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IDENTIFICATION OF AN INTESTINAL MICROBIOTA SIGNATURE ASSOCIATED WITH HOSPITALIZED PATIENTS WITH DIARRHEA

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As an important global health challenge, diarrhea kills nearly two million people each year. Postinfectious irritable bowel syndrome (IBS) usually manifests itself as the diarrhea-predominant subtype. Small intestinal bacterial overgrowth has been observed more frequently in patients with IBS compared to healthy controls. However, the pathophysiology of IBS is not fully understood, and based on recent evidences, altered gut microbiota is involved in the pathogenesis of IBS. Therefore, we aimed to compare the microbiome in hospitalized patients with diarrhea and healthy individuals. Thirty patients and 10 healthy controls were included into this case–control study. Microbial count was performed using quantitative real-time polymerase chain reaction method using bacterial 16S rRNA gene. *Clostridium cluster IV* and *Bacteroides* were significantly more frequent in the patients compared with the healthy individuals ($p = 0.02$ and 0.023, respectively). However, the quantity of *Enterococcus* and *Bifidobacterium* groups were significantly higher in healthy controls than in diarrheal group ($p = 0.000076$ and 0.001, respectively). The results showed that the number of bacteria in all bacterial groups was significantly different between healthy individuals and diabetic group, whereas the difference between the healthy group and IBS was not significant for *Bifidobacterium* group. The findings of this study outlined the relationship between diarrhea, IBS, and diabetes disease and bacterial composition. It could be concluded that modifying the bacterial composition by probiotics can be helpful in the control and management of the mentioned disease.

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Introduction

Recent reports focused on the reality of a positive relationship between human health and the microbial flora that colonize the human gut. Among the gut bacteria, which behave as a functional human organ, probiotics represent the Holy Grail because they are related to a broad spectrum of positive effects on host health, including positive effects on host longevity [1]. As a functional gastrointestinal (GI) disorder, irritable bowel syndrome (IBS) is characterized by abdominal pain and changes in the pattern of bowel movements associated with bowel habit change such as diarrhea and constipation without any evidence of underlying damage [2, 3]. Although IBS is not known as a serious disorder, 10%–15% of the adult population in the developed world has been observed affected with the disorder; however, the pooled prevalence of IBS varies considerably, both by geographical location and by applied diagnostic criteria [4–6]. It is more common in South America and less common in Southeast Asia and the high prevalence of IBS is joined by vast societal monetary burdens and negative impacts on the quality of life in the afflicted individuals with the disorder [7, 8]. IBS does not lead to dangerous conditions in most patients, but can be associated with increased side effects such as chronic pain and fatigue and reduced work productivity [9, 10]. Researchers have reported that the high incidence of IBS together with the associated comorbidities can increase social costs and can also greatly affect the patient's quality of life [8, 11].

Although the pathophysiology of IBS is not fully understood, several hypotheses have been proposed. Acute GI infections increase the risk of developing and expanding IBS. Prolonged fever, anxiety, and depression are the other factors that increase the likelihood of developing the syndrome. The biochemical signaling taken place between the GI tract and the central nervous system (the brain–gut axis) has been accepted as the major pathogenetic mechanism of IBS, which suggests that IBS occurs due to disturbances in the interoperability of the brain-intestinal axis and is correlated with a dysfunction of the GI autonomic nervous system [4]. Other theories including gut motility disorders, pain sensitivity, infections including small intestinal bacterial overgrowth, neurotransmitters, genetic factors, and food sensitivity have also known as the causes of the disorder [12].

Despite the considerable burden of IBS, treatment alternatives stay restricted and look into the etiology, and pathophysiology of this multifactorial disorder is continuous [13]. The growing evidences have indicated that IBS might present due to other potential mechanisms including gut microbiota and low-grade inflammation/immune activation [14, 15]. Comparisons of IBS patients with healthy

participant in several lines of literatures have shown that the microbiota differs significantly between groups, which demonstrate the putative role of gut microbiota in IBS [16, 17]. Postinfectious IBS; colonic fecal microbiota transplantation; and therapeutic effects of probiotics, prebiotics, synbiotics, and non-systemic antimicrobials have also supported the contribution of the gut microbiota to the pathophysiology of IBS [18–23].

Given the evidence that approximately 10% of IBS patients' symptoms began following an episode of infectious diarrhea and based on our knowledge of the relationship between alteration of gut microbiota and inflammation of gut, we encouraged to compare the microbiome in hospitalized patients with diarrhea and healthy individuals.

Materials and Methods

Participant and sampling

Thirty affected hospitalized participants with diarrhea (age: 56 ± 8 years) who referred to Institute of Endocrinology and Metabolism Research and Training Center, Iran University of Medical Sciences in Tehran, Iran were recruited into the study as the case group. Twenty non-diarrheal individuals, matched for age, gender, and their current living environment were recruited as the healthy participants. Stool samples from the patients and healthy individuals were collected. Sterile cups were used to instant stool sampling after defecation and brought to the laboratory within 2 h. Collected stool samples were instantly stored in microbiology laboratory at – 70 °C upon arrival.

Pregnancy, lactation, organic GI disease, severe systematic disease, major or complicated abdominal surgery, severe endometriosis, and dementia were the exclusion criteria for patients, and intestinal disturbances (including lactose intolerance and celiac disease), ongoing antibiotic treatment, and all exclusion criteria of the patients were considered as the exclusion criteria for controls.

This study was approved by research ethics committees of Iran University of Medical Sciences and according to Declaration of Helsinki. A signed informed consent form was received from each of the participant and they were ensured anonymity of all information.

DNA extraction

An amount of 200 mg of each fecal sample was used for bacterial total DNA extraction. Extraction of total DNA from all stool specimens was performed using

QIAamp® DNA Stool Mini Kit (Qiagen Retsch GmbH, Hannover, Germany) according to the manufacturer's instruction. Quality and quantity of the extracted DNA was measured by Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and the DNA integrity was assessed by 1% agarose gel-electrophoresis. Finally, entire extracted DNA samples were immediately transferred into −20 °C storage.

Microbial quantification by real-time polymerase chain reaction (PCR)

Briefly, all bacterial 16S rRNA sequences were extracted from SILVA High Quality Ribosomal RNA database [30], then converted into 16S rDNA, and specific probe and primer sequences were designed in several steps using various databases including NCBI, probebase, IDT and EMBL-EBI, and also AlleleID software (version 7.5, Humana Press, USA). Characteristics of primers and TaqMan probes are demonstrated in Table I.

Using 16S rDNA gene-specific primers and probes, amplification of target 16s rDNA was performed in Rotor-Gene 6000 real-time PCR cycler (Qiagen Corbett, Hilden, Germany) by real-time TaqMan quantitative PCR (qPCR). Each reaction mixture in a total volume of 20 µl, which contained 0.5 µl of forward primer, 0.5 µl of reverse primer, 0.5 µl of TaqMan probe, 12 µl of Probe Ex Taq (probe qPCR) Master Mix (Takara Bio, Shiga, Japan), 1 µl of template DNA, and 5.5 µl sterilized ultrapure water, was run to amplify the target region under following real-time qPCR cycling condition: an initial holding at 95 °C for 30s, followed by 40 cycles of denaturation at 95 °C for 5s, and annealing/extension at 60 °C for 30s. No template reaction was used as the negative control. *Bifidobacterium bifidum* ATCC 29521, *Lactobacillus acidophilus* ATCC 4356, and *Fusobacterium nucleatum* ATCC 25586 were provided from the American Type Culture Collection (ATCC) and were used as the bacterial standard strains. All qPCR runs were carried out in triplicate, and averaged numbers were used for calculation and analysis.

Bacterial count

Determination of the number *of Lactobacillus, Bifidobacterium, Fusobacterium*, and *Prevotella* groups in each sample was performed after construction of standard curves based on 10-fold serial dilutions of bacterial standard strains genomic DNA of known concentration from pure cultures, corresponding to 10^1–10^{10} copies per gram feces. According to Applied Biosystems tutorials, standard curves were created and were normalized to the copy number of the 16S rRNA gene for each species. If there was a copy number-unknown species of

Table I. Characteristics of 16S rDNA gene-targeted primers and probes

Target bacteria	Primer/probe*	Sequence (5′ → 3′)	Oligonucleotide size (bp)	Product size (bp)	Ref.
Enterococcus group	Primer-F	TGGGTAGCGGAGAAATTCCA	20	103	This study
	Primer-R	ACAGTGCTCTACCTCCATCA	20		
	Probe	CCGAGGCTAGCCCTAAAGCTATTTCGG	27		
Bifidobacterium group	Primer-F	AAGCGATGGACTTTCACACC	20	87	This study
	Primer-R	TACGTAGGGTGCAAGCGTTA	20		
	Probe	CGCGACGAACCGCCTACGAGC	21		
Bacteroides group	Primer-F	GTATGTCRCAAGCGTTATCC	20	100	This study
	Primer-R	AACGCAATACRGAGTTGAGC	20		
	Probe	TAGACGCGCTTTACGCCCAAT	21		
Clostridium cluster IV	Primer-F	CGAACAGGATTAGATACCC	19	134	This study
	primer R	CTTTGAGTTTCACCGTTG	18		
	Probe	AAACGATGGATGCCCGC	17		

Note: *Primers F (forward), R (reverse), and probes targeting the 16S rDNA gene.

16S rRNA operon, average operon numbers of the closest bacterial taxa obtained from the ribosomal RNA database rrnDB was used as the operon copy number. The threshold cycle values (Ct) obtained from the standard curves were applied to determine bacterial copy number per gram stool.

Sample size and statistical analysis

SPSS version 20.0 software (SPSS Inc., Chicago, IL, USA) and Minitab version 16.2.0 (State College, PA, USA) were used for statistical analysis. According to a predicted difference of at least 2×10^5 copies per gram of feces in the mean bacterial numbers between the healthy individuals and patients, the sample size of 20 subjects provided the sufficient power (80%), considering a type I error of 0.05 and effect size of 0.4176. For group comparison, independent sample *t*-test was used and the Pearson's correlation was assessed for linear correlation analysis. A $p < 0.05$ was considered statistically significant. All the descriptive data have been expressed as mean ± standard deviation. The Kolmogorov–Smirnov test was applied to test for a normal distribution.

Results

Participants

Thirty participants with diarrhea with the mean age of 56 ± 8 years and 10 healthy individuals with the mean age of 51 ± 4 years were recruited to the study; 14 participants with diarrhea were males and 16 were females. Healthy individuals included 4 males and 7 females.

qPCR analysis of bacterial groups

This case–control study qPCR analysis was aimed to assess the differences in composition of fecal microbiota in patients with diarrhea and healthy participant for four groups of bacteria including *Bacteroides*, *Clostridium cluster IV*, *Enterococcus*, and *Bifidobacterium* groups. As indicated in Table II, the results showed that fecal microbiota in diarrheal cases and healthy individuals were significantly different for all the studied bacterial groups. *Clostridium cluster IV* and *Bacteroides* were significantly more frequent in the patients compared with the healthy individuals ($p = 0.02$ and 0.023, respectively). However, the quantity of *Enterococcus* and *Bifidobacterium* groups was significantly higher in healthy controls than in diarrheal

Table II. Correlations between concentration of the bacterial species in healthy–diarrheal, healthy–diabetic, and healthy–IBS groups

	Control	IBS			Diarrhea			Diabetic		
Bacterial species	Copies/gram of fecal ($N = 10$)	Copies/gram of fecal ($N = 10$)	Z	p	Copies/gram of fecal ($N = 10$)	z	p	Copies/gram of fecal ($N = 10$)	z	p
Bacteroides group	53E9 ± 18E5	70E5 ± 61E3	−2.12	0.035	87E5 ± 15E3	−2.27	0.023	44E4 ± 79E2	−3.68	0.000
Enterococcus group	28E4 ± 35E2	25E8 ± 48E6	−3.64	0.000	70E10 ± 56E2	−3.85	0.000	79E7 ± E4	−3.80	0.000
Clostridium cluster IV	21E8 ± 28E3	21E5 ± 87E3	$t = 3.42$, $df = 10.80$	0.006	39E5 ± 24E1	−3.03	0.020	66E4 ± 71E2	$t = 4.74$, $df = 11.58$	0.001
Bifidobacterium group	58E6 ± 41E4	12E7 ± 83E4	−1.97	0.052	16E8 ± 21E5	3.19	0.001	26E8 ± 26E4	$t = -5.59$, $df = 18.0$	0.001

Note: IBS: irritable bowel syndrome.

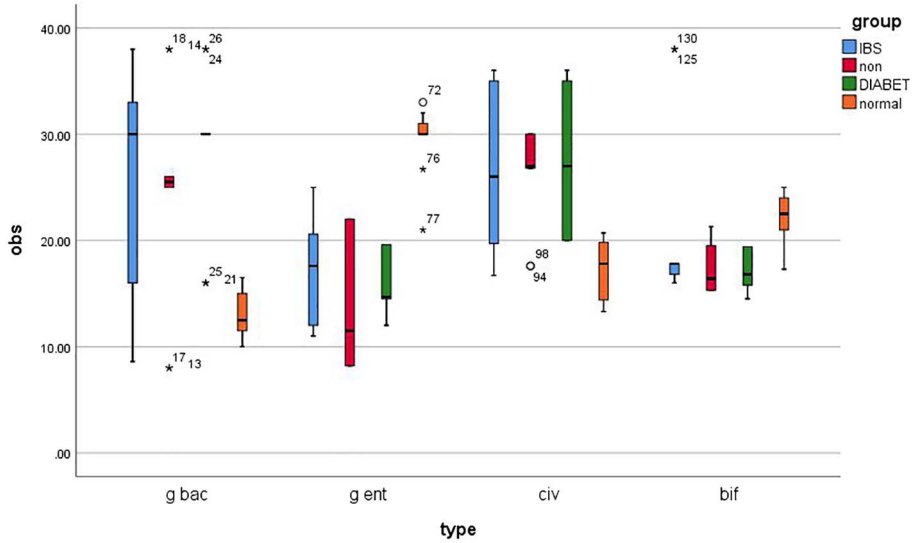

Figure 1. Bacterial groups quantified by real-time PCR and expressed as copy number of bacterial groups per gram stool in human adults with diarrhea (red boxes; $N = 10$), IBS (blue boxes; $N = 10$), diabetes (green boxes; $N = 10$), and healthy controls (orange boxes; $N = 10$). Boxes show the upper (75%) and the lower (25%) percentiles of the data. Whiskers indicate the highest and the smallest values. *Outlier points

group ($p = 0.000076$ and 0.001, respectively). Differences of intestinal bacterial genera in the study groups were represented in Table II and Figure 1.

The correlations between concentration of the bacterial species in healthy–diabetic and healthy–IBS groups

Some of the participants in this study had background diseases of diabetes and IBS; therefore, the correlation or, in other words, the difference in bacterial groups between healthy individuals and those with any of the mentioned diseases was investigated. The results showed that the number of bacteria in all bacterial groups was significantly different between healthy individuals and diabetic group, whereas the difference between the healthy group and IBS was not significant for *Bifidobacterium* group. The mean quantity of *Bacteroides*, *Clostridium cluster IV*, and *Bifidobacterium* groups was significantly higher in diabetic group than in non-diabetic individuals ($p = 0.000043$, $p = 0.001$, and $p < 0.001$, respectively). *Bacteroides*, *Clostridium cluster IV*, and *Bifidobacterium* were also significantly more frequent in the IBS patients compared with the healthy individuals ($p = 0.035$, 0.006, and 0.052, respectively). *Bacteroides* concentration was higher both in

diabetic group and IBS patients than in healthy group ($p = 0.000011$ and 0.000042, respectively). The correlations between concentration of the bacterial species in healthy–diarrheal, healthy–diabetic, and healthy–IBS groups have been indicated.

Discussion

Correlation between imbalance normal microbial community and GI conditions such as inflammatory bowel disease and IBS, and wider systemic manifestations of disease such as obesity, type 2 diabetes, and atopy have previously been reported in several reports [6, 13, 14]. Although intensive research over the past two decades has been carried to find the etiology of IBS, its pathophysiology remains poorly understood [19]. Diarrhea, constipation, or mixed type are the symptoms of IBS. Based on this knowledge, we hypothesized that intestinal microbiota composition in patients with diarrhea is different from healthy control participant. In this study, the number of four groups of bacteria including *Bacteroides*, *Clostridium cluster IV*, *Enterococcus*, and *Bifidobacterium* was measured using qPCR in patients and healthy participants. Our data showed that fecal microbiota in diarrheal cases and healthy individuals were significantly different for all the studied bacterial groups.

In comparison to healthy controls, our data showed that the copy number of *Bacteroides* and *Clostridium cluster IV* group was significantly higher in participants with diarrhea. In contrast with this study, Swidsinski et al. [24] using fluorescence *in situ* hybridization analysis (FISH) demonstrated that the fecal microbiota in idiopathic diarrhea was markedly different, marked by reduction in concentrations of habitual *Eubacterium rectale*, *Bacteroides*, and *Faecalibacterium prausnitzii* groups. Using FISH analysis, a study conducted on patients by Soko et al. also showed that a reduction of a major member of *Firmicutes*, *F. prausnitzii*, is associated with a higher risk of postoperative recurrence of ileal Crohn's disease (CD). They found that *F. prausnitzii* exhibits anti-inflammatory effects, partly due to secreted metabolites able to block NF-κB activation and IL-8 production; therefore, counterbalancing dysbiosis using *F. prausnitzii* as a probiotic can be a promising strategy in CD treatment [25].

In addition to the diarrhea, which is designated as the principal diagnosis, some patients also have underlying diseases of diabetes and IBS. Therefore, we also decided to evaluate the association of each of the studied bacterial group with the underlying diseases. In agreement with findings of Vrakas et al. [26], Maukonen et al. [27], and Rajilić-Stojanović et al. [28], our observation showed that the concentrations of *Clostridium cluster IV* and *Fusobacterium* groups were relatively higher in the IBS group compared to the healthy participant.

Consistently, Kerckhoffs et al. [29] showed that *bifidobacteria* levels in both fecal and duodenal brush samples of IBS patients were significantly lower (6 ± 0.6 vs. 19 ± 2.5, $p < 0.001$) compared to healthy participants, which indicate a role for microbiotic composition in IBS pathophysiology. They analyzed fecal samples for the composition of the total microbiota using FISH and analyzed both fecal and duodenal brush samples for the composition of *bifidobacteria* using qPCR. Similarly, Rajilić-Stojanović et al. [28] and Si et al. [30] showed meaningful lower concentration of *Bifidobacterium* in IBS patients compared to the controls. In this study, we found that the level of *Enterococcus* group was significantly lower in the patients with IBS compared to the healthy group. This observation was in contrast with the findings of Zhuang et al. [31].

In line with our results, the study conducted by Rajilić-Stojanović, the intestinal microbiota including a twofold increased ratio of *Bacteroidetes* ($p = 0.0002$), approximately 1.5-fold increase in numbers of *Clostridium* spp. ($p = 0.005$); a twofold decrease in the number of *Bacteroidetes* ($p = 0.0001$); a 1.5-fold decrease in numbers of *Bifidobacterium* and *Faecalibacterium* spp. ($p = 0.05$), of IBS patients differed significantly ($p = 0.0005$) from that of controls [28]. Characterization of the fecal microbiota using high-throughput sequencing of the 16S rRNA gene showed a significant increase of *Bacteroidetes* and *Proteobacteria* in the IBS group compared to the healthy participants [32]. Other report also confirmed our data and demonstrated that the mucosa-associated microbiota in patients with IBS is significantly different from healthy controls with increases in *bacteroides* and clostridia and a reduction in *bifidobacteria* in patients with IBS-D [33]. However, global and deep molecular analysis of microbiota signatures in fecal samples from patients with IBS disclosed a twofold decrease in the number of *Bacteroidetes* [33]. In this study, the level of *Bifidobacterium* was lower in IBS patients than controls, but this difference was not statistically significant.

This study revealed that the level of *Bacteroides*, *Bifidobacterium*, and *Clostridium cluster IV* in patients with underlying disease of diabetes was significantly higher compared with those in their healthy counterparts. Microbiome profile showed a high level of *Enterococcus* group in healthy participant than individuals with diabetes. On the contrary to this study, Remely et al. [34] and Sedighi et al. [6] found no significant differences in copy number of genus *Bifidobacterium* between the case and control groups; nonetheless, Murri et al. [35] reported a significant decrease in the number of *Bifidobacterium* in the children with diabetes. Consistent with our findings, Larsen et al. [36] found that the ratio of *Bacteroidetes* was increased in diabetes' cases; however, Lambeth et al. [37] did not observe differences in the abundances of phyla Bacteroidetes. On the contrary to this study, data reported by Remely et al. [34] and Larsen et al. [36] showed no significant difference in the level of *Clostridium cluster IV*

between patients with diabetes and healthy individuals. It is previously reported that total cholesterol and low-density lipoprotein cholesterol reduction are associated with dairy products enriched with *Enterococcus faecium* in participant having a normal lipid profile and participant with medium to moderate hypercholesterolemia [38].

Although participants included in this study were matched for age, gender, race, living environment, and non-interventions of medications and foods, which may affect the outcomes (such as different antibiotics, prebiotics, and probiotics), our findings were not completely consistent with previous reports, and controversial results regarding the dysbiosis of the gut microbiota in hospitalized patients with diarrhea as well as contradictory findings on the relationship between various bacterial groups and IBS and diabetes were observed. This discrepancy might be due to heterogeneity in various factors such as genetic background, ethnicity, geographical location, environmental and occupational exposures, medical history, possible underlying diseases/disorders, lifestyle, and diet habits of participant across studies. Non-significance in some of our results may have been related to a relatively small sample size.

On the whole, the results of this study indicate that diarrhea and the underlying disorders of IBS and diabetes in humans are associated with compositional changes in intestinal microbiota; however, we cannot conclude about the causality of this dysbiosis. Further studies are suggested to determine if microbial imbalance causes these medical conditions or changes in microbiota profile are a reflection of the disease state. Overall, it could be concluded that modifying the bacterial composition by probiotics can be helpful in the control and management of the mentioned disease.

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Conflict of Interest

The authors declare no conflict of interest.

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