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Defining Small Intestinal Bacterial Overgrowth by Culture and High Throughput Sequencing

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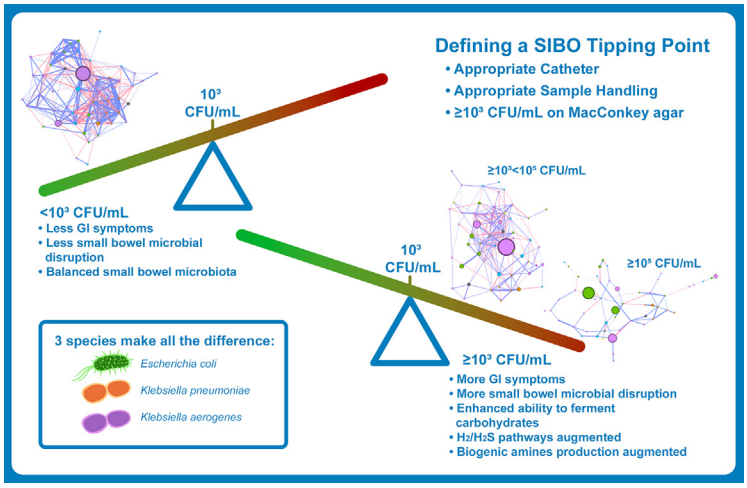

BACKGROUND& AIMS: **Despite accelerated research in small intestinal bacterial overgrowth (SIBO), questions remain regarding optimal diagnostic approaches and definitions. Here, we aim to define SIBO using small bowel culture and sequencing, identifying specific contributory microbes, in the context of gastrointestinal symptoms.**

METHODS: **Subjects undergoing esophagogastroduodenoscopy (without colonoscopy) were recruited and completed symptom severity questionnaires. Duodenal aspirates were plated on MacConkey and blood agar. Aspirate DNA was analyzed by 16S ribosomal RNA and shotgun sequencing. Microbial network connectivity for different SIBO thresholds and predicted microbial metabolic functions were also assessed.**

RESULTS: **A total of 385 subjects with $<10^3$ colony forming units (CFU)/mL on MacConkey agar and 98 subjects with $\geq 10^3$ CFU/mL, including $\geq 10^3$ to $<10^5$ CFU/mL (N = 66) and $\geq 10^5$ CFU/mL (N = 32), were identified. Duodenal microbial α-diversity progressively decreased, and relative abundance of *Escherichia/Shigella* and *Klebsiella* increased, in subjects with $\geq 10^3$ to $<10^5$ CFU/mL and $\geq 10^5$ CFU/mL. Microbial network connectivity also progressively decreased in these**

Abbreviations used in this paper: BMI, body mass index; CFU, colony forming unit; DA, duodenal aspirate; EGD, esophagogastroduodenoscopy; F, female; FC, fold change; GI, gastrointestinal; GLM, generalized linear model; IBS, irritable bowel syndrome; MAC, MacConkey agar; OTU, operational taxonomic unit; RA, relative abundance; rRNA, ribosomal RNA; SIBO, small intestinal bacterial overgrowth; SparCC, sparse correlations for compositional data.

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subjects, driven by the increased relative abundance of *Escherichia* ($P < .0001$) and *Klebsiella* ($P = .0018$). Microbial metabolic pathways for carbohydrate fermentation, hydrogen production, and hydrogen sulfide production were enhanced in subjects with ≥10^3 CFU/mL and correlated with symptoms. Shotgun sequencing (N = 38) identified 2 main *Escherichia coli* strains and 2 *Klebsiella* species representing 40.24% of all duodenal bacteria in subjects with ≥10^3 CFU/mL.

CONCLUSIONS: **Our findings confirm ≥10^3 CFU/mL is the optimal SIBO threshold, associated with gastrointestinal symptoms, significantly decreased microbial diversity, and network disruption. Microbial hydrogen- and hydrogen sulfide–related pathways were enhanced in SIBO subjects, supporting past studies. Remarkably few specific *E coli* and *Klebsiella* strains/species appear to dominate the microbiome in SIBO, and correlate with abdominal pain, diarrhea, and bloating severities.**

Keywords: Small Intestinal Bacterial Overgrowth; Small Intestinal Microbiome; Metabolic Pathways; *Escherichia*; *Klebsiella*.

Small intestinal bacterial overgrowth (SIBO) has been a recognized condition for more than 50 years.[1] Historically, a SIBO diagnosis was sought in patients with known structural risk factors such as blind loop syndrome and subjects with surgical antrectomy.[2] In those patients, symptoms of malabsorption, vitamin deficiency, and steatorrhea were often attributed to SIBO. More recently, SIBO has gained attention in patients with surgically naïve states.

Diagnosing SIBO is based principally on 2 approaches. The first and most common approach is carbohydrate breath testing.[3] This involves the detection of hydrogen (H_2), methane, and, more recently, hydrogen sulfide (H_2S) to provide indirect evidence for SIBO[3] and intestinal methanogen overgrowth.[4] Historically, standardization was lacking in the performance and interpretation of breath testing, but evidence-based consensus reports now provide clinicians and scientists guidance on the proper use and interpretation of breath testing.[3,5,6]

The second approach to diagnosing SIBO is small bowel culture.[7,8] Although this traditionally was considered the gold standard, there are many pitfalls that limit this approach. These include catheters that do not maintain sterility (leading to frequent sample contamination),[9] the lack of a standard location or approach to aspiration,[10] improper sample handling, and the inability to culture methanogenic archaea.[9] In fact, with current techniques, approximately 20% of aspirates yield results that are believed to be contaminants,[9] which cannot serve as a gold standard approach. In addition, there is no standard approach to quantitative culture. Many centers plate aspirates on both MacConkey agar and on blood agar and combine the counts on both agars, despite the fact that many bacteria can grow on both media, leading to double counting.[3–6] In addition, although the North American Consensus uses the threshold of ≥10^3 colony-forming units (CFU)/mL of aspirate on MacConkey agar to define SIBO,[3] other thresholds have been proposed. Lastly, technology has advanced far beyond culture with the advent of high-throughput sequencing, and a recent review commented that what is missing in understanding SIBO are studies to include high-throughput sequencing in conjunction with properly conducted culture of the small bowel, in the context of gastrointestinal symptoms, to define true diagnostic thresholds and clarify the role of SIBO.[11]

In this study, we aimed to characterize and define SIBO in the small bowel microbiome, using samples acquired with a validated double-lumen sterile protected catheter, by separately comparing the results of culture on 2 different media (MacConkey agar and blood agar) with the results of 16S ribosomal RNA (rRNA) gene sequencing, and correlating the results with gastrointestinal symptoms. In addition, metagenomic shotgun sequencing was performed in a subset of subjects to validate potential microbial determinants of SIBO, again in the context of gastrointestinal symptoms.

Methods

Study Subjects

Subjects aged 18 to 85 years undergoing esophagogastroduodenoscopy (EGD) for standard-of-care purposes were recruited prospectively for our Revealing the Entire Intestinal Microbiota and its Associations with the Genetic, Immunologic, and Neuroendocrine Ecosystem study.[12] Only subjects who were not taking antibiotics at the time of EGD were included in the analyses presented here. The study protocol was approved by the Cedars-Sinai Institutional Review Board, and all subjects provided informed written consent before participation.

Before EGD, all subjects completed a medical and family history questionnaire, including gastrointestinal (GI) disease, bowel symptoms, and medications. The severity of GI symptoms including abdominal pain, bloating, diarrhea, excess of gas, constipation, urgency with bowel movement, and straining during bowel movement were indicated using a visual analogue scale from 0 (absent symptom) to 100 (extremely severe).

Duodenal Aspirates

During the EGD procedure, duodenal luminal fluid samples (1–2 mL) were procured using a custom-designed, double-lumen, sterile aspiration catheter (Hobbs Medical, Inc) as previously described, passing the inner catheter through a protective bone wax cap to prevent any contamination from the proximal GI tract.[12]

Before culture, duodenal aspirates (DAs) were processed as described previously.[12] Serial dilutions were plated on MacConkey agar (MAC) at 36°C for 12 to 24 hours for aerobic culture and on blood agar (Becton Dickinson, Franklin Lakes, NJ) at 36°C for 12 to 24 hours for anaerobic culture.[12] If no growth was observed, plates were reincubated for an additional 24 hours under the same conditions. SIBO was defined as $\geq 10^3$ CFU/mL of aspirate on MAC plates, based on the North American Consensus.[3] The remaining DA was centrifuged and pellets were stored at -80°C.

DNA Extraction and 16S Sequencing

DNA extractions from DA pellets were performed using the MagAttract PowerSoil DNA KF Kit (cat. 27000-4-KF; Qiagen) as described previously,[12] using aliquots of 1× dithiothreitol as negative controls. 16S library preparation was performed and V3/V4 regions were amplified as described previously,[12] using gene-specific primers (S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21).[13] Operational taxonomic unit (OTU) clustering and taxonomic analyses were performed using CLC Genomics Workbench v.10.1.1 and CLC Microbial Genomics Module v.2.5 (Qiagen). Sequences were trimmed, merged, and clustered into OTUs at 97% sequence similarity with the Amplicon-Based OTU clustering tool (Qiagen). Taxonomic levels were assigned using CLC Microbial Genomics (Qiagen) default values by comparing against the SILVA Database v.132, 2019 release. Low-depth samples ($<$9000 sequences per sample) were removed, and α-diversity indexes were calculated. The weighted UniFrac metric and Bray–Curtis were used for the calculation of intersample diversity (β-diversity).

Library Preparation and Sequencing for Metagenomics Analysis

Libraries for whole DNA sequencing were prepared with the Illumina DNA Prep kit (Illumina) and IDT for Illumina–DNA/RNA UD Indexes (Illumina) following the manufacturer's instructions. Library qualities were checked using the High Sensitivity D5000 ScreenTape kit (Agilent) on a 2200 TapeStation instrument (Agilent). Only libraries greater than 0.48 nmol/L were sequenced. Paired sequencing was performed on NovaSeq (Illumina) using the NovaSeq 6000 S2 Reagent Kit v1.5 (300 cycles). Demultiplexed reads were analyzed using CLC Genomics Workbench 22.0.2/20.0.3 and Microbial Module (Qiagen). Reads were trimmed for leftover adapter removal and taxonomic profiling was performed after removal of host reads using the *Homo sapiens* genome reference consortium human build 38 as the genomic index. A curated Microbial Reference Database with selected references from GenBank optimized to 16 GB memory including all annotation tracks (coding sequences, genes, and so forth) of 4275 species was used as the reference database. Paired reads that could not map as an intact pair to the reference database were dismissed. Reads found to map to the reference database were first assigned to the lowest common ancestor of all mapping positions with the highest mapping score. After mapping, qualification and quantification of the abundance of each qualified taxon were performed. This qualification was performed to determine whether a particular taxon was represented in a sample. The qualification step was based on a confidence score that a reference did not receive its reads by pure chance. Any taxon with a confidence score less than 0.995 was ignored and the reads assigned to it were reassigned to its closest qualified ancestor. The confidence score was very close to 1.0. To confirm the identification of bacterial species and strains, a second taxonomic analysis using assembled reads to contigs also was performed. Host filtered reads were assembled to longer sequences using the De Novo Assembly tool. Reads were mapped back to contigs using a mismatch cost of 2, an insertion cost and deletion cost of 3, and a length fraction and

What You Need to Know

Background

Small intestinal bacterial overgrowth (SIBO) is associated with many disease states and other conditions. However, questions persist regarding optimal diagnostic approaches and thresholds for defining SIBO by culture.

Findings

Subjects with $\geq 10^3$ colony forming units/mL of duodenal aspirate on MacConkey agar exhibit increased *Escherichia/Shigella* and *Klebsiella*, decreased microbial diversity and network connectivity, and altered microbial metabolic pathways, correlating with gastrointestinal symptoms. Two specific *Escherichia coli* strains and 2 *Klebsiella* species predominate and correlate strongly with symptoms in these subjects.

Implications for patient care

These findings confirm that $\geq 10^3$ colony forming units/mL on MacConkey agar is the optimal diagnostic threshold and may represent a key microbial tipping point in SIBO. A few specific *E coli* and *Klebsiella* strains/species appear responsible for the majority of overgrowth and SIBO symptoms.

similarity fraction of 0.8. Contigs were extracted and filtered again against the *Homo sapiens* genome reference consortium human build 38. Host-filtered contigs were mapped using the microbial database described earlier.

Data and Statistical Analysis

Significant differentially abundant OTUs between groups were identified following published recommendations,[14,15] used when the average library size for each group was approximately equal and/or the fold difference between groups was low (>2–3 times on average). Specifically, if no differences in library size were observed between groups (fold difference, 1.06; non-SIBO median, 181,868; SIBO median, 193,637; $P = .18$), OTU tables were not rarefied for downstream analysis.

Multiple comparisons and statistical analyses for both 16S and shotgun data were performed with CLC Genomics Workbench v.20.0.3/22.0.2 and CLC Microbial Genomics Module v.20.1.1/22.1 (Qiagen). A negative binomial generalized linear model (GLM) model was used to obtain maximum likelihood estimates for the fold change (FC) of a feature between non-SIBO and SIBO, and the Wald test was used for determination of significance. False discovery rate was performed to correct the *P* values. Despite similar library sizes between groups, fold changes were calculated from the GLM, which corrects for differences in library size between the samples and the effects of confounding factors. Spearman R correlations, Mann–Whitney tests, and graph construction were performed with GraphPad Prism 7.02 (GraphPad Software, La Jolla, CA). An unbiased analysis was performed to compare differences in relative abundance (RA) between groups at the genus level. Rare genera (defined by a median abundance within a group of <1) were removed. A negative binomial GLM model was used to obtain maximum likelihood estimates for the FC of a feature between groups, and the Wald test was used for determination of significance. False discovery rate was performed to correct the *P* values. A similar approach was applied to metagenomics shotgun data.

Analysis of predicted microbial pathways and functions were performed for both 16S and shotgun data using the Functional Analysis tool available at CLC Workbench 22.0.2. Functional profiles were inferred using enzyme commission numbers and Clusters of Orthologous Genes terms, and pathways were identified using the MetaCyc Pathway Database (2022-05). Randomization analyses were performed with 1000 replicates, and superpathways were included. For the shotgun data, microbial functional analyses were performed with long assembled contigs (minimum length, 1000 base pairs) built with the De Novo Assemble Metagenome tool. Prokaryotic protein-coding regions were identified using the MetaGeneMark tool (Qiagen) and coding sequences were annotated with the Best DIAMOND hit tool (Qiagen) using UniProt database (UniRef90 subset with gene ontology and enzyme commission annotations, 7GB, 2020/11). Reads were mapped back to annotated contigs using the Map Reads to Reference tool (Qiagen) with default parameters, except the similarity fraction, which was adjusted for 0.8 (default, 0.5). Mapping files were used to build functional profiles and pathways were identified using the MetaCyc Pathway Database (2022-05).

Microbial networks were built using an Integrated Network Analysis Pipeline,[16] using the Sparse correlations for compositional data (SparCC) approach for the calculation of potential associations between species. SparCC correlation and *P* value calculations were performed in filtered OTU tables using default parameters to calculate the correlation values of SparCC: 20 as the number of inference iterations, 10 for exclusion iterations, 0.1 as the strength exclusion threshold, and 100 as the number of times shuffled. Networks were built using a threshold of 0.2 for correlations and $P < .01$ for significance. Visualization and analysis of the networks were performed with Gephi software (Gephi.org).[17] Node colors indicated different taxonomic groups (phylum level). Node diameters represented the average of each taxon's relative abundance at the genus level. Blue edges represented positive interactions between nodes, and red edges represented negative interactions between nodes. Edge thickness represented the strength of the interaction between nodes (thicker edges indicated stronger interactions). The number of triangles was estimated using Latapy's[18] algorithm.

Study Analysis

The North American Consensus defines SIBO as $\geq 10^3$ CFU/mL of aspirate on MAC plates.[3] Because some investigators combine counts from MAC and blood agar in their determination of SIBO,[3–6] we examined growth on MAC agar and growth on blood agar as potential approaches to determining SIBO, and also evaluated thresholds of both $\geq 10^3$ and $\geq 10^5$, because $\geq 10^5$ is another threshold used in the literature.[3] Counts on both MAC and blood agar were compared separately to sequencing results and tested for associations with SIBO symptoms to determine the ideal definition of clinical and microbiological SIBO.

Results

Subject Demographics and Determination of Small Intestinal Bacterial Overgrowth by Duodenal Microbial Culture

16S rRNA sequencing of the duodenal luminal microbiome was performed for 505 subjects (mean age, 56.5 ± 16.0 y; female [F], 55%). DA from 38 of these subjects (mean age, 59.0 ± 14.0 y; F, 60%) were selected randomly for shotgun sequencing (Table 1). Of 505 subjects, 483 (96%) had duodenal microbial culture results available and were used in this analysis. The

Table 1. Subject Demographics

	Subjects sequenced by 16S rRNA	Subcohort sequenced by shotgun	Whole cohort vs subcohort	
Subjects, n	505	38	*P* value	
Females, n	269	22	.599	
Mean age ± SD, *y*	56.5 ± 16.0	59.2 ± 14.0	.324	
Mean BMI ± SD, *kg/m²*	25.9 ± 6.5	25.5 ± 6.0	.835	
	$<10^3$ CFU/mL	$\geq 10^3$ CFU/mL	$\geq 10^3$ to $<10^5$ CFU/mL	$\geq 10^5$ CFU/mL
Groups defined by growth on MacConkey agar				
Subjects, n	385	98	66	32
Females, n	197	55	38	17
Mean age ± SD, *y*	55.0 ± 15.5	63.5 ± 14.0	62.0 ± 15.0	66.0 ± 11.5
Mean BMI ± SD, *kg/m²*	25.9 ± 6.5	25.7 ± 5.9	25.1 ± 6.0	26.9 ± 5.4
Groups defined by growth on blood agar				
Subjects, n	203	276	126	150
Females, n	111	138	63	75
Mean age ± SD, *y*	54.4 ± 15.9	58.5 ± 15.3	56.1 ± 16.2	60.5 ± 14.2
Mean BMI ± SD, *kg/m²*	25.4 ± 6.2	26.2 ± 6.5	26.6 ± 7.5	25.9 ± 5.4

BMI, body mass index; CFU, colony forming unit; rRNA, ribosomal RNA.

indications for endoscopy in these subjects are presented in Supplementary Table 1. A total of 385 subjects (79.7%; mean age, 55.0 ± 15.5 y; F, 53%) had $<10^3$ CFU/mL from DA on MAC (Table 1), and 98 subjects (20.3%; mean age, 63.5 ± 14.0 y; F, 57%) had $\geq 10^3$ CFU/mL on MAC (Table 1). Of these 98 subjects, 32 (mean age, 66.1 ± 11.5 y; F, 55%) had $\geq 10^5$ CFU/mL on MAC. Additional analyses were performed based on growth on a nonselective medium (blood agar). Of 505 subjects, 479 had successful blood agar cultures. Of these, 203 (40.2%; mean age, 54.4 ± 15.9 y; F, 57%) had $<10^3$ CFU/mL, 126 (25.0%; mean age, 56.1 ± 16.2 y; F, 50%) had $\geq 10^3$ to $<10^5$ CFU/mL, and 150 (29.7%; mean age, 60.4 ± 14.1 y; F, 52%) had $\geq 10^5$ CFU/mL on blood agar.

Colony Counts $\geq 10^3$ Colony Forming Units per Milliliter on MacConkey Agar Are Associated With Increased Microbial Disruptors in the Duodenum and Disconnected Microbial Communities

When subjects were grouped based on colony counts on MAC agar, subjects with $\geq 10^3$ CFU/mL on MAC had lower duodenal microbial α-diversity than subjects with $<10^3$ CFU/mL (Shannon $P = .09E\text{-}2$) (Supplementary Figure 1*A*). Furthermore, microbial diversity correlated inversely with total CFU number on MAC (Spearman r = -0.184; $P = .05E\text{-}3$), and progressively decreased in subjects with $\geq 10^3$ to 10^5 CFU/mL ($P = .03$) and $\geq 10^5$

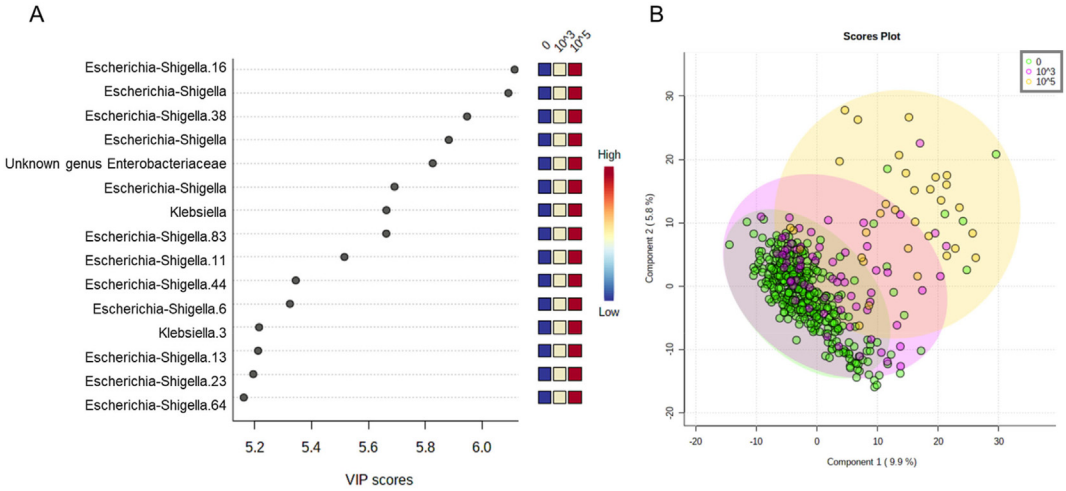

Figure 1. Variable importance in projection (VIP) scores (*A*) for bacterial features/operational taxonomic units associated with cluster formation after (*B*) partial least-squares discriminant analysis (PLS-DA) analysis of duodenal microbial β-diversity in subjects with $<10^3$ colony forming units (CFU)/mL (green), subjects with $\geq 10^3$ to $<10^5$ CFU/mL (pink), and subjects with $\geq 10^5$ CFU/mL (orange), based on colony counts on MacConkey agar.

CFU/mL ($P = .001$) compared with subjects with $<10^3$ CFU/mL (Supplementary Figure 1*B*).

In contrast, when subjects were grouped based on colony counts on blood agar, no differences in duodenal microbial diversity were observed between groups, independent of the threshold applied (Supplementary Figure 2), and no correlations were found between CFU on blood agar and microbial diversity ($P = .6$).

Duodenal microbial profiles differed significantly between subjects with $\geq 10^3$ CFU/mL on MAC and subjects with $<10^3$ CFU/mL (Supplementary Figure 3). Duodenal microbial β-diversity was significantly different between these groups (Bray–Curtis adjusted $P = .03$), and also was different in subjects with $\geq 10^5$ CFU/mL compared with those with $\geq 10^3$ to 10^5 CFU/mL (Bray–Curtis adjusted $P = .00015$) and those with $<10^3$ CFU/mL (Bray–Curtis adjusted $P = .00015$). When multivariate, dimensionality-reduction analyses were performed using partial least-squares discriminant analysis, the bacterial features/OTUs associated with differences in β-diversity between groups were mostly from the *Enterobacteriaceae* family (Figure 1*A*). The progressive increase in RA of *Enterobacteriaceae* taxa in subjects with $\geq 10^3$ to $<10^5$ CFU/mL and those with $\geq 10^5$ CFU/mL appeared to impact duodenal microbial composition and resulted in the formation of 3 distinct clusters (Figure 1*B*).

Duodenal RA of *Enterobacteriaceae* was increased in subjects with $\geq 10^3$ CFU/mL on MAC and in subjects with $<10^3$ CFU/mL ($\log_2$ FC, 2.43; adjusted $P =$ 2.66E-7) (Supplementary Figure 4). Importantly, *Enterobacteriaceae* RA was increased in subjects with $\geq 10^3$ to $<10^5$ CFU/mL vs subjects with $<10^3$ CFU/mL (FC, 1.92; adjusted $P = 1.00E\text{-}3$), but was not different between subjects with $\geq 10^3$ to $<10^5$ CFU/mL and those with $\geq 10^5$ CFU/mL (FC, -1.42; adjusted $P = .32$) (Supplementary Figure 4).

The RA of 21 genera was significantly different between subjects with $\geq 10^3$ CFU/mL on MAC and in subjects with $<10^3$ CFU/mL (Supplementary Table 2), including genera from *Enterobacteriaceae* previously associated with SIBO[19] such as *Escherichia/Shigella*, *Klebsiella*, and others (Figure 2). Increased RA of these genera was associated with decreased microbial diversity and with increased CFU counts on MAC (*Escherichia/Shigella* Spearman r = 0.280; $P = 4.1E\text{-}10$; *Klebsiella* Spearman r = 0.306; $P = 6.7E\text{-}12$; and *Enterobacter* Spearman r = 0.291; $P = 6.9E\text{-}11$), suggesting that recovery of these microbial DNAs could be from live duodenal bacteria.

Changes in the RA of *Enterobacteriaceae* taxa appeared to affect the resident duodenal microbiome in subjects with $\geq 10^3$ CFU/mL, leading to disturbances in microbial communities' networks and connectivity (Supplementary Figure 5). Although the number of nodes in the predicted networks for microbial communities in subjects with $\geq 10^3$ CFU/mL was similar to that in subjects with $<10^3$ CFU/mL (59 vs 51, respectively), the average connectivity per node (measured by the number of triangles [3 vertices with 3 edges] per node, indicating how nodes are embedded in their neighborhood) was

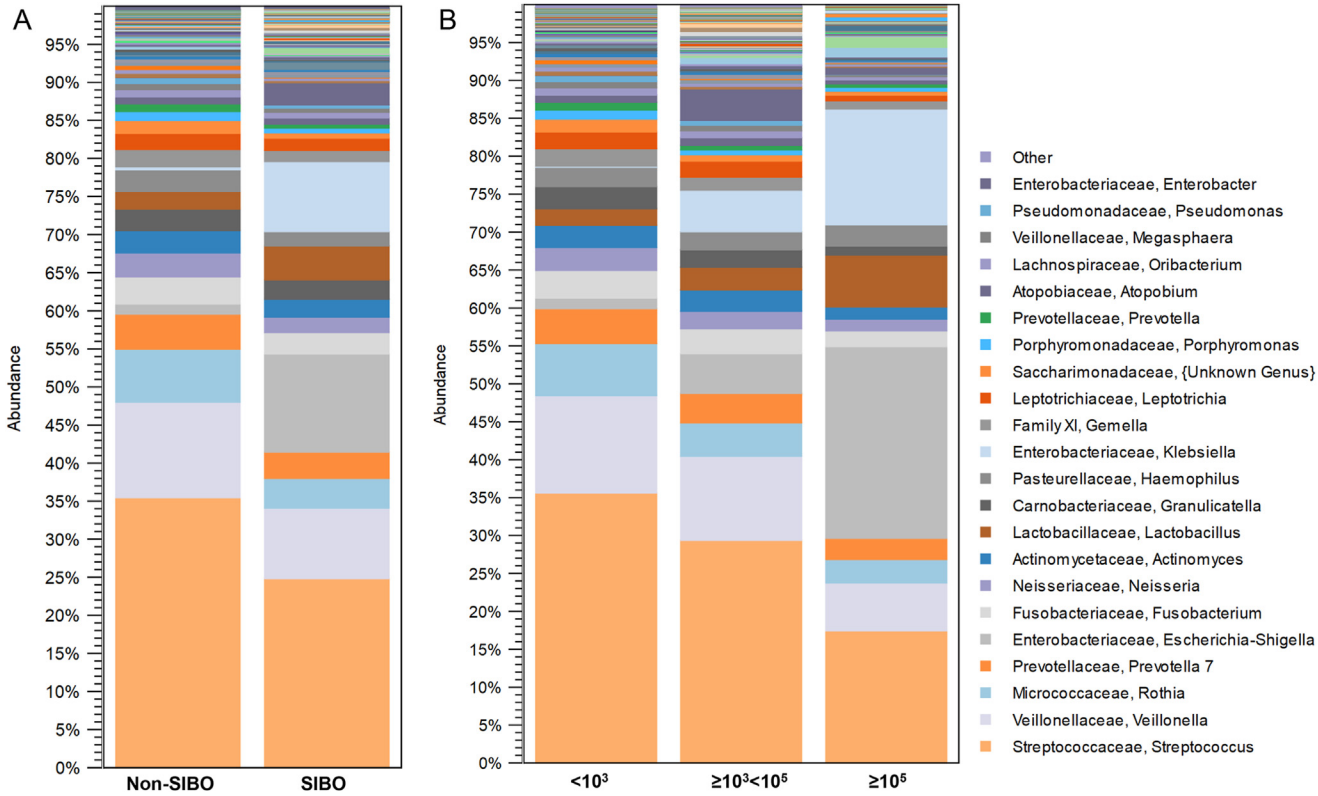

Figure 2. Duodenal microbial genus distribution in (*A*) subjects with $<10^3$ colony forming units (CFU)/mL and $\geq 10^3$ CFU/mL on MAC, and in (*B*) subjects with $<10^3$ CFU/mL, $\geq 10^3$ to $<10^5$ CFU/mL, and $\geq 10^5$ CFU/mL on MAC.

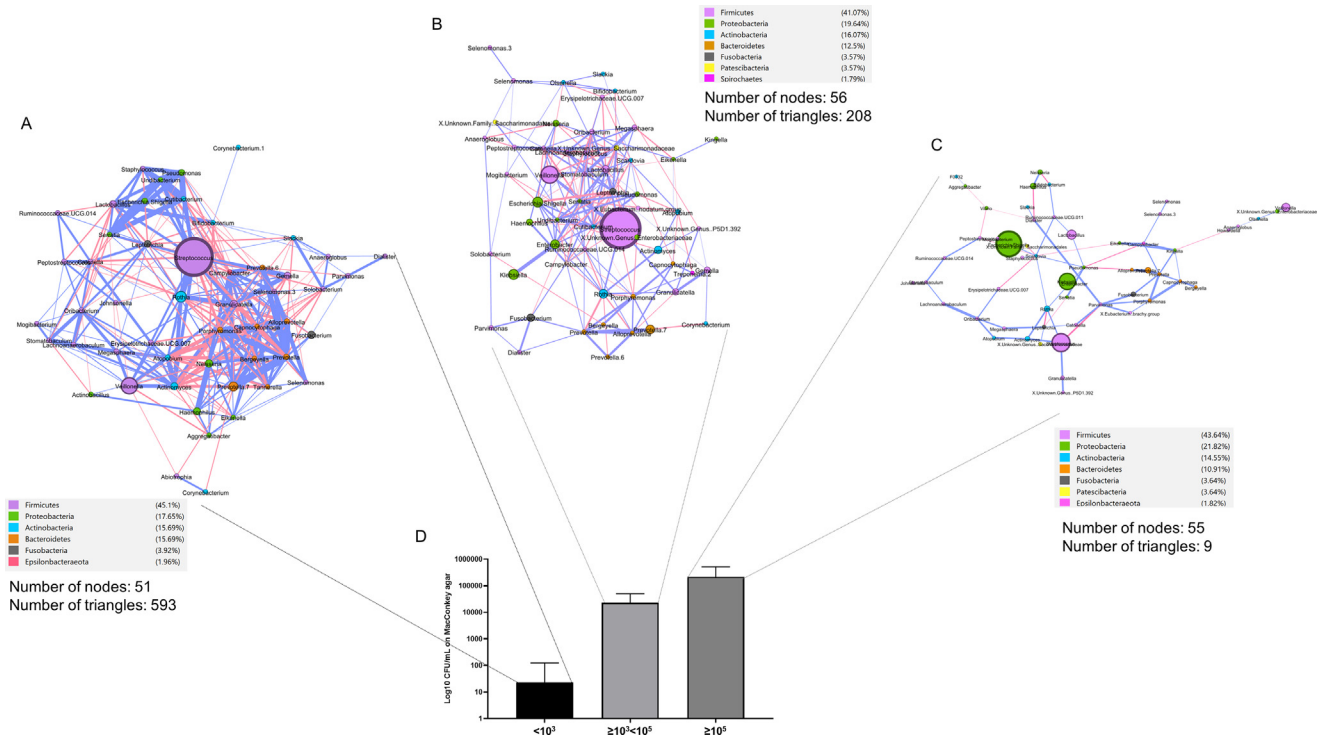

Figure 3. Visualization of duodenal microbial networks in subjects with (*A*) $<10^3$ colony forming units (CFU)/mL, (*B*) $\geq 10^3$ to $<10^5$ CFU/mL, and (*C*) $\geq 10^5$ CFU/mL on MacConkey agar. (*D*) Average CFU/mL on MacConkey agar ($\log_{10}$) per group.

decreased significantly in subjects with $\geq 10^3$ CFU/mL (total number of unique triangles, 425; average per node, 22) vs those with $<10^3$ CFU/mL (total number of unique triangles, 593; average per node, 35, $P = .0175$). When networks were built based on different thresholds for SIBO, microbial dysconnectivity already was evident in subjects with $\geq 10^3$ to $<10^5$ CFU/mL on MAC (total number of unique triangles, 208; average per node, 11) (Figure 3, Supplementary Figure 6), and was even more pronounced in subjects with $\geq 10^5$ CFU/mL, in whom it was evident that *Escherichia* and *Klebsiella* played a central role in the integration of the network (total number of unique triangles, 9; average per node, 0.5) (Figure 3, Supplementary Figure 6).

To investigate how the RA of *Escherichia* and *Klebsiella* could be associated with derangement of duodenal microbial communities, networks were constructed from 5 groups derived from terciles of *Escherichia* and *Klebsiella* RA (based on 16S) (Supplementary Figure 7): group 1: tercile 1 (low RA) for both *Escherichia* and *Klebsiella*, n = 76; group 2: *Escherichia* tercile 2 (moderate RA) and *Klebsiella* tercile 1 (low RA), n = 67; group 3: *Escherichia* tercile 3 (high RA) and *Klebsiella* tercile 1, n = 52; group 4: *Klebsiella* tercile 2 and *Escherichia* tercile 1, n = 23; and group 5: *Klebsiella* tercile 3 and *Escherichia* tercile 1, n = 22.

Higher duodenal RA of *Escherichia*, excluding *Klebsiella* effects, was associated with decreased microbial interconnectivity, as shown by a gradual reduction of the number of edges and triangles ($P < .0001$) (Supplementary Figure 7*B* and *C*). Sixty-four nodes and 797 unique triangles were detected in group 1, while 57 nodes and 174 unique triangles were detected in group 2 (Supplementary Figure 7*A* and *B*). The number of network triangles for group 2 (average/node, 9) was decreased compared with group 1 (average/node, 37; $P < .0001$) (Supplementary Figure 7*F*). There was more severe network disarray and dysconnectivity in subjects with high *Escherichia* RA (group 3) (Supplementary Figure 7*C*), associated with a reduced number of triangles/node (number of triangles, 44; average/node, 3.5) compared with group 1 ($P < .0001$) and group 2 ($P = .0021$) (Supplementary Figure 7*F*). Differences in *Klebsiella* RA were associated with the formation of even fewer unique triangles, resulting in even more disturbed networks than for *Escherichia* (Supplementary Figure 7*D* and *E*). The average number of triangles/node was 7 in group 4 and 0.9 in group 5 ($P = .0018$) (Supplementary Figure 7*F*).

Gas Production Pathways Are Augmented in the Duodenal Microbiome of Subjects With $\geq 10^3$ Colony-Forming Units per Milliliter on MacConkey

Changes in microbial profiles and networks, driven primarily by *Enterobacteriaceae* species, led to profound changes in duodenal microbial functional potential in subjects with $\geq 10^3$ CFU/mL (Supplementary Figure 8), with enrichments in more than 350 microbial pathways compared with subjects with $<10^3$ CFU/mL

(Supplementary Figure 9, Supplementary Table 3). These included pathways associated with the degradation of sugars and their derivatives through fermentation processes, and anaerobic/aerobic respiration through electron transport and hydrogen proton translocation (Supplementary Figure 10). Enrichment of these pathways was associated with increased severity of GI symptoms, particularly diarrhea (Supplementary File 1). Polyamine biosynthesis/metabolism pathways were also enriched in subjects with $\geq 10^3$ CFU/mL, and associated with increased symptom severity (Supplementary Figure 10, Supplementary File 1).

Enterobacteriaceae species produce H_2 from sugar fermentation and respiration, and increases in breath H_2 after glucose or lactulose ingestion are used to diagnose SIBO.[3] Microbial reactions known to produce or to consume H_2, including sugar fermentation pathways largely represented by pyruvate fermentation coupled with aerobic/anaerobic respiration, were analyzed. Twelve sugar pyruvate fermentation pathways were enriched in subjects with $\geq 10^3$ CFU/mL vs those with $<10^3$ CFU/mL (FC, >1.1; adjusted $P < .05$) (Supplementary Table 3), as was mixed acid fermentation (FC, 1.2; adjusted $P = 2.18E-25$) (Supplementary Table 3).

Aerobic cleavage of pyruvate was enriched in subjects with $\geq 10^3$ CFU/mL vs those with $<10^3$ CFU/mL, including pyruvate to cytochrome bd/bo oxidase electron transfer (FC, 1.48; adjusted $P = 2.09E-09$), which correlated with diarrhea (Spearman r = 0.252; $P = .0034$), bloating (Spearman r = 0.185; $P = .025$), and urgency (Spearman r = 0.205; $P = .0146$) (Supplementary File 1); and succinate to cytochrome bd/bo oxidase electron transfer (FC, 1.13; adjusted $P = .01$) (Supplementary Table 3), which correlated with bloating (Spearman r = 0.300; $P = .0006$) and abdominal pain (Spearman r = 0.194; $P = .0189$) (Supplementary File 1).

Breath H_2S is associated with stool microtypes enriched with H_2S producers, such as *Fusobacterium* and *Desulfovibrio*,[20] and is increased in diarrheal conditions. Therefore, H_2S production pathways were analyzed. Assimilatory sulfate reduction pathways (I, III, and IV) were enriched in subjects with $\geq 10^3$ CFU/mL on MAC vs those with $<10^3$ CFU/mL (FC, 1.541; adjusted $P = 8.25E-10$; FC, 1.6575; adjusted $P = 2.87E-11$; and FC, 1.115; adjusted $P = .020825$, respectively) (Supplementary Table 3), as was the sulfate assimilation and cysteine biosynthesis superpathway (FC, 1.14-fold; adjusted $P = 1.00E-07$). Furthermore, assimilatory pathways I and III correlated with diarrhea (Spearman r = 0.193; $P = .031$; Spearman r = 0.190; $P = .033$).

Specific Coliform Strains Are Associated With Gastrointestinal Symptoms in Small Intestinal Bowel Overgrowth Subjects

DA from a randomly selected subset of subjects with $\geq 10^3$ CFU/mL on MAC (N = 17; F = 11; mean age, 61 ± 16 y; mean body mass index [BMI], 26.45 ± 6.6) and subjects with $<10^3$ CFU/mL (N = 21; F = 12; mean age, 57 ± 12 y; mean BMI, 24.74 ± 5.6) underwent shotgun sequencing to identify species associated with GI symptom severity. No differences in age, sex, or BMI were observed between this subset and the whole cohort (Table 1). For validation of 16S results, the shotgun microbial RA distribution was compared with 16S profiles (matched subjects), and RA at all levels except species were similar (Supplementary Figure 11).

RA of 58 identified species were different between subjects with $\geq 10^3$ CFU/mL on MAC and subjects with $<10^3$ CFU/mL (Supplementary Table 4). Duodenal RA of specific *Enterobacteriaceae* species were increased in subjects with $\geq 10^3$ CFU/mL vs those with $<10^3$ CFU/mL, including *Escherichia coli* (FC, 6.55; adjusted $P = 4.8E-4$), *Klebsiella pneumoniae* (FC, 12.43; adjusted $P = 9.4E-12$), *Klebsiella aerogenes* (FC, 24.76; adjusted $P = 4.3E-11$), and *Enterobacter* species (FC, 22.11; adjusted $P = 7.1E-8$) (Supplementary Figure 12, Table 2). Associations between all identified significantly different species and strains, including *Escherichia* and *Klebsiella*, and symptoms were checked (Supplementary File 2), and only specific *E coli* strains, with genomic similarities to *E coli* BL21(DE3) and *E coli* strain K-12 substrain W3110, correlated with abdominal pain (Spearman r = 0.284, $P = .049$; and Spearman r = 0.337, $P = .02$, respectively), and diarrhea (Spearman r = 0.321, $P = .030$; and Spearman r = 0.298, $P = .04$, respectively); a specific *K aerogenes* strain, with genomic similarities to *K aerogenes* KCTC 2190, correlated with abdominal pain (Spearman r = 0.374; $P = .013$), diarrhea (Spearman r = 0.305; $P = .038$), and excess gas (Spearman r = 0.286; $P = .048$) (Table 2). Several differences in microbial functional pathways predicted by the 16S analyses also were identified when the shotgun sequencing data were analyzed (Supplementary File 3), including enrichment of sugar degradation (glucose, galactose degradation), aerobic respiration, and pyruvate fermentation pathways in subjects with $\geq 10^3$ CFU/mL on MAC when compared with subjects with $<10^3$ CFU/mL. Polyamine biosynthesis/metabolism pathways also were confirmed to be enriched in subjects with $\geq 10^3$ CFU/mL (Supplementary File 3).

Discussion

In this study, we present a number of important developments in understanding and defining SIBO. Using a specialized double-lumen catheter, we found that SIBO is relatively common in symptomatic patients undergoing upper endoscopy. Our findings suggest that only bacterial counts on MacConkey agar are needed to diagnose SIBO. Furthermore, based on impacts on microbial diversity, disruptions of bacterial networks, and associations with GI symptoms, we confirm that $\geq 10^3$ CFU/mL on MacConkey agar is the optimal threshold for defining

Table 2. Correlations Between Bacterial Species/Strains and Severity of GI Symptoms

	Relative abundance ($<10^3$ CFU/mL)	Relative abundance ($\geq 10^3$ CFU/mL)	Bloating	Excess gas	Incomplete evacuation	Abdominal pain	Constipation	Diarrhea	Urgency	Straining	Fatigue
Escherichia, all species/strains	1.05	21.44	0.008	0.180	-0.085	0.166	0.024	0.224	0.168	0.070	-0.214
E coli, BL21(DE3)	0.00	10.85	0.100	0.235	-0.034	0.284[a]	0.107	0.321[a]	0.186	0.110[a]	-0.182
E coli strain K-12	0.00	3.22	0.318[a]	0.348[a]	0.109	0.337[a]	0.187	0.298[a]	0.374[a]	0.129[a]	-0.116
Total for most important *E coli* strains	0.01	15.78									
Klebsiella, all species/strains	4.57	18.93	-0.073	0.121	0.096	0.203	-0.007	0.141	0.071	-0.019	-0.081
K aerogenes KCTC 2190	0.01	0.35	0.051	0.286[a]	0.104	0.374[a]	0.019	0.305[a]	0.262	0.032	0.009
K pneumoniae	4.54	18.45	-0.074	0.117	0.096	0.205	-0.013	0.139	0.071	-0.021	-0.079
Total for most important *Klebsiella* species	4.55	18.80									

CFU, colony forming unit; GI, gastrointestinal.
[a]Significant correlations ($P < .05$).

SIBO. This study also used shotgun sequencing of the luminal small bowel microbiome in a subset of subjects to identify bacterial species/strains potentially important in SIBO. These include 2 *E coli* strains and 2 *Klebsiella* species. This specific group of bacteria appears to have a tremendous influence on the duodenal microbiome and directly correlates with the severity of symptoms including bloating, diarrhea, and abdominal pain. Furthermore, increased relative abundances of *Escherichia* and *Klebsiella* were associated with significant disruptions of duodenal microbial networks and negatively impacted microbial metabolic pathways.

In the past 3 decades, SIBO has emerged as an important contributor to conditions that have no antecedent mechanical bowel problems, including scleroderma, diabetes, inflammatory bowel disease, and irritable bowel syndrome (IBS).[4] The 2 principal diagnostic approaches used to identify SIBO are breath testing and small bowel aspirate culture. However, unlike breath testing, small bowel aspirate culture still has significant associated challenges.[8–10] A recent study found that approximately 20% of samples obtained using an open single-lumen aspiration catheter were contaminated, and culture and breath test results agreed in only 63.5% of subjects.[9] This clearly shows that current aspirate culture techniques result in a poor gold standard, and could also result in significantly flawed study outcomes.

Some of the challenges surrounding sample contamination have been addressed through the use of a protected double-lumen catheter.[12] In this article, we address recently highlighted challenges in defining appropriate culture techniques and diagnostic thresholds for SIBO[11] using the validated catheter and sample collection techniques mentioned earlier. In 505 patients undergoing upper endoscopy, we identified SIBO in 20.3% of subjects using the definition of $\geq 10^3$ CFU/mL on MacConkey agar, and in 6.7% using $\geq 10^5$ CFU/mL. Furthermore, we found that SIBO is associated with a tremendous shift in microbial composition that already is evident at the $\geq 10^3$ CFU/mL threshold, and even more profound at $\geq 10^5$ CFU/mL. The $\geq 10^3$ CFU/mL threshold is associated with reduced duodenal microbial diversity, increased RA of coliforms, and disruptions in microbial networks (based on 16S rRNA sequencing), emphasizing the importance of this threshold. In addition, the $\geq 10^3$ CFU/mL threshold was associated with GI symptoms including abdominal pain, diarrhea, and bloating. Importantly, the associations between small bowel aspirate culture, microbiome changes, and symptoms were found only when SIBO was defined based on counts on MacConkey agar, whereas colony counts on blood agar showed no associations with any of these parameters. These findings indicate that although the use of other agars can be useful in identifying the types of bacteria present, culture on MacConkey agar alone is sufficient to diagnose SIBO.

High-throughput sequencing subsequently was performed, providing the clearest picture of SIBO to date.

The 16S rRNA sequencing, performed on the whole cohort, showed that 2 key genera, *Escherichia* and *Klebsiella*, account for the majority of bacterial overgrowth in SIBO. Moreover, *Escherichia* and *Klebsiella* are associated with disruptive changes to the microbiome proportional to their abundance, consistent with recent identification of *E coli* as a disruptor in the small bowel.[21] Here, we show that *Escherichia* and *Klebsiella* effect these changes both independently and synergistically. Regarding symptoms, *Escherichia* is associated with bloating and diarrhea, whereas *Klebsiella* has a greater association with abdominal pain, consistent with recent studies identifying *K aerogenes* as a major histamine producer potentially linked to visceral hypersensitivity.[22]

Shotgun sequencing also was performed in a randomly selected subset of subjects. The results supported and confirmed the findings from the 16S sequencing of the larger cohort and suggest that SIBO is accounted for primarily by 2 *E coli* strains and 2 *Klebsiella* species. This remarkably specific group of microbes account for 40.24% of duodenal bacteria in SIBO subjects, compared with 5.6% in non-SIBO subjects. In fact, the 2 main *E coli* strains identified in SIBO subjects—*E coli* BL21(DE3) and *E coli* strain K-12—were hardly detectable in the duodenum of non-SIBO subjects. This illustrates the disruptive power of these bacteria in SIBO.

Finally, analysis of duodenal microbial metabolic pathways using both the 16S and the shotgun sequencing data provides interesting insights for understanding SIBO. Pathways enriched in SIBO subjects include pathways involved in the degradation and fermentation of sugars, particularly pyruvate fermentation pathways coupled with aerobic/anaerobic respiration, as well as sulfate-reducing pathways. Changes in these pathways also correlated with GI symptoms in SIBO subjects, including diarrhea, bloating, urgency, and abdominal pain. Consistent with this, a recent study of subjects with IBS, a condition commonly linked to SIBO, found associations between increased breath hydrogen levels, increased oroanal transit times, and greater severity of postprandial GI symptoms in IBS subjects.[23] Furthermore, we recently found that increased breath H_2S correlated with increased predominance of H_2S producers, including *Fusobacterium* and *Desulfovibrio* species, in subjects with diarrhea-predominant IBS.[20] In addition, numerous sugar and other carbohydrate intolerances also have been noted in patients with functional GI disorders, including lactose, fructose, and highly fermentable foods.[24] Here, we found that sugar degradation and fermentation pathways leading to H_2 production are enhanced significantly in SIBO subjects, and associated with an increased severity of GI symptoms. This suggests that it is not just the simple interaction of SIBO microbes with carbohydrates that leads to GI symptoms, but that these microbes have exaggerated abilities to ferment carbohydrates to H_2 and to reduce sulfate to H_2S. In addition, the role of biogenic amines in abdominal pain is evolving. Colonic infection and subsequent immune responses mediated by mast cell degranulation and histamine release sensitizes visceral neurons and leads to increased visceral pain.[25] Although pathways directly related to histamine were not altered in our subjects, SIBO subjects showed enhanced polyamine metabolism pathways, including cadaverine biosynthesis. Cadaverine, a biogenic amine mainly produced by certain Gram-negative bacteria, inhibits the intestinal histamine-metabolizing enzyme histamine N-methyl transferase and diamine oxidase, facilitating histamine binding to mucin,[26] thereby potentially accentuating the pathophysiologic effects of luminal and extraluminal histamine in SIBO subjects.

A limitation of this study was that we used an undefined, heterogeneous population of patients with different indications for upper endoscopy. This actually may be a strength because in the real-world setting, patients present with nonspecific symptoms before being identified as having SIBO. Given the broad subject population, diet also was not standardized across the cohort, although this may not have been a significant limitation, at least for the duodenum.[27] An additional limitation was that the shotgun sequencing was performed on only a subset of subjects. Although the sample size for this was limited, the results correlated with and confirmed the 16S sequencing findings, giving additional confidence to the results from the larger data set. Lastly, it would have been ideal to have breath testing performed on these subjects. Nevertheless, microbial pathways for H_2 and H_2S production were increased significantly in the small bowel of subjects identified as having SIBO. This supports the argument that hydrogen on breath testing derives from the small intestine rather than rapid transit to the colon as previously suggested.[28]

In conclusion, SIBO is emerging as an important microbiome-associated condition. In this study, using well-validated catheters and techniques optimized for small bowel microbiome assessment, SIBO appears best defined using the threshold of $\geq 10^3$ CFU/mL, because this appears to be a key microbial tipping point both for disruption of the microbiome and for the development of GI symptoms. The most important disruptors in SIBO appear to be *E coli* and species of *Klebsiella*, and specific strains are likely responsible for the majority of overgrowth in SIBO. Metabolic data support augmentation of H_2- and H_2S-producing pathways in subjects with SIBO, defined by high-throughput sequencing, supporting current clinical use of breath testing. Identifying these specific microbes in SIBO could lead to better and more reliable approaches to managing these patients.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Clinical Gastroenterology and Hepatology* at www.cghjournal.org, and at http://doi.org/10.1016/j.cgh.2023.06.001.

References

1. Rao SSC, Bhagatwala J. Small intestinal bacterial overgrowth: clinical features and therapeutic management. Clin Transl Gastroenterol 2019;10:e00078.
2. Bures J, Cyrany J, Kohoutova D, et al. Small intestinal bacterial overgrowth syndrome. World J Gastroenterol 2010; 16:2978–2990.
3. Rezaie A, Buresi M, Lembo A, et al. Hydrogen and methane-based breath testing in gastrointestinal disorders: the North American Consensus. Am J Gastroenterol 2017;112:775–784.
4. Pimentel M, Saad RJ, Long MD, et al. ACG clinical guideline: small intestinal bacterial overgrowth. Am J Gastroenterol 2020; 115:165–178.
5. Ghoshal UC, Sachdeva S, Ghoshal U, et al. Asian-Pacific consensus on small intestinal bacterial overgrowth in gastrointestinal disorders: an initiative of the Indian Neurogastroenterology and Motility Association. Indian J Gastroenterol 2022;41:483–507.
6. Hammer HF, Fox MR, Keller J, et al. European guideline on indications, performance, and clinical impact of hydrogen and methane breath tests in adult and pediatric patients: European Association for Gastroenterology, Endoscopy and Nutrition, European Society of Neurogastroenterology and Motility, and European Society for Paediatric Gastroenterology Hepatology and Nutrition consensus. United European Gastroenterol J 2022;10:15–40.
7. Erdogan A, Rao SS, Gulley D, et al. Small intestinal bacterial overgrowth: duodenal aspiration vs glucose breath test. Neurogastroenterol Motil 2015;27:481–489.
8. Khoshini R, Dai SC, Lezcano S, et al. A systematic review of diagnostic tests for small intestinal bacterial overgrowth. Dig Dis Sci 2008;53:1443–1454.
9. Cangemi DJ, Lacy BE, Wise J. Diagnosing small intestinal bacterial overgrowth: a comparison of lactulose breath tests to small bowel aspirates. Dig Dis Sci 2021; 66:2042–2050.
10. Kastl AJ, Terry NA, Wu GD, et al. The structure and function of the human small intestinal microbiota: current understanding and future directions. Cell Mol Gastroenterol Hepatol 2020;9:33–45.
11. Bushyhead D, Quigley EM. Small intestinal bacterial overgrowth. Gastroenterol Clin North Am 2021;50:463–474.
12. Leite GGS, Morales W, Weitsman S, et al. Optimizing microbiome sequencing for small intestinal aspirates: validation of novel techniques through the REIMAGINE study. BMC Microbiol 2019;19:239.
13. Klindworth A, Pruesse E, Schweer T, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 2013;41:e1.
14. McMurdie PJ, Holmes S. Waste not, want not: why rarefying microbiome data is inadmissible. PLoS Comput Biol 2014;10: e1003531.
15. Weiss S, Xu ZZ, Peddada S, et al. Normalization and microbial differential abundance strategies depend upon data characteristics. Microbiome 2017;5:27.
16. Feng K, Peng X, Zhang Z, et al. iNAP: an integrated network analysis pipeline for microbiome studies. iMeta 2022;1:e13.
17. Bastian M, Heymann S, Jacomy M. Gephi: an open source software for exploring and manipulating networks. Proc Int AAAI Conf 2009;3:361–362.
18. Latapy M. Main-memory triangle computations for very large (sparse (power-law)) graphs. Theoretical Comp Sci 2008; 407:458–473.
19. Leite G, Morales W, Weitsman S, et al. The duodenal microbiome is altered in small intestinal bacterial overgrowth. PLoS One 2020;15:e0234906.
20. Villanueva-Millan MJ, Leite G, Wang J, et al. Methanogens and hydrogen sulfide producing bacteria guide distinct gut microbe profiles and irritable bowel syndrome subtypes. Am J Gastroenterol 2022;117:2055–2066.
21. Barlow JT, Leite G, Romano AE, et al. Quantitative sequencing clarifies the role of disruptor taxa, oral microbiota, and strict anaerobes in the human small-intestine microbiome. Microbiome 2021;9:214.
22. De Palma G, Shimbori C, Reed DE, et al. Histamine production by the gut microbiota induces visceral hyperalgesia through histamine 4 receptor signaling in mice. Sci Transl Med 2022;14:eabj1895.
23. Algera JP, Colomier E, Melchior C, et al. Associations between postprandial symptoms, hydrogen and methane production, and transit time in irritable bowel syndrome. Neurogastroenterol Motil 2022;35:e14482.
24. Dionne J, Ford AC, Yuan Y, et al. A systematic review and meta-analysis evaluating the efficacy of a gluten-free diet and a low FODMAPs diet in treating symptoms of irritable bowel syndrome. Am J Gastroenterol 2018;113:1290–1300.
25. Aguilera-Lizarraga J, Florens MV, Viola MF, et al. Local immune response to food antigens drives meal-induced abdominal pain. Nature 2021;590:151–156.
26. Ben-Gigirey B, Vieites Baaptista de Sousa JM, Villa TG, et al. Histamine and cadaverine production by bacteria isolated from fresh and frozen albacore (Thunnus alalunga). J Food Prot 1999; 62:933–939.
27. Shanahan ER, Kang S, Staudacher H, et al. Alterations to the duodenal microbiota are linked to gastric emptying and symptoms in functional dyspepsia. Gut 2023;72:929–938.
28. Yu D, Cheeseman F, Vanner S. Combined oro-caecal scintigraphy and lactulose hydrogen breath testing demonstrate that breath testing detects oro-caecal transit, not small intestinal bacterial overgrowth in patients with IBS. Gut 2011; 60:334–340.

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Conflicts of interest

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Data Availability

Data sets generated during this study are available at the National Center for Biotechnology Information BioProject Repository (https://www.ncbi.nlm.nih.gov/bioproject) under BioProject ID: PRJNA922536.