Molecular analysis of faecal and duodenal samples reveals significantly higher prevalence and numbers of *Pseudomonas aeruginosa* in irritable bowel syndrome

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Intestinal microbiota may play a role in the pathophysiology of irritable bowel syndrome (IBS). In this case-control study, mucosa-associated small intestinal and faecal microbiota of IBS patients and healthy subjects were analysed using molecular-based methods. Duodenal mucosal brush and faecal samples were collected from 37 IBS patients and 20 healthy subjects. The bacterial 16S rRNA gene was amplified and analysed using PCR denaturing gradient gel electrophoresis (DGGE). Pooled average DGGE profiles of all IBS patients and all healthy subjects from both sampling sites were generated and fingerprints of both groups were compared. The DGGE band fragments which were confined to one group were further characterized by sequence analysis. Quantitative real-time PCR (q-PCR) was used to quantify the disease-associated microbiota. Averaged DGGE profiles of both groups were identical for 78.2 % in the small intestinal samples and for 86.25 % in the faecal samples. Cloning and sequencing of the specific bands isolated from small intestinal and faecal DGGE patterns of IBS patients showed that 45.8 % of the clones belonged to the genus *Pseudomonas*, of which *Pseudomonas aeruginosa* was the predominant species. q-PCR analysis revealed higher levels ($P < 0.001$) of *P. aeruginosa* in the small intestine of IBS patients ($8.3 \% \pm 0.950$) than in the small intestine of healthy subjects ($0.1 \% \pm 0.069$). *P. aeruginosa* was also significantly ($P < 0.001$) more abundant ($2.34 \% \pm 0.31$) in faeces of IBS patients than in faeces of healthy subjects ($0.003 \% \pm 0.0027$). This study shows that *P. aeruginosa* is detected more frequently and at higher levels in IBS patients than in healthy subjects, suggesting its potential role in the pathophysiology of IBS.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a gastrointestinal disorder of unknown aetiology characterized by abdominal pain and change in bowel habit. Alteration in faecal microbiota composition and abnormal colonic fermentation imply that gastrointestinal microbiota may play a role in the pathogenesis of IBS (Spiller, 2007; Quigley, 2007). In 7–30 % of IBS patients, acute gastrointestinal infection has been proposed as trigger of the onset of IBS symptoms, and the inflammatory response to the infection may cause persistent sensory-motor dysfunction (Madden & Hunter, 2002; Rodríguez & Ruigómez, 1999; Parry *et al.*, 2003; Collins *et al.*, 2001). Moreover, antibiotic therapy and

probiotic supplementation have been shown to reduce IBS symptoms in a subset of IBS patients, by either eradication of small intestinal bacterial overgrowth or modulation of the composition of the microbiota (Pimentel *et al.*, 2000; Nobaek *et al.*, 2000; Niedzielin *et al.*, 2001; O'Mahony *et al.*, 2005). Antibiotic therapy may also be a risk factor for developing IBS symptoms due to changes in bowel microbiota and colonization of pathogenic bacteria (Maxwell *et al.*, 2002). It is well recognized that the microbial community is significantly altered in IBS (King *et al.*, 1998; Posserud *et al.*, 2007). Conventional culturing methods have shown that faeces of IBS patients contain higher numbers of facultatively anaerobic bacteria and members of the *Enterobacteriaceae* and lower numbers of lactobacilli and bifidobacteria than those of healthy subjects (Malinen *et al.*, 2005; Balsari *et al.*, 1982; Madden & Hunter, 2002; Si *et al.*, 2004).

Abbreviations: DGGE, denaturing gradient gel electrophoresis; IBS, irritable bowel syndrome; q-PCR, quantitative real-time PCR.

Detection and identification of gut bacteria by culturing is hampered by the limitations of culture conditions, and a large number of intestinal bacteria cannot be cultured (Mai & Morris, 2004; Furrie, 2006; Suau *et al.*, 1999). Molecular techniques based on 16S rRNA gene analysis allow a more comprehensive assessment of this complex microbial ecosystem (Vaughan *et al.*, 2000). Using PCR-based techniques, a significant difference in the predominant faecal groups has been reported in IBS patients (Malinen *et al.*, 2005; Kassinen *et al.*, 2007). Studies investigating the gut microbiota in IBS using molecular-based methods have been limited to the faecal microbiota and have not assessed mucosa-associated microbiota in the small intestine. The mucosa-associated bacteria are thought to have a stronger interaction than luminal bacteria with the host (Hooper & Gordon, 2001). The composition of luminal and mucosa-associated bacteria is not the same since the micro-environments are very different at the surface of the intestinal epithelium than in the lumen (Zoetendal *et al.*, 2002; Lepage *et al.*, 2005).

The aim of our research was to determine differences in mucosa-associated small intestinal and luminal faecal microbiota between IBS patients and healthy subjects using a molecular approach based on the sequence variability of the 16S rRNA gene.

METHODS

Subjects. Thirty-seven IBS patients who fulfilled Rome II criteria for IBS were included in this study. The IBS patient group consisted of 13 alternating IBS patients, 13 diarrhoea-predominant IBS patients and 11 constipation-predominant IBS patients. Twenty healthy subjects (control group) had neither intestinal complaints nor a history of bowel resection. All subjects had to stop antibiotics at least 1 month before endoscopy. Use of probiotics, proton pump inhibitors and antisecretory medication had to be stopped at least 3 weeks prior to endoscopy. The standard preparation for an upper endoscopy in our hospital is fasting 12 h before the endoscopy. At the request of a subject, Xylocaine spray is used to anaesthetize the throat or a sedative (Dormicum 5 mg) is given. The Human Ethics Committee of the University Medical Centre Utrecht approved the study and all subjects gave written informed consent.

Sampling, preparation and storage. A sterile cytology brush (Uno-Brush; Prince Médical) sheathed in a sterile catheter was placed through the endoscope biopsy channel and advanced under direct vision out beyond the endoscope tip (León-Barúa *et al.*, 1993). The duodenal mucosa was brushed three times to obtain mucosa-associated bacteria. Brush samples from each subject were obtained from the descending and horizontal part of the duodenum. After brushing, the brush was pulled back into the sheath of the catheter, which was removed and the brush was immediately cut off the catheter and placed into a sterile tube in liquid nitrogen and stored at -80°C until analysis.

Faecal samples were collected before the endoscopy and were stored at -80°C until analysis. Both faecal and brush samples were shipped in dry ice to the microbiological laboratory for analysis.

DNA extraction and PCR amplification. Faecal samples were thawed and DNA extraction was performed using the Fast DNA Spin kit (Qbiogene) from approximately 0.1 g faecal material. The frozen

brush samples were thawed and suspended in 180 μl ATL buffer (Qiagen) and vigorously vortexed to extract the attached bacteria. Subsequently, DNA was isolated from the mucosa-associated bacteria using the DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instructions. The isolated DNA solutions from both faeces and brush samples were stored at -20°C .

The extracted DNA was used as a template to amplify the V6–V8 regions of the 16S rRNA gene with primers U968-GC-f (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3'), which contains at its 5'-end a 40-base GC clamp, and U1401-r (Muyzer *et al.*, 1993; Muyzer & Smalla, 1998). PCRs were performed using a *Taq* DNA polymerase kit from Invitrogen. The reaction mixture consisted of 1 $\times$ PCR buffer, 3 mM MgCl_2 , 50 μM each deoxynucleotide triphosphate, 1.25 U *Taq* polymerase, 10 pmol each primer and 1 μl appropriately diluted template DNA in a final volume of 50 μl . Samples were amplified in a PTC-200 PCR system (MJ Research) with the following thermocycling program: 94°C for 5 min; 10 cycles of denaturation at 94°C for 1 min, annealing at $65\text{--}56^{\circ}\text{C}$ for 1 min (reduction of 1°C for each cycle) and extension at 68°C for 3 min; 33 cycles for faecal and 43 cycles for brush samples of 94°C for 1 min, 56°C for 1 min and 68°C for 3 min. Aliquots of 5 μl PCR product were analysed by electrophoresis on a 1.5% (w/v) agarose gel containing ethidium bromide (0.044%, v/v).

Denaturing gradient gel electrophoresis analysis of PCR amplicons. PCR amplicons were separated by denaturing gradient gel electrophoresis (DGGE) based on the protocol of Muyzer & Smalla (1998) using the DGGE Decode system (Bio-Rad Laboratories) with the following modifications. Polyacrylamide gels consisted of 8% (v/v) polyacrylamide (ratio of acrylamide/bisacrylamide, 37.5:1) and $0.5 \times$ Tris/acetate-EDTA (TAE; pH 8.0) buffer. Denaturing acrylamide of 100% was defined as 7 M urea and 40% formamide. The polyacrylamide gels were made with a denaturing gradient ranging from 32.5 to 72.5%. The gels were poured from the top using a gradient maker and a pump (Econo gradient pump; Bio-Rad Laboratories) set at a speed of 3 ml min^{-1} . Prior to the polymerization of the denaturing gel (gradient volume, 28 ml), a 7.5 ml stacking gel without denaturing chemicals was added, and the appropriate comb was subsequently inserted. Electrophoresis was performed first for 5 min at 200 V and then at 80 V for 17 h in $0.5 \times$ TAE buffer at a constant temperature of 60°C . The gels were stained with AgNO_3 as described by Sanguinetti *et al.* (1994) and dried overnight at 50°C .

Analysis of the DGGE profiles and generation of pooled profiles. The average curve feature 'show average curve' of GelCompar software (Applied Maths) was used to create averaged fingerprints. This script generates an averaged fingerprint using the densitometric and location results from the bands in the original gels. The intensity of the band in the average IBS patient or healthy subject profile is calculated by the intensity of the original bands in the original gels of all IBS patients or healthy subjects, respectively. From all of the DGGE gels from the IBS patient samples we created the averaged IBS profile and using all of the DGGE gels from the healthy subject samples we created the healthy subjects averaged profile. Percentage similarity between the averaged IBS profile and the averaged healthy subject profile is based on the number of identical bands and the numeric values of the band intensity (with 5% background subtraction) found in the two profiles. Similar band positions are removed, generating a graph with unique bands for healthy subjects and unique bands for IBS patients. Band differences in these average computer-generated profiles were determined and the bands which were unique for either group were localized in the individual original DGGE profiles. The most intense bands in the individual profiles were cut from the original gels. The DNA from

Fig. 1. DGGE analysis of the duodenal mucosa-associated and faecal bacteria. No evident clusters were shown in the clustering tree of the silver-stained DGGE gel of the V6–V8 regions of the 16S rRNA gene, obtained from duodenal samples (a) and faecal samples (b) of 20 healthy subjects (HS), 11 diarrhoea-predominant IBS patients (IBS-D), 13 alternating IBS patients

(IBS-A) and 10 constipation-predominant IBS patients (IBS-C). Dendrograms based on the DGGE gel for each individual were generated by using the Pearson correlation index for each pair of lanes within a gel and were used as a measure of similarity between the fingerprints. The scale bar indicates the DGGE similarity between profiles. The clustering of patterns was calculated using the unweighted-pair group method using average linkages.

these bands was used for a second DGGE analysis using a 46–51.6 % gradient. Bands of interest were subjected to sequencing as described below.

DGGE band sequence analysis. To identify the bacteria, a small piece from the middle of the selected band was cut from the DGGE gel with a sterile scalpel and then incubated in 50 µl sterile MilliQ water for 24 h at 4 °C to allow diffusion. The eluent containing DNA fragments was used for PCR reamplification with the same primers used earlier. To check whether the DNA of interest on the first DGGE gel and the reamplified DNA migrated to the same position, we carried out a second DGGE in order to compare the two samples. When the two bands co-migrated, DNA fragments were purified with the GenElute PCR DNA Purification kit (Sigma) and thereafter ligated into the pCR2.1-TOPO vector and transformed into *Escherichia coli* One Shot TOP10 competent cells (Invitrogen). Plasmids from colonies of kanamycin-resistant transformants were extracted with the Qiagen Plasmid Midi Purification kit. The extracted plasmids were screened for inserts of the correct size by performing a PCR with the M13 forward and reverse primers as well as with the 968f and 1401r primers. Insert PCR amplicons of selected transformants were purified and subjected to DNA sequence analysis (Baseclear). Sequence similarities of the clones were checked with the Basic Local Alignment Search Tool (BLAST) at the NCBI database (<http://www.ncbi.nlm.nih.gov/BLAST>) (Altschul *et al.*, 1990).

Quantitative real-time PCR analysis. Quantitative real-time PCR (q-PCR) was performed to determine the percentage of *Pseudomonas aeruginosa* in brush and faecal samples. Measurement of *P. aeruginosa* was performed as described by Pirnay *et al.* (2000) with some slight modifications. The detection limit of the assay was 10^3 c.f.u. g⁻¹ and 1–10 c.f.u. per reaction (Pirnay *et al.*, 2000). The fluorescent labels were changed from LC Red 640 to 6FAM and from fluorescein to TAMRA while the other ingredients and conditions for the described q-PCR stayed the same. The fluorescence signal was measured in the annealing phase on the ABI 7900HT Fast system (Applied Biosystems). The total bacterial load was determined as described by Nadkarni *et al.* (2002). The relative percentage of *P. aeruginosa* was subsequently calculated according to Liu & Saint (2002a, b). The efficiency of each amplification curve was calculated separately and used to determine the initial relative amounts of DNA. The amount used was in general between 100 and 200 ng DNA per reaction. Finally, the obtained ratios between the initial amounts of DNA were normalized against a monoculture of the same species, which was set at 100 %.

Statistical analysis. DGGE data were analysed by GelCompar II software (Applied Maths). Cluster analysis and calculation of the similarity indices between the different banding patterns were performed using Pearson product-moment correlation and the unweighted-pair group method using arithmetic averages.

P. aeruginosa levels in IBS patients and healthy subjects were analysed using independent samples *t*-test. *P. aeruginosa* levels in IBS subgroups were analysed using one-way ANOVA with Bonferroni correction. Data are expressed as mean $\pm$ SEM. SPSS 12.0.1 for Windows was used for analysis.

RESULTS

Study population

Healthy subjects (5 men, 15 women) were significantly ($P=0.005$) younger (32 ± 2.6 years) than the IBS patients (11 men, 26 women; 42 ± 2.3 years). No differences between both groups in gender distribution were observed.

Bacterial diversity of the dominant microbiota as assessed by DGGE

DGGE analysis of the PCR-amplified fragments of the V6–V8 region of the 16S rRNA gene of both duodenal mucosa-associated bacteria (Fig. 1a) and faecal bacteria (Fig. 1b) from IBS patients and healthy subjects was performed, showing no evident clusters between healthy subjects and IBS subgroup patients. Comparing the fingerprints of faecal and small intestinal samples for all subjects, a clear clustering was observed independently from the disease state (data not shown), indicating that the mucosa-associated microbiota is significantly different from the faecal microbiota, as also observed in a previous study (Zoetendal *et al.*, 2002). Based on the DGGE profiles, no clear difference between the IBS patients and healthy subjects could be detected for the most abundant microbiota at any sampling location. Moreover, statistical analysis of the DGGE profiles from both sampling sites (duodenal brush and faecal samples) did not reveal any specific core microbiota that could be distinguished between healthy subjects, IBS patients or IBS subgroup patients. However, some subclusters could be identified, suggesting overlap of specific microbial components. To identify possible microbial components specifically associated with healthy subjects or with IBS patients, the DGGE profiles generated from all IBS patients and those from healthy subjects were pooled in average profiles (Figs 2 and 3).

Comparing the generated average duodenal fingerprints of healthy subjects and IBS patients, 14 (78.2 %) of the bands were identical for both groups, 6 bands were specific for IBS patients (10 % of all IBS bands) while 27 bands were specific for healthy subjects (34 % of all healthy subject bands) (see Fig. 2). The band location and intensity in the average profiles were used to generate Fig. 2.

Fig. 3 shows the average faecal fingerprints of both groups, in which 21 (86.25 %) of the bands were identical, 33 bands were confined to healthy subjects (21 % of all healthy subject bands) and 17 bands were confined to IBS patients (10 % of all IBS bands). The DGGE band fragments that were of interest were further characterized by sequence analysis.

Sequence analysis of the dominant microbiota

The 16S rRNA genes from the isolated bands from the DGGE profiles of faecal and duodenal samples from the healthy subjects and IBS patients were amplified and cloned in *E. coli*. The V6–V8 region of the cloned 16S rRNA gene fragments from the cell lysates of transformants was amplified. In this way, 51 clones were generated, the plasmid DNA from the corresponding clones was purified and, subsequently, the nucleotide sequence inserts were determined and compared to the 16S rRNA databases using the BLAST tool (Zhang *et al.*, 2000). From the duodenal samples, a total of 19 clones, comprising 15 clones recovered from 4 unique bands from the IBS

patients and 4 clones from 1 unique band from the healthy subjects, were generated. A total of 32 clones retrieved from faecal samples, comprising 24 clones from 3 unique bands from IBS patients and 8 clones from 1 unique band from healthy subjects, were obtained.

Among the 51 sequenced clones retrieved from both groups, 35 shared 95 % or less 16S rRNA sequence identity with their nearest relatives based on a BLAST search. This indicated that the majority of the sequences were derived from new, as yet undescribed, phylotypes. Most cloned sequences retrieved from the IBS patients could be assigned to the class *Gammaproteobacteria* mainly to *Pseudomonas* species, and other clones retrieved essentially from the faecal samples were assigned to *Clostridium nexile*. However, in the healthy subjects, the cloned sequences were allocated to the two major phylotypes commonly encountered in human faecal clone libraries, namely *Clostridium* cluster XIVa and *Bacteroidetes*.

Brush samples. Out of the 15 clones retrieved from the brush samples from IBS patients, seven cloned sequences were affiliated with different *Pseudomonas* species with a sequence similarity ranging from 94 to 99 %. Remarkably, one cloned sequence was almost identical to *P. aeruginosa* (99 % sequence similarity). Three clones were closely related to *Klebsiella pneumoniae* with 96–97 % sequence similarity, one clone was almost identical to *Oribacterium sinus* (99 % sequence similarity) and there were two new phylotypes (90–96 % sequence similarity to an uncultured bacterium clone, rc2-18, isolated from rat faeces; Brooks *et al.*, 2003). In addition, one clone showed 96 % sequence similarity with an uncultured *Neisseria* sp. clone isolated from *P. aeruginosa*-colonized patients (Flanagan *et al.*, 2007) and shared only 91 % similarity with the closest cultured relative, i.e. *Kingella kingae* strain ATCC 23330. Another clone was related to uncultured *Neisseria* sp. clone 101C07 isolated from the oral cavity with only 91 % sequence similarity.

The four cloned sequences obtained from the healthy subjects were identified as *Serratia* sp., *Acinetobacter* sp., *Pantoea* sp. and an uncultured *Clostridiales* bacterium with 99, 98, 91 and 95 % sequence similarity, respectively.

Faecal samples. Out of the 24 clones retrieved from faecal samples from IBS patients, 11 cloned sequences were affiliated with different *Pseudomonas* species with a sequence similarity ranging from 85 to 100 %. Noticeably, one cloned sequence showed a perfect match with *P. aeruginosa* and three other sequences shared 88–95 % similarity with the same species. Furthermore, eight clones were affiliated with different *Clostridium* species. Among these, six clones were closely related to *C. nexile* with sequence similarity varying from 93 to 97 %, and two clones were identified as *Clostridium* sp. with sequence similarity varying from 92 % to 96 %. One clone was identified as *Desulfovibrio vulgaris* (98 % similarity), and one clone was related to an uncultured faecal bacterium clone isolated by

Li *et al.* (2008) with 94 % sequence similarity. In addition, two clones shared only 92 % and 91 % sequence similarity with *Burkholderia mallei* and finally one cloned sequence was related to *Alcaligenes faecalis* with 92 % sequence similarity.

Out of the eight clones from the faecal samples from healthy subjects, five cloned sequences were affiliated with different *Bacteroides* species with a sequence similarity ranging from 95 to 99 %, with the closest culturable species being *Bacteroides ovatus* (97 % sequence similarity), *Bacteroides vulgatus* (96 % sequence similarity) and *Bacteroides coprophilus* (99 % sequence similarity). Furthermore, one clone was almost identical to *Prevotella corporis* (99 % sequence similarity), one clone shared 95 % sequence similarity with *Prevotella oulorum* and one clone shared 97 % sequence similarity with *Clostridium butyricum*.

Quantification of *P. aeruginosa* using real-time PCR

Since the majority of IBS unique bands belonged to the genus *Pseudomonas*, of which *P. aeruginosa* was the most frequently identified species, real-time PCR was used to quantify *P. aeruginosa* in the brush and faecal samples.

The data in Fig. 4(a) show that the relative abundance of *P. aeruginosa* (% of total bacterial load) was significantly ($P < 0.001$) higher in IBS patients ($8.3 \% \pm 0.950$) than in healthy subjects ($0.1 \% \pm 0.069$). The C_t value of the positive controls, negative control (water), all IBS patients and healthy controls was <22 , >45 , 30.4 (SEM 0.84) and 36.6 (SEM 0.83), respectively. Moreover, the prevalence of *P. aeruginosa* in the duodenal samples was 97.3 % in IBS patients and only 40 % in healthy subjects.

The results illustrated in Fig. 4(b) reveal that the percentage of *P. aeruginosa* in faecal samples was also significantly higher ($P < 0.001$) in IBS patients ($2.34 \% \pm 0.31$) than in healthy subjects ($0.003 \% \pm 0.0027$). We also found that *P. aeruginosa* was detected in 97.2 % of the faecal samples from IBS patients whereas only 15.8 % of the healthy subjects were positive carriers of *P. aeruginosa*. No differences related to the abundance and prevalence of *P. aeruginosa* between IBS subgroups were detected in both sampling sites.

DISCUSSION

This study shows clearly that, analysing the entire faecal and small intestinal microbiota population using DGGE, there are specific DGGE bands for healthy subjects and specific bands for IBS patients. Focussing on the specific bands showed that most clones belonged to the genus *Pseudomonas*, of which *P. aeruginosa* was the predominant species. The most important finding of this study is the higher prevalence and levels of *P. aeruginosa*

Fig. 2. Average DGGE profiles of all fingerprints from IBS patients ($n=34$) and all fingerprints from healthy subjects ($n=20$) in duodenal samples constructed using GelCompar II software. IBS represents the average of 34 IBS patients and HS represents the average profile of 20 healthy subjects. The graph shows a numerical presentation of the average profile with the unique bands present in the healthy subjects on top and the unique bands for the IBS group on the bottom of the graph. AU, Arbitrary units.

in small intestinal and faecal samples from IBS patients than in those from healthy subjects, analysed by q-PCR.

Previously, Kassinen *et al.* (2007) also showed distinct microbiota based on their mol% G+C fractions in faecal samples from IBS subtypes and from controls using molecular tools. After faecal microbial genomes of IBS subtypes and controls were pooled, differences in the *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* were shown (Kassinen *et al.*, 2007). Our sequence analysis data showed that members of the *Proteobacteria* and *Firmicutes* were specific for IBS patients. *Pseudomonas* sp. was the most prevalent and q-PCR analysis showed a higher prevalence and levels of *P. aeruginosa* in small intestinal mucosa-associated and faecal samples from IBS patients than in those from healthy subjects.

P. aeruginosa, a Gram-negative aerobic rod, is known to predominantly infect immunosuppressed and cystic fibrosis patients (Oliver *et al.*, 2000; Fichtenbaum *et al.*, 1994; Baron & Hollander, 1993). The 15.8% prevalence of

P. aeruginosa in faecal samples from healthy subjects in this study is within the range of 0–24% reported in other studies (Bonten *et al.*, 1999; Speert *et al.*, 1993; Kessner & Lepper, 1967; Bleday *et al.*, 1993; Levy, 2000). Higher prevalence (97%) and levels of *P. aeruginosa* in IBS patients have not been reported in the literature to our knowledge. However, in functional dyspepsia patients, *Pseudomonas* was cultured as the predominant bacterium in the small intestine (León-Barúa *et al.*, 1993). Previous studies using molecular-based methods to determine the quantity of faecal microbiota in IBS patients and healthy subjects showed differences in microbiota composition (Kassinen *et al.*, 2007; Malinen *et al.*, 2005). However, *P. aeruginosa* levels were not tested in these studies.

The age differences between the healthy subjects and IBS patients in our study might be a confounding factor. The effect of the age difference between healthy subjects (mean age 32 years) and IBS patients (mean age 42 years) on bacterial DNA load, prevalence and quantity of *P. aeruginosa* is not known. Current literature does describe change in faecal microbiota composition in subjects older

Fig. 3. Average DGGE profiles of all fingerprints from IBS patients ($n=34$) and all fingerprints from healthy subjects ($n=20$) in faecal samples constructed using GelCompar II software. IBS represents the average of 34 IBS patients and HS represents the average profile of 20 healthy subjects. The graph shows a numerical presentation of the average profile with the unique bands present in the healthy subjects on top and the unique bands for the IBS group on the bottom of the graph. AU, Arbitrary units.

Fig. 4. Duodenal *P. aeruginosa* as a percentage of total bacterial load of healthy subjects (HS), IBS patients (IBS) and the IBS subgroups [alternating IBS patients (IBS-A), diarrhoea-predominant IBS patients (IBS-D) and constipation-predominant IBS patients (IBS-C)] in duodenal samples (a) or faecal samples (b). Values are represented as means $\pm$ SEM. * $P < 0.001$.

than 65 years of age compared to younger adults (Hopkins & Macfarlane, 2002; Woodmansey *et al.*, 2004; Mueller *et al.*, 2006). To evaluate the effect of age difference in adult subjects on microbiota composition, further investigations are needed.

The question arises whether differences in prevalence and quantity of *P. aeruginosa* might be epiphenomenal or be the cause of IBS symptoms. First of all, it is known that *P. aeruginosa* may occur as the sole potential pathogen in patients with diarrhoea and *P. aeruginosa* induces signs and symptoms of enteritis in antibiotic-treated rats (Adlard *et al.*, 1998). Also, a causative relationship between *P. aeruginosa* and diarrhoea has been found in immunocompromised individuals (Adlard *et al.*, 1998). However, in our study, *P. aeruginosa* was increased not only in diarrhoea-predominant IBS patients, but also in constipation-predominant and alternating IBS patients.

Secondly, proteases of *P. aeruginosa* are known to disable protease-activated receptor-2 (PAR-2) in the respiratory

tract (Dulon *et al.*, 2005). The effect of *P. aeruginosa* proteases on PAR-2 in the gastrointestinal tract is unknown. PAR-2 activation in the gastrointestinal tract has been shown to modify motility patterns, inflammatory mediator release, intestinal barrier integrity, ion transport and nociceptive functions, all functions which are part of gut physiology involved in generation of IBS symptoms (Vergnolle, 2005).

Indication that increased *P. aeruginosa* might be epiphenomenal is reported in a recent study which showed high levels of *Pseudomonas* species in pouchitis patients in remission. If the normal microbiota of the gastrointestinal tract is altered, potential pathogens can colonize or resident pathogens can multiply. The remission of the pouchitis patients was induced by antibiotic therapy (Kühbacher *et al.*, 2006). It has been shown that antibiotic treatment of mice increases the colonization potential of *Pseudomonas* species (George *et al.*, 1989). *P. aeruginosa* is naturally resistant to many antibiotics due to the permeability barrier of its outer membrane lipopolysaccharide, tendency to colonize surfaces in a biofilm form and maintenance of antibiotic-resistance plasmids (Mah *et al.*, 2003; Walters *et al.*, 2003; Masuda *et al.*, 1999; Livermore, 1992). Antibiotics and probiotics used more than a month before the start of the study are unlikely to have had an effect on the *P. aeruginosa* colonization since stabilization of the intestinal microbiota within 1–2 weeks after antibiotic therapy has been reported (Woodmansey *et al.*, 2004; Edlund & Nord, 2000; Hentges *et al.*, 1985; Levison, 1973; Gorbach *et al.*, 1988).

In a previous study, we showed a lower *Bifidobacterium catenulatum* level in both faecal and duodenal brush samples from IBS patients than in those from healthy subjects (Kerckhoffs *et al.*, 2009). This decrease in normal microbiota might give *P. aeruginosa* the opportunity to multiply. A major obstacle in relating microbial composition with a role in IBS pathophysiology is the fact that each individual is inhabited by a specific microbial community shaped by host and environmental factors (Eckburg *et al.*, 2005).

In conclusion, our data show no apparent difference in the diversity of predominant microbiota profiles between IBS patients and healthy subjects. However, using the pooled average profiles of the PCR-DGGE fingerprints allowed us to isolate and sequence specific bands from IBS patients and healthy subjects. IBS-specific bands were predominantly members of the genus *Pseudomonas*. q-PCR analysis confirmed that *P. aeruginosa* is found more frequently and in higher levels in IBS patients than in healthy subjects. Therefore, we propose that *P. aeruginosa* may be involved in the pathophysiology of IBS.

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