Altered gut microbiota in patients with small intestinal bacterial overgrowth

(Short title: Gut microbiota in SIBO)

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Abstract

Background: Small intestinal bacterial overgrowth (SIBO) is diagnosed by using quantitative culture of duodenal aspirates and/or a hydrogen breath test. However, few studies have analyzed bacterial microbiota in Japanese patients with SIBO. **Methods:** Twenty-four patients with any abdominal symptoms and suspected SIBO were enrolled. Quantitative culture of duodenal aspirates and a glucose hydrogen breath test were performed on the same day. SIBO was diagnosed based on a bacterial count $\geq 10^3$ CFU/mL or a rise in the hydrogen breath level of ≥20 ppm. The composition of the duodenal microbiota was analyzed by 16S rRNA gene sequencing. **Results:** SIBO was diagnosed in 17 of 24 patients (71%). The positive rates for the hydrogen breath test and quantitative culture of duodenal aspirates were 50% and 62%, respectively. Patients with SIBO showed significantly reduced α-diversity compared with non-SIBO patients, and analysis of β-diversity revealed significantly different distributions between SIBO and non-SIBO patients. In addition, the intestinal microbiome in SIBO patients was characterized by increased relative abundance of *Streptococcus* and decreased relative abundance of *Bacteroides* compared with non-SIBO patients. **Conclusions:** Duodenal dysbiosis was identified in patients with SIBO and may play a role in the pathophysiology of SIBO.

Keywords: Intestinal disorders, Small bowel, Clinical intestinal disorders

Introduction

The small intestine is responsible for the digestion of food and absorption of nutrients. In healthy individuals, bacterial growth in the small intestine is suppressed by peristalsis of the digestive tract, mucosal barrier function, digestive juices(e.g., gastric acid, bile, and protease), and anatomical structure (the ileocecal valve preventing reflux of colonic contents), thus keeping the bacterial count at 10^2-10^3 CFU/mL in the duodenum and jejunum[1]. Disruption of these factors results in abnormal bacterial growth, which causes small intestine bacterial overgrowth (SIBO).

Symptoms of SIBO include abdominal pain, bloating, flatulence, and diarrhea. However, because of the lack of SIBO-specific symptoms and of endoscopic and histological abnormalities, SIBO is often diagnosed as functional dyspepsia or irritable bowel syndrome. The prevalence of SIBO is not known, but the proportion of patients with SIBO is reported to be as high as 38% in irritable bowel syndrome[2], 14.7% in chronic pancreatitis[3], 61% in liver cirrhosis[4], 50% in nonalcoholic steatohepatitis[5], 46.8% after cholecystectomy[6], and 54% in hypothyroidism[7].

Quantitative culture of duodenal and jejunal aspirates is considered the gold standard for diagnosis of SIBO[8]. Historically, a cutoff value of 10^5 CFU/mL was used in the quantitative culture test, but now a cutoff value of 10^3 CFU/mL is recommended in guidelines from North America[8]. In addition to quantitative culture, a hydrogen breath test (HBT) is performed after the patient consumes a certain amount of glucose or lactulose. This method indirectly measures bacterial overgrowth in the small intestine, and SIBO is diagnosed when the hydrogen breath level is increased from baseline by ≥20 ppm within 90 min after glucose or lactulose consumption [9].

Streptococcus, *Bacteroides*, *Escherichia coli*, and *Lactobacillus* are commonly detected in the cultures of jejunal samples from patients with SIBO[10]. Bacterial overgrowth causes damage to the intestinal epithelium, primarily by invasive strains[11]. Facultative anaerobes can cause epithelial damage by adhering to the epithelium and producing enterotoxin. Aerobes produce enzymes and metabolites that are toxic to epithelial cells[12]. The involvement of free bile acids formed by bacterial deconjugation of bile acids in epithelial damage has also been suggested[13]. A recent 16S rRNA-based metagenomic study showed abnormalities in the intestinal microbiome in SIBO typically increased relative abundance of the phylum Proteobacteria[14].

Here, we performed quantitative culture of duodenum aspirates collected by upper gastrointestinal endoscopy and compared the results with those of HBTs performed on the same day as duodenal aspirates sampling. Although, as mentioned above, the North American guidelines recommend a cutoff value of 10^3 CFU/mL in quantitative culture, there are few reports of metagenomic analysis of bacterial flora according to bacterial count. In this study, microbiomes in collected duodenal aspirates were characterized by bacterial count and analyzed to identify abnormalities in the microbiome in SIBO.

Materials and Methods

Ethical considerations

This study was approved by the ethics committee of Shiga University of Medical Science (R2018-079) and registered with the University Hospital Medical Information Network Center (UMIN000033715). All participants were recruited from the Shiga

University of Medical Science Hospital, and written informed consent was obtained from each participant before enrollment.

Patients and symptom evaluation

Patients with any abdominal symptoms and suspected SIBO were included. Patients who had received antimicrobial therapy in the preceding 3 months were excluded. Twenty-four patients with suspected SIBO were enrolled (Table 1). To evaluate clinical symptoms, the Gastrointestinal Symptom Rating Scale (GSRS) was used[15]. In this study, SIBO was diagnosed based on a bacterial count $\geq 10^3$ CFU/mL in quantitative culture of duodenal aspirates or a rise in the hydrogen breath level of ≥20 ppm on the HBT[8]. The HBT and duodenal fluid collection using upper gastrointestinal endoscopy were performed on the same day, with duodenal fluid collection performed in the morning and the HBT in the afternoon.

Hydrogen breath test (HBT)

Patients fasted for 12 h before the glucose HBT. After baseline measurement of hydrogen breath level, they ingested 50 g of glucose and then hydrogen breath level was measured every 20 min up to 120 min using a Gastrolyzer® (Bedfont, Kent, UK). A positive breath test for SIBO was defined as a rise in hydrogen of ≥20 ppm above the baseline within 90 min[9].

Collection of duodenal fluids

After fasting overnight, patients underwent upper gastrointestinal endoscopy to obtain intestinal fluid from the second part of the duodenum. Duodenal fluid (1-2 mL) was aspirated using a sterile endoscopic catheter (ES-825H; Yasec Co., Ltd., Shiga, Japan). For patients with Roux-en-Y reconstruction, the samples were obtained from Roux-en-

Y anastomotic site. The intestinal fluids were quantitatively cultured and subjected to 16S rRNA metagenomic analysis.

Quantitative microbial culture

A 10-fold dilution of duodenal fluid was prepared by mixing 0.2 mL of the duodenal fluid with 1.8 mL of GAM broth, and 10-fold serial dilutions down to 10^{-6} were similarly prepared. Fifty microliters of diluted sample was applied to the corresponding culture medium and spread using a cell spreader: 10^{-1}-10^{-6} dilutions were used for bacterial counts, while 10^{-1}, 10^{-3}, and 10^{-5} dilutions were used for other purposes. Aerobic and anaerobic cultures were performed at 35 °C for 2-3 days and 3-5 days, respectively. The following culture media were used for the detection of bacteria: mannitol salt agar with egg yolk (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) for *Staphylococcus* species, CHROMagar Candida II (Becton, Dickinson and Company [BD], Franklin Lakes, NJ) for yeast-like fungi, EF agar (Apple Science Co., Ltd., Tokyo, Japan) for *Enterococcus* species, Columbia CNA agar with 5% sheep blood (BD) for *Streptococcus* species, cycloserine-cefoxitin fructose agar (BD) for *Clostridium* species, Bacteroides bile esculin agar (BD) for *Bacteroides* species, nalidixic acid cetrimide agar (Eiken Chemical Co., Ltd., Tokyp, Japan) for *Pseudomonas aeruginosa*, and CHROMagar Orientation (BD) for *Escherichia coli* and *Klebsiella pneumonia*. M58 agar with sheep blood (Eiken Chemical Co., Ltd.) and CHROMagar Orientation were used for total aerobic bacterial counts, while Anaero Columbia agar with rabbit blood (BD) was used for total anaerobic bacterial counts. The limit of detection was 2.0×10^2 CFU/mL.

DNA extraction from duodenal aspirates

DNA extraction was conducted as previously described[16], using an automated DNA isolation system (GENE PREP STAR PI-480; Kurabo, Osaka, Japan). The V3-V4 regions of bacterial and archaeal 16S rRNA were amplified using Pro341F/Pro805R primers and the dual-index method[16, 17].

16S rRNA gene sequencing and sequence curation

Barcoded amplicons were paired-end sequenced on a 2×284-bp cycle using the MiSeq system with MiSeq Reagent Kit version 3 (600 cycle) chemistry. Paired-end sequencing reads were merged using the fastq-join program with default settings[18]. The joined amplicon sequence reads were processed using QIIME 2 ver 2020.6[19]. Quality value scores of < 33 and chimeric sequences were filtered and representative sequences were created using DADA2 denoise-single plugin ver 2017.6.0[20]. Taxonomy of representative sequences was assigned using Greengenes database ver 13.8[21] by training a naive Bayes classifier using the q2-feature-classifier plugin.

Statistical analysis

Nonparametric data were compared between groups by the Mann-Whitney *U* test using Prism version 8.01 (GraphPad, San Diego, CA) and JMP software version 14.0 (SAS Institute, Cary, NC). Spearman's rank correlation coefficients between the parameters were evaluated. P-values were two-sided, with statistical significance set at $P < 0.05$. The observed species and the Chao1 and Shannon phylogenic diversity indices were calculated using MicrobiomeAnalyst[22, 23]. β-Diversity was estimated using the UniFrac metric to calculate the distances between the samples, and was then visualized using non-metric multidimensional scaling (NMDS) ordination and statistically analyzed by permutational multivariate analysis of variance (PERMANOVA) using MicrobiomeAnalyst[22, 23]. Linear discriminant analysis effect size (LEfSe) values of

operational taxonomic units (OTUs) were calculated using the Galaxy framework (http://huttenhower.sph.harvard.edu/lefse/)[24].

Results

HBTs were positive in 12 of the 24 patients (50%). Changes in the hydrogen breath level over time are shown in Fig. 1. The baseline level was <10 ppm in all patients, except for one with chronic idiopathic intestinal pseudo-obstruction (CIPO). All patients with a positive HBT result showed a ≥20 ppm rise in the hydrogen breath level 20 min after ingestion of glucose. The difference between the peak and the baseline values ranged from 37 ppm to 383 ppm.

The positive rate of quantitative culture of duodenal aspirates was 62% (15/24) when using 10^3 CFU/mL as a cutoff and 37% (9/24) when using 10^5 CFU/mL. Among 15 patients with a bacterial count $\geq 10^3$ CFU/mL, 13 were positive for aerobic bacterial overgrowth and 2 were positive for mixed anaerobic and aerobic overgrowth. None were positive for anaerobic bacterial overgrowth alone. The positive rate (bacterial count $\geq 10^3$ CFU/mL) according to type of bacteria is shown in Table 2. The positive rate was highest for *Streptococcus* (37%) followed by yeast (25%) and *Escherichia coli* (20%) (Table 2).

SIBO was diagnosed in 17 of 24 patients (71%), specifically 10 tested positive on both the HBT and a bacterial count $\geq 10^3$ CFU/mL in quantitative culture of duodenal aspirates, 2 were positive on the HBT only, and 5 were positive on duodenal culture only. Although there was no significant difference in baseline characteristics between SIBO and non-SIBO patients, all patients on proton pump inhibitors (PPIs), on probiotics, after Roux-en-Y reconstruction, and with CIPO were diagnosed as having

SIBO. Baseline characteristics were also analyzed according to bacterial count and HBT results (Supplementary Tables 1 and 2, respectively). There was no significant difference in baseline characteristics according to bacterial count or HBT results, except for a significantly higher incidence of intestinal resection in patients with a bacterial count $\geq 10^3$ CFU/mL.

Microbial analysis revealed a total of 4521 OTUs among 24 samples. A comparison of α-diversity in three groups according to quantitative culture results ($<10^3$ CFU/mL, 10^3-10^5 CFU/mL, and $>10^5$ CFU/mL) showed that observed species, Chao1 index, and Shannon index were significantly lower in the group with a bacterial count $>10^5$ CFU/mL than in the group with a bacterial count $<10^3$ CFU/mL (Fig. 2a-c). Meanwhile, when the patients were separated according to the results of the HBT, only the Shannon index (evenness markers) was significantly lower in the group with a positive HBT (Fig. 2 d-f). As a result, when patients were separated into SIBO and non-SIBO groups, the Shannon index was significantly lower in SIBO patients than in non-SIBO patients (Fig. 2i). However, there was no significant difference in the observed species and Chao1 index (richness markers) (Fig. 2g, h).

β-Diversity was evaluated using the UniFrac metric to calculate the distance between the samples. There were significant differences in β-diversity between the three groups divided according to quantitative culture results ($<10^3$ CFU/mL, 10^3-10^5 CFU/mL, and $>10^5$ CFU/mL; $P = 0.02$, PERMANOVA) (Fig. 3a). A significant difference in the distribution was observed between positive cases and negative cases according to the HBT results ($P = 0.01$, PERMANOVA) (Fig. 3b). A significant difference in the distribution was also observed between SIBO and non-SIBO patients ($P = 0.01$, PERMANOVA) (Fig. 3c).

As shown in Fig. 4, the relative abundance of the phylum Bacteroidetes was significantly lower in SIBO patients than in non-SIBO patients. The relative abundance of the phylum Saccharibacteria tended to be higher in SIBO patients, but the increase was not significant. There were no significant differences in the relative abundance of any other phyla between these two groups. Analysis of phylum levels stratified according to the results of quantitative culture and HBT was also performed (Supplementary Figs. 1 and 2). The trend for decrease in the phylum Bacteroidetes in patients with an increased quantitative culture count and positive HBT was the same as for the comparison of SIBO and non-SIBO patients. Meanwhile, abundance of the phylum Firmicutes was increased in patients who were positive on the HBT compared with those who were negative.

In a comparison of the SIBO and non-SIBO patients, representative taxa showing a significant difference in abundance are shown in Fig. 5. The relative abundance of the genera *Streptococcus* and *Actinomyces* was significantly higher in the SIBO group than in the non-SIBO group, while that of the genera *Bacteroides*, *Blautia*, and *Prevotella* was significantly lower in the SIBO group. Taxonomic composition was analyzed using LEfSe stratified according to the results of quantitative culture and the HBT was performed (Supplementary Figs. 3 and 4). Similar to the results above, increased abundance of the genera *Streptococcus* and *Actinomyces* was confirmed in patients with $\geq 10^3$ CFU/mL and a positive HBT. Also, abundance of the genera *Bacteroides* was significantly lower in patients with $\geq 10^3$ CFU/mL and a positive HBT.

Discussion

We performed quantitative culture of duodenal aspirates and an HBT in patients who visited our hospital with digestive symptoms such as abdominal bloating and confirmed that a high percentage of these patients had SIBO. Patients with SIBO showed significantly reduced α-diversity compared with non-SIBO patients, and analysis of β-diversity showed significantly different distributions between SIBO and non-SIBO patients. Also, the intestinal microbiome in SIBO patients was characterized by increased relative abundance of *Streptococcus* and decreased relative abundance of *Bacteroides* compared with non-SIBO patients.

The HBT clearly separated the positive patients from the negative patients. Patients with a positive HBT and bacterial count $<10^3$ CFU/mL showed a peak hydrogen breath level at 40 min after glucose ingestion (dash-dotted lines in Fig. 1), indicating the possibility that bacterial overgrowth was absent in the duodenum.

The results of quantitative culture showed predominantly aerobic bacterial overgrowth in our cohort. In contrast to our results, Saffouri et al. reported that anaerobic bacterial overgrowth was predominant in patients with SIBO[25]. The composition of bacteria in SIBO may differ between Japan and western countries. In quantitative culture, *Streptococcus* and yeast were detected most frequently in SIBO patients. This agrees well with the increased relative abundance of *Streptococcus* in SIBO patients confirmed by 16S rRNA gene sequencing. After *Streptococcus*, yeast was the second most increased microorganism. Jacobs et al. defined small intestinal fungal overgrowth (SIFO) as the detection of fungi in cultured duodenal aspirates, and reported that the causative fungus was Candida. Among patients with a bacterial count $\geq 10^3$ CFU/mL or the presence of fungi in cultured duodenal aspirates, SIFO was

observed in 66% of patients[26]. Given that fungi and bacteria are closely associated and influence each other[27, 28], the fungal microbiome needs to be analyzed in the future.

Analysis of the bacterial microbiome confirmed significant differences in α-diversity between the group with a bacterial count $<10^3$ CFU/mL and the group with a bacterial count $>10^5$ CFU/mL in quantitative culture, but there tended to be overlap in α-diversity between the group with a bacterial count $<10^3$ CFU/mL and the group with a bacterial count of 10^3-10^5 CFU/mL. The specimens for quantitative culture and those for 16S rRNA analysis were collected at the same time, and an increased bacterial count was closely associated with reduced α-diversity. On the other hand, a significant difference was observed in the evenness marker (i.e., Shannon index) but not in the richness markers (i.e., observed species and Chao1 index) in patients with SIBO diagnosed in this study.

The results of β-diversity analysis were strongly associated with the HBT results, as well as those of quantitative culture. Saffouri et al. diagnosed SIBO based only on quantitative culture of duodenal aspirates and reported that there was no difference in β-diversity between patients with symptomatic SIBO and those without SIBO[25]. This result differed from ours. In our study, we compared three groups that were stratified according to the number of bacteria in quantitative culture, and β-diversity showed a significantly different distribution between these groups. In their cohort, 45% of patients complained of diarrhea, which was the most frequent symptom[25]. In our cohort, the highest median GSRS values were found for “bloating” and “sensation of not completely emptying the bowels”. Therefore, the baseline characteristics of the patients may have affected the results.

Phylum level analysis showed that the relative abundance of Bacteroidetes was lower in SIBO patients than in non-SIBO patients. In comparison, Leite et al. examined changes in the duodenal microbiome and reported decreases in Firmicutes and increases in Proteobacteria in SIBO patients compared with non-SIBO patients[14]. Such changes were not observed in our study. On the other hand, a significant decrease in Bacteroidetes was observed in our cohort of SIBO patients but not in the study by Leite et al. ($p = 0.05$)[14]. The median relative abundance of Bacteroidetes in non-SIBO patients was 14.3% in our cohort but only 6% in their cohort. These findings could be attributed to the fact that the composition of the intestinal microbiome differed between the Japanese cohort in our study and the US cohort in the study by Leite et al[14].

Genus level analysis revealed increases in the relative abundance of aerobes and facultative anaerobes such as *Streptococcus*, *Actinomyces*, and *Granulicatella*, which are indigenous to the oral cavity, and decreases in the relative abundance of obligate anaerobes such as *Bacteroides*, *Blautia*, and *Prevotella* in SIBO patients compared with non-SIBO patients. Switching from a high-fiber diet to a low-fiber, high simple-sugar diet triggered gastrointestinal symptoms and increased intestinal permeability[25]. Such increased intestinal permeability may promote the expansion of aerobes and/or facultative anaerobes in SIBO patients.

This study has some limitations. First, all patients had some kind of digestive symptoms, and patients with SIBO were not compared with healthy individuals. It has been reported that dysbiosis is present in symptomatic patients without SIBO[25], therefore, a comparison with healthy individuals is a subject for future study. Nevertheless, we performed duodenal aspirate sampling and an HBT on the same day, and we believe that the diagnosis of SIBO was made appropriately. Second, the number

of patients enrolled in this study was limited. As a result, in our cohort, there was only one patient taking PPIs. Although the effect of PPIs has already been reported[29], this point was not investigated adequately in the present study. Third, we did not analyze the fungal microbiome, and this needs to be addressed in the future.

In conclusion, this study found duodenal dysbiosis in patients with SIBO. It was also revealed that increases in the relative abundance of *Streptococcus* and decreases in the relative abundance of *Bacteroides* were characteristics of the duodenal bacterial microbiome in SIBO patients compared with non-SIBO patients. These differences may play a role in the pathophysiology of SIBO. Further analysis of the duodenal microbiome, including the fungal microbiome, is necessary in the future.

Conflict of Interest

Akira Andoh is an Editorial Board member of the *Journal of Gastroenterology and Hepatology* and a co-author of this article. To minimize bias, he was excluded from all editorial decision-making related to the acceptance of this article for publication.

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Authors' contributions

Conceptualization: SB and AA. Methodology: SB, TI, MS, MO, SY, and KT. Formal analysis: SB. Investigations: SB, MO, AN, KT, and OI. Writing: AA. Supervision: TI, MS, and AA. Approval of final manuscript: all authors.

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Figure legends

Figure 1. Changes in hydrogen levels over time as measured by a glucose HBT. Dashed line indicates the results of a patient with chronic idiopathic intestinal pseudo-obstruction (CIPO). Dotted lines indicate the results of patients who had undergone Roux-en-Y reconstruction. Dashed-dotted lines indicate the results of patients with positive HBT results but a bacterial count of $<10^3$ CFU/mL.
CFU: colony forming unit; HBT: hydrogen breath test

Figure 2. Comparative analysis of α-diversity using duodenal samples. Observed species, Chao1 index, and Shannon index were used to analyze α-diversity, and the patients were stratified according to quantitative culture results (a-c), HBT results (d-f), or the presence or absence of SIBO* (g-i). The Mann-Whitney *U* test was used for analysis and P-values are shown above the corresponding bars compared in the graphs.
*SIBO was diagnosed based on a bacterial count $\geq 10^3$ CFU/mL in quantitative culture of duodenal aspirates or a rise in the hydrogen breath level of ≥20 ppm on an HBT.
CFU: colony forming unit; HBT: hydrogen breath test; SIBO: small intestinal bacterial overgrowth

Figure 3. Comparative analysis of β-diversity using duodenal samples. β-Diversity was estimated using the UniFrac metric and visualized using non-metric multidimensional scaling (NMDS) ordination. (a) Patients were stratified into three groups according to quantitative culture results: $<10^3$ CFU/mL (n = 9); 10^3-10^5 CFU/mL (n = 6); and $>10^5$ CFU/mL (n = 9) (P <0.01, PERMANOVA). (b) Patients were grouped according to HBT results: positive (n = 12) and negative (n = 12), (P = 0.01, PERMANOVA). (d)

Comparison between SIBO*(n = 17) and non-SIBO (n = 7) patients (P = 0.01, PERMANOVA).

*SIBO was diagnosed based on a bacterial count ≥10^3 CFU/mL in quantitative culture of duodenal aspirates or a rise in the hydrogen breath level of ≥20 ppm on an HBT.

CFU: colony forming unit; HBT: hydrogen breath test; PERMANOVA: permutational multivariate analysis of variance; SIBO: small intestinal bacterial overgrowth

Figure 4. Comparative analysis of the taxonomic composition of the microbial community at the phylum level in SIBO*(n = 17) and non-SIBO (n = 7) patients. (a) Actinobacteria. (b) Bacteroidetes. (c) Firmicutes. (d) Fusobacteria. (e) Proteobacteria. (f) Absconditabacteria. (g) Saccharibacteria. The Mann-Whitney *U* test was used for analysis and P-values are shown above the corresponding bars compared in the graphs.
*SIBO was diagnosed based on a bacterial count ≥10^3 CFU/mL in quantitative culture of duodenal aspirates or a rise in the hydrogen breath level of ≥20 ppm on an HBT.

CFU: colony forming unit; HBT: hydrogen breath test; SIBO: small intestinal bacterial overgrowth

Figure 5. Comparative analysis of the taxonomic composition of the microbial community at the genus level using linear discriminant analysis effect size (LEfSe) between SIBO*(n = 17) and non-SIBO (n = 7) patients.
*SIBO was diagnosed based on a bacterial count ≥10^3 CFU/mL in quantitative culture of duodenal aspirates or a rise in the hydrogen breath level of ≥20 ppm on an HBT.

c: class; CFU: colony forming unit; f: family; g: genus; HBT: hydrogen breath test; o: order; p: phylum SIBO: small intestinal bacterial overgrowth

Supplementary Figure 1. Comparative analysis of the taxonomic composition of the microbial community at the phylum level. Patients were stratified into three groups

according to quantitative culture results: $<10^3$ CFU/mL (n = 9), 10^3-10^5 CFU/mL (n = 6), and $>10^5$ CFU/mL (n = 9). (a) Actinobacteria. (b) Bacteroidetes. (c) Firmicutes. (d) Fusobacteria. (e) Proteobacteria. (f) Absconditabacteria. (g) Saccharibacteria. The Mann-Whitney *U* test was used for analysis and P-values are shown above the corresponding bars compared in the graphs.

CFU: colony forming unit

Supplementary Figure 2. Comparative analysis of the taxonomic composition of the microbial community at the phylum level. Patients were stratified into two groups according to HBT results: positive (n = 12) and negative (n = 12). (a) Actinobacteria. (b) Bacteroidetes. (c) Firmicutes. (d) Fusobacteria. (e) Proteobacteria. (f) Absconditabacteria. (g) Saccharibacteria. The Mann-Whitney *U* test was used for analysis and P-values are shown above the corresponding bars compared in the graphs.

HBT: hydrogen breath test

Supplementary Figure 3. Comparative analysis of the taxonomic composition of the microbial community at the genus level using linear discriminant analysis effect size (LEfSe). Patients were stratified into two groups according to quantitative culture results: $<10^3$ CFU/mL (n = 9) and $\geq 10^3$ CFU/mL (n = 15).

c: class; CFU: colony forming unit; f: family; g: genus o: order; p: phylum

Supplementary Figure 4. Comparative analysis of the taxonomic composition of the microbial community at the genus level using linear discriminant analysis effect size (LEfSe). Patients were stratified into two groups according to HBT results: positive (n = 12) and negative (n = 12).

c: class; f: family; g: genus; HBT: hydrogen breath test; o: order; p: phylum

Table 1. Background characteristics of the patients according to presence or absence of SIBO

Characteristics	**SIBO*** **(n = 17)**	**Non-SIBO** **(n = 7)**	**P-value**
Male / female	6/11	2/5	0.748[a]
Age (years), median (IQR)	59 (45-69)	46 (33-49)	0.119[b]
History of intestinal resection (yes/no)	4/13	0/7	0.079[a]
Patient status			
Symptoms only	14	7	
Roux-en-Y reconstruction	2	0	
CIPO	1	0	
Medication			
Probiotics	2		
Proton pump inhibitors	1		
GSRS			
Pain or discomfort in the upper abdomen or pit of the stomach, median (IQR)	1.7 (1.0-3.0)	4.0 (1.7-6.0)	0.105[b]
Heartburn, median (IQR)	2.0 (1.0-2.7)	2.0 (1.5-4.5)	0.318[b]
Acid reflux, median (IQR)	2.0 (1.0-3.0)	2.0 (1.0-4.5)	0.728[b]
Hunger pains, median (IQR)	2.0 (1.0-2.7)	3.0 (1.5-6.0)	0.110[b]
Nausea, median (IQR)	1.0 (1.0-2.0)	2.0 (1.0-3.5)	0.182[b]
Rumbling, median (IQR)	2.0 (1.0-4.0)	5.0 (2.0-6.0)	0.061[b]
Bloated, median (IQR)	3.0 (1.0-6.3)	7.0 (3.0-7.0)	0.149[b]
Burping, median (IQR)	1.5 (1.0-5.0)	4.0 (2.5-5.0)	0.287[b]
Passing gas or flatus, median (IQR)	2.5 (1.2-3.7)	5.0 (2.5-6.5)	0.084[b]

Constipation, median (IQR)	3.0 (2.0-4.0)	2.0 (1.5-5.0)	0.612[b]
Diarrhea, median (IQR)	1.5 (1.0-4.0)	2.0 (1.5-2.5)	0.759[b]
Loose stools, median (IQR)	2.0 (1.0-4.0)	3.0 (2.0-5.0)	0.146[b]
Hard stools, median (IQR)	2.0 (1.2-4.7)	2.0 (1.5-3.5)	0.766[b]
Urgent need to have a bowel movement, median (IQR)	2.0 (1.0-3.7)	2.0 (1.5-3.5)	0.897[b]
Sensation of not completely emptying the bowels, median (IQR)	3.5 (3.0-5.0)	4.0 (3.5-6.5)	0.310[b]

[a]Chi-square test, [b]Mann-Whitney *U* test

*SIBO was diagnosed based on a bacterial count $\geq 10^3$ CFU/mL in quantitative culture of duodenal aspirates or a rise in the hydrogen breath level of ≥20 ppm on an HBT.

CFU: colony forming unit; CIPO: chronic idiopathic intestinal pseudo-obstruction; GSRS: Gastrointestinal Symptom Rating Scale; HBT: hydrogen breath test; IQR: interquartile range; SIBO: small intestinal bacterial overgrowth

Table 2. Quantitative culture of duodenal aspirate

Characteristics	**N = 24**
Aerobes	
Staphylococcus	12% (3)
Enterococcus	0% (0)
Streptococcus	37% (9)
Pseudomonas aeruginosa	0% (0)
Escherichia coli	20% (5)
Klebsiella pneumonia	8% (2)
Yeast	25% (6)
Anaerobes	
Clostridium	4% (1)
Bacteroides	4% (1)

Concentration $\geq 10^3$ CFU/mL was considered positive.

CFU: colony forming unit; IQR: interquartile range

Supplementary Table 1. Background characteristics of the patients according to the results of quantitative culture

Characteristics	**≥10^3 CFU/mL (n = 15)**	**<10^3 CFU/mL (n =9)**	**P-value**
Male / female	6/9	2/7	0.363[a]
Age (years), median (IQR)	60 (43-70)	47 (37-53)	0.107[b]
History of intestinal resection (yes/no)	4/11	0/9	0.039[a]
Patient status			
Symptoms only	12	9	
Roux-en-Y reconstruction	2	0	
CIPO	1	0	
Medication			
Probiotics	1	1	
Proton pump inhibitors	1		
GSRS			
Pain or discomfort in the upper abdomen or pit of the stomach, median (IQR)	1.7 (1.0-3.2)	2.5 (1.0-6.0)	0.335[b]
Heartburn, median (IQR)	2.0 (1.0-3.0)	2.0 (1.0-3.0)	0.638[b]
Acid reflux, median (IQR)	2.0 (1.0-3.2)	2.0 (1.0-3.0)	0.937[b]
Hunger pains, median (IQR)	2.0 (1.0-2.2)	3.0 (1.0-6.0)	0.172[b]
Nausea, median (IQR)	1.0 (1.0-2.0)	2.0 (1.0-3.0)	0.196[b]
Rumbling, median (IQR)	2.0 (1.0-4.0)	2.0 (2.0-5.0)	0.282[b]
Bloated, median (IQR)	3.0 (1.0-6.1)	7.0 (1.0-7.0)	0.192[b]
Burping, median (IQR)	2.0 (1.0-5.0)	3.0 (2.0-4.0)	0.729[b]
Passing gas or flatus, median (IQR)	2.5 (1.0-4.2)	3.0 (2.0-6.0)	0.183[b]

Constipation, median (IQR)	3.0 (2.0-4.0)	3.0 (2.0-6.0)	0.760[b]
Diarrhea, median (IQR)	1.5 (1.0-4.0)	2.0 (1.0-2.0)	0.937[b]
Loose stools, median (IQR)	2.0 (1.0-4.0)	2.0 (2.0-4.0)	0.463[b]
Hard stools, median (IQR)	2.0 (1.0-4.2)	2.0 (2.0-4.0)	0.730[b]
Urgent need to have a bowel movement, median (IQR)	2.5 (1.0-4.0)	2.0 (1.0-2.0)	0.698[b]
Sensation of not completely emptying the bowels, median (IQR)	3.5 (2.7-3.5)	4.0 (3.0-7.0)	0.221[b]

[a]Chi-square test, [b]Mann-Whitney *U* test

CFU: colony forming unit; CIPO: chronic idiopathic intestinal pseudo-obstruction; GSRS: Gastrointestinal Symptom Rating Scale; IQR: interquartile range

Supplementary Table 2. Background characteristics of the patients according to HBT results

Characteristics	**HBT positive (n = 12)**	**HBT negative (n =12)**	**P-value**
Male / female	5/7	3/9	0.386[a]
Age (years), median (IQR)	59 (47-72)	47 (36-65)	0.105[b]
History of intestinal resection (yes/no)	2/10	2/10	1.000[a]
Patient status			
Symptoms only	9	12	
Roux-en-Y reconstruction	2	0	
CIPO	1	0	
Medication			
Probiotics	2	0	
Proton pump inhibitors	1		
GSRS			
Pain or discomfort in the upper abdomen or pit of the stomach, median (IQR)	2.0 (1.0-3.0)	2.5 (1.0-6.0)	0.533[b]
Heartburn, median (IQR)	2.0 (1.0-3.0)	2.0 (1.0-2.5)	0.970[b]
Acid reflux, median (IQR)	2.0 (1.0-3.7)	1.0 (1.0-2.5)	0.331[b]
Hunger pains, median (IQR)	2.0 (1.0-2.7)	2.0 (1.0-5.0)	0.298[b]
Nausea, median (IQR)	1.0 (1.0-2.0)	1.0 (1.0-2.5)	0.565[b]
Rumbling, median (IQR)	2.0 (1.0-4.0)	2.0 (1.0-5.0)	0.714[b]
Bloated, median (IQR)	3.0 (1.5-6.3)	5.0 (1.0-7.0)	0.465[b]
Burping, median (IQR)	1.5 (1.0-5.0)	3.0 (1.5-5.0)	0.463[b]
Passing gas or flatus, median (IQR)	2.5 (2.0-4.7)	3.0 (1.5-5.5)	0.690[b]

Constipation, median (IQR)	3.0 (2.0-3.7)	3.0 (1.5-5.0)	1.000[b]
Diarrhea, median (IQR)	2.0 (1.0-4.0)	2.0 (1.0-2.5)	0.598[b]
Loose stools, median (IQR)	2.0 (1.0-4.0)	2.0 (1.0-4.0)	0.912[b]
Hard stools, median (IQR)	2.0 (1.2-3.7)	2.0 (1.5-4.5)	0.912[b]
Urgent need to have a bowel movement, median (IQR)	3.0 (1.0-4.0)	2.0 (1.0-2.0)	0.184[b]
Sensation of not completely emptying the bowels, median (IQR)	3.5 (3.0-5.0)	4.0 (3.0-6.5)	0.560[b]

[a]Chi-square test, [b]Mann-Whitney *U* test

CIPO: chronic idiopathic intestinal pseudo-obstruction; GSRS: Gastrointestinal Symptom Rating Scale; HBT: hydrogen breath test; IQR: interquartile range

Figure 1

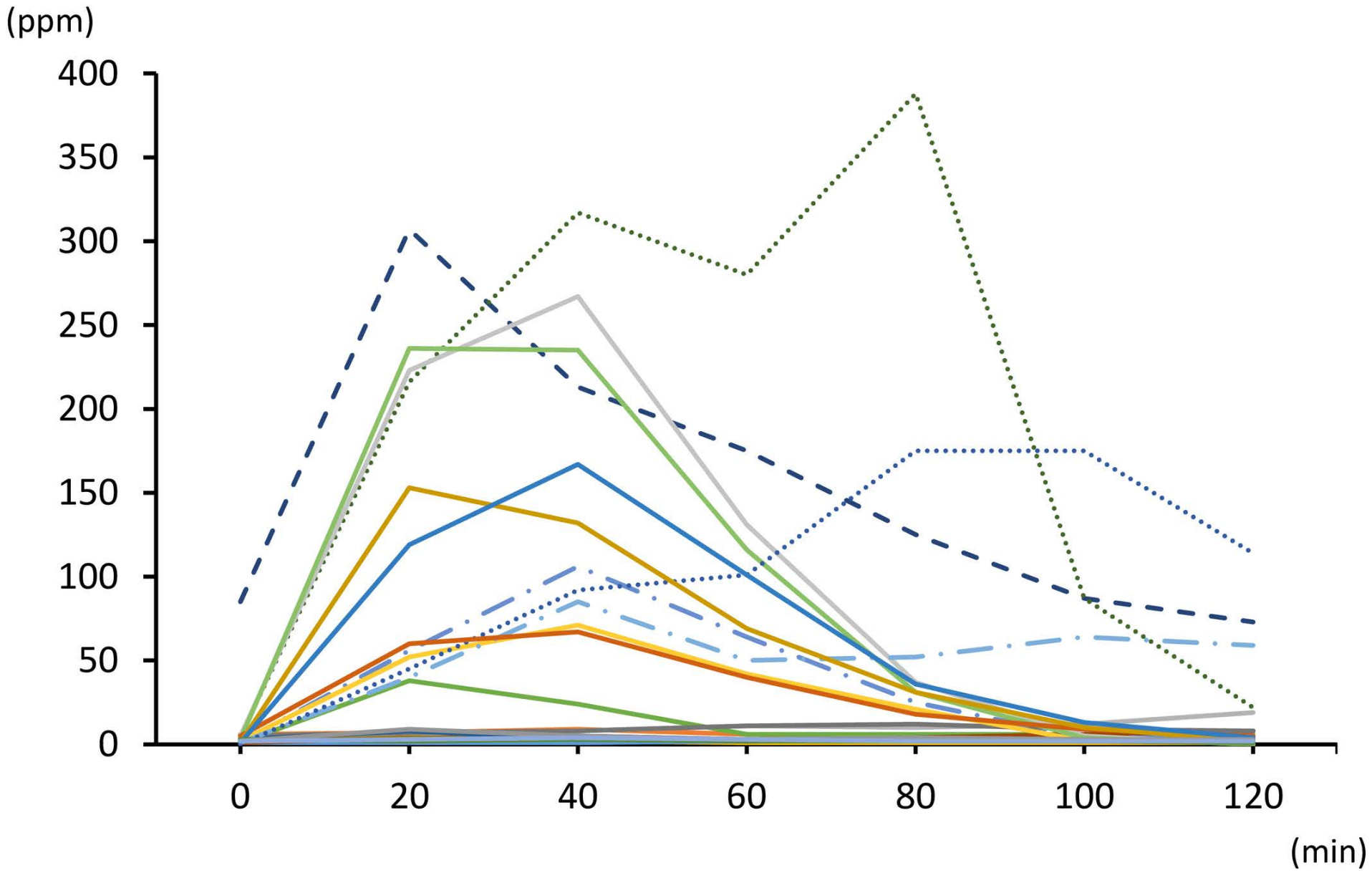


Figure 2

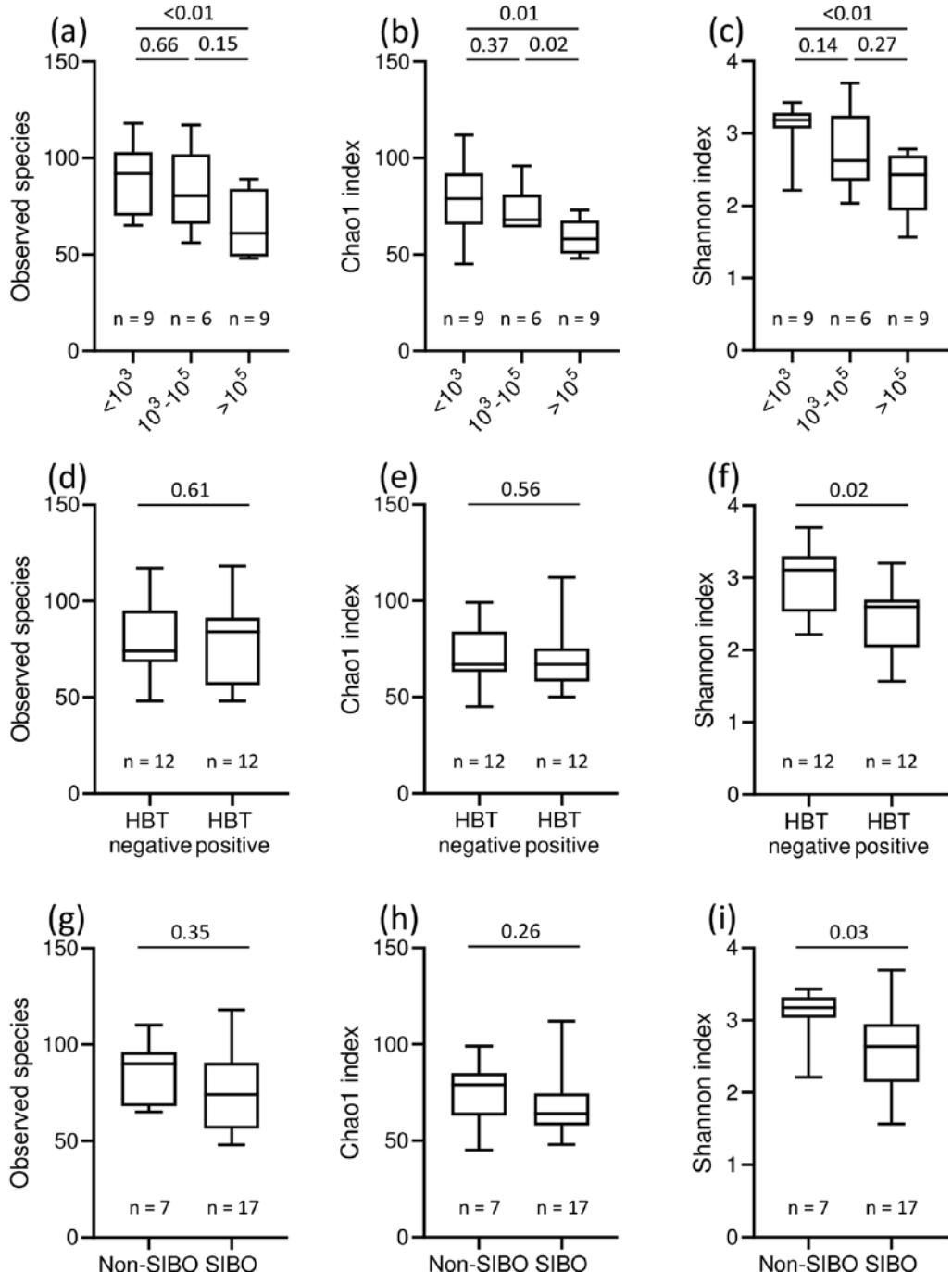

(a)
<0.01
0.66
0.15
Observed species
150
100
50
0
n = 9
n = 6
n = 9
<10^3
10^3-10^5
>10^5
(b)
0.01
0.37
0.02
Chao1 index
150
100
50
0
n = 9
n = 6
n = 9
<10^3
10^3-10^5
>10^5
(c)
<0.01
0.14
0.27
Shannon index
4
3
2
1
0
n = 9
n = 6
n = 9
<10^3
10^3-10^5
>10^5
(d)
0.61
Observed species
150
100
50
0
n = 12
n = 12
HBT negative
HBT positive
(e)
0.56
Chao1 index
150
100
50
0
n = 12
n = 12
HBT negative
HBT positive
(f)
0.02
Shannon index
4
3
2
1
0
n = 12
n = 12
HBT negative
HBT positive
(g)
0.35
Observed species
150
100
50
0
n = 7
n = 17
Non-SIBO
SIBO
(h)
0.26
Chao1 index
150
100
50
0
n = 7
n = 17
Non-SIBO
SIBO
(i)
0.03
Shannon index
4
3
2
1
0
n = 7
n = 17
Non-SIBO
SIBO

Figure 3

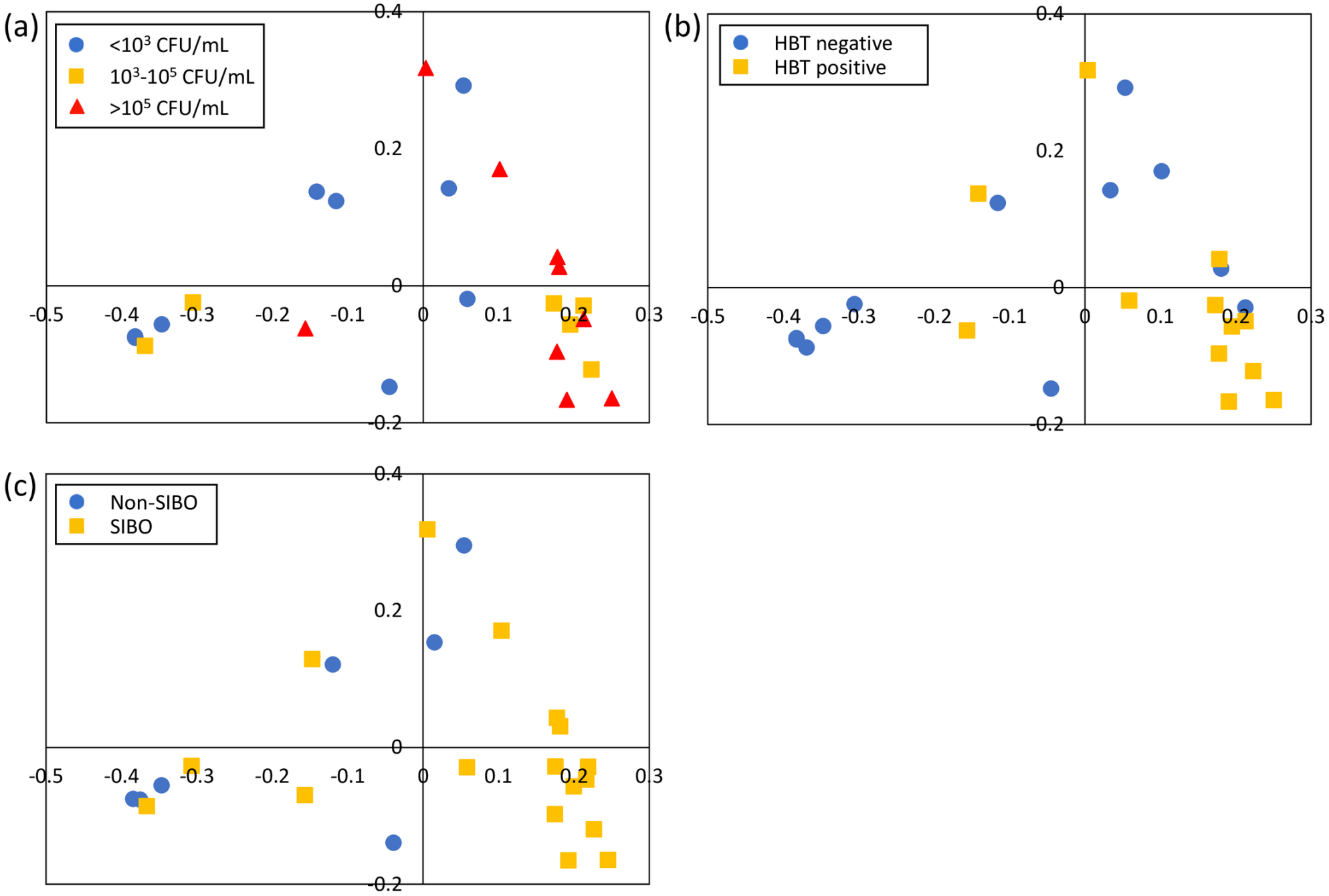

(a)
<10³ CFU/mL
10³-10⁵ CFU/mL
>10⁵ CFU/mL
(b)
HBT negative
HBT positive
(c)
Non-SIBO
SIBO
0.4
0.2
0
-0.2
-0.5
-0.4
-0.3
-0.2
-0.1
0
0.1
0.2
0.3

Figure 4

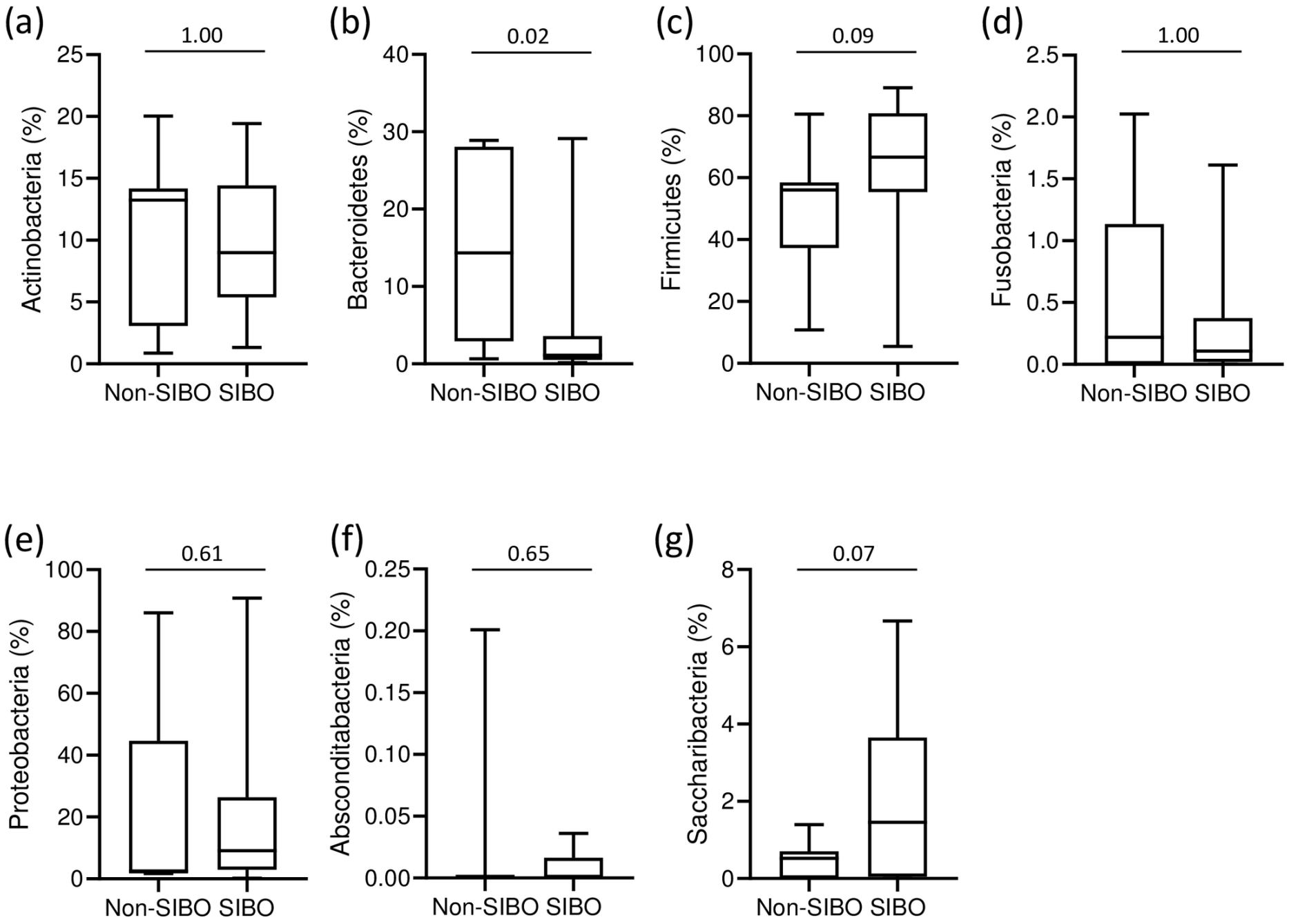
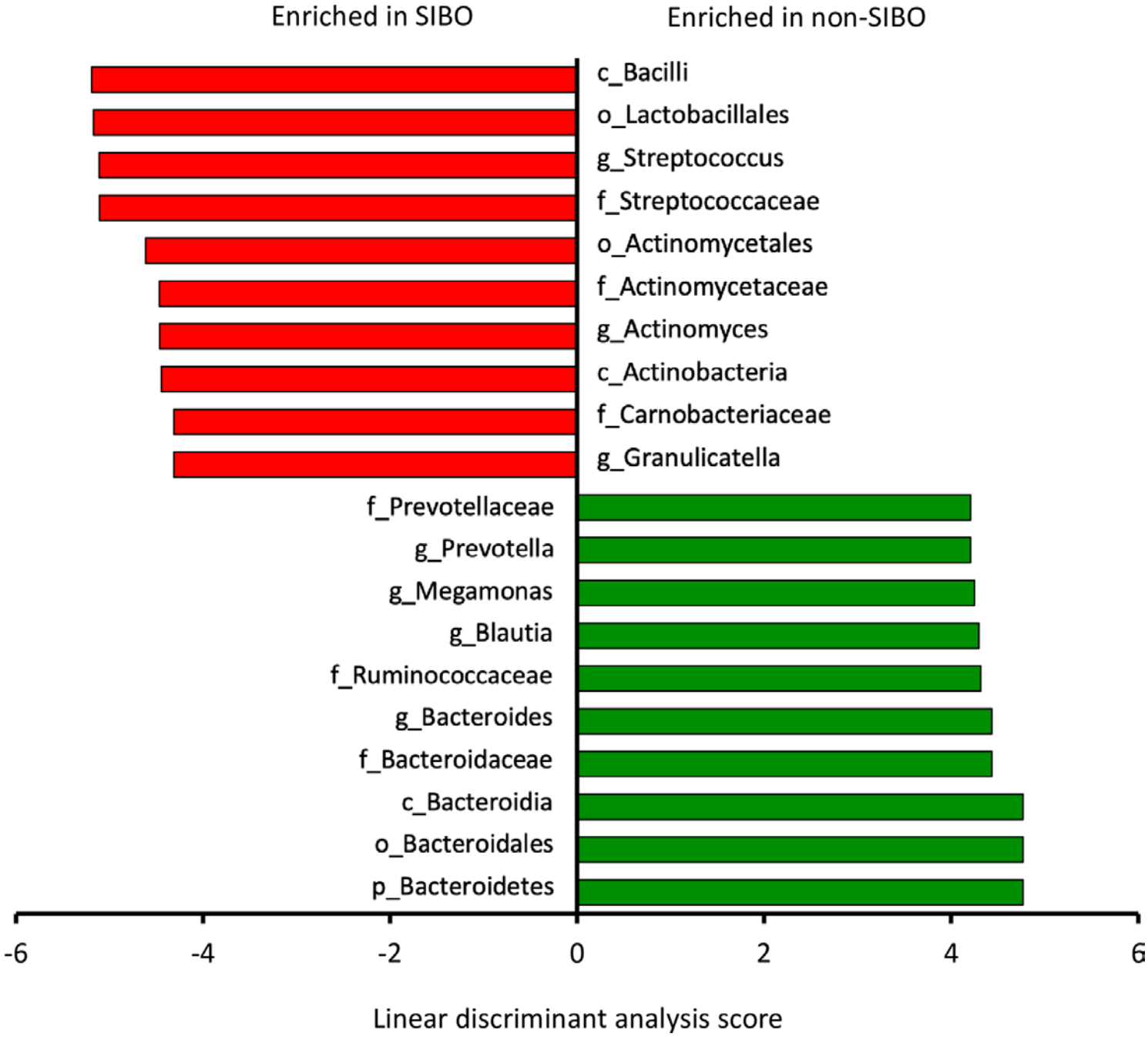
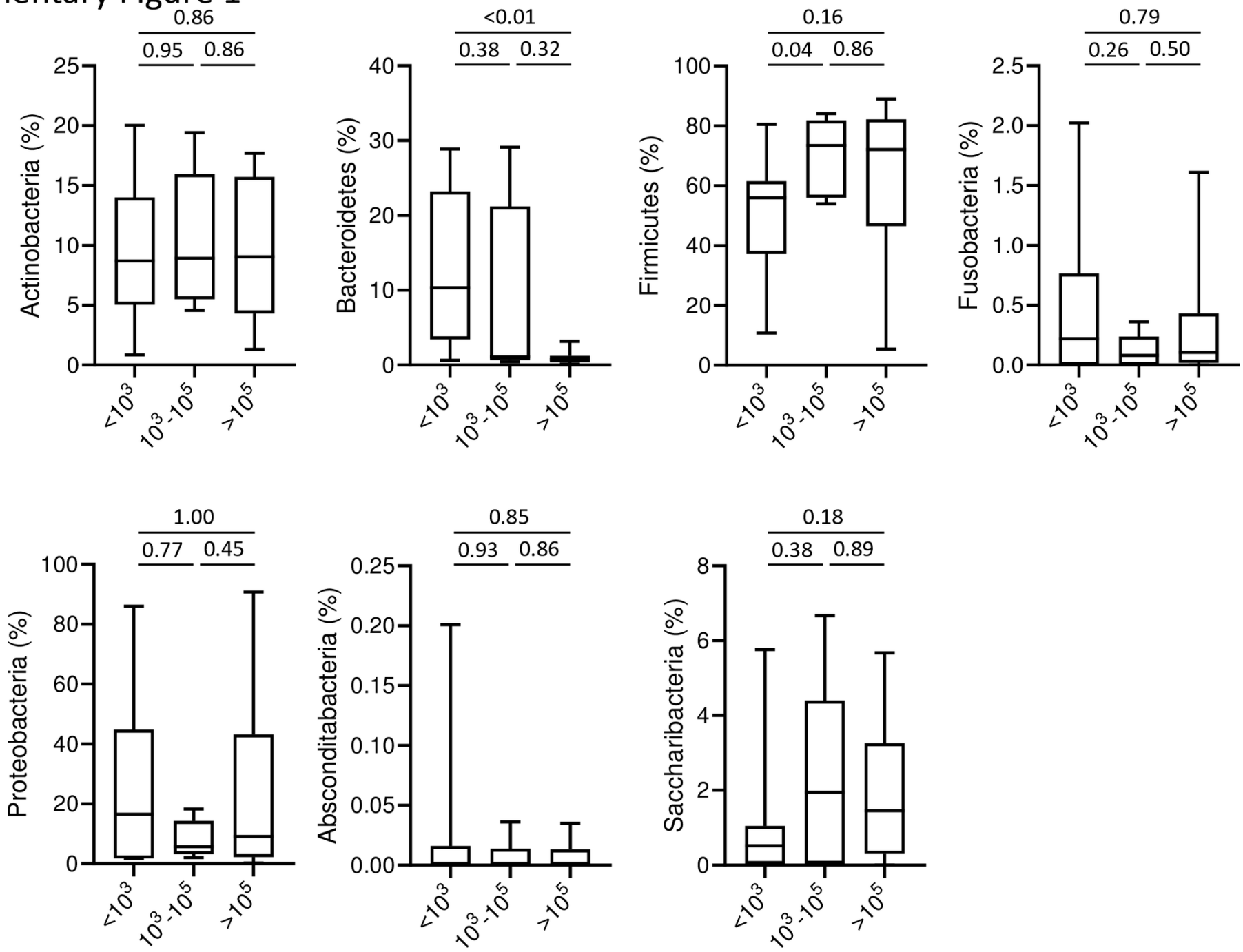

(a)
1.00
Actinobacteria (%)
25
20
15
10
5
0
Non-SIBO SIBO
(b)
0.02
Bacteroidetes (%)
40
30
20
10
0
Non-SIBO SIBO
(c)
0.09
Firmicutes (%)
100
80
60
40
20
0
Non-SIBO SIBO
(d)
1.00
Fusobacteria (%)
2.5
2.0
1.5
1.0
0.5
0.0
Non-SIBO SIBO
(e)
0.61
Proteobacteria (%)
100
80
60
40
20
0
Non-SIBO SIBO
(f)
0.65
Absconditabacteria (%)
0.25
0.20
0.15
0.10
0.05
0.00
Non-SIBO SIBO
(g)
0.07
Saccharibacteria (%)
8
6
4
2
0
Non-SIBO SIBO

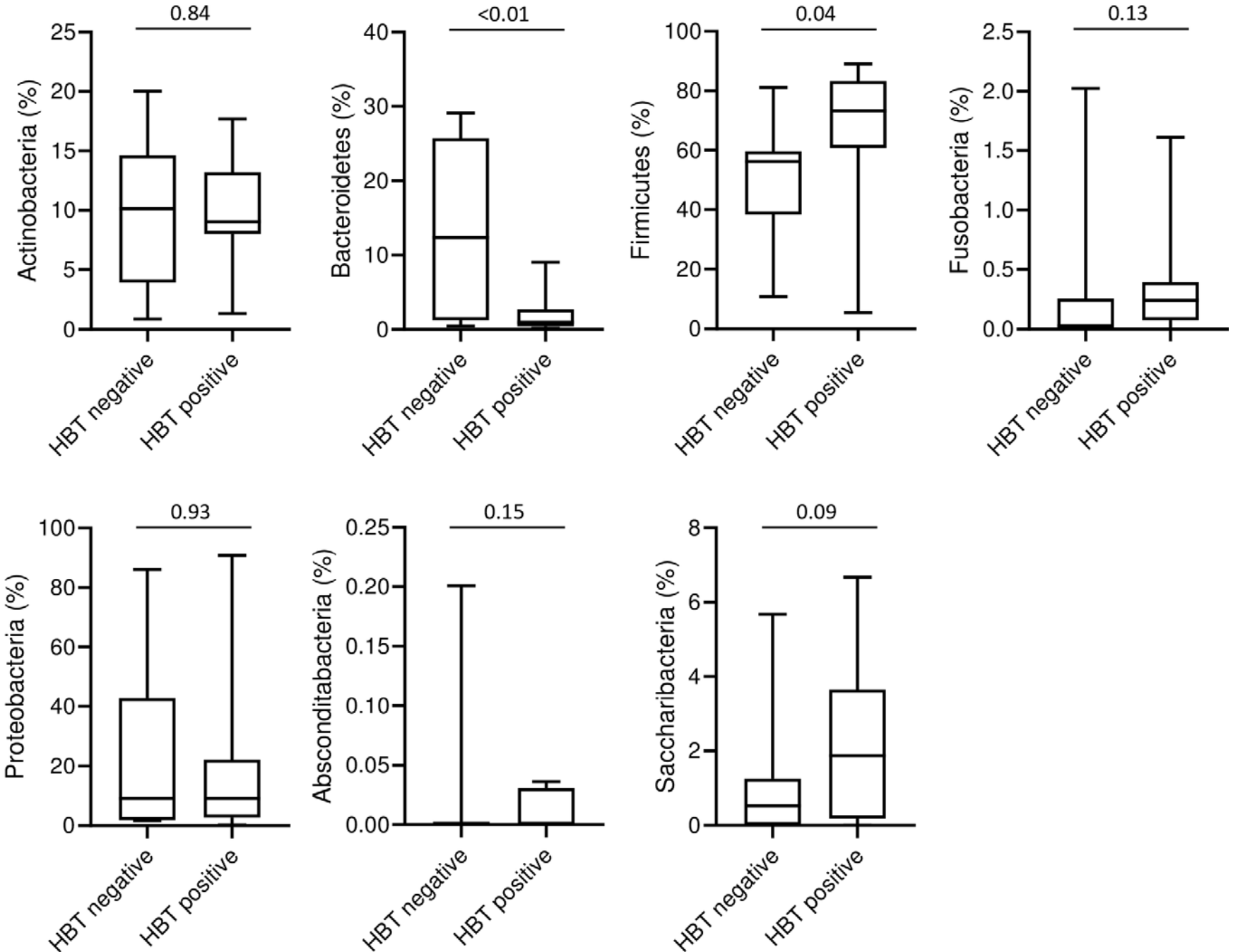
Figure 5


Enriched in SIBO
Enriched in non-SIBO
c_Bacilli
o_Lactobacillales
g_Streptococcus
f_Streptococcaceae
o_Actinomycetales
f_Actinomycetaceae
g_Actinomyces
c_Actinobacteria
f_Carnobacteriaceae
g_Granulicatella
f_Prevotellaceae
g_Prevotella
g_Megamonas
g_Blautia
f_Ruminococcaceae
g_Bacteroides
f_Bacteroidaceae
c_Bacteroidia
o_Bacteroidales
p_Bacteroidetes
-6
-4
-2
0
2
4
6
Linear discriminant analysis score

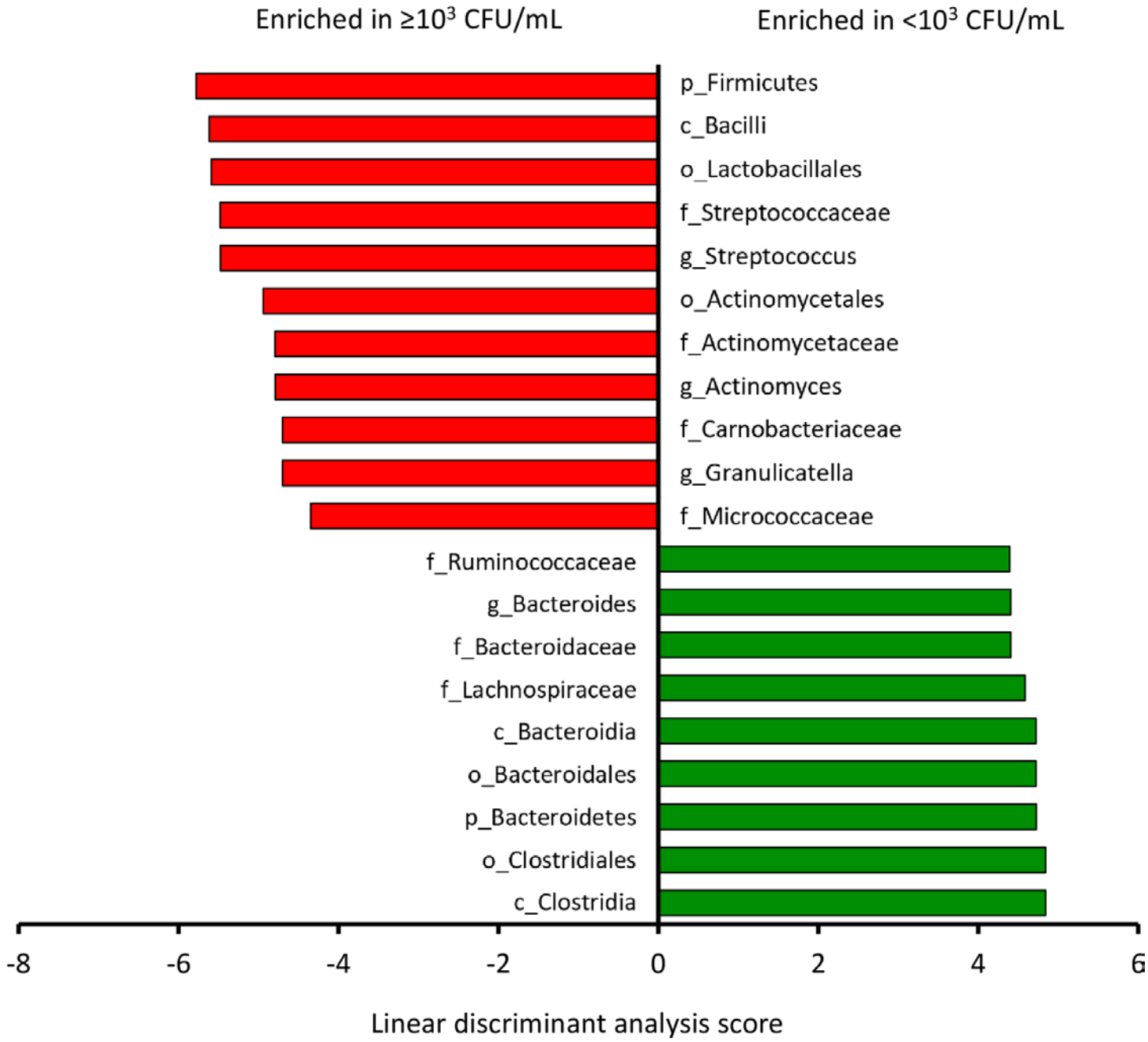
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4