ARTICLE I

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Prevalence and temporal stability of selected clostridial groups in irritable bowel syndrome in relation to predominant faecal bacteria

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The differences in faecal bacterial population between irritable bowel syndrome (IBS) and control subjects have been reported in several studies. The aim of the present study was to compare the predominant and clostridial faecal microbiota of IBS subjects and healthy controls by applying denaturing gradient gel electrophoresis (DGGE) and a recently developed multiplexed and quantitative hybridization-based technique, transcript analysis with the aid of affinity capture (TRAC). According to the results, the studied clostridial groups (*Clostridium histolyticum*, *Clostridium coccoides-Eubacterium rectale*, *Clostridium lituseburense* and *Clostridium leptum*) represented the dominant faecal microbiota of most of the studied subjects, comprising altogether 29–87 % of the total bacteria as determined by the hybridized 16S rRNA. The *C. coccoides-E. rectale* group was the dominant subgroup of clostridia, contributing a mean of 43 % of the total bacteria in control subjects and 30 % (constipation type) to 50 % (diarrhoea type) in different IBS symptom category subjects. The proportion of the *C. coccoides-E. rectale* group was found to be significantly lower in the constipation-type IBS subjects than in the control subjects. DNA-based PCR-DGGE and RNA-based RT-PCR-DGGE analyses targeted to the predominant bacterial population showed considerable biodiversity as well as uniqueness of the microbiota in each subject, in both control and IBS subject groups. The RT-PCR-DGGE profiles of the IBS subjects further indicated higher instability of the bacterial population compared to the control subjects. The observations suggest that clostridial microbiota, in addition to the instability of the active predominant faecal bacterial population, may be involved in IBS.

INTRODUCTION

The composition of the resident intestinal microbiota varies between individuals, and the predominant population is fairly stable under normal conditions (Zoetendal *et al.*, 1998; Harmsen *et al.*, 2002a; Vanhoutte *et al.*, 2004; Mättö *et al.*, 2005). However, several factors, such as antibiotic therapy, ageing and disease, may cause disturbances in the intestinal balance. Transient disturbance of the intestinal microbiota during antibiotic therapy has been shown in several studies (Edlund & Nord, 2000; Donskey *et al.*, 2003). Changes in the intestinal microbiota have also been suggested to occur in certain intestinal diseases and disorders, such as inflammatory bowel disease (IBD) (Seksik *et al.*, 2003) and irritable bowel syndrome (IBS) (Madden & Hunter, 2002). IBS is an intestinal disorder that involves continuous or recurrent intestinal pain or discomfort that is relieved during defecation. In addition, IBS symptoms include bloating, altered stool frequency, form or passage, and passage of mucus (Thompson *et al.*, 1999). The existence of abnormal colonic fermentation in IBS (King *et al.*, 1998) and alleviation of IBS symptoms by eradication of small intestinal bacterial overgrowth by antibiotic therapy (Pimentel *et al.*, 2000), suggest that the intestinal microbiota has a role in IBS. Some studies have reported differences in the faecal bacterial population between IBS and control subjects (Balsari *et al.*, 1982; Bradley *et al.*, 1987; Madden & Hunter, 2002; Mättö *et al.*, 2005; Malinen *et al.*, 2005). However, the results obtained in earlier studies are partly contradictory, and with the exception of those of Mättö *et al.* (2005) and Malinen *et al.* (2005), are based on the culture-based analysis of the microbiota. Therefore, the role of intestinal microbiota in IBS is still poorly known. Since IBS is often linked with extensive gas production in the colon (King *et al.*, 1998), and some bacterial groups are more prone to gas production than others (Cato *et al.*, 1986), the possible role of the composition and/or activity of clostridia and related bacteria in intestinal (im)balance warrants further study.

Abbreviations: DGGE, denaturing gradient gel electrophoresis; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; TRAC, transcript analysis with the aid of affinity capture.

The aim of the present study was to compare the diversity and temporal stability of the predominant faecal microbiota, with both DNA- and RNA-based denaturing gradient gel electrophoresis (DGGE), to reveal possible differences between IBS subjects and healthy controls. In addition, the composition, abundance and stability of selected clostridial groups were studied to further assess possible differences.

METHODS

Samples. Five male and 11 female subjects were included in the IBS subject group (age 24–64 years; mean 45 years). The IBS group consisted of subjects with diarrhoea-dominant (seven subjects), constipation-dominant (six subjects) and alternating (mixed) type (three subjects) symptoms. The control subject group consisted of four male and 12 female subjects that were 26–63 years of age (mean 45 years). The exclusion criterion for both groups was antimicrobial therapy during the 2 months prior to each sampling timepoint. Faecal samples from the 32 subjects were obtained on two occasions 6 months apart (0 and 6 months). More detailed information about the IBS and control subject groups, as well as sampling and sample handling, has been described previously by Mättö *et al.* (2005).

DNA extraction. DNA was extracted from 300 mg of faecal material using the FastDNA Spin Kit for Soil (QBIOgene) according to manufacturer's instructions, with the modification that the bacterial cells were broken with a Fast Prep instrument at 6·0 m s^{-1} for 60 s three times. The isolated DNA was stored at −20 °C until examined.

RNA extraction and biotinylation. RNA was extracted from faeces as described elsewhere (Zoetendal *et al.*, 1998) with minor modifications (Satokari *et al.*, 2005), and further purified by using the clean-up protocol of the RNeasy mini kit (Qiagen). Total RNA was quantified by A_{260} measurement, and the purity of RNA was evaluated by agarose gel electrophoresis. Purified RNA used for quantitative analysis (see below) was biotinylated by using Photoprobe biotin (Vector Laboratories). Biotinylation was performed by exposing the RNA to long-wave UV light (365 nm) for 30 min, and the subsequent purification of the RNA from free biotin was performed according to the manufacturer's instructions, with slight modifications according to Satokari *et al.* (2005).

Specific quantification of 16S rRNA. Multiplexed quantification of clostridial 16S rRNA was performed with the transcript analysis with the aid of affinity capture (TRAC) technique, according to Satokari *et al.* (2005). Specific 16S rRNA-targeted probes for different groups of clostridia and a universal bacterial probe were used in the study (Table 1). The probes were labelled with 6-FAM (6-carboxyfluorescein) at the 5′ end and HPLC-purified. Briefly, the TRAC analysis was performed as follows: RNA (maximum amount 5–10 ng) was denatured at 70 °C for 2 min and hybridized with all the oligonucleotide probes at 50 °C for 1 h. After hybridization, the biotin–nucleic acid probe complexes were captured on magnetic streptavidin-coated microparticles and washed. The hybridized probes were eluted, and their identity and quantity were determined by capillary electrophoresis with an ABI PRISM 3100 genetic analyser. The signal intensities of the recorded probes corresponded to the amount of target nucleic acid in the mixture, while the size indicated the target. Results were expressed as the percentage of total bacterial 16S rRNA that was detected by the Bact338-IIA probe, and as the mean ± SD of triplicate measurements.

PCR and RT-PCR. Partial 16S rRNA genes were PCR-amplified using primers U968-f+GC and U1401-r (Table 1), as described by Mättö *et al.* (2005). RT-PCR was performed with the Qiagen OneStep RT-PCR kit according to the manufacturer's instructions, with or without a Q-solution. Primers used for the RT-PCR were the same as those used for the PCR (Table 1). The PCR programme consisted of reverse transcription performed at 50 °C for 30 min,

Table 1. Probes and primers used

Target group	Short name	Probe/primer	Sequence (5′→3′)	Reference
C. lituseburense group*	Clit group	Clit135¶	GTTATCCGTGTGTACAGGG	Franks *et al.* (1998)
C. histolyticum group†	Chis group	Chis150¶	TTATGCGGTATTAATCTYCCTTT	Franks *et al.* (1998)
C. coccoides-E. rectale group‡	Erec group	Erec482-5A¶#	GCTTCTTAGTCARGTACCGAAAAA	Franks *et al.* (1998); Satokari *et al.* (2005)
C. leptum group§	Clept group	Clept1240¶	GTTTTRTCAACGGCAGTC	Sghir *et al.* (2000)
Bacteria		Bact338-IIA¶**	GCTGCCTCCCGTAGGAGTIIA	Amann *et al.* (1990); Satokari *et al.* (2005)
Bacteria‖		U968-f +GC††	CGCCCGGGGCGCGCCCCGGGCGG-GGCGGGGGCACGGGGGGAACGC-GAAGAACCTTA	Nübel *et al.* (1996)
Bacteria‖		U1401-r††	CGG TGT GTA CAA GAC CC	Nübel *et al.* (1996)

*Clostridial phylogenetic cluster XI (Collins *et al.*, 1994).
†Clostridial phylogenetic clusters I and II (Collins *et al.*, 1994).
‡Clostridial phylogenetic clusters XIVa and XIVb (Collins *et al.*, 1994).
§Clostridial phylogenetic cluster IV (Collins *et al.*, 1994).
‖Partial 16S rRNA gene (V_6–V_8 hypervariable region).
¶Used as a probe in the quantitative TRAC analysis.
#The Erec482 (Franks *et al.*, 1998) probe was tailed with an additional AAAAA sequence (Erec482-5A) at the 3′ end for size-distinction purposes.
**The Bact338 (Amann *et al.*, 1990) probe was tailed with an additional IIA sequence (Bact338-IIA, where I is inosine) at the 3′ end for size-distinction purposes.
††Used as a primer in PCR-DGGE and RT-PCR-DGGE.

followed by denaturation at 95 °C for 15 min. Thereafter, the RT-PCR programme was similar to the PCR programme: 35 cycles of denaturing at 94 °C for 30 s, primer annealing at 50 °C for 20 s and elongation at 72 °C for 40 s, and final extension for 10 min at 72 °C.

DGGE analysis of 16S rDNA fragments. DGGE analysis was performed as described by Mättö *et al.* (2005). The similarity of the PCR-DGGE profiles of the samples obtained from a single subject at different time-points was compared to evaluate the temporal stability of the predominant faecal bacterial population. The comparison of the profiles was performed by visual inspection of the gels by two researchers and by calculating the similarity percentage using BioNumerics software version 3.0 (Applied Maths BVBA). Clustering was performed with Pearson correlation and the unweighted pair group method with arithmetic mean (UPGMA). Amplicons with a total surface area of at least 1 % were included in the similarity analysis. Similarity value thresholds indicating stable, rather stable and unstable DGGE profiles were determined according to the visual inspection. A DGGE profile was defined as stable if profiles at different time-points were identical (had the same amplicons and no/minor intensity differences), rather stable if they were similar (had differences in presence and intensity of few amplicons), and unstable if the profiles were different (had differences in presence and intensity of several amplicons). Similarity value thresholds for RNA-based and DNA-based profiles were determined separately.

Statistical analysis. The mean and standard deviation were calculated for each experiment. Student's *t* test (two-sample assuming unequal variances) was used for the statistical analysis of the results.

RESULTS AND DISCUSSION

In this study, the predominant and clostridial faecal microbiota of IBS subjects and healthy controls were compared to reveal possible differences in the composition, abundance and stability of selected groups. Mättö *et al.* (2005) have shown by DNA-based analysis that IBS subjects have more instability in their predominant faecal microbiota than healthy controls. We broadened the study by examining whether the instability which was seen in the DNA-based analysis could also be seen in RNA-based analyses, both predominant microbiota and clostridia-targeted. In addition to stability, the composition and abundance of the selected clostridial groups were studied. Clostridia were selected as a target group in this study, since disturbed metabolism of intestinal gases, as well as increased sensitivity of the colon, has been associated with IBS symptoms (King *et al.*, 1998). Since clostridia are able to produce gas, they may play a role in IBS symptoms (Cato *et al.*, 1986).

Temporal stability of predominant bacterial populations

The temporal intraindividual stability of the predominant bacteria was studied with DNA-based PCR-DGGE and RNA-based RT-PCR-DGGE to determine whether the stability/instability of clostridial populations corresponded with the stability/instability of the predominant bacterial population, and if any differences could be observed between control and IBS subjects (Fig. 1). DNA-based PCR-DGGE and RNA-based RT-PCR-DGGE analyses targeted to the predominant bacterial population showed considerable intraindividual biodiversity, as well as uniqueness of the faecal microbiota in both control and IBS subject groups in this study. PCR-DGGE and RT-PCR-DGGE analysis with universal bacterial primers did not reveal any IBS-specific amplicons. PCR-DGGE and RT-PCR-DGGE profiles were heterogeneous, containing about 20–30 and 15–25 amplicons, respectively. The RT-PCR-DGGE profiles of IBS subjects (16 ± 5 amplicons) and control subjects (16 ± 4 amplicons) contained significantly fewer amplicons than the PCR-DGGE profiles (IBS subjects, 21 ± 5 amplicons; control subjects, 23 ± 5 amplicons) ($P < 0{\cdot}05$; Fig. 1). There were differences in all parts of the denaturing gradient. Some amplicons were more prominent in the RNA-derived profile than in the DNA-derived profile, and vice versa. In addition, some bands were missing from the RNA-derived profiles and some from the DNA-derived profiles. Furthermore, the amplicons with the highest GC content (migrating to the lower part of the DGGE gel) were mostly missing in the RT-PCR-DGGE profiles (Fig. 1). The clear difference between the DNA- and RNA-derived predominant faecal bacterial populations has also been reported by Tannock *et al.* (2004). The differences between the DNA- and RNA-derived profiles may be due to several reasons. The resolving power of RT-PCR is limited by the efficiency of RNA-to-cDNA conversion (Bustin & Nolan, 2004). In addition, DNA obtained from complex samples does not reflect metabolic activity (Josephson *et al.*, 1993), whereas rRNA is regarded as an indicator of total bacterial activity (Felske *et al.*, 1997). Therefore, the amplicons that are missing from

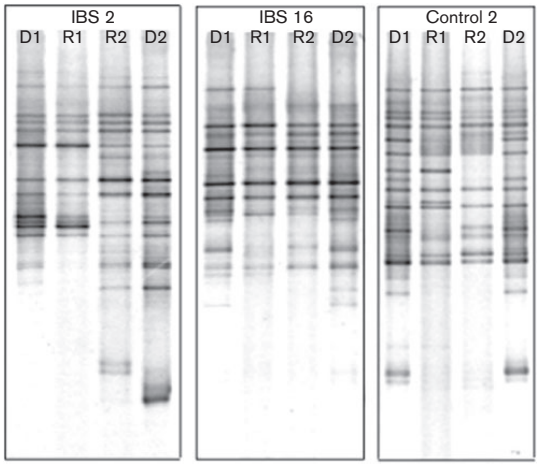

Fig. 1. Comparison of DNA-based PCR-DGGE and RNA-based RT-PCR-DGGE profiles during a 6 month time-period for two IBS subjects and one control subject. Lanes D1 correspond to DNA-based DGGE profiles at baseline, lanes R1 to RNA-based DGGE profiles at baseline, lanes R2 to RNA-based DGGE profiles 6 months later and lanes D2 to DNA-based DGGE profiles 6 months later. The panels are from different individuals with IBS (IBS2 and IBS16) and from a healthy control (Control 2).

the RNA-derived profiles may reflect a low activity in that part of the faecal microbiota.

When DGGE profiles derived from faecal RNA amplicons were compared to the DGGE profiles derived from faecal DNA amplicons in this study, the temporal intraindividual similarity values of RT-PCR-DGGE and PCR-DGGE profiles were significantly higher than the intraindividual similarity values between DNA- and RNA-based DGGE profiles at a given time-point in nearly all cases ($P < 0{\cdot}05$; Table 2). However, since each individual had unique PCR-DGGE and RT-PCR-DGGE profiles, the intraindividual RNA- and DNA-based DGGE profiles resembled each other more than the interindividual RNA-based profiles (Fig. 1). In the present study, the assessment of temporal stability of the predominant microbiota with DNA-based PCR-DGGE did not reveal more instability in the IBS subjects than in the control group subjects (Table 2), contrary to the findings of Mättö *et al.* (2005). However, less similarity (i.e. more instability) was observed in the DGGE profiles derived from faecal RNA amplicons of the IBS subjects than the control subjects (Table 2). In addition, the similarity value range of RNA-derived DGGE profiles of IBS subjects was much larger than that of the control subjects (Table 2). The main difference between the study group of Mättö *et al.* (2005) and the study group in this study was that we did not include subjects who had received antimicrobial therapy during the 2 months prior to the two sampling time-points, since antibiotic therapy can cause disturbances in the intestinal microbiota (Edlund & Nord, 2000; Donskey *et al.*, 2003), and may also affect the predominant bacterial groups (Bartosch *et al.*, 2004). Under normal circumstances, the predominant intestinal microbiota of an adult individual has been reported to be fairly stable (Zoetendal *et al.*, 1998, 2001; Seksik *et al.*, 2003; Vanhoutte *et al.*, 2004). The effect of disease on the stability of intestinal microbiota is clearly seen in the study of Seksik *et al.* (2003), in which a marked instability of the predominant faecal microbiota was observed in four IBD patients. In our study, temporal instability was observed in both IBS and control subjects (with both DNA- and RNA-based DGGE). In previous studies in which the long-term temporal stability of the predominant microbiota from healthy subjects has been assessed, the number of subjects has been limited (one to four individuals) (Zoetendal *et al.*, 1998, 2001; Seksik *et al.*, 2003; Vanhoutte *et al.*, 2004).

The intraindividual similarity values of PCR-DGGE and RT-PCR-DGGE profiles of IBS subjects between time-points 1 (baseline) and 2 (6 months) varied between 45 and 95 %, and between 39 and 95 %, respectively, whereas the similarity values of control subjects varied between 59 and 97 %, and between 68 and 94 %, respectively (Table 2). When IBS patients were divided into subgroups according to their symptoms in our study, the greatest intraindividual variability in DGGE similarity values within the subject group was seen in the diarrhoea-type subgroup and the smallest in the mixed-type subgroup (Table 2). The great intraindividual variation seen in the diarrhoea-type subgroup might be due to different phases of the syndrome. If a patient is experiencing a more symptomatic phase of IBS, the predominant bacterial population might be significantly different to that when symptom-free, in a similar manner to observations for IBD patients (Seksik *et al.*, 2003). However, we did not have data on the symptomatic activity of each IBS subject, so this conclusion remains speculative. Further studies are needed to assess the influence of the more symptomatic phase of the syndrome on the stability of the faecal microbiota. The smallest intraindividual variability in our study, shown by the mixed-type subject group, is at least partly explained by the small number of subjects (three), so further studies are needed with larger patient groups.

Proportion of clostridia

In the present study, the recently developed TRAC technique (Satokari *et al.*, 2005) allowed the multiplexed quantification of the clostridial groups. The sensitivity of the technique allows as little as 0·05 ng of total RNA to be detected, but the quantification of proportions is limited by the dynamic range of measurement. In practice, it is feasible to measure 100–200-fold differences in the amounts of individual target RNAs (Satokari *et al.*, 2005). According to our results, the studied clostridial groups [*Clostridium. histolyticum* (probe Chis150), *Clostridium coccoides-Eubacterium rectale* (probe Erec482), *Clostridium lituseburense* (probe Clit135) and *Clostridium leptum* (probe Clept1240) groups] represented the dominant faecal microbiota in most of the studied subjects (26 out of 32 subjects, including both IBS and control subjects), contributing altogether 29–87 %, as determined by the hybridized 16S rRNA (Table 3). This is in agreement with other studies, where several clostridial groups have been studied from clone libraries of adult faecal samples (Wilson & Blitchington, 1996; Suau *et al.*, 1999; Hayshi *et al.*, 2002; Mangin *et al.*, 2004). The proportion of each studied clostridial group showed marked interindividual variation (Table 3).

There were only small differences in the proportions of the *C. leptum*, *C. lituseburense* and *C. histolyticum* groups between IBS and control subjects (Table 3). It has been reported that the *C. lituseburense* and *C. histolyticum* groups contribute less than 1 % of the total faecal microbiota of healthy adults (Franks *et al.*, 1998; Harmsen *et al.*, 1999). Our results for the *C. lituseburense* group (mean 1·5 and 2·3 %, for the baseline and 6 month samples, respectively) were thus in accordance with the results reported elsewhere. However, we found that the members of the *C. histolyticum* groups contributed a mean of 7·4 and 5·5 % (for baseline and 6 month samples, respectively) of the faecal bacterial microbiota of the 16 healthy control subjects, and 6·2 and 6·5 % (for baseline and 6 month samples, respectively) of the faecal microbiota of the IBS subjects. Satokari *et al.* (2005) note in their study that the Chis150 probe and assay conditions employed give approximately 3 % background

Table 2. Similarity values of DGGE profiles of a given subject obtained at two different time-points (baseline and 6 months later)

The values are grouped according to the RNA similarity/stability. Abbreviations: S, stable; R, rather stable; U, unstable.

Subject	Qualitative DGGE analysis						Quantitative Δclostridia (%)†, 1 versus 2
	RNA-based similarity*, 1 versus 2		DNA-based similarity*, 1 versus 2		Similarity (%)*, RNA1 versus DNA1	Similarity (%)*, RNA2 versus DNA2	
IBS subjects, diarrhoea type							
IBS 1	94·2 %	S‡§	91·9 %	R‡‖	83·2	88·6	6·7
IBS 2	38·5 %	U	44·6 %	U	88·3	59·5	50·2
IBS 8	81·0 %	R	92·0 %	R	76·8	74·1	9·0
IBS 13	50·1 %	U	87·7 %	R	67·7	70·8	5·1
IBS 14	59·9 %	U	74·4 %	U	56·0	81·9	15·5
IBS 15	89·6 %	R	90·1 %	R	77·0	89·0	15·3
IBS 16	92·5 %	S	95·1 %	S	84·4	89·2	3·3
Mean ± SD	72·3 ± 22·6		82·3 ± 17·9		76·2 ± 11·1	79·0 ± 11·4	15·0 ± 16·2
Range	38·5–94·2		44·6–95·1		56·0–88·3	59·5–89·2	3·3–50·2
IBS subjects, constipation type							
IBS 3	43·1 %	U	68·0 %	U	75·3	84·8	22·3
IBS 4	80·5 %	R	84·3 %	R	70·6	76·4	8·0
IBS 5	79·0 %	R	81·3 %	R	72·0	78·8	0·5
IBS 6	93·4 %	S	89·4 %	R	70·4	62·9	11·1
IBS 9	66·1 %	U	81·8 %	R	56·2	59·4	28·9
IBS 11¶	−¶		83·1 %	R	65·0	−¶	6·9
Mean ± SD	72·4 ± 19·0		81·3 ± 7·1		68·3 ± 6·8	72·5 ± 10·8	13·0 ± 10·6
Range	43·1–93·4		68·0–89·4		56·2–75·3	59·4–84·8	0·5–28·9
IBS subjects, mixed type							
IBS 7	94·5 %	S	85·7 %	R	70·6	69·4	8·0
IBS 10	80·6 %	R	88·0 %	R	89·5	80·7	11·7
IBS 12	83·2 %	R	87·7 %	R	66·8	63·4	20·2
Mean ± SD	86·1 ± 7·4		87·1 ± 1·3		75·6 ± 12·2	71·1 ± 8·8	13·3 ± 6·3
Range	85·7–88·0		85·7–88·0		66·8–89·5	63·4–80·7	8·0–20·2
All IBS subjects together							
Mean ± SD	75·1 ± 19·0		82·8 ± 12·3		73·1 ± 10·0	75·3 ± 10·7	13·9 ± 12·2
Range	38·5–94·5		44·6–95·1		56·0–89·5	59·4–89·2	0·5–50·2
Control subjects							
Control 1	85·9 %	R	88·8 %	R	75·1	70·7	13·0
Control 2	66·0 %	U	91·3 %	R	73·2	64·6	29·8
Control 3	87·8 %	R	90·0 %	R	85·9	84·7	42·0
Control 4	92·9 %	S	86·7 %	R	83·6	86·4	10·5
Control 5	72·2 %	R	90·1 %	R	88·7	75·3	8·6
Control 6	94·2 %	S	88·4 %	R	81·4	72·0	7·3
Control 7	91·6 %	R	58·8 %	U	42·4	68·1	18·8
Control 8	69·4 %	U	63·0 %	U	76·5	73·4	10·1
Control 9	85·5 %	R	96·3 %	S	56·9	54·2	8·8
Control 10#	−#		64·1 %	U	66·9	−#	−#
Control 11	90·7 %	R	92·2 %	S	82·3	80·8	25·1
Control 12	92·4 %	S	90·4 %	R	56·1	64·8	5·7
Control 13	92·3 %	S	84·6 %	R	88·2	86·1	1·8
Control 14	69·9 %	U	96·7 %	S	61·9	76·7	14·1
Control 15	68·2 %	U	90·2 %	R	72·8	81·5	8·7
Control 16	92·4 %	S	85·4 %	R	31·1	42·4	4·8
Mean ± SD	83·4 ± 10·8		84·8 ± 11·8		70·2 ± 16·7	72·1 ± 12·2	13·9 ± 10·8
Range	68·2–94·2		58·8–96·7		31·1–88·7	42·4–86·4	1·8–42·0

with non-target organisms when compared to the bacterial signal of the Bact338-IIA probe. Assuming that the background signal with the Chis150 probe is more or less the same from all mixed bacterial populations, the *C. histolyticum* group contributed a mean of 3–5 % of the total population, which is still higher than the previously reported <1 %. The proportions of the *C. leptum* group found in control subjects (mean 12 and 9 %, for baseline and 6 month samples, respectively) and IBS subjects (mean 12 and 15 %, for baseline and 6 month samples, respectively) were in agreement with earlier studies (Sghir *et al.*, 2000; Marteau *et al.*, 2001; Hold *et al.*, 2002).

In the present study, the *C. coccoides-E. rectale* group was the dominant subgroup of faecal clostridia. It contributed a mean of 43 % (range 18–71 %) of the total bacteria in control subjects, and 30 % (constipation type) to 50 % (diarrhoea type) (range 14–68 %) in different IBS symptom category subjects. The mean percentage observed with our control subjects is somewhat higher than the previously reported 11–35 % (range 5–45 %) for healthy adults (Franks *et al.*, 1998; Jansen *et al.*, 1999; Sghir *et al.*, 2000; Marteau *et al.*, 2001; Harmsen *et al.*, 2002a, b; Zoetendal *et al.*, 2002; Hold *et al.*, 2003; Rigottier-Gois *et al.*, 2003; Rochet *et al.*, 2004). This highlights interindividual differences that are possibly due to diet and genetic background. In addition, the age distribution in both our subject groups was wider than that of most other studies. Furthermore, it should be noted that the smaller proportions of the *C. coccoides-E. rectale* group in other studies compared to our results have been obtained with fluorescent *in situ* hybridization (FISH) [except for the studies of Sghir *et al.* (2000) and Marteau *et al.* (2001)], whereas the results of Rigottier-Gois *et al.* (2003) obtained with dot-blot hybridization (mean 22 %) were more similar to those of our study (range 12–55 % versus 18–71 % in our study). The FISH analysis measures the proportion of the cells that contain a sufficient number of ribosomes to be detected, while rRNA dot-blot hybridization gives an index that reflects the ribosomal content of the cells and the general metabolic activity (Rigottier-Gois *et al.*, 2003), as does the TRAC technique. The amount of rRNA per cell varies according to the species and the metabolic activity of the bacterial cell (Klappenbach *et al.*, 2000). When the human faecal microbiota has been analysed with the 16S rDNA library method, bacteria from the *C. coccoides-E. rectale* group have contributed 10–59 % of the total faecal flora (Wilson & Blitchington, 1996; Suau *et al.*, 1999; Hayshi *et al.*, 2002; Hold *et al.*, 2002; Wang *et al.*, 2003), which is in accordance with our results.

No difference was observed in the present study in the proportions of the *C. coccoides-E. rectale* group when all IBS subjects together were compared to the control subjects (Table 3). However, when different IBS symptom categories were compared to the control subjects, the proportion of the *C. coccoides-E. rectale* group of clostridia was significantly lower in the constipation-type IBS subjects than in the control subjects, and also at the 6 months sampling time-point of the mixed-type IBS subjects ($P < 0{\cdot}05$) (Table 3). Similar differences have been observed by Malinen *et al.* (2005). In contrast to this, Rinttilä *et al.* (2004) have found with real-time PCR that the number of cells belonging to the *C. coccoides-E. rectale* group is somewhat lower in the IBS group than in the control group. However, their subject groups contain only three subjects. As a target clostridial group, the *C. coccoides-E. rectale* group (phylogenetic clusters XIVa and XIVb) is too large to detect subtle variations between the microbiota of control and IBS subjects, and therefore needs to be divided into smaller subgroups in further studies.

Temporal stability of clostridial populations

No clear differences in intraindividual temporal stability (change in proportions between the two sampling points) of the *C. leptum*, *C. lituseburense* and *C. coccoides-E. rectale* groups were observed between the IBS and control subjects. However, the *C. histolyticum* group was temporally more stable (the change in proportions of the *C. histolyticum* groups was smaller) in mixed-type IBS subjects than in the

Table 2. cont.

*Similarity values were counted with BioNumerics 3.0 software. Clustering was performed with Pearson correlation and the unweighted pair group method with arithmetic mean (UPGMA). Amplicons with a total surface area of at least 1 % were included in the similarity analysis.
†Quantitative Δclostridia (%) indicated the total change of clostridia as measured by the TRAC technique, which was defined as: absolute change (Δ; percentage at baseline minus percentage at 6 months) in the Clept-hybridized population + absolute change (Δ) in Clit-hybridized population + absolute change (Δ) in Chis-hybridized population + absolute change (Δ) in Erec-hybridized population. See Table 1 for meanings of abbreviations.
‡Similarity value thresholds indicating stable, rather stable or unstable DGGE profiles were determined according to the visual inspection of two researchers. Similarity value thresholds for RNA-based and DNA-based profiles were determined separately.
§RNA-based similarity thresholds: stable (>92·2 %), rather stable (70·0–92·1 %), unstable (<70·0 %).
||DNA-based similarity thresholds: stable (>92·2 %), rather stable (75·0–92·1 %), unstable (<75·0 %).
¶The RNA of the second sample (time-point 6 months) of the subject IBS 11 did not amplify with RT-PCR sufficiently (for DGGE), regardless of various modifications to the protocol.
#The quality and amount of RNA of the second sample (time-point 6 months) of the subject Control 10 was not sufficient either for RT-PCR or the quantitative TRAC analysis. RNA was extracted three times with two different methods.

Table 3. Proportions of studied groups of clostridia, as quantified with the TRAC technique from faecal samples of IBS subjects and control subjects on two sampling occasions

See Table 1 for meanings of abbreviations. Values show the percentage of the hybridized total bacteria (with Bact338-IIA probe), mean ± SD. Values in bold type show a statistically significant difference ($P < 0{\cdot}05$) compared to the control subject group.

Group (number of subjects)	Probe							
	Clept1240*		Clit135†		Chis150‡		Erec482§	
	Baseline	6 months	Baseline	6 months	Baseline	6 months	Baseline	6 months
Diarrhoea type (7)								
Proportion	11·0 ± 4·7	14·0 ± 4·3	1·1 ± 2·1	0·4 ± 0·4	6·7 ± 1·5	5·3 ± 1·1	49·8 ± 10·5	45·9 ± 10·1
Range (of proportion)	4·3–16·8	8·4–22·2	0·1–5·9	0·0–1·0	5·4–9·2	4·0–7·3	38·6–68·2	30·2–60·7
Change in proportion‖		4·0 ± 6·3		1·1 ± 2·0		1·4 ± 0·8		8·5 ± 10·2
Range (of change in proportion)		0·8–17·9		0–5·6		0·1–2·7		1·6–30·9
Total change of clostridia¶								15·0 ± 16·2
Constipation type (6)								
Proportion	14·5 ± 5·4	14·8 ± 8·1	1·6 ± 1·1	3·9 ± 2·5	5·9 ± 1·6	7·1 ± 3·0	**33·8 ± 5·2**	**29·5 ± 9·4**
Range (of proportion)	5·5–20·1	5·5–25·1	0·4–3·3	0·5–8·0	3·1–7·9	3·1–10·4	24·8–38·7	14·0–40·0
Change in proportion‖		2·6 ± 2·4		2·3 ± 2·6		1·6 ± 1·5		6·4 ± 8·5
Range (of change in proportion)		0–6·1		0·1–7·4		0–3·9		0·4–23·3
Total change of clostridia¶								13·0 ± 10·6
Mixed type (3)								
Proportion	11·5 ± 4·9	17·0 ± 10·6	2·2 ± 0·6	3·2 ± 0·2	5·6 ± 1·3	6·7 ± 0·8	33·6 ± 8·4	**30·7 ± 8·1**
Range (of proportion)	7·0–16·7	8·7–29·0	1·7–2·9	3·0–3·4	4·6–7·0	5·8–7·4	27·3–43·1	22·2–38·4
Change in proportion‖		5·5 ± 5·9		1·0 ± 0·8		1·2 ± 0·8		5·7 ± 2·1
Range (of change in proportion)		1·7–12·3		0·1–1·6		0·4–1·9		4·3–8·1
Total change of clostridia¶								13·3 ± 6·3
Overall mean of IBS subjects (16)								
Proportion	12·4 ± 4·9	14·9 ± 6·8	1·5 ± 1·6	2·3 ± 2·2	6·2 ± 1·5	6·5 ± 2·1	40·7 ± 11·4	36·9 ± 12·1
Range (of proportion)	4·3–20·1	5·5–29·0	0·1–5·9	0·0–8·0	3·1–9·2	3·1–10·4	24·8–68·2	14·0–60·7
Change in proportion‖		3·8 ± 4·9		1·5 ± 2·1		1·4 ± 1·0		7·2 ± 8·2
Range (of change in proportion)		0–17·9		0–7·4		0–3·9		0·4–30·9
Total change of clostridia¶								13·9 ± 12·2
Control subjects (16)								
Proportion	11·5 ± 5·5	9·4 ± 4·2	1·7 ± 2·0	1·2 ± 1·6	7·4 ± 3·9	5·5 ± 1·8	43·3 ± 16·5	42·3 ± 13·7
Range (of proportion)	4·6–23·6	2·9–18·1	0·1–7·0	0·1–4·8	3·7–19·5	2·4–9·2	17·7–71·2	20·9–66·9
Change in proportion‖		3·3 ± 4·1		1·0 ± 1·6		2·7 ± 2·9		9·2 ± 8·3
Range (of change in proportion)		0–13·2		0–6·4		0·4–12·6		0·2–24·6
Total change of clostridia¶								13·9 ± 10·8

*The Clept1240 probe (Sghir *et al.*, 2000) targets the *C. leptum* group, which corresponds to clostridial phylogenetic cluster IV (Collins *et al.*, 1994).

†The Clit135 probe (Franks *et al.*, 1998) targets the *C. lituseburense* group, which corresponds to clostridial phylogenetic cluster XI (Collins *et al.*, 1994).

‡The Chis150 probe (Franks *et al.*, 1998) targets the *C. histolyticum* group, which corresponds to clostridial phylogenetic clusters I and II (Collins *et al.*, 1994).

§The Erec482 probe (Franks *et al.*, 1998) targets the *C. coccoides-E. rectale* group, which corresponds to clostridial phylogenetic clusters XIVa and XIVb (Collins *et al.*, 1994).

‖The change was defined as: absolute change (Δ; percentage at baseline minus percentage at 6 months) in the population.

¶The total change of clostridia was defined as: absolute change (Δ; percentage at baseline minus percentage at 6 months) in Clept-hybridized population + absolute change (Δ) in Clit-hybridized population + absolute change (Δ) in Chis-hybridized population + absolute change (Δ) in Erec-hybridized population.

control subjects ($P = 0{\cdot}05$) (Table 3). Altogether, there was mostly smaller temporal variation in the studied clostridial groups than interindividual variation, and this has also been reported by Matsuki *et al.* (2004). However, the instability of predominant bacteria was also seen in the change of the proportions of the studied clostridial groups (Tables 2 and

3) in our study. Subjects whose predominant bacterial population (by RT-PCR-DGGE) was unstable over the studied time-period had a significant change in the proportions of the clostridial groups between the two sampling points (baseline and 6 months) when compared to the subjects whose predominant bacterial population was stable ($P < 0{\cdot}05$). This might be expected, since the studied clostridial groups represented the dominant faecal microbiota in these subjects. The same was also noticed with DNA-based DGGE profiles, but the difference with DNA-based profiles was not statistically significant ($P = 0{\cdot}06$).

Conclusions

The differences in faecal bacterial population between IBS and control subjects have been reported in several studies (Balsari *et al.*, 1982; Bradley *et al.*, 1987; Madden & Hunter, 2002; Mättö *et al.*, 2005). We found a marked difference in the proportions of the *C. coccoides-E. rectale* group of clostridia between constipation-type IBS subjects and control subjects. In addition, there was more instability in the DGGE profiles derived from faecal RNA amplicons of the IBS subjects than those of the control subjects. However, further studies are needed with larger subject groups to confirm these findings.

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