

# Patients with breath test positive are necessary to be identified from irritable bowel syndrome: a clinical trial based on microbiomics and rifaximin sensitivity

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## Abstract

**Background:** As a non-invasive and effective diagnostic method for small intestinal bacterial overgrowth (SIBO), wild-use of breath test (BT) has demonstrated a high comorbidity rate in patients with diarrhea-predominant irritable bowel syndrome (IBS-D) and SIBO. Patients overlapping with SIBO respond better to rifaximin therapy than those with IBS-D only. Gut microbiota plays a critical role in both of these two diseases. We aimed to determine the microbial difference between IBS-D overlapping with/without SIBO, and to study the underlying mechanism of its sensitivity to rifaximin.

**Methods:** Patients with IBS-D were categorized as BT-negative (IBSN) and BT-positive (IBSP). Healthy volunteers (BT-negative) were enrolled as healthy control. The patients were clinically evaluated before and after rifaximin treatment (0.4 g bid, 4 weeks). Blood, intestine, and stool samples were collected for cytokine assessment and gut microbial analyses.

**Results:** Clinical complaints and microbial abundance were significantly higher in IBSP than in IBSN. In contrast, severe systemic inflammation and more active bacterial invasion function that were associated with enrichment of opportunistic pathogens were seen in IBSN. The symptoms of IBSP patients were relieved in different degrees after therapy, but the symptoms of IBSN rarely changed. We also found that the presence of IBSN-enriched genera (*Enterobacter* and *Enterococcus*) are unaffected by rifaximin therapy.

**Conclusions:** IBS-D patients overlapping with SIBO showed noticeably different fecal microbial composition and function compared with IBS-D only. The better response to rifaximin in those comorbid patients might associate with their different gut microbiota, which suggests that BT is necessary before IBS-D diagnosis and use of rifaximin.

**Registration:** Chinese Clinical Trial Registry, ChiCTR1800017911.

**Keywords:** Irritable bowel syndrome; Small intestinal bacterial overgrowth; Breath test; Gut microbiota; Rifaximin

## Introduction

Irritable bowel syndrome (IBS) is a functional gastrointestinal (GI) disorder with a high prevalence worldwide.<sup>[1,2]</sup> IBS-like symptoms such as abdominal pain, discomfort, diarrhea, bloating, and flatulence are commonly observed in patients with small intestinal bacterial overgrowth (SIBO) as well.<sup>[3]</sup> Hydrogen and methane breath test (BT) is an effective and non-invasive diagnostic tool for SIBO.<sup>[4]</sup> In patients with IBS who meet Rome

criteria, BT positive rate is  $\geq 35.5\%$ .<sup>[5,6]</sup> Studies have confirmed that patients with IBS, especially those with a diarrhea-predominant subtype (IBS-D), are more likely to overlap with SIBO. However, the symptom profiles of IBS or SIBO are non-specific and clinical history alone cannot clearly distinguish the underlying cause.

Rifaximin, a broad-spectrum oral antibiotic with little absorption, is recommended for IBS treatment.<sup>[7,8]</sup>

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Rifaximin inhibits bacterial RNA synthesis by binding to the  $\beta$ -subunit of bacterial DNA-dependent RNA polymerase and against a variety of entomopathogens. Although rifaximin is more effective than placebo in patients with non-constipated IBS,<sup>[9]</sup> the efficacy was modest and symptoms recurred after treatment. In addition, high cost and insufficient efficacy rate limit the clinical use of rifaximin.<sup>[10]</sup> Patients with IBS overlapping with SIBO presented with higher abdominal bloating scores and severer GI symptoms.<sup>[6,11]</sup> Eradication of SIBO using absorbable or non-absorbable antibiotics reduces IBS-like symptoms; for example, rifaximin significantly relieves the clinical symptoms of SIBO.<sup>[12,13]</sup> Therefore, some experienced gastroenterologists<sup>[14,15]</sup> might recommend BT to exclude SIBO before making an IBS diagnosis or before a rifaximin treatment. However, there is neither any guideline in place that specifies the necessity to do so, nor any study that addresses the pathophysiological mechanism resulting in different curative effects of rifaximin on IBS overlapping with SIBO.

Gut microbiota plays a vital role in IBS. The small intestinal and fecal microbiota of IBS patients has been studied previously.<sup>[16-20]</sup> The fecal microbial community of IBS exhibits lower  $\alpha$ -diversity, with increasing abundance of Firmicutes and decreasing Bacteroidetes at the phylum level, and with increasing *Clostridia* and decreasing *Bacteroidia* at the lower taxonomic levels.<sup>[16,17]</sup> Microbial community changes in SIBO seem to contribute to IBS. Moreover, the changes in small intestinal microbiota may influence fecal flora.<sup>[19,20]</sup> However, previous studies on small intestinal microbiota of patients with IBS neglect to consider whether the patients have SIBO, and few studies have attempted to characterize the differences in fecal microbes in patients with IBS with or without SIBO.

In this study, we aimed to determine the microbial difference between IBS-D overlapping with/without SIBO, and to study the underlying mechanism of its sensitivity to rifaximin. We elucidated the necessity to eliminate SIBO from IBS diagnosis through BT to provide precise treatment options for better clinical efficacy.

## Methods

### Study design and subject's recruitment

The trial was performed from April 2015 to February 2019. IBS-D patients whose symptoms fulfilled the diagnostic criteria of Rome III were recruited from the Department of Gastroenterology, Peking University Third Hospital. Healthy volunteers without previous or current GI symptoms and infection were recruited at the same time though advertisements. Both patients and healthy volunteers aged between 18 and 65 years were recruited, as indicated in the flowchart shown in Supplementary Figure 1 [<http://links.lww.com/CM9/B141>]. After ingestion of 10 g of lactulose in a 20 mL water solution, lactulose, hydrogen, and methane breath test (LHMBT) was performed using the methane-hydrogen breathing analyzer (Quintron Instrument Company, Milwaukee, WI, USA). IBS-D patients were separated into two groups according to the results of

LHMBT: patients with BT negative termed as IBSN group, and patients with BT positive termed as IBSP group. Healthy volunteers with BT negative constituted the healthy control (HC) group. All subjects underwent colonoscopy with biopsies in distal ileum and sigmoid. Details are shown in Supplementary Methods [<http://links.lww.com/CM9/B141>]. Consecutive patients of IBSN and IBSP were administered with rifaximin (Xifaxan, Alfa Wassermann S.P.A., Italy) 0.4 g twice per day orally for 4 weeks. After 4 weeks, subjects completed LHMBT, IBS-SSS, and fecal sample collected again. During the intervention therapy, patients received a follow-up clinical interview once a week.

### Ethical approval

The study was conducted in accordance with the *Declaration of Helsinki* and was approved by the local ethics committee of Peking University Health Science Center (No. IRB00001052-14091). Informed written consent was obtained from all patients prior to their enrollment in this study.

### Clinical evaluation

Each subject received routine blood and stool tests to rule out local and systemic organic lesions before treatment. Daily bowel movement frequency and consistency were recorded based on the Bristol Stool Form (BSF) scale. Visceral sensitivity was evaluated by colon rectal distension (CRD) test using a barostat (Distender Series II; G&J Electronics, Ontario, Canada). These procedures have been described in detail in our previous studies.<sup>[11,21]</sup> GI symptom severity was evaluated by IBS Symptom Severity Scores (IBS-SSS) and Gastrointestinal Symptom Rating Scale (GSRS) before and after treatment. Half-year food frequency questionnaire (FFQ) (Chinese version) was used to estimate the dietary pattern.

### Experimental evaluation

Mucosa biopsy tissues from distal ileum and sigmoid were used for intestinal mucosal expression of interleukin-10 (IL10), interleukin-12 (IL12), and tight junction proteins Zona occludens 1 (ZO1) using immunohistochemistry.

Systematic inflammatory tone was assessed by measuring the ratio of IL10/IL12 in both serum and supernatant of peripheral blood mononuclear cells (PBMCs) culturing through Enzyme-Linked Immunosorbent Assay (ELISA) (EBioscience, Human IL10 Platinum ELISA Kit; Human IL12/IL23 p40 Platinum ELISA Kit, Vienna, Austria).<sup>[20]</sup> Further details can be found in the Supplementary Methods [<http://links.lww.com/CM9/B141>].

### Gut microbiota analysis

Details for DNA extraction, PCR amplification, and sequencing and processing of sequencing data are described in the Supplementary Methods [<http://links.lww.com/CM9/B141>].

Alpha diversity indices were calculated in Mothur (version 1.30.1, [https://www.mothur.org/wiki/Download\\_mothur](https://www.mothur.org/wiki/Download_mothur))

and compared using Student's *t* test. The rarefaction curves were plotted in R (version 3.6.0). Partial Least Squares Discriminant Analysis (PLS-DA) was analyzed and plotted using the *mixOmics* package in R. The ternary plot was performed in GGTERN (<http://www.ggtern.com/>) software. Difference analyses among groups in each level were performed using Kruskal–Wallis test.

All correlations were analyzed using Spearman's coefficient with pairwise comparisons. The correlations between genera and clinical indicator were visualized using Pheatmap package in R. The correlations between top 50 genera were exported for downstream analysis using Cytoscape (version 3.8.0) software (<https://cytoscape.org/>) to generate networks, with  $P < 0.05$  and  $|r| \geq 0.6$  considered significant.

### Statistical analysis

Statistical analysis was performed on SPSS 17.0 (SPSS Inc., Chicago, IL, USA) and R using public and inhouse packages. Comparisons of parametric data between more than two groups were performed using one-way analysis of variance (ANOVA), and otherwise, the Mann-Whitney *U* test was used. Non-parametric data were compared by chi-squared test. All tests were corrected for multiple testing using Benjamini–Hochberg method.  $P < 0.05$ , with false discovery rate corrected, was considered statistically significant (unless specified otherwise).

## Results

### IBSP and IBSN were similar with regard most clinical features except for fat-to-energy ratio

In total, 176 subjects were enrolled, including 49 BT-negative HC (33 males, aged  $32.28 \pm 11.33$  years) and 127 IBS-D patients (92 males, aged  $32.61 \pm 9.68$  years). In patients with IBS-D, 51 were BT-positive (IBSP group; 36 males, aged  $30.76 \pm 8.94$  years) and 76 were BT-negative (IBSN group; 56 males, aged  $33.84 \pm 10.02$  years); the BT-positive rate was 40%. The body mass index (BMI) in IBSP group was significantly lower than that in IBSN group ( $21.66 \pm 3.60$  vs.  $23.38 \pm 3.75$  kg/m<sup>2</sup>,  $P = 0.01$ ,  $t = 3.19$ ). For FFQ Supplementary Table 1 [<http://links.lww.com/CM9/B141>], more subjects in IBSP were found to be on a high-fat diet compared with IBSN (37% vs. 22%), and their diet contributes to a significant increasing of fat-to-energy ratio when compared with IBSN.

Clinical symptoms were evaluated using IBS symptom severity scale (IBS-SSS) and GSRS for the three groups [Figure 1 and Table 1]. In comparison with HC group, both IBSN and IBSP presented a significant increase in clinical symptom scores, such as abdominal pain and diarrhea [Figure 1A–C]. Watery stools were distinguished in IBSP and IBSN groups according to BSF scores [Figure 1D]. No significant difference was observed between IBSN and IBSP groups according to IBS-SSS and GSRS scores.

In CRD test, subjects in IBSN and IBSP showed a visceral hypersensitivity compared with HC, which presents as a significant decrease in thresholds for initial defecation,

and defecation urgency [Figure 1E]. Additionally, no significant difference was observed in visceral sensitivity between the IBSP and IBSN groups.

### IBSN presented a low-grade inflammation

The levels of IL10 and IL12 were used to evaluate the inflammatory condition. When compared with HC group, serum IL12 tended to elevate and IL10 tended to decrease in the IBSN group [Table 1], and thus the ratio of serum IL10/IL12 decreased ( $P = 0.051$ ; Figure 1G). The IL12 level in PBMC supernatant was significantly higher in IBSN than that in HC ( $P < 0.05$ ; Figure 1F). Neither the level of IL10 nor that of IL12 presented a significant change between IBSP and HC.

Tissue expression for IL12 differed from IBSP or IBSN to HC group. The level of IL12 increased significantly in IBSP group in ileum, whereas in colon, it showed a significant increase in IBSN group [Figure 1H and 1I].

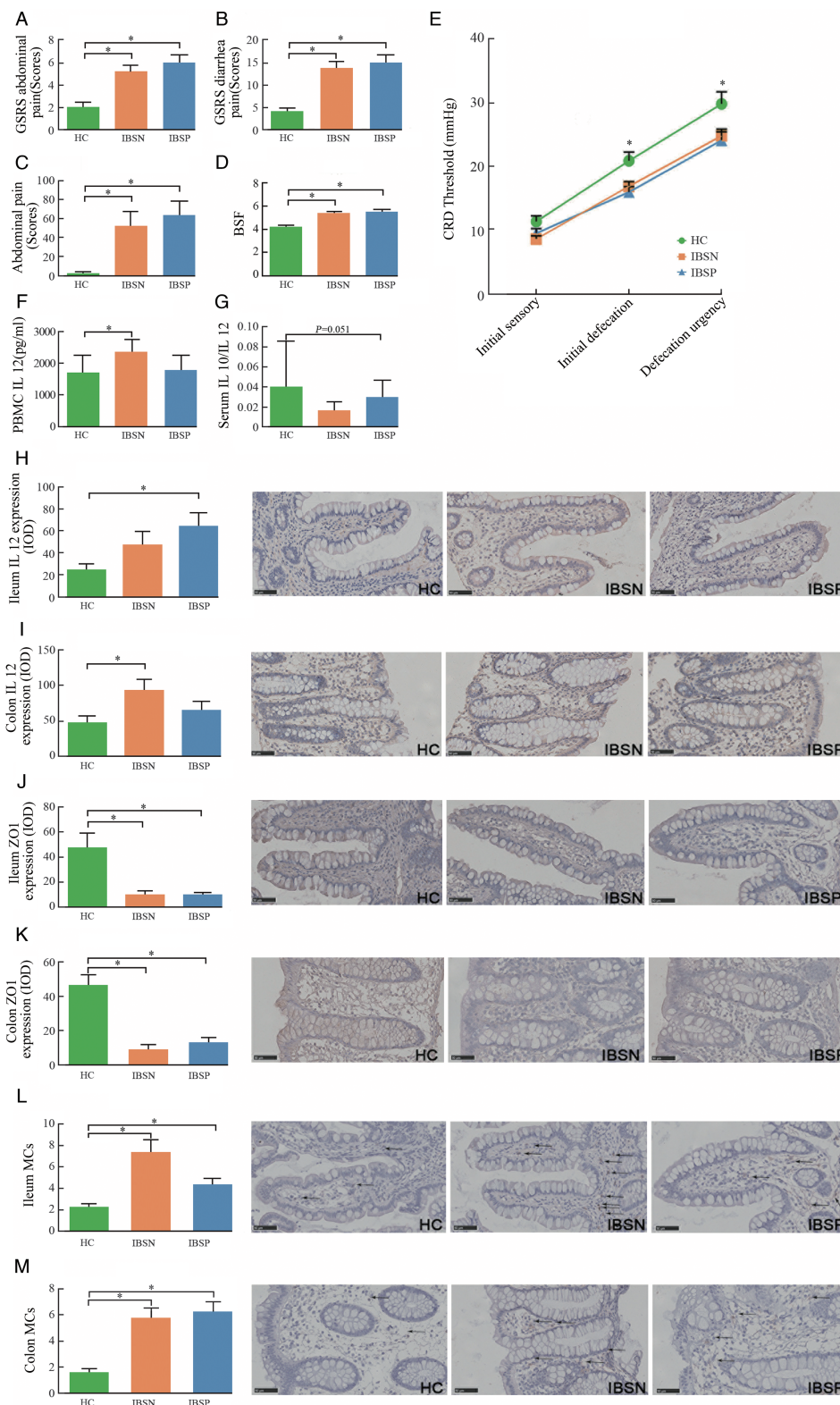
The level of tight junction protein ZO1, which is associated with gut barrier function, was observed to be significantly reduced in both the ileum and colon in IBSP and IBSN groups when compared with HC group ( $P < 0.001$ ; Figure 1J and 1K).

Both IBSN and IBSP groups had a higher count of mast cells in the ileum and colon ( $P < 0.05$ , separately) when compared with HC groups [Figure 1N and 1M].

No significant difference was found between IBSP and IBSN among those markers.

### Different fecal microbiota features in IBSN and IBSP groups

A total of 6,440,193 16S rRNA sequences were obtained from the V3 to V4 regions. The rarefaction curve indicated that a reasonable number of sequence samples were obtained Supplementary Figure 2A and 2B [<http://links.lww.com/CM9/B141>]. The community diversity [Figure 2A and 2B] and partial least squares discriminant analyses (PLS-DA; Figure 2D) indicated a compositional distinction of microbiota in IBSN, IBSP, and HC. IBSP had the highest abundance of gut microbes, presenting with the highest Shannon index and the lowest Simpson index among the three groups. There was significant difference among three groups in the beta-diversity evaluated using analysis of similarity ( $R = 0.0551$ ,  $P = 0.005$ ). The phylum Proteobacteria enriched significantly in IBSN group compared to HC and IBSP groups Supplementary Figure 2C and 2D [<http://links.lww.com/CM9/B141>]. The abundance of Enterobacteriaceae, Enterococcaceae, and Lachnospiraceae are increased, and Coriobacteriaceae, Desulfurellaceae and Verrucomicrobiaceae are decreased significantly at the family level in the IBSN group compared with HC group [Figure 2C]. The phylum Tenericutes was significantly enriched in the IBSP group compared with HC and IBSN groups. A higher abundance of Synergistaceae and lower Desulfurellaceae at the family level were found in the IBSP group compared with HC group. Overall, at the genus level, more differences were identified among the three groups [Figure 2E and Supplementary Table 2, <http://links.lww.com/CM9/>



**Figure 1:** Clinical symptom evaluations and inflammation factors among the three groups. (A,B) Abdominal pain and diarrhea scores evaluated by GRSRS; (C) Abdominal pain score evaluated by IBS-SSS; (D) BSF scores; (E) CRD test; \*IBSN vs. HC, Mann-Whitney *U* test,  $P < 0.05$ ; (F) IL12 in PBMCs culturing supernatant; (G) Ratio of IL10/IL12 in serum; (H,I) IL12 expression in ileum and colon; (J,K) ZO1 expression in ileum and colon; (L,M) MCs counts in ileum and colon, shows by arrows. \*IBSP or IBSN vs. HC, Mann-Whitney *U* test,  $P < 0.05$ . GRSRS: Gastrointestinal Symptom Rating Scale; IBS-SSS: IBS Symptom Severity Scores; BSF: Bristol stool form; CRD: Colon rectal distension; PBMCs: Peripheral blood mononuclear cells; IL10: Interlukin-10; IL12: Interlukin-12; ZO-1: Zona occludens 1; MCs: Mast cells; HC: Healthy control; IBS: irritable bowel syndrome; IBSP: breath test positive IBS patients; IBSN: breath test negative IBS patients; Bars: 50  $\mu$ m.

**Table 1: Clinical symptoms and inflammation factor analysis among healthy controls and IBS-D patients.**

Items	HC (n = 49)	IBSN (n = 76)	IBSP (n = 51)	P value
<b>IBS-SSS</b>				
Abdominal pain	0.00 (0.00, 0.00)	50.00 (0.00, 81.25)*	50.00 (33.75, 80.00)†	0.001
Pain frequency	0 (0.00, 0.00)	30.00 (30.00, 50.00)*	30.00 (10.00, 40.00)†	0.001
Abdominal bloating	0.00 (0.00, 0.00)	30.00 (0.00, 30.00)*	27.50 (0.00, 26.50)†	0.001
Dissatisfaction with bowel movement	20.00 (0.00, 70.00)	70.00 (30.00, 70.00)*	70.00 (40.00, 80.00)†	0.001
Disturbance to life	0.00 (0.00, 20.00)	70.00 (40.00, 70.00)*	68.00 (40.00, 70.00)†	0.001
<b>GSRs</b>				
Abdominal pain	2 (2, 2)	5.00 (4, 6)*	6.00 (5.00, 7.75)†	0.001
Abdominal bloating	4.00 (3.00, 4.00)	8.00 (5.00, 10.75)*	9.00 (5.00, 11.00)†	0.001
Diarrhea	4.00 (4.00, 5.00)	13.00 (9.00, 18.00)*	16.00 (9.25, 19.75)†	0.001
Constipation	2.00 (2.00, 2.00)	2.00 (2.00, 3.00)*	3.00 (2.00, 4.00)†	0.002
Satiety	2.00 (2.00, 2.00)	3.00 (2.00, 5.00)*	5.50 (3.25, 8.00)†	0.001
<b>Inflammation factors</b>				
Serum IL10	1.40 (0.94, 3.17)	1.34 (0.99, 2.02)	1.70 (1.12, 2.40)	0.120
Serum IL12	162.80 (113.10, 250.20)	212.10 (111.10, 335.30)	151.20 (85.69, 222.20)	0.130
Serum IL10/IL12	0.01 (0.01, 0.03)	0.01 (0, 0.02)*	0.01 (0.01, 0.02)	0.050
PBMC IL10	46.39 (19.43, 90.47)	38.96 (15.70, 76.88)	33.41 (10.00, 108.80)	0.930
PBMC IL12	1380.00 (781.70, 1862.0)	2282.00 (1163.00, 3096.00)*	1508.00 (769.40, 2516.00)	0.070
PBMC IL10/IL12	0.03 (0.01, 0.05)	0.02 (0.01, 0.04)	0.02 (0.01, 0.06)	0.330

Data were described as median (25% percentile, 75% percentile). P value: ANOVA test. \* IBSN vs. HC, M-W U test, P < 0.05. † IBSP vs. HC, M-W U test, P < 0.05. ANOVA: Analysis of variance; M-W U test: Mann-Whitney U test; GSRs: Gastrointestinal Symptom Rating Scale; HC: Healthy control; IBS: Irritable bowel syndrome; IBSP: breath test positive IBS patients; IBSN: breath test negative IBS patients; IL10: Interlukin-10; IL12: Interlukin-12; PBMC: Peripheral blood mononuclear cell.

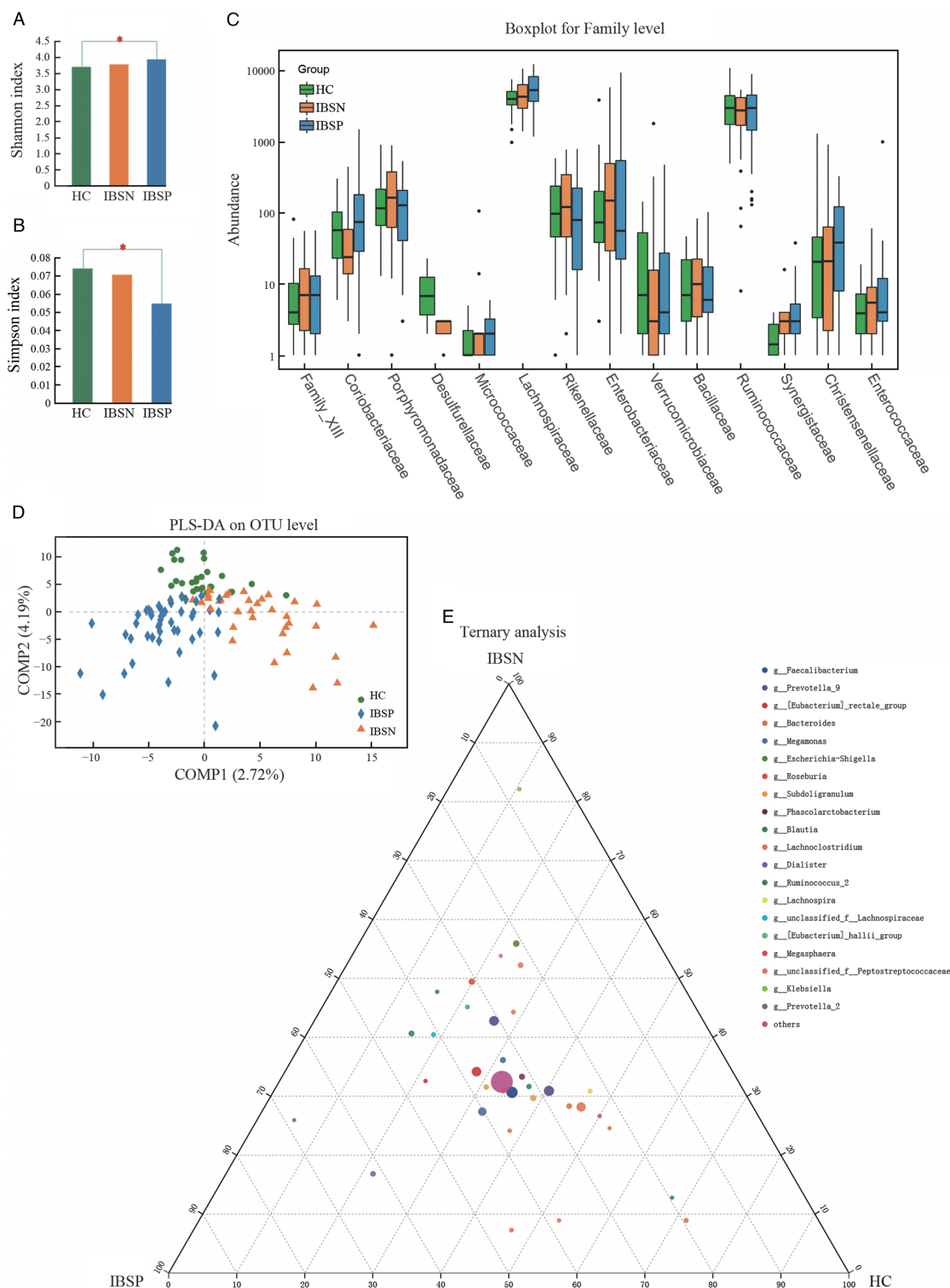
B141]. We found significant enrichments of *Escherichia-Shigella*, *Blautia*, *Klebsiella*, *Enterococcus*, *Citrobacter*, *Enterobacter*, and *Cronobacter* (top five relative abundance [RA]) in IBSN group compared with HC group, but depletion of *Lachnospiraceae* NK4A136 group, *Alistipes*, and *Ruminococcus\_1* in IBSN group compared with HC group. Meanwhile, higher abundance of *Blautia*, *Prevotella\_2*, *Lachnospiraceae* NC2004 group, *Cronobacter*, and *Romboutsia*, along with lower abundance of *Mitsuokella*, were found in IBSP group compared with HC group. Further analysis was applied to explore the differences in microbiota between IBSN and IBSP groups. The abundance of *Ruminococcaceae*\_UCG-002, *Parabacteroides*, *Ruminococcus\_1*, *Butyrivimonas*, *Lachnospiraceae*\_UCG-010, and *Odoribacter* (top five relative abundance [RA]) was significantly higher in IBSP group, while some pathogens such as *Lachnoclostridium*, *Escherichia-Shigella*, *Klebsiella*, *Enterococcus*, and *Cronobacter* (top five RA) were significantly higher in IBSN group. We used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) to infer the metagenomic function based on the microbial community profiles obtained from the 16S rRNA gene sequences. A comparison among the three groups is shown in Supplementary Table 3 [http://links.lww.com/CM9/B141]. We present a significant level 2 pathway for IBSN and IBSP groups in Figure 4A, where the ordinate represents the number of level 3 pathway belonging to level 2 pathway. In the IBSN group, the predicted Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways were significantly enriched in infectious diseases, energy metabolism, membrane transport, lipid metabolism, and metabolism of cofactors and vitamins [Figure 3A]. The enriched pathways in IBSP group were the same as those in IBSN group. In level 3 pathways, bacterial invasion of epithelial cells pathway was significantly increased in IBSN group compared with HC (P = 0.002) and IBSP groups (P = 0.001; Figure 3B). Besides, lipopolysaccharide (LPS) biosynthesis proteins and geraniol degradation were

significantly decreased in the IBSP group compared to HC group (P = 0.003), and it did not show a remarkable difference between IBSN and HC groups Supplementary Table 3 [http://links.lww.com/CM9/B141]. Microbial inner correlation in IBSP, IBSN, and HC groups is shown in Figure 3C, which suggests the interaction networks among the different fecal microbes. Microbial interaction in HC group was more complex than that in IBSN and IBSP; the most active microbes were Prevotellaceae, Lachnospiraceae, Bifidobacteriaceae, and Bacteroidaceae. In the IBSN and IBSP groups, their microbial inner correlation was less active and limited to one or two families such as Prevotellaceae and Fusobacteriaceae. To determine the association of microbiota and disease, the RA of the genera and all parameters were considered for the correlation analyses [Figure 3D]. Overall, the enriched genera in the IBSN group were positively associated with IBS-SSS and GSRs scores, and negatively correlated with CRD tolerance. On the other hand, those depleted genera in the IBSN group presented controversial correlation. In particular, enriched genera in the IBSN group, especially the Enterobacteriaceae family (*Enterobacter*, *Cronobacter*, *Citrobacter*, *Escherichia*, *Shigella*), were significantly positively correlated with IL12 level (in serum or in PBMC supernatant). *Sutterella* was positively correlated with IL10/IL12 in the PBMC supernatant.

**Rifaximin therapy**

**Clinical symptoms improvement after rifaximin treatment**

After rifaximin treatment for 4 weeks, 15 patients in the IBSN group (IBSNt) and 24 patients in IBSP group were recalled and re-donated stool samples. The GI symptoms for treated patients were relieved to varying degrees. The scores for abdominal pain, dissatisfaction with bowel movement, and disturbance to life in IBS-SSS, scores for abdominal pain and diarrhea in GSRs,

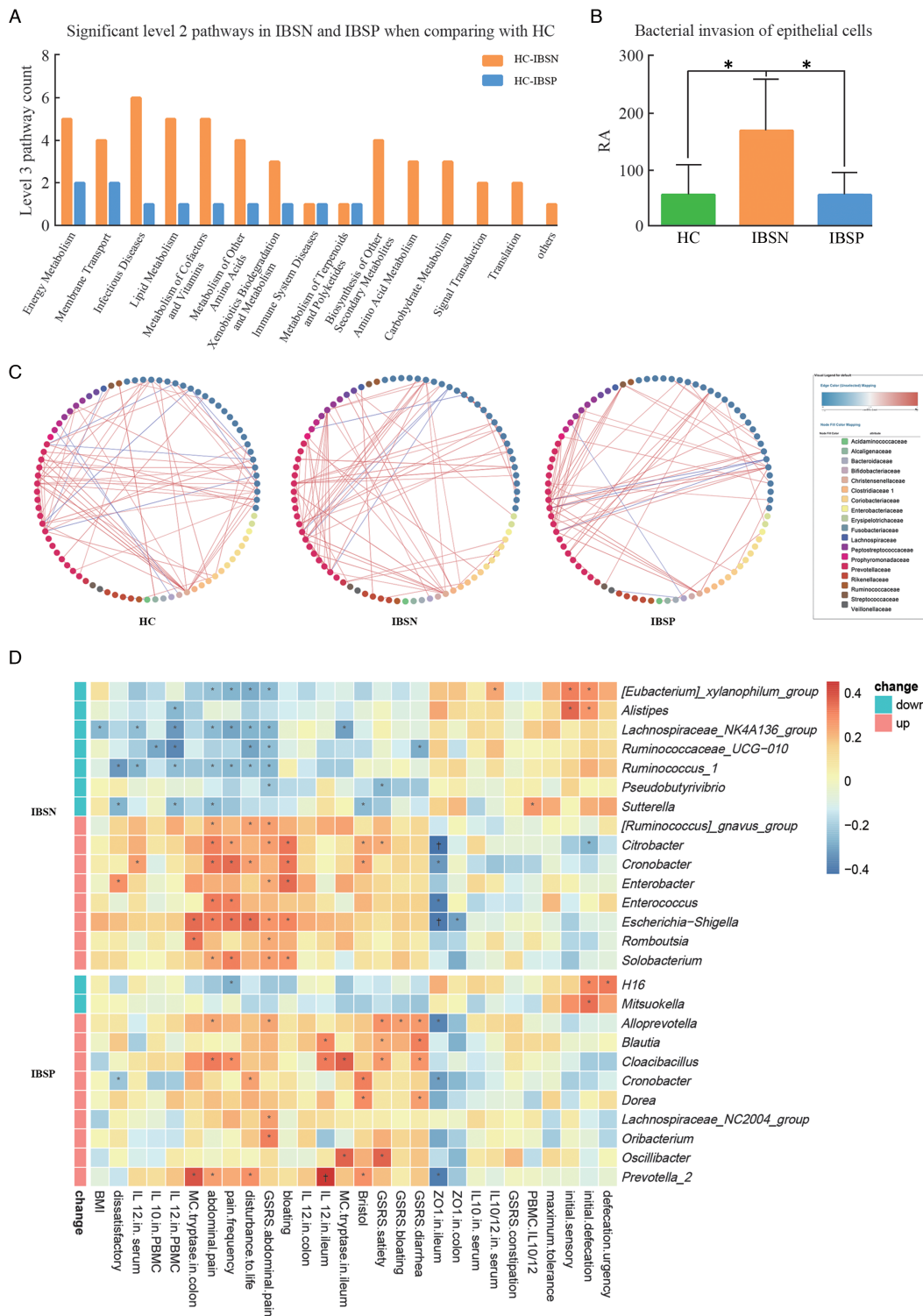


**Figure 2:** Different microbiota profiles in IBSN and IBSP. (A) Shannon index analysis, \* $P < 0.05$ ; (B) Simpson index analysis, \* $P < 0.05$ ; (C) Microbial analysis in family level (the family presented are significantly different among the three groups, Kruskal–Wallis test,  $P < 0.05$ ); (D) PLS-DA analysis on OTU level; (E) Ternary analysis in genus level; PLS-DA: Partial least squares discriminant analysis; HC: healthy controls; IBS: irritable bowel syndrome; IBSP: breath test positive IBS patients; IBSN: breath test negative IBS patients.

and BSF scores decreased significantly after treatment in IBSP group ( $P < 0.05$ , separately) [Table 2]. On the other hand, in IBSNt patients, only the IBS-SSS abdominal pain scores significantly decreased ( $P = 0.001$ ); and 0.41% BT-positive patients turned BT-negative after treatment.

### Different fecal microbial changes in IBSP and IBSN after rifaximin treatment

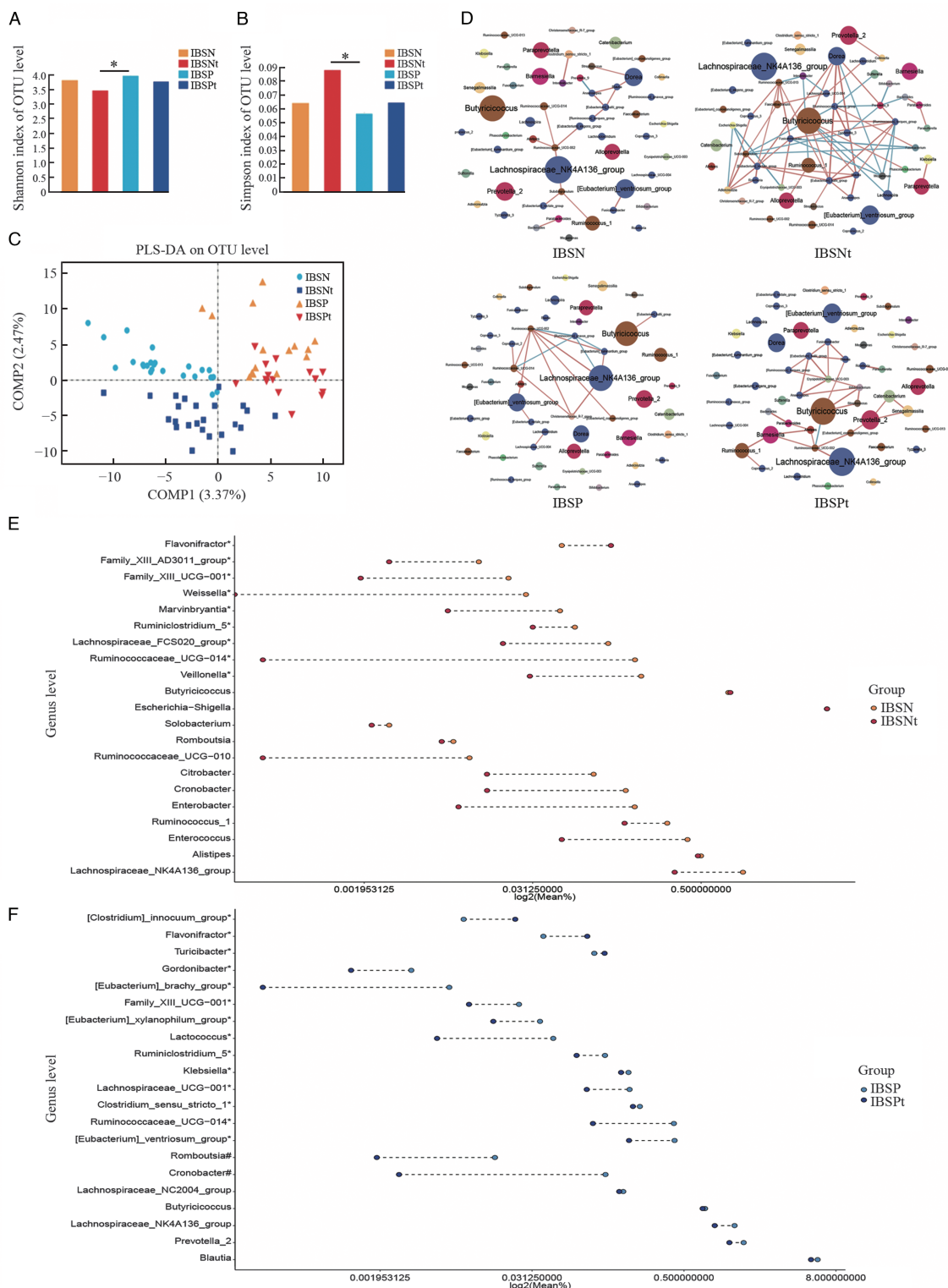
The alpha-diversity of bacterial communities (Shannon and Simpson indexes) decreased after therapy in both IBSN and IBSP groups [Figure 4A and 4B]. Following



**Figure 3:** Microbial function prediction, inner associations, and correlation of microbiota with clinical parameters. (A) Significant level 2 pathways in IBSN and IBSP in comparison with HC. Y-axis: the number of level 3 pathways belonging to level 2; (B) Comparison of the bacterial invasion of epithelial cells pathway in the three groups ( $^*P < 0.05$ ); (C) Associations among different genus in HC, IBSN, or IBSP; (D) Correlation of significant changing microbiota in IBSN and IBSP with clinical and experimental parameters: ( $^*P < 0.05$ ;  $^\dagger P < 0.01$ ). HC: Healthy control; IBS: irritable bowel syndrome; IBSP: breath test positive IBS patients; IBSN: breath test negative IBS patients; RA: Relative abundance.

therapy, a compositional separation was observed by PLS-DA analysis in both IBSN and IBSP groups [Figure 4C]. There was no significant difference at the phylum level after therapy in both IBSN and IBSP groups by taxonomic analysis, and the same results were found for Firmicutes/

Bacteroidetes (F/B) ratio. Analysis for the family level and genus level is shown in Supplementary Figure 3A and 3B [http://links.lww.com/CM9/B141]. As for genus level, IBSN-enriched genera such as *Escherichia-Shigella*, *Cronobacter*, and *Enterococcus* did not change after therapy



**Figure 4:** Microbiota profile changes before and after therapy. (A) Shannon index analysis, \*  $P < 0.05$ ; (B) Simpson index analysis, \*  $P < 0.05$ ; (C) PLS-DA analysis on OTU level; (D) Microbial interaction network of significant changing genus before and after therapy (Dot color: different phylum level; dot diameter: RA; red line: positive correlation; green line: negative correlation; line color shade: correlation coefficient); (E,F) Significantly changed genus in IBSN and IBSP groups after rifaximin therapy; each dot represents the median RA for genus, # significant different genus for IBSN or IBSP in comparison with HC; # significant different genus for IBSP in comparison with HC and significant changes after rifaximin therapy. HC: Healthy control; IBS: irritable bowel syndrome; IBSP: breath test positive IBS patients; IBSPt: IBSP after rifaximin treatment; IBSN: breath test negative IBS patients; IBSNt: IBSN after rifaximin treatment; PLS-DA: Partial least squares discriminant analysis; RA: Relative abundance.

[Figure 4E, Supplementary Table 5 [http://links.lww.com/CM9/B141]. IBSP-enriched genera *Romboutsia* and *Cronobacter* significantly decreased after rifaximin treatment, and

IBSP-depleted genera *Ateromonas* and *Dyella* increased after therapy [Figure 4F, Supplementary Table 5 [http://links.lww.com/CM9/B141]. Furthermore, IBSP-depleted *Gordo-*



**Table 2: Clinical symptoms after rifaximin treatment of the IBS patients.**

Clinical symptoms	IBSN (n = 76)	IBSNt (n = 15)	IBSP (n = 51)	IBSPt (n = 24)	P1	P2
IBS-SSS						
Abdominal pain	59.11 ± 5.29	22.86 ± 6.71	62.5 ± 7.02	20.91 ± 5.79	0.001	0
Pain frequency	40.20 ± 4.64	28.57 ± 8.84	33.08 ± 4.86	26.36 ± 7.41	0.326	0.505
Bloating	25.82 ± 2.86	15.71 ± 7.82	24.50 ± 3.49	15.91 ± 5.17	0.257	0.235
Dissatisfaction with bowel movement	64.44 ± 10.56	39.44 ± 9.73	70.00 ± 5.43	41.92 ± 6.19	0.101	0.002
Disturbance to life	60.13 ± 2.73	49.29 ± 8.76	59.15 ± 3.22	46.36 ± 4.91	0.213	0.036
Bristol	5.30 ± 0.13	4.79 ± 0.39	5.48 ± 0.12	4.20 ± 0.13	0.111	0
GSRs						
Abdominal pain	5.27 ± 0.22	4.29 ± 0.78	6.06 ± 0.33	4.50 ± 0.64	0.185	0.050
Bloating	7.81 ± 0.46	7.71 ± 1.13	8.54 ± 0.55	6.70 ± 0.91	0.949	0.156
Diarrhea	13.66 ± 0.75	11.71 ± 1.85	14.88 ± 0.87	9.50 ± 0.62	0.418	0
Constipation	2.72 ± 0.21	2.14 ± 0.14	3.46 ± 0.32	3.20 ± 0.47	0.387	0.726
Satiety	4.06 ± 0.32	3.86 ± 0.46	6.21 ± 0.51	4.70 ± 0.63	0.843	0.077

Data were described as mean ± standard deviation. P1: P value of IBSN vs. IBSNt; P2: P value of IBSP vs. IBSPt. GSRs: Gastrointestinal Symptom Rating Scale; IBS: Irritable bowel syndrome; IBSNt: Patients in IBSN after rifaximin treatment; IBSPt: Patients in IBSP after rifaximin treatment; IBS-SSS: IBS Symptom Severity Scores; IBSP: Breath test positive IBS patients; IBSN: Breath test negative IBS patients.

*nibacter*, *Butyricimonas*, and *Parabacteroides* increased after treatment.

In order to detect the influence of rifaximin therapy on the gut microbiota, genera were used to construct a microbial interaction network with correlation coefficient >0.6, as shown in Figure 4D. Rifaximin treatment changed the microbial inner correlation from *Lachnospiraceae* NK4A136 group-dominant to *Butyricimonas*-dominant in both IBSP and IBSN groups. Moreover, a stronger positive correlation was observed between *Gemmiger* and *Odoribacter*, *Lactococcus* and *Enterococcus*, and *Butyricimonas* and *Parabacteroides*, and a negative correlation was found between *Flavonifractor* and *Dorea*.

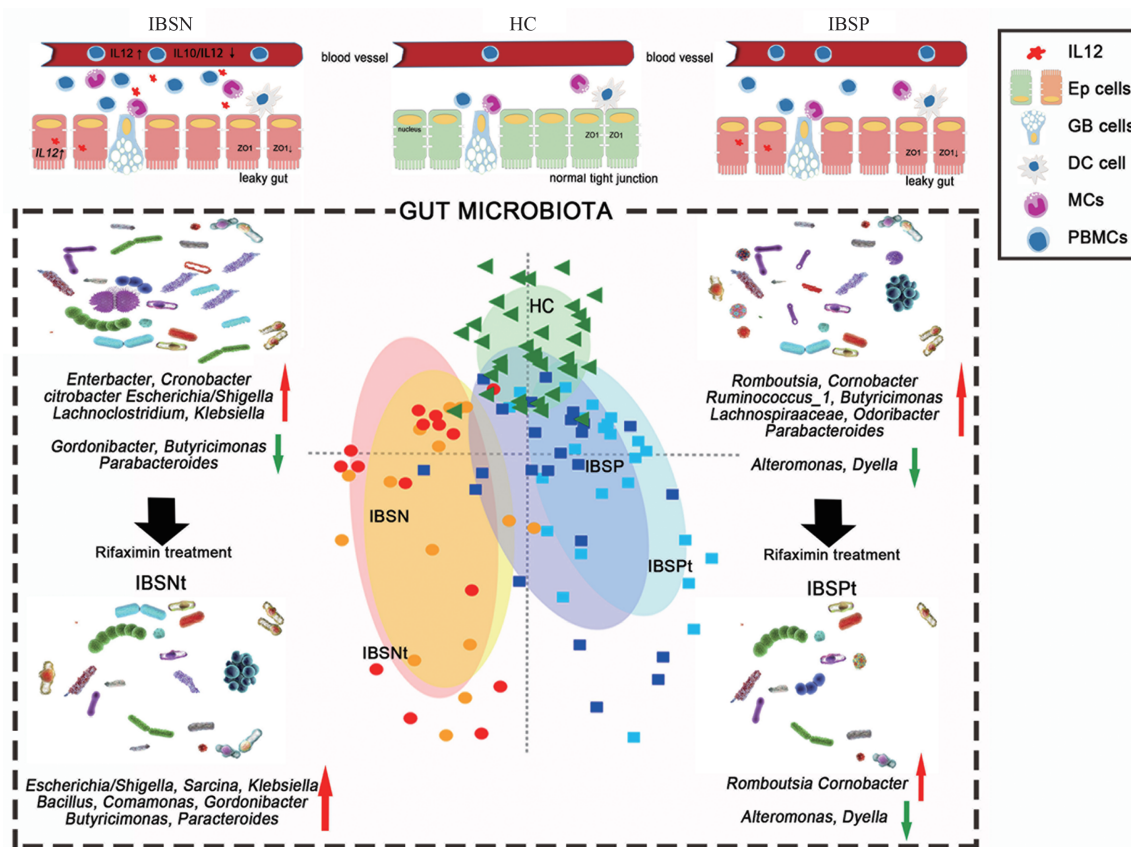
**Discussion**

With the use of BT, SIBO has been increasingly detected in patients with IBS. Patients with SIBO and some IBS patients with BT positive respond well to rifaximin, but in some cases those with IBS alone respond modestly.<sup>[11,22,23]</sup> The underlying reasons are not clear. In this study, we identified that the inflammation features and gut microbiota are different between BT-positive and BT-negative IBS-D (IBSP and IBSN). Fecal microbial variation might contribute to the different therapeutic response to rifaximin in these two groups [Figure 5].

It is challenging to distinguish whether IBS-D patients are BT positive or negative according to clinical symptoms (IBS-SSS and GSRs), or even using the visceral hypersensitivity status. These two groups were similar with regard most clinical features. However, we identified that systemic and colon inflammation were significantly activated in the BT-negative group presenting with a significant increase for IL12 level in PBMC and colon mucosa. The host gut microbiota contributes to the etiology and symptomology of IBS,<sup>[24,25]</sup> which are associated with increased epithelial permeability and aberrations in immunity. In our study, the microbiota of BT-negative patients presented a higher proportion of Proteobacteria, a phylum consisting of many pathogenic

bacteria. Proteobacteria enriching in the gut can represent an imbalanced and unstable microbial community structure or a state of disease of the host, such as obesity, inflammation, and cancer.<sup>[26]</sup> Specifically, enriched abundance of Enterobacteriaceae (*Enterobacter*, *Citrobacter*, *Escherichia-Shigella*, *Kluyvera*), Enterococcaceae, and Lachnospiraceae, most of which are gram-negative, were observed in IBSN group. The Enterobacteriaceae family is well known as enteric pathogens<sup>[27,28]</sup> and Lachnospiraceae are opportunistic pathogens<sup>[29]</sup> of the gut. The enrichment in these floras might lead to bacterial invasion of epithelial cells. The enriched genera in BT-negative patients, especially those belonging to the Enterobacteriaceae family (*Enterobacter*, *Cronobacter*, *Citrobacter*, *Escherichia-Shigella*), were significantly positively correlated with IL12 level (in serum or in PBMC supernatant). *Sutterella* was positively correlated with IL10/IL12 ratio in the PBMC supernatant. These bacteria are able to activate the immune system.<sup>[30]</sup> The systemic and local inflammatory processes induced by these bacteria can be activated by different pathways.<sup>[31-33]</sup> For example, gram-negative bacteria and their bacterial components, especially LPS, induce inflammation responses depending on the release of proinflammatory cytokines from monocytes and macrophages, while other bacteria become aggressive in the crypts, and then are recognized and engulfed by macrophages and dendritic cells or by transcytosis through M cells and active Peyer’s patches. These processes contribute a low-degree inflammation status to BT-negative patients. Interestingly, the level of IL12 was increased especially in the colon tissue, which demonstrates that the main local inflammation occurred in the colon rather than small intestine.

Some studies<sup>[21,34-36]</sup> suggest that SIBO may aggravate the inflammation status in patients with obesity, Crohn’s disease, cirrhosis, or even Parkinson’s disease. Interestingly, in this study, for IBS-D patients in BT-positive group, their ileum rather than systemic immunity is activated. Our data show that the IL12 level in the ileum was significantly raised in the BT-positive group. For their



**Figure 5:** The probable mechanism of different rifaximin therapeutic response in IBS-D overlapping with and without SIBO. HC: healthy controls; IBS: irritable bowel syndrome; IBSP: breath test positive IBS patients; IBSN: breath test negative IBS patients; SIBO: small intestinal bacterial overgrowth; IBSPt and IBSNt: Patients in IBSP and IBSN group after rifaximin treatment; Ep cells: Epithelium cells; GB cells: Gallbelt cells; DCs: Dendritic cells; MCs: Mast cells; PBMCs: Peripheral blood mononuclear cells.

microbial changes, patients had a higher abundance of *Blautia*, *Prevotella\_2*, *Lachnospiraceae\_NC2004* group, *Cronobacter*, and *Romboutsia*, and lower abundance of *Mitsuokella*. This result is consistent with the findings of Wu *et al.*<sup>[6]</sup> They found a remarkable increase in *Prevotella* in patients with IBS-D, especially those who were BT-positive. Moreover, we found that the microbial changes may influence LPS biosynthesis and geraniol degradation as per PICRUSt prediction. The increased abundance of *Blautia*, *Prevotella\_2*, and *Cloacibacillus* were positively correlated with IL12 level in the ileum.

In clinical work, it is of great significance to distinguish rifaximin responders from patients with IBS for precision medicine. Two clinical trials<sup>[8,37]</sup> have shown that rifaximin had a better therapeutic effect on patients with IBS and SIBO in adults and children; however, symptoms of some IBS patients<sup>[38]</sup> relapse after rifaximin treatment. As an oral GI-targeted antibiotic, rifaximin treatment decreases the total bacterial population in gut. Microbial dysbiosis seems to be the key factor in the efficacy of rifaximin. Our results are consistent with the report that BT-positive patients respond better to rifaximin treatment.<sup>[11,22,23]</sup> It is shown that in BT-positive group *Cronobacter* decreased significantly, while *Butyricimonas* increased after treatment. *Cronobacter*<sup>[39]</sup> is associated with outbreaks of life-threatening infections in neonates, which can invade human intestinal cells, replicate in

macrophages, and invade the blood–brain barrier. *In-vitro* studies<sup>[40,41]</sup> have shown that *Cronobacter* attachment to and invasion of mammalian intestinal cells, macrophage survival, and serum resistance are comparable with those of *Enterobacter cloacae* and *Citrobacter freundii* but are less than those of *Salmonella typhimurium*. *Butyricimonas* is a typical butyrate-producing bacterium.<sup>[42]</sup> Additional sodium butyrate intake through microencapsulation was reported to reduce the frequency of abdominal pain in patients with IBS, which confirms the protective effect of butyrate on IBS symptoms.<sup>[43]</sup> *Butyricimonas* might have a specific effect in GI diseases and this effect was reduced immediately after oral probiotic intake. We considered that rifaximin therapy was useful to BT-positive IBS-D patients by reducing the microbial community, suppression of harmful bacteria such as *Cronobacter*, and regulating the microflora for maintenance of the microecological equilibrium in the gut.

After 4 weeks of rifaximin therapy, enriched genera such as *Enterobacter* and *Enterococcus* in BT-negative IBS-D patients did not decrease significantly, and other IBSN-enriched microbes such as *Flavonifractor* even increased after therapy. As mentioned above, these bacteria are enteric pathogens that lead to intestinal and systemic inflammation and VH. This might be the reason for negligible recovery of symptoms in BT-negative group. We strictly excluded those participants who had a history of

GI infection over the past 3 years. However, there is a lack of studies that explore the cause of increase of bacteria belonging to the antimicrobial spectrum of rifaximin after therapy. We hypothesize that antibiotic resistance may have developed in those bacteria, that rifaximin is more efficacious against antagonistic bacteria, or that longer therapeutic time is needed.

There are some limitations that need to be addressed in future studies. First, more patients must be studied to verify the microbial results after therapy. Second, the inflammation status was represented by IL10 and IL12 in this study, but more inflammatory factors or inflammatory cells must be evaluated. Finally, long-term rifaximin therapy (>12 weeks) or diet management must be included in the evaluation, so that the changes in the microbiota can be tracked over time.

In summary, IBS-D patients who were BT-positive presented a different gut microbial composition compared with BT-negative patients. The former also responded better to rifaximin therapy. Thus, it is necessary to use BT to exclude SIBO before IBS diagnosis for precision medicine. Some patients with IBS-D may have responded poorly to rifaximin owing to the absence of obvious changes of Enterobacteriaceae after therapy. However, the detailed mechanism requires to be further analyzed.

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### Conflicts of interest

None.

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