Molecular analysis of the luminal- and mucosal-associated intestinal microbiota in diarrhea-predominant irritable bowel syndrome

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IRRITABLE BOWEL SYNDROME (IBS) is the most prevalent functional gastrointestinal (GI) disorder, affecting 10–20% of adults and adolescents (20). IBS can present as diarrhea-predominant IBS (D-IBS), constipation-predominant IBS, or mixed-bowel-habit IBS. These disorders are associated with a significantly reduced quality of life (17), psychological comorbidities (21), and a considerable economic burden (44). The lack of understanding of the factors associated with the pathogenesis of this complex group of disorders has resulted in limited effective treatment options for IBS patients. Recent studies have implicated new etiological factors in the pathogenesis of IBS, including alterations in the normal intestinal microbiota, genetic determinants, pathogenic bacterial infection, food sensitivity/allergy, altered enteric immune function, and intestinal inflammation (2, 30, 34).

The intestinal microbiota has been demonstrated to be important for normal GI motor and sensory functions (5, 14, 18, 34, 35), and alterations in the intestinal microbiota have been suggested as a possible etiological factor in the development of functional GI disorders, including IBS (34). Indeed, attempts to alter the composition of the intestinal microbiota using antibiotics, probiotics, or prebiotics have resulted in reduced IBS symptoms (8, 13, 27, 31–33, 35, 39, 43). Several studies have characterized a dysbiosis of the intestinal microbiota in patients with IBS. However, there is no consensus among these studies regarding the specific compositional changes of the intestinal microbiota associated with these disorders (4, 6, 9, 15, 16, 22, 23, 25, 26, 45). Identification of specific alterations in the intestinal bacterial groups that are associated with IBS symptoms is complicated by the heterogeneity of the disorders and the diversity of the intestinal microbiota (19). In addition, it is unclear whether symptoms of IBS are associated with compositional variation of microbial groups in the luminal and/or mucosal niches (49). Therefore, the possible association between IBS and the intestinal microbiota must be investigated in clinically relevant, well-defined subgroups of patients, and luminal and mucosal niches must be analyzed using techniques that explore the composition and diversity of these complex microbial communities. The present study used the molecular fingerprinting technique terminal-restriction fragment length polymorphism (T-RFLP) (7) to characterize and compare the microbiota in fecal and colonic mucosal samples obtained from a subgroup of D-IBS patients and healthy controls.

MATERIALS AND METHODS

Study population. We studied 16 patients that met the Rome III criteria for D-IBS and 21 healthy controls. Subjects were recruited from the general population of Chapel Hill, NC, with advertising and from the University of North Carolina (UNC) Hospitals outpatient clinics.

Inclusion criteria included subjects ≥18 yr of age and of any sex, race, or ethnicity. Healthy controls had no recurring GI symptoms. Subjects with a history of GI tract surgery other than appendectomy or cholecystectomy or a history of inflammatory bowel disease, celiac disease, lactose malabsorption, or any other diagnosis that could explain chronic or recurring bowel symptoms were excluded from the study. In addition, individuals were excluded if they had a history of...
treatment with antibiotics or anti-inflammatory agents, including aspirin or nonsteroidal anti-inflammatory drugs (or steroids), or if they had intentionally consumed probiotics 2 mo prior to the study.

All subjects were evaluated by a physician to exclude an alternative diagnosis to IBS. The study was approved by the UNC Internal Review Board, and all subjects provided written consent prior to participation in the study.

Sample collection and preparation. Fresh stool samples were collected from all 37 subjects on site during a single study visit at UNC. Each fecal sample was immediately transferred on ice to the laboratory, where it was homogenized, divided into aliquots, and stored at −80°C for future DNA extraction and molecular microbiological analysis.

Colonic mucosal biopsies (n = 8 per patient) were collected from each subject at a single study visit during an unsedated flexible sigmoidoscopy. To avoid possible effects of colonic preparation on the composition and diversity of the intestinal microbiota, all biopsies were collected from unprepared colons (i.e., subjects did not undergo a bowel cleansing prior to the procedure). Cold forces were used to take mucosal biopsy samples from the rectosigmoid colon. Taking biopsy samples from the rectosigmoid junction permits consistent collection of samples representative of the distal colon from all subjects and minimizes the discomfort associated with the procedure. Once removed from the colon, each biopsy sample was washed in 1 ml of sterile PBS to remove fecal material. The biopsy samples were then weighed, flash-frozen in liquid nitrogen, and stored at −80°C for further DNA extraction and molecular microbiological analysis.

Extraction of DNA. Bacterial DNA was extracted from one fecal and one mucosal sample from each of the subjects. DNA was isolated from 18 (7 D-IBS patients and 11 healthy controls) fecal and mucosal samples using a phenol-chloroform extraction method combined with physical disruption of bacterial cells and a DNA clean-up kit (DNeasy blood and tissue extraction kit, Qiagen, Valencia, CA). Briefly, a 100-ng sample 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, and 20 mg/ml lysozyme] and incubated at 37°C for 30 min. Then 40 μl of proteinase K (20 mg/ml) and 85 μl of 10% SDS were added to the mixture. After 30 min of incubation at 65°C, 300 mg of 0.1-mm zirconium beads (BioSpec Products, Bartlesville, OK) were added, and the mixture was homogenized in a bead beater (BioSpec Products, Bartlesville, OK) 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-isoamyl alcohol (25:24:1) and then by chloroform-isoamyl alcohol (24:1). After 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 (see above) according to the manufacturer’s instructions. Additional fecal and mucosal DNA from 19 subjects (9 D-IBS patients and 10 healthy controls) was obtained from samples collected in a previous study (6). The study population, inclusion and exclusion criteria, and sample collection and handling procedures were identical in both studies.

T-RFLP PCR. A complex mixture of bacterial 16S rRNA genes was amplified by PCR from each intestinal DNA sample using fluorescently labeled universal primers [carboxyfluorescein (FAM)-labeled 5’-AGAGTTTGTATCCTGGCTCAG-3’ (forward primer 8F) and hexachlorocarbocyanine (HEX)-labeled 5’-GGTACCTTGGTACGACTT-3’ (reverse primer 1492R)], as previously described (10, 40). PCR products were purified using a Qiagen PCR purification kit. PCR products of all fecal and mucosal samples were then digested with Hha I to generate T-RFs of varying sizes. All fecal samples were also digested separately with Hae III or Msp I (mucosal samples were not digested with Hae III or Msp I because of a limited supply of DNA). As a result of polymorphisms in the variable regions of the 16S rRNA gene, T-RF size corresponds to different bacterial groups. T-RFs were separated by capillary electrophoresis on a genetic analyzer (model 3100, Applied Biosystems, Carlsbad, CA). GeneMapper software (Applied Biosystems) was used to determine the size (terminal fragment length in base pairs), height (fluorescence intensity), and abundance (peak width × height) of each T-RF. To attribute specific bacterial groups to T-RFs, we compared the fingerprints generated from each sample with a database containing T-RF data from known and unknown bacterial groups [Microbial Community Analysis (MiCA) database] (41).

Statistical analysis. T-RF size and abundance data from GeneMapper were compiled into a data matrix using Sequenix software (Sequenix, Klein Raden, Germany). These data were normalized (individual T-RF peak area as a proportion of total T-RF peak area within that sample), transformed by square root, and compiled into a Bray-Curtis similarity matrix using PRIMER version 6 software (Primer-E, Ivybridge, UK). T-RF data were then subjected to multi-variante analysis, including hierarchical cluster analysis followed by analysis of similarity (ANOSIM). Using ANOSIM, we assessed the similarities between groups (D-IBS patients vs. healthy controls) and within groups. We computed the significance in PRIMER version 6 by permutation of group membership with 999 replicates. The test statistic R, which measures the strength of the results, ranges from −1 to 1: R = 1 signifies differences between groups, while R = 0 signifies that the groups are identical. The contribution of specific T-RFs to differences in bacterial composition between groups was assessed by similarity percentages (SIMPER). SIMPER results were used to generate pie charts of percent contribution of T-RFs within each group, after a 10% cutoff for low contributors. Biodiversity of each sample was measured by the Shannon-Weiner diversity index, while differences in biodiversity between groups were assessed by a non-parametric Mann-Whitney test.

RESULTS

Study population. A total of 37 subjects were investigated. All subjects provided a fecal and a colonic mucosal sample. The study population consisted of 75% females with a mean age of 36 yr. Demographics and body mass index were similar in the two study groups (Table 1).

A total of 261 different Hha I-generated T-RFs, which represent different bacterial groups, were found in fecal and colonic mucosal samples from all subjects. Hae III and Msp I generated 252 and 284 T-RFs from fecal DNA samples, respectively. Thirty-six of the Hha I-generated T-RFs contributed ~90% of the total T-RFs found in all subjects (Fig. 1, Table 2). The distribution of T-RFs differed between D-IBS patients and healthy controls and between intestinal niches (fecal vs. mucosal, Table 2). For example, 20 T-RFs found in fecal samples from healthy controls were not present in fecal samples from D-IBS patients (Table 2). The abundance (peak width × height) of 5’FAM-187 and 3’HEX-402 in the feces was significantly altered between D-IBS patients and healthy controls. In addition, 3’HEX-400 was present in mucosal samples from healthy controls but not D-IBS patients, while 5’

Table 1. Characteristics of D-IBS patients and healthy controls

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>D-IBS Patients (n = 16)</th>
<th>Healthy Controls (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>35.6 (23–52)</td>
<td>35 (21–60)</td>
</tr>
<tr>
<td>%Female</td>
<td>69</td>
<td>81</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.6 (20.9–40.6)</td>
<td>28.8 (18.1–53)</td>
</tr>
</tbody>
</table>

Values are means (ranges). D-IBS, diarrhea-predominant irritable bowel syndrome; BMI, body mass index.
FAM-189 and 3’ HEX-131 were found in mucosal samples from D-IBS patients but not healthy controls (Table 2). These data demonstrate compositional differences in the fecal- and mucosal-associated microbiota between D-IBS patients and healthy controls. To attribute bacterial groups to these T-RFs, the MiCA database was queried (41). This database provided bacterial grouping (phylum, class, order, or family) for the majority of T-RFs that contribute to the composition of the fecal- and mucosal-associated microbiota in D-IBS patients and healthy controls (Table 2). The majority of T-RFs represented several bacterial groups; however, comparison of bacterial groups identified by fecal T-RFs generated by three different enzymes [Hha I, Hae III, and Msp I (Tables 2 and 3)] revealed that the order Clostridiales and the family Planctomycetaceae were consistently associated with T-RFs that contributed 90% of fecal microbiota in healthy controls but not D-IBS patients.

**Biodiversity of T-RFLP profiles.** The Shannon-Weiner diversity index was used to determine the biodiversity (richness and evenness of T-RFs) of T-RFLP profiles from intestinal samples. A significant 1.2-fold decrease (2.7 ± 0.65 for D-IBS patients and 3.3 ± 0.86 for healthy controls, \( P = 0.008 \)) in biodiversity was found in Hha I-digested T-RFs from fecal samples from D-IBS patients compared with healthy controls (Fig. 2B). Hae III-generated T-RFs for 34 fecal samples [13 from D-IBS patients and 21 from healthy controls (3 samples from D-IBS patients failed to yield detectable T-RFs following digestion)] demonstrated a significant 1.06-fold decrease in biodiversity (3.47 ± 0.37 for D-IBS patients and 3.67 ± 0.60 for healthy controls, \( P = 0.015 \)) in fecal samples from D-IBS patients compared with healthy controls. A 1.04-fold decrease in biodiversity in Msp I-generated T-RFs from D-IBS fecal samples failed to reach statistical significance (0.979 ± 0.02 for D-IBS patients and 0.983 ± 0.02 for healthy controls, \( P = 0.296 \)). No significant differences were observed in biodiversity from mucosal samples between D-IBS patients and healthy controls (1.6 ± 0.89 and 1.6 ± 0.84, respectively, \( P = 1 \)) using Hha I-generated T-RFs.

We also compared the biodiversity of fecal and mucosal samples within each group. We observed a significant increase in biodiversity in the fecal niche compared with the mucosal niche in both groups, with a 1.7-fold increase (2.7 ± 0.65 for feces and 1.6 ± 0.89 for mucosa, \( P = 0.001 \)) in D-IBS patients and a 2.1-fold increase (3.3 ± 0.86 for feces and 1.6 ± 0.84 for mucosa, \( P = 0.001 \)) in healthy controls (Fig. 2, C and D).

**Multivariate analysis of T-RFLP profiles.** Nonmultidimensional scaling (nMDs) of T-RF profiles generated by Hha I-digested PCR products of samples obtained from all subjects (D-IBS patients and healthy controls) demonstrated that the community profiles in fecal and colonic mucosal niches are clearly distinct from one another (Fig. 3A). Hierarchical clustering analysis of T-RF profiles confirmed this observation (Fig. 3B). The degree of separation (as determined by \( R \) value, where \( R = 1 \) indicates complete separation between groups) between fecal and mucosal niches was more evident when these two niches were compared in healthy controls than in D-IBS patients, although both were statistically significant (\( R = 0.41, P = 0.001 \) in healthy controls; \( R = 0.22, P = 0.001 \) in D-IBS patients; Table 4).

No distinct separation by nMDs or hierarchical clustering was observed between D-IBS patients and healthy controls from fecal or mucosal niches (Table 4). T-RF profiles were also generated for fecal samples (13 from D-IBS patients and 21 from healthy controls) using Hae III or Msp I restriction enzymes (3 samples from D-IBS patients failed to yield detectable T-RFs following digestion). nMDs and hierarchical clustering of T-RFs generated by Hae III and Msp I did not detect a distinct separation between D-IBS patients and healthy controls from fecal samples (\( R = -0.035, P = 0.687 \) for Hae III; \( R = -0.001, P = 0.470 \) for Msp I).
Table 2. Percent contribution of predominant T-RFs in fecal and mucosal samples from healthy controls and D-IBS patients

<table>
<thead>
<tr>
<th>Predicted Bacterial Group</th>
<th>Mucosa</th>
<th></th>
<th>Feces</th>
<th></th>
<th>Predicted Bacterial Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Values are means ± SE of normalized terminal-restriction fragment (T-RF) peak abundance from top 90% of contributors (predominant contributors) within each group. Predicted bacterial group refers to bacterial phylum, class, order, or family assigned to a T-RF based on the resolution of the Microbial Community Analysis (MiCA) database (41). NBG, numerous bacterial groups (i.e., MiCA database provided a large number of bacterial groups for this T-RF that are too numerous to list; NC, no contribution (i.e., T-RF does not contribute to the top 90% of T-RFs in a sample). Underlined bacterial groups are those consistently identified by T-RFs generated from 3 restriction enzymes (Hha I, Hae III, and Msp I) that contributed 90% of microbiota in healthy controls but not D-IBS patients. Significantly different from % contribution in healthy controls: *P = 0.04; †P = 0.003.</td>
</tr>
</tbody>
</table>
D-IBS patients greater variation of denaturing gradient gel electrophoresis IBS patients. Similar to our findings, Codling et al. reported a to investigate the intestinal microbiota in a mixed population of groups within the intestinal microbiota. Codling et al. (9) identifies no differences in the mucosal-associated from D-IBS patients than in healthy controls. These two higher concentration of species in fecal samples

<table>
<thead>
<tr>
<th></th>
<th>Healthy Controls</th>
<th>D-IBS Patients</th>
<th>Predicted Bacterial Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%Contribution</td>
<td>%Positive</td>
<td>%Contribution</td>
</tr>
<tr>
<td><strong>Hae III T-RFs</strong></td>
<td></td>
<td></td>
<td>Hae III-</td>
</tr>
<tr>
<td>5’ FAM-316</td>
<td>2.4</td>
<td>95.4</td>
<td>NC</td>
</tr>
<tr>
<td>5’ FAM-297</td>
<td>1.2</td>
<td>91.3</td>
<td>NC</td>
</tr>
<tr>
<td>5’ FAM-221</td>
<td>1.2</td>
<td>82.6</td>
<td>NC</td>
</tr>
<tr>
<td>3’ HEX-218</td>
<td>0.9</td>
<td>69.6</td>
<td>NC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Msp I-</td>
</tr>
<tr>
<td>5’ FAM-478</td>
<td>0.7</td>
<td>62.5</td>
<td>NC</td>
</tr>
<tr>
<td>5’ FAM-221</td>
<td>1.0</td>
<td>79.2</td>
<td>NC</td>
</tr>
<tr>
<td>5’ FAM-67</td>
<td>0.8</td>
<td>70.8</td>
<td>NC</td>
</tr>
<tr>
<td>3’ HEX-84</td>
<td>2.9</td>
<td>95.8</td>
<td>NC</td>
</tr>
<tr>
<td>3’ HEX-68</td>
<td>1.6</td>
<td>87.5</td>
<td>NC</td>
</tr>
</tbody>
</table>

Underlined bacterial groups are those consistently identified by T-RFs generated from Hae III, Hha I, or Msp I that contributed 90% of microbiota in healthy controls but not D-IBS patients.

DISCUSSION

Several studies have investigated the composition of the intestinal microbiota in patients with IBS and healthy individuals (4, 9, 15, 16, 22, 23, 25, 26, 45). Although these studies demonstrated some differences between these two groups, the findings are not consistent. The majority of studies that characterized the intestinal microbiota in IBS patients investigated mixed populations of IBS patients (4, 42, 45) or focused on a single intestinal niche (4, 15, 16, 22, 23, 25, 45, 46). Recent reports demonstrating compositional and diversity differences between intestinal luminal- and mucosal-associated microbiota in humans highlight the importance of characterizing enteric microorganisms in both niches when investigating the role of the microbiota in intestinal diseases (11, 29). Only two studies have investigated the intestinal microbiota of luminal and mucosal niches in patients with IBS (6, 9). In a recent study, using culture and quantitative real-time PCR (6), we investigated the intestinal microbiota in fecal and colonic mucosal samples from D-IBS patients and healthy controls. We found a significantly lower concentration of aerobic bacteria and a higher concentration of Lactobacillus species in fecal samples from D-IBS patients than in healthy controls. These two techniques identified no differences in the mucosal-associated microbiota between the groups. However, this study was limited to the analysis of a finite number of predetermined bacterial groups within the intestinal microbiota. Codling et al. (9) used denaturing gradient gel electrophoresis (an alternate fingerprinting technique to the method used in our current study) to investigate the intestinal microbiota in a mixed population of IBS patients. Similar to our findings, Codling et al. reported a greater variation of denaturing gradient gel electrophoresis profiles in fecal samples from healthy controls than IBS patients. However, in contrast to our findings, Codling et al. reported no differences in the variability between the fecal- and mucosal-associated microbiota in IBS patients. The discrepancy between the two studies may relate to the mixed population, preparation prior to mucosal sample collection, or inclusion of only nine IBS patients and no healthy controls for the mucosal analysis in the study of Codling et al. Thus our current study is the first to use a molecular fingerprinting technique in a comprehensive investigation of fecal and unprepared colonic mucosal samples from a large group of patients with a specific subtype of IBS (D-IBS) and healthy controls.

Analysis of the contribution of T-RFs to the composition of the intestinal microbiota demonstrated a considerable overlap in the predominant T-RFs within the fecal- and mucosal-associated microbiota in IBS patients. The discrepancy between the two studies may relate to the mixed population, preparation prior to mucosal sample collection, or inclusion of only nine IBS patients and no healthy controls for the mucosal analysis in the study of Codling et al. Thus our current study is the first to use a molecular fingerprinting technique in a comprehensive investigation of fecal and unprepared colonic mucosal samples from a large group of patients with a specific subtype of IBS (D-IBS) and healthy controls.

Hae III T-RFs

Hae III- and Msp I-generated T-RFs that contribute to 90% of the fecal microbiota in healthy controls but not D-IBS patients.

5’ FAM-67 0.8 70.8 NC 57.1 Acidimicrobineae, Actinomycetales, Comamonadaceae

5’ FAM-237 1.2 82.6 NC 69.2 Ktedonobacteraceae, Burkholderiales, Xanthomonadales, Planctomycetaceae

3’ HEX-218 0.9 69.6 NC 76.9 Acidimicrobidae, Anaerolineaceae, Lachnospiraceae, Planctomycetaceae, Verrucomicrobiaceae

Hae III T-RFs

Hae III T-RFs

Hae III- and Msp I-generated T-RFs that contribute to 90% of the fecal microbiota in healthy controls but not D-IBS patients.

FAM-478 0.7 62.5 NC 71.4 Cytophagaceae, Chromatiaceae

FAM-67 0.8 70.8 NC 57.1 Acidimicrobinae, Actinomycetales, Solirubrobacterales, Ruminococcaceae, Planctomycetaceae, Rhodospirillales, Comamonadaceae

HEX-68 1.6 87.5 NC 57.1 Desulfovibacteraceae

HEX-84 2.9 95.8 NC 92.8 Actinomycetales

HEX-297 1.2 91.3 NC 84.6 Cyanobacteria, Synergistaceae, Planctomycetaceae, Sphingomonadaceae

FAM-221 1.2 82.6 NC 69.2 Ktedonobacteraceae, Burkholderiales, Xanthomonadales, Planctomycetaceae

FAM-316 2.4 95.4 NC 100 Clostridiales

FAM-297 1.2 91.3 NC 84.6 Cyanobacteria, Synergistaceae, Planctomycetaceae, Sphingomonadaceae
90% of the fecal microbiota in healthy controls but not D-IBS patients. Previous reports showed a reduction of \textit{Clostridium coccoides} in IBS patients that is in line with our findings (23). Interestingly, a reduction in the order Clostridiales has also been reported in ileal Crohn’s disease (47). The order Clostridiales encompasses many bacterial species, including the protective bacterium \textit{Faecalibacterium prausnitzii}. It is therefore speculated that the absence of protective bacterial species from the order Clostridiales may be associated with D-IBS. A more sensitive molecular technique that classifies the intestinal microbiota at a species level will enable further investigation of this finding. The family Planctomycetaceae is a low-abundance member of the normal intestinal microbiota (1). The importance of our finding of reduced abundance of this bacterial group in D-IBS patients is unclear, as the role of this group of organisms in the intestinal tract has not been investigated in depth.

Although we found compositional differences in the fecal microbiota between D-IBS patients and healthy controls with respect to specific T-RFs, we did not find a clear separation between these two groups with nMDs or hierarchical clustering. This suggests that the differences in composition of the microbiota between D-IBS patients and healthy controls are due to the relative abundances of specific bacterial groups that do not affect the overall composition of the intestinal microbiota. In support of this concept, it has been recently demonstrated that specific bacterial species (\textit{Klebsiella pneumoniae} and \textit{Proteus mirabilis}) that do not affect the overall composition of the intestinal microbiota independently drive colonic disease in the presence of the endogenous microbiota in a horizontally and vertically transmissible mouse model of ulcerative colitis (12).

Our study demonstrates a significantly lower level of fecal microbial biodiversity in patients with D-IBS compared with healthy controls. Additionally, our study demonstrates, for the first time, a significantly higher level of microbial biodiversity in fecal- than in mucosal-associated communities within D-IBS patients and healthy controls. The increase in biodiversity in fecal compared with mucosal samples is greater in the healthy controls than in D-IBS patients. This observation requires further investigation to determine whether the intestinal microbiota in D-IBS patients is distributed differently between the fecal and mucosal niches compared with the microbiota in a...
healthy intestine. The biodiversity in the human gut is a result of coevolution between host and microbe (3) and has been shown to be stable over time in healthy individuals (48). Thus a departure from the normal biodiversity of the intestinal microbiota may reflect a disease state in the gut. For example, a reduction in enteric bacterial biodiversity has been reported in inflammatory bowel diseases (24, 28, 37, 38). It can be speculated that a reduction in bacterial biodiversity allows for certain members of the intestinal microbiota to flourish and affect intestinal function, such as regulation of inflammation or motility, and therefore perpetuate GI symptoms.

Despite the meticulous efforts used in our study to preserve and characterize the microbiota from different intestinal niches, certain limitations were encountered. 1) T-RFLP was unable to provide the resolution necessary to identify the bacterial species in the intestinal microbiota that are altered between D-IBS patients and healthy controls. We were able to assign a bacterial “phylum, class, order, or family” to most T-RFs. Although this level of characterization provides interesting insight into the intestinal microbiota associated with D-IBS patients, bacterial families encompass many bacterial genera and species. Thus, on the basis of these data sets, it is difficult to make definitive conclusions regarding the association of specific bacterial groups with D-IBS. 2) It is important to remember that T-RFLP fingerprinting is limited to the analysis of only the predominant members (usually 30–50 T-RFs) of complex microbial communities (7). Not surprisingly then, our study found that 34 T-RFs contributed 90% of the fecal microbiota in healthy controls. Hence, it is possible that differences in less abundant bacteria were not detected by this technique. 3) The biopsies analyzed in this study were taken during unprepared flexible sigmoidoscopy to avoid the effects of bowel cleansing agents on the intestinal microbiota. The biopsies were taken from the distal colon just above the rectosigmoid junction. This site was selected because it provides a clear anatomic point to enable consistent collection of mucosal samples and to minimize the discomfort of the subject. However, the microbiota of this colonic region may not be representative of the microbiota of the whole colon, and we did not collect biopsies from more proximal colonic regions.

In conclusion, we have demonstrated compositional and biodiversity differences in the intestinal microbiota between patients with D-IBS and healthy controls. Our study does not address whether these differences are an etiological cause or an effect of the disorder, but it does provide a rationale for further investigation of the role of the intestinal microbiota in the pathogenesis of IBS. Our findings emphasize the importance of investigating the fecal- and mucosal-associated microbiota in this disorder, as well as the need for techniques with higher resolution (e.g., high-throughput sequencing of the 16S rRNA gene), which may enable deeper characterization of the intestinal microbiota and identification of the specific bacterial groups that are altered in IBS.

Table 4. Analysis of similarity test for global community composition

<table>
<thead>
<tr>
<th>Global Test (R)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feces: D-IBS patients (n = 16) vs. healthy controls (n = 21)</td>
<td>0.04</td>
</tr>
<tr>
<td>Mucosa: D-IBS patients (n = 16) vs. healthy controls (n = 21)</td>
<td>-0.02</td>
</tr>
<tr>
<td>D-IBS patients: feces (n = 16) vs. mucosa (n = 16)</td>
<td>0.22</td>
</tr>
<tr>
<td>Healthy controls: feces (n = 21) vs. mucosa (n = 21)</td>
<td>0.41</td>
</tr>
</tbody>
</table>

Data are from Hha I-generated T-RF length polymorphism fingerprints. *Statistically significant.

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GRANTS

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