rRNA gene was used as standard in the universal qPCR assay. qPCR: Quantitative real-time polymerase chain reaction.

Table 3 The average relative log10 amount of the 16S rRNA gene copies detected with qPCR assays in proportion to the universal qPCR results

qPCR assay	Control (*n* = 15)	IBS-C (*n* = 8)	IBS-D (*n* = 8)	IBS-M (*n* = 4)
Bacteroides intestinalis-like	-4.85 ± 1.52[1] (12)	-4.71 ± 1.42 (5)	-5.8 ± 1.32 (5)	-3.46 ± 1.26 (4)
Bifidobacterium catenulatum/Bifidobacterium pseudocatenulatum-like	-4.1 ± 2.22 (14)	-5.63 ± 2.52 (7)	-5.42 ± 2.63 (5)	-4.4 ± 2.54 (4)
Butyrivibrio crossotus-like	-6.2 ± 2.03 (8)	-6.5 ± 1.97 (3)	-7.34 ± 1.58 (0)	-6.04 ± 2.19 (2)
Clostridium cocleatum 88%	-1.7 ± 1.32 (15)	-2.36 ± 2.35 (8)	-2.69 ± 2.33 (8)	-0.72 ± 0.98 (4)
Clostridium thermosuccinogenes 85%	-3.33 ± 1.16[a] (15)	-3.7 ± 0.84 (8)	-4.08 ± 0.90[a,b] (8)	-3.08 ± 1.38[b] (4)
Collinsella aerofaciens-like	-2.45 ± 1.16 (15)	-2.9 ± 2.33 (7)	-4.63 ± 2.35 (7)	-1.73 ± 2.61 (4)
Coprobacillus catenaformis 91%	-4.72 ± 0.77 (15)	-4.41 ± 0.67 (8)	-4.79 ± 0.61 (8)	-4.71 ± 0.25 (4)
Coprococcus eutactus 97%	-5.44 ± 2.53 (9)	-5.91 ± 2.61 (3)	-6.55 ± 2.28 (2)	-4.09 ± 2.69 (3)
Ruminococcus bromii-like	-3.69 ± 2.42[c] (15)	-1.61 ± 1.83[c] (8)	-3.4 ± 2.49 (8)	-2.08 ± 1.56 (4)
Ruminococcus torques 91%	-3.13 ± 0.77 (15)	-2.87 ± 1.10 (8)	-2.58 ± 1.09 (8)	-2.83 ± 1.26 (4)
R. torques 93%	-2.41 ± 0.53[d] (15)	-2.61 ± 0.72 (8)	-2.65 ± 0.59 (8)	-2.92 ± 0.56[d] (4)
R. torques 94%	-4.02 ± 1.63[c] (14)	-3.39 ± 1.40 (8)	-2.43 ± 1.49[c] (8)	-3.82 ± 2.16 (3)
Slackia faecicanis 91%	-5.53 ± 2.26 (8)	-5.6 ± 2.33 (4)	-6.22 ± 2.16 (3)	-4.01 ± 2.28 (4)
Spiroplasma chinense 84%	-5.62 ± 2.04 (9)	-5.36 ± 2.20 (5)	-6.51 ± 1.98 (2)	-5.7 ± 2.22 (2)

The number of subjects with target 16S rRNA gene copies detected above the calculated threshold value in any of the three samples analysed are given in parentheses. [1]Values are presented as averages of log10-values ± SD from three time-points (0, 3 and 6 mo). [a]$P = 0.04$, [b]$P = 0.05$, [c]$P = 0.01$, [d]$P = 0.00$.

bacterial phylotypes were detected from all samples with the *C. cocleatum* 88%, *Coprobacillus catenaformis* 91%, *Clostridium thermosuccinogenes* 85%, *Ruminococcus bromii*-like, *R. torques* 91%, and R. *torques* 93% assays (Table 3).

Divergences in the intestinal microbiota in IBS

In a PCA of the 14 phylotype targeting assays and three time-points (0, 3 and 6 mo), the IBS-D group differed from the control group ($P = 0.01$), IBS-M ($P = 0.00$),
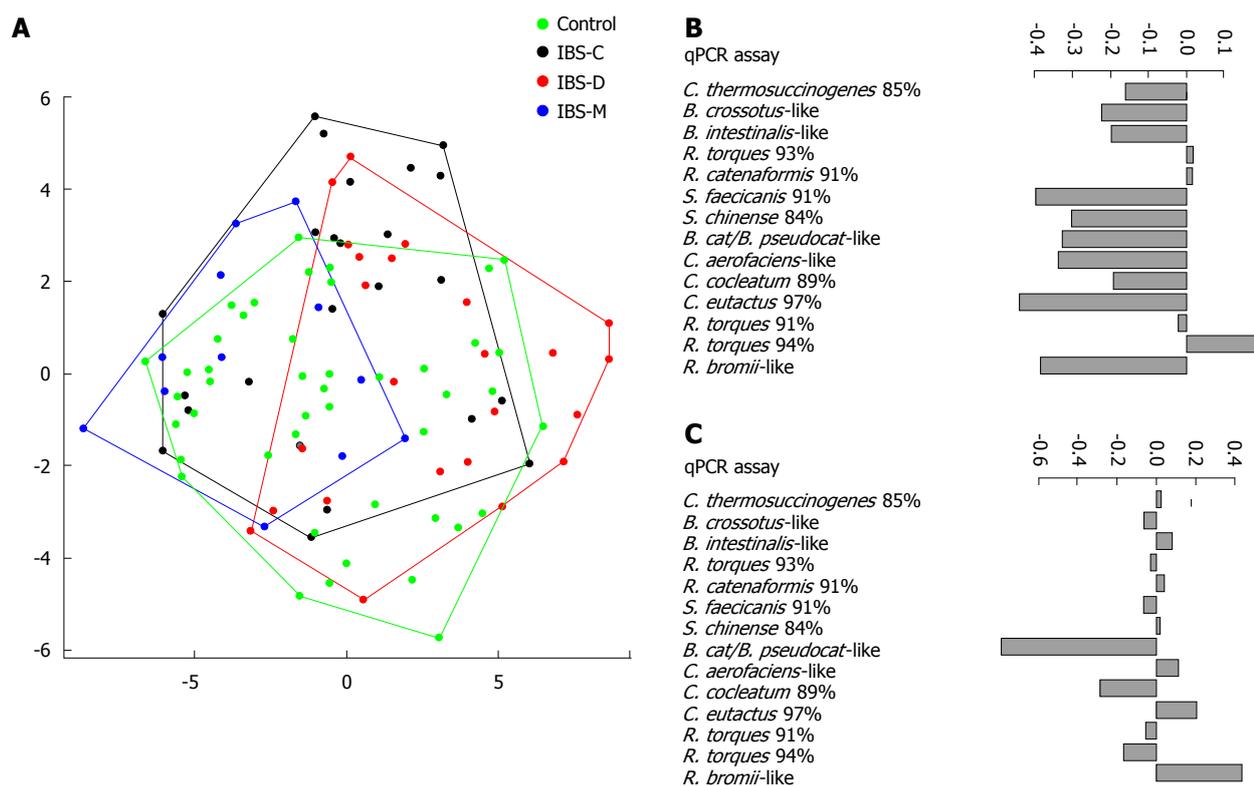
Figure 1 Principal component analysis (PCA) of fourteen 16S rRNA phylotypes quantified from faecal samples of irritable bowel syndrome (IBS) patients and healthy volunteers. A: The PCA plot with outermost data points within each sample group is outlined. The control samples are presented in green, the constipation-predominant IBS (IBS-C) in black, the diarrhoea-predominant IBS (IBS-D) in red and the mixed symptom-subtype IBS (IBS-M) in blue. Each time-point is presented as a separate point. To quantify the multivariate differences between the groups, linear mixed-effects models were applied to the first (x-axis) and the second (y-axis) principal component scores, which represent the dominant multivariate changes present in the data; B: The bars represent the relative contribution of each quantitative real-time PCR (qPCR) assay to the principal component 1 (PC1). On PC1 the IBS-D samples differed from the control ($P \leq 0.01$), IBS-M ($P \leq 0.01$), and IBS-C ($P \leq 0.05$) samples; C: The bars represent the relative contribution of each qPCR assay to the principal component 2 (PC2). On PC2, the IBS-C patients diverged from the control subjects ($P \leq 0.05$) and time-points. In addition, the second time-point (3 mo) diverged significantly from the first (0 mo, $P \leq 0.01$) and the third (6 mo, $P \leq 0.01$) time-points independent of sample group; The height of the bars in graphs in Figure 1B and C reflect the relative magnitude of the contribution and the direction the sign of the contribution (in relation to the other assays and to the axis in Figure 1A). For example, in PC1 (Figure 1B), the largest contributor is the *Coprococcus eutactus* 97% phylotype, while the samples on the right in Figure 1A (mostly IBS-D) tend to have higher concentrations of *Ruminococcus torques* 94% and lower concentrations of phylotypes with bars highly on the negative side. Similarly, on PC2 (Figure 1C) the samples with high PC2 value in the top part of the Figure 1A tend to have higher concentrations of the *Ruminococcus bromii*-like phylotype, and lower concentrations of the *Bifidobacterium catenulatum/Bifidobacterium pseudocatenulatum*-like phylotype. On PC2, the IBS-C patients diverged from the control subjects ($P \leq 0.05$) and time-points. In addition, the second time-point (3 mo) diverged significantly from the first (0 mo, $P \leq 0.01$) and the third (6 mo, $P \leq 0.01$) time-points independent of sample group. qPCR: Quantitative real-time polymerase chain reaction; IBS-C: Constipation-predominant irritable bowel syndrome; IBS-D: Diarrhoea-predominant irritable bowel syndrome; IBS-M: Mixed-subtype irritable bowel syndrome.

and IBS-C ($P = 0.05$) on the first principal component (PC1; Figure 1A and B). The *R. torques* 94% phylotype was unique in being more predominant in IBS-D (Figure 1A and B). On the second principal component (PC2), the IBS-C patients diverged from the control subjects ($P = 0.03$; Figure 1A and C). Time-points were significantly different on PC1 and PC2 (data not shown).

Quantities of *C. thermosuccinogenes* 85%, *R. bromii*-like, *R. torques* 93%, and *R. torques* 94% phylotypes diverged between different IBS symptom subtypes and healthy subjects independent of the effect of time (Table 3). Relatively high levels of the *C. thermosuccinogenes* 85% phylotype were associated with IBS-M patients and control subjects compared with IBS-D patients. The relative amount of *C. thermosuccinogenes* 85% 16S rRNA gene copies detected in proportion to the universal assay were 0.08%, 0.05%, and < 0.01% for the IBS-M, control, and IBS-D subjects, respectively. The *R. bromii*-like

phylotype was significantly ($P = 0.01$) more abundant in the IBS-C (relative abundance 2.45%) than in the control (relative abundance 0.02%) subjects' samples and the *R. torques* 93% phylotype was significantly ($P = 0.00$) more abundant in the control (relative abundance 0.39%) than in the IBS-M subjects' samples (relative abundance 0.12%). The lowest amount of *R. torques* 94% phylotypes was quantified in the control samples (relative abundance < 0.01%) significantly differing ($P = 0.01$) from the relative amount detected among the IBS-D patients' samples (relative abundance 0.37%).

Additional time-point dependent divergences between the sample groups were also detected (Supplementary Table 1): The *B. intestinalis*-like and *C. cocleatum* 88% phylotypes were relatively abundant in the IBS-M and control samples, and were detected in lower amounts in the IBS-D patients' samples. The relative amounts of *C. aerofaciens*-like phylotype detected were lowest in samples of the IBS-D patients, whereas the relative

amounts of *R. torques* 91% phylotype were lowest in the control subjects' samples.

DISCUSSION

The aim of this study was to test the capability of a set of qPCR assays targeting the 16S rRNA gene on a phylotype level to differentiate between IBS symptom subtypes and healthy controls. Eight novel and six previously published[11] qPCR assays were used to study faecal samples of 20 IBS patients grouped according to symptom subtype and 15 healthy controls at three time-points (0, 3 and 6 mo). None of the assays have previously been applied to samples from several time-points. The knowledge of putative alterations on phylotype level may be essential in association with health, as has been shown to be the case for *Faecalibacterium prausnitzii* in Crohn's disease[44,45].

In our approach, the selection of faecal bacteria phylotypes for analysis was based on a comparison of clone sequence libraries of IBS patient symptom subtypes and healthy controls[11]. The amount of bacterial 16S rRNA genes detected with the universal qPCR was in accordance with previous findings[46]. Quantities relative to the amount of bacterial 16S rRNA gene copies detected with the universal bacterial qPCR assay were used in data analyses. The diarrhoea-predominant symptom subtype diverged significantly from the other IBS symptom subtypes and healthy controls in a PCA of all 14 qPCR analyses and three time-points (Figure 1). In addition, *C. thermosuccinogenes* 85%, *R. bromii*-like, *R. torques* 93%, and *R. torques* 94% phylotypes diverged between different IBS symptom subtypes and healthy controls independent of the time-point analysed (Table 3). According to the results presented here and in previous studies[10-12,47,48], grouping of IBS patients based on their main symptom subtype is advisable in future studies.

The *C. thermosuccinogenes* 85% -phylotype represents an uncultured firmicute within the human GI microbiota. It was detected in significantly lower amounts in IBS-D patients' samples in comparison to healthy controls or IBS-C patients. The target sequence of the *C. thermosuccinogenes* 85% -assay has previously been found from human faecal samples in several studies[11,18,49] and from human mucosal biopsy samples taken from the caecum, descending and sigmoid colon, and the rectum[18], implying that the phylotype truly represents a human intestinal bacterium. However, the closest isolated strain has negligible similarity according to the 16S rRNA sequence (85% similarity with *Ruminococcus* sp. 16442 strain 16S rRNA sequence).

The *R. bromii*-like phylotype was significantly more abundant in IBS-C patients than in healthy controls samples. *R. bromii* is a common starch degrader of the human intestinal microbiota[50]. The amounts of *R. bromii* -related phylotypes have been shown to increase with a diet high in resistant starch[51]. In the present study, the possible effect of diet could not be ruled out, but it is more likely that the slowed colonic transit in IBS-C, rather

than a dietary effect, results in a favourable environment for the *R. bromii*-like phylotype associated with IBS-C.

Ruminococcus torques, a resident mucin-degrading member of the human GI microbiota[52], has been associated with the mucosa of Crohn's disease patients[53]. The specific target sequence of the *R. torques* 94% -assay applied in this study has been found from human faecal samples in several studies[19,54,55] and has also been associated with Crohn's disease[56]. In the present study, a comparatively higher abundance of *R. torques* 94% phylotype was linked with IBS-D in both the multivariate and assay specific analyses. The *R. torques* 91% phylotype was associated with IBS-D and IBS-M and the *R. torques* 93% phylotype was more abundant in IBS-M than in healthy controls. The target sequences of *R. torques* 91%, 93% and 94% are affiliated with *Lachnospiraceae* as is the 16S rRNA sequence of the strain A4 (DQ789118)[57] carrying the IBS associated flagellin Fla2[14].

As a further support to our previous results[11], a significantly lower abundance of the *C. aerofaciens*-like phylotype was associated with the IBS-C and IBS-D symptom subtypes at two of the time-points analysed. *Collinsella aerofaciens* (formerly *Eubacterium aerofaciens*) belongs to the order *Coriobacteriales* within the high G+C Gram-positive *Actinobacteria*. It is a prominent member of the endogenous human intestinal microbiota[58] and has previously been connected with a low risk of colon cancer[59].

Significantly lower levels of several 16S rRNA gene phylotypes within the genus *Bacteroides* (*B. ovatus*, *B. uniformis*, and *B. vulgatus*) have previously been discovered among IBS-C patients in comparison to healthy controls, but no effect was seen with the *B. intestinalis*-like phylotype targeting probes[12]. All samples analysed in this study have previously been analysed with a *Bacteroides-Prevotella-Porphyromonas* -group and a *B. fragilis* species-specific qPCR assay[10] without detecting any significant divergences. In this study, a *B. intestinalis*-like phylotype was quantified with qPCR and found to be least abundant in the IBS-D patient group and most abundant in the IBS-M patient group at the selected time-points. The seemingly contradictory results might be due to different specificities of the probes and primers used.

The qPCR assays presented here were based on a thorough analysis of IBS associated faecal bacterial 16S rRNA gene sequence data originating from the same samples and both the previously published partial 16S rRNA gene sequences. The qPCR assays detailed here will be valuable in upcoming IBS studies. A more thorough sequencing approach using novel high-throughput sequencing technologies[23] on IBS subjects' GI microbiota would be valuable in further investigating IBS-associated alterations within the GI microbiota.

The faecal microbiota of IBS patients has been associated with less temporal stability within individuals[47] and more variation between individuals[12] compared to that of the healthy controls. Therefore, the results of this study should be further confirmed with independent sample panels including both IBS subjects and healthy

controls. In addition, analyzing mucosal samples, in addition to luminal samples, would be of interest, since the mucosal and faecal microbiotas differ from each other[60]. Previously, IBS patients have been shown to have a slightly more abundant mucosal microbiota compared to that of healthy volunteers, but the difference was not statistically significant[27]. However, obtaining mucosal samples from IBS patients would require colonoscopy, which is not a regular procedure on IBS patients.

In conclusion, we observed alterations in the GI microbiota of IBS-D subjects with a multivariate analysis and several additional statistically significant differences were detected between the intestinal microbiotas of the different IBS subtypes and healthy controls in assay-specific analyses. Recovering the target bacteria of the *C. thermosuccinogenes* 85% and *R. torques* 94% qPCR assays would be essential for further analysis of their possible role in the human GI tract and their association to IBS. In the future, biomarkers associated to the GI microbiota could aid therapeutic trial follow-up, diagnosis and treatment of IBS patients.

ACKNOWLEDGMENTS

COMMENTS

Background

Irritable bowel syndrome (IBS) is a common gastrointestinal functional disorder that can greatly affect the patient's well being. Multiple interacting mechanisms, including alterations in the intestinal microbiota, are suspected to lie behind IBS aetiology.

Research frontiers

Alterations in the gastrointestinal microbiota in association to health and disease have become an essential field of research in gastroenterology. For instance, indications of dysbiosis have been detected in relation to Crohn's disease. In this study, assays for analyzing phylotype specific bacterial alterations in association to IBS were developed and applied.

Innovations and breakthroughs

The authors' results support the hypothesis of intestinal bacteria having a role in IBS, as significant phylotype specific alterations between the faecal microbiotas of IBS symptom subtype groups and healthy controls were detected. Furthermore, the results emphasize the importance of subgrouping IBS patients in future studies.

Applications

An IBS-associated 16S ribosomal RNA (rRNA) gene sequence library data was used to design the real-time polymerase chain reaction (PCR) assays capable of differentiating IBS symptom subgroups and healthy controls in the test sample panel. The detected altering phylotypes might be useful as targets in diagnostic, therapeutic and host-microbe interaction studies.

Terminology

The bacterial 16S rRNA gene is constructed from conserved and variable regions according to its phylogenetic origin. It enables the detection and quantification of microbes from environmental samples even when the bacteria cannot be cultivated. Real-time PCR targeting the 16S rRNA gene can be used to quantify bacterial subpopulations of 0.01% from faecal DNA samples.

Peer review

The authors examined faecal bacterial phylotypes in eight diarrhea-predominant, eight constipation-predominant, four mixed symptom subtype IBS patients, and 15 control subjects with quantitative real-time polymerase chain reaction assays. They found significant phylotype level alterations in the intestinal microbiotas of IBS patients.

REFERENCES

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

2   **Arebi N**, Gurmany S, Bullas D, Hobson A, Stagg A, Kamm M. Review article: the psychoneuroimmunology of irritable bowel syndrome--an exploration of interactions between psychological, neurological and immunological observations. *Aliment Pharmacol Ther* 2008; **28**: 830-840

3   **Drossman DA**, Camilleri M, Mayer EA, Whitehead WE. AGA technical review on irritable bowel syndrome. *Gastroenterology* 2002; **123**: 2108-2131

4   **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

5   **Spiller RC**, Jenkins D, Thornley JP, Hebden JM, Wright T, Skinner M, Neal KR. Increased rectal mucosal enteroendocrine cells, T lymphocytes, and increased gut permeability following acute Campylobacter enteritis and in post-dysenteric irritable bowel syndrome. *Gut* 2000; **47**: 804-811

6   **Liebregts T**, Adam B, Bredack C, Roth A, Heinzel S, Lester S, Downie-Doyle S, Smith E, Drew P, Talley NJ, Holtmann G. Immune activation in patients with irritable bowel syndrome. *Gastroenterology* 2007; **132**: 913-920

7   **Rodriguez LA**, Ruigomez A. Increased risk of irritable bowel syndrome after bacterial gastroenteritis: cohort study. *BMJ* 1999; **318**: 565-566

8   **Spiller RC**. Role of infection in irritable bowel syndrome. *J Gastroenterol* 2007; **42** Suppl 17: 41-47

9   **Maukonen J**, Satokari R, Mättö J, Söderlund H, Mattila-Sandholm T, Saarela M. Prevalence and temporal stability of selected clostridial groups in irritable bowel syndrome in relation to predominant faecal bacteria. *J Med Microbiol* 2006; **55**: 625-633

10 **Malinen E**, Rinttilä T, Kajander K, Mättö J, Kassinen A, Krogius L, Saarela M, Korpela R, Palva A. Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. *Am J Gastroenterol* 2005; **100**: 373-382

11 **Kassinen A**, Krogius-Kurikka L, Mäkivuokko H, Rinttilä T, Paulin L, Corander J, Malinen E, Apajalahti J, Palva A. The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects. *Gastroenterology* 2007; **133**: 24-33

12 **Rajilić-Stojanović M**. Diversity of the human gastrointestinal microbiota - novel perspectives from high throughput analyses. Wageningen: Wageningen University, 2007

13 **Gecse K**, Roka R, Ferrier L, Leveque M, Eutamene H, Cartier C, Ait-Belgnaoui A, Rosztoczy A, Izbeki F, Fioramonti J, Wittmann T, Bueno L. Increased faecal serine protease activity in diarrhoeic IBS patients: a colonic lumenal factor impairing colonic permeability and sensitivity. *Gut* 2008; **57**: 591-599

14 **Schoepfer AM**, Schaffer T, Seibold-Schmid B, Muller S, Seibold F. Antibodies to flagellin indicate reactivity to bacterial antigens in IBS patients. *Neurogastroenterol Motil* 2008; **20**: 1110-1118

15 **Spiller R**. Review article: probiotics and prebiotics in irritable bowel syndrome. *Aliment Pharmacol Ther* 2008; **28**: 385-396

16 **Kajander K**, Myllyluoma E, Rajilić-Stojanović M, Kyrönpalo S, Rasmussen M, Järvenpää S, Zoetendal EG, de Vos WM, Vapaatalo H, Korpela R. Clinical trial: multispecies probiotic supplementation alleviates the symptoms of irritable bowel syndrome and stabilizes intestinal microbiota. *Aliment Pharmacol Ther* 2008; **27**: 48-57

17 **Rajilić-Stojanović M**, Smidt H, de Vos WM. Diversity of the human gastrointestinal tract microbiota revisited. *Environ Microbiol* 2007; **9**: 2125-2136

18 **Eckburg PB**, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA. Diversity of the human intestinal microbial flora. *Science* 2005; **308**: 1635-1638

19 **Ley RE**, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. *Nature* 2006; **444**: 1022-1023

20 **Zoetendal EG**, Akkermans AD, De Vos WM. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Appl Environ Microbiol* 1998; **64**: 3854-3859

21 **Li M**, Wang B, Zhang M, Rantalainen M, Wang S, Zhou H, Zhang Y, Shen J, Pang X, Zhang M, Wei H, Chen Y, Lu H, Zuo J, Su M, Qiu Y, Jia W, Xiao C, Smith LM, Yang S, Holmes E, Tang H, Zhao G, Nicholson JK, Li L, Zhao L. Symbiotic gut microbes modulate human metabolic phenotypes. *Proc Natl Acad Sci USA* 2008; **105**: 2117-2122

22 **Turnbaugh PJ**, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, Egholm M, Henrissat B, Heath AC, Knight R, Gordon JI. A core gut microbiome in obese and lean twins. *Nature* 2009; **457**: 480-484

23 **Andersson AF**, Lindberg M, Jakobsson H, Backhed F, Nyren P, Engstrand L. Comparative analysis of human gut microbiota by barcoded pyrosequencing. *PLoS One* 2008; **3**: e2836

24 **Balsari A**, Ceccarelli A, Dubini F, Fesce E, Poli G. The fecal microbial population in the irritable bowel syndrome. *Microbiologica* 1982; **5**: 185-194

25 **Si JM**, Yu YC, Fan YJ, Chen SJ. Intestinal microecology and quality of life in irritable bowel syndrome patients. *World J Gastroenterol* 2004; **10**: 1802-1805

26 **Mättö J**, Maunuksela L, Kajander K, Palva A, Korpela R, Kassinen A, Saarela M. Composition and temporal stability of gastrointestinal microbiota in irritable bowel syndrome--a longitudinal study in IBS and control subjects. *FEMS Immunol Med Microbiol* 2005; **43**: 213-222

27 **Swidsinski A**, Weber J, Loening-Baucke V, Hale LP, Lochs H. Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. *J Clin Microbiol* 2005; **43**: 3380-3389

28 **Thompson WG**, Longstreth GF, Drossman DA, Heaton KW, Irvine EJ, Muller-Lissner SA. Functional bowel disorders and functional abdominal pain. *Gut* 1999; **45** Suppl 2: II43-II47

29 **Kajander K**, Hatakka K, Poussa T, Färkkilä M, Korpela R. A probiotic mixture alleviates symptoms in irritable bowel syndrome patients: a controlled 6-month intervention. *Aliment Pharmacol Ther* 2005; **22**: 387-394

30 **Krogius-Kurikka L**, Kassinen A, Paulin L, Corander J, Mäkivuokko H, Tuimala J, Palva A. Sequence analysis of percent G+C fraction libraries of human faecal bacterial DNA reveals a high number of Actinobacteria. *BMC Microbiol* 2009; **9**: 68

31 **Apajalahti JH**, Särkilahti LK, Mäki BR, Heikkinen JP, Nurminen PH, Holben WE. Effective recovery of bacterial DNA and percent-guanine-plus-cytosine-based analysis of community structure in the gastrointestinal tract of broiler chickens. *Appl Environ Microbiol* 1998; **64**: 4084-4088

32 **Ludwig W**, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Forster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, Konig A, Liss T, Lussmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH. ARB: a software environment for sequence data. *Nucleic Acids Res* 2004; **32**: 1363-1371

33 **Thompson JD**, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994; **22**: 4673-4680

34 **Hall TA**. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser* 1999; **41**: 95-98. Available from: URL: http://www.mbio.ncsu.edu/BioEdit/bioedit.html

35 **Nobaek S**, Johansson ML, Molin G, Ahrne S, Jeppsson B. Alteration of intestinal microflora is associated with reduction in abdominal bloating and pain in patients with irritable bowel syndrome. *Am J Gastroenterol* 2000; **95**: 1231-1238

36 **Schloss PD**, Handelsman J. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl Environ Microbiol* 2005; **71**: 1501-1506

37 **Rozen S**, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 2000; **132**: 365-386

38 **Zuker M**. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 2003; **31**: 3406-3415

39 **Labarga A**, Valentin F, Anderson M, Lopez R. Web services at the European bioinformatics institute. *Nucleic Acids Res* 2007; **35**: W6-W11

40 **Altschul SF**, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997; **25**: 3389-3402

41 **Wang Q**, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 2007; **73**: 5261-5267

42 **R Development Core Team**. R: A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing, 2008

43 **Pinheiro JC**, Bates DM. Mixed-Effects Models in S and S-Plus. New York: Springer, 2000

44 **Sokol H**, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, Gratadoux JJ, Blugeon S, Bridonneau C, Furet JP, Corthier G, Grangette C, Vasquez N, Pochart P, Trugnan G, Thomas G, Blottiere HM, Dore J, Marteau P, Seksik P, Langella P. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci USA* 2008; **105**: 16731-16736

45 **Willing B**, Halfvarson J, Dicksved J, Rosenquist M, Jarnerot G, Engstrand L, Tysk C, Jansson JK. Twin studies reveal specific imbalances in the mucosa-associated microbiota of patients with ileal Crohn's disease. *Inflamm Bowel Dis* 2009; **15**: 653-660

46 **Matsuki T**, Watanabe K, Fujimoto J, Takada T, Tanaka R. Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. *Appl Environ Microbiol* 2004; **70**: 7220-7228

47 **Maukonen J**, Mättö J, Satokari R, Söderlund H, Mattila-Sandholm T, Saarela M. PCR DGGE and RT-PCR DGGE show diversity and short-term temporal stability in the Clostridium coccoides-Eubacterium rectale group in the human intestinal microbiota. *FEMS Microbiol Ecol* 2006; **58**: 517-528

48 **Roka R**, Rosztoczy A, Leveque M, Izbeki F, Nagy F, Molnar T, Lonovics J, Garcia-Villar R, Fioramonti J, Wittmann T, Bueno L. A pilot study of fecal serine-protease activity: a pathophysiologic factor in diarrhea-predominant irritable bowel syndrome. *Clin Gastroenterol Hepatol* 2007; **5**: 550-555

49 **Gill SR**, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM, Nelson KE. Metagenomic analysis of the human distal gut microbiome. *Science* 2006; **312**: 1355-1359

50 **Leitch EC**, Walker AW, Duncan SH, Holtrop G, Flint HJ. Selective colonization of insoluble substrates by human

faecal bacteria. *Environ Microbiol* 2007; **9**: 667-679

51   **Abell GC**, Cooke CM, Bennett CN, Conlon MA, McOrist AL. Phylotypes related to Ruminococcus bromii are abundant in the large bowel of humans and increase in response to a diet high in resistant starch. *FEMS Microbiol Ecol* 2008; **66**: 505-515

52   **Hoskins LC**, Agustines M, McKee WB, Boulding ET, Kriaris M, Niedermeyer G. Mucin degradation in human colon ecosystems. Isolation and properties of fecal strains that degrade ABH blood group antigens and oligosaccharides from mucin glycoproteins. *J Clin Invest* 1985; **75**: 944-953

53   **Martinez-Medina M**, Aldeguer X, Gonzalez-Huix F, Acero D, Garcia-Gil LJ. Abnormal microbiota composition in the ileocolonic mucosa of Crohn's disease patients as revealed by polymerase chain reaction-denaturing gradient gel electrophoresis. *Inflamm Bowel Dis* 2006; **12**: 1136-1145

54   **Hayashi H**, Sakamoto M, Benno Y. Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods. *Microbiol Immunol* 2002; **46**: 535-548

55   **Mai V**, Greenwald B, Morris JG Jr, Raufman JP, Stine OC. Effect of bowel preparation and colonoscopy on post-procedure intestinal microbiota composition. *Gut* 2006; **55**: 1822-1823

56   **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**: 13780-13785

57   **Duck LW**, Walter MR, Novak J, Kelly D, Tomasi M, Cong Y, Elson CO. Isolation of flagellated bacteria implicated in Crohn's disease. *Inflamm Bowel Dis* 2007; **13**: 1191-1201

58   **Moore WE**, Holdeman LV. Human fecal flora: the normal flora of 20 Japanese-Hawaiians. *Appl Microbiol* 1974; **27**: 961-979

59   **Moore WE**, Moore LH. Intestinal floras of populations that have a high risk of colon cancer. *Appl Environ Microbiol* 1995; **61**: 3202-3207

60   **Zoetendal EG**, von Wright A, Vilpponen-Salmela T, Ben-Amor K, Akkermans AD, de Vos WM. Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. *Appl Environ Microbiol* 2002; **68**: 3401-3407

61   **Nadkarni MA**, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* 2002; **148**: 257-266

62   **Louis P**, Duncan SH, McCrae SI, Millar J, Jackson MS, Flint HJ. Restricted distribution of the butyrate kinase pathway among butyrate-producing bacteria from the human colon. *J Bacteriol* 2004; **186**: 2099-2106