

The efficacy and mechanism of fecal microflora transplantation in the treatment of slow transit constipation based on metabolomics anion mode

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Author contributions

Lu Han proposed the research topic and wrote the paper; Yu-Hong Bian and Chen Xu provided guidance and funding support; Lu-Lu Xie, Hai-Zhao Liu, Jia-Tong Liu, Yi-Yang Wang revised the paper; Yu-Wei Li, Yu-Tong Jin, Ji-Da Wang, Joseph Kofi Abankwah collected and sorted out the data.

Competing interests

The authors declare no conflicts of interest.

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Abbreviations

FMT, Fecal microbiota transplantation; STC, Slow transit constipation; PAC-SYM, Patient Assessment of Constipation-Symptoms; DF, defecation frequency; CBMs, complete bowel movements; OPLS-DA, orthogonal projections to Least Squares Discrimination Analysis; C-AMP, cyclic adenosine monophosphate; PKA, protein kinase A; 5-HT, 5-hydroxytryptamine; ECs, chromaffin cells.

Citation

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Abstract

Background: Fecal microbiota transplantation (FMT) based on the positive ion mode of metabolomics has a good therapeutic benefit for slow transit constipation (STC) patients. However, a piece of comprehensive metabolomics information is yet to be established. The aim of the study was to explore the efficacy and mechanism of FMT in the treatment of STC under metabolomics. **Methods:** Eight STC patients meeting the set inclusion and exclusion criteria were enrolled and treated with FMT (three times). The Patient Assessment of Constipation-Symptoms (PAC-SYM), weekly total defecation times, and defecation frequency scores of these STC patients were compared before and after treatment. Feces and serum of STC patients before and after treatment were analyzed using 16SrDNA and metabolomics. **Results:** After FMT treatment, the PAC-SYM score of constipated patients decreased [(5.00 ± 2.94) vs (5.20 ± 2.87)], while the number of complete defecations per week increased [(2.00 ± 1.79) vs (1.69 ± 1.80)]. The score of defecation frequency decreased [(0.83 ± 1.03) vs (0.86 ± 0.95)]. The metabolites in the feces and serum of patients receiving FMT changed significantly ($P < 0.05$). The results from 16SrDNA analysis showed that the α and β diversity of the fecal microbiome changed significantly ($P < 0.05$) after transplantation, and the contents of genera *Lactobacillus*, *Bacillus*, *Succinibacillus*, *Cellvibrio*, and *Escherichia* increased in FMT treated patients. **Conclusion:** FMT may treat STC by increasing the beneficial intestinal flora and metabolites in the anion mode of metabolomics.

Keywords: Fecal microbiota transplantation; Slow transit constipation; metabolomics; anion mode; *Lactobacillus*

Introduction

Slow transit constipation (STC) refers to the transit dysfunction of the colon and the difficulty in defecation caused by the slow transit of intestinal contents [1]. This lazy bowel syndrome is a common problem in many patients, especially in women of childbearing age, and its symptoms gradually worsen over time, seriously affecting patients' quality of life [2]. Studies have shown that STC significantly increases the incidence of colonic polyps and other related diseases [3] and is also an important factor in inducing heart failure, atherosclerosis, and other cardiovascular diseases [4]. At present, laxatives and stimulants are usually used to relieve STC symptoms clinically [5], but nearly half (47%) of the patients were dissatisfied with the current treatment due to factors such as insignificant efficacy and obvious side effects [6]. Studies have shown that the pathogenesis of STC is related to intestinal nerve cells, neurotransmitters and intestinal microbes [7, 8].

There are many kinds of microorganisms in the human digestive system which participate in the life activities of the host. These microorganisms are distributed in the small and large intestines and can affect the physiological function of the intestinal tract [9]. Studies have shown that prolonged fecal residence time in STC patients may lead to intestinal flora imbalance, which may affect intestinal immune function, peristalsis, and barrier function [10, 11]. Fecal microbiota transplantation (FMT) is the transfer of functional flora from the feces of healthy persons into the intestinal tract of patients by certain means, so as to regulate the imbalance of intestinal flora and rebuild the intestinal micro-ecosystem to normal function, and treat intestinal diseases. Gold juice, also known as gold water or feces clearing, is a traditional Chinese medicine formed after collecting the feces of healthy people, repeatedly washing them with good well water or underground spring water, and then sealing storage in a new earthen pot after being filtered by bamboo sieve and gauze, and buried underground for at least 1 year, generally 20 to 30 years later [12]. FMT uses fresh, healthy human feces, while golden juice is the product of normal human feces buried underground for years. In a sense, gold juice is the product of deep-buried anaerobic fermentation of fecal bacteria transplant solution, but with the improvement of modern fecal bacteria transplant technology, the preparation process of fecal bacteria transplant solution is more clear. FMT, this form of treatment, was included in the Clinical Medical Guidelines of the United States in 2013 as a new way of reconstructing intestinal flora and has achieved good efficacy in the treatment of chronic constipation and other diseases [13]. FMT transplants functional flora in the stool of healthy people into the intestinal tract of patients in a special way, re-establishing a new intestinal flora, balancing the composition and function of intestinal microbes, regulating the intestinal mucosal immune system, stabilizing the intestinal barrier function, promoting the recovery of gastrointestinal motility, and thus improving the symptoms of constipation in patients. However, the mechanism of FMT treatment of STC remains unclear. Due to individual differences and complex interaction mechanisms between intestinal flora, FMT poses a great challenge to clinical development. As a new therapy, FMT still needs to be repeatedly demonstrated and tested for a long time to confirm its effectiveness and safety.

The previous results of our research group showed that FMT had a good therapeutic effect on STC patients. It was further proved by 16S rDNA gene sequencing and metabolomics that FMT can improve the intestinal flora, and *L-Arginine* and other metabolites closely related to constipation were detected in the positive ion mode of metabolomics. KEGG analysis showed that these metabolites participate in protein digestion and absorption pathways and produce a large number of sodium ions. These sodium ions not only improve intestinal electrophysiology and promote intestinal peristalsis, but also increase intestinal osmotic pressure, promote intestinal mucosa to secrete more mucus and form loose stools, thus relieving constipation [14]. Based on previous research results, we found that anion pattern metabolites, such as ketoisocaproic acid and benzoic acid, which are

closely related to STC, also changed. In order to obtain a piece of comprehensive information on metabolomics, the metabolites of the anion mode of this research have to be investigated so as to supplement existing literature.

Materials and Methods

Inclusion criteria

(1) Refer to the Roman IV standard [15]; (2) Constipated patients with poor therapeutic effects of dietary intervention, enema, and catharsis in the past six months.

Exclusion criteria

(1) Congenital and secondary Hirschsprung's disease; (2) Patients with secondary constipation (drug, endocrine, metabolic, or neurological diseases); (3) Patients with a history of abdominal surgery or perianal surgery; (4) People with a history of the digestive system (such as a tumor, inflammatory bowel disease, etc.); (5) Those who have a history of intestinal pathogen infection; (6) Pregnant or lactating women.

Donor requirements

(1) The volunteers were healthy adults without any other diseases, especially digestive diseases, except for pregnant women; (2) No history of taking antibiotics, probiotics, or other probiotics within six months; (3) Infectious diseases (hepatitis B, C, syphilis, AIDS, etc.) negative; (4) Stool test (*Clostridium difficile*, dysentery, *Shigella*, *Campylobacter*, and parasites, etc.) was negative.

Preparation of fecal suspension

Refer to the international Amsterdam preparation specification [16]. Briefly, 100 g of fresh feces was collected from a standard healthy donor and transferred into a sterile beaker. A volume of 500 mL sterile normal saline was added and stirred evenly using a stirrer. The stirred fecal bacteria solution was passed through a screen with a diameter of 2.0 mm and 0.5 mm successively to remove small particles. A 10% sterile glycerol was added to the filtered suspension and the supernatant was stored in a -80°C refrigerator or immediately given to the patient.

FMT treatment

Patients were orally administered vancomycin (500 mg, twice a day, for 3 consecutive days). The nasal jejunal tube was placed in the proximal jejunum of the patient under an endoscope. Prior to FMT, the patient stopped other conventional constipation treatments for three days and did not eat anything for eight hours. After antibiotic treatment, sodium phosphate (90 mL) was given to empty the intestine. After a 12 hour rest, 50 mL fecal suspension was slowly injected into the patient through a nasal jejunal tube within 5 minutes. The nasojejunal tube was then rinsed with 15 mL saline solution. This was done for six consecutive days, once every two weeks, and repeated three times. Stool and serum samples were collected from patients on the day before (baseline-B1) and after (B2, B3, B4) FMT.

Efficacy evaluation

The PAC-SYM is a regularly adopted questionnaire in clinical studies to assess the constipation symptom score [17] and quantify it from best to worst. This questionnaire investigates the subjects' constipation symptoms, which include fecal character, abdominal symptoms, and crissum rectum symptoms. The five-point scale, which assigns no, mild, moderate, severe, and extreme cases to the points 0 to 4, respectively, was used. The dimension scores for the average of all the entries of dimensions and the average score of the total score for all items was obtained to assess the degree of constipation. The higher the score, the heavier the degree of constipation. In the assessment of the number of complete bowel movements (CBMs) per week, a score of the defecation frequency (DF) was used. Briefly, 0–4 points were assigned to 1–2 times every 1–2 days, 2 times per week, 1 time per week, less than 1 time per week, and less than 1 time per

month, respectively. The higher the score, the lower the frequency and the more severe the degree of constipation.

Metabolite extraction and UPLC-Q-TOF-MS detection

After the samples were thawed slowly at 4 °C, a mixture of the stool (100 µL), methanol/acetonitrile solution (400 µL) (1:1, V/V), was vortexed, made to stand at -20 °C for 30 min before it was then centrifuged (14,000 g at 4 °C for 20 min). The supernatant was taken and vacuum dried for mass spectrometry analysis. Briefly, the supernatant was redissolved in 100 µL acetonitrile solution (acetonitrile: Water = 1:1, V/V), vortexed, and centrifuged (14,000 g at 4 °C for 15 min). The supernatant was sampled for analysis [18]. The samples were separated by Agilent 1,290 Infinity LC ULTRA High-Performance Liquid chromatography (UHPLC) HILIC column [19]; The positive and negative ionization modes of electrospray ionization (ESI) were used for detection. The samples were separated by UHPLC and analyzed using a Triple TOF6600 mass spectrometer (AB SCIEX).

Genomic DNA extraction and 16SrDNA gene sequencing

The genomic DNA of the samples was extracted using a cationic detergent or anionic detergent (CTAB or SDS, respectively), and the purity and concentration of DNA were detected using the agarose gel electrophoresis. Appropriate samples were placed in a centrifuge tube, and the samples were diluted to 1 ng/µL with sterile water. Genomic DNA was extracted using CTAB or SDS, and the purity and concentration of DNA were detected. According to the selection of sequencing regions, specific primers with Barcode and high-fidelity DNA polymerase were used for PCR amplification of selected V3-V4 variable regions. Library construction was performed using the NEB Next® Ultra™DNA Library Prep Kit. The constructed library was inspected using Agilent Bioanalyzer 2,100 and Qubit, and the library was sequenced on the machine after passing the inspection.

Statistical analysis

The SPSS version 20.0 software was used for the data analysis. The measured data was expressed as mean ± SD ($\bar{X} \pm SD$), and the continuous variables of normal distribution were determined using the Shapiro-Wilk test of normality. The counting data were expressed by rate, and the comparison was performed using the χ^2 test. The R-value was used to evaluate the degree of correlation and $P < 0.05$ was considered statistically significant.

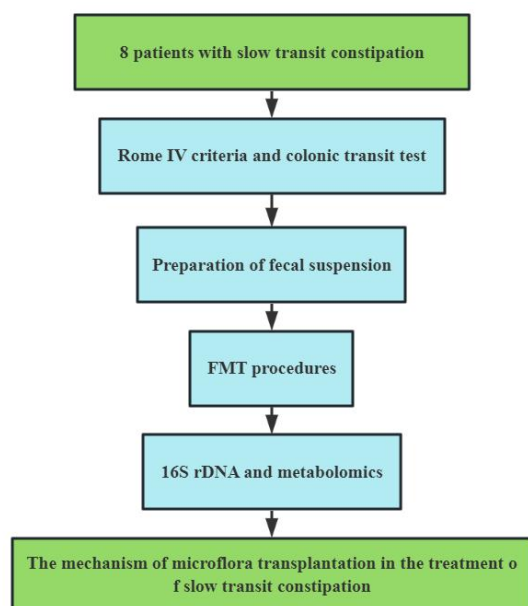


Figure 1 Experimental design

Table 1 Observation results of 8 patients with slow transit constipation before and after FMT treatment

	PAC-SYM	CBMs	DF
B1	5.20 ± 2.87	1.69 ± 1.80	0.86 ± 0.95
B4	5.00 ± 2.94 [#]	2.00 ± 1.79 [#]	0.83 ± 1.03 [#]

Data are expressed as the M ± SD

PAC-SYM: constipation symptom score, the higher the score, the more severe the degree of constipation; CBMs: Number of complete bowel movements per week; DF: Score of defecation frequency, the higher the score, the lower the frequency, the more severe the degree of constipation; B1: before FMT treatment; B4: after the third FMT treatments.

[#] $P < 0.05$ and the difference was statistically significant compared with B1 (before the treatment) values.

Ethics approval

Our research group started the study on the efficacy and safety of FMT in the treatment of STC in 2018, which has been reviewed and approved by the Ethics Committee of Tianjin People's Hospital (approval number: (2018) lun review no. (C04).

Clinical trial registration number

In 2020, the registration in the Chinese Clinical Trial Registry was officially completed and approved, registration number: ChiCTR2000033227.

Results

Evaluation results

Based on the research group's previous research [14], this study supplemented the relevant evaluation indexes of FMT efficacy. The results showed that the PAC-SYM constipation symptom score of constipation patients decreased after FMT treatment, the number of complete defecation per week increased, and the defecation frequency score decreased when compared with the before treatment (Table 1).

Screening and identification of potential metabolic differentials

In metabolomics anion mode, we used the orthogonal projections to Least Squares Discrimination Analysis (OPLS-DA) score plot, Volcano plot, significant differences metabolites hierarchy clustering heat maps, and multiple differences metabolites expression analysis method to analyze patients before and after the FMT feces and the change of the negative ion mode in serum metabolites. We found significant differences in fecal and serum metabolites in patients with constipation after FMT treatment. Multivariate statistical analysis of OPLS-DA showed that metabolite clusters in the feces and serum of patients were significantly separated before and after FMT treatment (Figure 2). Volcano plots and significantly different metabolite hierarchical cluster heat maps also showed metabolic changes in patients before and after FMT treatment (Figures 3 and 4). Multiple analyses of differential metabolite expression showed that the contents of Ketoisocaproic acid, Benzoic acid, Malonic acid, N-Acetyl-L-glutamate, and other metabolites in fecal metabolites of patients after FMT treatment were higher than the before treatment group (Figure 5A). Meanwhile, serum L-Threonine and N-Acetyl-L-Histidine levels were higher after FMT treatment than before (Figure 5B).

Effects of FMT treatment on intestinal flora

To explore the effects of FMT treatment on the intestinal flora, we investigated the changes in the increase of the number of transplants at class and order levels by using the relative abundance of species quantified by the 16SrDNA gene amplicon sequencer. The relative abundance of species showed that the abundance of intestinal flora in FMT patients before and after treatment changed significantly ($P < 0.05$). The quantities of *Lactobacillus*, *Bacillus*, *Succinilasticum*, *Cellvibrio*, and *Escherichia* increased, while the quantities of *Ruminococcus* and *Bacteroides* decreased (Figure 6).

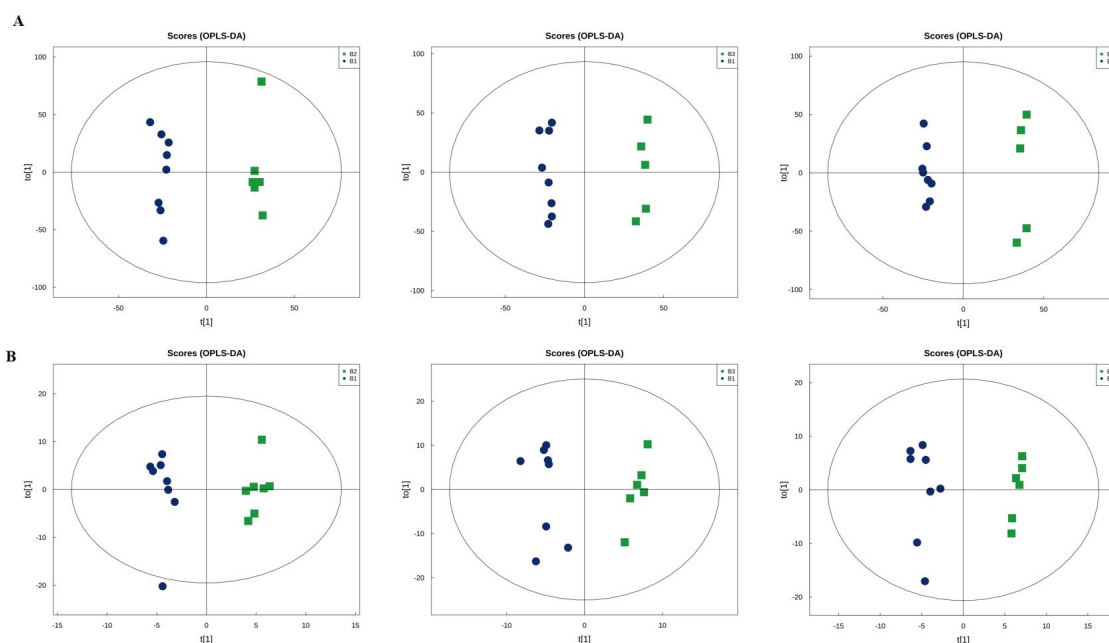


Figure 2 The OPLS-DA in stool (A) and serum (B) showed a clear separation of the metabolite clusters both at baseline and after FMT

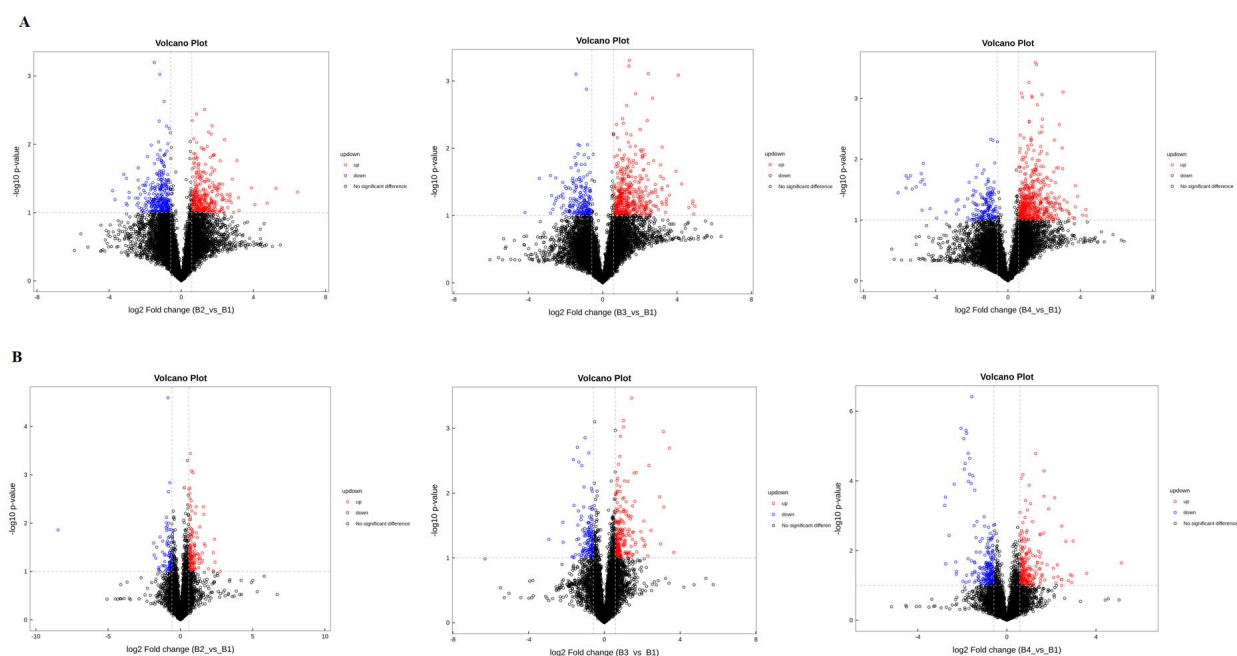


Figure 3 Univariate statistical analysis (volcano chart) showed significant changes in the stool (A) and serum (B) metabolites before and after the FMT treatment.

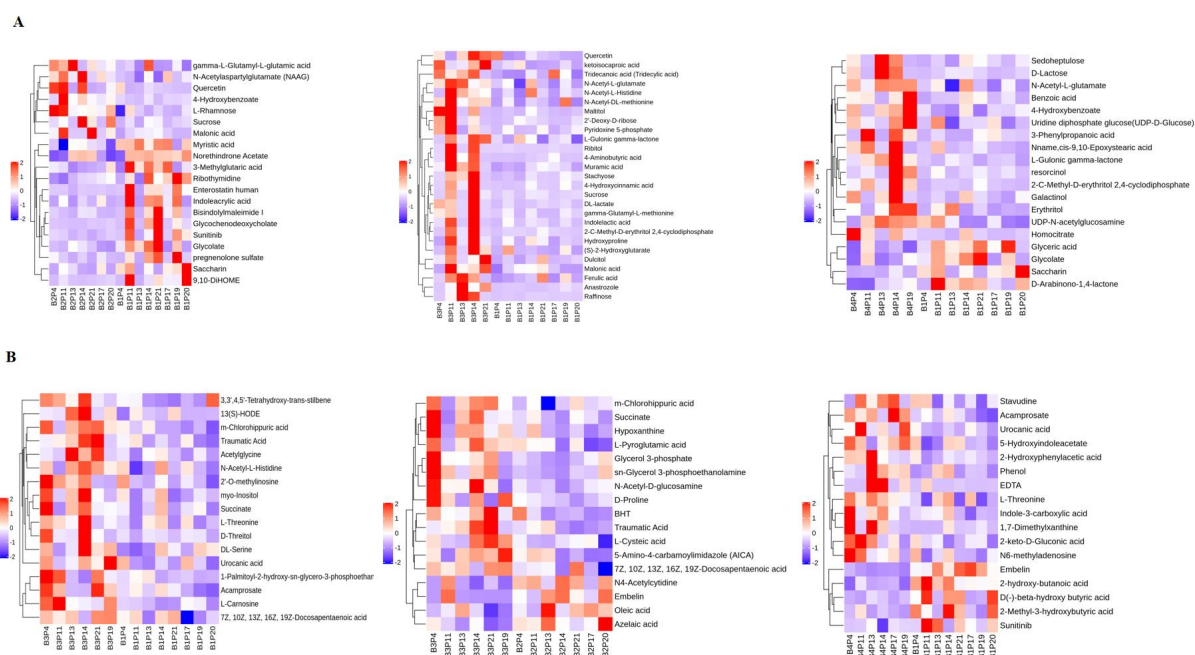


Figure 4 Significant difference metabolite hierarchical cluster heat map analysis showed changes in metabolites in feces (A) and serum (B) before and after patients receiving FMT

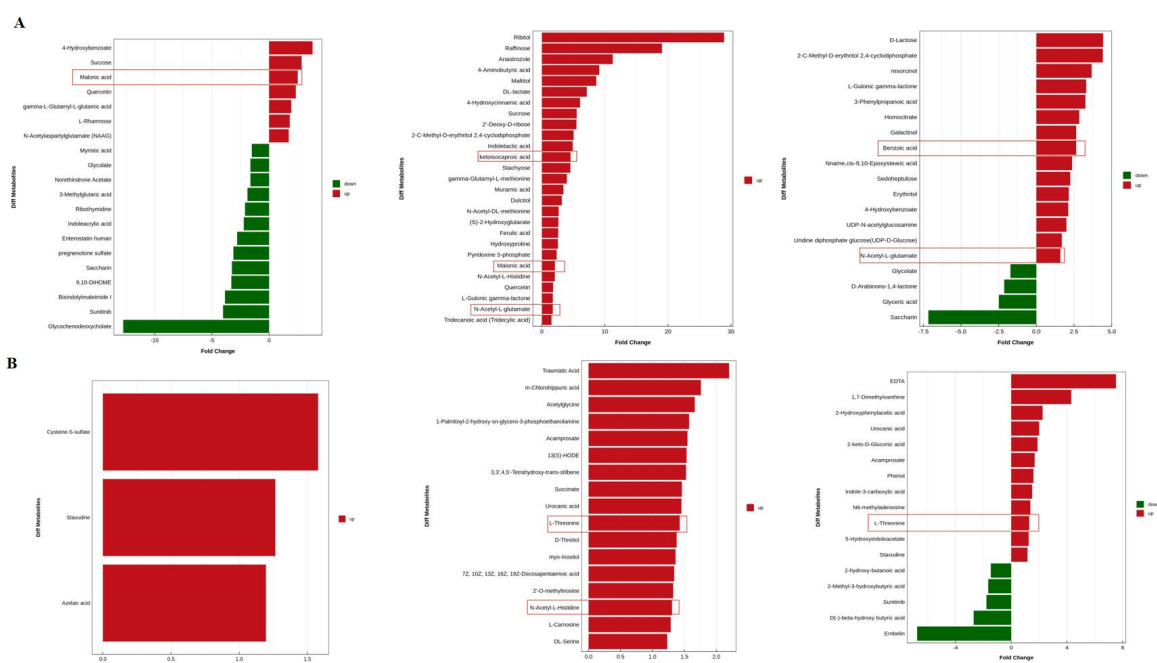


Figure 5 The differential fold analysis of significant differential metabolite expression showed that stool (A) and serum (B) differed from baseline in patients receiving FMT

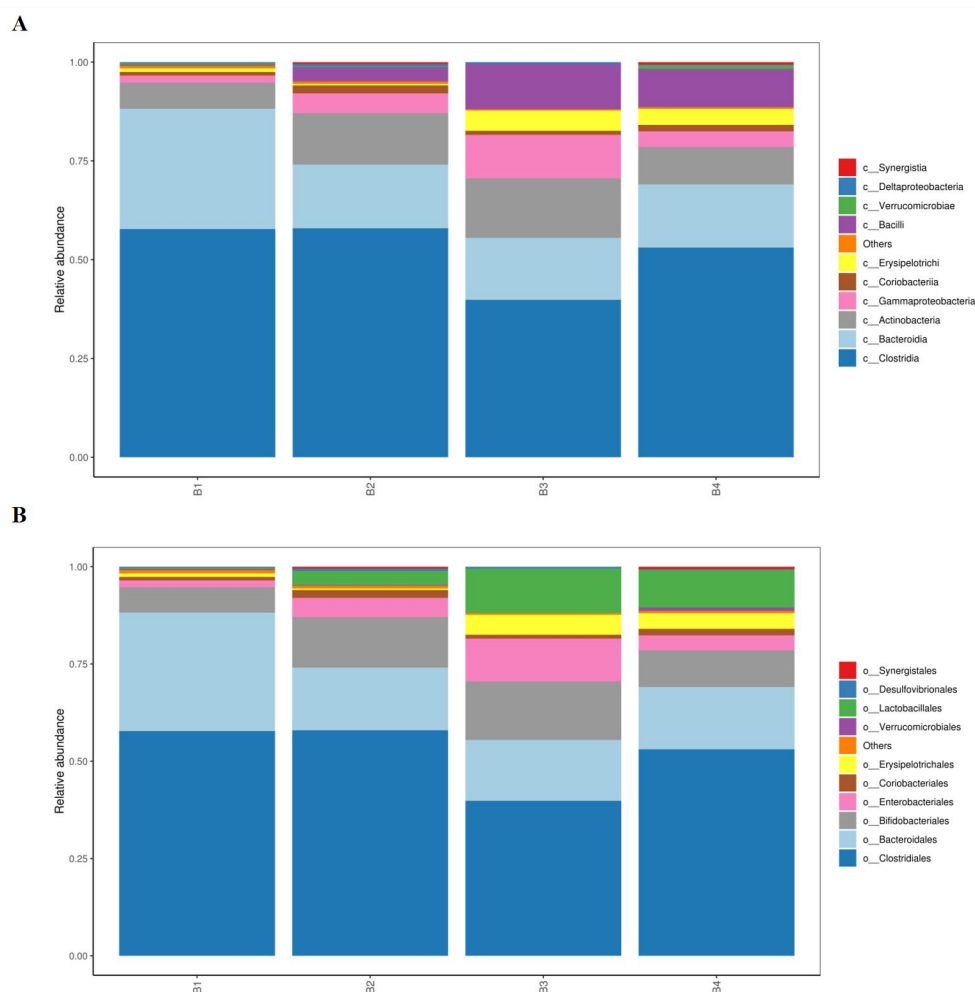


Figure 6 Class level (A) and order level (B) abundance of gut flora in FMT patients before and after treatment

Association analysis between intestinal flora and metabolic differences

To further explore the relationship between intestinal flora and metabolic differences, we conducted a correlation analysis between them. The heat map and scatter plot of the correlation coefficient matrix showed that the significant difference between the flora and the significant difference between the metabolites were closely related in FMT patients before and after treatment (Figures 7 and 8). For example, Ketoisocaproic acid and *Lactobacillus* ($r = 0.212$), *Microbacterium* ($r = 0.586$), *Escherichia* ($r = 0.555$), *Succiniclaticum* ($r = 0.758$) were positively correlated with *Mycoplana* ($r = 0.612$) and *Anaerostipes* ($r = 0.385$), while negatively correlated with *Mycoplana* ($r = 0.612$) and *Anaerostipes* ($r = 0.385$). Benzoic acid was positively correlated with *Cellvibrio* ($r = 0.613$). Interestingly, the preliminary study of our research group proved that the metabolite L-arginine screened out under the positive ion mode was also positively correlated with *Lactobacillus*. It can be seen that the significantly different metabolites screened out under the positive and negative ion modes of metabonomics were closely related to *Lactobacillus*.

Discussion

Li Zhongzi, in his account of the Processing of medicinal Properties of Lei Gong, said, "(Golden juice) was made in the winter month by taking a bamboo basket and placing it on a VAT, covering it with millet husk, adding several layers of papyri, pouring excrement on it, pouring the juice on the VAT, storing it in a new VAT, sealing it with

salt and mud, burying it deep enough to clear the water and smell no dirty odor" [20]. The microbiological study of gold juice is similar to that of modern flora transplantation. At present, as a safe and effective treatment with few side effects, FMT is effective in treating STC [21]. In this study, PAC-SYM scores, weekly total defecation times, and defecation frequency scores of patients after FMT treatment were significantly improved compared to those before treatment. Metabolites detected in the feces and serum of STC patients before and after receiving FMT in metabolomic anion mode were significantly changed. After FMT treatment, the contents of metabolites such as Ketoisocaproic acid, Benzoic acid, Malonic acid, and N-Acetyl-L-glutamate in the feces of patients were higher than those before treatment. Serum levels of L-Threonine and N-Acetyl-L-Histidine increased compared with those before treatment. Studies have shown that Ketoisocaproic acid can significantly increase intracellular cyclic adenosine monophosphate (C-AMP) levels, thereby activating C-AMP-dependent protein kinase A (PKA), and then opening voltage-sensitive calcium channels [22]. This causes the influx of Ca^{2+} ions leading to esophageal smooth muscle contraction and accelerated intestinal peristalsis. Hence, Ketoisocaproic acid can play a vital role in improving STC symptoms. A similar study supported the idea that Ketoisocaproic acid increases gastrointestinal motility through the increase of intracellular Ca^{2+} levels induced by PKA activity [23]. Benzoic acid is widely used in food and feed as an antifungal preservative, and many recent studies have shown that Benzoic acid can improve intestinal digestion, absorption, and barrier functions [24, 25]. Based on the similarity of human and pig intestinal

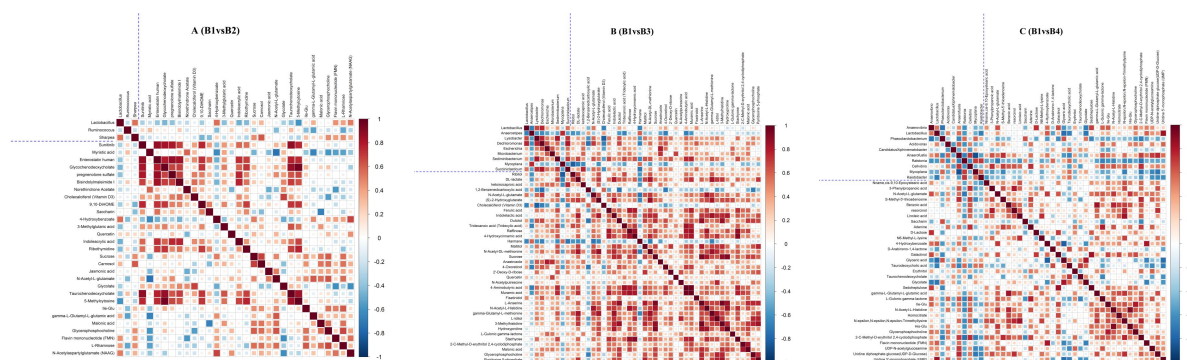


Figure 7 Heat map of correlation coefficients of significant differential flora versus significant differential metabolites before and after patients with FMT treatment

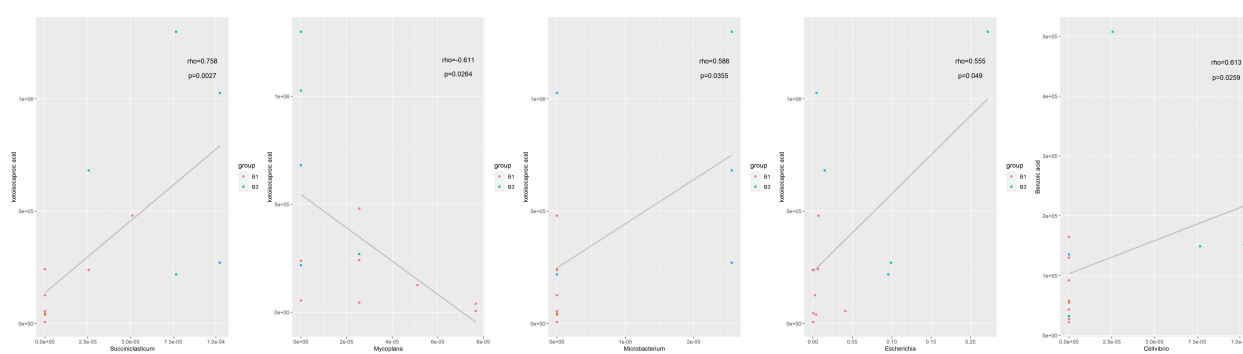


Figure 8 Scatter plots show the relationship between differential flora and metabolites

physiology, studies using piglet and pig intestinal epithelial cells as models suggest that appropriate levels of Benzoic acid may improve intestinal function by regulating enzyme activity, redox reaction, immunity, and microbiota [24]. At the same time, Benzoic acid, as an organic acidifier, can not only improve the digestibility of nutrients but also inhibit harmful microorganisms in the digestive tract (such as *Escherichia coli*) [26]. Therefore, Benzoic acid has been recognized as an intestinal health supplement for humans and animals. The 5-hydroxytryptamine (5-HT) regulates bowel movement and fluid secretion, which can also trigger gastrointestinal peristalsis and secretion reflex, coordinate the vasodilation and contraction of the intestinal wall, and regulate visceral sensation [27]. In recent years, a large number of human and animal experiments have proved that 5-HT is related to intestinal motility [28] and L-Threonine is important for the synthesis of 5-HT, which is closely related to vitamin B6 [29]. It can be seen that the differential metabolites screened out by metabolomics in negative ion mode are closely related to STC.

Recent meta-analyses have demonstrated that many gut microbes can reduce colon transport time, increase the frequency of bowel movements, and improve constipation-related symptoms [30]. We analyzed the intestinal microbiota of the patients by 16SrDNA gene sequencing and found that the ecological diversity and abundance of the fecal microbiota of the patients were different from that before FMT treatment, indicating that constipation was closely related to the changes in intestinal microbiota. The results showed that the contents of the genera *Lactobacillus*, *Bacillus*, *Succiniblasticum*, *Cellvibrio*, and *Escherichia* increased and the contents of *Ruminococcus* and *Bacteroides* decreased in FMT patients. These results suggest that FMT has a therapeutic effect on STC. Previous studies have shown that *Lactobacillus* can cause intestinal growth by improving the barrier function of the intestinal epithelium. *Lactobacillus paracasei* can increase the content of short-chain fatty acids, which is conducive to intestinal motility, thus treating constipation [31, 32]. *Lactobacillus* can accelerate Na^+/H^+ transport by stimulating butyrate absorption

and thereby increasing intestinal electrolytes [33, 34]. *Bacillus* has been reported to induce intestinal peristalsis by promoting the release of 5-HT from chromaffin cells (ECs) in the colon [35]. *Bacillus* can tolerate high temperatures, high pressure, and an acidic environment of the intestinal tract, which helps it to maintain the balance of intestinal flora, repair mucosal barrier, regulate immunity, and inhibit the development of colitis and colon cancer. It was found that *Bacillus* could reverse intestinal peristalsis dysfunction in the STC mouse model by increasing 24-hour defecation weight, fecal moisture, and intestinal transport rate and alleviating pathological damage to the colon [36].

Correlation analysis of metabolites and intestinal flora showed that Ketoisocaproic acid was positively correlated with *Lactobacillus*, while Benzoic acid was positively correlated with *Cellvibrio*. The preliminary study of our research group proved that the metabolite L-arginine screened out under the positive ion mode was also positively correlated with *Lactobacillus*. It can be seen that the significantly different metabolites screened out under the positive and negative ion modes of metabolomics are closely related to *Lactobacillus*. This further confirmed the conclusion that FMT can treat STC by changing intestinal flora and its metabolites. *Lactobacillus* is an important probiotic, which can not only directly promote intestinal motility, but also promote the colonization and reproduction of exogenous bacteria, increase the proportion of probiotics, and further improve the symptoms of STC. The use of probiotics to treat STC is not easy to cause adverse reactions and has attracted more attention [37]. Yoon et al. [38]. have shown that *Lactobacillus* and other probiotics can significantly improve the fecal consistency of STC patients. Dimidi et al. [39]. have also found that probiotics such as *Lactobacillus* can shorten intestinal transport times and increase bowel movements. Therefore, probiotics can be used to treat STC patients by accelerating their colon transport function, without the side effects of drugs and surgical treatment, which is more acceptable to patients and worthy of clinical promotion.

Conclusions

FMT improved STC symptoms by improving intestinal flora and its metabolites. The focus of our future research will be to continue to optimize the treatment of FMT and explore how to shift from whole fecal transplantation to using specific flora transplantation to treat STC.

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