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Yunpi Tongbian Fang alleviates slow transit constipation induced by loperamide by regulating intestinal microbiota and short-chain fatty acids in rats

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ABSTRACT

Slow transit constipation (STC) is a prevalent chronic colonic dysfunction disease that significantly impairs the quality of life for affected individuals. Yunpi Tongbian Fang (YPTBF), a traditional Chinese medicine compound, has demonstrated promising clinical efficacy; however, its underlying mechanism remains elusive. In order to assess the laxative properties of YPTBF, which encompasses the influence on gut microbiota, gut metabolites, gut neurotransmitters, and colon histology, an oral administration of YPTBF was conducted for a duration of two consecutive weeks on STC rats induced by loperamide hydrochloride. The results showed that YPTBF improved the symptoms of STC, alleviated the decrease in total fecal volume and fecal water content caused by loperamide-induced constipation, restored intestinal transport function, and HE staining showed the recovery of pathological damage to the colon mucosa. In addition, YPTBF increased the concentrations of 5-HT and ACHE, while reducing the concentrations of VIP and NO. YPTBF adjusted the diversity and abundance of gut microbiota in STC rats, enabling the recovery of beneficial bacteria and promoting the production of acetic acid, propionic acid, and butyric acid. We found that YPTBF can improve constipation in STC rats, possibly by regulating the intestinal microbiota structure and improving SCFAs metabolism.

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Introduction

Slow transit constipation (STC) is a chronic colonic dysfunction disease with a high incidence rate. The main causes are weakened colonic transmission function and slow transmission of intestinal contents. With the accelerated pace of life and the increased pressure of work and life, the incidence rate of STC is increasing year by year, which not only affects the patients' physical health but also causes excessive mental pressure, leading to anxiety, depression and other symptoms, seriously affecting the quality of life of patients. The main purpose of STC treatment is to alleviate constipation symptoms, and restore normal intestinal motility and bowel function (1, 2). At present, the treatment of STC in clinical practice mainly focuses on Western medicine, including general therapy, medication and non-medication, surgery, and other methods. Stimulating laxatives have good short-term efficacy, but poor long-term efficacy. Once stopped, symptoms worsen. Therefore, many patients seek traditional Chinese medicine (TCM) treatment.

Traditional Chinese medicine compounds are widely used to treat STC. Studies have shown that traditional Chinese medicine (including traditional Chinese patent medicines and simple preparations and decoction) can effectively alleviate the symptoms of chronic constipation (3). Compared with Western medicine, traditional Chinese medicine can treat diseases through multiple components, pathways, targets, and mechanisms. Traditional Chinese medicine has the advantages of fewer side effects, a low recurrence rate, and significant therapeutic effects (4). The clinical efficacy of Yunpi Tongbian Formula (YPTBF) is significant, but there is still a lack of systematic research on the therapeutic effect of STC, and the potential mechanism of regulating intestinal peristalsis to improve STC is still unclear. It is necessary and urgent to study the therapeutic effect and mechanism of YPTBF on STC, which can provide new ideas for the treatment of STC.

More and more clinical and animal experimental evidence suggests that changes in gut microbiota are closely related to the pathophysiology and clinical symptoms of STC (5). Changes in the structure of gut microbiota can affect gastrointestinal motility, such as imbalanced microbiota or a lack of purely beneficial microbiota, which can weaken gastrointestinal peristalsis and promote constipation (6). Short-chain fatty acids are the main metabolites of intestinal bacteria, and their content significantly affects the homeostasis of the intestinal environment. Studies have shown that the levels of butyrate, acetate, and propionate in patients with constipation are significantly reduced (7).

Therefore, we attempt to investigate the laxative effect and potential mechanisms of YPTBF, as well as whether YPTBF improves STC by regulating the gut microbiota.

Materials and Methods

Animals

30 male SD rats, aged 6-8 weeks, with a body weight of 180-200g, were purchased from SPF (Beijing) Biotechnology Co., Ltd and raised in the Basic Pharmacology Labo-

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ratory of Nanjing University of Traditional Chinese Medicine Affiliated Hospital with license number SYXK (Su) 2012-0047. During the experiment, they were allowed to eat and drink freely 24 hours a day, with temperature controlled at 23 ± 1 ℃, humidity controlled at 50 ± 5%, and lighting system set at 12 hours per day. All experimental plans were conducted in accordance with the guidelines of the Ethics Committee.

Drugs and Reagents

All drugs are purchased and placed at the Affiliated Hospital of Nanjing University of Traditional Chinese Medicine. The preparation method of YRD containing raw medicinal herbs is as follows: 70g of Atractylodes macrocephala, 30g of Fructus Aurantii, 15g of Salvia miltiorrhiza, 15g of Salvia miltiorrhiza, 15g of Codonopsis pilosula, 10g of almonds, 10g of Platycodon grandiflorum, 10g of perilla leaves, 10g of black plum, 5g of tangerine peel, and 3g of licorice. Add 10 times the amount of water for the first time, and 8 times the amount of water for the second time, and decoct for 30 minutes each time. Combine the two decoctions, let them stand, filter, and concentrate the filtrate to 3g/mL. Set the dosage according to the grouping, dilute with double distilled water to the corresponding concentration, and refrigerate for later use. Prucalopride was purchased from Jiangsu Haosen Pharmaceutical Group Co., Ltd. (batch number: 133211101). Loperamide was purchased from Xian-Janssen Pharmaceutical Ltd. (batch number: LKY2050). When applied to rats, all drugs were dissolved in sodium chloride injection (NS0.9%).

Experimental Design

STC rat modeling method: Loperamide suspension (10mg/kg/d) was orally administered once a day for 10 consecutive days. To maintain the stability of the STC model, the administration of loperamide suspension (10mg/kg/d) should be maintained during subsequent drug interventions. After one week of basic adaptive feeding, rats were randomly divided into 5 groups, with 6 rats in each group. Specifically, the blank group (NC) did not undergo any special treatment and consumed water and food normally; The model group (MC) was treated with loperamide suspension (10mg/kg/d); The positive control group (PC) was treated with loperamide suspension and Prucalopride (0.018mg/100g); The low-dose group (L-YRD) was treated with loperamide suspension and YRD (17.23g/kg); The high-dose group (H-YRD) was treated with loperamide suspension and YRD (34.46g/kg). The L-YRD dose is equivalent to a human clinical dose, while the H-YRD group received twice that dose. All intervention drugs were administered once a day for 14 consecutive days.

24-hour fecal volume and fecal moisture content

Collect the total number of feces within 24 hours and weigh them once a week. Weigh fresh feces and record them as wet weight (A). Fresh feces are dried in an oven for 3 hours to obtain dry weight (B). Fecal moisture content=(A-B) /A × 100%.

Intestinal propulsion rate

Rats fasted for 24 hours and couldn't help but water. They were given 5% charcoal Arabic gum suspension by gavage. After 20 minutes, the rats were euthanized. Then, the abdominal cavity was quickly exposed, and all the intestines from the pylorus to the anus at the bottom of the stomach were removed. The excess mesentery was removed, and after being smoothed in a relaxed state, it was placed on the operating table. The distance between the entire pylorus to the anus intestinal tract and the length of charcoal powder advancement (black staining) was measured, Then calculate the percentage of charcoal advance according to the following formula: intestinal advance rate=(charcoal advance length/intestinal length) × 100%.

Histological analysis

Fix a portion of the fresh colon in 4% paraformaldehyde for more than 24 hours, then dehydrate it for paraffin treatment, and cut it into 5mm thick slices. Stain the histopathological features of colon tissue with hematoxylin-eosin (HE).

Enzyme-linked immunosorbent assay

Collect blood samples and centrifuge at 3000rpm for 20 minutes to obtain serum. According to the manufacturer's instructions, use an ELISA kit to determine the levels of 5-hydroxytryptamine (5-HT, MLBIO, article number ml028308), vasoactive intestinal peptide (VIP, MLBIO, article number 936598), nitric oxide (NO, BOXBIO, article number AKNM005M), and acetylcholine (ACHE, BOXBIO, article number AKFA005M).

16S rRNA gene sequencing

Yang et al. (8) described the method for sequencing the 16SrRNA gene in their report. Microbial classification metagenomic sequencing 16SrRNA gene sequencing was conducted by Shanghai Biotree Biotechnology Co., Ltd.

Quantitative detection of SCFAs by gas chromatography-mass spectrometry

Lin et al. (9) described the SCFAs analysis method in their report. Gas chromatography-mass spectrometry (GCMS) was conducted by Shanghai Biotree Biotechnology Co., Ltd.

Statistic analysis

All data was processed and analyzed using GraphPad-Prism9 statistical software and plotted. The measurement data is represented by the mean soil standard error. After conducting normal distribution and homogeneity of variance tests on the experimental data of each group, a one-way analysis of variance is used for inter-group comparison, and LSD is used for intra-group comparison. $P<0.05$ indicates a statistically significant difference.

Results

YPTBF Improving constipation in STC rats

Observe the intestinal motility of STC rats from the 24-hour fecal volume, fecal water content, and intestinal propulsion rate. The intestinal propulsion rate (Figure 1A, 1C) in the MC group was significantly lower than that in the NC group. Compared to the NC group, the PC group, L-YRD group, and H-YRD group all increased, and the H-YRD group had better effects. The 24-hour fecal volume (Figure 1B) in the MC group was significantly lower than that in the NC group, and compared to the NC group, the PC group, L-YRD group, and H-YRD group all increased. The fecal water content (Figure 1D) of the MC group was

significantly lower than that of the NC group, and compared to the NC group, the PC group, L-YRD group, and H-YRD group all increased. The results indicate that YPTBF can improve the symptoms of STC and effectively restore intestinal motility function.

YPTBF Improving the structural changes of colon tissue in STC rats

The colon tissue of the NC group showed abundant glands, intact columnar epithelial cells, clear U-shaped crypts and goblet cell structures, and no obvious abnormalities. Compared with the NC group, the MC group had poor mucosal integrity, loose glands, disappearance of U-shaped crypts and goblet cell structures, infiltration of inflammatory cells into the colon, and tissue breakage. The congestion and edema of mucosal tissue in the PC group, L-YRD group, and H-YRD group were improved compared to the model group, with improved glandular arrangement and reduced inflammatory cell infiltration. The improvement was more significant in the high-dose group. (Figure 2).

YPTBF Effects on gut neurotransmitters in STC rats

Compared with the NC group, the serum levels of 5-HT and ACHE in rats decreased, while VIP and NO levels increased, with statistically significant differences(P<0.01). Compared to the MC group, the levels of 5-HT and SP in the PC group, L-YRD group, and H-YRD group increased, while VIP and NO levels decreased, with statistically significant differences(P<0.05). There is a statistical difference in neurotransmitter levels between the H-YRD group and the L-YRD group compared within the group(P<0.05) (Figure 3).

YPTBF Effects on the gut microbiota of STC rats

In order to investigate the effect of Yunpi Tongbian Formula on gut microbiota in STC rats, 16SrDNA detection was performed on the cecal contents of five groups: NC, MC, PC, L-YRD, and H-YRD. (Figure 4-A) A total of 7278 OTUs were detected in the above 5 groups, including 2330 in the NC group, 2966 in the MC group, 3004 in the PC group, 2590 in the L-YRD group, and 2449 in the H-YRD group. The dilution curves of each group of samples (Figure 4-B) tend to flatten out as the number of sequencing increases, with only a few OTUs detected, indicating a reasonable sequencing depth. (Figure 4-C) The principal component coordinate analysis PCoA plot was obtained by applying the Bray-Curtis clustering method. The PCoA plot showed that different groups of samples were clustered separately, with obvious inter-group differentiation. Moreover, when different doses of drugs were used for treatment, the overall microbial status of the two groups of samples was farther from the model group and closer to the positive drug group, indicating that the drug has a certain effect on improving intestinal microbiota. The system cluster tree (Figure 4-D) shows significant differences between the groups, with low and high-dose groups and model groups clustered separately, indicating that the low and high-dose group can improve intestinal microbiota imbalance in STC rats. α Diversity analysis showed that the microbial diversity of the model group (Chao1 index 1002.13, Shannon index 7.70) was significantly higher than that of the normal group (Chao1 index 790.45, Shannon index 6.75). Compared to the model group, the low-dose group increased microbial diversity (low-dose group: Chao1 index 928.46, Shannon index 7.43; high-dose group: Chao1 index 852.99, Shannon index 7.13). This result indicates that the treatment group improved the intestinal microbiota imbalance caused by the model group.

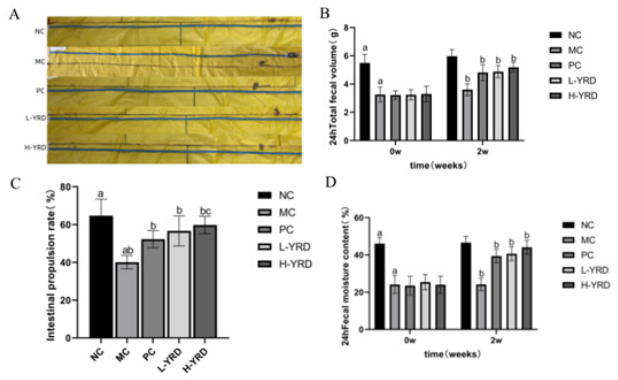

Figure 1. YPTBF improves constipation in STC rats. (A) The intestinal propulsion status of rats in each group. (B) The 24-hour fecal volume of rats in each group. (C) Intestinal propulsion rate of rats in each group. (D) The fecal water content of rats in each group. (Note: Compared with NC, P^a<0.01; compared with MC, P^b<0.01;compared with PC group, P^c<0.05).

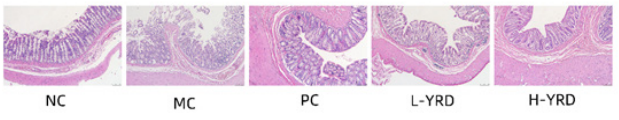

Figure 2. HE staining of colon tissue in each group (× 100).

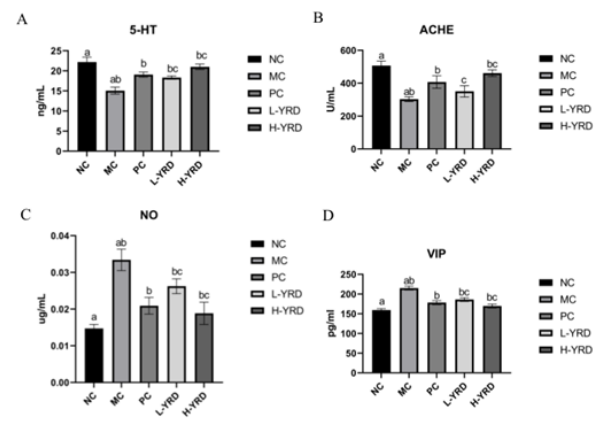

Figure 3. Effect of YPTBF on gut neurotransmitters in STC rats (A): Relative content of 5-HT in serum of STC rats. (B) The relative content of ACHE in the serum of STC rats. (C) The relative content of NO in the serum of STC rats. (D) The relative content of VIP in the serum of STC rats. (Note: Compared with NC, P^a<0.01; compared with MC, P^b<0.05; compared with L-YRD group, P^c<0.05).

YPTBF Effects on gut microbiota and biological communities in STC rats

We analyzed the effects of each group on the abundance of gut microbiota species in STC rats at the phylum and genus levels (Figure 5-AB). At the gate level, the microbial communities identified in this study mainly include Firmi-

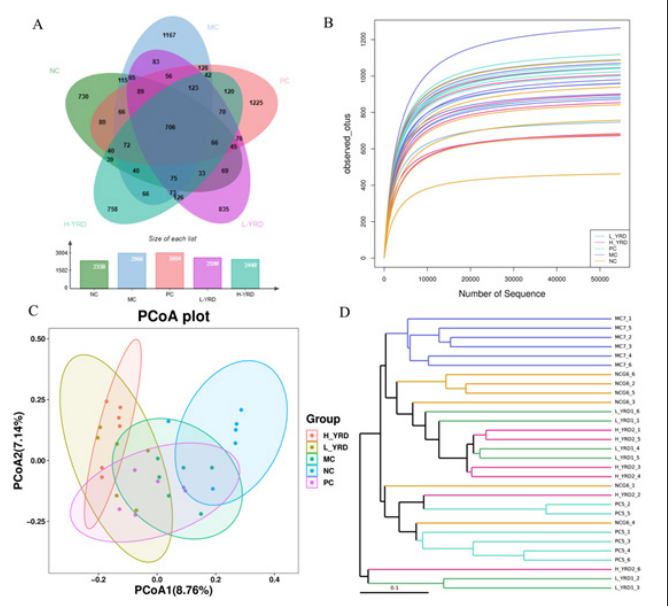

Figure 4. Effect of YPTBF on gut microbiota in STC rats. (A) VENN diagram, indicating the number of common or unique OUTs in each group of samples. (B) Dilution curves of each group of samples. (C) Based on the Bray Curtis clustering method, the principal component coordinate analysis (PCoA) graph was obtained. (D) The system cluster diagram was obtained from UPGMA hierarchical clustering analysis.

cutes, Bacteroidota, Actinobacteriota, etc. Among them, Firmicutes account for the largest proportion. Cluster analysis at the gate level showed that the microbiota of mice treated with high-dose drugs was closer to that of normal mice compared to the positive and low-dose drug groups, indicating that high-dose drugs have a better regulatory effect on the gut microbiota. At the genus level, it can be observed that the model group has significant differences in abundance compared to the normal group in bacterial genera such as UCG − 005, Romboutsia, Lactobacillus, Muribaculaceae (P<0.05). However, after high-dose drug treatment, the corresponding bacterial abundance will return to close to the normal group, showing a difference in abundance compared to the model group ((P<0.05), indicating that the high-dose treatment group has the effect of regulating the intestinal microbiota in mice. (Figure 5-C) These results indicate that Yunpi Tongbian Fang can reverse the imbalance in the biological community structure caused by the model group.

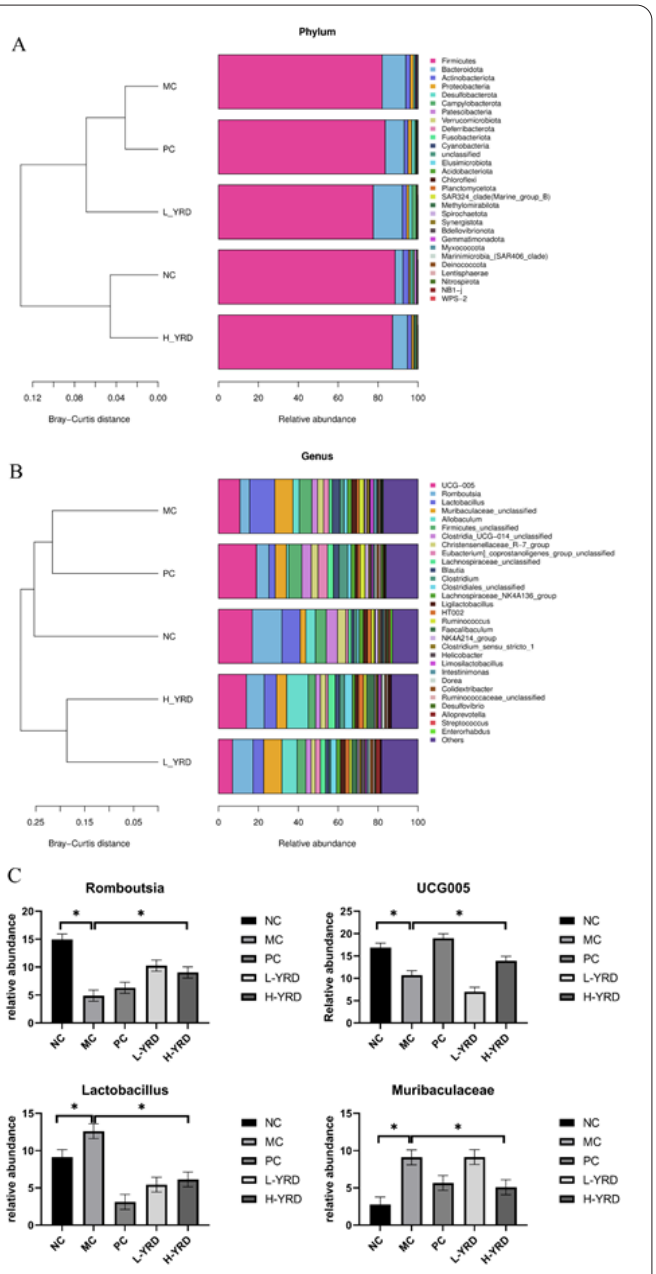

Figure 5. Effect of YPTBF on the gut microbiota of STC rats. (A) The impact of changes in rat microbiota at the phylum level in each group. (B) The impact of changes in rat microbiota at the genus level in each group. (C) Comparison of the abundance of UCG-005, Romboutsia, Lactobacillus, and Muribaculaceae in the cecal contents of each group. *P**<0.05.

YPTBF Effects on Short Chain Fatty Acids (SCFAs) in STC Rats

In order to study the effect of Yunpi Tongbian Formula on SCFAs in STC rats, we measured the levels of main SCFAs in the cecal contents of each group of rats, including acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valerenic acid, caproic acid, heptanic acid, octanoic acid, nonanoic acid, and decanoic acid. The results showed that the model group could significantly reduce the levels of acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valerenic acid, and decanoic acid (P<0.05), and significantly increase the levels of hexanoic acid and heptane acid (P<0.05). However, after drug intervention, the levels of acetic acid, butyric acid, and decanoic acid increased, and the levels of hexanoic acid and heptane acid decreased, which was different from the model group (P<0.05). (See Figure 6).

K-Means analysis shows that compared with the normal group, the content of acetic acid, butyric acid, nonanoic acid, valerenic acid, octanoic acid, decanoic acid, propionic acid, isobutyric acid, and isovaleric acid in the model group decreases, while the content of heptanoic acid and caproic acid increases. However, after high-dose drug intervention, the levels of octanoic acid and decanoic acid increased, while the levels of heptanoic acid, butyric acid, and caproic acid decreased, all of which were closer to the levels of the normal group. (See Figure 7).

By using the Spearman algorithm to calculate the correlation between the abundance of differential microbiota at the level of gut microbiota in rats and the level of SCAFs in the cecal contents of rats, a correlation heat map was drawn. It can be seen that compared with the normal group, the differential microbiota in the model group led

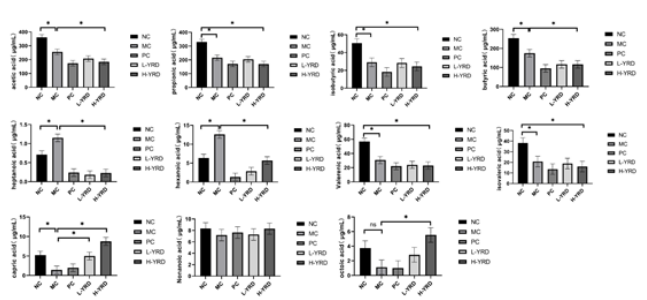

Figure 6. Determination of Short Chain Fatty Acids in Rat Cecal Content by Gas Chromatography-Mass Spectrometry (GC-MS) (*P**<0.05).

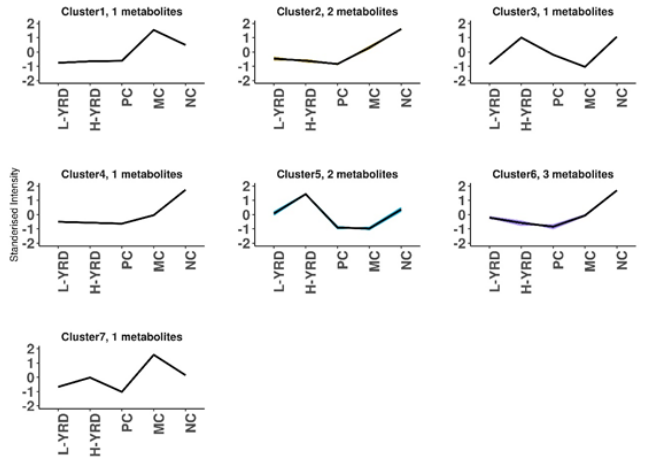

Figure 7. K-Means shows the trend of relative content changes of metabolites in different groups (note: Cluster17 includes 1-heptanoic acid, 2-acetic acid, butyric acid, 3-nonanoic acid, 4-valerenic acid, 5-octanoic acid, decanoic acid, 6-propionic acid, isobutyric acid, isovaleric acid, 7-hexanoic acid).

to a downregulation of some short-chain fatty acid levels, while after intervention with the Yunpi Tongbian Formula, the levels of these short-chain fatty acids were upregulated. The relative abundance of lactic acid bacteria is positively correlated with the content of acetic acid, propionic acid, and butyric acid, while the relative abundance of Clostridium is negatively correlated. (See Figure 8AB).

Discussion

STC is a disease characterized by delayed colonic transport, 24-hour fecal volume, and fecal water content, and ITR can be used as a representative diagnostic indicator (10). This study found that after the intervention, the fecal water content, intestinal transport function, and pathological damage to the colon mucosa of STC rats were restored, with the high-dose group having the most significant improvement effect. In addition, YPTBF increased the expression of Ache and 5-HT in the serum of STC rats and reduced the expression of VIP and NO. In addition, YPTBF can adjust the intestinal microbiota structure of STC rats, restore beneficial bacteria in the model group, promote the generation of short-chain fatty acids, and improve the intestinal transport function of STC rats, thus achieving therapeutic goals. Therefore, it can be believed that YPTBF promoting intestinal transport function in STC rats may be related to regulating intestinal microbiota and regulating intestinal neurotransmitters.

Studies have shown that SP, Ache, and 5-HT are the main excitatory neurotransmitters, while NO and VIP are the main inhibitory neurotransmitters in the intestinal nervous system (11). Neurotransmitter levels are closely related to gastrointestinal motility (11). VIP can relax gastrointestinal smooth muscle, thereby inhibiting gastrointestinal peristalsis (12), Yuxuan Liang et al (13) research has shown that ROS significantly reduces VIP levels and regulates intestinal microbiota to alleviate constipation. The autonomic nervous system regulates intestinal peristalsis. When the parasympathetic nervous system is excited, Ache is released to activate gastrointestinal peristalsis. In neurotransmitters, 5-HT and dopamine (DA) play a role in the release of Ach: when the 5-HT receptor is stimulated, the degree of Ach release increases; When DA receptors are stimulated, the degree of Ach release decreases, leading to a decrease in gastrointestinal peristalsis. Yutaka Makizakiet al (14) In a rat model of constipation induced by subcutaneous administration of loperamide, BBG9-1 can improve ecological imbalance, prevent a decrease in butyric acid concentration in the intestine, increase 5-HT, and inhibit the increase in serum DA and decrease in Ach. The regulation of colonic peristalsis depends on the integrity of intestinal inhibitory neurotransmission mediated by nitric oxide (NO), purine neurotransmitters, and neuropeptides (15).

The dominant phyla of gut microbiota in healthy adults are mainly Firmicutes, Bacteroidetes, and Actinobacteria, while the changes in gut microbiota in constipation patients are mainly reflected in the ratio of Firmicutes to Bacteroidetes (16). Studies have shown that the abundance of Actinobacteria and Firmicutes is related to the acceleration of colonic transport, while the larger proportion of Firmicutes and Bacteroidetes is related to the acceleration of colonic transport (17). A study on the characteristics of gut microbiota changes in patients with slow transit constipation (STC) in Fuzhou, China has shown that at the phylum level, patients with chronic constipation and healthy individuals are mainly composed of Firmicutes and Bacteroidetes, which is consistent with research results at home and abroad; At the genus level, the relative abundance of beneficial bacteria such as Lactobacillus,

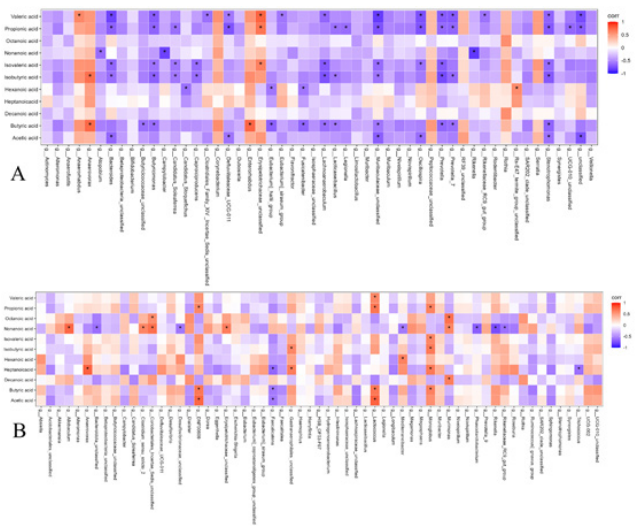

Figure 8. Thermodynamic map of the correlation between the abundance of gut microbiota and the levels of SCAFs in the cecal contents of rats (A) NC vs MC. (B) H-YRD vs MC.

Bifidobacterium, and Brucella in patients with slow transit constipation decreased compared to the normal control group, while the abundance of conditionally pathogenic bacteria such as Enterobacteriaceae and Erythromycin increased (18). This shows that the abundance and diversity of intestinal microbiota in patients with slow transit constipation decreased, and the structure of the microbiota changed, indicating a change in intestinal microbiota in patients with slow transit constipation, Their imbalance in quantity and composition can disrupt the homeostasis of gut microbiota, affect the development and growth of the intestinal nervous system, and ultimately lead to secretion and dysfunction of the gastrointestinal tract, leading to the occurrence of slow transit constipation. The results of this experiment show that YPTBF can downregulate the abundance of Firmicutes in the gut microbiota of rats, consistent with relevant research results. However, after drug intervention, the proportion of main bacterial groups tends to be normal, indicating that YPTBF can improve the composition of STC microbiota to achieve therapeutic purposes. Short-chain fatty acids are the main metabolites of intestinal bacteria, and their content significantly affects the homeostasis of the intestinal environment (19). SCFAs play an important role in the physiological metabolism process of the body. SCFAs can stimulate the absorption of water and electrolytes, enhance the proliferation of epithelial cells, affect gastrointestinal motility, and increase mesenteric blood flow (19). Acetic acid acts to upregulate the barrier function of host intestinal epithelial cells, while propionate can reduce fat production, serum cholesterol levels, and carcinogenic effects in other tissues (2). Butyrate is the main source of metabolic energy in the large intestine, helping to maintain its integrity, control intestinal inflammation, and support genomic stability (21). According to reports, butyric acid directly stimulates colon motility by stimulating the release of 5-HT or promoting the cholinergic pathway (22). Recent studies have shown that the gut microbiota acts on intestinal chromaffin cells using SCFAs as mediators, regulating the synthesis and release of 5-HT. SCFAs promote the secretion and release of 5-HT and induce peristaltic reflex, thereby enhancing colonic contraction and accelerating colonic transport (23). Therefore, butyrate-producing bacteria in feces are associated with rapid colonic transport. Butyrate has a biphasic effect on colon motility, stimulating movement at low concentrations and inhibiting movement at high concentrations. The increase in butyrate production may lead to the onset of constipation through various mechanisms (24). High concentrations of butyrate may inhibit the secretion of mucin by testicular goblet cells(24), and can also reduce fecal volume by stimulating the absorption of water and electrolytes in the colon (25). Moreover, butyrate can inhibit the contraction of colonic smooth muscle, leading to slow colonic transport (26). Studies have shown that (27,28), Transplanting gut microbiota into small trees without gut microbiota resulted in significantly lower levels of butyrate in mice from STC donors compared to mice from healthy donors. After supplementation with butyrate, the results of mice from STC donors were reversed in terms of particle frequency, water percentage, and colonic contractility (2). The results of this experiment show that after drug intervention, butyric acid, acetic acid, propionic acid, etc. increase, which is consistent with existing research. This indicates that YPTBF Formula can adjust the gut microbiota environment and the levels of metabolites and short-chain fatty acids in STC rats to achieve the goal of treating STC.

Conclusions

This study found that in the STC rat model, YPTBF reversed intestinal peristalsis dysfunction, manifested as increased 24-hour stool weight, fecal water content, and intestinal transport rate, and alleviated pathological damage to the colon. In addition, YPTBF can regulate the expression of gut neurotransmitters, regulate the structure of gut microbiota, and improve the secretion of SCFAs to improve constipation in STC rats. The results of this study can provide an experimental basis for the clinical application of YPTBF in the treatment of STC from the perspective of gut microbiota.

Conflicts of Interest

The authors report no conflict of interest.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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