Characterization of Proximal Small Intestinal Microbiota in Patients With Suspected Small Intestinal Bacterial Overgrowth: A Cross-Sectional Study

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OBJECTIVES: The composition of the small intestinal microbiota has not yet been characterized thoroughly using culture-independent techniques. We compared small intestinal microbial communities in patients with and without small intestinal bacterial overgrowth (SIBO) using culture-dependent and culture-independent bacterial identification approaches.

METHODS: Small bowel aspirate and mucosal samples were collected from patients with suspected SIBO. The aspirates were cultured to diagnose SIBO, defined as $\geq 10^4$ colony-forming units/mL coliform or $\geq 10^5$ colony-forming units/mL upper aerodigestive tract bacteria. Bacteria in the aspirates and mucosa were identified using 16S rRNA gene sequencing. We compared small intestinal microbiome composition between groups with and without a culture-based SIBO diagnosis.

RESULTS: Analysis of the aspirate and mucosal microbial communities from 36 patients revealed decreased $\alpha$-diversity but no differences in $\beta$-diversity in patients with SIBO compared with those without SIBO. There were no significant differences in the relative abundance of individual taxa from the aspirates or mucosa after adjustment for false discovery rate between patients with and without SIBO. Subgroup analysis revealed significant differences in mucosal $\beta$-diversity between the coliform and upper aerodigestive tract subgroups. Relative abundances of a mucosal Clostridium spp. ($P = 0.05$) and an aspirate Granulicatella spp. ($P = 0.02$) were higher in coliform SIBO vs non-SIBO subgroups. The microbial composition and relative abundance of multiple taxa significantly differed in the mucosal and aspirate specimens.

DISCUSSION: Culture-based results of small bowel aspirates do not correspond to aspirate microbiota composition but may be associated with species richness of the mucosal microbiota.

SUPPLEMENTARY MATERIAL accompanies this paper at http://links.lww.com/CTG/A89

INTRODUCTION
Small intestinal bacterial overgrowth (SIBO) has historically been described in the context of maldigestion and malabsorption in patients with anatomic abnormalities (e.g., fistulae, strictures, bowel reconstruction, bowel obstruction), underlying dysmotility secondary to diabetes or scleroderma, and hypochlorhydria (1). This syndrome has also been implicated in the pathophysiology of numerous gastrointestinal and hepatobiliary diseases including common conditions such as irritable bowel syndrome (IBS) (2) and functional dyspepsia (3). Typical SIBO-associated symptoms include bloating, diarrhea, abdominal pain, and gas. SIBO symptoms may overlap with those of functional gastrointestinal disorders. However, the role of SIBO in the pathogenesis of functional gastrointestinal disorders remains controversial given that its definition relies on tests, including breath test and culture of small bowel aspirates, which have not been appropriately validated (4). Indirect techniques such as breath testing have important limitations related to heterogeneous testing methods and lack of standardization in interpretation. To address these limitations, guidelines for breath testing indications, performance, and
interpretation were recently published in a North American consensus document (5). Yet, there remain important knowledge gaps in how these tests reflect the microbial landscape of the human small intestine.

Small bowel aspirates and culture were previously considered the gold standard for the diagnosis of SIBO. SIBO has traditionally been defined by abnormally high amounts of bacteria in the small intestine and more precisely by >10^5 colony-forming units (CFU)/mL of colonic-type bacteria from jejunal aspirates (6). However, the validity of this approach has been questioned as diagnostic of SIBO (5), especially if coliforms are present (8). Standard culture-based techniques are limited in their ability to characterize fully the complex diversity of the small intestinal microbiome, particularly as a large proportion of bacterial species remain uncultured (9). In light of increasing evidence supporting the hypothesis that the intestinal microbiome serves a key role in host physiology and gastrointestinal disease, better characterization of the small intestinal microbiome will be a critical step in developing novel diagnostic and therapeutic strategies in a number of human diseases and disorders.

In the recent years, the introduction of culture-independent techniques has dramatically expanded our ability to characterize the complexity of the intestinal microbiota. It also has increased our knowledge of the culture conditions needed to detect and identify previously difficult to culture organisms (10). Integration of rapid molecular approaches (11) for the assessment of SIBO in conditions such as IBS has yielded promising data including evidence for decreased microbial diversity from duodenal aspirates in patients with IBS compared with control subjects (12). However, these findings are preliminary. Overall, there are limited data reporting detailed characterizations of the human small intestinal microbiome. It may be hypothesized that alterations in the small intestinal microbiome are associated with culture-proven SIBO, which in turn could suggest that although imperfect, culture-proven SIBO is at least a useful surrogate marker of small intestinal dysbiosis. However, further investigation is needed to (i) explore the role of microbial diversity in SIBO using culture-independent approaches and (ii) describe the interactions between the host and small intestinal microbiome in the context of gastrointestinal disease. This study characterizes small intestinal microbial composition of small bowel aspirates and mucosal specimens in patients with and without a diagnosis of SIBO defined by qualitative and quantitative culture of proximal small bowel aspirates.

**METHODS**

**Study design and participants**

We conducted a cross-sectional study comparing microbial profiles of small bowel specimens in patients with and without a culture-based diagnosis of SIBO presenting to the Motility and Neurogastroenterology Clinic at Indiana University from March 2013 to November 2015. The study protocol was approved by the Indiana University School of Medicine Institutional Review Board. Participants were prospectively enrolled after signing informed consent at the time of small bowel enteroscopy. All patients with suspected SIBO based on clinical symptoms and undergoing small bowel enteroscopy for diagnostic evaluation were eligible for inclusion regardless of clinical history. We excluded patients using antibiotics, probiotics, or colon cleansing preparations in the last 30 days before enteroscopy and those who were pregnant or could not provide informed consent.

**Variables**

Demographic data (age, gender, race/ethnicity) and anthropometric assessments (body mass index [BMI], kg/m^2/) were collected at the time of enrollment. Clinical history was reviewed to assess for the presence of abdominal distension, diarrhea, and the risk factors for SIBO including conditions (e.g., proton pump inhibitor use) and surgeries associated with decreased acid production (e.g., Billroth I or II, vagotomy, gastric bypass for obesity) (13). Clinical symptoms were further assessed using the validated Patient Assessment of Gastrointestinal Disorder-Symptoms Severity Index questionnaire (14). All demographic and clinical data were collected on enrollment and before the assessment of SIBO status to minimize potential for information bias. Outcomes of interest were (i) quantitative and qualitative cultures of jejunal aspirates and (ii) microbial composition of jejunal aspirates and mucosal biopsy specimens.

**Study procedures**

Study participants underwent upper enteroscopy using a pediatric colonoscope (11.3 mm diameter) or a small caliber upper enteroscope (9.2 mm diameter) for the collection of luminal aspirate and mucosal biopsy specimens within the proximal jejunum using a standardized protocol as previously detailed (13). Procedure details are outlined in the supplementary material (see Table, Supplemental Digital Content, http://links.lww.com/CTG/A89), which outlines the procedures for enteroscopy. The enteroscope was advanced past the ligament of Treitz to the maximum extent of reach into the jejunum, and an aspiration catheter was introduced through the working channel to collect at least 2 mL of luminal fluid. After the collection of luminal aspirates, 2 mucosal biopsies were obtained from the proximal jejunum on withdrawal of the endoscope. Mucosal biopsy samples were stored at -80 °C for subsequent 16S rRNA analysis. Jejunal aspirate samples were split at the time of collection to be sent for quantitative and qualitative bacterial cultures and to be stored at -80 °C for subsequent polymerase chain reaction analysis.

**Quantitative culture of jejunal aspirates**

Small bowel aspirates were cultured for both aerobic and anaerobic bacteria using standard techniques. Aspirate specimens were plated on blood agar, MacConkey agar, chocolate agar, and colistin and nalidixic acid agar plates, and incubated for a minimum of 48 hours. Bacterial isolates were identified by species and quantified as CFU/mL. Given the lack of a universally accepted cutoff for defining SIBO at the time this study was conducted, we used the following thresholds to define coliform and upper aerodigestive tract (UAT) SIBO, respectively: (i) presence of ≥10^4 CFU/mL Gram-negative aerobic and anaerobic colonic-type bacteria (e.g., Escherichia coli spp, Klebsiella spp, Proteus mirabilis, Acinetobacter spp, Enterobacter spp, Citrobacter spp, Bacteroides spp, or Clostridium spp) and (ii) presence of ≥10^5 CFU/mL Gram-positive aerobes or facultative anaerobes or other bacteria characteristic of the proximal gut and oropharynx (e.g., Streptococcus spp, Staphylococcus spp, Enterococcus spp, Lactobacillus spp, Fusobacterium spp, or Peptostreptococcus spp) (4,15,16). Participants were classified as having SIBO if the culture results revealed evidence of either UAT or coliform SIBO.
Molecular analysis of jejunal aspirates and mucosal biopsy specimens

Luminal aspirate samples and mucosal biopsy samples were thawed on ice. Total nucleic acids were extracted using the DNeasy tissue kit (Qiagen, Venlo, The Netherlands), and the eluted DNA was stored at −20 °C. Gel electrophoresis and fluorescent assay (Qubit assay kit; Life Technologies, Carlsbad, CA) were used to determine the quality and quantity of isolated genomic DNA (gDNA). gDNA specimens were stored at 4 °C for immediate use in 16S rRNA sequencing. Polymerase chain reaction amplification of V1–V3 regions of 16S rRNA alleles was performed using degenerate 16S rRNA primers with a barcoding strategy previously validated by our laboratory. Reagent controls were processed in parallel to monitor for contamination. Normalized amplicon libraries were pooled and sequenced using the Illumina MiSeq sequencer (Illumina, San Diego, CA) using paired 300 bp reads to generate 4,765,249 total reads. 16S sequences were processed and fed into the Mothur package (v1.37.4) to generate lists of microbial taxa and relative loads. Subsampling at a depth of 1,563 total reads was performed for each sample to correct for biases caused by differential sequencing depth. Consensus sequences or contigs were identified by overlapping paired end reads. The standard operating procedure of Mothur for MiSeq data processing was used to trim primers and barcodes from each read and to remove chimeric and low-quality sequences. Operational taxonomic units (OTUs) were identified with a sequence similarity cutoff at 0.97, and the representative OTUs were taxonomy classified using the RDP classifier (v2.11) (17) at the genus level and Bayesian Lowest Common Ancestor method (18) at the species level. The R software environment (https://www.r-project.org/) was used to calculate standard ecological α-diversity (Observed taxa, Chao1 metrics; S, ACE; Shannon; Simpson; Pielou evenness) with vegan package. Principal coordinate analysis (ape package in R) (19) and nonmetric dimensional scaling method were applied to cluster samples based on Bray-Curtis dissimilarities (ecodist package in R) (20) in anticipation of comparative analyses of β-diversity.

Study endpoints

The primary study endpoint was overall microbial diversity. Secondary endpoints were relative abundance and prevalence of specific bacterial taxa.

Study size

Sample size was determined by the number of available cases during the study period. No formal a priori calculation for sample size was performed for this pilot investigation.

Statistical analysis

Pairwise comparisons of α-diversity indexes between cohorts were performed using the Wilcoxon rank sum test. Principal coordinate analysis and nonmetric dimensional scaling were used to visualize and compare Bray-Curtis dissimilarity, and comparisons between groups were performed using the PERMANOVA approach adjusting...
for BMI. We also uncovered associations between clinical cohorts and specific bacterial taxa by comparing relative taxa abundance between groups using a linear model adjusting for BMI. A negative binomial regression with mixed model for repeat measurements was applied to analyze the differences in relative abundance of specific bacterial taxa between the groups. Differences in community composition across sampling sites (aspirate vs mucosa) were examined by Bray-Curtis dissimilarities using the PERMANOVA approach. To explore potential heterogeneity related to cultured bacterial types, we also conducted predefined subgroup analyses comparing patients with a diagnosis of coliform SIBO, UAT SIBO, and no SIBO.

Participants with missing data were excluded from the analyses; all statistical tests were performed using the R software environment (http://www.r-project.org). Analyses were confined to taxa with relative abundance of $>0.1\%$ in any one of the cohort groups. Two-sided $P$-values $<0.05$ were regarded as significant. Reported $P$ values were corrected for multiple testing using the Benjamini-Hochberg procedure. All sequences and associated metadata were deposited to the NCBI Sequence Read Archive under the BioProject ID PRJNA472002.

RESULTS

Patient characteristics

Among the 86 patients who signed informed consent, 76 completed upper enteroscopy with luminal aspiration. 16S sequencing data were obtained in 32 and 34 patients, respectively. In one case, sequencing data were available from only the aspirate or the biopsy specimen but not both. We were unable to amplify bacterial 16S sequences from 2 of the 32 aspirate specimens and 1 of the 34 biopsy specimens.

Table 1. Demographics and baseline characteristics among patients with and without SIBO by the culture of jejunal aspirates

| Factor                                | SIBO (n = 15)       | No SIBO (n = 21)     | P value |
|---------------------------------------|--------------------|----------------------|---------|
| Age (yr)                              | 53.00 ± 13.58      | 48.05 ± 12.00        | 0.27    |
| Female patients, n (%)                | 13 (86.7)          | 20 (95.2)            | 0.56    |
| Race (% white)                        | 13 (86.7)          | 20 (95.2)            | 0.56    |
| Body mass index (kg/m²)               | 28.42 ± 6.96       | 25.16 ± 7.05         | 0.18    |
| Condition associated with decreased gastric acid production (e.g., PPI use), n (%) | 10 (66.7)          | 8 (38.1)             | 0.18    |
| Surgery associated with decreased gastric acid, n (%) | 4 (26.7)           | 1 (4.8)              | 0.14    |
| Small bowel motility failure (e.g., chronic intestinal pseudo-obstruction, radiation enteropathy, visceral neuropathy, or myopathy), n (%) | 3 (20)             | 2 (9.5)              | 0.63    |
| Diarrhea, n (%)                       | 6 (40)             | 7 (33.3)             | 0.74    |
| Daily abdominal distention, n (%)     | 8 (53.3)           | 12 (57.1)            | 0.35    |
| PAGI-SYM total score                  | 2.81 ± 0.94        | 3.09 ± 0.87          | 0.39    |
| PAGI-SYM subscale scores              |                    |                      |        |
| Heartburn/regurgitation                | 2.04 ± 1.43        | 2.13 ± 1.35          | 0.85    |
| Nausea and vomiting                   | 2.45 ± 1.34        | 3.03 ± 1.30          | 0.22    |
| Postprandial fullness/early satiety   | 3.07 ± 1.09        | 3.47 ± 1.10          | 0.31    |
| Bloating                              | 3.64 ± 1.51        | 3.55 ± 1.53          | 0.86    |
| Upper abdominal pain                  | 2.92 ± 1.43        | 3.57 ± 1.00          | 0.17    |
| Lower abdominal pain                  | 2.36 ± 1.47        | 2.83 ± 1.12          | 0.32    |

Data show mean ± SD values unless otherwise stated. Comparisons carried out using t-test for continuous variables and Fisher exact test for categorical variables.

PAGI-SYM, patient assessment of upper gastrointestinal symptom severity index; PPI, proton pump inhibitor; SIBO, small intestinal bacterial overgrowth.
Significant differences were also observed in the relative abundance of multiple individual taxa (Table 3) between the aspirates and mucosa at both genus and OTU levels.

**Subgroup analysis of SIBO cohorts by culture type**

Significant differences in mucosal microbiota β-diversity were observed between patients with coliform and UAT SIBO (Figure 3). There were no differences in mucosal microbiota α-diversity among subgroups (no SIBO, UAT SIBO, and coliform SIBO). Comparisons of taxonomic-level datasets (Table 4) showed an increase in the relative abundance of a *Clostridium* spp. in mucosal specimens of patients with coliform SIBO vs patients without SIBO ($P = 0.05$) after correcting for FDR. *Granulicatella* spp. was also enriched in the aspirates of patients with coliform SIBO vs patients without SIBO ($P = 0.02$) after adjusting for BMI and correcting for FDR. OTU level analyses showed a higher abundance of a *Clostridium perfringens*-assigned OTU in the mucosa of patients with coliform SIBO compared with patients without SIBO before ($P = 0.001$) and after ($P = 0.03$) adjusting for BMI and correcting for FDR. A *Granulicatella*-assigned OTU was enriched in the aspirates of patients with coliform SIBO vs patients without SIBO after adjusting for BMI and correcting for FDR ($P = 0.01$). Prevalence of individual bacterial taxa from the mucosal specimens was not significantly associated with culture diagnosis. Prevalence of *Clostridium sensu stricto* in the luminal aspirates of patients with coliform SIBO ($24\%$) was higher ($P = 0.04$) than in patients without SIBO ($0.01\%$) or UAT SIBO ($0\%$). There were no significant differences in the relative abundance of individual taxa in the aspirates or mucosa between patients with UAT SIBO and those with coliform SIBO nor between patients with UAT SIBO and patients without SIBO at either taxonomic level. Relative abundances of individual bacterial taxa from each individual are shown in Figure 4.

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**Figure 2.** Small intestinal mucosal microbiota demonstrates lower α-diversity in patients with SIBO vs patients without SIBO. SIBO, small intestinal bacterial overgrowth. Alpha diversity determined using Obs (a), Chao1 (b), ACE (c), Shannon (d), Simpson (e), and Pielou (f) indices.

**DISCUSSION**

In this pilot investigation, we compared luminal aspirate and mucosal microbial communities from patients with and without a culture-based diagnosis of SIBO of the proximal gastrointestinal tract. To date, there are only a handful of studies that have examined the small intestinal microbiome. Relatively little is known regarding its role in symptom generation, although recent data have suggested that the small intestinal microbiome, but not SIBO based on quantitative culture of small bowel aspirates, is associated with gastrointestinal symptoms (21). In our study, we made several interesting observations. First, comparison of microbial communities revealed lower α-diversity (Chao1, ACE index) at the genus level in the mucosal microbiome of patients with SIBO compared with patients without SIBO ($P = 0.047$).

**Comparisons of community composition across sampling sites**

Significant differences in β-diversity (Bray-Curtis dissimilarity) were observed between the aspirate and mucosal microbiota ($P = 0.001$) at the genus level. These differences remained significant after including subject and BMI as confounding factors.

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Second, analysis by subgroup went on to reveal significant differences in mucosal microbiota β-diversity between patients with coliform SIBO and those with UAT SIBO, suggesting that there may be differences in the community composition of the mucosal microbiota between these 2 SIBO subgroups. These observations may further suggest that the overall SIBO cohort encompasses 2 heterogeneous groups (coliform and UAT SIBO). By combining these groups using broad culture-based cutoffs, we may be diluting our ability to appreciate important differences in the community composition of the luminal and mucosal microbiota between patients with and without bacterial overgrowth.

Single taxon-based testing suggested that the relative abundance of multiple taxa in the aspirate and mucosa of patients with SIBO differed from those of patients without SIBO before but not after adjustment for FDR. Overall results support the long-standing concern that cultivating bacteria in the laboratory does not clearly represent pathologic shifts in the members of the microbial community and is largely insufficient for characterizing the complex microbial composition of the gut.

Subgroup analyses further demonstrated enrichment of Clostridium spp. and a C. perfringens-assigned OTU in the mucosa of patients with coliform SIBO compared with those without SIBO as well as enrichment of Granulicatella spp. and a Granulicatella-assigned OTU in the luminal aspirates of patients with coliform SIBO compared with those without SIBO. Enterotoxinogenic C. perfringens is an important cause of food-borne illnesses and other gastrointestinal diseases such as antibiotic-associated diarrhea (22,23). Higher levels of fecal C. perfringens have been described in elderly individuals residing in long-term care. The presence of C. perfringens in these individuals was associated with significant changes in microbiota composition including decreased levels of Bifidobacterium spp., suggesting that C. perfringens recovery in the stool may reflect a less healthy microbiota (24). Granulicatella spp. are Gram-positive facultative anaerobes that are considered a normal part of the oral bacteria but have been shown to be increased in patients with cirrhosis with hepatic encephalopathy (25) compared with patients with cirrhosis without encephalopathy. Granulicatella spp. have also been shown to coaggregate with other bacterial species implicated in dental plaque biofilm formations, such as Aggregatibacter actinomycetemcomitans and Fusobacterium nucleatum (26). Although preliminary, our findings may suggest increased relative abundance of mucosal Clostridium spp. and/or luminal Granulicatella spp. may serve as a microbial marker for disturbances in the small intestinal microbiota in patients with coliform SIBO. Findings could also indicate that patients with coliform SIBO may have increased susceptibility to infection by pathogenic microorganisms or abnormal commensal expansion. Lack of significant differences with single-taxon analyses between patients with UAT and coliform SIBO or between patients with UAT and no SIBO may suggest considerable overlap between these particular culture-based diagnostic subgroups.

Last, comparison of small intestinal microbiota composition from the luminal aspirates and mucosal biopsies showed significant differences in β-diversity (Bray-Curtis dissimilarity) and relative abundance of multiple bacterial taxa. Higher abundances of members belonging to Prevotella and Fusobacterium were observed in the mucosal microbiota compared with the luminal microbiota, whereas higher abundances of the genera Streptococcus and Actinomyces and the Lactobacillus gasseri-assigned OTU were observed in the luminal aspirate microbiota. These differences emphasize the distinct stratification of microbial community composition in the gastrointestinal tract.
communities within the lumen and mucosa, which have previously been described by others (27,28). It should be noted that there remains a paucity of data describing the changes in microbial composition from the lumen to the mucosa within the human small intestine, which may be directly influenced by aspects of perfusion and oxygen diffusion that are unique to this site (29).

The relative importance of the luminal microbiota compared with the mucosa-associated microbiota in disease pathogenesis and the interaction between these 2 communities will require further study. This study has several strengths including prospective enrollment of study participants with careful assessment of several factors associated with the gut microbiota (age, gender, BMI), use of a standardized technique for the collection of study specimens by 2 experienced endoscopists, and concomitant application of both traditional culture-based methods and culture-independent molecular approaches for the characterization of the small intestinal bacteria. The proportion of patients meeting diagnostic criteria for SIBO in this study was high, which was not unexpected given that our site serves as a tertiary referral center and participants were recruited from among those in whom a diagnosis of SIBO was suspected based on the clinical history and symptoms. Study limitations include the lack of nonmicrobial-defined patient groups and the inability to study SIBO of the mid- and distal gastrointestinal tract. It is also possible that culture techniques were limited in their ability to facilitate the growth of anaerobic organisms due to potential exposure of specimens to air and may, in some cases, have been inadequate for the assessment of SIBO. Because the participants were recruited among patients receiving clinical care at the gastrointestinal motility clinic, study physicians could not be blinded to the clinical history or results of previous testing, as this information was pertinent to patient care. However, patient groups were defined based on objective laboratory testing rather than a clinical diagnosis, and investigators sought to enroll patients both with and without a culture-based diagnosis of SIBO. Furthermore, outcome assessments (i.e., laboratory-based culture results and small intestinal microbiota based on culture-independent techniques) were performed by study team members and by members of

### Table 3. Significant differences observed in relative abundance of specific bacterial taxa in the aspirate and mucosal specimens in the overall cohort

| Factor          | Aspirate specimens | Mucosal specimens | P-value | FDR-adjusted P-value |
|-----------------|--------------------|-------------------|---------|----------------------|
| **Genus**       |                    |                   |         |                      |
| Prevotella      | 1.07 ± 3.87        | 9.14 ± 10.16      | <0.0001 | <0.0001              |
| Streptococcus   | 58.85 ± 24.67      | 31.60 ± 21.47     | <0.0001 | 0.0008               |
| Fusobacterium   | 0.81 ± 1.45        | 2.24 ± 4.39       | 0.0007  | 0.0043               |
| Actinomyces     | 6.41 ± 8.99        | 2.91 ± 2.87       | 0.0096  | 0.0353               |
| Lactobacillus   | 2.71 ± 7.22        | 0.69 ± 2.16       | 0.018   | 0.0619               |
| Neisseria       | 2.10 ± 12.06       | 2.14 ± 9.92       | 0.032   | 0.0942               |
| **OTU index**   |                    |                   |         |                      |
| 003 (Prevotella melaninogenica) | 1.04 ± 3.74       | 10.16 ± 11.36     | <0.0001 | <0.0001              |
| 011 (Fusobacterium nucleatum) | 0.80 ± 1.38        | 2.51 ± 4.61       | <0.0001 | <0.0001              |
| 001 (Streptococcus mits) | 61.13 ± 22.60     | 31.73 ± 21.89     | <0.0001 | 0.0001               |
| 021 (Porphyromonas pasten) | 0.10 ± 0.38        | 1.00 ± 1.89       | 0.00017 | 0.0007               |
| 015 (Lactobacillus gassen) | 2.15 ± 6.50        | 0.25 ± 0.64       | 0.001   | 0.0039               |
| 004 (Actinomyces odontolyticus) | 6.48 ± 8.87       | 3.27 ± 2.92       | 0.0094  | 0.0291               |
| 013 (Lachnoanaerobaculum orale) | 1.72 ± 2.20       | 0.99 ± 1.06       | 0.012   | 0.0334               |
| 006 (Neisseria perflava) | 2.22 ± 12.82       | 2.18 ± 9.80       | 0.019   | 0.0474               |

Comparisons carried out using negative binomial regression with mixed model for repeat measures. Correction for multiple testing applied using Benjamini-Hochberg Procedure. OTU annotation performed by Bayesian Lowest Common Ancestor software. Data represent mean relative abundance (%) ± SD. FDR, false discovery rate; OTU, operational taxonomic unit.

Figure 3. Significant differences in mucosal microbiota β-diversity between patients with coliform SIBO (black circles) and those with UAT SIBO (white circles) at both (a) genus level and (b) OTU levels. SIBO, small intestinal bacterial overgrowth; UAT, upper aerodigestive tract.
the microbiology laboratory who were not directly involved with patient care. Therefore, the impact of a potential selection bias on study recruitment and outcomes due to lack of blinding is overall felt to be minimal. Interpretation of findings are also limited by small sample size and limited generalizability given that our institution serves a tertiary referral center. We did not assess for methanogenic archaea, which may be pathogenic contributors to SIBO (30). However, post hoc analysis did not reveal any significant differences in the relative abundance of methanogenic organisms such as Methanobrevibacter spp. between those with and without SIBO. This is not unexpected as comparison groups were defined based on standard culture results and not on the results of breath testing for methane.

In conclusion, our study suggests that in patients with evidence of proximal SIBO based on culture of small bowel aspirates, luminal overgrowth is not correlated with luminal microbiota composition. Instead, patients with a culture-based diagnosis of proximal SIBO exhibit decreased mucosal microbial richness (e.g., α-diversity). Additionally, mucosal community diversity (e.g., β-diversity) differs between patients with coliform SIBO vs those with UAT SIBO by culture, suggesting that they are distinct microbiologic groups that should be examined separately to better define the clinical utility of culture-based testing. Further prospective investigations that examine the correlation between symptom profiles and the small intestinal aspirate and mucosal microbiota, incorporate deep sequencing techniques, and consider functional and metabolomics profiling will be required to better characterize the relevant microbial communities of the small intestine and to identify future microbial targets for improved diagnosis and management of SIBO.

**CONFLICTS OF INTEREST**

Guarantor of the article: Andrea Shin, MD, MSc.

Specific author contributions: Andrea Shin, MD, MSc, and Xiang Gao, PhD, MS share co-first authorship. Andrea S. Shin, MD, MSc and Quinfeng Dong, PhD share co-corresponding authorship. A.S.S.: co-investigator, preparation of biologic specimens for molecular analysis, and writing of the manuscript. X.G.: senior biostatistician, data analysis, and writing of the manuscript. M.B.: co-investigator, recruitment, collection of biologic specimens, and writing of the manuscript. A.G.: study coordinator, recruitment, and data collection. D.N.: co-investigator, molecular analysis of biologic specimens, and writing of the manuscript. H.L.: biostatistician, data analysis, and

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**Table 4.** Significant differences observed in relative abundance of specific bacterial taxa in the aspirate and mucosal specimens in coliform SIBO vs no SIBO subgroups

| Factor          | Specimen | Subgroups of SIBO | P value | Adjusted P value |
|-----------------|----------|-------------------|---------|------------------|
|                  |          | Coliform SIBO     | No SIBO |                  |
| Genus           | Mucosa   | 2.76 ± 6.87       | 0.31 ± 0.40 | 0.001  | 0.05  |
| Clostridium spp.| Aspirate | 5.34 ± 5.42       | 1.92 ± 2.80 | 0.001  | 0.02  |
| Granulicatella spp. | Aspirate | 5.58 ± 6.58 | 2.24 ± 2.94 | 0.001  | 0.01  |
| OTU index       |          |                   |         |                  |
| 016 (Clostridium perfringens) | Mucosa | 2.69 ± 6.34 | 0.38 ± 0.42 | 0.001  | 0.035 |
| 008 (Granulicatella adiacens) | Aspirate | 5.58 ± 6.58 | 2.24 ± 2.94 | 0.001  | 0.01  |

Comparisons carried out using negative binomial regression with mixed model for repeat measures. Correction for multiple testing applied using Benjamini-Hochberg Procedure. OTU annotation performed by Bayesian Lowest Common Ancestor software. Data represent mean relative abundance (%) ± SD. OTU, operational taxonomic unit; SIBO, small intestinal bacterial overgrowth.

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**Figure 4.** Relative abundances of individual bacterial taxa at the genus level in (a) aspirates and (b) mucosa from each participant. Participants along the X-axis are labeled by final SIBO diagnosis as defined by culture results (0 = no SIBO, 1 = upper aerodigestive tract SIBO, 2 = coliform SIBO). SIBO, small intestinal bacterial overgrowth.

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writing of the manuscript. Q.D.: bioinformatician, data analysis, and writing of the manuscript. E.T.: co-investigator and preparation of biologic specimens for molecular analysis. S.T.: co-investigator, fellow, data collection, and editing of the manuscript. R.S.: co-investigator, recruitment, and editing of the manuscript. J.W.: principal investigator, study conceptualization, writing of the protocol, recruitment, and writing of the manuscript.

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Study Highlights

**WHAT IS KNOWN**

- SIBO remains a poorly defined clinical syndrome, and the validity of currently available techniques to evaluate for abnormal bacterial expansion in the small intestine has been questioned.
- The complexity and composition of the small intestinal microbiome are incompletely understood.

**WHAT IS NEW HERE**

- Patients with a culture-based diagnosis of SIBO show no differences in aspirate microbiota composition but exhibit decreased mucosal alpha-diversity, suggesting that decreased mucosal microbial richness is associated with luminal bacterial overgrowth.
- Comparisons of patients with a culture-based diagnosis of coliform SIBO and UAT SIBO suggest that the types of bacterial species cultured are associated with distinct changes in the community composition of the mucosal microbiome.
- Differences in the relative abundance of individual taxa between patients with coliform and those with UAT SIBO suggest that there may be abnormal expansion of specific bacteria SIBO which are not directly identified by culture-based testing.

**TRANSITIONAL IMPACT**

- Small intestinal mucosal microbiota and aspirate microbiota are distinct in both community profile and relative abundance of individual taxa, and studying the role of both will be important in understanding the relative contribution of these 2 communities to clinical symptoms.

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