

Effect of Soothing Liver and Invigorating Spleen on Intestinal Permeability and Intestinal Flora in IBS-D Rats

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ABSTRACT

BACKGROUND: The changes in intestinal permeability as important pathogenesis of irritable bowel syndrome (IBS), and involve the disorder of intestinal flora. IBS is a clinical syndrome caused

by multiple factors and multiple target points. Liver depression and spleen deficiency type IBS has become the most common clinical syndrome. This study investigated the therapeutic target and therapeutic effects of Pingwei capsule on IBS-D rats with liver depression and spleen deficiency by detecting the expression of aquaporin and intestinal flora to observe the effects of soothing liver and spleen and the underlying mechanisms.

METHODS: The IBS-D rat model was established with acetic acid enema and stress binding method, and the drug intervention was carried out for 14 days after successful establishment of the model and collection of experimental specimens. ELISA was performed to determine D-lactic acid (D-LA) in rat plasma. IHC and qRT-PCR were used to detect the expression of AQP3, AQP4, AQP8, and ZO-1 content in the proximal colon tissues and the influence of related drugs on their expression. 16S rRNA gene sequencing was used to analyze the changes of fecal microbial community richness and diversity in rats.

RESULTS: The plasma D-LA concentration in IBS-D model rats showed a significant increase ($p < 0.05$). Hepatic soothing and invigorating spleen caused up-regulation of AQP3, AQP4, AQP8, and ZO-1 expression in rats, thus regulating the changes in intestinal permeability ($p < 0.05$). The results of 16S rRNA gene sequencing showed that the method of soothing liver and invigorating spleen could improve the diversity and physiological function of intestinal flora.

CONCLUSIONS: Soothing liver and invigorating spleen are desirable treatment methods for IBS-D rats with liver stagnation and spleen deficiency. This can effectively relieve the symptoms of diarrhea in patients, and has a certain regulatory effect on intestinal permeability and intestinal flora disorders, playing a therapeutic role in IBS-D.

Key words: Irritable bowel syndrome; Intestinal permeability; Intestinal flora; Soothing liver and invigorating spleen

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BACKGROUND

The prevalence of gastrointestinal diseases is increasing with the individual needs and urbanization. IBS has become a common digestive system disease in clinics, which is characterized by repeated abdominal pain, diarrhea, and changes in defecation habits. This is regarded as a functional disease without any conventional medical examination to detect the organic lesions. According to the statistics, the prevalence of IBS in North America and Europe is as high as 15%^[1], and the overall prevalence of IBS in Southeast Asian countries is about 7.0%, all these occupy a considerable proportion of attention^[2]. The prevalence of IBS in women is globally dominant. Although IBS is not associated with increased mortality, it brings huge burden for the sufferers^[3]. In China, IBS cases accounted for 40%-70% of all the remaining digestive diseases in the medical clinics, and the life quality of patients is greatly reduced due to repeated occurrence of symptoms^[4]. However, there is still no clear conclusion to elaborate on pathogenesis. The second Asian consensus on IBS^[5] stated it as a multi-dimensional disease, which includes intestinal dysbiosis, increased intestinal permeability, altered gut-brain interaction, gastrointestinal dyskinesia, visceral hypersensitivity, food intolerance, and psychosocial factors. Also the increase in intestinal permeability is one of the important causes of diarrhea, and there are certain hidden dangers. Therefore, the changes in intestinal permeability have become a hot topic in the current research field. A study has shown^[6] that aquaporin (AQP) channels are involved in regulating fluid homeostasis in the colon, and warrants conduction of further studies on the potential role of AQPs in the pathophysiology of diarrhea in IBS-D. So, by studying the changes of AQP expression in IBS-D colon tissues of rats with liver depression and spleen deficiency, this study investigated the mechanism of changes in the permeability of colon tissues and the intervention mechanism of soothing liver and invigorating spleen.

However, the gastrointestinal diseases have to be linked to the intestinal flora, which is a large microecological system with hundreds of millions of different species. Changes in the gut environment affect the human health, in which the gut bacteria produce metabolites that can affect gut flora, acts as intestinal barrier, causes immune response, and visceral sensation^[7]. Homeostasis of intestinal flora promotes the integrity of intestinal mucosal structure, provides essential nutrients, improves body's immunity, and protects the body from various pathogens^[8]. Moreover, intestinal epithelial barrier plays an important role in preventing bacterial translocation, and disruption of the barrier increases intestinal permeability, causing an immune response to various diseases^[9-10]. Although the pathogenesis of IBS is unclear, intestinal flora are still considered to play an important role in its mechanism. Intestinal epithelial cells provide an effective umbrella to prevent the direct exposure of immune cells to the microbiota, preventing the bacteria from entering the intestinal mucosa and invading the homeostasis of the gut microbiota^[11]. When this umbrella is destroyed, the bacteria then penetrate through the intestinal wall, causing a series of immune responses involved in the pathophysiological changes of IBS^[12]. Therefore, we believe that altered intestinal permeability causes disruption of intestinal mucosa and environmental homeostasis plays a key role in the development of IBS. Therefore, 16S rRNA gene sequencing on the feces of rats with liver depression and spleen deficiency type IBS-D was conducted to observe the diversified changes of rat fecal colonies to further explore the correlation between them.

In traditional Chinese medicine, IBS-D belongs to the category of

“abdominal pain”, “diarrhea”, in which abdominal pain, and diarrhea are the main symptoms. Traditional Chinese medicine believes that diarrhea occurs mainly due to foreign bodies, diet, emotional disorders, and long-term body deficiency. More and more studies have shown that IBS-D involves abdominal pain and diarrhea as the main symptoms, the clinical study of liver depression and spleen deficiency type is regarded as the main syndrome, and then traditional Chinese medicine treatment of IBS-D takes soothing liver and invigorating spleen as the main principle. Liver depression forms a pathological basis throughout the entire course of IBS-D disease, and so soothing liver makes the liver sparsely diarrhea to achieve normal function, causing abdominal pain relief. Liver catharsis is normal, which causes the spleen and stomach to rise and reduce the turbidity function recovery, and according to traditional Chinese medicine “seek the basis of treatment”. In spleen and stomach damage, the spleen and stomach can be transported, and eliminate the water and dampness. The spleen transport causing normal intestinal transformation, and diarrhea can also be alleviated.

Pingwei capsule is a clinical observation, and it is experimentally studied for spleen and stomach diseases in the affiliated hospital of the Gansu University of traditional Chinese medicine for many years. Based on PingWeiSan in Song dynasty, its curative effect was remarkable, which made up for a single treatment principle of western medicine, and highlighted the advantages of relieving of the overall symptoms of the patients. The whole prescription to soothe the liver and strengthening spleen, eliminating dampness and antidiarrheal, has been approved in 2012 by the Gansu University of traditional Chinese Medicine affiliated hospital preparation (license number: Z120022224). Previous studies have confirmed^[13] that Pingwei is a multi-targeted capsule and involve multi-dimensional treatment approach to functional dyspepsia of the liver depression and spleen deficiency, and soothing liver and invigorating spleen are the treatments of liver depression and spleen deficiency of gastrointestinal disease. Nowadays, with the development and popularization of traditional Chinese medicine, many scholars have devoted themselves to the concept of treating diseases, starting from different targets to exploring the pathogenesis and treatment approaches. With the continuous development and improvement of molecular biology research, major scholars were involved in conducting animal experiments, which acts as the leading scientific research, and strived to bring a breakthrough in clinical research by using advanced scientific research technology and preferred experimental methods. The main purpose of this study was to investigate the effect of Pingwei capsule using AQP as therapeutic target in improving the intestinal permeability and relieving of the symptoms of diarrhea. After that the treatment target and key links of Pingwei capsule were elucidated to provide the research ideas and scientific basis for the treatment of the disease and the related pathogenesis.

MATERIALS AND METHODS

Forty eight healthy male Wistar rats with a weight of (200±20) g were provided by the experimental animal center of Lanzhou University. The animal qualification number: N0.62000800000058, experimental animal production license number: SCXK (gan) 2018-0003. Kits for D-LA ELISA in rats (JL20792 JiangLai China), anti-AQP3 antibody (ab125219 Abcam Britain), anti-AQP4 antibody (GB11529 WuHan China), anti-AQP8 antibody (ab203682 Abcam Britain), Goat Anti-Rabbit IgG(h+L)HRP(S0001 affinity USA) and β -actin antibody were obtained. qPCR SYBR Green Master Mix was purchased (11195ES03 ShangHai China).

Drugs

Pingwei Capsule: 0.5g/capsule, (composed of *Atractylodes*, *Magnolia officinalis*, *Fructus Auranti*, *Bupleurum*, *Chenpi*, *radices saussureae*, etc.). For hospital preparation in the affiliated hospital of Gansu University of traditional Chinese Medicine (Approval code: Z120022224), the traditional Chinese Medicine was prepared by modern technology. Pinaverium Bromide Tablets: 50mg/pill (Dicetel operative norm: Registration criteria for imported drugs JX20160115; license number: H20160396; manufacturing enterprise: MYLAN LABORATORIES SAS.)

Animal groups and Animal model

After one week of adaptive feeding, all 48 healthy male Wistar rats were divided into normal group, IBS-D model group, Pividonium Bromide Tablets group (PWB), and low- (PWI), medium- (PWII), and high-doses (PWIII) Pingwei capsule groups (with 8 rats in each group). Routine feeding was performed in the normal group and acetic acid and stress binding enema were used for the rats in the model group according to the internationally accepted method, and the stress-bound model-making method established a model of hepatic-depression-type in IBS-D rats for 21 days^[14]. No mice died during modeling.

Treatment medication

After the model was established, The doses administered through a gastric tube were determined using the body surface area conversion ratio between rats and humans, which amounted to 13.75, 27.5 and 55mg/mL Pingwei capsule suspension for the low-, medium-, and high-dose Pingwei capsule groups, respectively; 2.5mg/ml suspension for the Pividonium Bromide Tablets group, and physiological saline for the control and IBS-D model groups. Each dose was 1 ml/100g by gavage twice a day for 14 days, at least for an 8h interval.

Collection of specimens

After treatment, 2ml blood was collected in the cardiac blood by anticoagulation, and then centrifuged (4°C, 4000 rpm, 20min) after standing for 10 min. The upper plasma was taken and stored in an enzyme-free centrifugation tube at -80°C for storage to be tested. Two segments from the proximal colon tissues were taken, and one segment of the colon tissue was preserved at 4°C or at room temperature after 4% paraformaldehyde fixation for immunohistochemistry, and the other segment of the colon tissue was stored at -80°C after rapid freezing under liquid nitrogen for qPCR experiments.

Dry-wet specific gravity of feces

The stools of rats were collected from 8 a.m. to 12 a.m., wet weight was weighed, dried in an oven (75°C, 2.5h) and then the dry weight was weighed. The Dry-wet specific gravity of feces was calculated using the following formula: Dry-wet specific gravity = (wet weight - dry weight) / wet weight × 100%^[15].

Sugar-water preference

Each group was given two bottles of water, the two bottles of 1% sucrose water 100ml within the first 24 hours, one bottle of 1% sucrose water 100ml and another bottle of purified water 100ml within the second 24 hours. After fasting for 24h, a sugar water/pure water partiality experiment was conducted in rats. Each group of rats was given two bottles of water in advance: one bottle of pure water and another bottle of sucrose water 100ml each. An hour later, the two bottles of water were taken and weighed, and the sugar water consumption, the pure water consumption and the sugar water bias were

calculated using the following formula: Sugar water bias rate = (sugar water consumption / total liquid consumption) × 100%^[16].

ELISA

The levels of D-LA in the serum were quantitated by ELISA. The OD values were measured after 30 min using an enzyme marker (MA-100A, HangZhou) at 450 nm wavelength according to the recommendations of the kit instructions. The concentration of D-LA in the plasma was calculated according to the measured data.

quantitative real-time PCR (qRT-PCR) mRNA assay

Total RNA extraction kit of animal tissues was selected to extract the total RNA of proximal colon of rats. The primers were synthesized by Shanghai Shengong Bioengineering Co. Ltd (Table 1). The reaction system was shown in the table below (table 2). The amplification conditions are operated according to the instructions, and the qRT-PCR instruments and ASA-9600 system software were used to set the program, obtained the data and the relative expression level of the target protein was calculated using the 2- $\Delta\Delta C_t$ method.

IHC staining

Streptavidin-alkaline phosphatase labeling system of indirect immunohistochemistry (SP-IHC) was used to observe the expression of AQP3, AQP4 and AQP8 in the colon tissues of rats to observe the changes of intestinal permeability in the colon mucosa of rats in each group. The tissue was first fixed in 4% paraformaldehyde solution, embedded in paraffin wax, cut into 4 μ m thickness tissue sections, and then baked at 60°C in an incubator for 3h. For xylene dehydration and gradient ethanol hydration, 3% dioxygen deionized water was inactivated for 10 min, goat serum was added for about 15min, and then incubated overnight at 4°C with the primary antibody (AQP3 dilution ratio of 1:300, AQP4 dilution ratio of 1:1200, AQP8 dilution ratio of 1:300). Again incubated at 37°C with the secondary antibody for 15 min. The sections were stained with DAB color for 3-10min, and then with ethanol gradient hydration. Finally, The sections were sealed using neutral gum and photographed under a microscope at $\times 200$.

16S rRNA gene sequencing

The genomic DNA of the sample was extracted by CTAB method, and then the purity and concentration of the DNA were detected by agarose gel electrophoresis. A proper amount of the DNA sample was taken in a centrifugation tube and the sample was diluted to 1ng/ μ l using sterile water. The microbiota 16SrRNA (V3-V4) region primers were used for the diluted genomic DNA as a template. The forward primer was 341F 5'-CCTAYGGGRBGCASCAG-3', and reverse the primer was 5'-806R GGACTACNNGGGTATCTAAT-3' for PCR purification and amplification. PCR products were detected by electrophoresis using a 2% agarose gel, followed by library construction and computer sequencing. According to sequence alignment, the operational taxonomic units (OTUs), which are the representative sequences are constructed using the software MUSCLE (Version 3.8.31) for phylogenetic relationship. Using Uparse (v7.01001) software to cluster all clean reads of all samples, the sequence is clustered into OTUs by defaulting with 97% consistency. According to its algorithm principle, the sequences with the highest occurrence frequency in OTUs are selected as the representative sequences. The OTU sequences were annotated, and finally the data were analyzed and processed by community taxonomic composition, alpha diversity, beta diversity and so on. There were parameter test and non-parameter test in the analysis of Alpha Diversity Index.

T-test and wilcoxon test were used between the two groups, and Tukey's test and wilcoxon test of agricolae package were used for many groups.

Statistical Analysis

SPSS 24.0 software was used for statistical analyses. All experimental data were expressed as means±SD. Student's t-test was used for comparison between the two groups, and multi-group statistics were performed using univariate analysis to calculate significant differences between 95% confidence interval measurement and control group. The statistically significant threshold was set as $p < 0.05$.

RESULTS

Rat Behavior

During the course of the experiment, the general condition of all rats was observed. The rats during the adaptive feeding period were in good condition, had smooth glossy hair, good activity, without lethargy and significant difference in the body weight. After modeling, the rats in the model groups had poor spirit, were lethargic, had dark yellow hair, decreased activity, reduced body weight, loose stools, like to pile up, irritable and other typical symptoms of liver depression and spleen deficiency. After treatment, the condition has been improved, the somnolence state was obviously improved, hair was smooth, became active gradually, weight was increased, had normal stools.

The results showed that the fecal water content of the rats was obviously higher than that of the normal group after modeling ($p < 0.05$). In the course of the experiment, the changes in the fecal morphology of rats showed dilute stools in rats. This showed that this model was established successfully. The results showed that the treatment effect in rats of the Pingwei capsule group was better than that of Pivian ammonium bromide group ($p < 0.01$). The therapeutic effect of different doses of Pingwei capsule group was not obvious. Therefore, traditional Chinese medicine has some advantages in improving the symptoms of diarrhea (Figure 1A).

The results showed that the partial rate of sugar-water was lower than that of the normal group ($p < 0.01$). During the course of the experiment, the symptoms of hepatic depression and spleen deficiency such as like to pile up, irritability, diet reduction and so on were observed. However, the symptoms of hepatic depression and spleen deficiency after treatment were improved, but there was no significant difference between Pingwei capsule group and Pivian ammonium bromide group ($p < 0.01$), (Figure 1B).

Expression of D-LA

The results of ELISA showed that the content of D-LA in the rat model was higher than that in the normal group ($p < 0.05$), and this reflected the change in intestinal permeability of rats. After treatment, the content of D-LA in the rat plasma was decreased, and showed no significant difference between the spleen and stomach ammonium group and the Pingwei capsule group ($p < 0.05$), (Figure 1C).

qRT-PCR mRNA assay

The results of quantitative qRT-PCR showed that the expression of AQP and ZO-1 in colon tissues of rats in the model group was significantly lower than that in normal group, and the data showed statistical significance ($p < 0.001$). After treatment, the expression of AQP and ZO-1 was up-regulated in rat colon tissues, and the effect of different drugs and different doses of treatment varied. Data analysis showed that the expression of AQP3, AQP8, ZO-1 mRNA was significantly up-regulated in PWBj and PWII groups; the expression

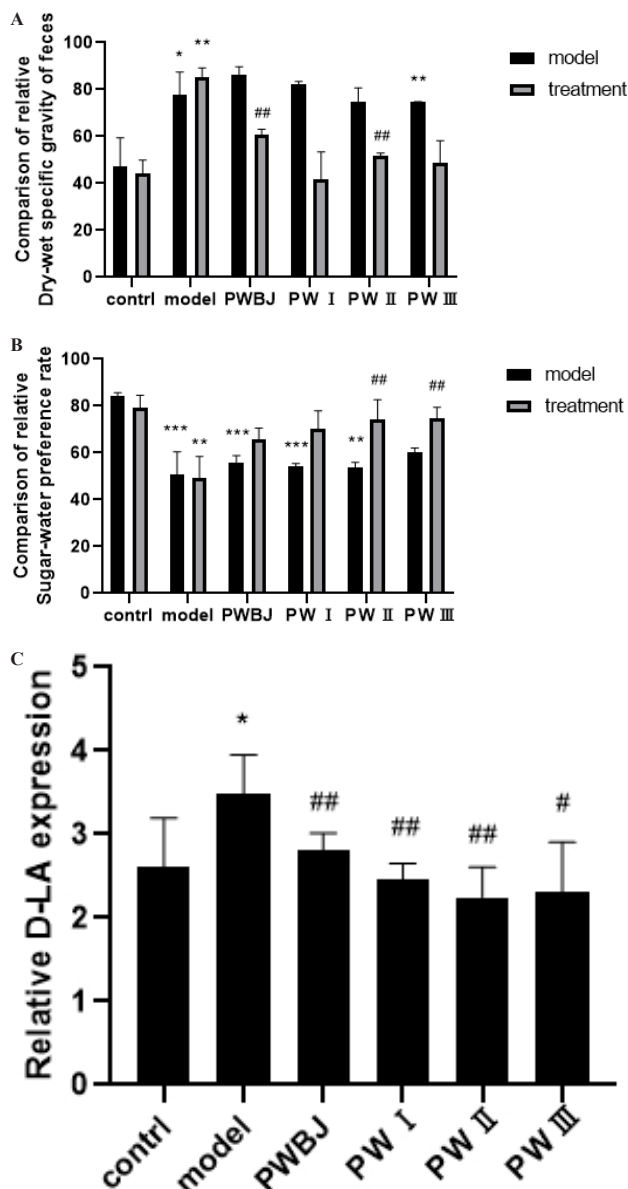


Figure 1 Before and after the intervention of soothing the liver and invigorating the spleen, the behavior of rats and the change of D-LA in plasma were analyzed. (A) Changes in the Dry-wet specific gravity of feces in the six groups of rats: control, model, PWBj, PWI (low-dose Pingwei capsule groups), PWII (medium-dose Pingwei capsule groups), and PWIII (high-dose Pingwei capsule groups). (B) The variation of sugar water preference in the six groups of rats: control, model, PWBj, PWI, PWII and PWIII. (C) ELISA was used to detect the changes of D-LA content in the plasma of six groups of rats: control, model, PWBj, PWI, PWII, and PWIII. Comparison with control * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Comparison with model # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ indicating statistical significance of the data.

of AQP4 mRNA was significantly up-regulated in PWBj, PWI and PWIII; Therefore, the effect of Pinaverium Bromide Tablets and Medium dose of Pingwei Capsule on up-regulating the expression of AQP in rat colon was better, showing statistical significance ($p < 0.05$).

IHC staining assay of AQP

The change of AQP expression in the colon tissues of rats by immunohistochemical SP method showed that the expression of AQP in colon tissues of rats in model group was significantly lower than that in normal group. Compared with rats in the model group, The expression of aquaporin in colon tissues of rats after treatment was increased. AQP3 was mainly expressed on the top membrane of the colon mucosa-absorbing epithelial cells, and a small amount of

expression was observed in the cytoplasm, and the brown granules are considered as positive expression (Figure 3A). AQP4 was mainly expressed on the cell membrane of the deep intestinal gland cells of the colon mucosa, and all these were absorbed cells, and brown

granules were considered as positive expression (Figure 3B). AQP8 was mainly expressed in the colon mucosal epithelial cells and goblet cells, and the brown granules were considered as positive expression (Figure 3C).

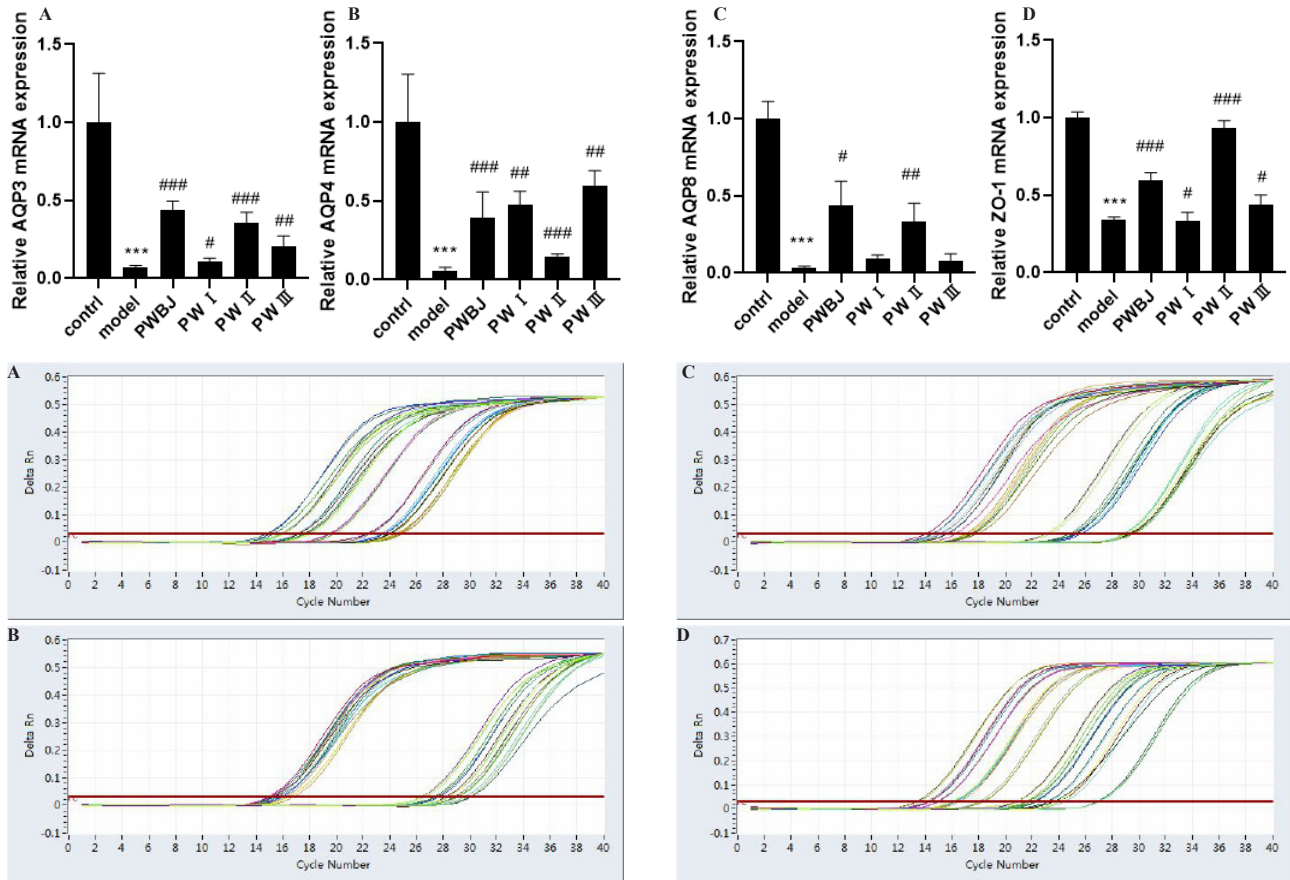


Figure 2 qRT-PCR was performed to detect the expression of AQP3, AQP4 and AQP8 mRNA in the colon of rats before and after intervention of soothing liver and invigorating spleen. (A) Changes of AQP3 mRNA in the colon tissues of rats; (B) Changes of AQP4 mRNA in the colon tissues of rats; (C) Changes of AQP8 mRNA in the colon tissues of rats. Comparison with control; (D) Changes of ZO-1 mRNA in the colon tissues of rats. Comparison with control * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Comparison with model # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ indicating statistical significance of the data.

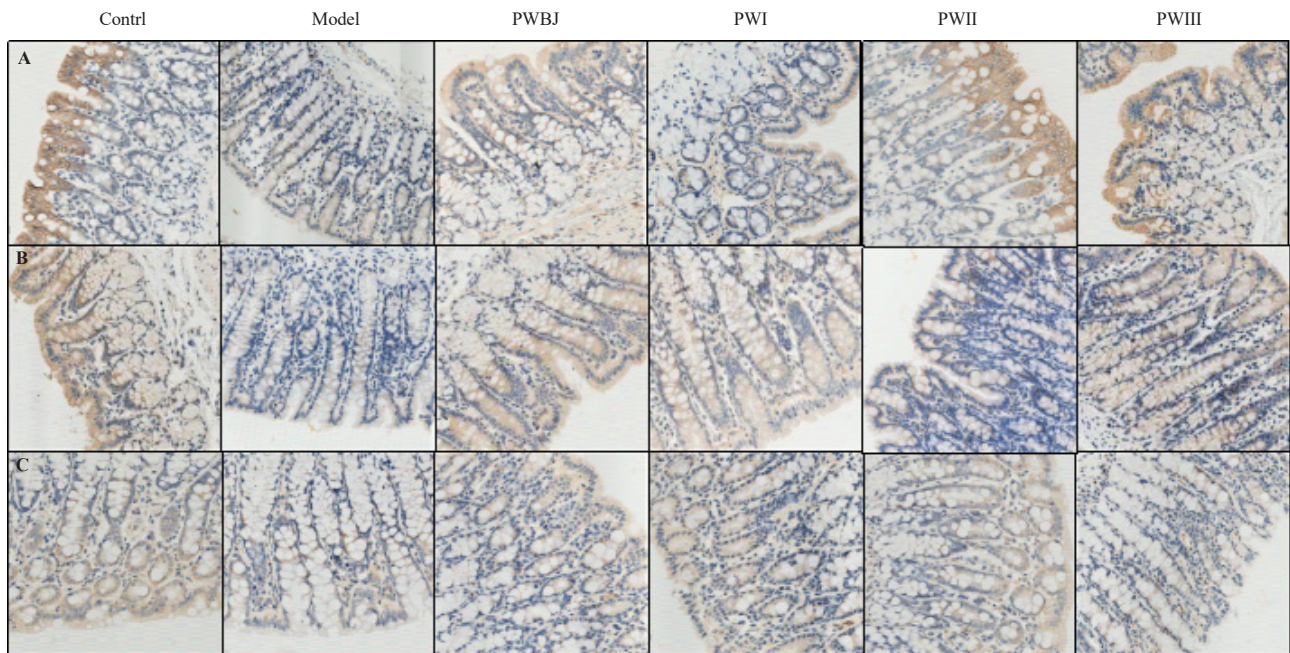
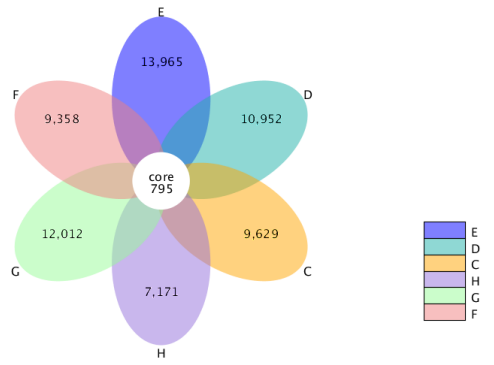
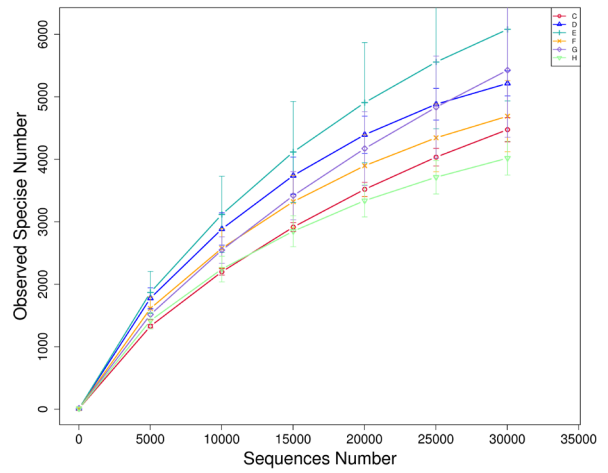


Figure 3 The expression of AQP3, AQP4 and AQP8 proteins in the colon tissues of rats before and after the intervention of soothing the liver and invigorating the spleen. (A) Changes in the expression of AQP3 protein in the colon tissues of each group of rats; (B) Changes in the expression of AQP4 protein in the colon tissues of each group of rats; (C) Changes in the expression of AQP8 protein in the colon tissues of each group of rats.

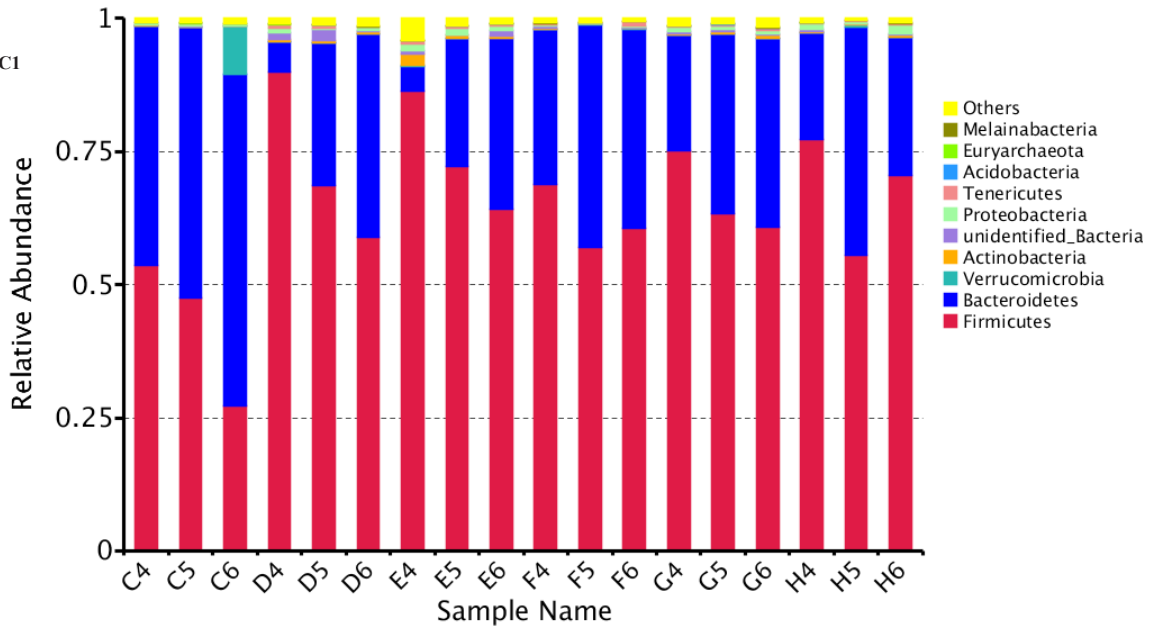
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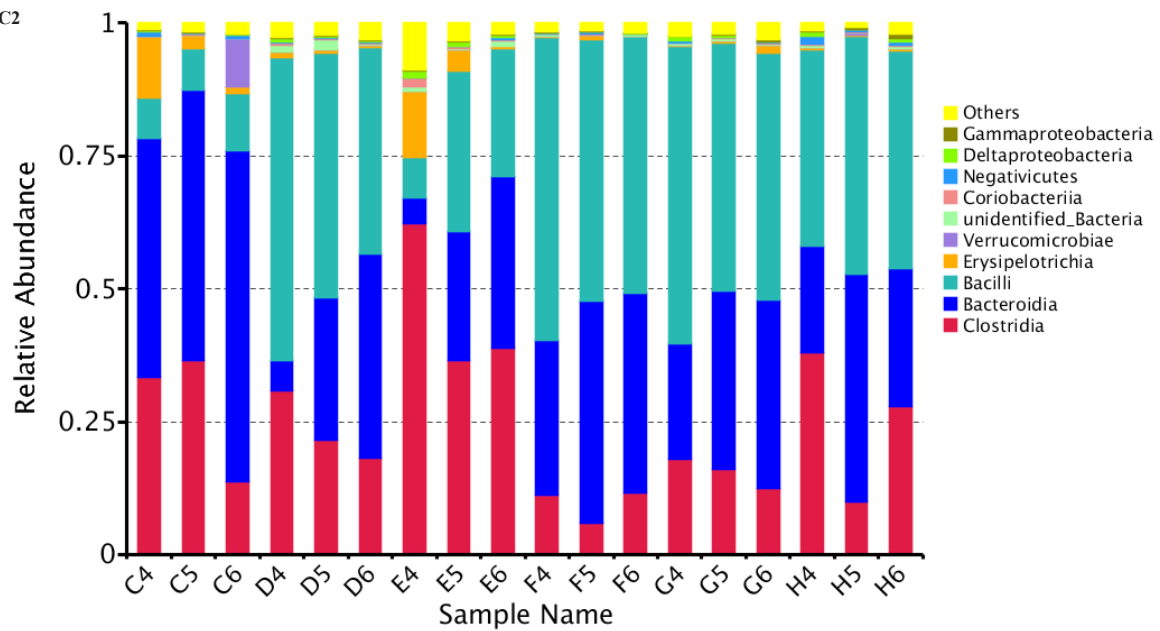
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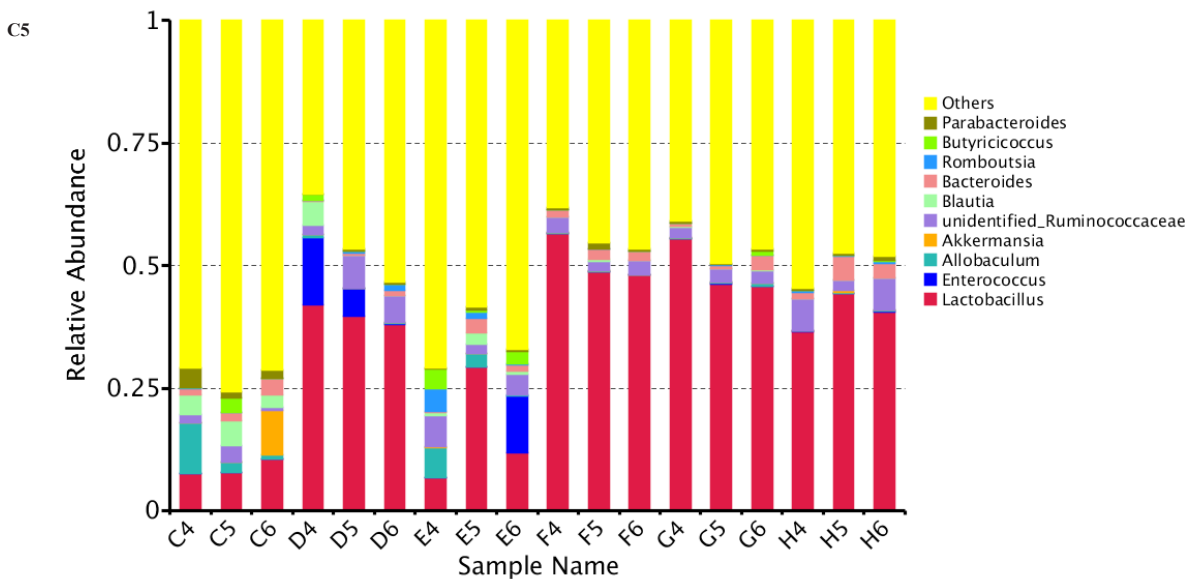
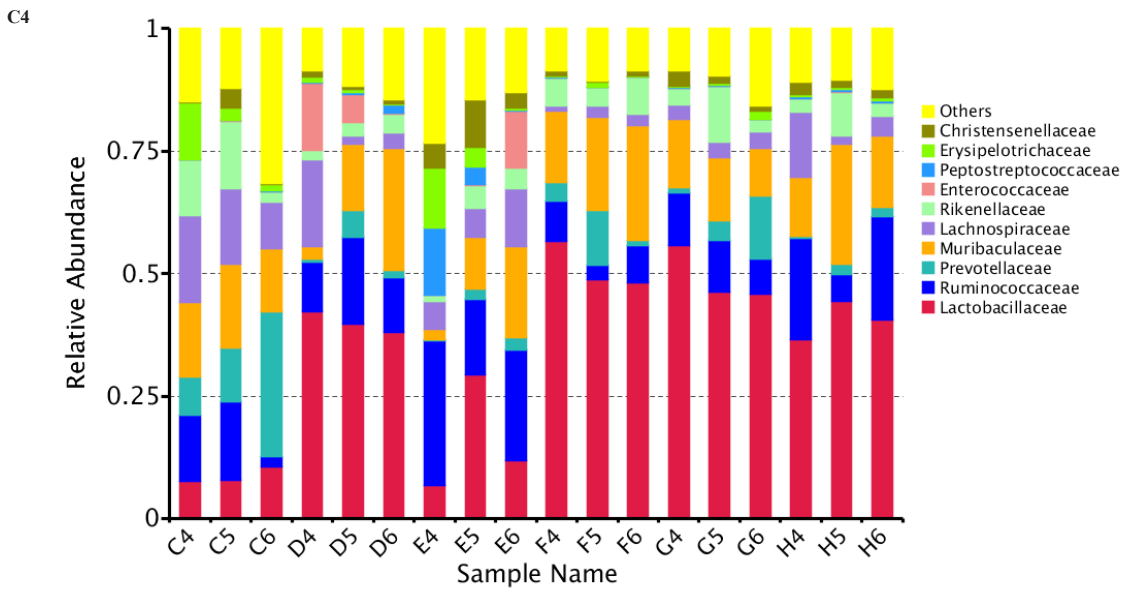
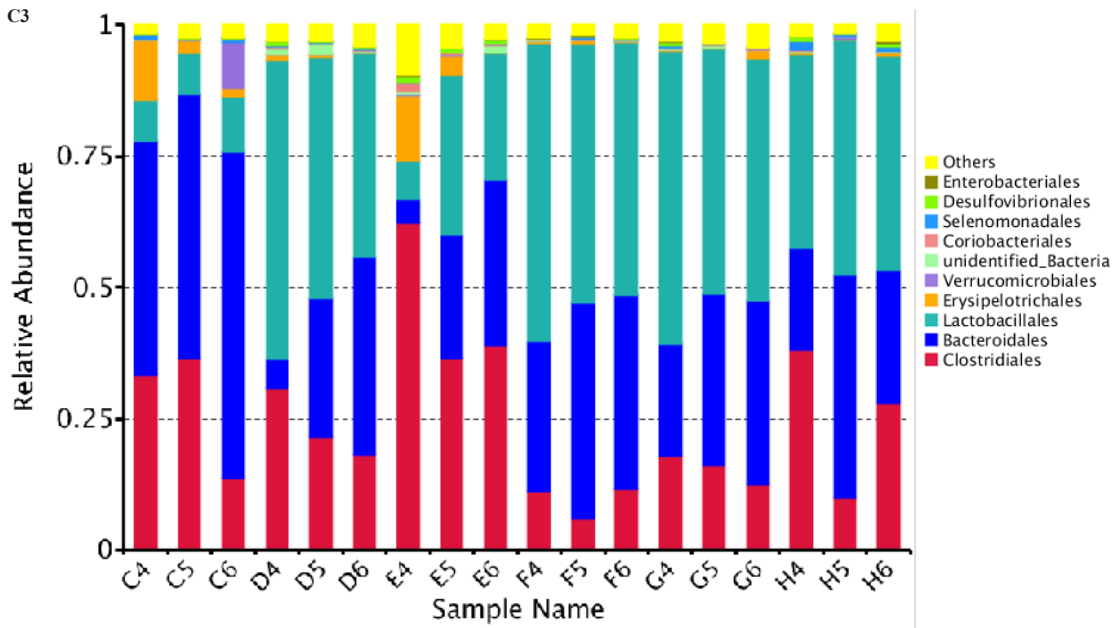


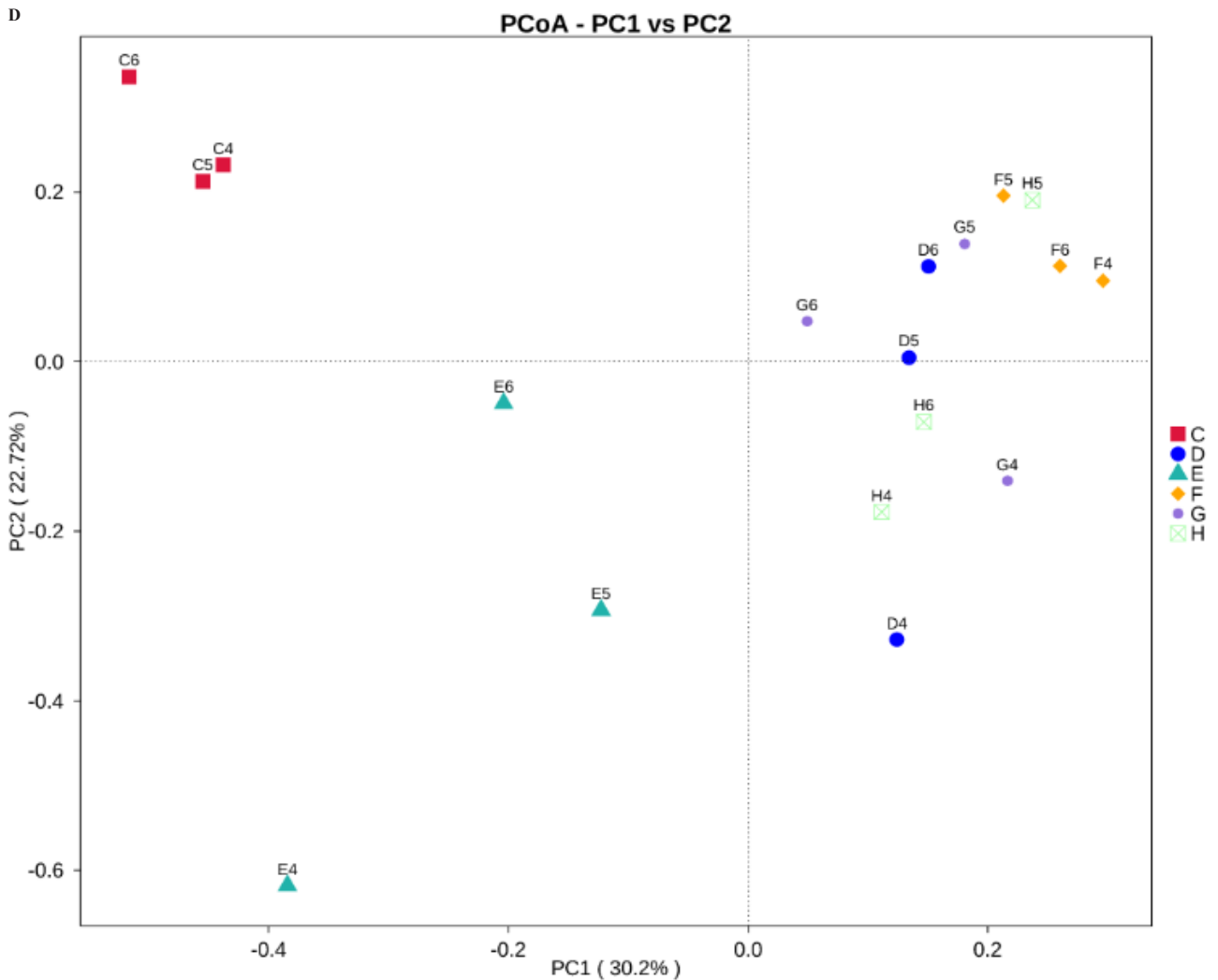
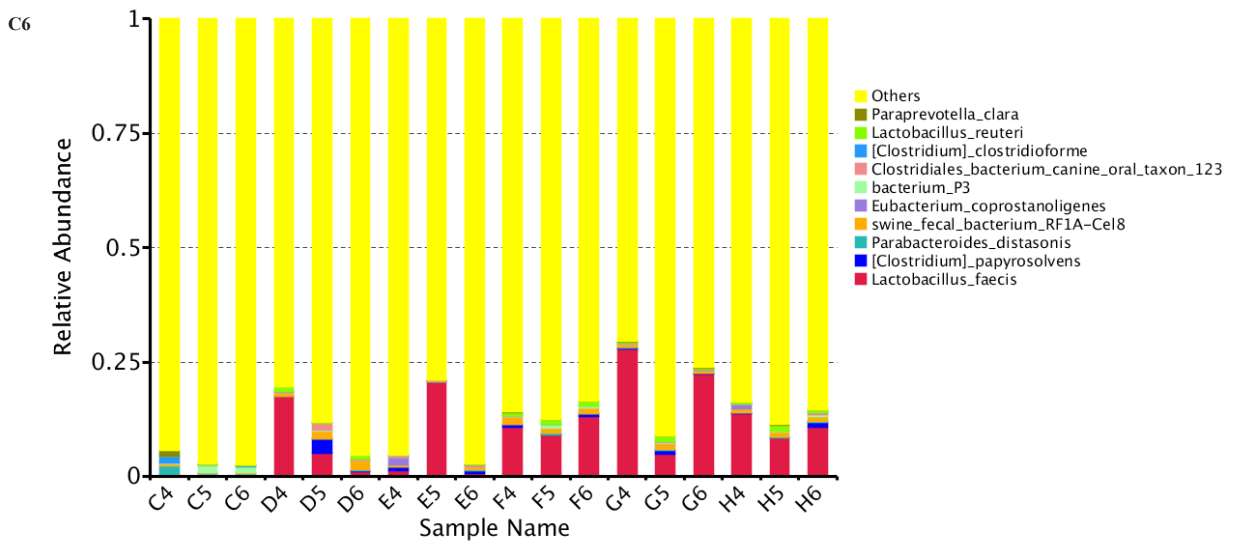
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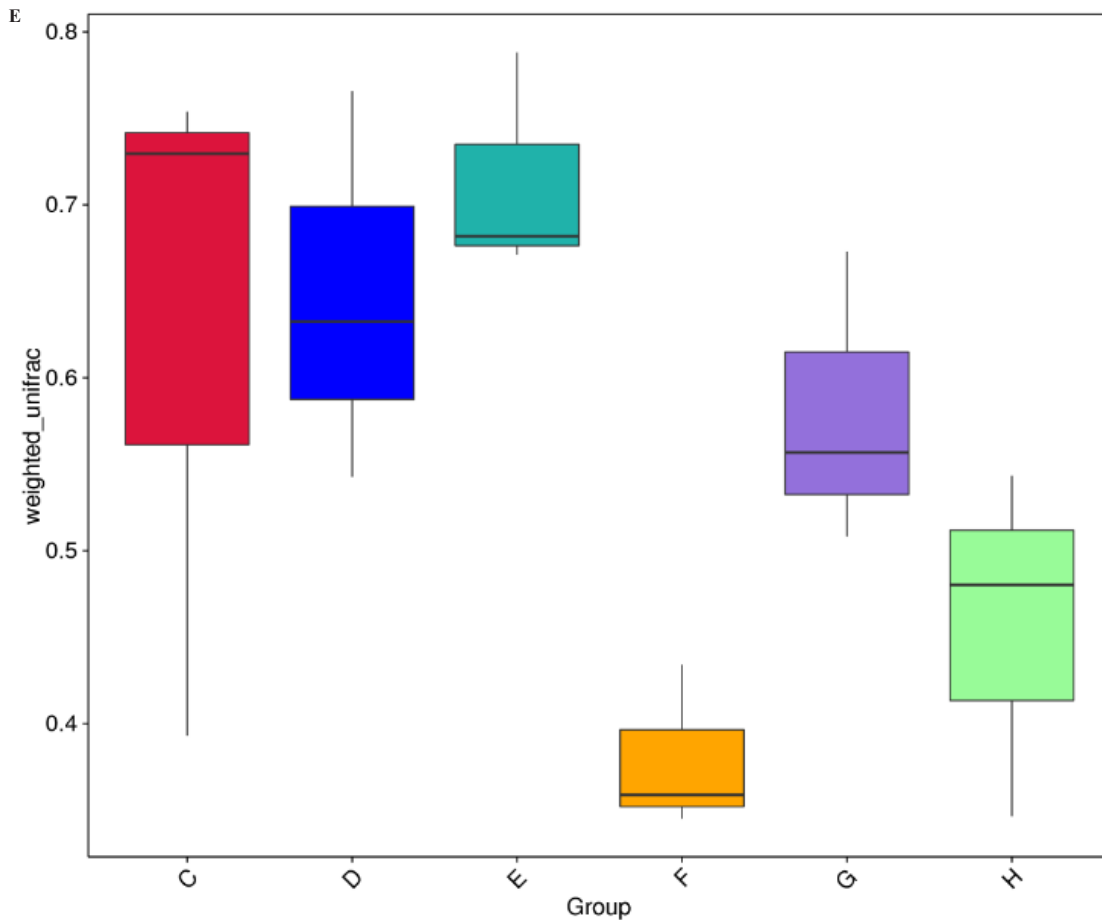


Figure 4 The flower plot intestinal flora in rat from each group (D:control; E:model; C:PWBJ; H:PWI; G:PWII; F:PWIII); B: dilution curve of alpha diversity of intestinal flora in rats of each group; C: Relative abundance of differences of species in the intestinal flora of rats in each group (C1:phylum; C2:class; C3:order; C4:family; C5:genus; C6:species). D: PCoA analysis of gut bacterial communities; E: Boxes based on Weighted Unifrac beta diversity.

16S rRNA gene sequencing

Three samples from each group were analyzed to get the OTU data, and were presented by the way of petal map. There were 795 OTUs in six groups, 10952 in Control, 13965 in Model, 9629 in PWB, 7171 in PWI, 12012 in PWII and 9358 in PWIII (Figure 4A). The results showed that the OTUs in the Model were more than the Control, and the OTUs in each group were decreased after treatment. The dilution curve was used to reflect the sequencing depth, which directly reflects the rationality of the sequencing data quantity, and indirectly reflects the richness of the species in the sample. When the curve tends to be flat, it shows that the sequencing data quantity was gradual and reasonable, and more data quantity produces only a small number of new species (Figure 4B). Based on the species annotation results of OTUs, the species with the highest abundance in each sample at the phylum, class, order, family, genus, species levels that are ranked top 10 were selected, and the relative abundance columnar cumulative graph was generated to observe each sample at different classification levels, and the species with high relative abundance and its proportion. The results showed that the intestinal flora of the Model had fewer species at the phylum, class, family, genus, species, and OTUs levels when compared to Control (Figure 4C). The composition of microbial community structure of intestinal flora in six groups of rats mainly included Firmicutes, Bacteroides and Proteobacteria at phylum level, in which the relative abundance of Model Firmicutes was increased and its relative abundance was decreased after treatment. The relative abundance of Bacteroides in

PWB group was higher than that of Model. At genus level, it was mainly composed of Lactobacillus, Enterococcus, and Bacteroides. Among these, the relative abundance of probiotics Lactobacillus decreased significantly in Model, and the relative abundance of Lactobacillus in different groups of Pingwei capsules were significantly higher than that of the Model. To analyze the extent of differences in the composition of intestinal flora OTUs in the samples of all groups, the principal co-ordinates analysis (PCoA) based on the Weighted Unifrac (a new phylogenetic method for comparing microbial communities)^[17] distance extracted the most important elements and structures from the multidimensional data through a series of eigenvalues and eigenvector ranking, with an abscissa contribution of 30.2% and an ordinate contribution of 22.7%. The results showed that the difference of colony composition between Model samples was large, and the difference of colony composition between the samples after drug intervention was small, indicating that Pingwei capsule and PWB could significantly affect the intestinal flora of rats (Figure 4D). Some studies have revealed that the microbial colony composition changes with the change of collection time and environment^[18]. Therefore, it is necessary to expand the sample size for further validation analysis.

DISCUSSION

IBS-D is a well-known functional gastrointestinal disease. The changes in intestinal permeability and the imbalance in intestinal flora play an important role in its pathogenesis, and there is a certain

correlation between them. Previous studies have shown that intestinal permeability plays a vital role in the prevention and control of ectopic bacteria, and the structural damage of intestinal mucosal barrier increases intestinal permeability, resulting in the disorder of ectopic intestinal bacterial flora, which might in turn lead to varied immune responses^[10,19]. When the intestinal environmental homeostasis is destroyed, the function of intestinal mucosal biological barrier is affected, further leading to the damage of mechanical as well as immune barriers. The disorder between the intestinal immune system and the intestinal symbiotic flora disrupts the dynamic balance of the intestinal microbiota, resulting in dysbiosis, impaired integrity of the intestinal barrier, increased intestinal permeability, and leads to two-way regulating exacerbation of the imbalance^[20], thus forming a vicious cycle state. Therefore, effective treatment of diseases in early stages is considered particularly important.

Studies have shown^[21-22] that damage to the intestinal mucosal barrier increases the intestinal epithelial cell permeability, whereas increased levels of D-LA in blood reflect the changes in intestinal mucosal permeability. In other words, the levels of D-LA can explain the extent of damage to the intestinal mucosal barrier function. Therefore, this study adopted the content of D-LA into the plasma of model rats to determine the change of intestinal permeability. Human intestinal tract is a complex microecological system and D-LA acts as a metabolite of natural bacteria in the gastrointestinal tract, producing and releasing into the blood. The results showed that the plasma D-LA levels were increased, which suggested abnormal changes in intestinal permeability^[23]. In short, various barriers that make up the intestinal mucosa interact with various co-biotypes of the gut in promoting the imbalance of intestinal osmotic pressure and thus causing symptom manifestations that cause changes in intestinal permeability.

Scientific studies have proved^[24-25] that rapid transmembrane transport of water molecules is achieved through a water channel protein on the cell membrane. Physiology studies have shown that AQPs are found in many organisms, such as animals and plants, and play an important role in maintaining normal life activities. AQPs are important membrane proteins that assist in maintaining the balance of human body fluids, which is involved in regulating the homeostasis of intestinal fluid and providing selective pores for rapid movement of water on different cell membranes, and is inseparable from intestinal water metabolism. AQP3 is a typical glycerol channel protein that assists in transportation of macromolecular substances such as water and urea. AQP4 is classified as a water selective classical AQP gene and acts as a regulator of cross-cell flow, wherein rapid water exchange enables tissues and organs to secrete and absorb water. AQP8 is an ammonia channel protein that transports water, urea, and ammonia with selective permeability into water^[26-27]. They maintain the balance of osmotic pressure. It has been reported^[28] that AQPs showed certain correlation with the expression of tight junction proteins, which in turn play an important role in intestinal mucosal barrier. The main role of it is to link transmembrane proteins to the actin cytoskeleton, and the changes in their function and structure can cause tight junction dissociation. ZO-1, as a scaffold molecule, mediates the connection between transmembrane junction and actin cytoskeleton and plays an indispensable role in protecting the integrity of intestinal mucosal barrier and regulating intestinal permeability^[29-30]. Therefore, in this study, the therapeutic effect of Pingwei capsule on liver depression and spleen deficiency type IBS-D model rats was investigated from three aspects: molecular level, pathological expression and microecology. As a result, the expression of AQP in colon tissues of rats with liver depression and spleen deficiency

of IBS-D was decreased significantly. Pingwei capsule promotes the expression of AQP3, AQP4, AQP8, and tightly linked ZO-1, suggesting that the method of soothing the liver and invigorating the spleen might reduce the permeability of intestinal tract and improve the tight junction structure of model rats by upregulating the expression of AQP and tight junction protein. This has a certain repair effect on the destruction of intestinal mucosal barrier function in model rats, which improves the symptoms of diarrhea in rats, and alleviates further development of the disease. In addition, this study used 16S rRNA gene sequencing to compare and analyze the changes of intestinal microbiota diversity in rats with hepatic depression and spleen deficiency IBS-D. The results showed that the intestinal flora structure of IBS-D rats was in an imbalanced state, and the bacterial flora diversity decreased to a certain extent. Pingwei capsule might regulate the intestinal flora by increasing the diversity of microbial colonies, reducing the relative abundance of pathogenic bacteria Firmicutes, and increasing the relative abundance of probiotic bacteria Lactobacillus. The analysis of sequencing results also revealed that the three classification systems along with the highest levels of intestinal mucosal flora in the six groups were the same at the phylum level, namely, Firmicutes, Bacteroides and Proteobacteria, but there were differences in bacterial abundance. The treatment of soothing the liver and invigorating the spleen reduced the abundance of Firmicutes and increased the abundance of Bacteroidetes and Lactobacillus, indicating that Pingwei capsule can regulate the imbalanced state of intestinal flora to a certain extent, and also improved the barrier function of intestinal mucosa, thus reducing the intestinal permeability. This suggested a therapeutic role of Pingwei capsule in IBS-D, bringing more convenience to clinical treatment.

PingWei capsule is mainly composed of *Rhizoma atractylodis*, *Mangnolia officinalis*, *Fructus Auranti*, *Bupleurum*, *Aucklandiae Radix*, *Chenpi* and other 16 kinds of traditional Chinese medicine. Among them, *Rhizoma atractylodis* is a traditional medicine with anti-inflammatory, antibacterial, anti-virus, anti-cancer activities, and protects from gastrointestinal injury and nerve^[31]. *Magnolia officinalis* is widely used as a traditional medicine for gastrointestinal motility disorders in Asian countries to eliminate distention. Some scholars have carried out various pharmacological activity studies on *Magnolia officinalis* and its components^[32-33]. *Magnolia officinalis* has the effects of anticancer, anti-stress, anti-anxiety, anti-depression, anti-oxidation, anti-inflammatory and liver protection, but its pharmacological mechanism still remained unclear. *Fructus Aurantii* is an essential medicine used for regulating qi, and it also has the effects of anti-ulcer, anti-inflammatory, anti-oxidation and anti-cancer, and has a considerable therapeutic effect on gastrointestinal diseases^[34]. *Bupleurum* soothes the liver and regulates qi, increasing intestinal peristalsis. In addition to analgesic, anti-inflammatory and anti-cancer effects, it is also shown to be beneficial to in protecting the gallbladder and liver, and for treating liver depression syndrome. Liver protection is mainly reflected in anti-liver injury and anti-liver fibrosis^[35-36]. *Aucklandiae radix* promotes qi circulation to relieve pain, and warms neutralization of the stomach. Scientific research has confirmed several biological properties of woody incense, including antioxidant, anti-ulcer, anti-cancer, anti-virus, anti-microbial and hepatoprotective effects^[37-38]. The traditional effects and modern pharmacological studies of the above several major drugs showed that the traditional Chinese medicine compound preparation uses systematic methodology to study the complex whole. Traditional Chinese medicine is the research object of complex science. In the new century, it is difficult to obtain the desired effect of single factor antagonism and supplement it for multiple causes of complex dis-

eases. Traditional Chinese medicine treatment combines the efficacy of medicine within the human body organically, and applies the whole thought of traditional Chinese medicine to treat the individual as a whole, while western medicine mainly treats the individual with the idea of reductionism. Secondly, under the influence of recent global COVID-19, Chinese medicine has played a great role, but excavation of Chinese medicine and the combination of western medicine requires further development to initiate greater benefits to human health.

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