ORIGINAL ARTICLE

Fecal and Mucosa-Associated Intestinal Microbiota in Patients with Diarrhea-Predominant Irritable Bowel Syndrome

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Abstract

Background Irritable bowel syndrome (IBS) has been associated with changes in the intestinal microbiota. Only a few studies have explored differences in the mucosa-associated microbiota between IBS patients and healthy controls (HC).

Aims To characterize and compare the microbiota in mucosal and fecal samples from carefully selected patients with IBS-D and HC.

Methods The cohort was composed of 23 diarrhea-predominant IBS (IBS-D) patients and 24 HC. Fresh stool samples were collected from participants prior to the collection of colonic mucosal samples from an unprepped bowel. After DNA extraction, 16S rRNA genes were sequenced by 454 pyrosequencing and analyzed using the QIIME pipeline.

Results The fecal microbiota (luminal niche) of IBS-D patients was found to have reduced enteric richness compared to HC ($P < 0.05$), whereas no differences were observed between the two groups within the mucosal microbiota. Within the luminal niche, the relative proportions of *Faecalibacterium* genus were found to be lower in IBS-D than in HC and the *Dorea* genus was higher in IBS-D. None of the taxa proportions were significantly different in IBS-D patients versus HC using an FDR of ≤ 0.1 when analyzing samples that appeared in > 25% samples of either niche.

Conclusion Fecal and mucosal microbiota of IBS-D patients and HC are very similar and are not sufficient to explain the reported altered physiology and symptomatology of IBS-D. Future studies should investigate intestinal microbiome-dependent functional activity in addition to the fecal and mucosal-associated microbial composition.

Keywords Fecal microbiota · Mucosal microbiota · Microbiome · Irritable bowel syndrome · Diarrhea

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Introduction

The intestinal microbiota has attracted considerable attention for its role in the maintenance of human health and the pathogenesis of disease, in particular as related to the gastrointestinal tract [1]. Previous studies have found differences in microbial diversity and composition between the fecal microbiota (FM) and mucosal-associated microbiota (MAM) of the intestinal microbiota in healthy individuals [2, 3]. In a recent study using deep sequencing analysis in healthy volunteers, we reported differences between fecal and mucosal-associated microbiotas [4]. These findings, which were also supported by other groups [5, 6], demonstrated that the intestinal FM and MAM are two distinct microbial niches. This points to the need to investigate both the FM and MAM to better understand the role of the intestinal microbiota in different diseases.

Irritable bowel syndrome (IBS) is the most common functional bowel disorder and is characterized by abdominal pain or discomfort associated with changes in bowel habits and stool characteristics. The etiology of IBS is multifactorial, and the pathophysiology is not completely understood. Nevertheless, several studies over the past few years have found qualitative and quantitative alterations in the intestinal microbiota of patients with IBS. This, together with the reported beneficial effects of interventions targeting the intestinal microbiota (e.g., low carbohydrate diet, antibiotics, and probiotics), has suggested that the intestinal microbiota may play a role in the pathogenesis of the disorder [7, 8]. However, most studies investigating the intestinal microbiota in IBS have been conducted on fecal samples [9–14] and only a few have investigated the differences in the MAM of patients with IBS compared to healthy controls (HC) [5, 10, 15, 16]. Investigation of the degree of microbial variability in fecal and mucosal samples collected from unprepped bowels of IBS and HCs would also enable a more accurate exploration and a better understanding of the importance of microbial dysbiosis in the pathogenesis of the disorder. The goal of this study was to characterize and compare the microbiota in mucosal and fecal samples from carefully selected patients with diarrhea-predominant IBS (IBS-D) and HC.

Methods

Study Population

The subjects were at least 18 years of age, and were recruited without screening for gender, race, or ethnicity. All the IBS patients met the Rome III criteria for the diagnosis of IBS and were examined by a physician prior to enrollment in the study to confirm the diagnosis and rule out other possible diagnoses. Only patients with diarrhea-predominant IBS (IBS-D) were included. Subjects must have been evaluated by a physician to exclude alternative diseases that could explain the symptoms and had no evidence of enteric pathogens on routine stool cultures. Patients with constipation-predominant IBS (IBS-C) or mixed type IBS were excluded. The HC had no significant chronic or recurring gastrointestinal (GI) symptoms and were also evaluated by a physician to exclude GI disease. Exclusion criteria for both groups included age < 18 years, a history of GI tract surgery other than appendectomy or cholecystectomy, a history of inflammatory bowel diseases (IBD), lactose malabsorption, celiac disease, or other diagnoses that could potentially disrupt the intestinal microbiome. Participants were also excluded if they intentionally consumed probiotics or had been treated with antibiotic 2 months prior to enrollment in the study. Participants were recruited through advertisements from the Chapel Hill general population and from the University of North Carolina (UNC) at Chapel Hill outpatient clinics. To accurately determine eligibility and diagnosis of IBS-D, patients were carefully screened at baseline and underwent a 2-week run-in period during which they completed a daily symptom diary. The study was approved by the UNC Internal Review Board and all subjects signed a consent form prior to participation in the study.

Sample Collection and Preparation

Fresh stool samples were collected from participants on site immediately prior to a flexible sigmoidoscopy, or at home the same morning as the procedure. For some participants, only stool was collected without a mucosal sample. Following collection, each fecal sample was immediately transferred on ice to the laboratory where it was homogenized, divided into aliquots, and stored at $-80\,^{\circ}\text{C}$ for DNA extraction, sequencing, and qPCR analysis. Fecal samples were collected and handled in compliance with a validated protocol [13]. Colonic mucosal samples were collected from subjects during an un-sedated flexible sigmoidoscopy. To avoid the possible effects of a colonic cleansing preparation on the mucosal-associated microbiota, all endoscopic procedures were performed on unprepped colons. Colonic mucosal biopsies were taken from the distal colon just above the recto-sigmoid junction using cold biopsy forceps. Once removed from the colon, each biopsy was washed and swirled in 1 ml of sterile PBS to remove non-adherent fecal material. The biopsy samples were then weighed, flash-frozen in liquid nitrogen and stored at $-80\,^{\circ}\text{C}$ for further DNA extraction and molecular microbiological analysis.

Extraction of DNA

Bacterial DNA of healthy individuals was isolated from paired mucosal and fecal samples and from IBS-D samples. In five patients, mucosal samples were not available for technical reasons. Some of these samples had been previously analyzed in a different study comparing fecal and mucosal microbiota in HCs [4] and comparing the fecal microbiota of HC and IBS patients [13]. Similarly, bacterial DNA was isolated from a total of 12 paired mucosal and fecal samples from the IBS study group, and from 11 additional IBS-D patients only stool was available. DNA extraction was done using a phenol/chloroform extraction method combined with physical disruption of bacterial cells and a DNA clean-up kit [Qiagen DNeasy® Blood and Tissue extraction kit (Qiagen, 69504)] as previously described [17]. In brief, 100 mg of frozen feces or a mucosal biopsy was suspended in 750 µl of sterile bacterial lysis buffer (200 mM NaCl, 100 mM Tris [pH 8.0], 20 mM EDTA, 20 mg mL^{-1} lysozyme) and incubated at 37 °C for 30 min. Next, 40 µl of proteinase K (20 mg mL^{-1}) and 85 µl of 10% SDS was added to the mixture and incubated at 65 °C for 30 min. Three hundred mg of 0.1 mm zirconium beads (BioSpec Products, 11079101z) was then added, and the mixture was homogenized in a bead beater (BioSpec Products, 112011) for 2 min. The homogenized mixture was cooled on ice and then centrifuged at 14,000 rpm for 5 min. The supernatant was transferred to a new 1.5-ml microfuge tube, and fecal DNA was further extracted by phenol/chloroform/iso-amyl alcohol (25:24:1) and then chloroform/iso-amyl alcohol (24:1). Following extraction, the supernatant was precipitated by absolute ethanol at − 20 °C for 1 h. The precipitated DNA was suspended in DNase free H2O and then cleaned using the DNeasy® Blood and Tissue extraction kit (Qiagen, 69504) from step 3 as per the manufacturer's instructions.

Pyrosequencing of 16S rRNA Genes

Bacterial community composition in isolated DNA samples was characterized by amplification of the V1-3 (forward, 8f:5′AGAGTTTGATCMTGGCTCAG-3′; reverse 518r: 5′-ATTACCGCGGCTGCTGG-3′) variable regions of the 16S rRNA gene by polymerase chain reaction (PCR). Forward primers were tagged with 10 bp unique barcode labels at the 5′ end along with the adaptor sequence (5′-CCATCT CATCCCTGCGTGTCTCCGACTCAG-3′) to allow multiple samples to be included in a single 454 GS FLX Titanium sequencing plate as previously described [18, 19]. 16S rRNA PCR products were quantified, pooled, and purified for the sequencing reaction. 454 GS FLX Titanium sequencing was performed on a 454 Life Sciences Genome Sequencer FLX machine (Roche, Florence, SC) at the microbiome core at UNC-Chapel Hill (http://www.med.unc.edu/microbiome).

Analysis of 16S rRNA Sequences Using the QIIME Pipeline

The 16S rRNA sequence data generated by the 454 GS FLX Titanium sequencer were processed by the Quantitative Insights into Microbial Ecology (QIIME) pipeline [20]. Briefly, sequences that were less than 200 bp or greater than 1000 bp in length, contained incorrect primer sequences, or contained more than 1 ambiguous base were discarded. Operational taxonomic units (OTUs) were selected using BLAST and the Greengenes reference database at a level of 97% similarity [21]. UniFrac analysis was used to calculate the distance between OTUs/bacterial communities on a phylogenetic tree and principal coordinates were generated using unweighted and weighted UniFrac distances for all samples [22, 23]. Weighted and unweighted UniFrac distances represent compositional dissimilarity/heterogeneity (which is often referred to as beta diversity). However, the unweighted analysis only considers the presence or absence of bacterial groups; thus, all bacterial groups have a similar impact on the UniFrac distances. In a weighted analysis, the relative abundance of the bacterial groups within the communities is accounted for such that more abundant bacterial groups have a correspondingly greater impact on UniFrac distances. Principal coordinate Analysis (PCoA) plots were used to visualize the similarities and dissimilarities between the fecal and MAM that best represented the pairwise distances between groups. Statistical differences for the weighted and unweighted UniFrac distances between groups (beta diversity) were tested using an analysis of similarity (ANOSIM—available through QIIME) by permutation of group membership with 999 replicates. The test statistic R, which measures the strength of the results, ranges from − 1 to 1 where $R = 1$ signifies differences between groups, and $R = 0$ signifies that the groups are identical. The number of observed bacterial species, the Shannon diversity index, and the α diversity (measures of diversity taking into account the richness and evenness of OTUs) [18] were compared using a Student's t test following rarefaction of the OTUs. The proportions of core bacterial taxa [taxa that appeared in at least 25% of samples within either healthy or IBS for each niche (mucosal or luminal)] were normalized as previously described [19] and compared using a student's t test. The normalized taxa abundances from both niches were correlated with each other and when using Pearson correlations. All results were corrected for multiple comparisons and were considered significant if the false discovery rate (FDR) was < 0.1.

Linear discriminant analysis with effect size estimation (LEfSe) [24] was performed on genus-level OTU tables

using the online analysis tool available from http://hutte nhower.sph.harvard.edu/galaxy/. The LEfSe algorithm allows identification of taxa that most strongly differentiate one group from another (i.e., biomarkers). A P value < 0.05 was considered significant. For multiple comparisons, a false detection rate (FDR) < 0.1 was considered significant.

We used PICRUSt to predict and categorize the gene function of mucosal and fecal bacteria. PICRUSt is a bioinformatics software package designed to predict metagenome functional content from 16S rRNA surveys. The correlations between inferred and metagenomically measured gene content averaged ~ 0.8 [25]. We compared categories of bacterial gene function of the mucosa to the stool after setting a threshold of > 2 fold differences between niches with $P < 0.5$. Significant differences were reported if an FDR < 0.1 was found.

Statistical Analysis

Continuous variables were summarized using the mean and standard deviation (SD).

We used a two-tailed significance test of $P < 0.05$ to determine a statistical significance. The SPSS statistical package (Version 15, SSPS Inc., Chicago, IL), was used for all analyses.

Results

A total of 23 IBS-D patients and 24 HC participated in this study. The two groups were similar in terms of age, gender and body mass index (BMI). The IBS-D group consisted of 74% females with a mean age of 35 ± 11.8 (29–50) years and a BMI of 27.8 ± 7.8 (18.7–43.8) kg/m^2. The HC group consisted of 79.2% females with a mean age of 34 ± 11.8 (21–58) years and a BMI of 26.3 ± 8.2 (18.1–53) kg/m^2.

Fecal Microbiota

Fecal bacterial DNA samples were available and analyzed for HC ($n = 19$) and IBS participants ($n = 23$).

Analysis of the Microbial Richness

Rarefaction curves showed reduced fecal enteric richness in IBS-D patients compared to the HC ($P < 0.05$) (Fig. 1a). However, compositional investigation of the fecal microbiota using UniFrac analysis (beta diversity) did not reveal significant group differences between IBS-D and HC (Fig. 2).
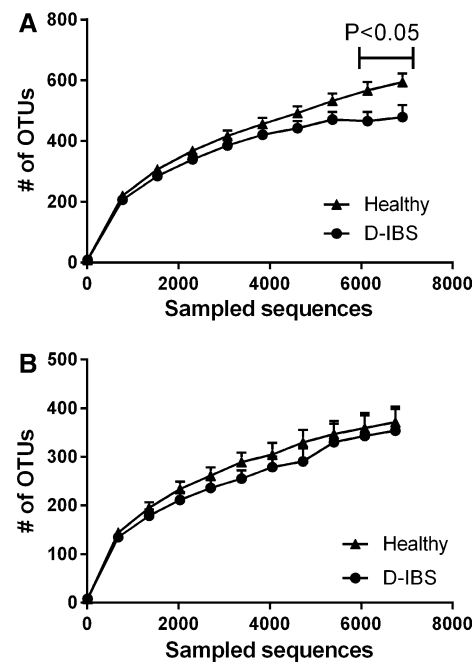

Fig. 1 Fecal and mucosal microbial richness and diversities (rarefaction curves) in samples from IBS-D patients and healthy controls. Rarefaction curves demonstrating (**a**) an increased fecal enteric diversity in healthy controls versus IBS-D patients, and (**b**) no difference in mucosal microbial diversity between the groups

Taxa Analysis

Proportions of various phyla comprising the fecal enteric microbiota were similar for the HC and IBS patients (Fig. 3a) including Firmicutes (92.7 vs. 91.2%, respectively), Bacteroidetes (4.8 vs. 5.5%, respectively), Actinobacteria (1.9 vs. 2.3%, respectively), Proteobacteria (0.4 vs. 0.7%, respectively), Verrucomicrobia (0.1 vs. 0.2%, respectively).

At the genus level, using LEfSe analysis, the IBS-D patients differed from the HC for 6 microbial genera (Fig. 4a). However, a genus analysis of samples that appeared in more than 25% of samples in either niche revealed only one genus (classified as *cc_115;* phylum-Firmicutes; family-*Erysipelotrichaceae*) significantly (FDR $= 0.022$) differed between the two groups. This genus was absent in the fecal samples from the IBS-D group and was present in 11 out of the 24 HC samples (the average proportion in these samples was 0.0018).

Further analysis of the samples, using a biased approach toward a few of the taxa that have been previously reported to be altered in IBS, revealed that the *Faecalibacterium* genus was prevalent in fecal samples of all the participants; however, its relative proportion in the fecal samples of IBS-D patients was significantly lower than in the HC (0.039 vs. 0.054, respectively, $P = 0.022$). In contrast, the proportion of an un-annotated genus from

Fig. 2 Compositional analysis of enteric microbiota in healthy controls and IBS patients. Analysis (beta diversity) of fecal enteric microbiota (**a**, **b**) and mucosal-adherent microbiota (**c**, **d**) in healthy controls and IBS-D patients demonstrated no difference between the groups in a weighted analysis (**a**, **c**) and an unweighted analysis (**b**, **d**) in either niche
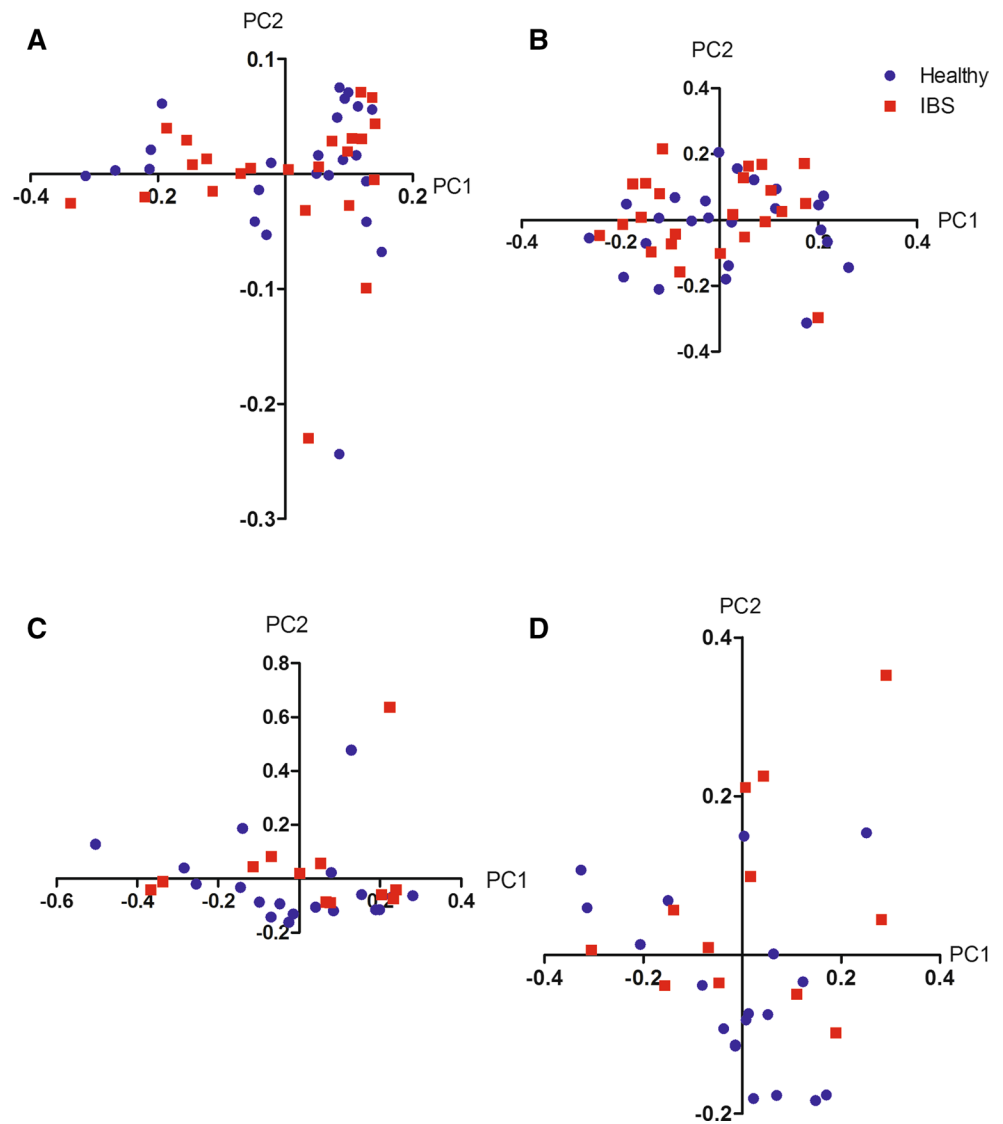
the *Enterobacteriaceae* family was higher in IBS-D patients than in the HC (0.0033 vs. 0.00059, respectively, $P = 0.028$), in that this genus was found in only 33% of the samples of the HC and in 56% of samples of IBS-D patients. Similarly the *Dorea* genus (*Lachnospiraceae* family) was prevalent in the fecal samples of all the participants but its relative proportion was higher in the IBS-D patients (0.03 vs. 0.02, respectively, $P = 0.005$) (Fig. 5).

Mucosa-Associated Microbiota

Mucosal samples were retrieved from the unprepped colon of 20 subjects ($n = 20$) in the HC group and 12 subjects ($n = 12$) from the IBS-D group. One sample from the HC group was not included in the analysis due to a low OTU count (< 1000).

Microbial Richness

Unlike the reduced microbial richness in fecal samples from IBS-D patients reported above, the rarefaction curves of the mucosal samples revealed no differences in microbial richness between IBS-D patients and the HC (Fig. 1b). Similar to the observation in the luminal niche, compositional UniFrac analysis of the mucosal enteric microbiota of the HC and IBS patients showed no statistically significant differences (Fig. 2c, d).

Taxa Analysis

Proportions of the various phyla comprising the mucosal microbiota were similar in the IBS-D patients and the HC (Fig. 3b). However, a LEfSe analysis identified 8 microbial genera that were different in abundance between the two

Fig. 3 Proportions of bacterial taxa in fecal and colonic mucosal samples of healthy individuals and IBS patients. The proportions of the phyla comprising both the fecal enteric microbiota (**a**) and mucosal microbiota (**b**) were similar in the HCs and IBS patients
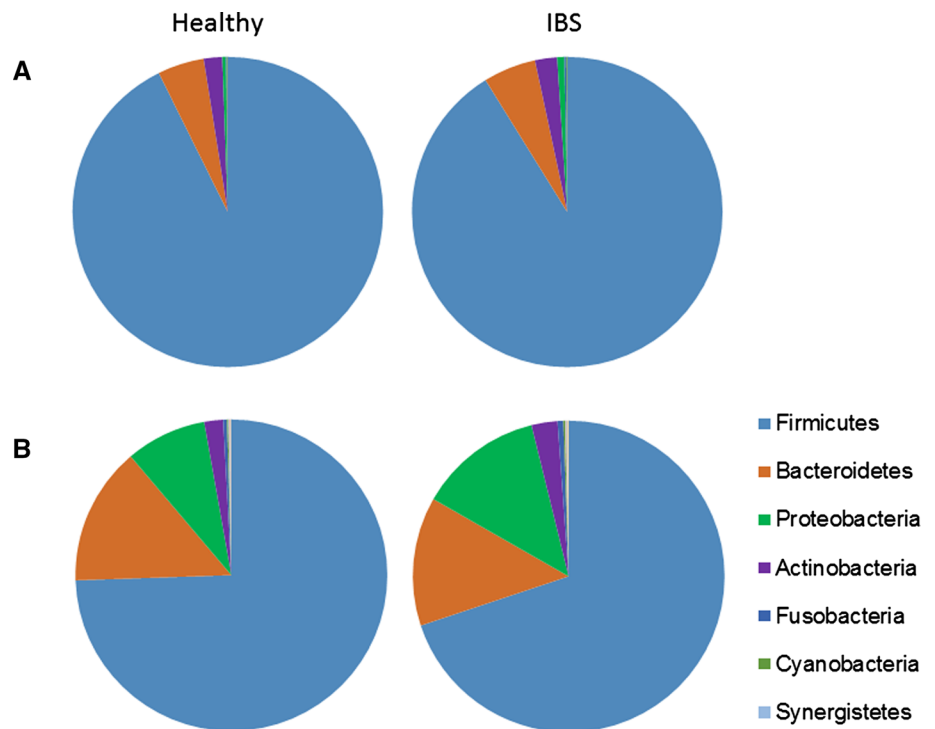
groups (Fig. 4b). None of the taxa proportions were significantly different for the IBS-D patients as compared to the HC using an FDR of ≤ 0.1 when analyzing samples that appeared in > 25% samples of either niche.

Functional Gene Analysis

In an attempt to reveal possible metabolic differences between groups, we used PICRUST software [25] to predict functional content from the 16S rRNA; however, no differences in fecal or mucosal samples in the IBS-D patients and the HC were found.

Discussion

Most studies that have investigated the intestinal microbiota in patients with IBS have focused on fecal microbiota and have been composed of heterogeneous group of patients. Although previous studies have reported an intestinal dysbiosis in IBS patients [12, 26, 27], there is still no general consensus regarding specific compositional alterations.

In this study, which is an extension of our previous work in healthy volunteers, we investigated and compared the intestinal microbiota of fecal samples and mucosal biopsies from a cohort of carefully selected patients with IBS-D and HC. In the fecal microbiota, there was significantly lower microbial diversity and differences in abundance of 6 microbial genera in the IBS-D and HC groups.

In the mucosa-associated microbiota, there were no significant differences in diversity between IBS-D and HC, but we identified differences in abundance of 8 microbial genera between the two groups.

Diversity measures provide useful information about the general composition of microbial populations. Consistent with other studies investigating the biodiversity of intestinal microbiota in IBS [13, 28, 29], we found lower levels of α-diversity (rarefaction analysis) in fecal samples of IBS-D patients compared to the HC, suggesting lower fecal microbial richness (based on the number of observed species) in patients with IBS-D. A decrease in microbial diversity has also been reported in association with intestinal inflammation [30], which is hypothesized to also occur to a lesser extent in some patients with IBS [31, 32]. Regarding the MAM, consistent with previous studies, we found no differences in mucosal microbial diversity between IBS-D patients and the HC [28, 29]. These findings may imply that alterations in the luminal microbiota (as represented by fecal microbiota) may be more important in the pathogenesis of IBS-D than alterations in the mucosal-adherent microbiota. However, it should be noted that in this study, the luminal microbiota was investigated by fecal samples which represented the overall luminal communities throughout the GI tract and possibly some shaded mucus microbes. Similarly, the MAM was examined solely in the recto-sigmoid colon and therefore may not represent possible alterations in other locations of the GI tract [33].
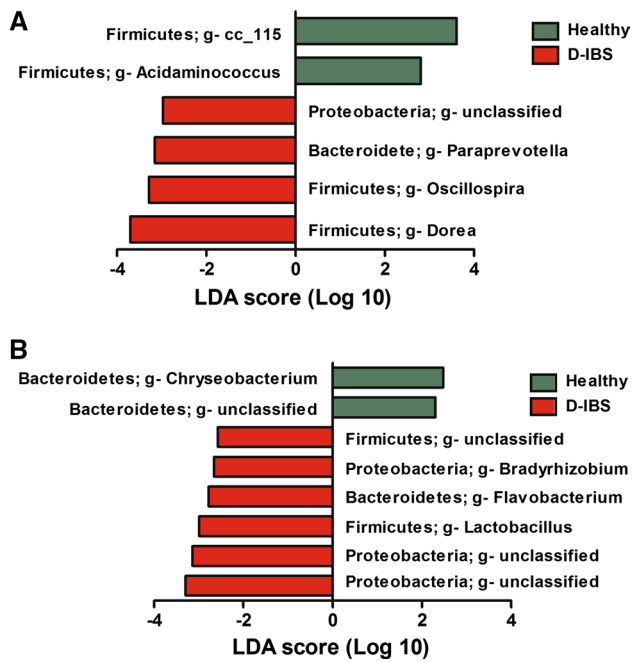
Fig. 4 Comparison of enteric microbial genera between samples from IBS-D patients and healthy controls in terms of linear discriminant analysis (LDA) effect size. Taxa are represented by their phylum and genera (g). (a) Fecal bacteria differed by 6 taxa between the groups. Two taxa (green) were more abundant in samples from healthy controls and four in samples from IBS-D patients (red). (b) Mucosal-adherent bacteria differed by 8 taxa between healthy controls (green more abundant in healthy) and IBS-D patients (Red- more abundant in IBS patients). LDA score is based on a pattern recognition and machine learning to find a linear combination of features that characterizes or separates two or more classes of objects or events. The higher the score is, the higher is the difference between the two groups

Fig. 5 Taxa differences in IBS and healthy controls in fecal samples. The luminal proportions of *Faecalibacterium* (**a**), *Dorea* (**b**) and an annotated bacterial group of the *Enterobacteriaceae* family, were significantly different (unadjusted P value < 0.05) between healthy controls and IBS-D patients

In addition, we found no differences in either the weighted or unweighted UniFrac distance analysis between IBS-D patients and HC for either fecal or mucosal microbiota. This suggests that despite having decreased microbial richness, the fecal samples of IBS-D patients were very similar to those of HC in terms of the compositional heterogeneity of the OTUs. This overall similarity between the microbiotas of IBS and HC participants is in line with a recent publication by Tap et al. [5] despite the two different populations (American and Swedish) recruited to the two studies. In that study, however, there were community differences that were associated with disease severity which our cohort was not large enough to capture. Taxonomic discrepancies between the studies can be attributed at least partially to the different genetic background, dietary habits and weight between the two populations. In addition, methane production was higher in IBS-C patients, implying that microbiome function may be more important than composition for the pathogenesis of IBS.
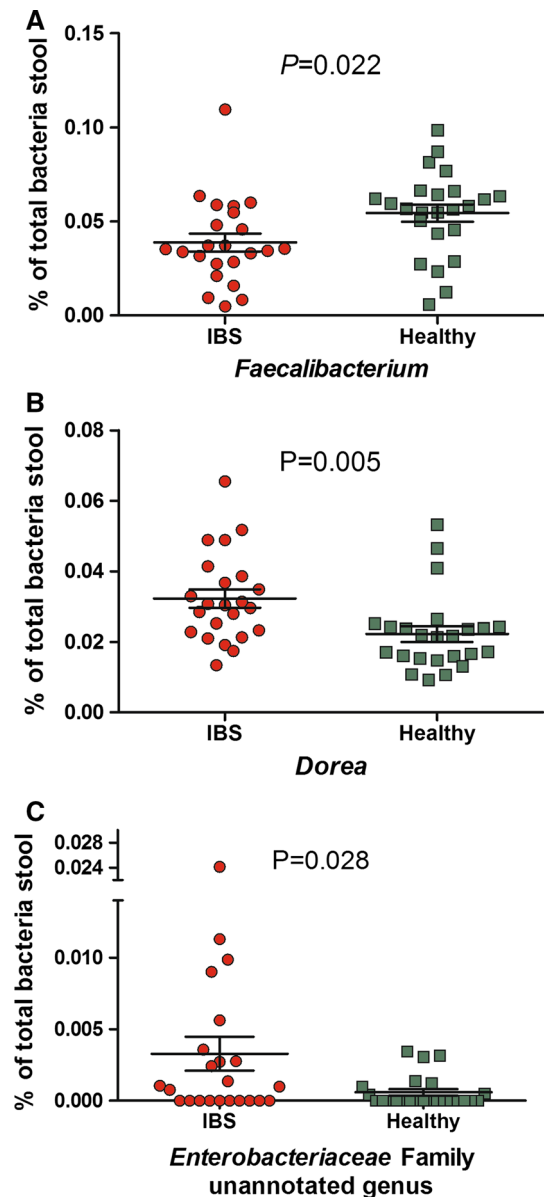
We found the relative proportions of the main bacterial phyla comprising both the FM and MAM to be similar in the two groups. Firmicutes, Bacteroidetes, and Actinobacteria were the predominant phyla within the fecal samples of both IBS-D patients and HC, whereas Firmicutes, Bacteroidetes, and Proteobacteria were the predominant phyla within the mucosal samples of both groups. These findings further illustrate the differences between fecal- and mucosal-associated microbiota and suggest that at least at the phylum level, IBS-D patients and HC do not differ significantly in

their fecal and mucosal-associated microbial populations. In contrast to our findings, Rangel et al. [29] recently reported that the relative abundance of Bacteroidetes was significantly higher and that of Actinobacteria and Proteobacteria were significantly lower in the luminal niche of HCs as compared to IBS patients. The differences can be attributed to differences in samples since Rangel et al. investigated a heterogeneous IBS group (a mix of IBS-D, IBS-C, and IBS-M subtypes), whereas the current study only examined IBS-D. Furthermore, the method of microbiota analysis—phylogenetic microarray (HITChip) analysis in the Rangel et al. study as compared to 16S rRNA pyrosequencing in our study may account for the discrepant findings.

The LEfSe analysis revealed some additional differences in abundance of specific bacterial genera in the two groups for both the mucosal and fecal microbial populations. We found that the fecal samples of IBS-D patients had an increased abundance of *Dorea* spp. compared to those of the HC. These findings are consistent with previous studies involving children [34] and adult patients with IBS [9]. The *Dorea* genus includes carbohydrate utilizing species that are major gas producers. Increased gas production has been associated with abdominal pain and flatulence typical of IBS-D. The *Dorea* genus was also associated with increased intestinal permeability, which is thought to contribute to the IBS pathophysiology [35].

Similar to other studies that have found alterations in bacterial groups belonging to the Firmicutes phylum [15, 36], LEfSe analysis revealed a significantly lower relative abundance of the genus *cc_115* of the Firmicutes phylum in the fecal samples of IBS-D patients compared to HC, but the functions of this specific genus are relatively unknown.

The finding of increased abundance of *Lactobacillus* genus in the mucosal samples of the IBS-D patients compared to the HC is in line with previous studies that found an increase in the concentration of *Lactobacillus* species in fecal samples of IBS-D patients compared to HCs [15, 37]. Interestingly, higher counts of *Lactobacillus* have also been observed in patients with ulcerative colitis [38]. These findings may be attributed to the consumption of *Lactobacillus* species-rich probiotics, by which IBS patients tend to consume.

Previous studies have shown significantly reduced concentrations of *Faecalibacterium* in IBS-D patients compared to HCs. In two separate studies, both Rangel et al. [29] and Rajilic-Stojanovic et al. [9] used a 16S rRNA gene microarray to compare the fecal microbial population of a mixed group of IBS patients to that of HCs. A negative correlation was also reported between the abundance of *Faecalibacterium*-related bacteria and IBS symptoms [9]. The finding of lower levels of *Faecalibacterium* in both of these studies is congruent with the results of our studies using 454 pyrosequencing [13]. The consistent

finding of low levels of *Faecalibacterium* despite the differences in populations (a mixed group of IBS patients in the microarray studies and IBS-D patients in our 454 studies) suggests that lower levels of these beneficial bacteria may be an important etiological factor in the pathogenesis of IBS. *Faecalibacterium* has been previously reported to have anti-inflammatory and protective effects against chronic low-grade inflammation [39] and low levels of this commensal bacterium, specifically *Faecalibacterium prausnitzii*, have been observed in inflammatory conditions, particularly Crohn's Disease [40]. Likewise, low-grade inflammation has been suggested to play a role in the pathophysiology of IBS [31, 32]. Furthermore, our LEfSe analysis also revealed a significant increase in an unclassified genus of the *Enterobacteriaceae* family in IBS-D fecal samples as compared to HCs. This family and some of its genera have been consistently shown to be associated with enteric inflammation in IBS and IBD patients [41, 42]. Further, it should be noted that the microbial alterations found IBS-D compared to HC may be the result of rapid intestinal transit and bowel evacuation. Finally, IBS patients frequently alter their diet, which may be associated with both alterations in transit times as well as with alterations of the enteric microbiota.

This study has several strengths including the careful selection of subjects, the collection of biopsies from a similar location (recto-sigmoid junction) of an unprepared colon and the collection of the fecal and mucosal samples during the same visit, thus ensuring an unbiased comparison of the fecal and mucosal niches. However, it also has a few limitations: (1) Although the number of fecal samples taken from IBS-D patients and the HCs corresponded (23 and 24, respectively), the bacterial DNA of mucosal samples retrieved from the two groups were smaller (12 and 19, respectively). The lower mucosal sampling rate can be attributed to several factors: some of the patients did not undergo sigmoidoscopy and in some of the samples, bacterial DNA retrieval was not sufficient to be included in the analyses; (2) the biopsies were taken solely from the distal (recto-sigmoid) colon given that in unprepped bowel it is usually risky to proceed proximal to the sigma. It has been shown that the MAM differs along the colon [33]. Therefore, samples from the recto-sigma junction may not have been representative of the entire colonic MAM; (3) although LEfSe analysis revealed differences in the abundance of several microbial genera between groups, this method has some inherent limitations and may be less stringent than other statistical analyses [24]; (4) the primers we used to amplify the V1–V3 regions of the fecal bacterial communities, although commonly used, could not detect certain microbial groups, such as, the *Methanobacteriales* class, which have been reported to be reduced in IBS-D patients [43]; and (5) we did not have data on the dietary habits of participants. Diet has

been shown to affect the intestinal microbiota and therefore could have altered the results.

In conclusion, by focusing on the subgroup of patients with IBS-D, we confirmed previous reports from our group and others of lower microbial diversity in patients with IBS-D as compared to HCs. However, there were no significant differences between the groups in terms of diversity in the mucosa-associated niche at the recto-sigma. The causality and importance of this lower fecal microbial diversity needs further investigation.

Given the findings of increased genera *Dorea* and *Lactobacillus* and the reduction in the *Faecalibacterium* genus, it can be speculated that the intestinal microbial dysbiosis in IBS-D is associated with an imbalance in the ratio of detrimental to beneficial bacterial groups.

Our high-throughput sequencing analysis also identified alterations in bacterial groups not yet reported or thoroughly studied in IBS-D patients. The pathophysiological role of these organisms in the etiology of IBS-D remains to be determined.

The lack of significant differences between the groups in overall microbial composition by UniFrac analysis and in the relative proportions of the main bacterial phyla in both FM and MAM indicates that the enteric microbial composition were not very different in IBS-D and HC. These findings highlight the need for future studies to investigate whether IBS patients are unique in their intestinal microbiome-dependent functional activity, including the production of neurotransmitters, gas molecules or other metabolic compounds, in addition to their microbial luminal and mucosal-associated microbial composition throughout the colon and small intestine.

Compliance with ethical standards

Conflict of interest None of the authors report conflict of interests.

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