

Fecal Microbiota in Patients with Irritable Bowel Syndrome Compared with Healthy Controls Using Real-Time Polymerase Chain Reaction: An Evidence of Dysbiosis

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Abstract

Background Dysbiosis may play a role in irritable bowel syndrome (IBS), hitherto an enigmatic disorder. We evaluated selected fecal microbes in IBS patients and healthy controls (HC).

Methods Fecal 16S rRNA copy number of selected bacteria was studied using qPCR in 47 patients with IBS (Rome III) and 30 HC.

Results Of 47 patients, 20 had constipation (IBS-C), 20 diarrhea (IBS-D), and seven unclassified IBS (IBS-U). Relative difference in 16S rRNA copy number of *Bifidobacterium* ($P = 0.042$) was lower, while those of *Ruminococcus productus-Clostridium coccoides* ($P = 0.016$), *Veillonella* ($P = 0.008$), *Bacteroides thetaiotamicron* ($P < 0.001$), *Pseudomonas aeruginosa* ($P < 0.001$), and Gram-negative bacteria (GNB, $P = 0.001$) were higher among IBS patients than HC. Number of *Lactobacillus* ($P = 0.002$) was lower, while that of *Bacteroides thetaiotamicron* ($P < 0.001$) and segmented filamentous bacteria (SFB, $P < 0.001$) was higher among IBS-D than IBS-C. Numbers of *Bacteroides thetaiotamicron* ($P < 0.001$), *P. aeruginosa* ($P < 0.001$), and GNB ($P < 0.01$) were higher among IBS-C and IBS-D than HC. Quantity of SFB was higher among IBS-D ($P = 0.011$) and lower among IBS-C ($P = 0.002$) than HC. Number of *Veillonella* species was higher among IBS-C than HC ($P = 0.002$). *P. aeruginosa* was frequently detected among IBS than HC (46/47 [97.9 %] vs. 10/30 [33.3 %], $P < 0.001$).

Abdominal distension ($n = 34/47$) was associated with higher number of *Bacteroides thetaiotamicron*, *Clostridium coccoides*, *P. aeruginosa*, SFB, and GNB; bloating ($n = 22/47$) was associated with *Clostridium coccoides* and GNB. Microbial flora was different among IBS than HC on principal component analysis.

Conclusion Fecal microbiota was different among IBS than HC, and different sub-types were associated with different microbiota. *P. aeruginosa* was more frequent and higher in number among IBS patients.

Keywords Functional gastrointestinal disorder · Quantitative real-time polymerase chain reaction · Dysbiosis · Gastrointestinal microbiota

Introduction

Irritable bowel syndrome (IBS), a common functional gastrointestinal disorder, is associated with recurrent abdominal pain or discomfort, bloating, incomplete evacuation, altered bowel habit, and stool form [1, 2]. Pathogenesis of IBS still remains enigmatic. Several hypotheses have been proposed about the pathogenesis of IBS; these include dysregulation of brain–gut axis, visceral hypersensitivity, psychological factors, abnormal level of gastrointestinal neuropeptides and hormones, and imbalance between commensal and pathogenic bacteria in the gut (dysbiosis) [3, 4]. Moreover, improvement of symptoms in IBS by manipulation of gut microbiota using probiotics, prebiotics, or antibiotics further suggests that dysbiosis may play a potential role in the pathogenesis of IBS [5–7]. However, data on gut dysbiosis in patients with IBS are scanty.

Normal gut flora resists the entry of pathogenic bacteria, enhances the host immune response, and maintains a

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healthy luminal microenvironment [8, 9]. Alteration in the normal gut flora, which may be due to either increased number of invasive bacteria like *Pseudomonas aeruginosa* and *Campylobacter jejuni* or less number of beneficial bacteria like *Lactobacillus* and *Bifidobacterium* species, impairs the gastrointestinal functions and disease susceptibility and may cause mucosal inflammation in the gastrointestinal tract (GIT) [10–12]. Low-grade inflammation has been reported in a subgroup of patients with IBS, particularly those with IBS-D [13, 14]. Inflammatory bowel disease, which is associated with more severe gut inflammation, is known to be associated with dysbiosis that includes less number of Gram-positive and more of Gram-negative bacteria [15]. In addition, segmented filamentous bacteria (SFB) were also found to be higher in chronic inflammatory intestinal diseases [16]. Most of the bacterial species residing in the GIT are non-culturable, obligate anaerobes [8]. Their detection using advanced molecular techniques such as quantitative real-time polymerase chain reaction (qPCR) has replaced the traditional cultural approaches. In this study, we used culture-independent qPCR for quantitative determination of selected fecal microbiota in patients with IBS and healthy controls (HC).

Methods

Study Participants

Patients with IBS diagnosed using Rome III criteria were recruited from the outpatient clinic of the Department of Gastroenterology of a multi-level teaching hospital in northern India. Patients were classified into three sub-types using Rome III criteria: constipation predominant IBS (IBS-C), diarrhea predominant (IBS-D), and un-subtyped (IBS-U) [17]. Patients who had prior history of gastrointestinal surgery, inflammatory bowel disease, and celiac disease were excluded. Moreover, patients with IBS who used antibiotics, probiotics, or prokinetics within last 4 weeks of inclusion into the study were also excluded. All the patients were subjected to investigations such as routine stool microscopy, occult blood test, hemogram, thyroid function tests, and proctosigmoidoscopy. Age- and gender-matched HC were included as controls. Written informed consent was obtained from each participant, and the study protocol was approved by the institutional ethics committee.

Evaluation of Clinical Symptoms

A translated and validated Rome III [18] diagnostic questionnaire was used to obtain the clinical information. In addition, demographic details and information about diet were obtained. Vegetarian was defined as non-uptake of

food products of animal origin, except milk and its products, and non-vegetarian as uptake of both vegetarian and non-vegetarian food products.

Sample Collection and Extraction of DNA

Three consecutive fresh stool samples were collected from each study participant. Stool sample from each subject was homogenized and divided into three aliquots. All stool samples were stored immediately at $-80\text{ }^{\circ}\text{C}$ for DNA extraction. DNA was extracted from stool sample using the QIAamp Qiagen mini stool kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions with some modifications. DNA concentration was quantified by NanoDrop ND—2000 Spectrophotometer (NanoDrop products, Wilmington, DE, USA).

Positive Controls for qPCR: Culture Condition of ATCC Bacterial Strains

Lactobacillus acidophilus (ATCC 4356), *Bifidobacterium breve* (ATCC 15700), *Bifidobacterium catenulatum* (ATCC 27539), *Bacteroides thetaiotamicron* (ATCC 29741), *Clostridium coccooides* (ATCC 29236), *Veillonella parvula* (ATCC 10790), and *Ruminococcus productus* (ATCC 27340) were cultured under anaerobic condition at $37\text{ }^{\circ}\text{C}$ in Wilkins–Chalgren media for 48 h. *Enterococcus faecium* (ATCC 27270) was cultured on blood agar under aerobic condition for 24 h. *P. aeruginosa* (ATCC 27853) was cultured on Muller–Hinton agar under aerobic condition. These ATCC strains were used as positive controls for qPCR, and standard graphs were generated. Genomic DNA was extracted from ATCC bacterial strain using heat extraction method [19] and quantified by NanoDrop spectrophotometer. Standard graph was used to calculate the number of copies of 16S rRNA gene for each bacterium in samples. Standard graphs were generated via serial dilution of control DNA (100 ng) to tenfold (corresponding to approximately 10^1 – 10^{10} 16S rRNA copies/ μl). Real-time PCR was performed to estimate the 16S rRNA copy number of each serially diluted DNA. The efficacy of qPCR for each bacterium was calculated from the standard graph using the Rotor gene 6000 software.

Quantitative Real-Time PCR

qPCR was performed in Corbett Research 6000 Q-PCR instrument (Rotor gene 6000 software, Sydney, Australia). Power SYBR[®] Green PCR master mixture (Applied Biosystems, Carlsbad, USA) was used to amplify the gene of specific bacterial group. The primers used to amplify the specific bacteria are listed in Table 1 [8, 20–28]. Each PCR was carried out in a final volume of 25 μl , comprising of

12.5 µl of Power SYBR[®] Green PCR master mixture, 0.25 µl of 10 pmol of forward and reverse primers, and 100 ng of DNA. Each set of reaction was run with positive and negative controls. qPCR was run in duplicate for each sample. The following thermal cycling parameters were used for amplification of DNA: reaction cycle at 95 °C for 10 min to activate the AmpliTaq Gold[®] DNA polymerase followed by 40 cycles of initial denaturation at 95 °C for 15 s, 30 s of annealing at optimal temperature for each bacterium (Table 1), elongation at 72 °C for 30 s. The quality of qPCR product was confirmed by melting curve analysis with increasing temperature from 60 to 95 °C (at regular increment of 0.5° for 5 s) to confirm that the fluorescence signal was due to specific PCR product and not from primer dimer or nonspecific PCR products. The qPCR amplified amplicon was further confirmed by applying on 2 % agarose gel electrophoresis along with 100-base pair ladder. Additionally, qPCR products were sequenced in 10 % of total samples.

Statistical Analysis

The ratio of copy number of 16S rRNA of specific bacteria to total 16S rRNA copy number of universal bacteria was calculated to find out relative difference of each bacterium. The value of relative difference was transformed into log₁₀ and presented as median and interquartile range (IQR). All statistical analysis was performed using SPSS version 15.0 (SPSS, Inc., Chicago, IL, USA) and Graph Prism version 5.0 (GraphPad software, Inc.). Categorical and continuous data were analyzed using Chi-square and Mann–Whitney *U* test, respectively. Kruskal–Wallis test was used for comparisons of more than two variables, and post hoc analysis was performed using Mann–Whitney *U* test. Bonferroni correction was applied for multiple comparisons. *P* value <0.05 was considered as significant. The principal component analysis (PCA) was used to find out the association between the studied bacterial species and

Table 1 Real-time PCR primers for specific bacterial groups

Name of microorganisms	Primer sequence (5'–3')	Annealing temp. (°C)	Amplicon size (bp)	References
<i>Lactobacillus species</i>	FP: AGCAGTAGGGAATCTTCCA RP: CACCGCTACACATGGAG	57.5	341	[20]
<i>Bifidobacterium species</i>	FP: GGGTGGTAATGCCGGATG RP: TAAGCGATGGACTTTCACACC	57.5	442	[20]
<i>Bifidobacterium catenulatum</i>	FP: CGGATGCTCCGACTCCT RP: CGAAGGCTTGCTCCCGAT	65	285	[21]
<i>Clostridium coccooides</i>	FP: AAATGACGGTACCTGACTAA RP: CTTTGAGTTTCATTCTTGCGAA	48	429	[22]
<i>Pseudomonas aeruginosa</i>	FP: CCTGACCATCCGTCGCCACAAC RP: CGCAGCAGGATGCC ACG CC	79	222	[23]
<i>Enterococcus faecium</i>	FP: AGCTTGCTCCACCGAAAAAGA RP: ATCCATCAGCGACACCCGAA	65	163	[24]
<i>Bacteroides thetaiotamicron</i>	FP: GGCAGCATTTTCAGTTTGCTTG RP: GGTACATACAAAATTCCACACGT	61	423	[25]
<i>Veillonella species</i>	FP: ACCAACCTGCCCTTCAGA RP: CGTCCCGATTAACAGAGCTT	58	343	[8]
<i>Ruminococcus productus-Clostridium coccooides</i>	FP: GGTGGCAAAGCCATTCGGT RP: GTTACGGGACGGTCAGAG	61	182	[8]
Gram-positive bacteria	FP: GAYGACGTCAARTCMTCATGC RP: AGGAGGTGATCCAACCGCA	69	400	[26]
Gram-negative bacteria	FP: AYGACGTCAAGTCMTCATGG RP: AGGAGGTGATCCAACCGCA	52	400	[26]
Segmented filamentous bacteria	FP: AGGAGGAGTCTGCGGCACATTAGC RP: TCCCCACTGCTGCCTCCCGTAG	55	139	[27]
Universal bacteria	FP: TCCTACGGGAGGCAGCAGTG RP: GGACTACCAGGGTATCTAATCCTGTT	65	446	[28]

various subgroups of IBS and HC using XLSTAT version 7.5.2 and SIMCA-P⁺ version 12.

Results

Demographic and Clinical Symptoms in Patients with IBS and HC

Patients with IBS (diagnosed using Rome III criteria, $n = 47$) and HC ($n = 30$) were comparable in age (mean 34 years [19–68] vs. 35 years [21–69], $P = 0.925$) and gender (39/47 [83 %] vs. 22/30 [73 %] male, $P = 0.309$). Clinical profiles of IBS and HC are summarized in Table 2. Patients with IBS were classified into IBS-C (20) and IBS-D (20) using Rome III criteria. Seven patients could not be classified into either category using Rome III criteria (IBS-U).

Quantitative Determination of Fecal Microbiota in Patients with IBS and HC

Relative difference in 16S rRNA copy number of *Bifidobacterium* species was lower among patients with IBS than HC (Table 3), whereas those in 16S rRNA copy numbers of *R. productus-C. coccoides*, *Veillonella* species, *Bacteroides thetaiotamicron*, Gram-negative bacteria, and *P. aeruginosa* was higher among patients with IBS than HC (Table 3; Fig. 1). Interestingly, *P. aeruginosa* was more frequently detected among patients with IBS as compared to HC (46/47 [97.9 %] vs. 10/30 [33 %], $P < 0.001$). Prevalence of *P. aeruginosa* among IBS-C, IBS-D, and IBS-U was comparable (20/20 [100 %], 19/20 [95 %], and 7/7 [100 %], respectively).

Comparisons of Fecal Microbes Among Different Subtypes of IBS and HC

16S rRNA copy number of *Lactobacillus* was lower among IBS-D than IBS-C (Table 4). However, fecal levels of *Bacteroides thetaiotamicron*, *P. aeruginosa*, and Gram-negative bacteria were higher among patients with IBS-C and IBS-D than HC (Table 4). 16S rRNA copy numbers of *R. productus-C. coccoides* and *Veillonella* species were higher among IBS-C than HC. Copy number of *Bacteroides thetaiotamicron* and SFB was higher among IBS-D than IBS-C. In addition, copy number of SFB was higher in IBS-D and lower in IBS-C than HC (Fig. 1). Relative difference in 16S rRNA copy number of fecal bacteria among subgroups of IBS (IBS-C, IBS-D, and IBS-U) and HC is shown in Table 4. Copy numbers of *Lactobacillus* species, *Bifidobacterium catenulatum*, *R. productus-C. coccoides*, *Veillonella* species, *Bacteroides thetaiotamicron*, *P. aeruginosa*, Gram negative, and SFB were

significantly different among subgroups of IBS and HC (Table 4).

Association Between IBS Symptoms and Different Species of Fecal Microbiota

Abdominal distension (34/47, 72 %) was associated with greater number of *Bacteroides thetaiotamicron*, *Clostridium coccoides*, *P. aeruginosa*, Gram negative, and SFB (Table 5). Abdominal bloating or discomfort (22/47, 47 %) was associated with greater number of *Clostridium coccoides* and Gram-negative bacteria (Table 5).

Principal Component Analysis

On PCA of qPCR assay, mean value of 16S rRNA copy number of target bacteria was different among different sub-types of IBS (Fig. 2a). First principal component analysis (PC1) showed 54 % variation of fecal microbiota among different subgroups of IBS. On PC1, IBS-C and IBS-D samples differed from the control subjects (Fig. 2b, c). The linear bars show the relative contribution of each target bacterium. *Veillonella* species, *R. productus-C. coccoides*, *Bacteroides thetaiotamicron*, *P. aeruginosa*, and GN bacteria were dominant among IBS-C, while *P. aeruginosa*, *Bacteroides thetaiotamicron*, and GN bacteria were dominant among IBS-D with respect to HC on PC-1.

Impact of Diet on Bacterial Load

16S rRNA copy numbers of specific bacterial groups were comparable between vegetarian and non-vegetarian patients with IBS.

Discussion

The present study shows that (1) relative difference in 16S rRNA copy numbers of *Bifidobacterium* species was lower, while those of *Bacteroides thetaiotamicron*, *R. productus-C. coccoides*, *Veillonella* species, *P. aeruginosa*, and Gram-negative bacteria were higher among patients with IBS than HC; (2) 16S rRNA copy numbers of *Lactobacillus* species were lower, while those of *Bacteroides thetaiotamicron* and SFB were higher among patients with IBS-D than IBS-C; (3) *P. aeruginosa* was frequently detected and higher in numbers in fecal samples of patients with IBS than HC; (4) patients with visible abdominal distension had greater number of *Bacteroides thetaiotamicron*, *Clostridium coccoides*, *P. aeruginosa*, Gram negative, and SFB; and abdominal bloating/discomfort was associated with greater number of *Clostridium coccoides* and SFB.

Table 2 Demographic and clinical symptoms in patients with IBS and healthy controls

Parameters	IBS (<i>n</i> = 47)	HC (<i>n</i> = 30)	<i>P</i> value
Age (years): median (range)	34 (19–68)	35 (21–69)	0.925*
Gender: male (%)	39 (83 %)	22 (73 %)	0.309
Predominant bowel habits (Rome III criteria)			
IBS-C	20 (43 %)	–	–
IBS-D	20 (43 %)	–	–
IBS-U	7 (14 %)	–	–
Clinical symptoms			
Visible abdominal distension	34 (72 %)	2 (7 %)	<0.001
Abdominal bloating/feeling of abdominal distension >¼ of days	22 (47 %)	0	<0.001
Abdominal pain	35 (74 %)	1 (3 %)	<0.001
Abdominal discomfort	47 (100 %)	2 (7 %)	<0.001
Relief of pain/discomfort with bowel movement	46 (98 %)	2 (7 %)	<0.001
More frequent stool at onset of pain	13 (28 %)	0	0.001
Loose stool at onset of pain	14 (30 %)	0	0.001
Urgency	17 (36 %)	0	<0.001
Passage of mucus	35 (74 %)	1 (3 %)	<0.001
Passage of mucus >1/4 of defecation	14 (28 %)	0	0.001
Feeling of incomplete evacuation	45 (96 %)	3 (10 %)	<0.001
Feeling of incomplete evacuation >1/4 of defecation	29 (62 %)	0	<0.001
Straining during defecation	27 (57 %)	1 (3 %)	<0.001
Irregular stool form	33 (70 %)	1 (3 %)	<0.001
Irregular stool frequency	39 (83 %)	1 (3 %)	<0.001
Diet			
Vegetarian	14 (30 %)	12 (40 %)	ns
Non-vegetarian	33 (70 %)	18 (60 %)	ns

IBS Irritable bowel syndrome, IBS-C constipation predominant IBS, IBS-D diarrhea predominant IBS, IBS-U un-subtyped IBS, ns not significant
 $P < 0.05$ was significant

* Nonparametric independent sample test. Chi-square test was used for all the other categorical data

Table 3 Relative difference in 16S rRNA copy number of fecal bacteria between patients with IBS and healthy controls

Target organism	HC (<i>n</i> = 30) Median (IQR)	IBS (<i>n</i> = 47) Median (IQR)	<i>P</i> value*
<i>Lactobacillus</i> species	5.1 (3.1–7.0)	5.6 (4.0–6.5)	0.653
<i>Bifidobacterium</i> species	4.1 (3.3–5.2)	3.6 (2.1–4.7)	0.042
<i>Bifidobacterium catenulatum</i>	3.1 (1.6–3.4)	2.4 (1.5–3.4)	0.680
<i>Clostridium coccoides</i>	5.1 (4.3–6.2)	5.4 (4.0–6.1)	0.921
<i>Ruminococcus productus-Clostridium coccoides</i>	2.6 (1.2–3.6)	3.2 (2.5–4.2)	0.016
<i>Veillonella</i> species	4.3 (3.5–5.0)	5.2 (4.2–6.3)	0.008
<i>Bacteroides</i> species	3.4 (2.4–4.8)	5.6 (4.7–7.3)	<0.001
<i>Enterococcus faecium</i>	5.3 (1.7–7.9)	5.0 (3.7–6.0)	0.896
<i>Pseudomonas aeruginosa</i>	0.0 (0.0–4.2)	5.2 (4.4–6.3)	<0.001
Gram-positive bacteria	2.4 (2.1–3.1)	2.0 (1.5–3.3)	0.284
Gram-negative bacteria	2.3 (1.4–3.1)	3.6 (2.0–4.8)	0.002
Segmented flagellated bacteria	3.4 (2.8–4.0)	3.0 (2.3–4.4)	0.383

* Mann–Whitney *U* test.
 $P < 0.05$ was considered as significant

Evidence of dysbiosis in patients with IBS has been supported by a few earlier studies [29, 30]. Alleviation of symptoms of IBS by antibiotic [5] and probiotic [31]

treatment also supports the concept of dysbiosis. Different phenotypes of IBS such as IBS-C and IBS-D are expected to have different pathogenesis [30]. Our finding of

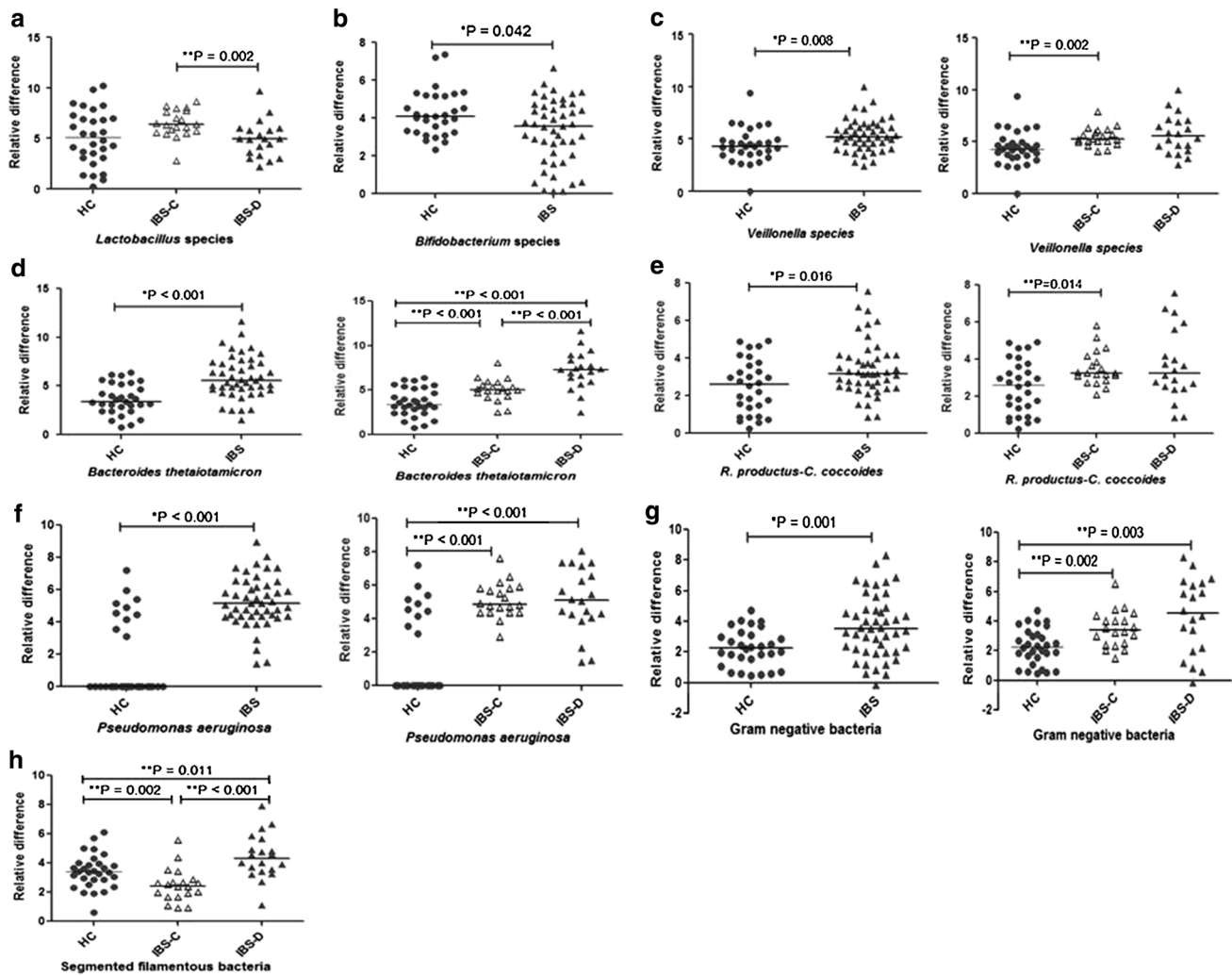


Fig. 1 **a** *Lactobacillus* species, **b** *Bifidobacterium* species, **c** *Veillonella* species, **d** *Bacteroides thetaiotamicron*, **e** *Ruminococcus productus-Clostridium coccoides*, **f** *Pseudomonas aeruginosa*, **g** Gram-negative bacteria, **h** Segmented filamentous bacteria. Mann–Whitney *U* test was used for pairwise comparison between

IBS subgroups and healthy controls. Post hoc Bonferroni correction was used for multiple comparisons. **P* value <0.05 was considered as significant. **Bonferroni-corrected *P* value <0.017 was considered as significant for subgroup analysis

difference in fecal microbiota among patients with IBS-C and IBS-D is in accordance with this hypothesis. We found that 16S rRNA copy number of *Lactobacillus* species was lower among patients with IBS-D than IBS-C. This finding is consistent with the study of Malinen et al. [8]. Our finding showing lower number of copies of *Bifidobacterium* species among patients with IBS than HC is consistent with a previous study that showed low counts of *Lactobacillus* species, *Bifidobacterium* species, and *Coliforms* in patients with IBS using culture methods [12]. In a recent study, level of *Bifidobacteria* was reduced twofold among patients with IBS than HC [32]. Both *Lactobacilli* and *Bifidobacteria* produce SCFAs but not gases, via fermentation of carbohydrates [33]. SCFAs turn the luminal environment acidic inhibiting adherence of invasive bacteria

[34, 35]. Therefore, reduction in numbers of these bacterial species favors colonization by pathogenic bacteria. Colonization by *Clostridium* species is associated with excess gas production, which is associated with IBS [36]. This observation is in accordance with our finding, showing that greater quantity of *Clostridium coccoides* was associated with abdominal discomfort and bloating among patients with IBS.

Colonic dysbiosis may lead to impaired fermentation patterns in patients with different phenotypes of IBS [37]. Recently, *Bacteroides* species has been identified as a member of enterotype 1 and helps in the fermentation of starch [38]. Most of *Bacteroides* species including *Bacteroides thetaiotamicron* have cell-envelope-associated multi-protein system, termed as “starch utilization system

Table 4 Relative difference in 16S rRNA copy number of fecal bacteria in subgroups of patients with IBS and healthy controls

Target organism	HC (n = 30) Median (IQR)	IBS-C (n = 20) Median (IQR)	IBS-D (n = 20) Median (IQR)	IBS-U (n = 7) Median (IQR)	P value*
<i>Lactobacillus</i> species [‡]	5.1 (3.1–7.0)	6.4 (5.6–7.7)	5.0 (3.7–6.0)	3.3 (2.9–4.0)	0.002
<i>Bifidobacterium</i> species	4.1 (3.3–5.2)	4.1 (2.7–4.7)	3.0 (1.1–4.4)	3.1 (2.8–4.7)	0.108
<i>Bifidobacterium catenulatum</i>	3.1 (1.6–3.4)	2.3 (1.8–3.9)	2.0 (1.2–2.8)	4.0 (2.8–4.6)	0.011
<i>Clostridium coccooides</i>	5.1 (4.3–6.2)	5.3 (3.5–6.1)	5.1 (3.9–6.0)	5.8 (5.4–6.6)	0.438
<i>Ruminococcus productus-Clostridium coccooides</i> [#]	2.6 (1.2–3.6)	3.2 (2.9–4.1)	3.3 (2.4–5.3)	2.3 (1.9–4.0)	0.035
<i>Veillonella</i> species [#]	4.3 (3.5–5.0)	5.2 (5.0–6.0)	5.6 (4.3–7.0)	3.7 (2.8–6.1)	0.006
<i>Bacteroides</i> species ^{#,†,‡}	3.4 (2.4–4.8)	5.0 (4.4–5.8)	7.3 (6.1–8.7)	4.6 (2.5–5.6)	<0.001
<i>Enterococcus faecium</i>	5.3 (1.7–7.9)	4.9 (3.9–6.0)	5.1 (3.2–6.6)	4.1 (3.6–5.6)	0.969
<i>Pseudomonas aeruginosa</i> ^{#,†}	0.0 (0.0–4.2)	4.9 (4.4–5.8)	5.1 (4.1–7.0)	6.0 (4.8–6.8)	<0.001
Gram-positive bacteria	2.4 (2.1–3.1)	2.0 (1.3–3.3)	2.4 (1.4–3.6)	1.9 (1.7–2.8)	0.680
Gram-negative bacteria ^{#,†}	2.3 (1.4–3.1)	3.4 (2.4–4.3)	4.6 (2.0–6.5)	1.9 (1.2–3.2)	0.001
Segmented flagellated bacteria ^{#,†,‡}	3.4 (2.8–4.0)	2.4 (1.7–2.8)	4.3 (3.5–5.5)	2.5 (2.2–3.0)	<0.001

* Kruskal–Wallis post hoc analysis. P < 0.05 was considered as significant

P < 0.017 between IBS-C and HC

† P < 0.017 between IBS-D and HC

‡ P < 0.017 between IBS-C and IBS-D

Table 5 Association of IBS symptoms in relation to bacterial load

Target organism	Visible abdominal distension			Abdominal bloating/abdominal discomfort >¼ days		
	Present (34/47)	Absent (13/47)	P value	Present (22/47)	Absent (25/47)	P value
<i>Bacteroides thetaiotamicron</i> (Median and IQR)	6.0 (4.9–7.8)	4.3 (2.6–6.0)	0.007	6.1 (4.9–7.6)	5.4 (4.2–6.5)	0.176
<i>Clostridium coccooides</i> (Median and IQR)	5.8 (4.7–6.5)	3.9 (3.4–5.4)	0.007	6.0 (4.8–6.7)	4.7 (3.6–5.6)	0.004
<i>Pseudomonas aeruginosa</i> (Median and IQR)	5.6 (5.0–6.5)	4.4 (3.9–5.0)	0.013	5.5 (4.6–6.7)	4.8 (4.0–6.1)	0.125
Gram-negative bacteria (Median and IQR)	4.1 (2.8–5.7)	2.2 (1.9–3.7)	0.046	4.5 (2.9–5.7)	3.2 (1.9–4.0)	0.043
Segmented filamentous bacteria (Median and IQR)	3.3 (2.4–4.8)	2.6 (1.4–3.3)	0.046	3.2 (2.3–4.8)	2.8 (2.3–4.0)	0.327

Mann–Whitney U test was used to find out the association between IBS symptoms and bacterial load. P < 0.05 was considered as significant

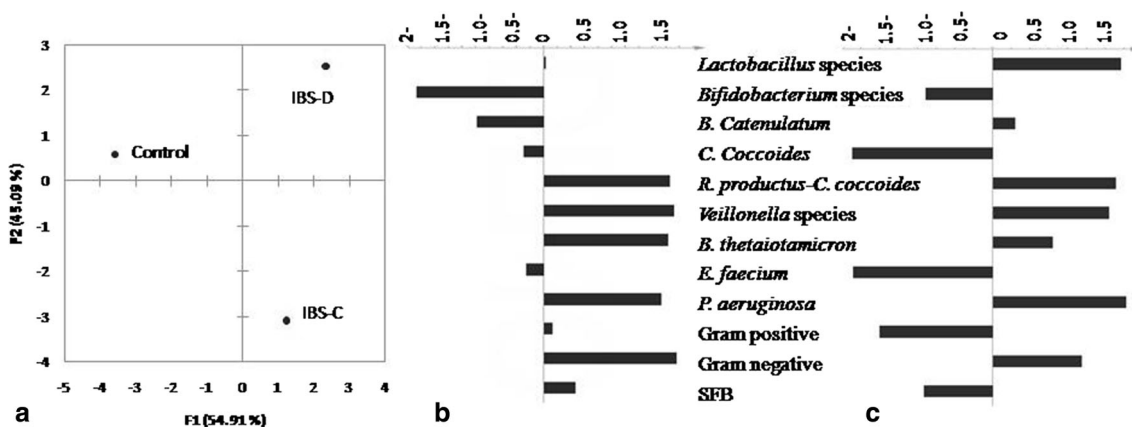


Fig. 2 Principal component analysis of qPCR datasheet. Mean value of 16S rRNA copy number for target bacteria among IBS-C, IBS-D, and HC (a). Relative contribution of each bacterium among IBS-C (b) and IBS-D (c) with respect to HC

(SUS)” that enables the *Bacteroides* species to degrade carbohydrates and proteins [39]. Increased number of *Bacteroides* species may be associated with production of organic acids in excess via fermentation [40], which might be associated with IBS symptoms. In the present study, increased level of *Bacteroides thetaiotamicron* was found to be associated with abdominal distension. Reduced number of *Bacteroides* species following probiotic administration is known to improve the symptoms of IBS [41]. Thus, greater copy number of *Bacteroides* species among patient with IBS in the present study strengthens the earlier hypothesis [41]. Altered fermentation of diets leads to the production of excess hydrogen, methane gases, and organic acids, suggesting an association of varied range of symptoms with different subgroups of IBS [36, 42]. IBS-C and IBS-D were associated with methane and hydrogen gas, respectively, as compared to HC [35]. This might be due to altered gut bacteria in different subgroups of IBS. Recent studies reported increased copy numbers of *Veillonella* species and *R. productus-C. coccoides* among patients with IBS, mainly in IBS-C [8], which is concordant with the present study. Both *R. productus-C. coccoides* and *Veillonella* species produce acetate and succinate [43]. Elevated amount of acetate was found to be associated with mucosal lesion and abdominal cramp in animal model [36]. Higher levels of *Veillonella* species enhance concentration of organic acids such as butyric acid, which is associated with the altered gastrointestinal motility and poor quality of life [36]. Increased level of butyrate has been reported in patients with IBS-D [44] and also shown to induce visceral hypersensitivity in rat model, thus leading to progression of IBS symptoms [45].

Altered luminal microbiota favors the adherence of enteric pathogens like *P. aeruginosa* to the enterocytes [35, 46]. In the present study, higher prevalence and copy number of *P. aeruginosa* were detected among patients with IBS-C and IBS-D than HC. This finding is in accordance with the previous study, which reported that prevalence of *P. aeruginosa* was 97.3 % in mucosal sample from small intestine and 97.2 % in fecal samples of patients with IBS [10]. *P. aeruginosa* has several virulence factors such as protease and elastase, which degrade the epithelial cells, reduce the integrity of tight junction proteins, enhance the cellular permeability, and confer a hostile environment for colonization of *P. aeruginosa* in the gut [10, 47]. Recently, a close association has been reported between colonization of *P. aeruginosa* in the gut and abnormal gastrointestinal motility [48]. Proteases of *P. aeruginosa* inhibit the protease-activated receptor-2 (PAR-2) in the respiratory tract [47]. However, the role of these proteases in the gut has not been clearly understood. Activation of PAR-2 in gut induces the cellular and paracellular permeability, visceral hypersensitivity, altered gastrointestinal motility, and release of

inflammatory mediators that may lead to progression of IBS symptoms [48]. These observations support the current finding that greater number of *P. aeruginosa* is associated with abdominal distension. However, it is not clear whether *P. aeruginosa* is the cause of IBS or its consequences. Gram-negative bacteria, particularly *Bacteroides thetaiotamicron*, *P. aeruginosa*, and SFB, were higher among patients with IBS-D than HC, which suggests low-grade inflammation in a subgroup of IBS. However, the cause of inflammation has not been understood so far, but altered gut flora may be involved [49, 50].

It is worth mentioning a few limitations of the present study. We used SYBR green-based qPCR for qualitative and quantitative determination of fecal flora rather than more sophisticated techniques such as deep sequencing. However, qPCR is also a quite sensitive, specific, cost-effective, and reproducible technique. The main limitation of qPCR is its ability to analyze only the selected microbial groups.

We found that IBS-D was associated with different fecal microbiota than IBS-C. However, stool consistency may itself result in variation in its composition including proportion of fecal microbiota. Hence, our findings showing difference in fecal microbiota among patients with IBS-D and IBS-C may not establish a cause and effect relationship between dysbiosis and different sub-types of IBS. More studies are needed to show whether manipulation of microbial composition by specific intervention leads to change in stool consistency before a cause and effect relationship between dysbiosis and different sub-types of IBS can be proposed.

Alterations in the normal gut flora among different sub-types of IBS than HC might be related to other sociocultural and demographic factors including age, gender, geographical origin, environmental factors, food hygiene, alcohol consumption, and diet [22, 51]. Therefore, our study of fecal microbiota from tropical and subtropical regions may not be entirely applicable in other geographical regions. However, in the present study, patients with IBS and HC were recruited from the similar sociocultural region. Therefore, our findings that patients with IBS have different gut flora from healthy controls are unlikely to be explained by these sociocultural and demographic factors only.

In conclusion, the findings of the present study support the hypothesis that different phenotypes of IBS were associated with distinct gut microbiota. IBS-C was associated with greater number of *Lactobacillus*, while IBS-D was associated with *Bacteroides thetaiotamicron*, Gram negative, and SFB. Gram-positive bacteria mainly *Bifidobacterium* species were reduced, and Gram-negative bacteria, particularly *P. aeruginosa*, *Bacteroides thetaiotamicron*, and *Veillonella* species, were increased in patients with IBS

than HC. Frequency of *P. aeruginosa* was higher in all IBS patients than HC, suggesting that it may play a role in pathophysiology of IBS. Thus, this study supports that gut microbiota may play a potential role in the pathophysiology of IBS and confers a new dimension in management of IBS.

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