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Original Article

Use of 16s RNA and metabolomics to investigate the therapeutic effect of Zhuyang Tongbian Decoction on mice with functional constipation

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Abstract: Objective: To explore the therapeutic effects of Zhuyang Tongbian Decoction (ZTD) on vasoactive intestinal peptide (VIP) and 5-hydroxytryptamine receptor (5-HTR) in colon tissues, intestinal flora, and fecal metabolites in mice with functional constipation (FC). Methods: A total of 36 BALB/c mice were divided into six groups: control, model, positive (Cisapride), and ZTD groups with three dosages (1.5 g/mL, 3 g/mL, and 6 g/mL). All mice, except those in the control group, were induced with FC by gavage using the compound diphenoxylate. After establishing the model, each group received the respective treatments by gavage for two weeks. The laxative effect was evaluated by comparing changes in body weight, fecal weight, fecal water content, and the percentage of carbon powder propulsion in the small intestine. Immunohistochemistry was used to assess the distribution and expression of VIP and 5-HTR in proximal colon tissues. Additionally, 16S rRNA sequencing and liquid chromatography with quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) non-targeted metabolomics analysis were used to examine the effects of ZTD on intestinal flora composition and metabolites in FC mice. Results: ZTD treatment not only alleviated FC symptoms but also increased the number of VIP and 5-HTR-positive cells in colon tissues. Furthermore, ZTD improved the diversity and abundance of intestinal flora, significantly increasing the relative abundance of Prevotellaceae, Bacteroidales_S24-7_group, Ruminococcaceae, and Roseburia while reducing the abundance of Proteobacteria, Desulfovibrionaceae, Rikenellaceae, Porphyromonadaceae, and Erysipelotrichaceae. In terms of metabolites, ZTD significantly elevated the levels of deoxyadenosine and adenine, while significantly lowering the levels of L-leucine, L-threonine, succinate, tyramine, L-tyrosine, and dopamine. Conclusions: This study provides a theoretical basis for the treatment of FC with ZTD. ZTD increased levels of the intestinal neurotransmitters VIP and 5-HTR and promoted the colonization of beneficial bacteria, including the dominant butyric acid-producing bacterium Roseburia. Additionally, ZTD reduced fecal dopamine levels, indicating its value as a therapeutic approach for FC.

Keywords: Zhuyang Tongbian Decoction, functional constipation, vasoactive intestinal peptide, 5-hydroxytryptamine, intestinal flora, metabolite

Introduction

Functional constipation (FC) is mainly characterized by symptoms such as infrequent bowel movements, straining during defecation, incomplete evacuation, anorectal obstruction, and hard stools [1, 2]. These symptoms are persistent and can lead to anal diseases, including hemorrhoids and anal fissures, as well as an increased risk of rectal cancer and cardiovascular conditions like hypertension [3]. Additionally, patients with FC often experience psychological comorbidities, such as anxiety, depression, and obsessive-compulsive disorder [4], which significantly affect their quality of life. In 2021, the global incidence of FC in adults was estimated to range between 6.6% and 11.7% [5]. The condition is more common in female patients, with a prevalence ratio of approximately 1.5:1 [6]. The exact cause of FC is not fully understood, but potential factors include abnormalities in interstitial cells of Cajal

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(ICC) within colon tissue, altered expression of aquaporins (AQP), dysfunction of intestinal neurotransmitters, and imbalances in intestinal flora [7, 8].

Vasoactive intestinal peptide (VIP) acts as an inhibitory neurotransmitter that regulates peristalsis and intestinal secretion [9]. Another neurotransmitter, 5-hydroxytryptamine (5-HT), plays a critical role in initiating gastrointestinal motility and secretory reflexes [10]. The role of intestinal flora in maintaining intestinal function is well established, as imbalances in the flora can lead to gastrointestinal disorders such as osmotic diarrhea and, in severe cases, colon cancer, in addition to FC [11, 12]. Current research suggests that the expression and secretion of VIP and 5-HT are regulated by the intestinal flora [13, 14]. Furthermore, metabolites produced by the intestinal flora, such as short-chain fatty acids (SCFAs), bile acids, and amino acids, promote intestinal peristalsis and contribute to alleviating FC symptoms [15, 16].

Current clinical treatments for FC include both pharmacologic and non-pharmacologic approaches. Non-pharmacologic methods, such as biofeedback therapy, surgical interventions, and fecal flora transplantation, have limitations, including poor patient compliance and high cost, making them less widely accepted and not a first choice for treating FC [17]. Although various pharmacologic agents, including stool softeners, stimulant or osmotic laxatives, and prokinetic drugs, are available for managing FC, these medications often cause side effects like abdominal discomfort, flatulence, and headache. These limitations reduce their clinical utility [18]. Traditional Chinese medicine (TCM) is recognized for its fewer side effects, lower recurrence rates, and higher efficacy, making it a valuable option for FC due to its multi-targeted and multi-level regulation. JiChuanJian (JCJ), a formula first documented in the "Jingyue Quanshu" during the Ming Dynasty, has been used for centuries to treat FC. Zhuyang Tongbian Decoction (ZTD), based on JCJ, is a traditional Chinese herbal formula known for its laxative effects through intestinal lubrication. ZTD contains *Cistanche deserticola* Y. C. Ma, *Cinnamomum cassia* Presl, *Citrus aurantium* L., *Magnolia officinalis* Rehd. et Wils, *Epimedium brevicornu* Maxim, Morus alba L., *Atractylodes macrocephala* Koidz, *Dioscorea opposita* Thunb, *Prunus japonica* Thunb, *Achyranthes bidentata* Bl., and *Sesamum indicum* L. *Cistanche deserticola* Y. C. Ma has been shown to relieve FC by enhancing colonic sensitivity through the upregulation of the SCF/c-kit pathway in colonic ICC [19]. Morus alba L. alleviates constipation by improving intestinal flora characteristics and SCFA levels [20]. *Atractylodes macrocephala* Koidz mitigates loperamide-induced constipation in rats by modulating tryptophan metabolism [21]. Semen Pruni oil reduces tumor necrosis factor alpha (TNF-α) and interleukin-1beta (IL-1β) expression, as well as extracellular regulated protein kinases (ERK), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (P38), and P65 phosphorylation in colonic tissues of constipated mice, resulting in significant symptom improvement [22].

In this study, we established a functional constipation mouse model and administered ZTD to assess its therapeutic effects. The aim of this study was to explore the effects of ZTD on the levels of VIP and 5-HTR-positive cells in colon tissue, the characteristics of intestinal microbiota, and the levels of fecal metabolites in constipated mice in order to gain insight into the mechanism of action.

Materials and methods

Chemicals and reagents

ZTD (raw drug concentration: 3.0 g/mL) was obtained from the pharmacy of the Third Affiliated Hospital of Liaoning University of Traditional Chinese Medicine (Z20210310). Compound diphenoxylate tablets were purchased from Jilin Wantong Group Co. (China, Cat. No. 160403) and prepared as a 0.5 mg/mL suspension in distilled water. Cisapride tablets were acquired from Xian-Janssen Pharmaceutical Ltd. (H10960289) and prepared as a 0.25 mg/mL suspension in distilled water. Normal saline was sourced from Shijiazhuang No. 4 Pharmaceutical Co., Ltd. (China, Cat. No. N20210304). Pentobarbital sodium was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). VIP Rabbit Anti-Mouse Primary Antibody, Sheep Anti-Rabbit Secondary Antibody, and anti-5HT receptor were supplied by Beijing Biosynthesis Biotechnology Co., Ltd. (China, Cat. No. A20211125, A20211029). Activated carbon was obtained from Sinopharm Chemical

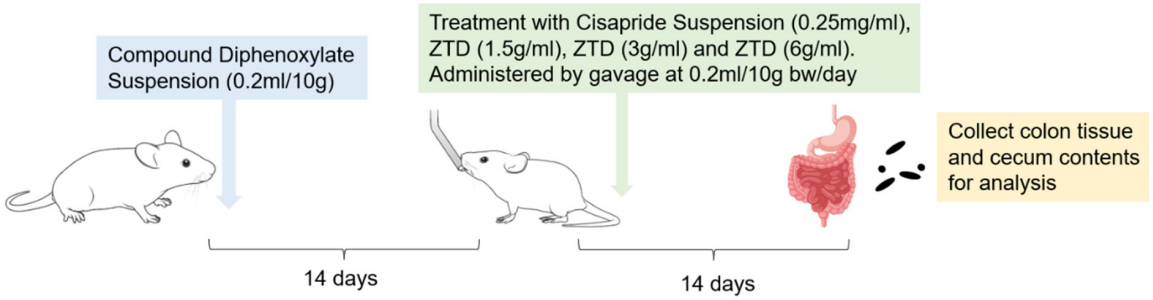


Figure 1. Induction of the FC model, ZTD treatment, and specimen collection process.

Reagent Co., Ltd. (China, Cat. No. F20220706). Methanol and acetonitrile were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Animals

Male BALB/c mice (20 ± 2 g) were procured from Liaoning Changsheng Biotechnology Co., Ltd. (License No. SCXK (Liaoning) 2020-0001). The mice were housed under SPF-class conditions at 18-22°C and 40-60% relative humidity. The Animal Ethics Committee of Liaoning University of Traditional Chinese Medicine approved this study under reference number 21000042020038.

Induction and treatment of FC

The FC model was developed with minor modifications based on a previous method [23]. Briefly, mice were acclimated and fed for five days, after which 36 male mice were randomly divided into six groups. Except for the control group, all mice received a gavage dose of 0.2 mL/10 g of compound diphenoxylate suspension once daily for 14 days (**Figure 1**). Successful modeling was confirmed by the presence of a thin appearance, reduced mobility, curled posture, yellow urine, dry stool surface, and stools formed as fine granules or globules [24]. After successful modeling, all treatment groups received 0.2 mL/10 g body weight per day via gavage for 14 days. The same dose of saline was administered to the control and model groups.

Collection and detection of specimen

At the end of the experiment, each mouse was housed in an individual metal cage for fecal collection every hour for 24 hours. The weight of fecal matter was recorded after collection, and the water content was calculated by drying samples at 60°C for 12 hours. The formula used was: (wet weight - dry weight)/wet weight × 100%. On the last day of the study, the mice underwent a 12-hour fast before receiving the prepared ink (0.02 mL/g) by gavage 30 minutes after the final drug dose. After 20 minutes, the mice were anesthetized and euthanized by intraperitoneal injection of 3% pentobarbital sodium (30 mg/kg). The abdominal cavity was immediately opened, and the large and small intestines were separated. The intestines from the pylorus to the ileocecum were placed on white paper to calculate the intestinal propulsion rate of the ink for each group (percentage of carbon powder propulsion in the small intestine = length of ink propulsion/total length of the small intestine × 100%). A segment of the proximal colon, approximately 15 mm, was cut and placed in a vial containing fixative. Cecal contents (3-5 g) were collected in a sterile tube, sealed, and stored at -80°C.

Immunohistochemistry procedure

The colon tissues in the fixative were routinely dehydrated (Model LEICA 300, Leica, Germany), embedded in paraffin (Model EG-1150, Leica, Germany), and sectioned (Model LEICA RM2235, Leica, Germany). Antibodies were sequentially added to the sections, which were then stained with hematoxylin and sealed with neutral gum. After staining, well-stained areas were selected for image acquisition and analysis using a Leica Q550CW system. Specific brownish-yellow particles on a clear background indicated a positive result, while the negative control showed no specific coloring. The mean optical density (MOD) values of VIP and 5-HTR were then measured.

16S rRNA high-throughput sequencing

For the comparison of ZTD's laxative effect in FC mice, we selected ZTD (3 g/mL), which demonstrated superior efficacy, as the treatment group. We then performed 16S rRNA high-throughput sequencing and liquid chromatography with quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) non-targeted metabolomics analysis on samples from the control, model, and ZTD groups.

Bacterial DNA was extracted from the cecum contents of mice in all three groups (control, model, and ZTD with 3 g/mL). After extraction, 16S rDNA V3-V4 fragments were amplified and purified to create sequencing libraries, which were then sequenced using the MiseqPE300 platform. The resulting sequences required further processing to obtain operational taxonomic units (OTUs). Based on OTU clustering and annotation, Venn diagrams, dilution curves, and Shannon-Wiener curves were plotted. α diversity analysis was conducted to evaluate the diversity within each group's intestinal flora, while β diversity analysis used principal component analysis (PCA) to visualize differences between groups. Linear discriminant analysis Effect Size (LEfSe) analysis was performed to identify differential species.

LC-QTOF-MS non-targeted metabolomic analysis

A mixed mouse feces sample (30 mg) was extracted by adding 500 μL of a methanol: acetonitrile: water solution (2:2:1) and vortexed for 30 seconds. After thorough mixing, the sample was centrifuged at 13,000 r/min for 15 minutes at 4°C, yielding 350 μL of supernatant. This supernatant was evaporated with 200 μL of acetonitrile: water (1:1) and then centrifuged again at 13,000 r/min for 10 minutes at 4°C. The final supernatant (50 μL) was transferred to the injection vial for LC-QTOF-MS analysis. The analysis was performed using electrospray ionization in both positive (POS) and negative (NEG) ion modes. A Waters ACQUITY UPLC BEH Amide column (1.7 μm, 2.1 mm × 100 mm) was used for liquid gradient elution at a flow rate of 0.5 mL/min and a temperature of 40°C. The electrospray ionization temperature was set to 650°C, with a voltage of 5500 V for positive ions and -4500 V for negative ions. The de-clustering voltage was set to 60 V, and the ion source gases (Gas1 and Gas2) were maintained at 60 psi, with the curtain gas (CUR) at 30 psi. The structural characterization of metabolites was performed using MasterView (SCIEX, USA) following data acquisition.

Statistical analysis

Statistical analysis was conducted using SPSS 24.0. Measured data were first tested for normality. Data that conformed to a normal distribution were presented as mean ± standard deviation ($\bar{x}\pm s$) and analyzed using univariate analysis (t-test). Non-normally distributed data were presented as median with interquartile range (median, Q1-Q3) and analyzed using the Wilcoxon rank sum test. Tukey's test was employed to determine differences in alpha diversity indices between groups. Correlation analyses between intestinal flora and metabolites were performed using Spearman's correlation. Metabolites with a VIP > 1 and *P*-value < 0.05 were identified as differential metabolites. Results with *P*-values below 0.05 were considered significant.

Results

ZTD improved symptoms in mice with FC

As shown in **Figure 2A**, after five days of acclimatization, the body weights of the mice in all groups were approximately similar. After the modeling period, body weights decreased in all groups except for the control group. Following 14 days of drug administration, body weights significantly increased in each treatment group. As shown in **Figure 2B-D**, ZTD notably increased fecal weight and water content in FC mice. ZTD also significantly boosted the percentage of carbon powder propulsion in the small intestine, indicating an improvement in intestinal motility in FC mice in a dose-dependent manner.

ZTD improved the distribution and expression of VIP and 5-HTR in the colonic tissues of mice with FC

The immunohistochemical analysis, shown in **Figure 3A**, indicated variable numbers of positive cells in all sections. These cells were predominantly round or oval with cytoplasmic granules showing yellow or brownish-yellow stain-

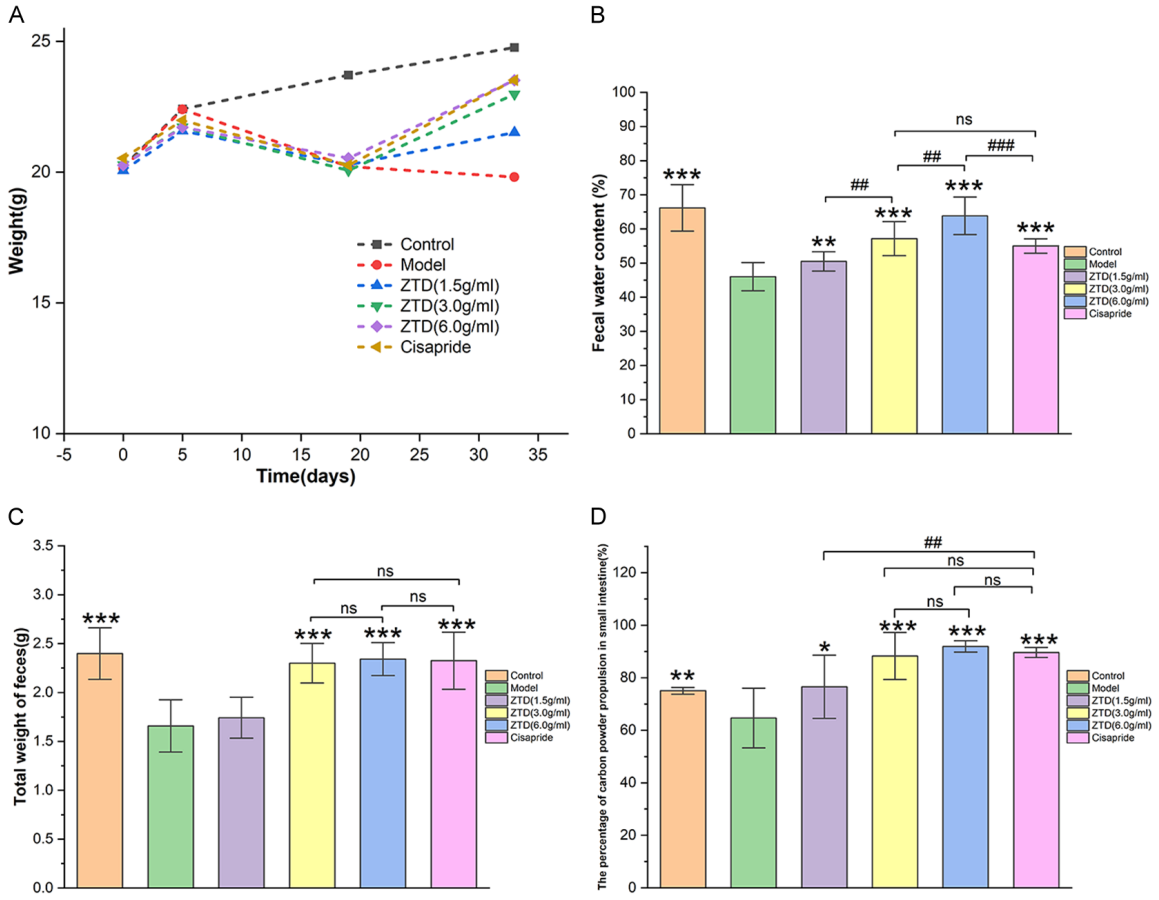


Figure 2. Improvement of symptoms in FC mice by ZTD. A. Changes in body weight of mice. B. Fecal water content. C. Total weight of feces. D. The percentage of carbon powder propulsion in small intestine. *P < 0.05, **P < 0.01, ***P < 0.001 vs. Model. ##P < 0.01, ###P < 0.001, ns: Not significant.

ing. The model group had a lower number of positive cells and a sparser distribution compared to the treatment groups, which displayed a denser arrangement of positive cells. The MOD values for VIP and 5-HTR were significantly reduced in the model group after induction (**Figure 3B**, **3C**), indicating a reduction in VIP-positive and 5-HTR-positive cells in the colons of constipated mice. All treatment groups showed increased MOD values for VIP and 5-HTR, with the 3 g/mL and 6 g/mL ZTD groups showing the most significant improvements, approaching the levels of the control group.

Effects of ZTD on the intestinal flora of FC mice

Sequences were grouped into OTUs based on similarity, with groups having 97% similarity typically used for bioinformatic analysis. The results showed 1,014 identical OTUs across all three groups (**Figure 4A**). The ZTD group had 87 unique OTUs, while the model and control groups had 36 and 24 unique OTUs, respectively. As shown in **Figure 4B**, the rarefaction curve indicated that the observed OTUs stabilized as the sequencing data increased, confirming the adequacy of the sequencing depth. **Figure 4C** demonstrates that the Shannon index plateaus with increased sequencing depth, suggesting sufficient data to capture most bacterial colony information. **Figure 4D** shows that alpha diversity indices decreased in the model group compared to the control group but increased after ZTD treatment. PCA analysis (**Figure 4E**) revealed significant differences in the structural composition of intestinal flora between the control, model, and ZTD groups.

In **Figure 5A**, the dominant phyla in each group were Firmicutes, Bacteroidetes, Proteobacteria, and Saccharibacteria. The Firmicutes to Bacteroidetes (F/B) ratio was 1.20, 1.62, and

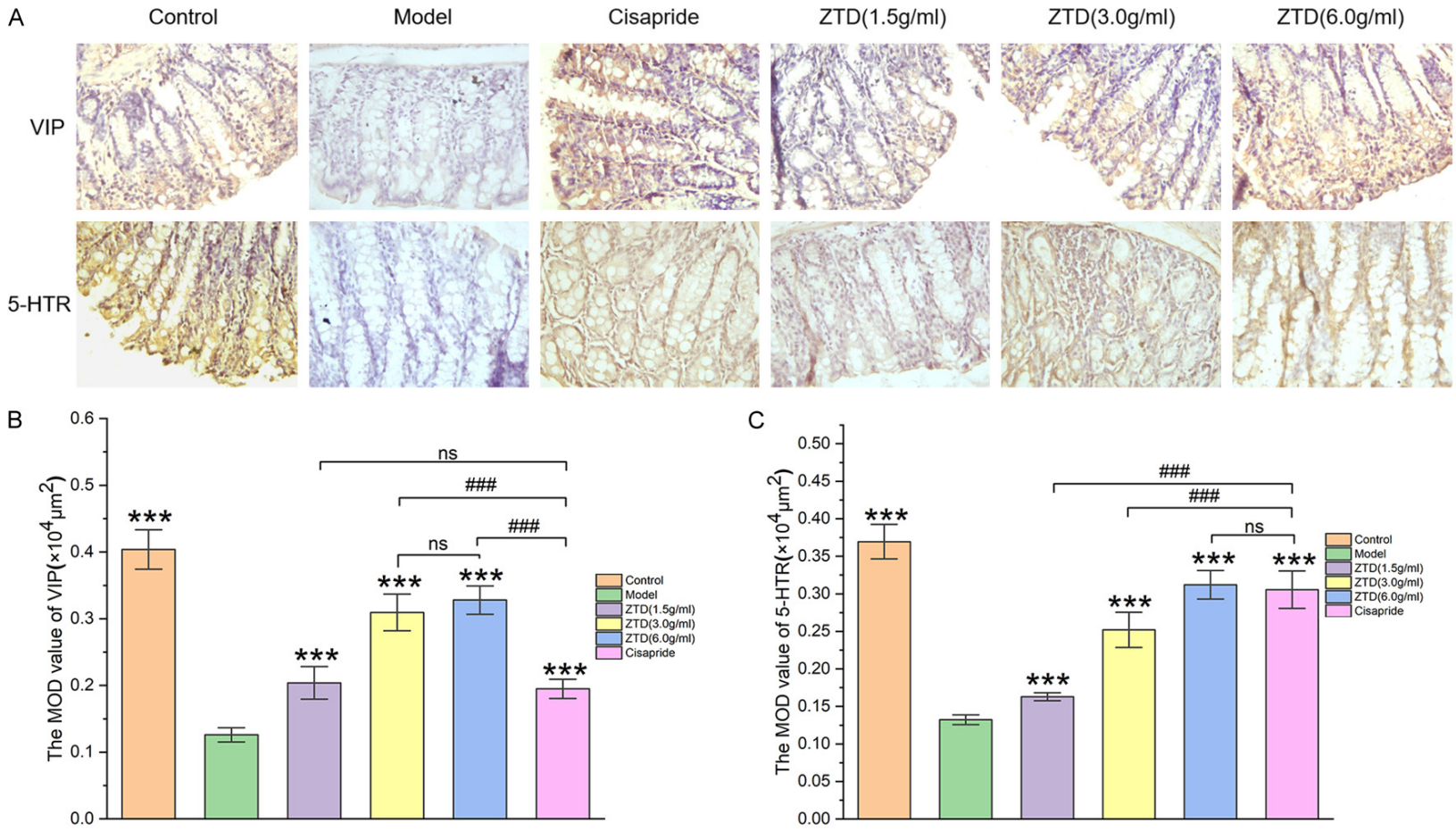


Figure 3. ZTD improved the distribution and expression of VIP and 5-HTR in the colonic tissues of mice with FC. A. The immunohistochemistry results of VIP and 5-HTR (400×). B. The MOD value of VIP. C. The MOD value of 5-HTR. ***P < 0.001 vs. Model. ###P < 0.001, ns: Not significant.

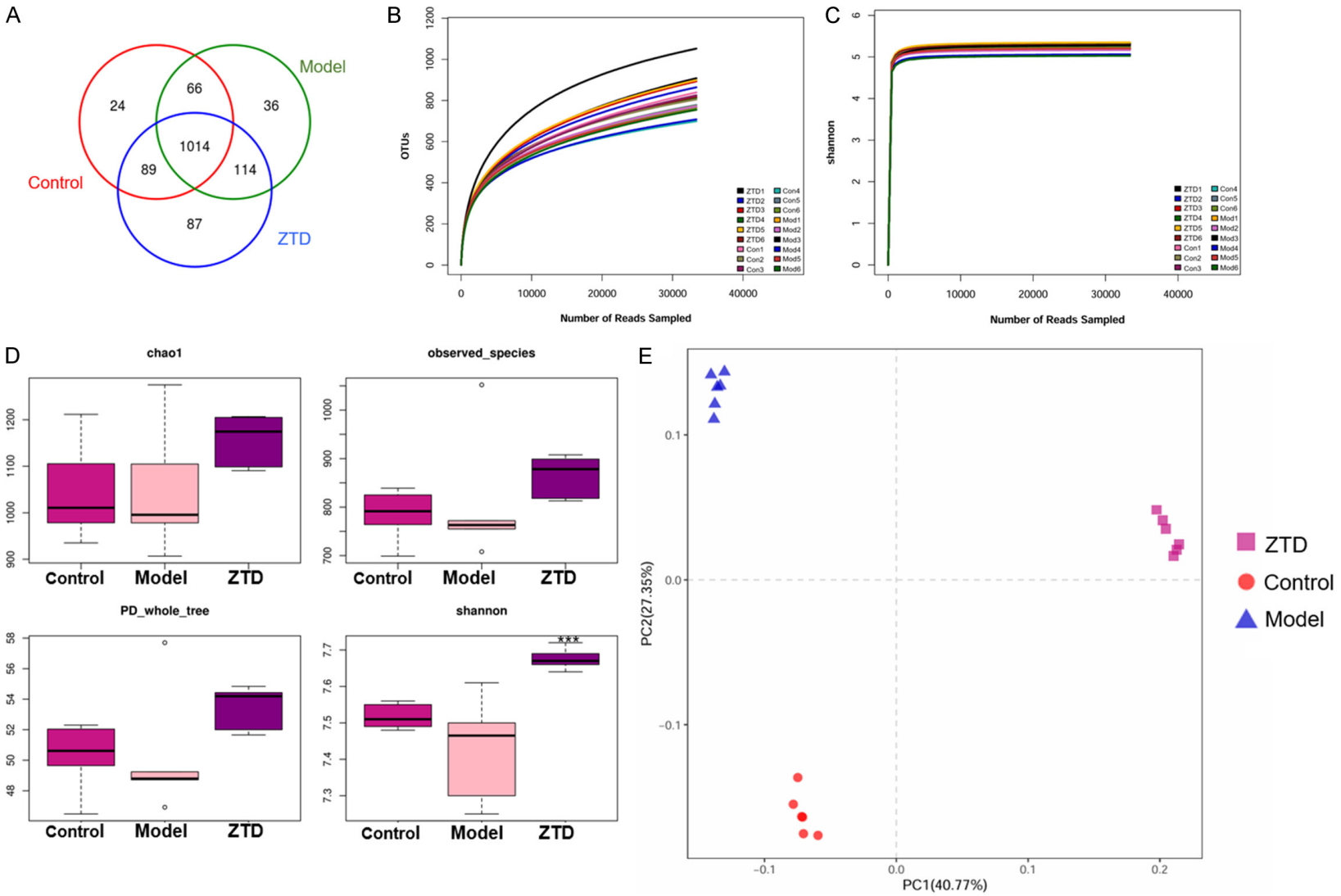


Figure 4. Bioinformatic analysis. A. Venn diagram of OTUs. Control group was denoted by the color red, model group was denoted by the color green, ZTD group was denoted by the color blue. B. Sample Rarefaction Curve. C. Shannon-Wiener Curve. D. α Diversity Index. ***P < 0.001 for ZTD vs. Model. E. Differential analysis of colony structure. The differences are well explained by the 40.77% contribution of PC1 and the 27.35% contribution of PC2.

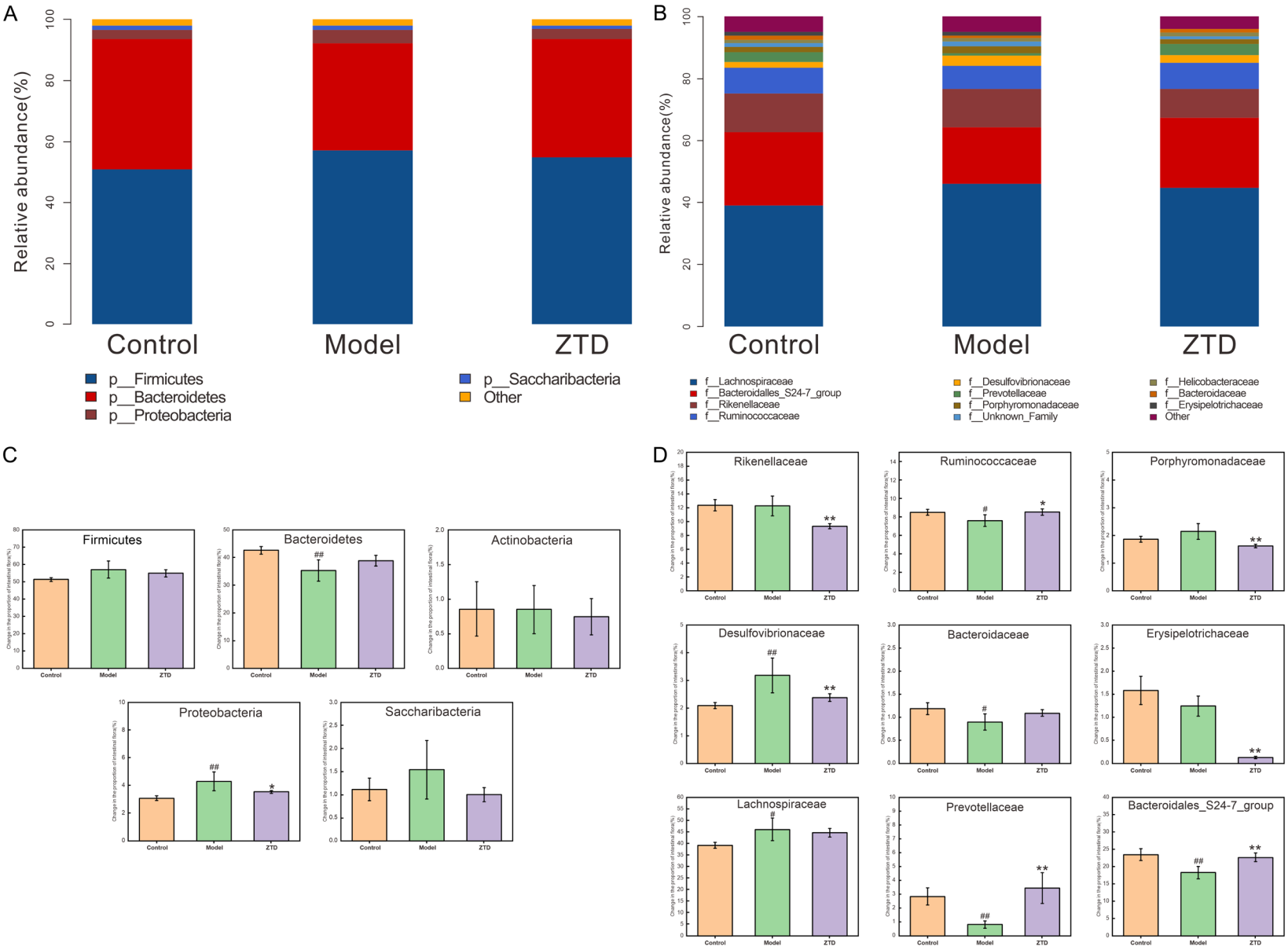


Figure 5. Comparison of flora abundance before and after treatment. A. The relative abundance of flora at the phylum. B. The relative abundance of flora at the family. C. Therapeutic effects of ZTD on intestinal flora at the phylum. D. Therapeutic effects of ZTD on intestinal flora at the family. *P < 0.05, **P < 0.01 for ZTD vs. Model. #P < 0.05, ##P < 0.01 for Model vs. Control.

1.41 in the control, model, and ZTD groups, respectively. After modeling, the relative abundance of Proteobacteria increased, while Bacteroidetes decreased. Treatment with ZTD notably reduced Proteobacteria levels (**Figure 5C**). At the family level (**Figure 5B**), the dominant families across all groups were Lachnospiraceae, Rikenellaceae, Bacteroidales_S24-7_group, and Ruminococcaceae, accounting for more than 80% of the total flora. After modeling, Desulfovibrionaceae and Lachnospiraceae increased significantly, while Bacteroidaceae, Prevotellaceae, Ruminococcaceae, and Bacteroidales_S24-7_group decreased. Post-treatment, Bacteroidales_S24-7_group, Prevotellaceae, and Ruminococcaceae significantly increased, whereas Desulfovibrionaceae, Rikenellaceae, Porphyromonadaceae, and Erysipelotrichaceae significantly decreased (**Figure 5D**). LDA Effect Size analysis (**Figure 6A**) identified 16 significantly enriched floras in the ZTD group, including Prevotellaceae, Roseburia, Ruminococcaceae, Alloprevotella, and Eubacterium.

As shown in **Figure 6B**, based on the LDA analysis results and Spearman test, we selected the top 20 genera with the highest absolute abundance for inter-correlation analysis. We excluded results with $P > 0.05$ or a correlation value $|R| < 0.6$. Roseburia, which was of particular interest, showed a positive correlation with Lachnospiraceae_UCG_006 and Lachnoclostridium, and a negative correlation with Prevotellaceae_UCG_001, Ruminococcaceae_UCG_014, and Alistipes.

Effects of ZTD on the fecal metabolite levels of FC mice

Figure 7A illustrates the results of PCA analysis, showing clear differences between the three groups. Metabolites with VIP ≥ 1 and $P < 0.05$ were identified as significant differential metabolites. As depicted in **Figure 7B-E**, we screened the top 20 significantly up- or down-regulated metabolites in both positive and negative ion patterns at $P < 0.05$, ranked by ascending VIP values. The results indicated that L-leucine, His-Leu, L-threonine, and D-aspartic acid were significantly up-regulated, while deoxyadenosine, adenine, glycitein, and hydroxyisocaproic acid were significantly down-regulated in FC mice, with ZTD effectively reversing these changes. **Figure 8A** outlines the process for obtaining matched data for differential metabolites, followed by a search in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database and subsequent pathway analysis. A comparative analysis identified primary bile acid biosynthesis, taurine and hypotaurine metabolism, tyrosine metabolism, valine, leucine, and isoleucine biosynthesis, and alanine, aspartate, and glutamate metabolism as the main differential pathways in the model group compared to the control group. In contrast, tyrosine metabolism, valine, leucine, and isoleucine biosynthesis, and phenylalanine, tyrosine, and tryptophan biosynthesis were the key differential pathways between the ZTD and model groups.

We found that the pathways of valine, leucine, and isoleucine biosynthesis, along with tyrosine metabolism, were closely related to ZTD's effects on FC. Therefore, we further analyzed the levels of differential metabolites involved in these pathways, including L-leucine and L-threonine in valine, leucine, and isoleucine biosynthesis, and succinate, tyramine, L-tyrosine, and dopamine in tyrosine metabolism. The results (**Figure 8B**) showed that ZTD significantly regulated these metabolites in FC mice.

As shown in **Figure 8C**, we explored the correlation between differential metabolites and differential flora. Roseburia was negatively correlated with xanthosine, propionic acid, and taurine. Additionally, Lactobacillus showed a positive correlation with L-valine, eicosapentaenoic acid, valeric acid, L-glutamate, cholic acid, and L-arginine, and a negative correlation with myristic acid and tridecanoic acid.

Discussion

ZTD regulates the expression and distribution of VIP, 5-HTR for the treatment of FC

The experiment evaluated fecal weight, water content, and the proportion of carbon powder propulsion in the small intestine of FC mice to assess how ZTD affected intestinal function and treated FC. The results showed that different dosages of ZTD effectively alleviated constipation symptoms in the mice. These findings suggest that ZTD enhances smooth muscle contraction and boosts intestinal activity, leading to the effective relief of FC.

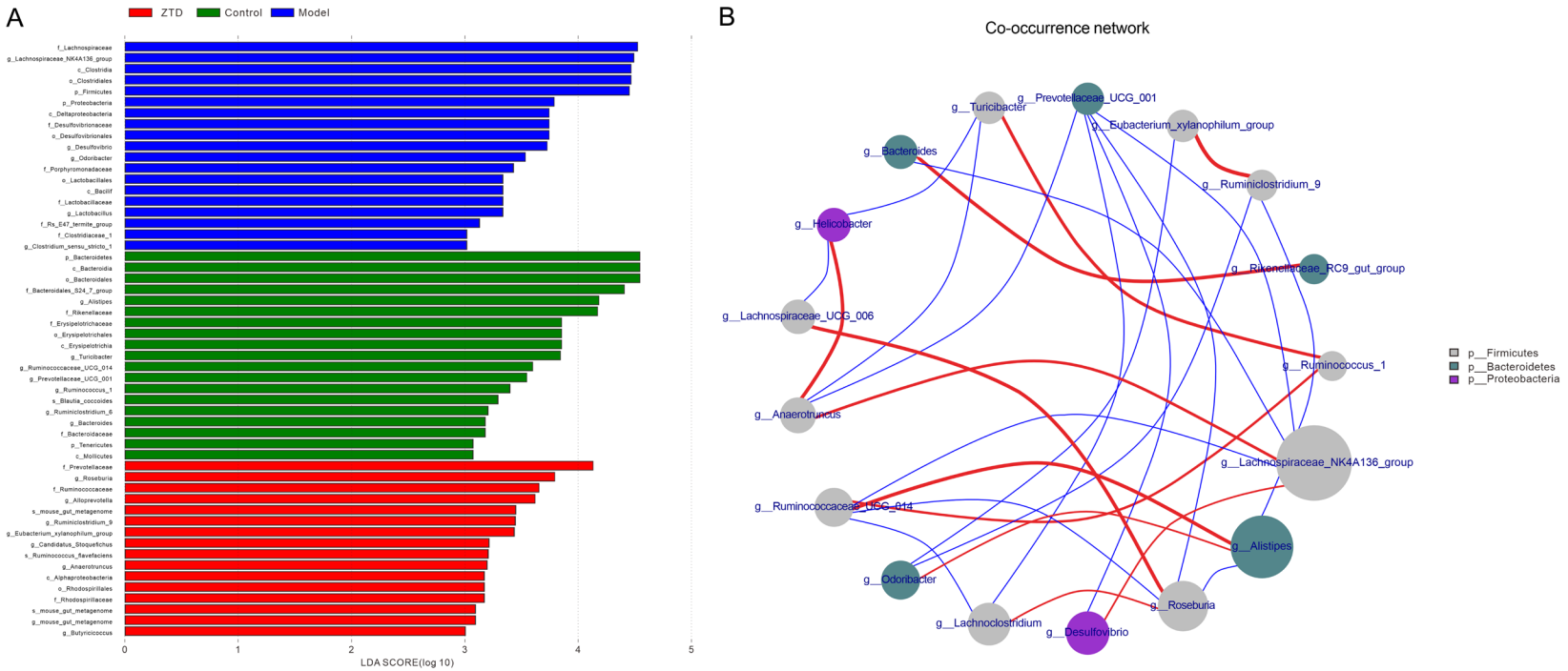


Figure 6. Correlation analysis of differential flora. A. The analysis of differential floras at different levels of classification. B. The correlation analysis of intestinal flora. The red line denotes a positive correlation, while the blue line signifies a negative correlation. Dot size indicates abundance magnitude, and line thickness indicates correlation strength.

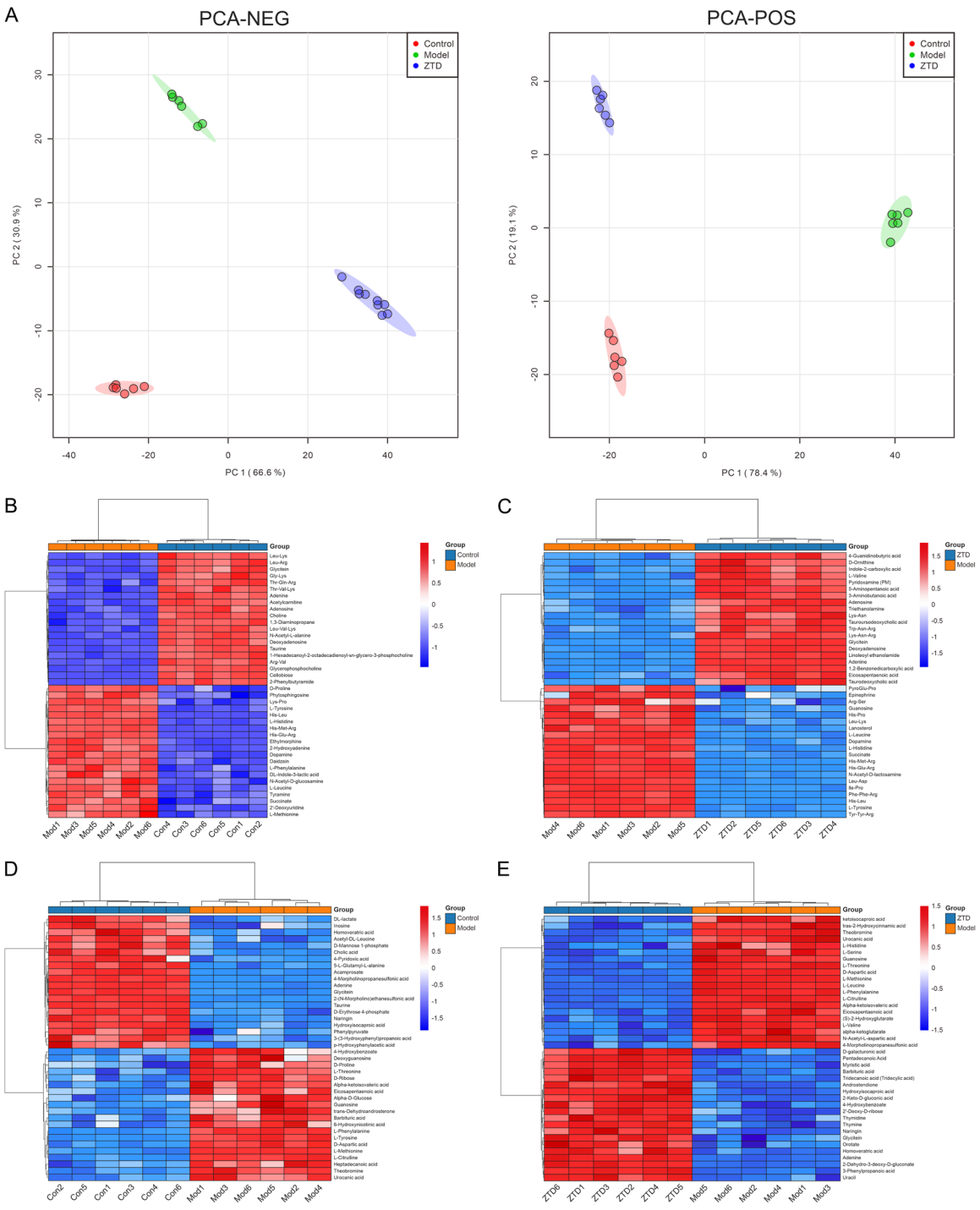
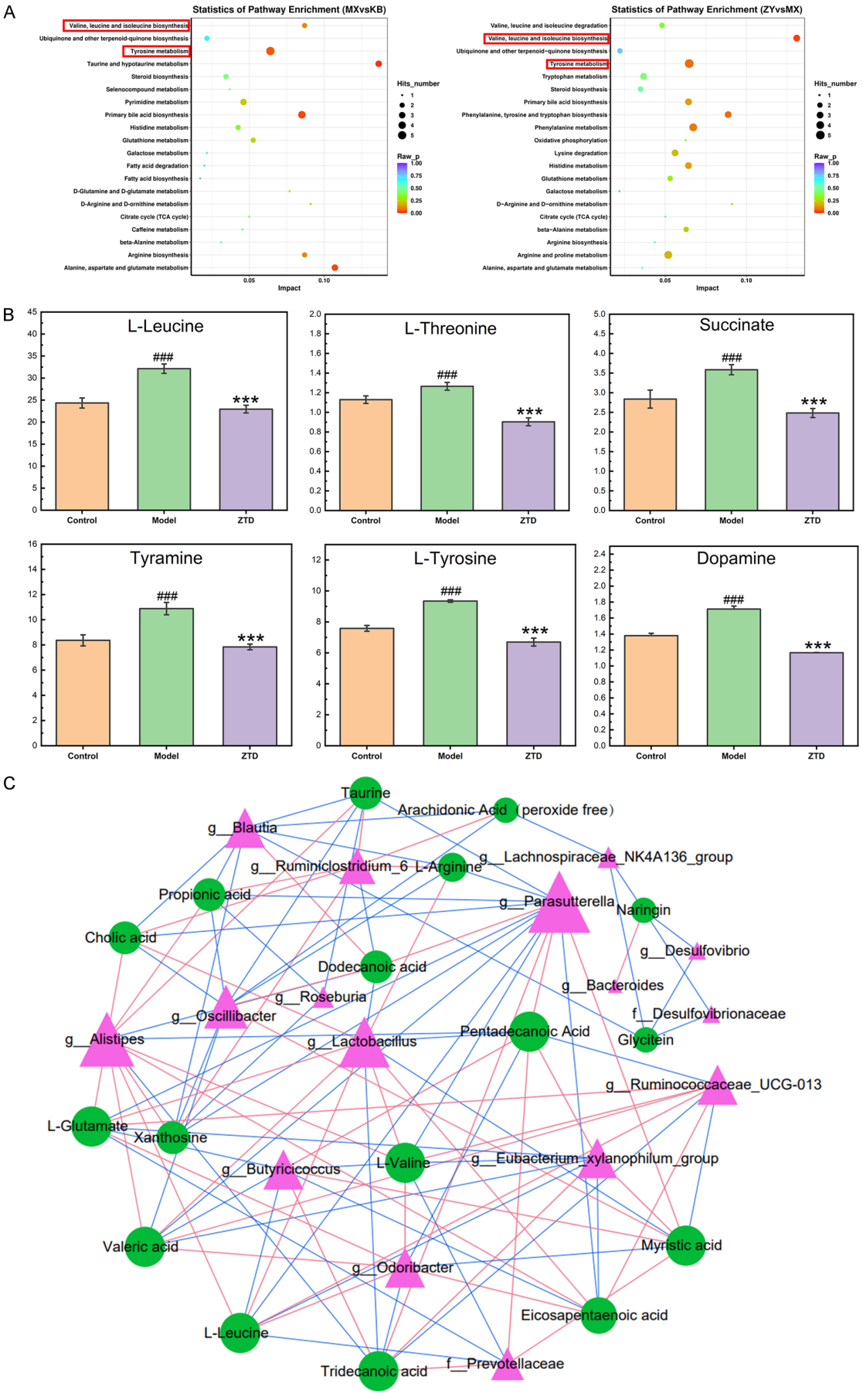


Figure 7. Metabolic profiles and changes in key metabolites. A. The PCA analysis of negative and positive ion modes. B. Control vs. Model in positive ion mode. C. Model vs. ZTD in positive ion mode. D. Control vs. Model in negative ion mode. E. Model vs. ZTD in negative ion mode.

This study demonstrated that VIP and 5-HTR levels were significantly reduced in the colon tissues of constipated mice, while the number of VIP and 5-HT receptor-positive cells increased significantly after ZTD treatment. The relationship between enteric neurotransmitters and FC has been a key area of interest. VIP is an important regulator that relaxes the gastro-


A
Statistics of Pathway Enrichment (MXvsKB)
Valine, leucine and isoleucine biosynthesis
Ubiquinone and other terpenoid-quinone biosynthesis
Tyrosine metabolism
Taurine and hypotaurine metabolism
Steroid biosynthesis
Selenocompound metabolism
Pyrimidine metabolism
Primary bile acid biosynthesis
Histidine metabolism
Glutathione metabolism
Galactose metabolism
Fatty acid degradation
Fatty acid biosynthesis
D-Glutamine and D-glutamate metabolism
D-Arginine and D-ornithine metabolism
Citrate cycle (TCA cycle)
Caffeine metabolism
beta-Alanine metabolism
Arginine biosynthesis
Alanine, aspartate and glutamate metabolism
Impact
Hits_number
Raw_p
Statistics of Pathway Enrichment (ZYvsMX)
Valine, leucine and isoleucine degradation
Valine, leucine and isoleucine biosynthesis
Ubiquinone and other terpenoid-quinone biosynthesis
Tyrosine metabolism
Tryptophan metabolism
Steroid biosynthesis
Primary bile acid biosynthesis
Phenylalanine, tyrosine and tryptophan biosynthesis
Phenylalanine metabolism
Oxidative phosphorylation
Lysine degradation
Histidine metabolism
Glutathione metabolism
Galactose metabolism
D-Arginine and D-ornithine metabolism
Citrate cycle (TCA cycle)
beta-Alanine metabolism
Arginine biosynthesis
Arginine and proline metabolism
Alanine, aspartate and glutamate metabolism
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Succinate
Tyramine
L-Tyrosine
Dopamine
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ZTD
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Taurine
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g__Ruminiclostridium_6
L-Arginine
g__Lachnospiraceae_NK4A136_group
Propionic acid
g__Parasutterella
Naringin
Cholic acid
g__Desulfovibrio
Dodecanoic acid
g__Bacteroides
g__Roseburia
g__Oscillibacter
f__Desulfovibrionaceae
g__Alistipes
g__Lactobacillus
Pentadecanoic Acid
Glycitein
g__Ruminococcaceae_UCG-013
L-Glutamate
Xanthosine
g__Butyricicoccus
L-Valine
g__Eubacterium_xylanophilum_group
Valeric acid
Myristic acid
g__Odoribacter
Eicosapentaenoic acid
L-Leucine
Tridecanoic acid
f__Prevotellaceae

Figure 8. Analysis of key metabolites. A. KEGG enrichment analysis bubble plots. B. Valine, leucine and isoleucine biosynthesis and Tyrosine metabolism pathway related differential metabolite levels. ***P < 0.001 for ZTD vs. Model. ###P < 0.001 for Model vs. Control. C. Correlation analysis of differential flora with differential metabolites. Red lines indicate positive correlation; blue lines indicate negative correlation.

intestinal tract and controls intestinal motility [13]. Reduced levels of VIP in the gastrointestinal tract can lead to impaired intestinal transit and decreased fluid secretion, which in turn causes or worsens FC [25]. Similarly, 5-HT, mainly secreted by enterochromaffin cells, binds to 5-HTR to promote peristalsis and stimulate glandular secretion [26, 27], effectively alleviating FC symptoms. ZTD increased the levels of VIP and 5-HTR in colonic tissues, promoting intestinal peristalsis, which may be one of the mechanisms by which ZTD treats FC.

ZTD improves intestinal flora imbalance in the treatment of FC

The study found that ZTD reversed the Firmicutes to Bacteroidetes (F/B) ratio in the intestines of constipated mice, enhancing beneficial bacteria while reducing harmful bacteria. The F/B ratio is a key indicator of gut flora balance, and an elevated ratio can disrupt carbohydrate metabolism by gut microbiota, reducing colonic water content and leading to FC [28]. Increased levels of harmful bacteria are also closely linked to FC. Studies have shown that Desulfovibrionaceae can reduce sulfate in the colon, producing high concentrations of hydrogen sulfide, which inhibits intestinal peristalsis [29]. Analyzing the intestinal flora structure in patients with chronic FC revealed that a high abundance of Prevotellaceae is crucial for treating FC [30, 31]. Prevotellaceae can promote blood vessel formation in the intestinal mucosa, improve immunity [32], and help maintain intestinal microecological balance. They also produce beneficial SCFAs [33], which may regulate intestinal motility and alleviate FC symptoms through multiple pathways.

It is important to highlight that this study focused on Roseburia, which was identified as a candidate probiotic in 2014 [34]. Recent studies suggest that a reduction in the abundance of this genus may be a key factor contributing to FC [35]. Roseburia is a dominant butyric acid-producing bacterium. Butyric acid, mainly present in the colon and cecum, is primarily utilized by colonic cells. It stimulates colonic smooth muscle contraction by inducing the release of 5-HT or binding to G protein-coupled receptors [36], thus promoting intestinal motility. Some studies [37] also reported that butyric acid can regulate intestinal motility and relieve constipation symptoms through the AKT-NF-κB pathway. Primary metabolites produced by bacteria such as Bifidobacterium, Lactobacillus, and Bacteroidetes through the degradation of indigestible carbohydrates can be cross-fed to Roseburia and Ruminococcaceae to enhance butyrate production [38]. Based on our correlation analysis results, we hypothesize that Roseburia may have a similar cross-feeding relationship with Lachnospiraceae_UCG_006 and Lachnoclostridium. Additionally, Ruminococcaceae, another butyric acid-producing bacterium, positively influences intestinal barrier function, reduces inflammation, and metabolizes complex polysaccharides into SCFAs [39]. Although changes in fecal butyric acid levels were not detected in this experiment, we observed that the relative abundances of the dominant butyric acid-producing bacteria Roseburia and Ruminococcaceae were significantly reduced in constipated mice, but ZTD effectively reversed this imbalance. In conclusion, ZTD corrected the imbalance of intestinal flora during FC pathogenesis, which may be another mechanism by which it treats FC.

ZTD improves fecal metabolite levels to treat FC

Feces are the final metabolic products of an organism, resulting from the interaction between the host and gut microbiota. They provide a better reflection of an individual's gastrointestinal function. Our findings showed that constipated mice had higher levels of amino acids like L-threonine, L-leucine, and L-tyrosine in their feces. Abnormal levels of L-threonine can impact the restoration of epithelial barrier function and apoptosis regulation. Additionally, L-leucine, along with L-isoleucine and L-valine, is involved in muscle repair, blood sugar regulation, and energy supply to body tissues [40].

Disruptions in amino acid metabolism in constipated mice affect these normal physiological functions. We also found that dopamine levels were increased in the feces of constipated mice. Dopamine is a key monoamine neurotransmitter that plays crucial roles in the central nervous system, including regulating movement, cognition, emotion, and memory. It also functions in peripheral tissues, particularly in the gastrointestinal tract, where it influences mucosal ionic secretion and gastrointestinal dynamics [41, 42]. Dopamine inhibits gastric and distal colonic motility by acting on D2 and D1 receptors on smooth muscle [43, 44]. Interestingly, L-tyrosine, a precursor for dopamine synthesis, and tyramine, an intermediate metabolite, were also significantly elevated in constipated mice. Tyrosine is decarboxylated by aromatic L-amino acid decarboxylase to form tyramine, which can then be further hydroxylated to produce dopamine [45, 46]. Studies have shown that Enterococcus faecalis can metabolize dopamine precursors L-dopa and L-tyrosine into tyramine [47], thereby affecting dopamine synthesis. Based on previous findings, we hypothesize that specific intestinal flora are involved in dopamine synthesis by utilizing L-tyrosine, thereby affecting intestinal motility. It is reassuring that ZTD effectively corrected these metabolite changes while also improving the intestinal flora imbalance.

Based on the identification of differential metabolites significantly associated with constipation, metabolic pathways like valine, leucine, and isoleucine biosynthesis, and tyrosine metabolism were found to be disrupted. These disturbances may either contribute to or result from FC. Our study showed that ZTD effectively alleviated these disruptions; however, the specific pathways and treatment targets require further detailed investigation.

In conclusion, this study demonstrated that ZTD significantly increased VIP and 5-HTR levels in colonic tissue, restored the balance of intestinal flora, and normalized fecal metabolite levels. These effects may represent a possible mechanism by which ZTD treats FC.

Conclusion

This study provided a theoretical basis for using ZTD to treat FC through a comprehensive approach, utilizing immunohistochemistry, 16S rRNA intestinal flora analysis, and LC-QTOF-MS non-targeted metabolomics. Our findings indicated that ZTD increased enteric neurotransmitters like VIP and 5-HTR to enhance effective peristalsis in the colon. It also promoted the growth of beneficial bacteria, including the dominant butyric acid-producing bacterium Roseburia, to correct the imbalance in intestinal flora, and reduced fecal metabolites like dopamine to address FC.

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Disclosure of conflict of interest

None.

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