

Lower *Bifidobacteria* counts in both duodenal mucosa-associated and fecal microbiota in irritable bowel syndrome patients

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CONCLUSION: Decreased bifidobacteria levels in both fecal and duodenal brush samples of IBS patients compared to healthy subjects indicate a role for microbiotic composition in IBS pathophysiology.

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Abstract

AIM: To determine the composition of both fecal and duodenal mucosa-associated microbiota in irritable bowel syndrome (IBS) patients and healthy subjects using molecular-based techniques.

METHODS: Fecal and duodenal mucosa brush samples were obtained from 41 IBS patients and 26 healthy subjects. Fecal samples were analyzed for the composition of the total microbiota using fluorescent *in situ* hybridization (FISH) and both fecal and duodenal brush samples were analyzed for the composition of bifidobacteria using real-time polymerase chain reaction.

RESULTS: The FISH analysis of fecal samples revealed a 2-fold decrease in the level of bifidobacteria (4.2 ± 1.3 vs 8.3 ± 1.9 , $P < 0.01$) in IBS patients compared to healthy subjects, whereas no major differences in other bacterial groups were observed. At the species level, *Bifidobacterium catenulatum* levels were significantly lower (6 ± 0.6 vs 19 ± 2.5 , $P < 0.001$) in the IBS patients in both fecal and duodenal brush samples than in healthy subjects.

INTRODUCTION

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder characterized by abdominal pain or discomfort and altered bowel function. Alterations in psychosomatic factors, gastrointestinal motility, visceral hypersensitivity and microbiotic composition have been suggested to play a role in the pathophysiology of IBS^[1]. Alterations in fecal and small intestinal microbiotic composition in IBS patients have been reported and studies revealed a somewhat higher bacterial count in jejunal juice of IBS patients and lower numbers of fecal coliforms, lactobacilli and bifidobacteria than in healthy subjects^[2,3]. More specifically, molecular-based methods showed that in IBS patients levels of members of the *Clostridium coccoides* subgroup, *Lactobacillus*, *Collinsella* and *Bifidobacterium catenulatum* groups are different from that of healthy subjects^[4,5]. These differences in the fecal microbiotic composition may underlie

symptom generation by promoting abnormal colonic fermentation^[6]. However, mucosa-associated bacteria might be more relevant in the symptom generation of IBS, since fecal and jejunal juice samples are only representing the composition of luminal microbiota. Alterations in luminal bacteria composition may change the commensal microbiota and affect the microbiota adhering to the mucosa. Microorganisms adhering to the intestinal wall are more likely to affect the host's immune, physiological or neuronal system or vice versa. The composition of luminal and mucosa-associated bacteria are not the same since the micro-environments are different at the surface of the intestinal epithelium and the lumen^[7,8]. Therefore, we aimed to determine the composition of fecal luminal and mucosa-associated microbiota in IBS patients using molecular identification and quantification techniques.

MATERIALS AND METHODS

Subjects

Twelve male and 29 female IBS patients included in this study fulfilled the Rome II criteria for IBS and were categorized as diarrhea predominant (IBS-D), constipation predominant (IBS-C) or alternating IBS subgroup (IBS-A)^[9]. The IBS population consisted of 14 IBS-D subjects, 11 IBS-C subjects and 16 IBS-A subjects. The control group consisted of 8 male and 18 female healthy subjects from the general population, devoid of GI symptoms or major abdominal surgery. The healthy subjects were significantly ($P < 0.001$) younger (31 ± 2.06 years) than the group of IBS patients (42 ± 2.12 years). Subjects taking medication known to influence bacterial composition and gastrointestinal motility, especially antimicrobial medications and/or probiotics were excluded from the study. The Human Ethics Committee of the University Medical Centre Utrecht approved the study and all subjects gave written informed consent.

Sampling

To obtain small intestinal mucosa-associated material a sterile cytology brush (Uno-Brush, Prince Médical, Ercuis, France) sheathed in a sterile catheter was placed through the endoscope biopsy channel and advanced under direct vision out beyond the endoscope tip^[10]. The duodenal mucosa was brushed three times and then pulled back into the sheath of the catheter. The catheter 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. Fecal samples, obtained before endoscopy, were collected and stored at -80°C until further handling.

Fluorescent in situ hybridization (FISH) analysis of fecal samples

The total number of bacteria present in the fecal samples was determined with the EUB 338 probe which targets all bacteria^[11]. FISH analysis was essentially

Table 1 Genus-specific probes used for FISH analysis

Probe	Target group	Ref.
EUB 338	Total bacteria	[44]
Bac 303	<i>Bacteroides-Prevotella</i> group	[45,46]
Bif 164	<i>Bifidobacterium</i>	[12]
Erec 482	<i>Clostridium coccooides-Eubacterium rectale</i> group	[47]
Chis 150	<i>Clostridium histolyticum</i> group	[47]
Clit 135	<i>Clostridium lituseburense</i> group	[47]
Cld 73	<i>Clostridium difficile</i>	This study: CGCCGC TCITTACCGAAGT
Fprau 645	<i>Faecalibacterium prausnitzii</i>	[46,48]
Lab 158	<i>Lactobacillus-Enterococcus</i> group	[49]

performed as described previously with the genus-specific probes listed in Table 1^[12]. Approximately 0.5 g of homogenized feces was suspended in 4 mL of 0.2 mm-pore-size-filtered PBS and 0.5 mL 37% formaldehyde and thoroughly mixed by vortexing for 3 min. After incubation for 4 h at 4°C the suspension was vortexed again for 2 min. Debris was removed by a short spin at 80 g for 1 min. In an eppendorf tube, 300 μL of the supernatant was collected and the fixed cells were washed twice with PBS. For FISH analysis of the *Lactobacillus-Enterococcus* group the cells were first permeabilized by resuspending the pellet in 100 μL Proteinase-K solution (180 kU/L) (Sigma-Aldrich, Zwijndrecht, The Netherlands) and incubated for 10 min at 37°C . The cells were washed as described above and resuspended in 300 μL PBS/ethanol (1:1 v/v). After one hour of storage at -20°C , the cell suspension was diluted 1:10 in hybridization buffer at the required temperature for hybridization, and 5 ng labeled probe was added. Cells were hybridized for 16 h at the prescribed hybridization temperature. After resuspension in 4 mL washing buffer, cells were filtered on a 0.2 mm pore size Isopore polycarbonate membrane filter (Millipore Corporation) and washed with 5 mL of 50°C washing buffer. Filters were mounted on microscope slides with Vectashield (Vector Laboratories, Burlingame, CA, USA), and hybridized cells were counted visually using an Olympus BX60 epifluorescence microscope using a FITC or Cy3-specific filter. All microscopic counts were determined in duplicate, with a minimum of 300 cells counted per assay.

DNA extraction and polymerase chain reaction (PCR) amplification of fecal and duodenal brush samples

Brush and fecal samples were thawed on ice cooled water. DNA was extracted using the DNeasy Tissue kit (Qiagen, Venlo, The Netherlands) or the Fast DNA Spin kit (Qbiogene, Irvine, USA) from the brush and fecal samples respectively. The eluted DNA samples were stored at -20°C . The integrity of the isolated DNA was determined visually after electrophoresis on a 1.0% agarose gel containing ethidium bromide.

Real-time PCR

Quantification of *Lactobacilli* genera and species

specifically belonging to bifidobacteria was performed using a 5' nuclease (TaqMan) assay as described previously^[13,14]. Briefly, a 20 µL PCR amplification mixture containing 10 µL TaqMan Fast Universal Master Mix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), optimized concentrations of primers and probes and 2.0 µL isolated DNA was prepared. The temperature profile for the amplification consisted of 20 s at 95°C and 45 cycles of 1 s at 95°C and 20 s at 60°C (ABI 7900 HT Fast; Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The percentages of the different bacteria were subsequently calculated as described by Liu *et al.*^[15,16].

Statistical analysis

FISH results with microbial numbers below the detection limit ($8.3 \times 10^5/g$) were excluded from statistical analysis. Nonparametric FISH microbiota data were compared using Mann-Whitney tests or Kruskal-Wallis test for subgroup analysis. Independent samples *t*-test was used to compare differences in real-time PCR microbiota data between IBS patients and healthy subjects. One-way ANOVA with Bonferroni correction was used for analysis of microbiota in the IBS subgroups.

P-values less than 0.05 were considered statistically significant. All statistical analysis was performed using commercially available software (SPSS 12.0.1 for Microsoft Windows). Data are expressed as mean ± SE.

RESULTS

Characterization of the fecal microbiota of IBS patients and healthy subjects

The mean percentages of all bacterial groups measured are presented in Table 2. The results show that *F. prausnitzii*, *E. rectale/C. coccoides* and bifidobacteria are the most abundant groups in both IBS patients and healthy subjects. The levels of bifidobacteria were significantly lower ($P < 0.05$) in IBS patients ($4.2\% \pm 1.3\%$) than in healthy subjects ($8.3\% \pm 1.9\%$). No significant differences were observed between IBS-D, IBS-C and IBS-A subgroups. The *C. lituseburense* group was detected in significantly lower levels ($P < 0.01$) in IBS patients compared to healthy subjects; however, *C. lituseburense* reached the detection limit only in 14 healthy subjects and 18 IBS patients. The proportions of *Lactobacillus* spp, *C. coccoides*, *C. histolyticum*, *C. difficile*, *Bacteroides* and *F. prausnitzii* showed no differences between IBS patients and healthy subjects. This set of probes covered 44% and 32% of the total fecal microbiota in the healthy subjects and IBS patients, respectively. The low coverage is predominantly due to low counts in *Bacteroides*.

Characterization of the fecal bifidobacteria microbiota of IBS patients and healthy subjects

In healthy subjects, the proportion of bifidobacteria identified as *Bifidobacterium catenulatum* ($19.31\% \pm 2.5\%$) was significantly ($P < 0.001$) higher compared to IBS patients ($6.24\% \pm 0.6\%$). The low proportion of *B. catenulatum* was consistent in all IBS subgroups (Table 3,

Table 2 FISH analysis of the composition of the fecal microbiota of HS, IBS patients and IBS subgroups

Probe	HS	IBS	IBS-A	IBS-D	IBS-C
Fprau 645	12.0 ± 2.1	9.2 ± 0.80	10.6 ± 1.6	8.2 ± 1.4	8.6 ± 1.2
Erec 482	16.6 ± 5.4	11.7 ± 2.5	6.4 ± 1.3	7.1 ± 1.2	20.5 ± 5.8
Bif 164	8.3 ± 1.9	4.2 ± 1.3 ^a	1.7 ± 0.63	7.9 ± 5.2	4.5 ± 0.94
Lab 158	4.7 ± 0.88	4.0 ± 0.78	2.0 ± 0.38	4.0 ± 1.7	6.0 ± 1.5
Chis 150	2.4 ± 0.43	2.1 ± 0.57	2.0 ± 0.61	1.6 ± 0.37	2.4 ± 1.4
Bac 303	1.5 ± 0.89	3.6 ± 1.5	0.41 ± 0.21	4.4 ± 3.3	5.7 ± 2.8
Cld73	0.88 ± 0.28	0.43 ± 0.09	0.61 ± 0.16	0.32 ± 0.15	0.21 ± 0.08
Clit 135	0.39 ± 0.10	0.09 ± 0.03 ^a	0.06 ± 0.03	0.09 ± 0.04	0.11 ± 0.06
Sum	44.4 ± 6.85	32.1 ± 3.27	22.7 ± 2.30	28.3 ± 5.90	43.5 ± 5.89

^a $P < 0.05$ vs HS. Data are expressed as percentage, mean ± SE.

Table 3 Real time PCR analysis of fecal bifidobacteria in HS, IBS patients and IBS subgroups

	HS	IBS	IBS-A	IBS-D	IBS-C
<i>B. catenulatum</i>	19.31 ± 2.5	6.24 ± 0.6 ^b	6.57 ± 1.1 ^b	5.67 ± 0.8 ^b	6.49 ± 1.2 ^b
<i>B. adolescentis</i>	17.05 ± 2.5	15.96 ± 1.6	16.86 ± 3.4	16.73 ± 1.8	14.06 ± 3.05
<i>B. bifidum</i>	2.1 × 10 ⁻⁴	9.1 × 10 ⁻⁴	3.3 × 10 ⁻⁴	1.9 × 10 ⁻³	4.5 × 10 ⁻⁴
	±	±	±	±	±
	1.4 × 10 ⁻⁴	6.3 × 10 ⁻⁴	2.2 × 10 ⁻⁴	1.8 × 10 ⁻³	3.1 × 10 ⁻⁴
<i>B. longum</i>	7.11 ± 1.4	7.30 ± 0.8	6.68 ± 1.4	8.71 ± 1.7	6.45 ± 1.33

^b $P < 0.001$ vs HS. Data are expressed as percentage, mean ± SE.

Figure 1A). The proportions of the other species (*Bifidobacterium adolescentis*, *Bifidobacterium bifidum* and *Bifidobacterium longum*) were not significantly different between healthy subjects, IBS patients and IBS subgroups. Low levels of *B. bifidum* were detected in fecal samples of all subjects as compared to the other *Bifidobacterium* species and as compared to the *B. bifidum* level in duodenal samples (Tables 3 and 4). The bifidobacterial species covered by these Q-PCR assays were only 43% and 29.5% of the total bifidobacteria population for healthy subjects and IBS patients, respectively.

Characterization of the duodenal microbiota of IBS patients and healthy subjects

In healthy subjects, *B. catenulatum* level as percentage of total bifidobacterial load ($17.04\% \pm 2.3\%$) was significantly ($P < 0.001$) higher when compared to IBS patients ($4.85\% \pm 0.5\%$). The significantly lower proportion of *B. catenulatum* was observed in all IBS subgroups (Table 4, Figure 1B). The levels of *B. adolescentis*, *B. bifidum* and *B. longum* as percentage of total bifidobacterial load were comparable between healthy subjects, IBS patients and IBS subgroups (Table 4). With the set of probes used, the total percentage of bifidobacteria of the bifidobacterial load which could be detected is 46% for healthy subjects and 31% for IBS patients.

Characterization of *B. catenulatum* in age-matched IBS patients and healthy subjects

Since the patients and healthy subjects were not matched, the age difference between the healthy subjects and IBS patients may be a confounding factor. In a subset of the subjects, 19 IBS patients (33 ± 2.8) matched for age

Table 4 Duodenal mucosa-associated bifidobacteria in HS, IBS patients and IBS subgroups

	HS	IBS	IBS-A	IBS-D	IBS-C
<i>B. catenulatum</i>	17.04 ± 2.3	4.85 ± 0.5 ^b	5.26 ± 0.9 ^b	4.26 ± 0.7 ^b	5.04 ± 1.0 ^b
<i>B. adolescentis</i>	15.68 ± 2.3	13.90 ± 1.4	14.66 ± 2.7	14.58 ± 1.7	12.21 ± 3.0
<i>B. bifidum</i>	6.09 ± 0.9	5.13 ± 0.8	5.36 ± 1.7	4.43 ± 1.1	5.68 ± 1.3
<i>B. longum</i>	6.88 ± 1.3	7.27 ± 0.8	5.84 ± 1.3	9.01 ± 1.6	6.88 ± 1.40

^b*P* < 0.001 vs HS. Data are expressed as percentage, mean ± SE.

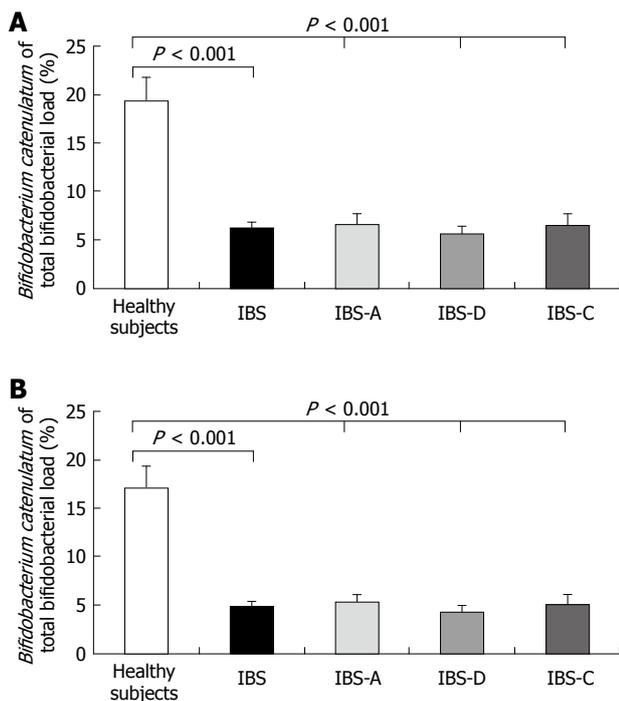


Figure 1 Percentage of *Bifidobacterium catenulatum* as percentage of total bifidobacterial load in fecal samples and duodenal samples of healthy subjects, IBS patients and IBS subgroups (mean ± SE). A: Fecal samples; B: Duodenal samples.

with 19 healthy subjects (33 ± 2.7), decreased levels of *B. catenulatum* were also shown in duodenal as well as in fecal samples of the IBS patients. The mean percentage of *B. catenulatum* of total bifidobacterial load in duodenal samples was significantly ($P < 0.001$) lower in IBS patients ($5.48\% \pm 0.60\%$) compared to healthy subjects ($17.19\% \pm 2.43\%$). Percentage of *B. catenulatum* of total bifidobacterial load in the fecal samples was significantly ($P < 0.001$) lower in IBS patients ($6.98\% \pm 0.69\%$) compared to healthy subjects ($19.50\% \pm 2.67\%$).

DISCUSSION

Composition of gastrointestinal microbiota is known to be relatively stable and composed of permanent, the so-called core phyla, and transient species which contribute to gastrointestinal health and disease^[17-19]. The presence of beneficial microbes in the intestine prevents colonization by potentially pathogenic microbes, referred to as colonization resistance^[20,21]. Imbalances in the microbiota are characterized by a decrease in beneficial anaerobic bacteria and increases in aerobic bacteria,

fungi and harmful anaerobic bacteria^[20].

In this study, we showed, using FISH analysis, that IBS patients have significantly lower fecal levels of bifidobacteria but no differences in the other major bacterial groups. Previous studies have also shown microbial alterations in fecal samples of IBS patients using both culturing and molecular-based techniques^[2,4]. Using culturing techniques, Balsari *et al*^[2] have already shown in 1982 a decrease in bifidobacteria, coliforms and lactobacilli in IBS patients. Using molecular-based techniques decreased *B. catenulatum*, *C. coccoides*, *Lactobacillus* and *Collinsella* counts in the fecal samples of IBS patients were found^[4,5]. These studies were limited to the fecal flora. We broadened the study by examining bifidobacteria levels in duodenal mucosa-associated samples.

Differences in microbiotic composition between luminal and mucosa-associated bacteria have been shown^[7,22-25]. The different micro-environment of the epithelium compared to the lumen might lead to a different microbiotic composition^[7,26]. Bacteria that attach to the mucosa may exert greater influence on innate immune processes in the intestine^[24,27]. In addition, the adhesion of non-pathogenic bacteria to the epithelial surface may contribute to the barrier that effects host resistance to pathogenic bacteria^[24].

In this study, we showed that the percentages of *B. bifidum* of the total bifidobacterial counts were lower in the fecal samples than in the duodenal mucosa-associated samples in both IBS patients and controls. This might be due to high hydrophobicity of *B. bifidum* which is related to its ability to adhere to surfaces^[28,29]. Furthermore, *B. catenulatum* counts were decreased in duodenal mucosa-associated samples as well as in fecal samples of IBS patients compared to controls. The effect of *B. catenulatum* on the health of the host is unknown. However as a group, bifidobacteria are considered beneficial for the host, as they produce lactic and acetic acids that decrease pH and inhibit the growth of potential pathogenic bacteria^[30-32].

Moreover, *Bifidobacterium* spp prevent diarrhea and intestinal infections, alleviate constipation and stimulate the immune system^[31]. The lower levels of bifidobacteria might be epiphenomenal or develop as a consequence of altered gastrointestinal motility or genetic makeup of IBS patients rather than being the cause of IBS symptoms^[31].

Since the patients and healthy subjects were not matched, the age difference between the healthy subjects and IBS patients might have been a confounding factor. It was reported that elderly (> 65 years old) have lower fecal levels of *B. catenulatum*^[33,34]. The elderly were not included in our study. The effect of the age difference between healthy subjects (mean age 32 years) and IBS patients (mean age 42 years) on *B. catenulatum* levels is not known. However, in age-matched IBS patients and healthy subjects statistically significantly decreased levels of *B. catenulatum* were also seen in duodenal as well as in fecal samples of the IBS patients.

An imbalanced microbiotic composition may lead to a different fermentation pattern, especially with an increased hydrogen production resulting in bloating^[6,35].

Both antibiotics and probiotics have been shown to reduce IBS symptoms, which further suggests that microbial imbalance may underlie symptom generation in IBS patients^[36-40]. Previously, a therapeutic trial suggested that particular *B. infantis* species were efficacious in the treatment of IBS symptoms^[41]. No effects of *B. infantis* on stool consistency or frequency could be observed which implies that this therapeutic approach may be applicable to all IBS patients irrespective of their stool pattern^[41]. *B. breve* in combination with *L. plantarum* has been shown to decrease pain and the severity of symptoms in IBS patients^[42]. Prebiotics, oligofructose and inulin might reduce symptoms in IBS-C patients as they selectively stimulate bifidobacteria which results in increased stool frequency^[43].

In conclusion, lower bifidobacteria levels were found both in duodenal mucosa-associated samples as well as in fecal samples of IBS patients when compared to healthy subjects. Specifically, *B. catenulatum* was found to be reduced in duodenal mucosa-associated bacteria as well as in the feces of IBS patients. The relevance of specific *Bifidobacterium* spp in relation to IBS symptoms is unknown; however modulation of the gut microbiota by means of prebiotics or bifidobacteria-containing probiotics to restore a balanced microbiotic composition may have a therapeutic role.

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COMMENTS

Background

Alterations in fecal and small intestinal microbiotic composition in irritable bowel syndrome (IBS) patients have been reported and studies revealed a somewhat higher bacterial count in jejunal juice of IBS patients and lower numbers of fecal coliforms, lactobacilli and bifidobacteria than in healthy subjects.

Research frontiers

The aim of the study was to determine the composition of both fecal and duodenal mucosa-associated microbiota in IBS patients and healthy subjects using molecular-based techniques.

Innovations and breakthroughs

Decreased bifidobacteria levels in both fecal and duodenal brush samples of IBS patients compared to healthy subjects indicate a role for microbiotic composition in IBS pathophysiology.

Peer review

The authors investigated the fecal and duodenal mucosal-associated microbiota in patients with IBS. This topic is of interest since, as the authors mentioned, alterations in intestinal flora have been suggested as a potential etiological factor in the pathogenesis of this disorder.

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