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Study on the effect of Xi-Feng-Hua-Shi granules on intestinal flora in patients with diarrhea-predominant irritable bowel syndrome based on 16S rDNA sequencing

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Author contributions

Hui Li are the principal investigator and takes responsibility for the study and the manuscript. Xiao-Mei Xu, Zu Yang and Yong-Shuang Wang participated in planning and designing the study and recruiting. Zhen-Tao An supported the data management and statistical analysis. Xiao-Mei Xu and Zu Yang contributed to the manuscript writing. Yao-Zhou Tian was responsible for revising the manuscript. All the authors reviewed the final manuscript.

Competing interests

The authors declare no conflicts of interest.

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Abbreviations

IBS-D, irritable bowel syndrome; TCM, traditional Chinese medicine; XFHS, Xi-Feng-Hua-Shi; DADA2, Differential Amplication Denoising Algorithm; ASVs, Amplication Sequence Variables; OTU, Operational Taxonomy Units.

Citation

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Abstract

Background: Diarrhea-predominant irritable bowel syndrome (IBS-D) is a common functional gastrointestinal disease, that significantly impacts the quality of life and social interactions of patients, and also consumes a significant amount of medical resources. Xi-Feng-Hua-Shi (XFHS) granule is an empirical formula developed by Professor Yao-Zhou Tian, a famous TCM doctor in Jiangsu Province. Clinical experience has shown that XFHS granules are effective at relieving the symptoms of IBS-D patients. Therefore, this study used 16S rDNA sequencing to determine the species and richness of microflora in order to explore the overall efficacy and changes in the intestinal microflora of IBS-D patients after treatment with XFHS granules, and to further understand the mechanism of drug action as a potential new therapeutic method for the clinical treatment of IBS-D. **Methods:** A total of 66 IBS-D patients who were treated at Jiangsu Provincial Hospital of Integrated Traditional Chinese and Western Medicine from June 2021 to January 2022 were recruited and randomly divided into a reatment group and a control group. The treatment group was given XFHS granules and the control group was given placebo granules for 4 weeks. Traditional Chinese medicine (TCM) syndrome scores of patients before and after treatment were recorded, and fresh fecal samples of some patients before and after treatment were collected to analyze the changes of intestinal flora by 16S rDNA sequencing method. **Results:** The effective rate in the treatment group was 92.86%, which was significantly higher than the control group ($P < 0.05$). The bacterial diversity of IBS-D patients did not change significantly after treatment with XFHS granules, but the species composition analysis showed that the abundance of Firmicutes, Proteobacteria and Cyanobacteria decreased, while the abundance of Bacteroidetes, Actinobacteria and Bifidobacteria increased. **Conclusion:** XFHS granules can effectively improve the clinical symptoms of IBS-D patients, and its therapeutic effect may be related to regulating the structure and abundance of intestinal flora.

Keywords: diarrhea-predominant irritable bowel syndrome; Xi-Feng-Hua-Shi granules; efficacy observation; intestinal flora

Background

Diarrhea-predominant irritable bowel syndrome (IBS-D) is a functional gastrointestinal disease associated with disorder of the intestinal microflora, brain-gut axis, and visceral hypersensitivity. It is mainly characterized by recurrent abdominal pain and diarrhea[1]. Currently, most clinical treatments for IBS-D use spasmolytic drugs and antidiarrheal drugs to relieve symptoms, but there is no reliable specific drug. Traditional Chinese medicine(TCM) treatment has the advantages of fewer drug reactions and a lower recurrence rate. Xi-Feng-Hua-Shi(XFHS) granules is an empirical prescription of Professor Yao-Zhou Tian, a famous TCM doctor in Jiangsu Province. It focuses on softening the liver and extinguishing wind, strengthening the spleen and dampening the dampness, and has a remarkable effect on the treatment of IBS-D patients with stagnation of liver Qi and spleen deficiency. Previous studies have confirmed that XFHS granules can improve the loose stool rate of IBS-D rats by regulating brain and intestinal peptides and reducing visceral hypersensitivity[2, 3], but its effect on intestinal flora has not been studied. Therefore, this study used XFHS granules to treat IBS-D patients and its clinical efficacy was observed. In addition, 16S rDNA sequencing was performed on feces of some patients before and after treatment to explore the effects of XFHS granules on intestinal microflora of IBS-D patients.

Methods

Participants
The IBS-D patients with stagnation of liver Qi and spleen deficiency who received treatment in Jiangsu Provincial Hospital of Integrated Traditional Chinese and Western Medicine from June 2021 to January 2022 were selected. The XFHS granules and placebo granules were numbered according to the random number table generated by SPSS 25.0 software, and drugs were randomly distributed and registered according to the enrollment order of patients.

Diagnostic criteria
According to the diagnostic criteria for IBS-D in the Rome IV standard for functional gastroenterology [4], a diagnosis of IBS-D can be made if the following criteria are met: recurrent abdominal pain at least one day per week in the last three months, accompanied by two or more of the following: related to defecation; changes in frequency of defecation during attacks; changes in the appearance of feces during attacks. Symptoms must have appeared at least six months before diagnosis and persisted for nearly three months. At least 25% of bowel movements will be watery or loose, and less than 25% will be hard or lumpy.

TCM syndrome diagnosis criteria
It is formulated according to Expert Consensus on TCM Diagnosis and Treatment of Irritable Bowel Syndrome [5] approved by Spleen and Stomach Diseases Branch of China Association of Traditional Chinese Medicine in 2017. The main symptoms of stagnation of liver Qi and spleen deficiency IBS-D include abdominal pain with diarrhea, relief of pain after diarrhea, and attacks often related to emotions, as well as irritability and a tendency to sigh. Secondary symptoms include swelling in the hypochondriac region, anorexia, and fatigue. The tongue may appear light and fat, with possible tooth marks and thin white moss, and the pulse may be thin. To diagnose stagnation of liver qi and spleen deficiency IBS-D, at least two main symptoms and two secondary symptoms must be present, along with the characteristic tongue and pulse.

Inclusion criteria
Eligible participants for this study included those who meet the diagnostic criteria for IBS-D and the TCM syndrome criteria, are between the ages of 18 and 70 years old, and provide informed consent to participate. Participants must be willing to undergo testing.

Exclusion criteria
Exclusion criteria for this study included individuals with organic lesions of the gastrointestinal tract, serious dysfunction of important organs, mental anomalies, pregnancy or lactation, and those who have taken drugs that may affect the results of this study within one month prior to enrollment. Participants who are currently participating in other clinical studies were also excluded.

Fall-off criteria
Other reasons for exclusion from the study included poor compliance or drug allergies, voluntary requests to stop the study after enrollment, and cases lost to follow-up.

Ethics approval
The study was approved by the Ethics Committee of Jiangsu Provincial Hospital of Integrated Traditional Chinese and Western Medicine (ethics approval No.: 2020LWKY038) and was registered on the Registry Platform for Evidence-Based Traditional Chinese Medicine (study No.: ChiMCTR2100004795).

Treatment method
The treatment group was given XFHS granules orally, with the following composition: 15 g of *Radix Alba*, 6 g of *Aucklandiae Radix*, 3 g of *Coptidis Rhizoma*, 3 g of *Zingiberis Rhizoma*, 6 g of *Glycyrrhizae Radix*, 6 g of *Scutellariae Radix*, 10 g of U*ncariae Ramulus Cum Uncis*, 15 g of *Fructus Tribuli*, 15 g of *Herba Patriniae*, 6 g of *Citri Reticulatae Pericarpium*, 6 g of *Saposhnikoviae Radix*, 10 g of *Rhizoma Atractylodis Macrocephalae*, and 10 g of *Granati Pericarpium*. The control group was given placebo granules orally, which were similar in appearance, shape, color, and taste to the XFHS granules. Both the XFHS granules and placebo granules were produced by Jiangyin Tianjiang Pharmaceutical Co., Ltd., in accordance with relevant national standards. The recommended dosage was 2 bags twice daily, taken with boiling water, for a total treatment duration of 4 weeks.

Observation indexes

TCM symptom score
The main symptoms (abdominal pain with diarrhea, relief of pain after diarrhea; irritability or sighing) and secondary symptoms (swelling in the hypochondriac region, anorexia, fatigue) were recorded using a grading system of 0, 2, 4, and 6 points for no, mild, moderate, and severe, respectively. The secondary symptoms were recorded using a grading system of 0, 1, 2, and 3 points for no, mild, moderate, and severe, respectively. The sum of individual symptom scores for these TCM syndromes was used as the total syndrome score. The therapeutic effect was evaluated using the nimodipine method, as described in the Guiding Principles for Clinical Research of New Chinese Medicine [6]: Ineffective: symptoms and signs were not significantly improved or were even aggravated, with a symptom score reduction of less than 30%; Effective: symptoms and signs improved, with a symptom score reduction of 30% to less than 70%; Obvious effect: symptoms and signs significantly improved, with a symptom score reduction of 70% to less than 95%; Clinical recovery: main symptoms and signs disappeared or were significantly improved, with a symptom score reduction of 95% or more.

Sample collection, DNA extraction, and 16S rDNA sequencing
Fifteen patients from each treatment group and control group were randomly selected. Fresh fecal samples were collected before and after treatment using sterilized cotton swabs and sterile cryotubes, and immediately stored in a freezer at -80 ℃ for DNA extraction, PCR amplification and machine sequencing by Hangzhou Lianchuan Biotechnology Company.

Bioinformatics analysis
The raw on-off numbers were paired, spliced, and underwent quality control and chimerism filtering. The Differential Amplication Denoising Algorithm (DADA2) was called using qiime dada2 denoise-paired for length filtering and denoising. Based on this, the

concept of Amplication Sequence Variables (ASVs) was used to construct an Operational Taxonomy Units (OTU) table, resulting in the final ASV feature table and feature sequence. Further diversity analysis, species classification annotation, and difference analysis were performed.

Statistical analysis

All data were statistically analyzed using SPSS25.0 software. Measurement data that conformed to a normal distribution were analyzed using a T-test and expressed as the mean ± standard deviation (mean ± SD). Non-parametric rank sum tests were used for measurement data that did not conform to a normal distribution. Chi-square tests were used for count data, and nonparametric rank sum tests were used for rank comparisons. $P < 0.05$ was considered statistically significant.

Results

Enrollment and baseline characteristics

Sixty-six patients with IBS-D were treated at our hospital from June 2021 to January 2022. Nine cases dropped out due to the epidemic, use of antibiotics, poor compliance, and other reasons, leaving a final sample of 28 cases in the treatment group and 29 cases in the control group, with 15 cases in each group selected for determination of intestinal flora. There were no significant differences in gender composition, age, or course of disease between the two groups. No adverse reactions or other events occurred during the trial. The study process are presented in Figure 1.

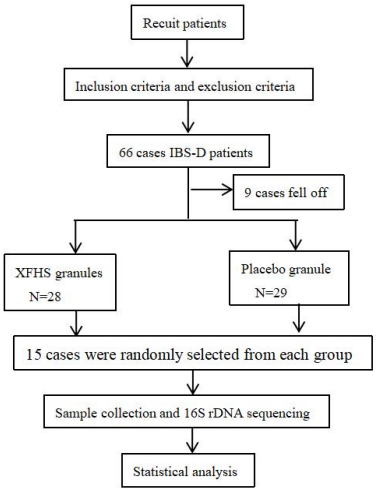


Figure 1 Flow chart of study

Clinical efficacy evaluation

After treatment, the total effective rate of the treatment group and the control group were 92.86% and 58.62%, respectively. The results show that the therapeutic effect of XFHS granules in the treatment of IBS-D patients was significantly higher than that of the placebo granule, with a statistically significant difference ($P < 0.05$). The details are shown in Table 1.

Comparison of TCM symptom scores

After treatment with XFHS granules, the single symptoms of IBS-D patients, such as abdominal pain with diarrhea and relief of pain after diarrhea, irritability or sighing, swelling in the hypochondriac region, anorexia, and fatigue, were significantly improved compared to before treatment, with statistical significance ($P < 0.01$). After treatment with placebo granules, the symptoms of abdominal pain with diarrhea and relief of pain after diarrhea, irritability or sighing, were significantly improved compared to before treatment ($P < 0.01$), while fatigue was improved compared to before treatment ($P < 0.05$), but there was no significant improvement in the symptoms of swelling in the hypochondriac region and anorexia ($P > 0.05$). Among them, the XFHS granules showed better improvement in the symptoms of abdominal pain with diarrhea and relief of pain after diarrhea, anorexia, and fatigue in IBS-D patients compared to placebo ($P < 0.05$). The details are shown in Table 2.

Microflora analysis results

Number of ASVs. The concept of ASV was used to construct the class OUT table, and species classification annotation, diversity analysis, difference analysis, etc. were performed. A Venn diagram was created based on the abundance of ASVs in each group. The total number of ASVs in the XFHS granules group before and after treatment was 921, with 1477 unique ASVs before treatment and 1041 unique ASVs after treatment. The total number of ASVs in the placebo granule group before and after treatment was 1213, with 1463 unique ASVs before treatment and 1346 unique ASVs after treatment. The details are shown in Figure 2.

Alpha diversity and beta diversity. Alpha diversity analysis of the two groups before and after treatment showed that the community contained the number of species (Chao1 and Observed species), the microbial coverage (Goods_coverage) and the shannon index, and no statistically significant differences were observed after rank and test ($P > 0.05$), as shown in Figure 3.

Beta diversity before and after treatment in the two groups: principal coordinate analysis of PCoA results showed that the distribution of each sample point in this study was relatively discrete, and the same group of samples did not show obvious clustering phenomenon, indicating that there was no significant difference in Beta diversity after treatment. The details are shown in Figure 4.

Table 1 Comparison of clinical efficacy (cases)

Group	Number of cases	Clinical recovery	Obvious effect	Effective	Ineffective	Effective rate
Treatment group	28	3	6	17	2	92.86%$^{\rho}$
Control group	29	0	4	13	12	58.62%

Compared with the control group, $^{\triangle}P < 0.05$.

Table 2 Comparison of single scores of TCM syndromes between the two groups

Single syndrome	Treatment group		Control group	
	Before treatment	After treatment	Before treatment	After treatment
abdominal pain refers to diarrhea, pain reduction after diarrhea	6(6,6)	2(0.5,2)$^{\#\rho}$	6(4,6)	2(2,5)$^{\#}$
irritability or sighing	4(4,6)	2(0,2)$^{\#}$	4(2,6)	2(0,3)$^{\#}$
two hypochondriac swelling	1(1,2)	0(0,1)$^{\#}$	1(1,2)	1(0,2)
anorexia	1(0,2)	0(0,1)$^{\#\rho}$	1(0,2)	1(0,3)
fatigue	2(1,2)	1(0,1)$^{\#\rho}$	2(1,2)	1(1,2)*

Compared with before treatment, $^{\#}P < 0.01$, $^{*}P < 0.05$; Compared with the control group, $^{\triangle}P < 0.05$

Structural analysis of intestinal flora. At the phylum level, as shown in Figure 5, Firmicutes and Bacteroidetes were the dominant bacteria in patients with IBS-D. The others are Proteobacteria, Actinobacteria and Fusobacteria, etc. The relative abundance of Firmicutes decreased after treatment with XFHS granules, but increased after treatment with placebo. The relative abundance of Bacteroidetes increased after treatment with XFHS granules, but decreased after treatment with placebo. After treatment, the abundance of Proteobacteria and Clostridium decreased, while the abundance of Actinobacteria increased in both groups, with no statistical significance ($P > 0.05$).

At the genus level, as shown in Figure 6, the relatively high abundance is Faecalibacteriu, Bacteroides, Bifidobacterium, Escherichia-Shigella, Megamonas , streptococcus, Prevotella_9, etc. The abundance of Lachnoclostridium, active Ruminococcus and Lutispora decreased after treatment with XFHS granules, and the difference was statistically significant ($P < 0.05$). The abundance of Enterobacter and Oscillibacter decreased after treatment with placebo granules, while the abundance of Oribacterium increased, both of which were statistically significant ($P < 0.05$). The abundance of Bifidobacterium increased in both groups after treatment, but there was no statistical difference ($P > 0.05$).

LEfSe analysis results showed that there were 13 different bacteria (LDA > 3, $P < 0.05$) before and after the treatment of XFHS granules, and those that were enriched after treatment included: General Bacteria, Rickettsiales, Mitochondria, and Mitochondria-Unclassified species; Enrichment before treatment includes: Lachnospirales, Lachnospiraceae, Cyanobacteria, and active Ruminococcus, as shown in Figure 7. The bacteria enriched after treatment with placebo granules were Enterobacter (LDA > 3, $P < 0.05$), as shown in Figure 8.

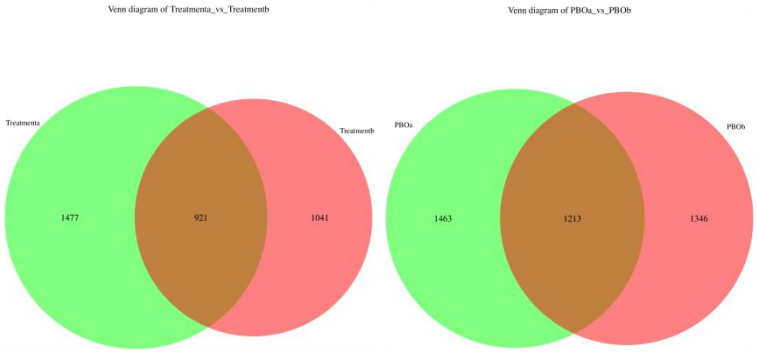
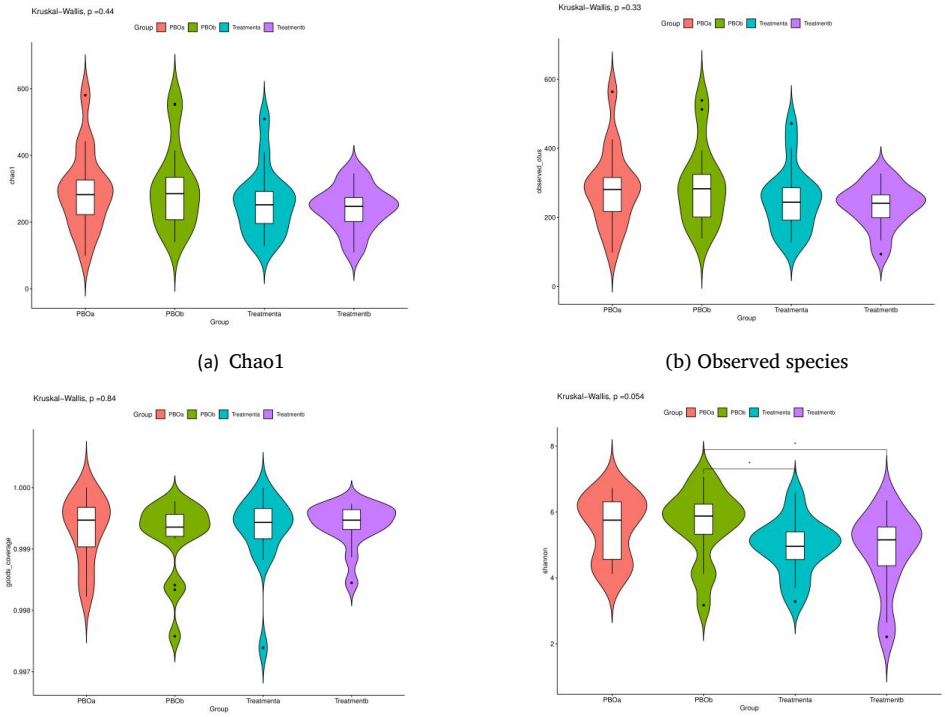


Figure 2 Venn diagram. Treatmenta represents before the treatment of XFHS granules, Treatmentb represents after the treatment of XFHS granules, PBOa represents before the treatment of placebo granules, and PBOb represents after the treatment of placebo granules, the same below.



(a) Chao1 (b) Observed species

(c) Goods_coverage (d) shannon

Figure 3 Alpha diversity violin chart

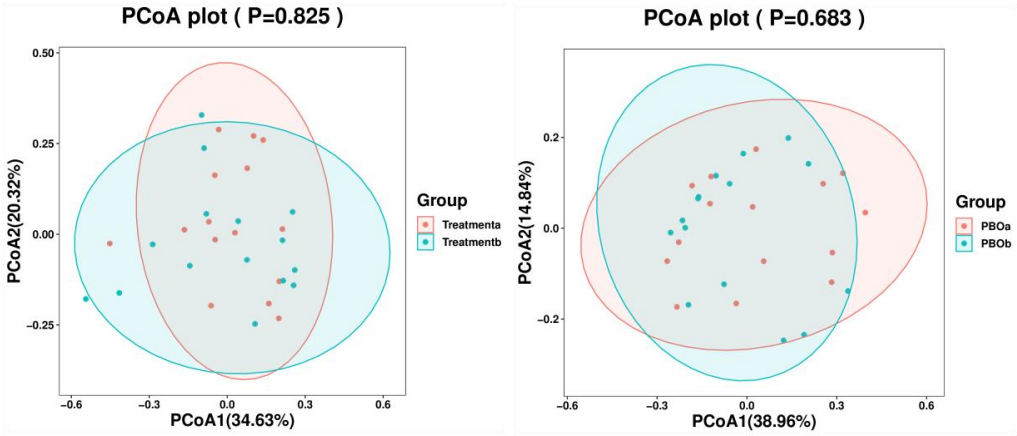


Figure 4 PCoA diagram of Beta analysis. The percentage of the horizontal and vertical coordinates represents how well the first and second axes explain the sample differences

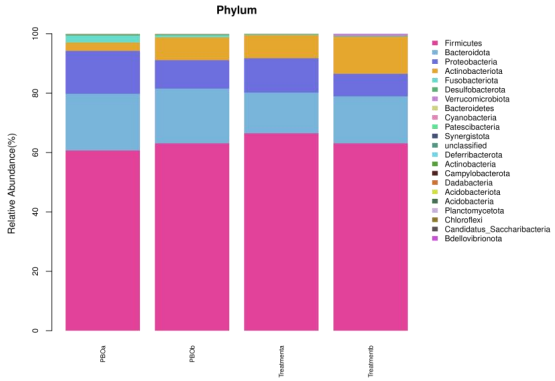


Figure 5 Composition and abundance distribution of intestinal flora in each phylum level group

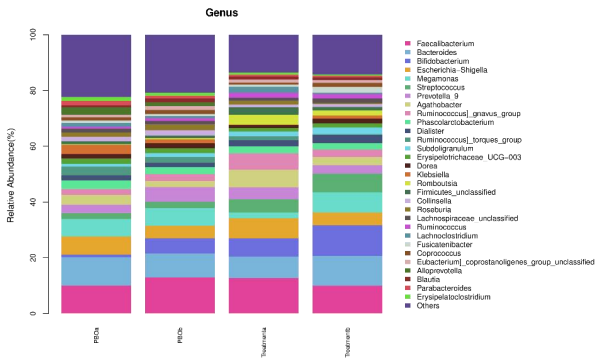


Figure 6 Composition and abundance distribution of intestinal flora at genus level

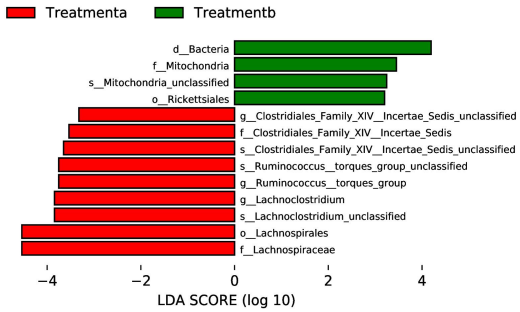


Figure 7 LEfSe analysis of XFHS granules

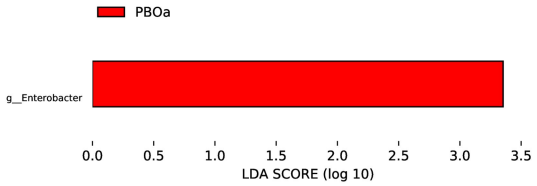


Figure 8 LEfSe analysis of placebo group

Discussion

The main clinical manifestations of IBS-D are abdominal pain and diarrhea, and the disease is difficult to treat. According to TCM theory, the liver wind moving internally and the wind wood restricting the spleen can cause abdominal pain, while the spleen deficiency causes excessive dampness and diarrhea. The dampness becomes turbid and sticky over time, and heat is easily induced. The pathogenesis is complicated. Professor Yao-Zhou Tian therefore developed the XFHS granules by combining the methods of softening the liver, strengthening the spleen, and calming the endogenous wind with the methods of eliminating dampness and clearing heat. Fructus Tribuli is particularly effective at softening the liver, soothing the liver, and extinguishing wind. Radix Alba is essential for relieving pain. Uncariae Ramulus Cum Uncis is useful for treating internal liver wind movement. The three return to the liver meridian, a total of sovereign

medicine; Infection by dampness, liver depression is easy to fire, fear of dampness and heat accumulation, so *Citri Reticulatae Pericarpium*, *Saposhnikoviae Radix*, strengthening spleen, dry dampness and dispelling wind; *Scutellariae Radix*, *Coptidis Rhizoma* clear the dampness and heat of the spleen, stomach and large intestine, *Herba Patriniae* to thicken the intestine to stop diarrhea, a total of minister medicine; With *Rhizoma Atractylodis Macrocephalae*, *Aucklandiae Radix*, *Zingiberis Rhizoma*, strengthen the spleen, activating Qi and stop diarrhea, at the same time to raise the spleen Yang to prevent bitter cold too; *Granati Pericarpium* acid astringent anti diarrhea, *Glycyrrhizae Radix* used to harmonize various medicines. Throughout the whole prescription, the combination of various methods, so that the liver depression solution, spleen Qi transport, liver wind can be flat, heat and humidity, pain diarrhea can be solved. The results of this study showed that the clinical effective rate was as high as 92.86% in the treatment of IBS-D patients with XFHS granules, which could effectively improve the symptoms of "abdominal pain refers to diarrhea, pain reduction after diarrhea", "anorexia", "fatigue" and so on in IBS-D patients.

It has been proved that the intestinal flora of IBS-D patients is different from that of healthy people, as demonstrated by stool and mucosal samples [7]. Many studies have shown that TCM compounds and extracts can regulate intestinal flora, including promoting the reproduction of beneficial bacteria, inhibiting the growth of pathogenic bacteria, and improving the diversity of bacteria at the same time [8-10]. Yan-Xia Zhang treated IBS-D patients with Qiwei Baizhu powder combined with umbilical moxibustion, and found that the stool conditions of patients were improved and the duration of clinical symptoms was shortened, while the intestinal flora of Bifidobacteria and Lactobacillus increased, and Enterobacteria and Enterococcus decreased [11].

16S rDNA analysis has shown that the abundance of Cyanobacteria in the intestinal flora of IBS patients is significantly higher than in healthy individuals, and this may be a key factor in the development of IBS [12]. Previous studies generally believed that compared with healthy volunteers, fecal Firmicutes in IBS patients were higher, Bacteroidetes were reduced, and F/B ratio was increased, which was positively correlated with the severity of intestinal symptoms [13, 14]. The results of this study showed that the abundance of Firmicutes and Cyanobacteria decreased, the abundance of Bacteroidetes increased, and the ratio of F/B decreased after the treatment with XFHS granules. It is therefore speculated that XFHS granules can significantly improve the symptoms of IBS-D patients by regulating the F/B ratio of the intestinal flora in these patients.

It has been reported that, in general, the overall abundance of Proteobacteria is low, and the large number of Proteobacteria in the intestine often reflects the imbalance of intestinal microbial community structure, and the unrestricted proliferation will further promote inflammation or the invasion of exogenous pathogens [15]. Actinobacteria contains Bifidobacteria, Collins bacteria and other recognized beneficial bacteria. Bifidobacteria is believed to be able to prevent diarrhea and intestinal infection, relieve constipation and stimulate the immune system. Many studies have shown that, compared with the healthy control group, the relative proportion of Bifidobacteria and Bacteroidetes in the stool of IBS-D patients is reduced [16, 17]. This study found that the abundance of Proteobacteria decreased in IBS-D patients after treatment with XFHS granules, while the abundance of Actinobacteria, Bacteroidetes, and Bifidobacteria increased. This suggests that the treatment with XFHS granules may be related to the reduction of harmful bacteria such as Proteobacteria and the increase of beneficial bacteria such as Actinobacteria, Bacteroidetes, and Bifidobacteria in IBS-D patients.

There are several limitations to this study. The impact of the epidemic made it difficult to confirm the conditions of some patients after treatment on site, and the sample size included in the study was small. The location of case collection was limited, and the intestinal flora is affected by factors such as diet, region, and specimen collection, which may have influenced the results. Additionally, the clinical symptom judgment and scale filling of patients may have been affected by subjective factors, reducing the accuracy of the results. The treatment time was short and no follow-up was conducted, so the full effect of the drugs on the intestinal flora may not be reflected. While no adverse reactions were observed in the patients during the trial, there was no physical or chemical examination of the patients, so the safety of the treatment could not be fully evaluated. Furthermore, some bacterial groups in the experiment have not even been named due to a lack of relevant data and literature, making it difficult to accurately describe their functions. In the future, it is necessary to conduct large-sample, multi-area studies using metagenomics and other methods to further study the impact of the intestinal flora and search for other possible mechanisms of action.

Conclusion

In conclusion, the efficacious XFHS granules have a definite effect on IBS-D patients and can regulate the structure of the bacterial community and species abundance, which may be one of the mechanisms of its effectiveness.

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