Characteristics of Gut Microbiota in Diarrhea-predominant Irritable Bowel Syndrome of Han Nationality in Southwest China

Chengjiao Yao
Chengdu University of Traditional Chinese Medicine Affiliated Hospital
Yilin Li
Chengdu University of Traditional Chinese Medicine Affiliated Hospital
Lihong Luo
Chengdu University of Traditional Chinese Medicine Affiliated Hospital
Fengjiao Xie
Chengdu University of Traditional Chinese Medicine Affiliated Hospital
Qin Xiong
Chengdu University of Traditional Chinese Medicine Affiliated Hospital
Tinglin Li
Chengdu University of Traditional Chinese Medicine Affiliated Hospital
Chunrong Yang
Chengdu University of Traditional Chinese Medicine Affiliated Hospital
Peimin Feng (✉ 14807219@qq.com)
Chengdu University of Traditional Chinese Medicine Affiliated Hospital https://orcid.org/0000-0002-7716-368X

Research

Keywords: Gut microbiota, Functional prediction, Environmental factors

Posted Date: September 9th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-871688/v1

Abstract

Backgroud To study of gut microbiota in IBS-D of Han nationality in Southwest China, explore its relationship with environmental factors and differential expression function of gut microbiota in patients.

Methods 120 cases of IBS-D group and 63 cases of HCs group were recruited, baseline data such as age, height, weight, monthly income, work intensity, eating habits and exercise were collected. HAMA and HAMD scores were performed on the two groups. IBS-D group was scored with IBS-SSS and IBS-QOL, and the differences in gastrointestinal symptoms, mental and psychological status and quality of life were compared. The laboratory tests were performed such as blood routine and liver function. Fresh feces of the two groups were collected for 16S rDNA sequencing. Then analyze the differences of gut microbiota between the two groups, and look for typical biomarkers of each. According to the dominant species and differentially expressed species at different classification levels, FAPROTAX was used to predict the functional differences of gut microbiota between the two groups. Taking the above scores and laboratory tests as environmental factors, spearman correlation analysis was conducted at the phylum level to explore the impact of environmental factors on the occurrence and development of gut microbiota and IBS-D.

Results IBS-D patients are mostly concentrated in low-medium income people with medium-high labor intensity. The sleep time, exercise time, intake of vegetables and fruits of IBS-D are significantly lower than those of HCs, and the proportion of IBS-D who like to eat pepper is significantly higher than those of HCs. The scores of HAMA and HAMD, Urea nitrogen, AST, ALT , GGT in IBS-D were significantly higher than those in HCs.The richness of gut microbiota in IBS-D was significantly lower than that in HCs, but not diversity and eveness. The beta diversity index of gut microbiota community was significantly different between the two groups. The biomarkers of IBS-D were Prevotella, Clostridiales and Roseburia, the biomarkers of HCs were Veillonellaceae, Bacteroides-coprocola, Bifidobacteriales, Bifidobacteriumde, etc. The functions of gut microbiota in IBS-D were significantly up-regulated in cellulolysi, xylanolysis, and significantly down-regulated in fermentation, methanogenesis, nitrite_ammonification, nitrate_reduction. Correlation analysis showed that multiple intestinal microorganisms were closely related to HAMA, IBS-SSS, IBS-QOL, inflammatory indexes and liver enzymes.

Conclusion There are significant differences in richness of gut microbiota, flora structure and flora function between IBS-D and HCs in Southwest China. These differences may be closely related to environmental factors such as eating habits, living habits and mental and psychological factors.

The trial was registered and approved in China Clinical Trial Registry (Registration No.ChiCTR2100045751)

1. Introduction

Irritable bowel syndrome (IBS) is a chronic functional bowel disease characterized as recurrent abdominal pain or discomfort with altered bowel habits[1]. As a heterogeneous disease, the etiology,

clinical manifestations and pathogenesis of IBS show great heterogeneity in different regions of population. According to Rome IV diagnostic criteria, patients with IBS were divided into four types: IBS with diarrhea (IBS-D), IBS with constipation (IBS-C), IBS with constipation/diarrhea (IBS-M), and IBS unclassifiable (IBS-U)[2]. Epidemiological investigation have demonstrated that the incidence rate of IBS is 10–15 percent worldwide[3]. The incidence rate and the proportion of IBS-D varies from district to distric greatly. IBS is most prevalent in South America at approximately 21 percent[3]. The incidence rate of IBS in USA is about 7percent, of which IBS-D is 52.6percent[4]. The incidence rate of IBS in Africa is about 31.6percent, but IBS-D accounts for only 13percent[5]. The incidence rate of IBS in Korea is about 6.6percent, and IBS-D is 32.9percent[6]. The incidence rate of IBS in Japan is about 6.1percent, and IBS-D is about 31.3percent[7]. The prevalence of irritable bowel syndrome in China is 4.6 ~ 6.8percent, of which IBS-D accounts for about 40 ~ 60percent [8]. There are also differences in the proportion of IBS-D in different regions of China, 27percent in Hong Kong[9], 49.3percent in Qingdao[10], 48.3percentin Zhejiang Province, 26.1percent in Shanxi Province, and 39.9percent in Jiangxi Province[11].

As a heterogeneous disease, almost no single pathogenesis can fully explain all the symptoms of IBS. The current research shows that the imbalance of gut microbiota, visceral hypersensitivity, abnormal gastrointestinal motility, dysfunction of brain-intestinal axis, dysfunction of intestinal mucosal barrier, mental and psychological factors are closely related to the development of IBS[12]. With 16S rDNA amplicon sequencing rapidly progressed, multiple studies have found that the gut microbiota of IBS is significantly different from that of healthy controls[13]. A relatively common observation is that the number and activity of intestinal dominant bacteria in fecal samples from IBS is lower than in those from healthy control subjects, as well as the intestinal microbial diversity and the intestinal microecological stability[14.15] .On the contrary, the ratio of Firmicutes,Bacteroidetes and the number of potential intestinal pathogenic bacteria ,such as Escherichia coli, Streptococcus, Rumen cocci, Clostridium increased significantly[16.17]. Fecal microbiota transplantation(FMT)[18], probiotics[19] and antibiotic[20] therapy can effectively improve the symptoms of IBS patients, which also demonstrate the important role of gut microbiota in the pathogenesis of IBS. However, there still were some studies which found no significant difference in gut microbiota from IBS[21] and FMT was ineffective in the treatment of IBS[22].

Considering the regional differences of clinical symptoms, incidence rate and curative effect, we infer that the role of gut microbiota in pathogenesis is also different. Therefore, we selected IBS-D which was the largest proportion in this region as the research object to study the characteristics of gut microbiota in IBS-D of Han nationality in Southwest China using 16S rDNA amplicon sequencing. Through this experiment, we aim to answer the following three questions: Is there any difference in the gut microbiota between IBS-D and healthy people? Which functions of gut microbiota may affect the occurrence and development of IBS-D? Is there any correlation between different gut microbiota and environmental factors?

2. Materials And Methods

2.1 Participants

In this study, 120 patients of IBS-D and 63 healthy control (HCs) aged from 18 to 65 were recruited in the same period. Patients with IBS-D came from outpatients and inpatients in the Department of gastroenterology of Hospital of Chengdu University of Traditional Chinese Medicine from June to August 2021. The HCs were from the physical examination center of Hospital of Chengdu University of Traditional Chinese Medicine. The trial was approved by the ethics committee of Hospital of Chengdu University of Traditional Chinese Medicine (Approval No.2019KL-024). The trial was registered and approved in China Clinical Trial Registry (Registration No. ChiCTR2100045751), and all participants signed informed consent.

2.2 Diagnostic criteria

All patients with IBS-D meet the Rome IV criteria:1) abdominal pain occurred repeatedly in the last 3 months, with an average of at least 1 day per week; 2) meet 2 or more of the following symptoms (related to defecation, with changes in defecation frequency and fecal properties);3) at least 6 months before diagnosis; 4) stool traits Bristol 6–7 at least 25%, Bristol 1–2 less than 25%.

2.3 Inclusion criteria

IBS-D group: 1) age from 18 to 65 years; 2) meet the Rome IV criteria of IBS-D; 3)sign informed consent. HCs group: 1) age from 18 to 65 years; 2) blood routine, urine routine, stool routine, liver and kidney function, abdominal ultrasound, colonoscopy and ECG were normal; 3□sign informed consent.

2.4 Exclusion criteria

The IBS-D and the HCs should exclude IBS-C□IBS-U□IBS-M and other digestive system diseases, should exclude infectious diarrhea, inflammatory bowel disease, colorectal cancer and diarrhea caused by systemic diseases, should exclude diabetes, hyperthyroidism, and severe primary diseases such as heart, brain, liver, kidney and hematopoietic system. Patients who had taken immunosuppressants, anticholinergic drugs, antidiarrheal agents, acid inhibitors, driving agents, antidepressants, anxiolytics, antibiotics and intestinal flora regulating drugs within 3 months were excluded.

2.5 Clinical data collection

The baseline data of gender, age, nationality, marital status, personal monthly income, height, weight, BMI, exercise, allergy history, previous treatment history and complications of IBS-D and HCs groups were collected. The fecal properties of the two groups were recorded according to Bristol classification. The timetable, dietary tastes, intake of vegetables, fruits, meat, coffee, tea, smoking and drinking of the two groups were collected.

After detailed explanation to the patients by gastroenterologists, the patients were scored with IBS symptom severity scale (IBS-SSS) and IBS quality of life (IBS-QOL). IBS-SSS is a table for clinical used to measure the extend of abdominal pain, number of days having pain, extend of abdominal distension,

satisfaction of bowel habits and affect or interfere with life[23]. The score range of each item is 0-100, with a total score of 500. The higher the score, the more serious the condition is. Scores < 175 represent mild IBS, 175–300 represent moderate IBS, and > 300 represent severe IBS[24]. IBS-QOL is an IBS patient specific scale compiled by Patrick which from literature review and direct interview with IBS patients[25]. It is divided into 34 questions, which are respectively aimed at dysphoria, body image, interference with activity, food avoidance, health worry, sexual dysfunction, social reaction, and relationships[26]. Each problem is divided into five grades: asymptomatic, mild, moderate, heavy and serious, with 5, 4, 3, 2 and 1 points respectively. The score of each aspect is converted by formula to make its value within the range of 0-100. Theoretically, the higher the score, the better the quality of life. Conversion score = (Original Score - lowest possible score) / possible score range* 100. The items to which each dimension belongs and the possible score range of the initial score are shown in the Table 1.

Table 1
The subitem of IBS-QOL

IBS-QOL	Item	Lowest/highest possible score	Possible score range
dysphoria	1,6,7,9,10,13,16,30	8,40	32
body image	5,21,25,26	4,20	16
interference with activity	3,18,19,22,27,29,31	7,35	28
food avoidance	11,23,28	3,15	12
health worry	4,15,32	3,15	12
sexual dysfunction	12,20	2,10	8
social reaction	2,14,17,34	4,20	16
family relationships	8,24,33	3,15	12

The patients were scored with Hamilton Anxiety Scale (HAMA) and Hamilton Depression Scale (HAMD) by strictly trained psychiatrists. HAMA includes 14 items such as anxiety, tension, fear, insomnia, cognitive function and somatization symptoms. The higher the score, the more serious the anxiety[27]. HAMD includes 24 items such as depression, guilt, suicide, difficulty falling asleep, early awakening, hypochondriasis, weight loss and insight[28]. The higher the score, the heavier the degree of depression.

2. 6 Routine laboratory index determination

Collected 4 ml of fasting peripheral venous blood (heparin anticoagulation) and 4 ml of peripheral venous blood (EDTA anticoagulation). 13 biochemical indexes such as alanine aminotransferase(ALT), aspartate aminotransferase(AST), alkaline phosphatase(ALP), serum albumin, uric acid (UA), creatinine (Crea), triglyceride (TG), cholesterol (TC) were detected by automatic biochemical instrument. Blood cell analyzer was used to detect routine blood indexes such as leukocytes, red blood cells and platelets.

Collect 3ml of clean middle urine of all subjects, and use urinary sediment analyzer to detect routine urine.

2. 7 Fecal collection and fecal bacterial genomic DNA extraction

Use a special spoon in the fecal collector to take samples from at least 3 parts in the depth of fresh feces, and the appropriate amount of samples is 3-5g. Avoid mixing urine, water, plants, soil, sewage and other components into fecal samples. The fecal samples shall be frozen in liquid nitrogen for 4 hours within 3 minutes after sampling, and then transferred to - 80 ℃ refrigerator for storage.The bacterial DNA of fecal samples was extracted by cetyltrimethyl ammonium bromide(CTAB)[29]. The purity and concentration of DNA were detected by agarose gel electrophoresis. The qualified DNA was placed in centrifuge tube and diluted with aseptic water to 1ng/ul.

2.8 16S rDNA sequencing

The variable region of 16S rDNA V4 was selected, and specific primers with barcode (515f GTGYCAGCMGCCGCGGTAA 806R GGACTACNVGGGTWTCTAA), phusion ® High fidelity PCR master Mix with GC Buffer of New England Biolabs, high fidelity enzyme were used for PCR amplification. PCR products were detected by electrophoresis with agarose gel of 2% concentration. The qualified PCR products were purified by enzyme beads. The samples were mixed according to the concentration of PCR products. The PCR products were detected by 2% agarose gel electrophoresis. Construction of library used Truseq® DNA PCR Free Sample Preparation Kit. After the constructed library was quantified by Qubit and Q-PCR, Illumina novaseq6000 was used for computer sequencing.

2.9 Bioinformatics analysis

2.9.1 Sequencing data processing

Raw Tags are obtained by splicing the original data with FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/)[30], and high-quality Clean Tags are obtained after strict filtering[31]. According to the tags quality control process of Qiime (V1.9.1 http://qiime.org/scripts/split_libraries_fastq.html)[32], Tag interception and tag length filtering are carried out in turn. After removed the chimera,the Effective Tags were obtained.

2.9.2 OTU clustering and species annotation

Uparse algorithm is used to cluster all effective tags, and the sequences with 97% consistency are clustered into OTUs[33]. The species annotation analysis (with a threshold of 0.8-1) was carried out by the Mathur and the SSUrRNA database of SILVA138(http://www.arb-silva.de/)[34,35]. The taxonomic information was obtained, and the community composition of each sample was counted at the level of Kingdom, phylum, class, order, family, genus and species.

2.9.3 Sample complexity and multi sample analysis

Qiime software (version 1.9.1) was used to calculate the flora abundance index and diversity index, including Observed OTUs, Chao1, Shannon, Simpson, ACE, Goods coverage and PD_whole_ For tree. R software was used to draw Dilution curve and Rank variance curve, and also to analyze the difference between groups of alpha diversity index. Qiime software was used to calculate UniFrac distance. R software was used to draw PCoA and NMDS diagrams, and to analyze the differences between beta diversity index groups.

2.9.4 Analysis of species differences between groups

The MetaStat method was used to test the hypothesis of species abundance data between IBS-D and HCs, and the *p* value was obtained. Through the correction of *P* value, the *q*-value was obtained. Finally, the species with significant differences were screened according to the *q*-value, and the abundance distribution box map of different species among groups was drawn. LDA Effect Size (LEfSe) analysis were used to find biomarkers with statistical differences between IBS-D and HCs. LEfSe software was used for LEfSe analysis, and the screening value of LDA score was 3.5. The significance of species difference between IBS-D and HCs was analyzed at levels of phylum, class, order, family, genus and species. The contribution of species to the difference was quantified by Simper analysis.

2.9.5 Function prediction and environmental factor correlation analysis

FAPROTAX was used to predict the functional differences of gut microbiota between the two groups. Taking the above scores and laboratory tests as environmental factors, spearman correlation analysis was conducted at the phylum level to explore the impact of environmental factors on the occurrence and development of gut microbiota and IBS-D.

2.9.6 Statistical analysis

Spss 22.0 software was used for statistical analysis. The measurement data were expressed ± S. T-test was used for conforming to normal distribution, Nonparametric test was used for non conforming to normal distribution, Chi-square test was used for counting data, and Rank-sum test was used for grade data. $P < 0.05$ was regarded as statistical significance.

3. Results

3.1. Comparison of baseline data between IBS-D and HCs groups

- A total of 120 cases of IS-D and 63 cases of HCs were recruited. The baseline data of gender, age, marital status, income, work intensity, height, weight and dietary preference of all subjects are shown in Table 2. We noticed that there was a large difference in personal monthly income between the two groups. Among them, the personal monthly income of HCs is mostly concentrated at the level of 5000–10000 RMB per month, accounting for 58.7% of the HCs, which is much higher than 37.5% of

IBS-D. The proportion of low-income people in HCs group is 4.8%, which is far lower than 12.5% in IBS-D group. Similar to personal monthly income, the labor intensity of HCs was also significantly different from that of IBS-D. The labor intensity of IBS-D is mainly concentrated in medium and high intensity, accounting for 67.5% of the total, while the work intensity of HCs group is mostly concentrated in low and medium intensity, accounting for 79.3%. There was still significant difference in sleep time between the two groups. Among them, 47 patients in IBS-D group slept less than 6 hours a day, accounting for about 39.2%, which was much higher than 22.2% in HCs group. Significant difference also existed in exercise time between the two groups. Among them, 75 patients in IBS-D group take exercise less than 30min, accounting for 62.5%, which was significantly higher than that of patients in HCs, accounting for 36.5%. On the contrary, the proportion of people who exercise 30–60 minutes a day in HCs was 50.8%, which was much higher than 32.5% in IBS-D. No significant difference between the two groups was found in favourvite taste, but it can be seen from the statistical data that 69.2% of patients in IBS-D liked spicy food, which was much higher than 30.2% of patients in HCs. There were also significant differences in vegetables and fruits between the two groups. The proportion of eating vegetables less than 2.5Kg per week in IBS-D was 45.8%, which was higher than 28.6% in HCs; The proportion of eating 1.25 Kg of fruit per week in IBS-D was 78.3%, which was much higher than 36.1% in HCs group.

- Still, we found that there was no significant difference between the two groups in marital status, working hours, height, weight, BMI, staple food, oil, meat, coffee, Liquor and cigarettes.

Table 2
The baseline data of IBS-D and HCs

Baseline data	IBS-D	HCs	*P*-Value
Number	120	63	
Gender			0.880
Male	49(40.8%)	25(39.7%)	
Female	71(59.2%)	38(60.3%)	
Age (years)	41.672 ± 11.740	36.921 ± 11.830	0.936
Marital status			0.967
Unmarried	17(14.2%)	9(14.3%)	
Married	102(85%)	53(84.1%)	
Divorced	1(0.8%)	1(1.6%)	
widowed	0	0	
Personal monthly income			0.016*
Low-income(<2000RMB/Month)	15(12.5%)	3(4.8%)	
Medium income (2000-5000RMB/Month)	49(40.8%)	15(23.8%)	
High income (5000-10000RMB/Month)	45(37.5%)	37(58.7%)	
Higher income (> 10000RMB/Month)	11(9.2%)	8(12.7%)	
labour intensity			0.002**
Low	19(15.8%)	29(46%)	
Medium	49(40.8%)	21(33.3%)	
High	32(26.7%)	11(17.5%)	
Extremely High	20(16.7%)	2(3.2%)	
Working hours			0.195
< 8 hours/d	27(22.5%)	22(34.9%)	
8–12 hours/d	68(56.7%)	35(55.6%)	
> 12hours/d	25(20.8%)	6(9.5%)	
Sleep duration			0.018*
< 6 hours/d	47(39.2%)	14(22.2%)	
6–10 hours/d	65(54.1%)	45(71.4%)	

Baseline data	IBS-D	HCs	*P*-Value
> 10hours/d	8(6.7%)	4(6.4%)	
Exercise duration			0.003**
< 30min/d	75(62.5%)	23(36.5%)	
30-60min/d	39(32.5%)	32(50.8%)	
> 60min/d	6(5%)	8(12.7%)	
Height (cm)	164.706 ± 8.823	163.438 ± 6.429	0.420
Weight (Kg)	63.226 ± 11.183	60.281 ± 11.949	0.063
BMI (Kg/m^2)	23.185 ± 2.893	22.488 ± 3.823	0.086
Favourvite taste (multiple choice)			0.394
Sour	28(19.2%)	20(31.7%)	
Sweet	14(11.7%)	15(23.8%)	
Salty	1(0.8%)	3(4.8%)	
Light	21(17.5%)	21(33.3%)	
Spicy	83(69.2%)	19(30.2%)	
Bitter	0	0	
Food preferences			0.172
Cold food	2(1.7%)	1(1.6%)	
Hot food	15(12.5%)	1(1.6%)	
Normal temperature food	103(85.8%)	61(96.8%)	
Fat intake			0.735
< 20g/d	8(6.7%)	5(7.9%)	
20-30g/d	94(78.3%)	48(76.2%)	
> 30g/d	18(15%)	10(15.9%)	
Vegetable intake			0.002**
Never	0	0	
< 2.5Kg/w	55(45.8%)	18(28.6%)	
> 2.5Kg/w	65(54.2%)	45(71.4%)	
Fruit intake			0.003**

Baseline data	IBS-D	HCs	*P*-Value
Never	0	0	
< 1.25Kg/w	94(78.3%)	36(57.1%)	
> 1.25Kg/w	26(21.7%)	27(42.9%)	
Meat intake			0.151
Never	0	0	
< 50g/d	3(2.5%)	3(4.8%)	
50-100g/d	59(49.2%)	18(28.6%)	
> 100g/d	58(48.3%)	42(66.6%)	
Staple food			0.704
Rice	114(95%)	59(93.7%)	
Wheat	6(5%)	4(6.3%)	
Coffee			0.680
Never	86(71.7%)	46(73.0%)	
Occasionally	24(20%)	15(23.8%)	
1-2Cup/d	8(6.7%)	2(3.2%)	
> 2Cup/d	2(1.6%)	0	
Liquor			0.782
Never	41(34.2%)	24(38.1%)	
Occasionally	49(40.8%)	26(41.3%)	
< 15g/d	16(13.3%)	9(14.2%)	
15-30g/d	8(6.7%)	2(3.2%)	
30-60g/d	4(3.3%)	2(3.2%)	
> 60g/d	2(1.7%)	0	
Cigarettes			0.120
Never	49(40.8%)	34(54.0%)	
Occasionally	2(1.7%)	5(7.9%)	
< 10 cigarettes/d	29(24.2%)	8(12.7%)	
10–20 cigarettes/d	33(27.5%)	15(23.8%)	

Baseline data	IBS-D	HCs	*P*-Value
> 20 cigarettes/d	7(5.8%)	1(1.6%)	

3.2 Comparison of IBS-SSS and IBS-QOL in IBS-D group

- The total score and five sub-scores of IBS-SSS in IBS-D are shown in the Table 3 below. According to IBS-SSS score, there were 101 patients with moderate IBS-D, 16 patients with severe IBS-D and 3 patients with mild IBS-D. In IBS-SSS, the item of number of days having pain and affect or interfere with life were the highest. The total scores of IBS-QOL and the scores of 8 main question setting options are shown in the table below. IBS-QOL table is the score for patients' quality of life. The item scores of dysphoria and interference with activity were the highest, and the scores of food avoidance were the lowest.

Table 3
The score of IBS-SSS and IBS-QOL in IBS-D group

Baseline data	IBS-D
IBS-SSS	
Abdominal pain	41.250 ± 16.962
Number of days having pain	55.952 ± 22.670
Abdominal distension	43.541 ± 19.218
Satisfaction of bowel habits	46.608 ± 16.986
Affect or interfere with life	58.417 ± 20.270
Total score	248.770 ± 44.912
IBS-QOL	
dysphoria	73.411 ± 18.082
body image	62.321 ± 23.098
interference with activity	72.887 ± 22.476
food avoidance	60.764 ± 23.605
health worry	69.792 ± 22.456
sexual dysfunction	63.083 ± 21.125
social reaction	68.177 ± 24.496
family relationships	69.375 ± 24.016
Total score	469.565 ± 57.009

3.3 Comparison of HAMA and HAMD between IBS-D and HCs

- In HAMD, there were 9 mild depression, 5 moderate depression in IBS-D and 1 mild depression in HCs. The score of HAMD in IBS-D was significantly higher than that in HCs, the difference was statistically significant. In HAMA, there were 12 mild anxiety, 7 moderate anxiety in IBS-D and 2 mild anxiety in HCs. The score of HAMD and HAMA in IBS-D was significantly higher than that in HCs, the difference was statistically significant (Table 4).

Table 4
The score of HAMA and HAMD in IBS-D and HCs

Item	IBS-D	HCs	*P*-Value
HAMA	6.650 ± 4.524	5.853 ± 2.320	0.005*
HAMD	9.083 ± 4.857	7.016 ± 2.926	0.007*

3.4 Comparison of laboratory test indexes between IBS-D and HCs

- In blood routine indexes, monocyte count, erythrocyte count, hemoglobin and platelet count were significantly different between the two groups. In biochemical indexes, there were significant differences in Urea, AST, ALT and GGT between the two groups (Table 5).

Table 5
Comparsion the routine laboratory tests between IBS-D and HCs

Item	IBS-D	HCs	*P*-Value
Routine blood indexes			
WBC	5.449 ± 1.381	5.920 ± 1.788	0.105
NE	3.271 ± 1.063	3.574 ± 1.620	0.416
Lymph	1.736 ± 0.531	1.843 ± 0.482	0.103
Mono	0.311 ± 0.108	0.346 ± 0.127	0.021*
RBC	4.899 ± 0.462	4.639 ± 0.523	0.002*
HGB	150.4 ± 14.59	141 ± 19.21	0.001*
PLT	194 ± 58.6	217 ± 53.8	0.009*
Routine urine			
Urine PH	6.086 ± 0.585	6.238 ± 0.679	0.172
Urine occult blood	negative	negative	
Urine protein	negative	negative	
Biochemical indexes			
Urea	5.06 ± 3.82	4.230 ± 1.180	0.040*
Crea	65.265 ± 13.277	62.3836 ± 11.918	0.138
UA	316.548 ± 44.326	305.724 ± 40.278	0.269
ALB	43.257 ± 6.342	46.890 ± 4.862	0.146
AST	28.1 ± 24.4	20.9 ± 16.3	0.002*
ALT	25 ± 12	22.2 ± 7.4	0.018*
ALP	77.3 ± 21.5	71.6 ± 19.7	0.083
GGT	28.521 ± 39.403	19.967 ± 12.021	0.029*
DBil	4.844 ± 2.189	5.115 ± 2.102	0.328
IBil	11.374 ± 4.229	12.339 ± 5.238	0.494
TBil	16.322 ± 5.865	17.454 ± 7.095	0.344
TG	1.14 ± 0.51	1.06 ± 0.47	0.452
TC	3.68 ± 2.57	3.9 ± 2.4	0.473

3.5 Quality analysis of 16S rDNA sequencing

- 16S rDNA high-throughput sequencing was performed on 120 IBS-D and 63 HCs collected. Raw Tags were obtained by splicing the original data with FLASH. A total of 11699364 Raw Tags were obtained in IBS-D group, with an average of 97495 Raw Tags per sample. A total of 6396847 Raw Tags were obtained in HCs group, with an average of 101537 Raw Tags per sample. Effective Tags were obtained through tag interception, length filtering and chimera removal. The average length of effective tags of all samples was 253bp. A total of 7634994 Effective Tags were obtained in IBS-D group, with an average of 63625 Effective Tags per sample, and the effective rate of quality control was 65.25%. In the HCs group, 4092712 Effective Tags were obtained, with an average of 64964 Effective Tags per sample. The effective rate of quality control was 63.98%.

3.6 OTU clustering

- The sequence was clustered into OTUs with 97% consistency, and a total of 2931 OTUs were obtained. Venn(http://bioinformatics.psb.ugent.be/webtools/Venn/)was used to get the intersection of OTUs in IBS-D and HCs groups. The number of common OTUs between IBS-D and HCs group were 1399, and there were 1201 proper OTUs in IBS-D group and 196 proper OTUs in HCs group(Fig. 1A). Goods_Coverage is an index of sequencing depth. The higher its value, the lower the probability that the sequence in the sample is not detected. The Goods_coverage is greater than 0.99 (Fig. 1B), indicating that the sequencing depth covered almost all species in fecal samples.

3.7 Species annotation

- OTUs sequences were annotated with Silva 138 database. Among the annotation results, 2926 (99.82%) of 2931 OTUs were annotated to the kindom level, 2638 (90.00%) to the phylum level, 2579 (87.99%) to the class level, 2404 (82.02%) to the order level, 2142 (73.08%) to the family level, 1407 (48.0%) to the genus level and 314 (10.71%) to the species level. At the phylum level, the dominant flora of the two groups are Firmicutes, Bacteroidota, Proteobacteria, Actinobacteriota and Fusobacteriota in proper order. At the class level, the dominant flora of the two groups are Clostridia, Bacteroidia, Negativicutes and Gammaproteobacteria in proper order. At the order level, the dominant flora of the two groups are Bacteroidales, Lachnospirales, Oscillospirales and Veillonellales selenomonadales. At the family level, the dominant flora of the two groups are Lachnospiraceae, Bacteroidaceae and Ruminococcaceae. At the genus level, the dominant flora are Bacteroides, Faecalibacterium, Prevotella and Megamonas. The dominant species in IBS-D group were Faecalibacterium_ prausnitzii、Prevotella_copri、Bacteroides_plebeius、Escherichia_Coli in proper order, the dominant species in HCs group were Faecalibacterium_prausnitzii、Bacteroides_plebeius、 Escherichia_coli and Bacteroides_coprocola in proper order. Taking the phylum level as examples, we show the histogram of the relative abundance of the top 10 flora in the HCs and IBS-D groups(Fig. 2). According to the species annotation and abundance information of the sample, we select the top 35 flora at the genus level to cluster and draw the heat map. The Fig. 3 shows the species abundance clustering of the top 35 in HCs and IBS-D groups and samples at the genus level.

3.8 Sample complexity (Alpha diversity)

- Calculate the microbial community diversity index in the sample based on the species and abundance of OTUs, including Observed species, Chao1, Shannon, Simpson, ACE, Goods coverage and PD_whole_Tree index. Observed OTUs, Chao1 and ACE reflect the richness of microbial communities in the sample. Shannon, Simpson and PD_whole_Tree index reflects the diversity and eveness of microbial community in the sample. The smaller the Simpson value, the higher the species diversity in the sample, and the size of other indexes is directly proportional to the diversity or richness.
- It can be seen that the richness of microbial community in IBS-D group is significantly lower than that in HCs group from the Table 6. However, there was no significant difference in diversity and eveness between the two groups. Then, we draw Rarefaction curve and Rank Abundance according to the OTUs results of clustering. As can be seen from the Fig. 4 below, the Rarefaction curve gradually flattens with the increase of sequencing data, indicating that the current sequencing depth is sufficient to reflect the microbial diversity contained in the community samples, which is mutually confirmed with the above Goods-coverage results. In the Rank Abundance, it can be seen that the decline of the curve is gentle, the horizontal axis span is large, which indicating the species richness and average degree in the sample are good.

Table 6
Comparison of Alpha diversity index between IBS-D and HCs

Group	Observed-species	Chao1	ACE	Shannon	Simpson	Goods-coverage	PD_whole_tree
IBS-D	510.74 ± 74.57	754.96 ± 333.64	594.17 ± 108.67	5.61 ± 0.60	0.936 ± 0.44	0.9979 ± 0.0006	24.09 ± 4.42
HCs	520.30 ± 60.24	759.81 ± 108.65	610.07 ± 66.61	5.61 ± 0.65	0.94 ± 0.41	0.9978 ± 0.0002	24.46 ± 2.50
P-Value	0.1814	0.002947*	0.02785*	0.9917	0.8162	9.64E-06*	0.02181*

3.9 Multi sample analysis (Beta diversity)

- Multi sample analysis is to compare the similarity of microbial community diversity from different samples. Firstly, we select the matrix heat map of unweighted UniFrac distance to represent the difference coefficient between samples(Fig. 5A). The smaller the data, the smaller the diversity difference between the two samples. And then we found that there was significant difference in beta-diversity index between IBS-D and HCs group (Fig. 5B).

- Principal coordinate analysis(PCoA) is an analysis method to reduce the dimension of multidimensional data. We use the unweighted UniFrac for PCoA and show it in a two-dimensional PCoA diagram(Fig. 5C). The farther the distance between the two points in the diagram, the greater

the gap between species composition and structure. Therefore, those with high similarity tend to gather together, and those with large differences tend to be far apart. In order to avoid the influence of linear model on data analysis, we choose nonlinear model NMDS for analysis(Fig. 5D). In the NMDS diagram, the species information contained in the sample is reflected in the multi-dimensional space in the form of points. The degree of difference between different samples can be reflected by the distance between points.

- In order to measure the similarity between samples and show the species relative abundance within samples, we conducted cluster analysis on the samples based on the unweighted UniFrac, and integrated the cluster results with the species relative abundance of each sample at the phylum level. From the clustering tree of UPGMA formed by clustering, we can see that HCs group and IBS-D group show better aggregation effect(Fig. 6). Finally, we used Adonis method to test the significance of community structure difference between two groups. The results showed that R^2 statistic was greater than 0, indicating that the difference between IBS-D and HCs groups was greater than that within the group (R^2 = 0.02746, P = 0.001), and the grouping was more reasonable.

3.10 Analysis of species differences between groups

- We used MetaStat to screen different species between IBS-D and HCs groups. The results showed that at phylum level(Fig. 7). Fusobacteriota, Desulfobacterota and Euryarchaeota in IBS-D group were significantly lower than those in HCs group, while Gemmatimonadetes, Bdellovibrionota, Chloroflexi, Myxococota and Deferibacters were significantly higher than those in HCs group. In order to further analyze the statistical significance and biological correlation, we use LEfSe to find statistically significant biomarkers(Fig. 7). The results showed that the biomarkers of IBS-D included Prevotella, Clostridiales and Roseburia, and the biomarkers of HCs included Veillonellaceae, Bacteroides-coprocola, Bifidobacteria, Bifidobacterium De, etc.

- **3.11 Function prediction**
- FAPROTAX is a manually constructed database based on published literature. It corresponds the classification and metabolism of microorganisms. At present, more than 80 functional groups of more than 4600 microorganisms and more than 7600 functional annotation information are collected. In order to better understand the function of gut microbiota, we used FAPROTAX database to predict the function of sample flora. We selected the top 10 function for display. As shown in the Fig. 8, the functions of the two groups mainly involve animal_Paracites, symbols, chemoheterotrophy, mediation, etc. According to the functional annotation and abundance information of samples in the database, the top 35 functions in abundance and their abundance information in each sample are selected to draw a heat map(Fig. 9). The results showed that the function of animal_ parasites, symbionts, cellulolysis, aliphatic_non_methane_hydrocarbon_Degradation and xylanolysis were significantly up-regulated, in induction and nitrite_ammonification, nitrate_reduction, aromatic_compound_Degradation and methanogenesis decreased significantly in IBS-D group.

- **3.12 Environmental factor correlation analysis**
- In order to explore the impact of environmental factors on intestinal flora species and clinical manifestations, we introduced the environmental factors and species abundance at the phylum level for Spearman correlation analysis, and displayed it in the form of correlation heat map. Spearman correlation analysis between routine laboratory indexes and species abundance at phylum level (Fig. 10A) showed that Bdellovibrionota was significantly negatively correlated with leukocyte count, neutrophil count and monocyte count, Cyanobacteria was negatively correlated with platelets, significantly correlated with ALT and AST, and Euryarchaeota was negatively correlated with Urea, Actinobacteriota was negatively correlated with neutrophil count, hemoglobin and Urea, Fusobacteriota was negatively correlated with AST and conjugated bilirubin, Proteobacteria was negatively correlated with urinary PH, but negatively correlated with monocyte count and alkaline phosphatase, Bacteroidota was negatively correlated with GGT. Spearman correlation analysis between HAMD \HAMA and species abundance at phylum level showed that Verrucomicrobiota was negatively correlated with HAMD (Fig. 10B).Spearman correlation analysis between IBS-SSS\IBS-QOL and species abundance at phylum level (Fig. 10C) shows that the subitem of IBS-SSS:extend of dominant pain was positively correlated with Fusobacteriota; The total score of IBS-SSS was positively correlated with Synergistata and negatively correlated with Chloroflex. Dysphoria and body image in IBS-QOL scale were negatively correlated with Euryarchaeota; Interaction with activity was negatively correlated with Cyanobacteria. Food avoidance was negatively correlated with Chloroflex; Sexual dysfunction was positively correlated with Chloroflexi and negatively correlated with Desulfobacterota.

- A:extend of abdominal pain, B:number of days having pain, C:extend of abdominal distension, D:satisfaction of bowel habits E: affect or interfere with life F:IBS-SSS;G:dysphoria□H:body image□I:interference with activity□J:food avoidance□K:health worry□L:sexual dysfunction□M:social reaction□N:family relationships

4. Discussion

At present, many studies on the pathogenesis of IBS-D have emerged, the focus of them is mostly on the pathogenesis of gut microbiota. Multiple studies suggest that gut microbiota plays an important role in the occurrence and development of IBS-D. However, there are still great heterogeneity in the research results of gut microbiota and IBS-D all over the world. The study found that the abundance of Firmicutes, Clostridium and Actinomycetes in patients with IBS-D in Nanchang, China decreased significantly, and Proteus increased significantly[36]. A meta-analysis which include 13 related literatures found that the abundance of Lactobacillus and Bifidobacterium decreased significantly in patients with IBS-D[37]. But a study from the United States believes that fecal and mucosal microbiota of IBS-D patients and HCs are very similar and their difference are not sufficient to explain the altered physiology and symptomatology of IBS-D[38]. Another study from Southeast Asia Thailand also found that there was no significant difference in intestinal flora between IBS-D patients and HCs[21]. Therefore, it is necessary to strengthen

the research on the pathogenesis of IBS-D in different regions, climate, nationality, race, diet, belief and other factors. At present, there are few research data on IBS-D gut microbiota in China, especially in Southwest China. Southwest China has unique topographic and climatic characteristics, mainly basin and hilly terrain, and the climate is relatively humid, which has created a special eating habit of the people living in Southwest China for a long time. The staple food of people in Southwest China is rice, and they like hot pepper and sichuan pepper very much. Animal experiments showed that intragastric administration of capsaicin could significantly increase the abundance of Faecalibacterium in mouse feces and lead to weight gain[39]. Another experiment found that the content of Akkermansia in the intestine of mice fed with capsaicin decreased, and their body weight increased[40]. Doubanjiang fermented with pepper as the main component is an important seasoning material in the diet of people in Southwest China. It is found that the core microorganisms of traditional doubanjiang in Southwest China include Proteus, Firmicutes and Cyanobacteria. Long term eating is bound to affect the gut microbiota structure of one resident[41]. So it is necessary to study the gut microbiota in patients with IBS-D from Southwest China. Through this study, we can now answer the three questions raised above.

Significant differences in gut microbiota between IBS-D and HCs in Southwest China

Our study found that the richness of gut microbiota in IBS-D was significantly lower than that in healthy people, but there was no significant difference in diversity and eveness between two groups. There was significant difference in beta-diversity index between two groups. At the phylum level, Fusobacteriota, Desulfobacterota and Euryarchaeota in IBS-D were significantly lower than those in HCs, while Gemmatimonadetes, Bdellovibrionota, Chloroflexi, Myxococcota and Deferribactes were significantly higher than those in HCs. The biomarkers of IBS-D include Prevotella, Clostridiales and Roseburia. Prevotella is an opportunistic pathogen that often inhabits the digestive tract and female reproductive tract. At present, several research results from Shandong China[42], Chongqing China[43], Sweden[44], the United States[45] and Australia[46] all unanimously suggest that the abundance of Prevotella in IBS-D increases significantly. When studying gut microbiota, anxiety and depression, Simpson found that Prevotella not only increased significantly in IBS-D, but also was associated with anxiety and depression comorbidity[46]. Liu's results are similar to Simpson,Prevotella's expression in IBS-D is increased, and the gut microbiota structure is similar to that of patients with depression[42]. When screening gut microbiota associated with the severity of IBS-D,Tap found IBS symptom severity was associated negatively with Prevotella[47]. Wu[43] found that Prevotella increased significantly in IBS-D, especially in IBS patients with intestinal bacterial overgrowth, and its abundance was positively correlated with the severity of IBS-SSS, which was similar with Tap's results. Animal experiments also confirmed that Prevotella abundance was closely related to visceral hypersensitivity in IBS-D mice[48]. Studies from Sweden found that Prevotella abundance was positively correlated with abdominal pain[44].

Fusobacteriota is a common gram-negative bacterium in the digestive tract. In previous studies, the abundance of intestinal Fusobacteriota in IBS-D patients in Nanchang, China was lower than that in healthy controls[36]. A meta-analysis including 24 studies also support our results[49]. But two other

studies from Zhejiang China[50] and Los Angeles[51], yielded the opposite results. The results from Los Angeles also showed that Clostridiales, one of the IBS-D biomarkers identified by us, increased significantly in their data of IBS-D[51]. Another study from Hong Kong found that Clostridiales could promote the secretion of excessive bile acids in IBS-D patients, which is one of the important pathogenesis of IBS-D[52]. A study of Clostridiales and mental illness found that depletion of Clostridiales probably mediated different psychiatric diseases by dysfunction of intestinal amino acid metabolism and SCFA production[53]. Our baseline data do show that the prevalence of anxiety and depression in IBS-D patients is much higher than that in healthy people.

Roseburia is also the biomarker of IBS-D we identified. A study found that Roseburia was negatively correlated with the severity of IBS in Shandong[42]. In Italy, Roseburia abundance was significantly reduced in IBS-D patients[54]. Wang et al. found that Roseburia was positively correlated with the expression of melatonin in colonic mucosa, and Melatonin plays a beneficial role in gut motility and immunity[55].

Through species annotation and statistical analysis, we believe that the gut microbiota of IBS-D in Southwest China is significantly different from that of healthy people. Our experimental results have some of the same trends as other studies around the world, and some of the opposite results. Whether this difference is caused by region, race, diet and so on is worthy of further study. In conclusion, these represents the characteristics of gut microbiota of IBS-D in Southwest China.

Significant differences in the prediction of gut microbiota function between IBS-D and HCs in Southwest China

- We predicted that the function of animal_ parasites□symbionts□cellulolysis□ aliphatic_non_methane_hydrocarbon_Degradation and xylanolysis were significantly up-regulated, but induction and nitrite_ammonification□nitrate_reduction□aromatic_compound_Degradation and methanogenesis decreased significantly in IBS-D group. In the past, more attention was paid to the biological function of methane in intestinal tumors and IBS-C. Studies believed that excessive methane could produce a large number of cholate derivatives and caused cancer with the enhancement of anaerobic function in the colon. On the other hand, excessive methane can inhibit intestinal peristalsis and increase the risk of constipation, so the methane production function of gut microbiota in IBS-C is too strong[56]. Combined with our functional prediction results, we speculate that the methane production function of gut microbiota in IBS-D is reduced, and then the intestinal peristalsis is too strong, resulting in typical symptoms such as abdominal pain, diarrhea and irregular stool. We also noticed that the functions of cellulose decomposition and xylan decomposition were strengthened, while the fermentation function was weakened. This trend is the embodiment of FODMAP diet therapy widely recommended to IBS patients. Low FODMAP diet recommend that reducing the intake of oligosaccharides, disaccharides, monosaccharides and polyols, reducing food fermentation reaction and hydrogen, methane, carbon dioxide and other gases produced by fermentation, and avoiding inducing or aggravating intestinal symptoms of IBS

patients[57]. Residents in Southwest China like hot pepper, bean sprouts, green vegetables, Chinese cabbage, cucumber, tomato, rice, which belong to the food recommended by FODMAPs. Interestingly, we also found in the baseline data that the proportion of IBS-D patients who like to eat pepper is higher than that of healthy people, but the consumption of vegetables and fruits in IBS-D is far lower than that of healthy people. We speculate that the long-term low FODMAPS for IBS-D patients in Southwest China leads to the gut microbiota function tend to cellulose decomposition and xylan decomposition, while the fermentation function is weakened; On the other hand, the fermentation function is too weakened, resulting in the reduction of methane in fermentation products, and then the typical symptoms of IBS-D such as abdominal pain, diarrhea and irregular stool. Combined with the statistical results of baseline data, we recommend IBS-D patients to increase the consumption of vegetables and fruits with low FODMAP.

Effect of environmental factors on gut microbiota and IBS-D in Southwest China

The species and quantity of gut microbiota are extremely rich. It can be regarded as a diversified ecosystem with the functions of reproduction, regeneration and balance. This huge ecosystem maintains a complex and precise symbiotic relationship with various organs, tissues and intestinal contents of the human body[58]. On the one hand, the intestinal flora is affected by factors such as heredity, age, diet, sleep, exercise, emotion and disease. On the other hand, it can participate in the occurrence and development of IBS, inflammatory bowel disease, tumor, depression, Parkinson's disease, rheumatoid arthritis, schizophrenia, metabolic syndrome and other diseases[59]. We collected the baseline data and living habits of the two groups. The results show that there are significant differences in monthly income and work intensity between IBS-D and HCs. IBS-D tends to occur in low-income people with medium and high labor intensity. We also sorted out the subjects' dietary preferences. The proportion of IBS-D who like to eat pepper is much higher than that of healthy controls, and those who like to eat sour taste are much lower than that of healthy controls. Sour food in Southwest China mostly comes from vinegar added to food. Vinegar is an acidic flavoring agent made from grain by adding a variety of microorganisms and enzymes through a complex fermentation process[60]. Microorganisms in vinegar mainly include mold, yeast, bacillus, lactic acid bacteria and acetic acid bacteria[61]. Among them, yeast, bacillus and lactic acid bacteria are probiotics, which can maintain human health through immune regulation[62], antioxidation[63] and inhibition of pathogenic bacteria[64]. Pepper also affects the structure of gut microbiota to a great extent. Animal experiments showed that the relative abundance of lachnospiraceae and ruminococcaceae in the intestine of mice fed with capsaicin increased significantly. Capsaicin can inhibit pathogenic bacteria such as Bacillus cereus, Bacillus subtilis, Clostridium sporogenes and Clostridium tetani[65], but increase the relative abundance of Prevotella, Lactobacillus and akkermansia in rat intestine[66]. The amount of vegetables and fruits consumed by IBS-D was much lower than that of healthy controls. Fruits and vegetables are rich in dietary fiber. The content of dietary fiber can determine the abundance of the dominant flora Bacteroides and Prevotella in the gut microbiota[67], regulate the production of short chain fatty acids, and futher affect the occurrence and development of IBS-D.

Psychological factors play an important role in the pathogenesis of IBS-D. Previous studies have found that the rate of IBS with mental problem is as high as 91%[68], and the structure of IBS-D gut microbiota was similar to that of depression[69]. In the HAMA and HAMD scores we collected, IBS-D was significantly higher than healthy population. Sleep status is a very important evaluation index both in HAMA and HAMD. Our study found that the proportion of IBS patients who sleep less than 6 hours a day is much higher than that of healthy people, and the exercise time is much lower than that of healthy people. Sleep status and aerobic exercise can affect the function of brain-intestinal-bacteria-axis by regulating the secretion of neurotransmitters such as brain-derived neurotrophic factor(BDNF) and serotonin(5-HT), and futher changing the structure of gut microbiota[70]. In the correlation analysis, we found that Verrucomicrobiota was negatively correlated with HAMD score. At present, there is no research on Verrucomicrobiota. But Akkermansia, which belongs to Verrucomicrobiota, is considered to be a very potential probiotics[71]. Akkermansia is rich in the gut microbiota of healthy individuals. It has the role of prevention and treatment of obesity, type 2 diabetes and other metabolic disorders. The decrease of Akkermansia is related to the development of obesity, type 2 diabetes, inflammatory bowel disease (IBD), autism, and many other diseases[71].

We also performed IBS-SSS and IBS-QOL scores on IBS-D patients. The statistical results of IBS-SSS showed that number of days having pain and interfere with life were the highest. IBS-QOL statistics showed that patients had the highest scores of dysphoria and interference with activity. It can be seen from the score that the social, family relations, anxiety and depression of IBS patients caused by gastrointestinal symptoms have a great impact on the quality of life of patients and their families. Our study also found that the subitem extend of dominant pain in IBS-SSS was positively correlated with Fusobacteriota, the total score of IBS-SSS was positively correlated with Synergistata and negatively correlated with Chloroflex. Dysphoria and body image in IBS-QOL scale were negatively correlated with Euryarchaeota, Interaction with activity was negatively correlated with Cyanobacteria. Food avoidance was negatively correlated with Chloroflex, sexual dysfunction was positively correlated with Chloroflexi and negatively correlated with Desulfobacterota. This suggests that different bacteria have their own physiological functions in the pathogenesis of IBS-D. Euryarchaeota may play an important role in the pathogenesis of IBS-D and may be the key factor leading to the occurrence of IBS-D. Fusobacteriota may play an adverse role in IBS-D progression and affect the prognostic function and Chloroflex and Cyanobacteria may be the key factors of emotion regulation. This is consistent with the previous research results of Yusof et al[72]. Yusof found that Fusobacteriota can be used as a biomarker for IBS with obvious abdominal pain when studying the gut microbiota of IBS patients after flood[72]. Euryarchaeota contains a variety of methanogens, such as Methanobrevibacter Smithii, which is the main methanogens in patients with IBS-C[73]. We can speculate that the low expression of Euryarchaeota leads to the significant reduction of methane production function of IBS-D gut microbiota, the enhancement of intestinal peristalsis, and then finally cause serious gastrointestinal symptoms, anxiety and depression.

We detected several laboratory indexes of IBS-D and healthy control at the same time. The results showed that there were significant differences in monocyte count, erythrocyte count, hemoglobin, urea

nitrogen, aspartate aminotransferase, alanine aminotransferase and glutamyltranspeptidase between the two groups. At present, multiple studies have shown that abnormal bile acid secretion is an important link in the pathogenesis of ibs-d[74]. The abnormal secretion of bile acids is bound to affect the production of important enzymes in the hepatobiliary system such as glutamic oxaloacetic transaminase, alanine transaminase and glutamyltranspeptidase, so it is significantly different from the healthy control. Bdellovibrionota was significantly negatively correlated with leukocyte count, neutrophil count and monocyte count. Proteobacteria was negatively correlated with urinary pH, monocyte count and alkaline phosphatase, and Bacteroidota was negatively correlated with GGT. Bdellovibrionota is a kind of parasitic bacteria, which can effectively cleave pathogenic bacteria such as typhoid bacillus, paratyphoid bacillus and Vibrio cholerae. We speculate that Bdellovibrionota reduces the level of inflammation and leads to the decrease of inflammatory indexes in the process of cracking pathogens. Proteobacteria is one of the main pathogens causing opportunistic urinary tract infection[75], and urinary pH > 7 is one of the risk factors of opportunistic urinary tract infection. Interestingly, this study shows that Proteobacteria is negatively correlated with urinary pH, which is different from previous studies and needs further research.

4 Conclusion

In conclusion, our study found that there were significant differences in richness and flora structure between IBS-D patients and healthy controls in Southwest China. Prevotella, Clostridiales and Roseburia were biomarkers of IBS-D. The functions of fermentation and methane production of gut microbiota decreased, and the functions of cellulose decomposition and xylan decomposition strengthened. Diet, sleep time, exercise time, work intensity, consumption of vegetables and fruits, anxiety and depression have an important impact on the gut microbiota and IBS-D.

However, this study also has some shortcomings: 1. Due to the strict screening criteria for healthy controls, the number of healthy control is not enough; 2. Quantifiable spicy index was not introduced into pepper consumption, resulting in limited statistical analysis; 3. When making statistics on diet, the intake of FODMAPs have not be subdivided. In the later stage, we will increase the number of samples and refine relevant indicators to further explore the important role of gut microbiota in the pathogenesis of IBS-D in Southwest China.

Abbreviations

IBS-D: Irritable bowel syndrome with diarrhea; HCs: Healthy controls; NMDS: Nonmetric multi-dimensional scaling; OTUs: Operational taxonomic units; PCoA: Principal component analysis; FMT: Fecal microbiota transplantation; IBS-SSS: IBS symptom severity scale; IBS-QOL:IBS quality of life; HAMA: Hamilton Anxiety Scale; HAMD: Hamilton Depression Scale; ALT:Alanine aminotransferase; AST:Aspartate aminotransferase; ALP:Alkaline phosphatase; UA:Uric acid; Crea: Creatinine; TG:Triglyceride;TC:Cholesterol;CTAB:cetyltrimethyl ammonium bromide;BDNF:brain-derived neurotrophic factor; 5-HT:serotonin.

Declarations

The trial was registered and approved in China Clinical Trial Registry (Registration No.ChiCTR2100045751).

All the authors listed have approved the manuscript that is enclosed.

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

No conflict of interest exits in the submission of this manuscript.

The authors acknowledge financial support from National Natural Science Foundation of China（NO. 81673854）.

Authors contribution: Recruitment of research subjects and write the first draft:Chengjiao Yao; Recruitment of research subjects and collecting data:Yilin Li, Lihong Luo; Collecting data:Fengjiao Xie.Qin Xiong,Tinglin Li; Experimental design and overall control:Peimin Feng.

Acknowledgments

The authors acknowledge financial support from National Natural Science Foundation of China（NO. 81673854）.

References

1. Lacy BE, Pimentel M, Brenner DM, et al. ACG Clinical Guideline: Management of Irritable Bowel Syndrome. Am J Gastroenterol. 2021;116(1):17–44.
2. Palsson OSW, Van Tilburg WE, Chang MAL, et al. Development and validation of the Rome IV diagnostic questionnaire for adults. Gastroenteology. 2016;150:1481–91.
3. Occhipinti K, Smith JW. Irritable bowel syndrome: a review and update. Clin Colon Rectal Surg. 2012;25(1):46–52.
4. Andrews EB, Eaton SC, Hollis KA,et al. Prevalence and demographics of irritable bowel syndrome: results from a large web-based survey. Aliment Pharmacol Ther. 2005;22(10):935–42.
5. Okeke EN, Ladep NG, Adah S, et al. Prevalence of irritable bowel syndrome:a community survey in an African population. Ann Afr Med. 2009;8(3):177–80.
6. Han SH, Lee OY, Bae SC, et al. Prevalence of irritable bowel syndrome in Korea: population-based survey using the Rome II criteria. J Gastroenterol Hepatol. 2006;21(11):1687–92.
7. Kumano H, Kaiya H, Yoshiuchi K, et al. Comorbidity of irritable bowel syndrome, panic disorder, and agoraphobia in a Japanese representative sample. Am J Gastroenterol. 2004;99(2):370–6.
8. Jin LIANG, Qian CHEN,ZHANG Xuan.Changes of Gastrointestinal Hormones in Diarrhea Predominant Irritable Bowel Syndrome Treated by Tongxie Sishen Decoction and Its Clinical Significance.World

Chinese Medicine,2020,15(1):71–77.

9. Kwan AC, Hu WH, Chan YK,et al. Prevalence of irritable bowel syndromein Hong Kong. J Gastroenterol Hepatol. 2002;17(11):1180–6.
10. JiangYP, Zhang CP, Zhang Q, et al. Evaluation of clinical characteristics of irritable bowel syndrome in coastal areas of Qingdao. Chin J Gastroenterol. 2010;15(2):109–11.
11. Liu Jie X, Qili F. Yan,et al.The Prevalence and Risk Factors of the Adult Irritable Bowel Syndrome in Ganzhou City.Chinese Journal of Clinical Medicine,2010,17(3):359–361.
12. Gwee KA, Ghoshal UC, Chen M. Irritable bowel syndrome in Asia: Pathogenesis, natural history, epidemiology, and management. J Gastroenterol Hepatol. 2018;33(1):99–110.
13. Mishima Y, Ishihara S. Molecular Mechanisms of Microbiota-Mediated Pathology in Irritable Bowel Syndrome. Int J Mol Sci. 2020;21(22):8664.
14. Drago L, Valentina C, Fabio P. Gut microbiota, dysbiosis and colon lavage. Dig Liver Dis. 2019;51(9):1209–13.
15. Simpson CA, Mu A, Haslam N, et al. Feeling down? A systematic review of the g**ut m**icrobiota in anxiety/depression and irritable bowel syndrome. J Affect Disord. 2020;266(1):429–46.
16. Duan R, Zhu S, Wang B. **et al**. Alterations of Gut Microbiota in PatientsWith Irritable Bowel Syndrome Based on 16S rRNA-Targeted Sequencing: A Systematic Review. Clin Transl Gastroenterol. 2019;10(2):e00012.
17. Jalanka -TJ, Salojarvi J, Salonen A. **et al**.**Faecal mi-crobiota composition and host-microbe cross-talk following gastroenteritis and in post-infectious irritable bowel syndrome.Gut**. 2014,63(**11**):**1737–1745**.
18. Holvoet T, Joossens M, Vázquez-Castellanos JF. **et al**. Fecal Microbiota Transplantation Reduces Symptoms in Some Patients With Irritable Bowel Syndrome With Predominant Abdominal Bloating: Short- and Long-term Results From a Placebo-Controlled Randomized Trial. Gastroenterology. 2021;160(1):145–57.
19. Xu H, Ma C, Zhao F. **et al**. Adjunctive treatment with probiotics partiallyalleviates symptoms and reduces inflammation in patients with irritable bowel syndrome. Eur J Nutr. 2021;60(5):2553–65.
20. Black CJ, Ford AC. How effective are antibiotics for the treatment of irritable bowel syndrome? Expert Opin Pharmacother. 2020;21(18):2195–7.
21. Jandee S, Chuensakul S, Maneerat S. No distinction in the gut microbiota between diarrhea predominant-irritable bowel syndrome and healthy subjects: matched case-control study in Thailand. Gut Pathog. 2021;13(1):16.
22. Aroniadis OC, Brandt LJ, Oneto C. **et al**. Faecal microbiota transplantation for diarrhoea-predominant irritable bowel syndrome: a double-blind, randomised, placebo-controlled trial. Lancet Gastroenterol Hepatol. 2019;4(9):675–85.
23. Francis CY, Morris J, Whorwell PJ. The irritable bowel severity scoring system: a simple method of monitoring irritable bowel syndrome and its progress. Aliment Pharmacol Ther. 1997;11(2):395–402.

24. Jandee S, Chuensakul S, Maneerat S. No distinction in the gut microbiota between diarrhea predominant-irritable bowel syndrome and healthy subjects: matched case-control study in Thailand. Gut Pathog. 2021;13(1):16.
25. Patrick DL, Drossman DA, Frederick IO. **et al**. Quality of life in persons with irritable bowel syndrome: development and validation of a new measure. Dig Dis Sci. 1998;43(2):400–11.
26. Huang WW, Zhou FS, Bushnell DM. **et al**. Cultural adaptation and application of the IBS-QOL in China: a disease-specific quality-of-life questionnaire. Qual Life Res. 2007;16(6):991-6.
27. Thompson E. Hamilton Rating Scale for Anxiety (HAM-A). Occup Med (Lond). 2015;65(7):601.
28. **HAMILTON M**. A rating scale for depression. J Neurol Neurosurg Psychiatry. 1960;23(1):56–62.
29. Yee W, Abdul-Kadir R, Lee LM. **et al**. A simple and inexpensive physical lysis method for DNA and RNA extraction from freshwater microalgae. 3 Biotech. 2018;8(8):354.
30. Magoč T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics. 2011;27(21):2957-63.
31. Bokulich NA, Subramanian S, Faith JJ. **et al**. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat Methods. 2013;10(1):57 – 9.
32. Caporaso JG, Kuczynski J, Stombaugh J. **et al**. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7(5):335–6.
33. Haas BJ, Gevers D, Earl AM. **et al**. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Res. 2011;21(3):494–504.
34. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods. 2013;10(10):996-8.
35. Wang Q, Garrity GM, Tiedje JM. **et al**. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007;73(16):5261–7.
36. Mei L, Zhou J, Su Y. **et al**. Gut microbiota composition and functional prediction in diarrhea-predominant irritable bowel syndrome. BMC Gastroenterol. 2021;21(1):105.
37. Liu HN, Wu H, Chen YZ. **et al**. Altered molecular signature of intestinal microbiota in irritable bowel syndrome patients compared with healthy controls: A systematic review and meta-analysis. Dig Liver Dis. 2017;49(4):331–7.
38. Maharshak N, Ringel Y, Katibian D. **et al**. Fecal and Mucosa-Associated Intestinal Microbiota in Patients with Diarrhea-Predominant Irritable Bowel Syndrome. Dig Dis Sci. 2018;63(7):1890–9.
39. Wang F, Huang X, Chen Y. **et al**. Study on the Effect of Capsaicin on the Intestinal Flora through High-Throughput Sequencing. ACS Omega. 2020;5(2):1246–53.
40. Yassour M, Lim MY, Yun HS. Sub-clinical detection of gut microbial biomarkers of obesity and type 2 diabetes. Genome Med. 2016;8(1):17. **et al**.
41. Li Z, Dong L, Huang Q, Wang X. Bacterial communities and volatile compounds in Doubanjiang, a Chinese traditional red pepper paste. J Appl Microbiol. 2016;120(6):1585–94.

42. Liu T, Gu X, Li LX. **et al**. Microbial and metabolomic profiles in correlation with depression and anxiety co-morbidities in diarrhoea-predominant IBS patients. BMC Microbiol. 2020;20(1):168.
43. Wu KQ, Sun WJ, Li N. **et al**. Small intestinal bacterial overgrowth is associated with Diarrhea-predominant irritable bowel syndrome by increasing mainly Prevotella abundance. Scand J Gastroenterol. 2019;54(12):1419–25.
44. Brunkwall L, Ericson U, Nilsson PM. **et al**. Self-reported bowel symptoms are associated with differences in overall gut microbiota composition and enrichment of Blautia in a population-based cohort. J Gastroenterol Hepatol. 2021;36(1):174–80.
45. Rigsbee L, Agans R, Shankar V. **et al**. Quantitative profiling of gut microbiota of children with diarrhea-predominant irritable bowel syndrome. Am J Gastroenterol. 2012;107(11):1740-51.
46. Simpson CA, Mu A, Haslam N. **et al**. Feeling down? A systematic review of the gut microbiota in anxiety/depression and irritable bowel syndrome. J Affect Disord. 2020;266:429–46.
47. Tap J, Derrien M, Törnblom H. **et al**. Identification of an Intestinal Microbiota Signature Associated With Severity of Irritable Bowel Syndrome. Gastroenterology. 2017;152(1):111–23.
48. Su T, Liu R, Lee A. **et al**. Altered Intestinal Microbiota with Increased Abundance of Prevotella Is Associated with High Risk of Diarrhea-Predominant Irritable Bowel Syndrome. Gastroenterol Res Pract. 2018;2018:6961783.
49. Pittayanon R, Lau JT, Yuan Y. **et al**. Gut Microbiota in Patients With Irritable Bowel Syndrome-A Systematic Review. Gastroenterology. 2019;157(1):97–108.
50. Chao G, Zhang S. The characteristics of intestinal flora of IBS-D with different syndromes. Immun Inflamm Dis. 2020;8(4):615–28.
51. Labus JS, Hollister EB, Jacobs J. Differences in gut microbial composition correlate with regional brain volumes in irritable bowel syndrome. Microbiome. 2017;5(1):49.
52. Zhao L, Yang W, Chen Y. **et al**. A Clostridia-rich microbiota enhances bile acid excretion in diarrhea-predominant irritable bowel syndrome. J Clin Invest. 2020;130(1):438–50.
53. Li J, Ma Y, Bao Z. **et al**. Clostridiales are predominant microbes that mediate psychiatric disorders. J Psychiatr Res. 2020;130:48–56.
54. Sciavilla P, Strati F. **et al**. Gut microbiota profiles and characterization of cultivable fungal isolates in IBS patients. Appl Microbiol Biotechnol. 2021;105(8):3277–88., **Di** Paola.
55. Wang B, Zhu S, Liu Z. Wet al. Increased Expression of Colonic Mucosal Melatonin in Patients with Irritable Bowel Syndrome Correlated with Gut Dysbiosis. Genomics Proteomics Bioinformatics. 2020;18(6):708–20.
56. Takakura W, Pimentel M. Small Intestinal Bacterial Overgrowth and Irritable Bowel Syndrome - An Update. Front Psychiatry. 2020;11:664.
57. Halmos EP, Power VA, Shepherd SJ. **et al**. A diet low in FODMAPs reduces symptoms of irritable bowel syndrome. Gastroenterology. 2014;146(1):67–75.

58. Adak A, Khan MR. An insight into gut microbiota and its functionalities. Cell Mol Life Sci. 2019;76(3):473–93.
59. Sebastián Domingo JJ, Sánchez Sánchez C. From the intestinal flora to the microbiome. Rev Esp Enferm Dig. 2018;110(1):51–6.
60. Lu ZM, Wang ZM, Zhang XJ. Microbial ecology of cereal vinegar fermentation: insights for driving the ecosystem function. Curr Opin Biotechnol. 2018 **Feb**;49:88–93.
61. Jing HUANG, Weiwei YAN, Shuliang LIU,**et al**. **Recent Progress in Functional Microbial Community and Quality Evaluation of Vinegar Daqu**.Food Science,2020,41(**07**):**322–329**.
62. YUE Y, YE K, LU J. **et al**. Probiotic strain Lactobacillus plantarum YYC-3 prevents colon cancer in mice by regulating the tumour microenvironment. Biomed Pharmacother. 2020;127:110159.
63. PAN Y, WANG H, TAN F. **et al**. Lactobacillus plantarum KFY02 enhances the prevention of CCl4-induced liver injury by transforming geniposide into genipin to increase the antioxidant capacity of mice. Journal of Functional Foods. 2020;73:104128.
64. LIM S-M, LEE N-K, PAIK H-D. Antibacterial and anticavity activity of probiotic Lactobacillus plantarum 200661 isolated from fermented foods against Streptococcus mutans. LWT - Food Science Technology. 2020;118:108840.
65. Cichewicz RH, Thorpe PA. The antimicrobial properties of chile peppers (Capsicum species) and their uses in Mayan medicine. J Ethnopharmacol. 1996;52(2):61–70.
66. Baboota RK, Murtaza N, Jagtap S. **et al**. Capsaicin-induced transcriptional changes in hypothalamus and alterations in gut microbia count in high fat diet fed mice. J Nutr Biochem. 2014;25(9):893–902.
67. Wu GD, Chen J, Hoffmann C. **et al**. Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes. Science. 2011;334(6052):105–8.
68. Lsckner JM, Ma CX, Keefer L. **et al**. Type, rather than number, of mental and physical comorbidities increases the severity of symptoms in patients the severity of symptomes in patients with irritable bowel syndrome. Clin Gastroenterol Hepatol. 2013;11:1147–57.
69. Ford AC, Lacy BE, Talley NJ. Irritable Bowel SyndromeNEnglJMed. 2017;376(26):2566–78.
70. Matenchuk BA, Mandhane PJ, Kozyrskyj AL. Sleep, circadian rhythm,and gut microbiota. Sleep Med Rev. 2020;53:101340.
71. Zhang T, Li Q, Cheng L. **et al**. Akkermansia muciniphila is a promising probiotic. Microb Biotechnol. 2019;12(6):1109–25.
72. Yusof N, Hamid N, Ma ZF. **et al**. Exposure to environmental microbiota explains persistent abdominal pain and irritable bowel syndrome after a major flood. Gut Pathog. 2017;9:14. ., :**75**.
73. Kim G, Deepinder F, Morales W. **et al**. Methanobrevibacter smithii is the predominant methanogen in patients with constipation-predominant IBS and methane on breath. Dig Dis Sci. 2012;57(12):3213-8.
74. Jeffery IB, Das A, O'Herlihy E. **et al**. Differences in Fecal Microbiomes and Metabolomes of People With vs Without Irritable Bowel Syndrome and Bile Acid Malabsorption. Gastroenterology. 2020;158(4):1016–28.e8.

75. Smith SN. Independent Transurethral Urinary Tract Inoculation in a Murine Model of Ascending Infection with Proteus mirabilis. Methods Mol Biol. 2019;2021:159–72.

Figures

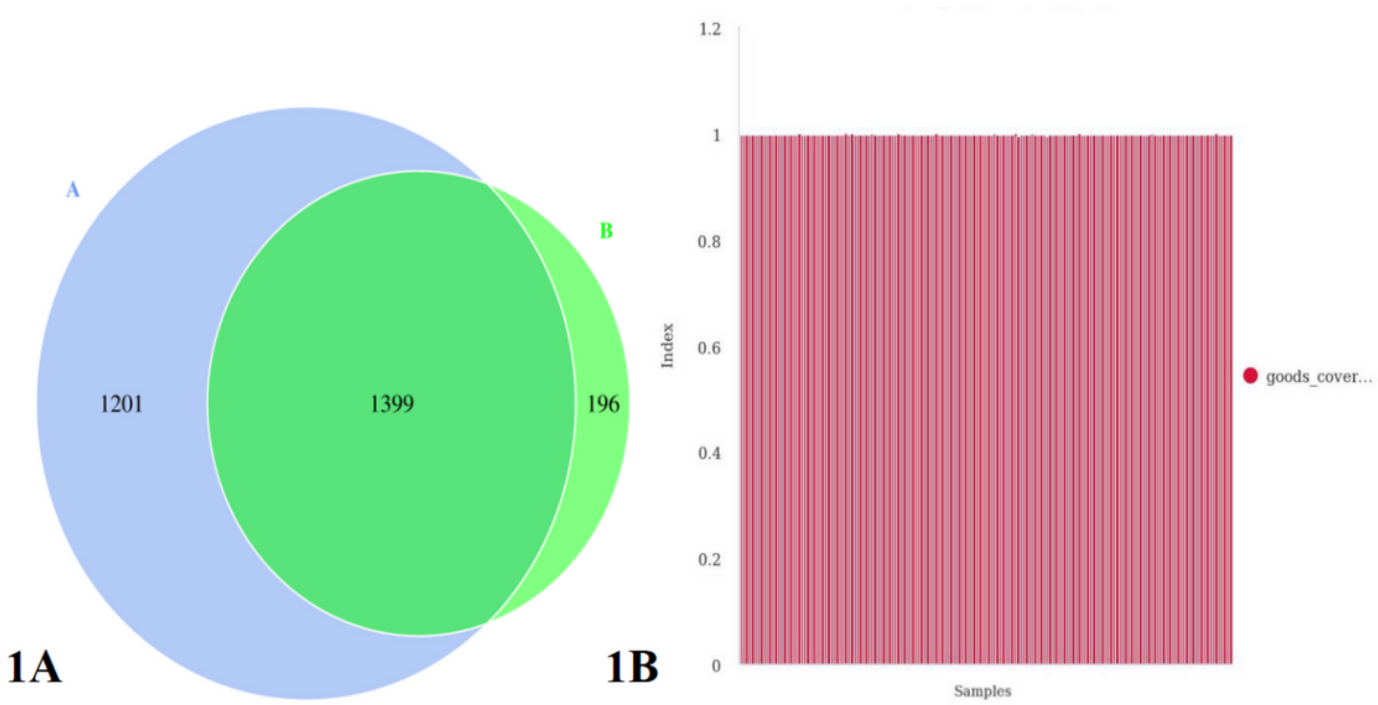

Figure 1

OTU clustering and quality analysis of 16S rDNA sequencing (1A) Intersection of OTUs with IBS-D and HCs. A:IBS-D group; B:HCs group. (1B) The Goods_coverage of all samples. The horizontal axis represents the sample and the vertical axis represents the sequencing depth.

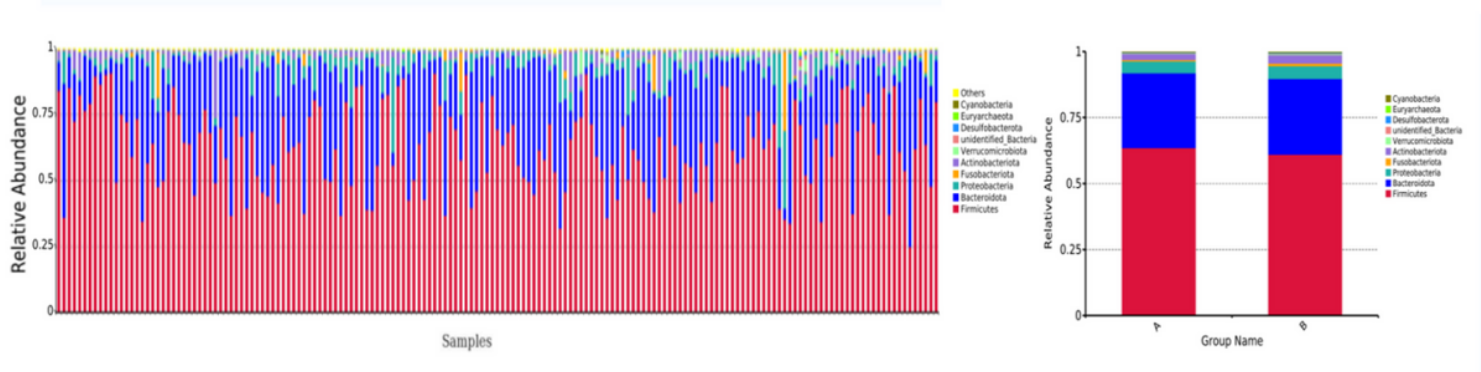

Figure 2

Top10 histogram of relative abundance of species (phylum level) A: IBS-D, B: HCs. The horizontal axis are the samples or groups, the vertical axis represents the relative abundance, and “others” represent

represents the sum of the relative abundance except the top10 in the figure.

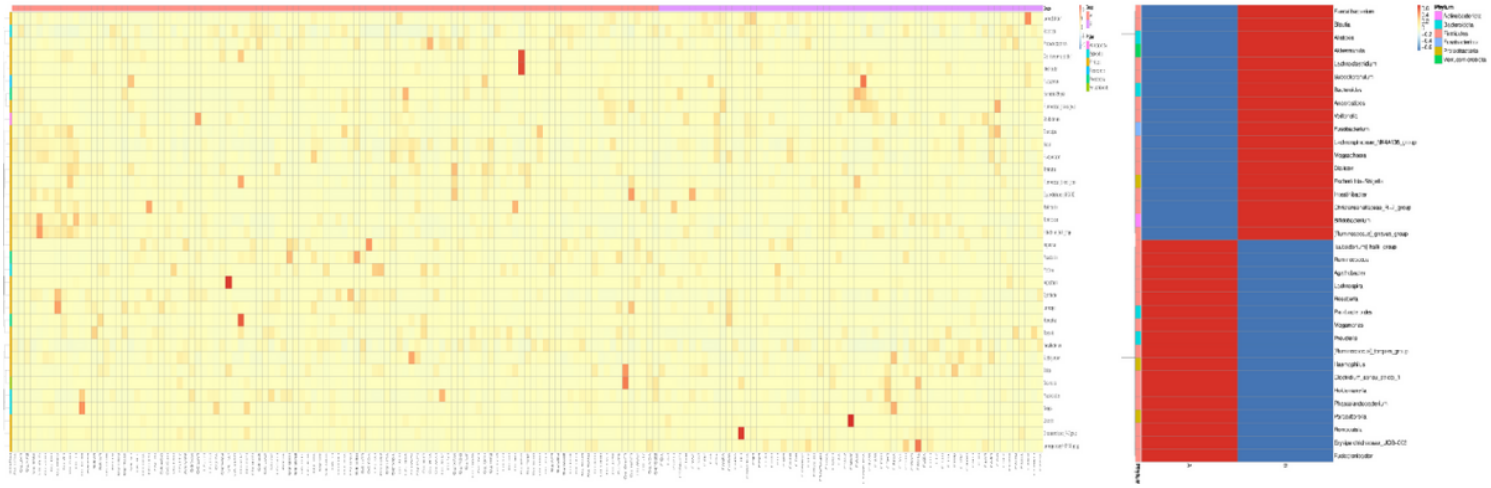

Figure 3

Top35 heat map of species abundance clustering (genus level) A: IBS-D, B: HCs. The horizontal axis are the samples or groups, the vertical axis represents the species annotation information. The cluster tree on the left is a species cluster tree, and the contrast between the two groups is on the right.

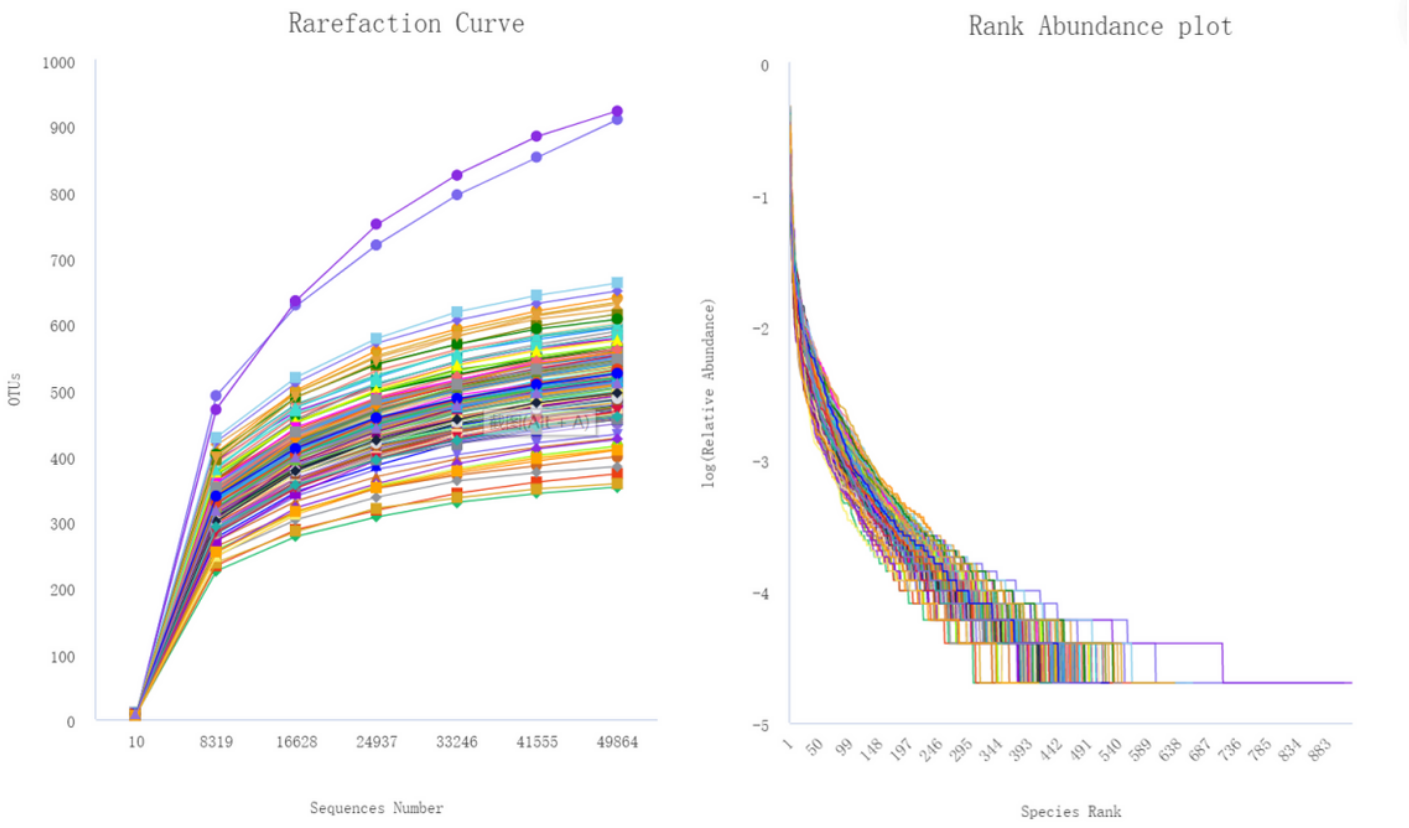

Figure 4

Rarefaction curve and Rank Abundance of all samples The horizontal axis is the sequence number sorted by OTUs abundance, and the vertical axis is the relative abundance of corresponding OTUs. Different samples are represented by broken lines of different colors.

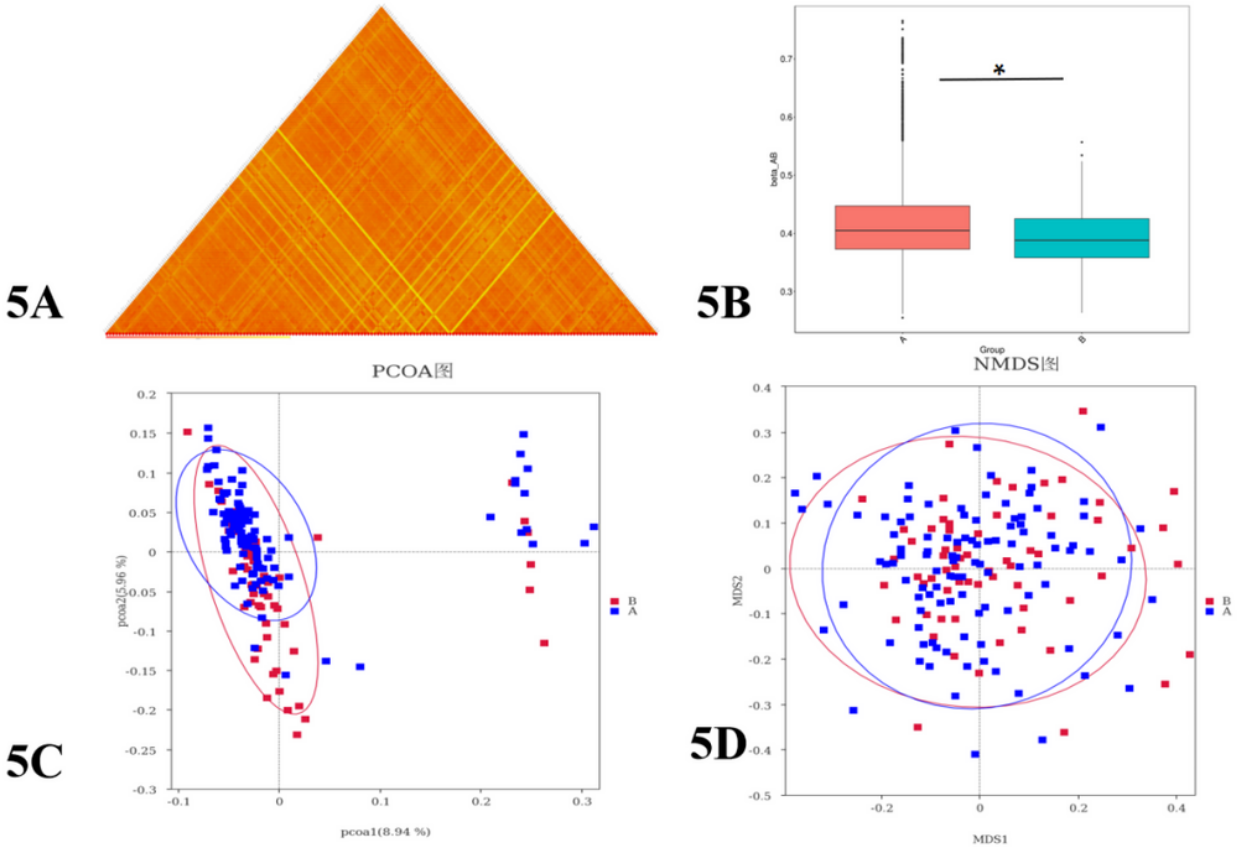

Figure 5

Beta diversity index between IBS-D and HCs (5A) Distance matrix heat map based on unweighted UniFrac. The number in the square is the difference coefficient between two samples. (5B) Significant difference in beta-diversity index between two groups. A:IBS-D group; B:HCs group. (5C) PCoA analysis. A: IBS-D, B:HCs. The x-axis represents one principal component, the y-axis represents another principal component, and the percentage represents the contribution of the principal component to the sample difference. Each point in the diagram represents one sample, and samples from the same group are represented in the same color. (5D) NMDS analysis. A: IBS-D, B: HCs. Each point in the diagram represents one sample, and samples from the same group are represented in the same color.

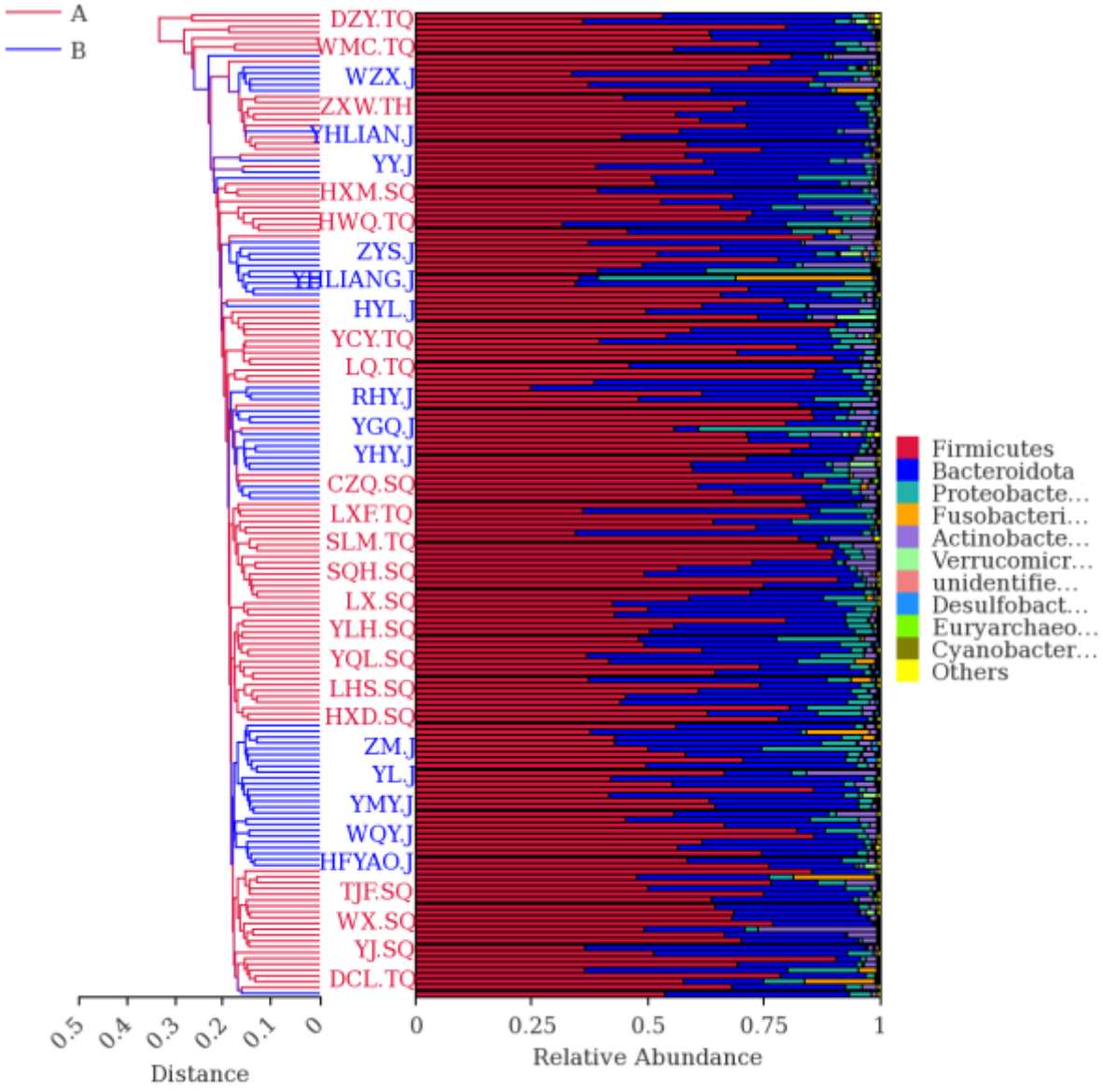

Figure 6

Clustering tree of UPGMA A:IBS-D group; B:HCs group.

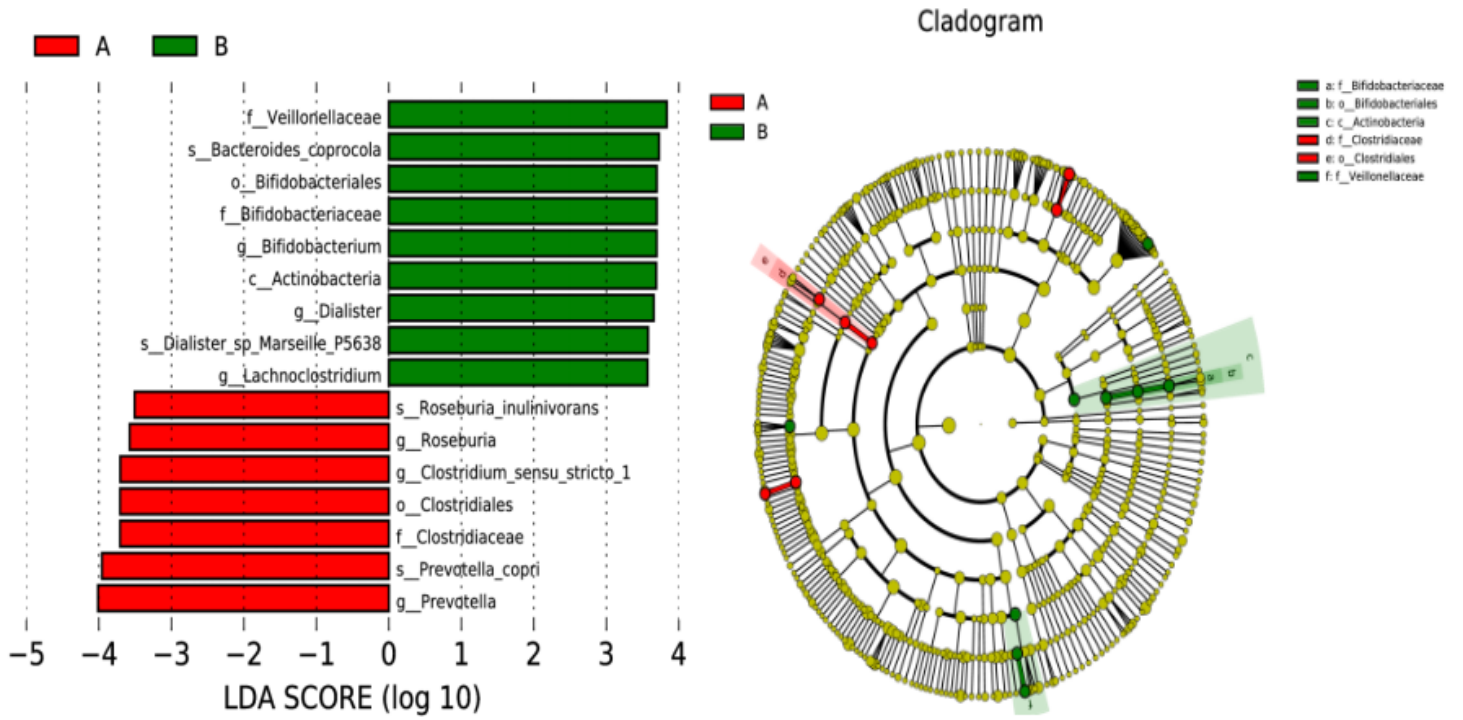

Figure 7

Distribution histogram of LDA score and cladogram of species A: IBS-D,B:HCs. The length of the bar chart on the left represents the infuence of diferent species. On the right, the circles radiating from the inside out represent taxonomic levels from phylum to genus (or species); each small circle at a diferent classifcation level represents a classifcation at that level, and the diameter of the small circle is proportional to the relative abundance. Coloring principle: red is the IBS-D, green is HCs, and yellow is the species with no signifcant diference.

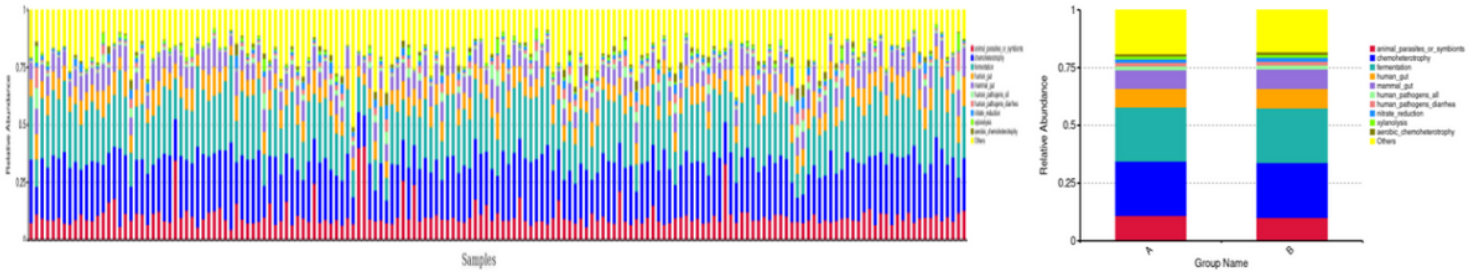

Figure 8

Histogram of functional annotation relative abundance A:IBS-D, B:HCs. The horizontal axis are the samples or groups, the vertical axis represents the relative abundance, and “others” represent represents the sum of the relative abundance except the top10 in the figure.

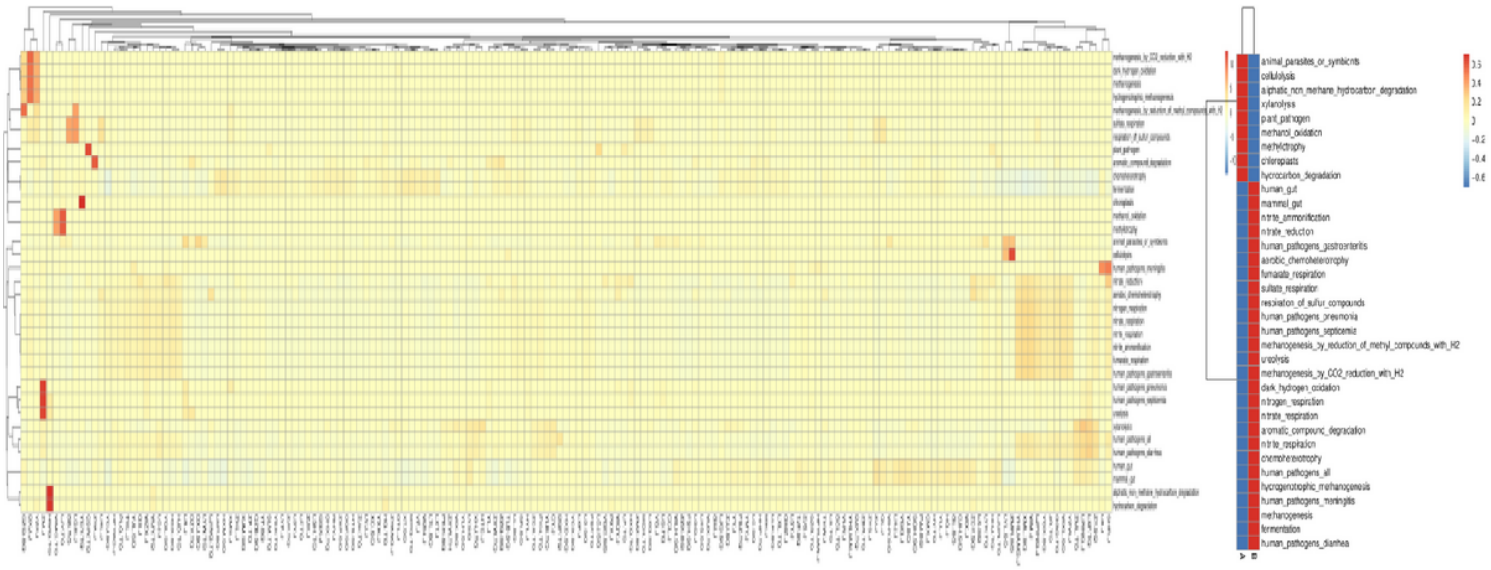

Figure 9

Top 35 heat map of functional annotation relative abundance A: IBS-D, B: HCs. The horizontal axis are the samples or groups, the vertical axis represents the functional annotation relative abundance. The cluster tree on the left is based on the samples comparsion, and the contrast between the two groups is on the right.

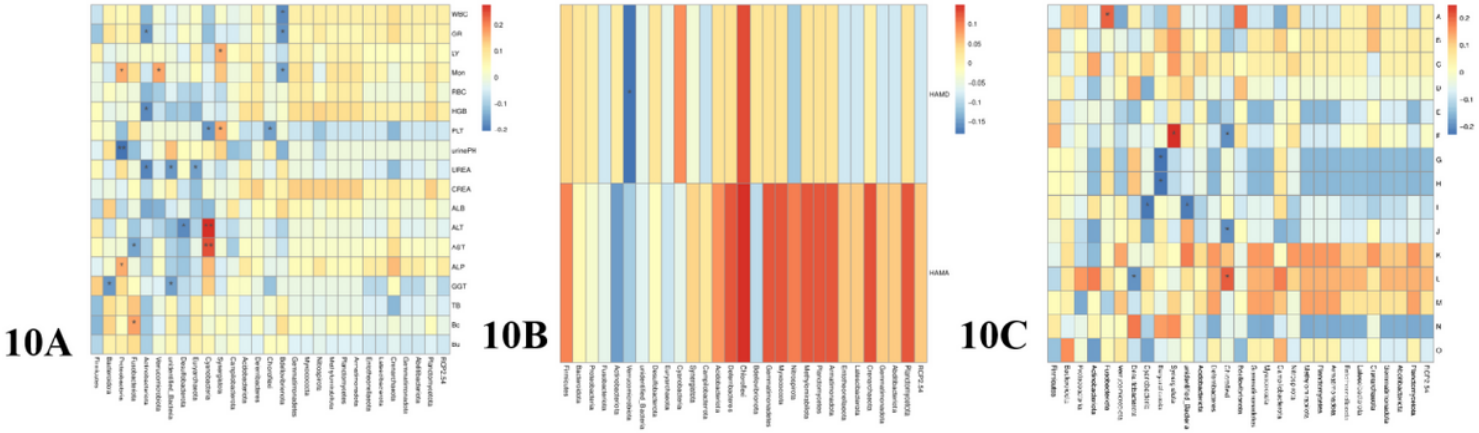

Figure 10

Heat map of correlation analysis between environmental factors and gut microbiota (10A) Correction between routine laboratory indexes and species abundance. (10B) Correction between HAMA/ HAMD and species abundance. (10C) Correction between IBS-SSS\IBS-QOL and species abundance. A:extend of abdominal pain. B:number of days having pain. C:extend of abdominal distension. D:satisfaction of bowel habits. E: affect or interfere with life. F:IBS-SSS. G:dysphoria. H:body image. I:interference with activity. J:food avoidance. K:health worry. L:sexual dysfunction. M:social reaction. N:family relationships. Red bex represents positive correlation, blue box represents negative correlation. The darker the color, the stronger the correlation. * represents p <0.05, * * represents p <0.01.