Alterations in the Composition of the Gut Microbiota in Celiac Disease, Non-Coeliac Gluten Sensitivity and Irritable Bowel Syndrome

Kaveh Naseri
Shahid Beheshti University of Medical Sciences
Mohammad Amin Shahrbaf
Shahid Beheshti University of Medical Sciences
Meysam Olfatifar
Shahid Beheshti University of Medical Sciences
Abbas Yadegar
Shahid Beheshti University of Medical Sciences
Mona Soheilian-Khorzoghi
Shahid Beheshti University of Medical Sciences
Hossein Dabiri
Shahid Beheshti University of Medical Sciences
Mohammad Rostami Nejad (m.rostamii@gmail.com)
Shahid Beheshti University of Medical Sciences
Amir Sadeghi
Shahid Beheshti University of Medical Sciences
Mohammad Reza Zali
Shahid Beheshti University of Medical Sciences

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Abstract

Background and aims: Some chronic intestinal disorders, including irritable bowel syndrome (IBS), coeliac disease (CD), and non-coeliac gluten sensitivity (NCGS), can make some changes to the gut microbiota composition and cause dysbiosis. This study aimed to determine the gut microbiota alterations in CD, NCGS, and IBS patients among the Iranian population compared to healthy controls.

Materials and Methods: In this prospective study, 72 patients, including 15 healthy controls (HC), 30 IBS, 12 NCGS, and 15 CD patients were included. IBS, CD, and NCGS were diagnosed based on the Rome IV diagnostic criteria, Modified Marsh classification, and gluten challenge test. Stool samples were collected from patients, and after DNA extraction, quantitative real-time PCR (qPCR) was performed for assessing the relative abundance of *Firmicutes*, *Bacteroidetes*, *Bifidobacterium spp.*, and *Lactobacillus spp.*

Results: *Firmicutes* and *Lactobacillus spp*. were the most and the least abundant phylum in all samples, respectively. In CD patients, *Firmicutes* phylum was the most significant relative abundance. *Bacteroidetes* phylum had a significant relative abundance in CD ($P<0.01$) and NCGS ($P<0.05$) patients. The relative abundance of *Bifidobacterium spp*. was statistically lower in CD ($P<0.05$) and IBS patients ($P<0.001$) compared to the HCs. The *Firmicutes* to *Bacteroidetes* ratio was statistically significant in NCGS and CD patients compared to the HCs ($P = 0.05$).

Conclusion: Chronic intestinal diseases, including IBS, CD, and NCGS, can alter the gut microbiota composition.

Introduction

Gut microbiota are microorganisms including bacteria, archaea, and fungi, which live in the gastrointestinal (GI) tract of human- beings and other animals [1]. The gut microbiota has the largest population of bacteria and the most significant number of species compared to other body areas [2]. The microbial composition of the gut microbiota is different across the GI tract with low bacterial species in the stomach and small intestine and high microbial density (up to 10^{11} cells per gram) in the large intestine [3, 4]. Typically, the gut microbiota composition is established one to two years after birth, at the end of the intestinal epithelium and the intestinal mucosal barrier development [5]. However, its composition changes over time based on the body's dietary habits and overall health status [6]. The four dominant bacterial phyla in the human gut microbiota are *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* [7]. Furthermore, most bacteria in the gut microbiota belong to the genera *Bacteroides*, *Clostridium*, *Faecalibacterium*, *Eubacterium*, *Ruminococcus*, *Peptococcus*, *Peptostreptococcus*, and *Bifidobacterium* [8].

Gut microbiota composition depends on various factors, including age [9], diet [10], geography [11], malnourishment [12], race, and ethnicity [13], and socioeconomic status [14]. In addition, some chronic intestinal diseases can alter the gut microbiota composition [15]. For instance, irritable bowel syndrome (IBS), which is a result of stress and its symptoms including abdominal pain, changes in the bowel

movements, can decrease the diversity of the microbiome with low levels of fecal *Lactobacilli* and *Bifidobacteria*, high levels of facultative anaerobic bacteria such as *Escherichia coli*, and increased ratios of *Firmicutes* to *Bacteroidetes* [16, 17]. In addition, inflammatory responses in coeliac disease (CD) and non-coeliac gluten sensitivity (NCGS) which are caused by interferon-gamma (IFN-γ), interleukin-17 (IL-17), and tumor necrosis factor-alpha (TNF-α) can stimulate the innate immune response and alter the microbiome composition [18–20]. These dissimilarities with a healthy microbiome are affective in GI symptoms in these patients [21].

Alterations in the gut microbiota composition in many diseases have been reported in several studies; however, the results are conflicting. Moreover, there is not enough relevant evidence among the Iranian population. This study was aimed to evaluate the gut microbiota in CD, NCGS, and IBS patients compared to healthy control in one of the referral centers of GI diseases in Tehran, the Capital of Iran.

Materials And Methods

Study design

This prospective study was conducted on IBS, CD, and NCGS patients recruited to the Research Institute for Gastroenterology & Liver Diseases of Taleghani Hospital, Tehran, Iran from March 2020 to November 2020. Convenience sampling was used for patients' selection. Patients with anatomical GI diseases were excluded from the study after physical examination and colonoscopy. For entering patients into the study, Rome and Marsh criteria were used. IBS patients were included based on the Rome IV diagnostic criteria [22], and Modified Marsh classification [23] was used for diagnosis of the CD after confirming CD with intestinal biopsy. NCGS patients were included after the gluten challenge test and rule out of the CD [24]. Furthermore, patients with a history of inflammatory bowel disease (IBD), liver diseases, gastrointestinal surgery, cancer, use of non-steroidal anti-inflammatory drugs (NSAIDs), excessive alcohol consumption, systemic use of immunosuppressive agents, and poorly controlled psychiatric diseases in addition to the patients with the history of broad-spectrum antibiotics and probiotics consumption (less than two weeks) were also excluded.

Fecal samples collection

Stool samples were collected from all patients at the baseline of the study. Homogenization of the stool samples was conducted through agitation by using a vortex. Afterward, stool samples were divided into three aliquots within 3 hours of defecation. The aliquots were quickly frozen and stored at -80°C in screw-capped cryovial containers for DNA extraction.

DNA extraction from fecal samples

QIAamp® DNA Stool Mini Kit (Qiagen Retsch GmbH, Hannover, Germany) was used for DNA extraction. DNA concentration was quantified by NanoDrop ND-2000 Spectrophotometer (NanoDrop products, Wilmington, DE, USA). In addition, Nanodrop (DeNovix Inc., USA) was used for assessing the

concentration and purity of the extracted DNA. Extracted DNA samples were stored at -20°C until further analysis.

Microbiota analysis by quantitative real-time PCR (qPCR)

qPCR assay was performed to enumerate four bacterial phyla including *Firmicutes*, *Bacteroidetes*, *Bifidobacterium* spp. and *Lactobacillus* spp. The qPCR was conducted by SYBR Green chemistry using universal and group-specific primers based on the bacterial 16S rRNA sequences presented in Table 1. All PCRs were performed in a volume of 25 µL, comprising 12.5 µL of SYBR green PCR master mix (Ampliqon, Odense, Denmark), 1 µL of 10 pmol of forward, and reverse primers, and 100 ng of the DNA template.

Table 1. The taxon-specific primers used in this study.

Target taxon	Primer name	Primer sequence (5'-3')	Amplicon length (bp)	Reference
Eubacteria	UniF340 UniR514	ACTCCTACGGGAGGCAGCAGT ATTACCGCGGCTGCTGGC	~ 200 bp	[1]
Lactobacillus spp.	Lacto-F Lacto-R	TGGATGCCTTGGCACTAG AAATCTCCGGATCAAAGCTTAC	~ 89 bp	[2]
Bifidobacterium spp.	Bifid-F Bifid-R	GGGATGCTGGTGTGGAAGAG TGCTCGCGTCCACTATCCAG	~ 200 bp	[2]
Bacteroidetes	Bac960-F Bac1100-R	GTTTAATTCGATGATACGCG TTAAGCCGACACCTCACG	~ 137 bp	[3]
Firmicutes	Firm934-F Firm1060-R	GGAG**Y**ATGTGGTTTAATTCGAAGCA AGCTGACGACAACCATGCAC	~ 129 bp	[3]

The nucleotides in bold type represent: Y, C or T; K, G or T; M, A or C; R, A or G; W, A or T.

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Rotor-Gene® Q (Qiagen, Germany) real-time PCR system was used for the PCR amplification. The amplification reaction parameters were assumed as: 95°C for 10 min and 40 cycles at 95°C for 20 and 30 seconds for each primer (Table 1) and 72°C for 20s. Melting curve analysis was conducted to assess amplification accuracy by increasing temperature from 60 to 95°C (0.5°C increase in every 5s). The relative abundance of studied taxa was evaluated based on the ratio of 16S rRNA copy number of the specific bacteria to total 16S rRNA copy number of all bacteria. In addition, the average Ct value for primers was reported as the percentage values using the following formula:

$$X = \frac{(\text{Eff. Univ})^{\text{Ct univ}}}{(\text{Eff. Spec})^{\text{Ct spec}}} \times 100$$

The percentage of 16S taxon-specific copy numbers was indicated by “X”. Furthermore, “Eff. Univ” and “Eff. Spec” represents the efficiency of the universal primers (2 = 100% and 1 = 0%) and the efficiency of the taxon-specific primers respectively. The threshold cycles registered by the thermocycler was indicated by “Ct univ” and “Ct spec”.

Statistical analysis

Analysis of collected data was done using SPSS version 25. Quantitative variables were reported as mean ± standard deviation (SD) and qualitative variables were reported as numerical (%) data. ANOVA test was used for the assessment of the relative abundance differences between two phyla. In addition, we used R software for assessing dissimilarity and Principal Coordinate Analysis (PCoA) in this study. The PCoA was calculated based on the Bray Curtis dissimilarity method [25].

Ethical and regulations consideration

This study protocol was approved and registered by the Ethical Review Committee of RIGLD at Shahid Beheshti University of Medical Sciences with the registration number of: IR.SBMU.RIGLD.REC.1396.154. The study was performed according to the revised Declaration of Helsinki 2013 [26] and informed consents were obtained from all subjects and/or their legal guardians prior to sample collection.

Results

Demographics

Seventy-two samples were enrolled in this study. Thirty-three patients were male (45.8%), and the mean age of the patients was 35.5 ± 6.4. Fifteen patients (20.8%) were in the HC group, 30 (41.7%) in the IBS group, 12 (16.6%) in the NCGS group, and 15 (20.8%) in the CD group. The baseline characteristics of the patients are presented in Table 2.

Table 2
Baseline characteristics of study participants at enrollment

Variables	HC (n = 15)	IBS (n = 30)	NCGS (n = 12)	Coeliac (n = 15)	Total (n = 72)
Age (years)	32.8 ± 12.2	37.8 ± 10.7	31.8 ± 6.4	40.1 ± 8.2	35.5 ± 6.4
Males (n%)	7 (46.7%)	15 (50%)	5 (41.7%)	6 (50%)	33 (45.8%)
Females (n%)	8 (53.3%)	15 (50%)	7 (58.3%)	6 (50%)	39 (54.2%)
Smoking (n%)	4 (26.6%)	9 (30%)	4 (33.3%)	2 (13.3%)	19 (26.4%)
HC: Healthy Control; IBS: Irritable Bowel Syndrome; NCGS: Non-celiac gluten sensitivity					

Microbiota relative abundance analysis

Relative abundance analysis indicated that *Firmicutes* followed by *Bacteroidetes* and *Bifidobacterium spp*. are the most common phylum in all study groups. Furthermore, *Lactobacillus spp.* was the least common phylum among the studied patients. Our results demonstrated that the relative abundance of *Firmicutes* phylum in CD patients was statistically higher than the HC group ($P<0.05$). In addition, *Bacteroidetes* phylum was significantly lower in CD patients ($P<0.01$) and NCGS ($P<0.05$) patients. In addition, *Bifidobacterium* spp. was statistically lower in Coeliac ($P < 0.05$) and IBS patients ($P < 0.001$) comparing with the HC group. Moreover, *Lactobacillus* spp. was significantly lower in Coeliac ($P < 0.05$) and IBS patients ($P < 0.01$) in comparison to the healthy group. The results for the median abundance are presented in Table3 and Figure 1.

Table 3
The mean of the relative abundance for taxonomical groups in each group of the study participants

Taxonomical Group	HC (n = 15)	IBS (n = 30)	NCGS (n = 12)	Coeliac (n = 15)
Firmicutes	29.5 ± 13.9	31.2 ± 13.6	28.6 ± 11.4	46.2 ± 14.0
Bacteroidetes	18.0 ± 11.9	12.0 ± 7.9	7.3 ± 4.0	12.4 ± 9.5
Bifidobacterium spp.	4.4 ± 3.3	0.5 ± 0.5	2.6 ± 1.1	2.1 ± 2.3
Lactobacillus spp.	1.7 ± 2.1	0.3 ± 1.1	0.7 ± 0.4	0.3 ± 0.6
HC: Healthy Control; IBS: Irritable Bowel Syndrome; NCGS: Non-celiac gluten sensitivity				

Firmicutes **to** Bacteroidetes **ratio**

The ratio of *Firmicutes* to *Bacteroidetes* (F/B) was significantly higher in NCGS and Coeliac patients than the HC individuals ($P = 0.05$). However, F/B had no statistical difference in IBS patients in comparison to the HC group. The results for the F/B ratio analysis are presented in Figure 2.

Dissimilarity and PCoA

We measured the extent of fit of the ordination by plotting the observed dissimilarity (as calculated by the dissimilarity matrix) to the ordination distance using a shepherd plot, which yielded a $R^2 = 0.996$, indicating a good fit between the ordination distance and the observed dissimilarity, as calculated by Bray-Curtis index (Figure 3). The dissimilarity between the microbiome of different groups is shown in Figure 4. PCoA suggested that IBS and NCGS patients are more similar to HCs in the context of gut microbiota. In addition, CD patients had more dissimilarity compare to the other groups.

Discussion

In the present study, fecal samples of the three major GI disease patients were analyzed for the gut microbiota and there was a significant difference in *Firmicutes, Bacteroidetes, Bifidobacterium spp.*, and *Lactobacillus spp.* in CD patients. In addition, the relative abundance of *Bifidobacterium spp.* and *Lactobacillus spp.* in IBS patients and *Bacteroidetes* in NCGS were statistically lower than HC group. Furthermore, *Firmicutes* to *Bacteroidetes* ratio assessment was another goal of this study, which was statistically higher in NCGS and CD patients than HCs.

Recent studies suggested that the alteration of gut microbiota composition is associated with CD [27–29]. It was observed that increased abundance of *Bacteroidetes* and reduced abundance of *Bifidobacterium spp.* and *Lactobacillus spp.* are the most often hallmarks of CD microbiota [30]. As it has been discussed in several studies, *Bifidobacterium spp.* and *Lactobacillus spp.* have protective effects on the intestinal epithelial cells from gliadin damage [31], it was suggested that the fecal transplant which can cause an increment in *Bifidobacterium spp.* could reverse the inflammatory pathway in CD patients [32]. In the study of Golfetto et al., the concentration of *Bifidobacterium spp.* in CD patients was significantly lower in compare to the HCs [33]. In contrast, Nylund et al. suggested a higher abundance of *Bifidobacterium spp.* in CD patients compared with HCs that was associated with the higher fecal acetate in HCs [34]. In the study of Nistal et al., *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* were the most frequent gut microbiota [35]. Furthermore, in the study of Bodkhe et al., *Actinobacteria*, *Bacteroides*, *Euryarchaeota*, *Firmicutes* and *Proteobacteria* were the major phyla in the duodenal microbiota of CD patients [36]. The current study was largely associated with the previous results. In our study, *Firmicutes* phylum was dominant in the gut microbiota of all studied groups. In addition, *Bacteroidetes*, *Bifidobacterium spp.* and *Lactobacillus spp.* had significantly lower abundance in CD patients compared to the HCs. The cause of lower abundance of *Bacteroidetes* in this study that is inconsistent with the previous results may be associated with genetic determinants, ethnic background, geographical diversity, and different diet habits which can affect the gut microbiota composition [37–39].

Gut microbiota dysbiosis in the IBS patients has been reported in several studies [40–42]. In fact, gastrointestinal dysbiosis in these patients is associated with the intestinal hypersensitivity, mucosal immune activation and chronic inflammation, which are the three important pathophysiology factors in this disease [43, 44]. Several studies suggested the higher amounts of *Bacteroidetes* and the lower amounts of *Firmicutes* in the IBS patients in compare to HCs [45, 46]. In addition, it has been suggested that IBS is associated with the lower relative abundance of *Bifidobacterium spp.* and *Lactobacillus spp.*

[47, 48]. This study was completely in line with the previous results. It was demonstrated that the relative abundance of *Bifidobacterium spp.* and *Lactobacillus spp.* in the IBS patients is significantly lower in compare to HCs. However, Maccaferri et al. observed an increase in the relative abundance of *Bifidobacterium spp.* and *Lactobacillus* among IBS subjects [49]. It seems that further evidences are needed for confirming these results.

Dysbiosis in the NCGS patients is one of the important issues which can cause constipation, diarrhea, chronic inflammation, intestinal hypersensitivity and immune dysfunction [50]. Garcia-Mazcorro et al. reported a high relative abundance of *Firmicutes* and low relative abundance of *Bacteroidetes* in the fecal microbiota of the NCGS individuals [51]. Furthermore, Dieterich et al. suggested low relative abundance of *Porphyromonadaceae* (from *Bacteroidetes* phylum) in the NCGS patients and high relative abundance of *Sphingobacteria* (from *Bacteroidetes* phylum) in the HCs [52]. This study was in line with the previous results. The relative abundance of *Bacteroidetes* phylum in the NCGS patients was significantly lower in compare to the HCs.

The human gut microbiota is majorly composed of two bacterial phyla and their subdominants that included in *Firmicutes* and *Bacteroidetes* [53]. The *Firmicutes/Bacteroidetes* ratio has an important role in gastrointestinal homeostasis. In fact, increased or decreased F/B ratio which states as dysbiosis, can observe in the chronic intestinal conditions like inflammatory bowel disease (IBD) [54, 55]. In this study, F/B ratio was significantly higher in the CD and NCGS patients compared to other studies groups. This issue has been discussed in the previous studies, especially for the CD therapeutic purpose. In the study of Quagliariello et al., three-month administration of *Bifidobacterium breve* significantly decreased the F/B ration in the CD patients [56]. In addition, in the study of Primec et al., three months of treatment with probiotic had a significant effect on F/B ratio in the CD patients [57]. The results of this study suggested an F/B abnormality in the CD and NCGS patients, which is a useful factor for the assessment of the outcomes of the CD and NCGS treatment.

Analysis of the dissimilarity in this study suggested that CD patients have more dysbiosis as compared to the other chronic GI patients. This issue can justify the alteration in the symptoms in this disease in comparison with the other GI diseases. Furthermore, it has been suggested that the microbiome composition in the IBS and NCGS patients are similar to the healthy microbiome and this issue can suggest better outcome of this disease in comparison to the CD.

Easy access to the patients and conducting the study in a referral center for gastrointestinal disease was one of the positive points of this study. In fact, accurate diagnosis of the diseases such as NCGS requires a gluten challenge test, which may not be available in many medical centers. On the other hand, low sample size and lack of mucosal microbiome evaluation were the major limitation of this study. It would be better for future studies to conduct in a larger sample size and as multicentric studies and examination of fecal microbiome and mucosal microbiome simultaneously to have a better prospective for the differences between mucosal microbiome and fecal microbiome.

Conclusion

In conclusion, this study assessed the relative abundance of the fecal microbiota in IBS, CD and NCGS. In this study, significant alteration was observed in the gut microbiota composition of IBS (low relative abundance of *Bifidobacterium* spp. and *Lactobacillus* spp.), CD (high relative abundance of *Firmicutes* and low relative abundance of *Bacteroidetes*, *Bifidobacterium* spp. and *Lactobacillus* spp.), and NCGS patients (low relative abundance of *Bacteroidetes*). However, some microbial alteration in this study was not concomitant with the previous results, which is may attributed to genetics, geographical pattern, ethnics and diet habits.

Declarations

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Conflict of interest

The authors declare that they have no conflicts of interest.

Author contributions

KN and MSK collected the samples and KN performed the real-time PCR analysis; MRN and HD designed and supervised the study; KN and MO participated in data analysis; KN, and MAS wrote the manuscript; MRN, AY, AS, HD, and MRZ critically revised the manuscript. All authors approved the final version of the manuscript.

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Figures

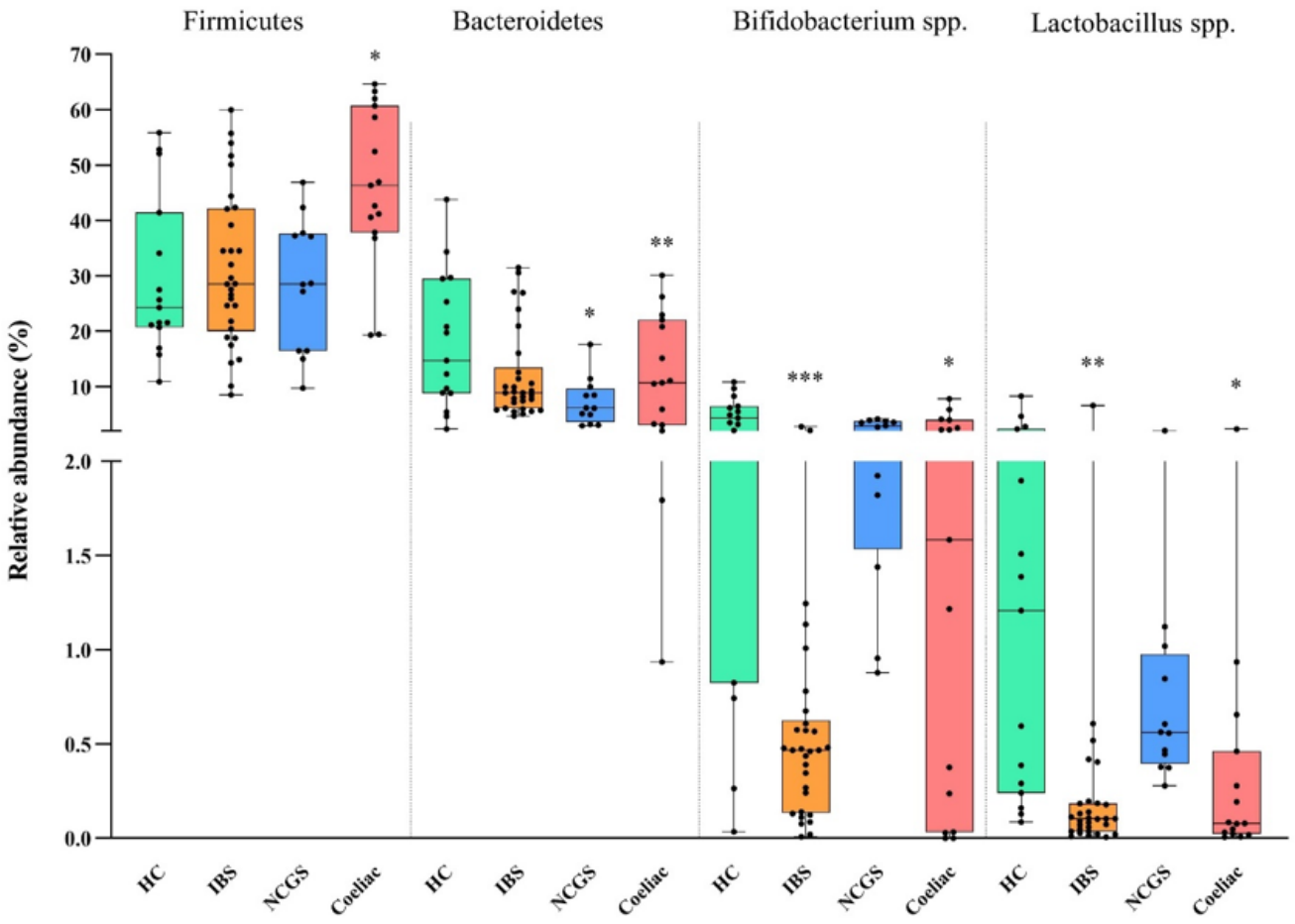

Figure 1

Box plot for the distribution of the selected bacterial taxa by the median abundance that constitutes the fecal microbiota in each group of the study population. Differences in each group of the patients were compared to the healthy control (HC) and were considered to be statistically significant when *P < 0.05, **P < 0.01, and ***P < 0.001.

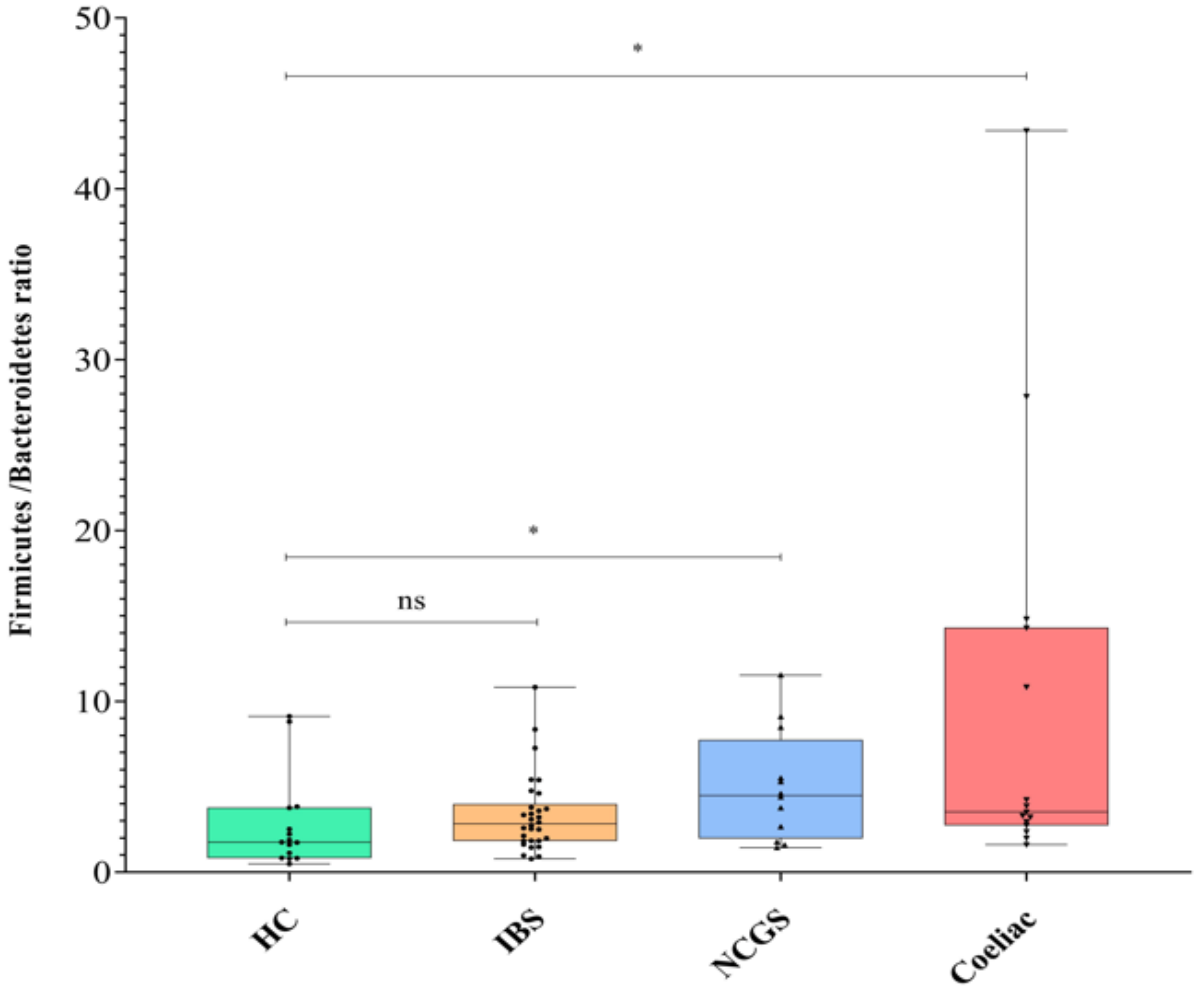

Figure 2

Box plots showing the Firmicutes to Bacteroidetes (F/B) I each group of participants. This ratio was significantly (*P = 0.05) increased in the NCGS and coeliac patients but non-significant in the IBS patients compared with the healthy controls (HC).

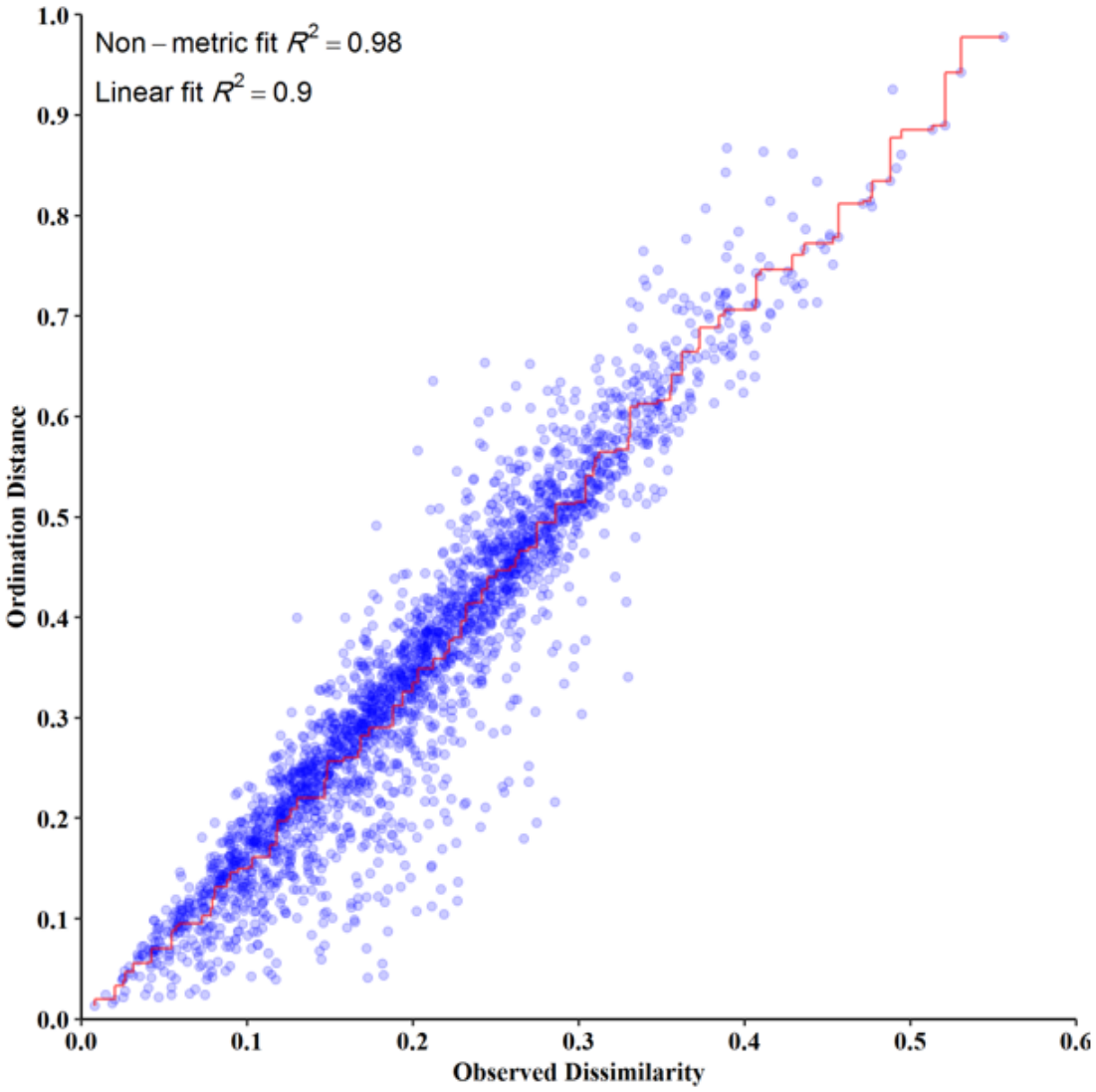

Figure 3

Shepherd plot showing the correlation between the distance from the dissimilarity matrix and the coordination distance for NMDS analysis

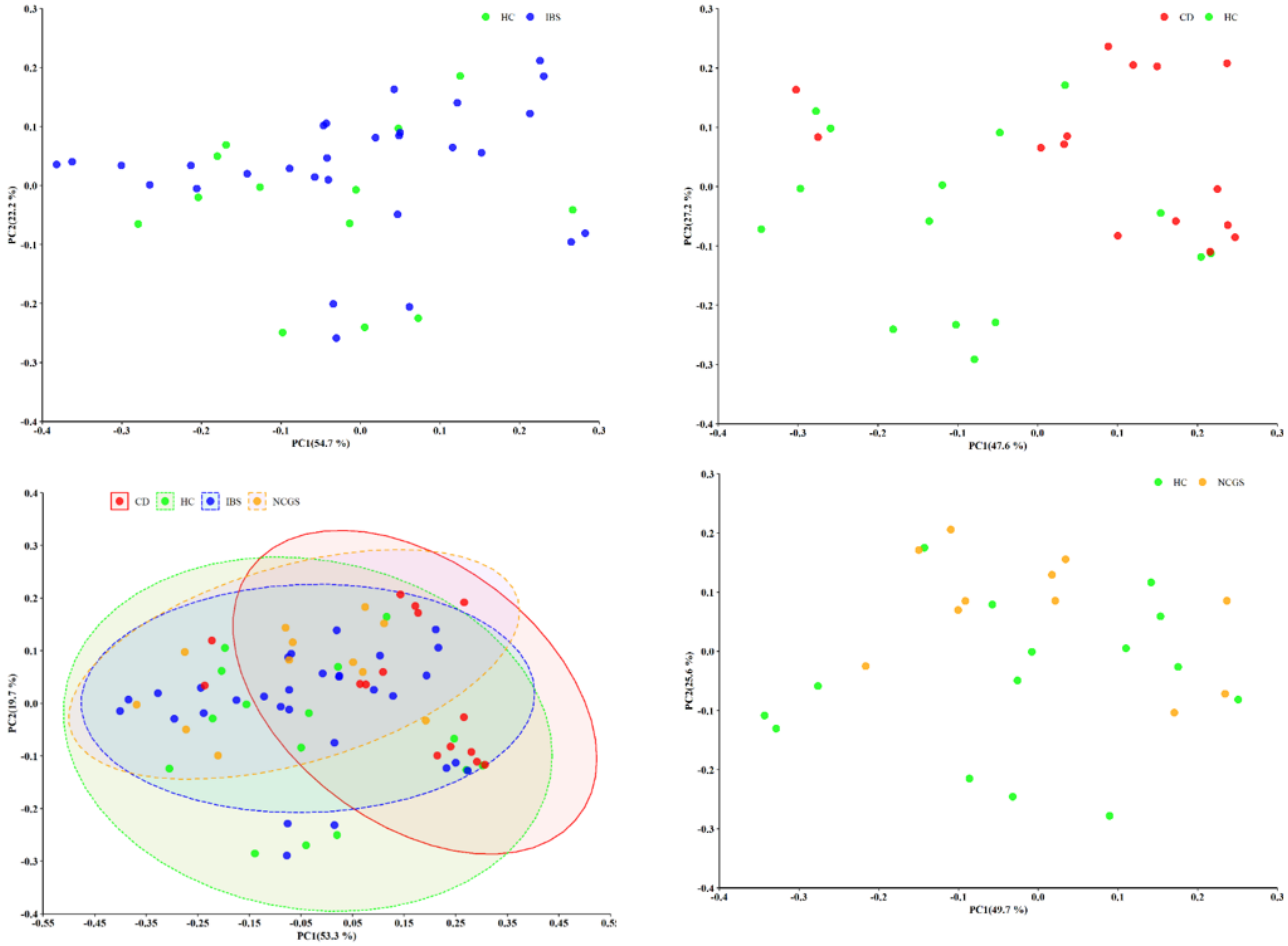

Figure 4

Bray-Curtis dissimilarity metric plotted in PCoA space comparing the microbial communities from different patient groups (CD, IBS, NCGS, and HC). Each circle representing a participant colored according to the studied group.