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ORIGINAL RESEARCH

Therapeutic Mechanism of Zhuyang Tongbian Decoction in Treating Functional Constipation: Insights from a Pilot Study Utilizing 16S rRNA Sequencing, Metagenomics, and Metabolomics

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Purpose: To explore the mechanism of Zhuyang Tongbian Decoction (ZTD) in treating functional constipation (FC) by observing its effects on intestinal flora composition, the metabolic function of gut microbiota, fecal short-chain fatty acid (SCFA) levels, and serum concentrations of TLR4, NF-κB, TNF-α, and IL-6 in patients with FC.

Patients and Methods: 40 patients with FC were randomly divided into the control group and the treatment group, 20 cases in each group. And 20 healthy volunteers were recruited during the same period. The control group was administered lactulose, while the treatment group was treated with ZTD. 16s RNA sequencing technology was used to compare the changes in the structure and diversity of the intestinal flora of patients before and after treatment. Changes in the levels of SCFAs in faeces and the levels of TLR4, NF-κB, TNF-α and IL-6 in serum were analysed. Metagenomics sequencing assessed microbiota metabolic functions.

Results: The treatment group showed a significant increase in the relative abundance of beneficial bacteria, including Bifidobacterium, Lactobacillus, and Faecalibacterium_prausnitzii ($P < 0.05$), whereas Desulfobacterota and Ruminococcus were significantly reduced ($P < 0.05$). Notably, fecal acetic and propionic acid levels were significantly higher in the treatment group ($P < 0.05$). Serum biomarkers TLR4, NF-κB, TNF-α, and IL-6 decreased significantly ($P < 0.05$). Metagenomics sequencing showed that Carbohydrate metabolism, Metabolism of cofactors and vitamins, and C5- Branched dibasic acid metabolism were significantly increased in functional abundance ($P < 0.05$).

Conclusion: ZTD notably improves intestinal flora composition and gut microbiota metabolic function, regulates SCFA levels, and reduces inflammation markers in FC patients. The strain Faecalibacterium_prausnitzii shows significant potential in regulation of intestinal inflammation and may play a crucial role in the treatment efficacy of ZTD for FC.

Keywords: Zhuyang Tongbian Decoction, functional constipation, intestinal flora, metabolite, intestinal inflammation

Introduction

Functional constipation (FC) is a common digestive disease, mainly manifested by symptoms such as low frequency of defecation, straining to defecate, feeling of incomplete defecation, anorectal obstructive sensation, and hard stools.[1,2] The global prevalence of FC is estimated to be approximately 15.3%, with an increasing trend observed over time.[3–5] The aetiology of FC remains unclear. Potential contributing factors include abnormalities in the function of Cajal

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mesenchymal stromal cells within colonic tissue, alterations in the expression of aquaporin (AQP), dysfunction of intestinal neurotransmitters, and an imbalance of intestinal flora.[6,7]

The intestinal flora interacts with the host's metabolism, nervous system, immune system, and endocrine system, and plays an important role in maintaining intestinal health,[8] and dysbiosis can cause a variety of changes in the pathophysiology of the host, which can lead to functional gastrointestinal disorders, especially FC.[9] Previous studies have shown a significant decrease in the abundance of intestinal Bifidobacteria and Lactobacillus in FC patients and an increase in the abundance of potentially pathogenic bacteria such as E. coli.[10] Metagenomics sequencing has shown that the gut microbiome of FC patients has lower levels of functional genes related to carbohydrate, fatty acid and lipid metabolism than healthy individuals.[11] As one of the metabolites of intestinal flora, short-chain fatty acids (SCFAs) affect intestinal motility through a variety of mechanisms. There is a significant difference in intestinal SCFAs levels between patients with FC and the healthy population.[12,13] Furthermore, a pro-inflammatory environment in the gut has been linked to the development of intestinal dyskinesias such as constipation.[14] There is growing evidence that intestinal flora may play a role in regulating the intestinal inflammatory response.[15] Despite the contribution of these studies in revealing, among other things, changes in the intestinal flora of FC patients and their underlying mechanisms, there is still a lack of multi-omics-based mechanistic studies.

A plethora of pharmaceutical agents are available for the treatment of FC, including stool softeners, stimulant or osmotic laxatives and prokinetics. However, these drugs are often accompanied by adverse effects such as headache, flatulence and abdominal pain, which largely limit their clinical application.[16] In comparison, Chinese medicines have fewer side effects, lower recurrence rates, and significant efficacy, which are of great advantage and important value for FC in terms of multi-targeted, multi-level action and regulation. Single herbs such as *Hericium erinaceus* have been shown to be effective for a variety of gastrointestinal diseases.[17,18] 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. The formula is comprised of *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. The research group's findings indicated that the effective rate of ZTD for FC was 90%, and the long-term efficacy was better than that of lactulose. Furthermore, animal studies have demonstrated that it can enhance the expression of colonic mucosal protein 2 (MUC2) and aquaporin 3 (AQP3) in constipation model mice, thereby improving colonic dynamics and alleviating constipation symptoms through the up-regulation of the VIP-cAMP-PKA-AQP3 pathway.[19–21] However, research on the underlying mechanisms of its efficacy remains limited.

In the present study, we employed a comprehensive range of analytical techniques, including 16s RNA high-throughput sequencing, metagenomics sequencing, targeted metabolomics and multi-omics joint analysis methods, to investigate the correlation between intestinal flora, SCFAs and TLR4/NF-κB. The objective was to conduct a comprehensive investigation into the mechanism of action of ZTD in the treatment of FC. This would facilitate the development of a more comprehensive theoretical basis for the clinical application of this formula in the treatment of FC.

Materials and Methods

Recruitment of Study Subjects

The FC patients were randomly divided into treatment group (FC_PreZY group) and control group (FC_PreCG group). Patient inclusion criteria were as follows: (a) meeting the Rome IV diagnostic criteria for FC[22] (b) aged 18–75 years old, both male and female (c) eating a regular diet, not eating extreme diets such as vegetarian diets. Patient exclusion criteria were as follows: (a) probiotics, antibiotics and any other therapies that may disturb the intestinal microbiota were used in the 4 weeks prior to faecal collection (b) suffering from other gastrointestinal diseases (c) the patients were unconscious or had cognitive dysfunction that may affect the questionnaire (d) accompanied by organic diseases of certain vital organs (e) women in pregnancy or breastfeeding period.

The inclusion criteria for the 20 healthy volunteers (NC group) in the same period were as follows: (a) aged 18–75 years (b) Bristol faecal character score of 4 according to the scoring criteria (c) no abdominal pain or other discomforts in defecation (d) not suffering from intestinal diseases or other illnesses that may affect the intestinal flora. The exclusion criteria for healthy volunteers were as follows: (a) patients previously diagnosed with constipation (b) pregnant or breastfeeding women (c) people with mental disorders and cognitive impairment. The enrolled patients and healthy volunteers were aware of the contents of this study and signed an informed consent form. This study was performed in line with the principles of the Declaration of Helsinki, and was approved by the Ethics Committee of the Third Affiliated Hospital of Liaoning University of Traditional Chinese Medicine (LLPJ-ZY-GC-2021-001-01).

Intervention

The FC_PreCG group was treated with lactulose (manufacturer: Fresenius Kabi Austria GmbH, Austria) orally, while the FC_PreZY group was treated with ZTD (raw drug concentration: 3.0 g/mL), which was provided by the Preparation Centre of the Third Affiliated Hospital of Liaoning University of Traditional Chinese Medicine. The two groups were administered the treatments twice daily, once in the morning and once in the evening, with the course of treatment lasting for a period of two weeks.

16S rRNA Sequencing

Feces were collected from healthy volunteers, treatment and control groups before and after treatment for 16S rRNA sequencing. Genomic DNA was extracted from the samples using the E.Z.N.A. Stool DNA Kit (D4015-02, Omega Biotek, Inc., USA) in strict accordance with the instructions provided by the manufacturer. The quality and concentration of the extracted DNA were then checked using the Nanodrop 2000 (Thermo Fisher Scientific, Inc., USA). The DNA samples were stored at −20°C for subsequent analysis. Subsequently, the highly variable region V3-V4 of the bacterial 16S rRNA gene was amplified by PCR using universal primers (Applied Biosystems, Inc., USA), and the resulting PCR products were purified automatically using the Agencourt AMPure XP (A63882, Beckman Coulter, Inc., USA) nucleic acid purification kit. The purified, amplified fragments were used to construct sequencing libraries, which were then subjected to high-throughput sequencing and database comparison and annotation using the MiSeq PE300 platform (Illumina, USA).

Targeted Detection of Faecal SCFAs Levels

Collect 2mL of faeces from each group in an EP tube, add 1mL of purified water, vortex for 10s, further grind and centrifuge, add 0.1mL of 50% sulfuric acid, 0.8mL of extraction solution (containing internal standard 2-methylvaleric acid, 25mg/L, methyl tert-butyl ether), vortex for 10s, oscillate for 10min, and sonicate for 10min (in an ice-water bath), then centrifuge the samples at 4°C and 10000rpm for 15min, then remove the supernatant from the injection bottle and test on the machine. Instrument: Shimadzu GC2030-QP2020NX gas chromatography-mass spectrometer.

Serum TLR4, NF-κB, TNF-α and IL-6 Levels Detection

Five mL of fasting venous blood was drawn from each group, and the levels of NF-κB, TLR4, TNF-α and IL-6 in serum were quantified using an enzyme-linked immunosorbent assay (ELISA). The experiment was conducted with the use of standard, sample, and blank wells. 50 μL of standards of varying concentrations were introduced to the standard wells, 50 μL of the samples to be tested were added to the sample wells, and no samples were included in the blank wells. A 100 μL volume of HRP-labelled detection antibody was added, and the mixture was incubated for 60 minutes at 37°C. Following this, the mixture was washed. Chromogenic substrates A and B were then added to each well, and the mixture was incubated for a further 15 minutes. Termination solution was then added, and the colour change was observed. The final results were detected by 450 nm enzyme marker. Kit name: Human ELISA KIT (NF-κB, TLR4, TNF-α and IL-6, specification: 96T). Enzyme labeled analyzer: Rayto RT-6100 (Rayto Life and Analytical Sciences Co., Ltd. China).

Metagenomics Sequencing

Groups of faeces were collected and after extraction of faecal genomic DNA, quality testing, fragment interruption and purification were performed (Covaris S220). The purified DNA fragments were screened on a 2% agarose gel, and gel blocks of 400–450 bp fragments were cut and further purified, followed by PCR enrichment to complete the construction of HiSeq libraries. Finally, different libraries were sequenced on the Illumina HiSeq platform (NovaSeq Reagent Kits 300 cycles, Illumina, USA) according to their effective concentration and the amount of downstream data required. The raw downstream data is in Illumina fastq format, which needs to be split, quality clipped, and host contaminants removed to obtain high-quality sequences. Next, quality sequences were spliced and assembled using splicing software, and gene prediction was performed. The predicted genes were functionally annotated and classified, and based on the results, sample similarity clustering, sequencing test and statistical analysis of variance were carried out.

Statistical Processing

SPSS 24.0 software package was used for statistical analysis, and the measurement data were tested for normality, and the measurement data obeying normal distribution were expressed as mean ± standard $\bar{x} \pm s$ deviation and analysed using the independent samples *t*-test; those not obeying normal distribution were expressed as median with interquartile range (median,Q1-Q3) and were tested using the Tukey's test or Wilcoxon rank-sum test to compare the differences between groups, and the difference was statistically significant at $P < 0.05$.

Results

General Characteristics of the Study Cohort

40 patients with FC who visited the Third Hospital Affiliated to Liaoning University of Traditional Chinese Medicine from December 2021 to December 2022 were included in this study, and were randomly divided into a control group and a treatment group, with 20 patients in each group. The results are presented in Table 1. No significant differences were observed between the treatment and control groups with regard to gender ($P = 0.733$), age ($P = 0.307$), or duration of disease ($P = 0.698$).

Comparison of the Structure and Diversity of Intestinal Flora

As illustrated in Figure 1A, an increase in sample size results in a flattening of the species accumulation curve, indicating that the species present in the environment do not increase significantly with the expansion of the sample size. This suggests that the sampling conducted for this experiment is sufficient for subsequent data analysis. The Venn diagram in Figure 1B demonstrates that the number of OTUs common to each group was 2232, in addition to the different number of endemic OTUs in each group. The PD_whole_tree index (Figure 1C) was significantly lower in the FC_PreZY group than in the NC group, and significantly higher after treatment with ZTD (ProZY). The results of PCoA (Principal Co-ordinates Analysis) demonstrated that the samples from the ProZY group were situated between those from the NC and FC_PreZY groups, which further suggests that ZTD can enhance the structure of the intestinal flora of FC patients in a manner that is similar to that observed in the NC group (Figure 1D).

Specifically, at the phylum level (Figure 2A), the relative abundance of p__Firmicutes was significantly higher in the ProZY group compared to the FC_PreZY group, and the relative abundance of p__Bacteroidota was significantly lower.

Table 1 General Characteristics of the Study Cohort

Group	Sex (M/F)	Age (yrs)	Disease Duration (yrs)
Treatment group (n=20)	7/13	48.53±14.72	4.55±5.04
Control group (n=20)	6/14	47.83±14.00	4.40±4.37
NC group (n=20)	7/13	47.25±16.84	–

Abbreviations: M, male; F, female; yrs, years.

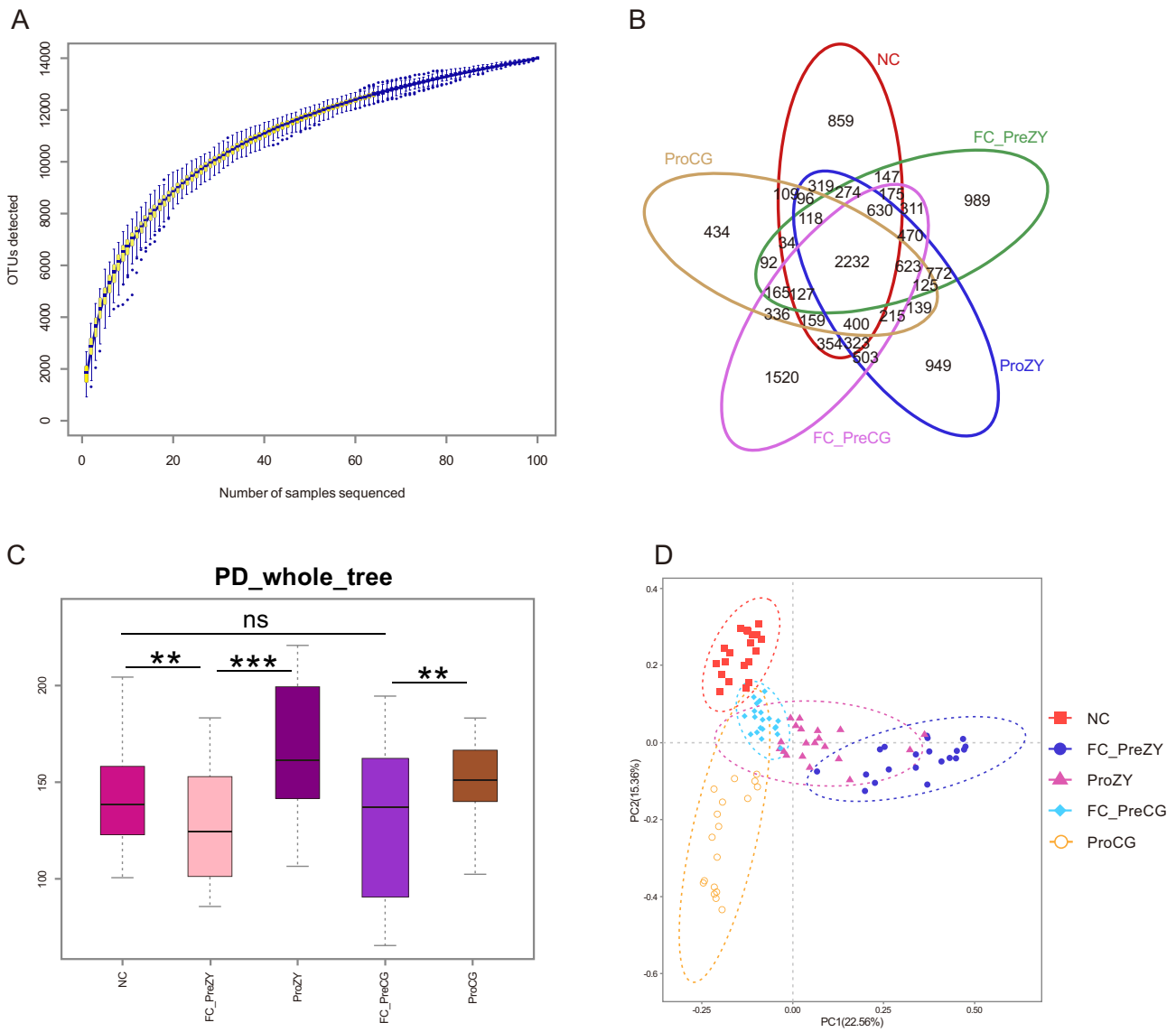

Figure 1 Diversity analysis of bacterial groups. **(A)**. Species accumulation curves; **(B)**. Venn plots, different colours represent different groups; **(C)**. PD_whole_tree index, **P<0.01, ***P<0.001, ns: No significant difference; **(D)**. PCoA analysis.

At the genus level (Figure 2B), g__Bacteroides, g__Dialister, g__Blautia, g__Eubacterium_coprostanoligenes_group and g__Faecalibacterium were the dominant genera in each group, and we selected the top 20 species in relative abundance at the genus level with significant differences to plot a Heat map ($P < 0.05$), compared with the NC group, g__Collinsella, g__Romboutsia, g__Ruminococcus, etc. increased significantly in the FC_PreZY group, and g__Fusicatenibacter, g__Faecalibacterium, etc decreased significantly. In contrast, the ProZY group exhibited a marked improvement in the relative abundance of these genera (Figure 2C). In addition, we further analysed the changes in the abundance of Bifidobacterium spp. and Lactobacillus spp. before and after the treatment of the patients, and the relative abundance of Bifidobacterium spp. and Lactobacillus spp. was significantly lower in the FC_PreZY group compared with the NC group, whereas the relative abundance of the two spp. in the ProZY group was significantly increased compared with that of the FC_PreZY group, and was closer to the level of the NC group (Figure 3A). At the species level, the relative abundance of s__Faecalibacterium_prausnitzii, s__Bifidobacterium_longum, s__Bifidobacterium_adolescentis and s__Bifidobacterium_pseudocatenulatum under the genus Bifidobacterium was significantly lower in the FC_PreZY

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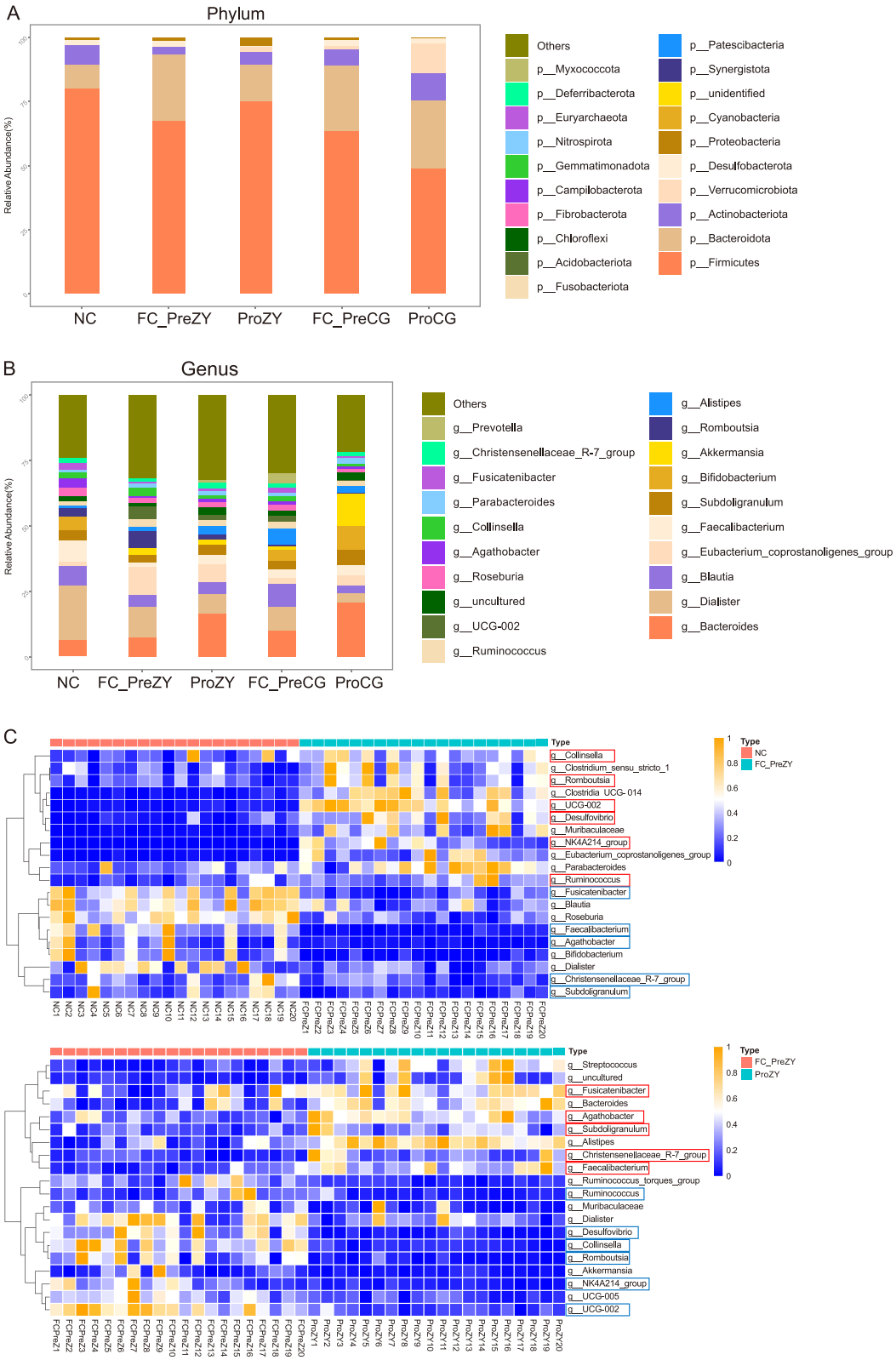

Figure 2 Structural analysis of the composition of the intestinal flora. **(A)**. Comparison of the relative abundance of the flora in each group at the phylum level; **(B)**. Comparison of the relative abundance of the flora in each group at the genus level; **(C)**. Comparison of significantly different flora before and after treatment with ZTD at the genus level.

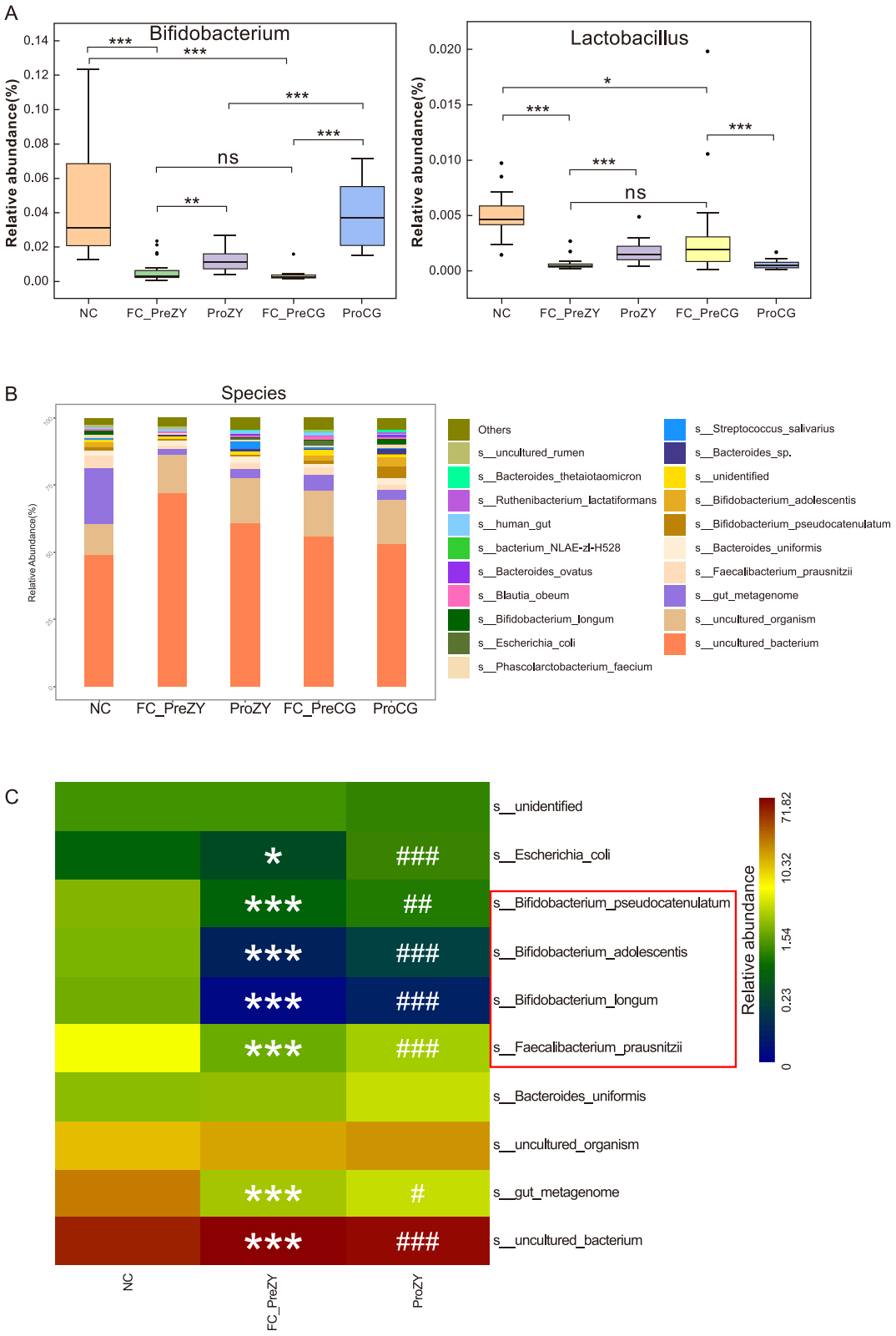

Figure 3 Comparison of relative abundance of specific flora. **(A)** Comparison of the abundance of Bifidobacterium spp. and Lactobacillus spp. in each group, *P<0.05, **P<0.01, ***P<0.001, ns: No significant difference; **(B)** Comparison of the relative abundance of each group of flora at species level; **(C)** Comparison of significantly different flora before and after treatment with ZTD at the species level, *P<0.05, **P<0.01, ***P<0.001 vs.NC, #P<0.05, ##P<0.01, ###P<0.001 vs. FC_PreZY.

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group, whereas the relative abundance of these beneficial bacterial species in the ProZY group increased significantly and tended to favour the NC group (Figure 3B and C).

To further characterise the significant changes in the intestinal microorganisms of FC patients after administration of ZTD, we performed a linear discriminant analysis effect size (LEfSe) and plotted histograms of LDA distributions based on taxonomic information. This identified statistically significant differences in bacterial populations at different taxonomic levels (Figure 4). O__Oscillospirales, F__Oscillospiraceae, P__Desulfobacterota and its subordinate C__Desulfovibrionia, O__Desulfovibrionales, etc. were significantly enriched in the FC_PreZY group, but after treatment with ZTD, the significantly enriched flora became c__Bacilli, f__Streptococcaceae, o__Lactobacillales, o__Clostridia_UCG_014 and its subordinate f__Clostridia_UCG_014 and g__Clostridia_UCG_014.

Comparison of Faecal SCFA Levels

To further explore the mechanism of the role of intestinal flora in the treatment of FC with ZTD, we determined the changes in the levels of SCFAs, the metabolites of the flora, before and after the treatment of FC patients using chromatography-mass spectrometry. The results showed that compared with the NC group, the levels of faecal acetic acid and propionic acid in the FC_PreZY group were significantly reduced, and the levels of butyric acid, isobutyric acid, valeric acid and isovaleric acid were significantly increased. The levels of acetic acid and propionic acid in the ProZY group were significantly increased compared with the FC_PreZY group, while the levels of butyric acid, isobutyric acid, valeric acid and isovaleric acid were significantly reduced and close to the level of the NC group (Figure 5).

Comparison of Serum TLR4, NF-κB, TNF-α and IL-6 Levels

Furthermore, an analysis of the differential flora revealed that FC patients exhibited a notable increase in the abundance of genera such as Faecalibacterium and Bacteroides following the administration of ZTD. It has been demonstrated that genera such as g__Faecalibacterium and g__Bacteroides are capable of regulating intestinal inflammatory responses via Toll-like receptor (TLR) receptor-mediated signalling pathways.[23,24] The activation of the TLR4/NF-κB signalling pathway results in the release of pro-inflammatory factors, including tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), which induce inflammatory responses and further exacerbate FC symptoms.[15] Consequently, an additional examination was conducted to investigate the alterations in serum TLR4, NF-κB, TNF-α and IL-6 levels in FC patients before and after treatment. The results demonstrated a notable elevation in the serum levels of TLR4, NF-κB, TNF-α and IL-6 in the FC_PreZY group in comparison to the NC group. Following the administration of ZTD, a discernible reduction in the aforementioned indexes was observed, culminating in a convergence towards the level observed in the NC group. The results demonstrated that ZTD (Figure 6) could inhibit the release of pro-inflammatory factors by regulating the TLR4/NF-κB pathway, thereby effectively alleviating the intestinal inflammation of FC patients.

Comparison of Metabolic Functions of the Gut Microbiota

In order to deeply explore the mechanism of gut microbes through their metabolic functions in maintaining host intestinal SCFA levels, regulating immune responses and other therapeutic FC, we performed metagenomics sequencing as shown in Figure 7A, and the results of the Anosim analysis showed that there were significant differences in functional abundance among the various groups of microbiota. Based on the classification information of genes in Kyoto Encyclopedia of Genes and Genomes (KEGG), combined with the abundance of genes in individual samples, we obtained a heat map of relative abundance of KEGG at level 2 top 50 and a functional abundance difference map at level 3 top 10. At level 2 (Figure 7B), compared with the NC group, the abundance of pathway-related genes such as Signaling molecules and interaction, Translation was significantly increased in the FC_PreZY group, and the abundance of Cell growth and death, Metabolism of cofactors and vitamins, and Carbohydrate metabolism involving SCFAs production were significantly reduced, while the abundance of the above functions in the ProZY group was found to be similar to that observed in the NC group. At the level 3 (Figure 7C), the ProZY group demonstrated enhanced

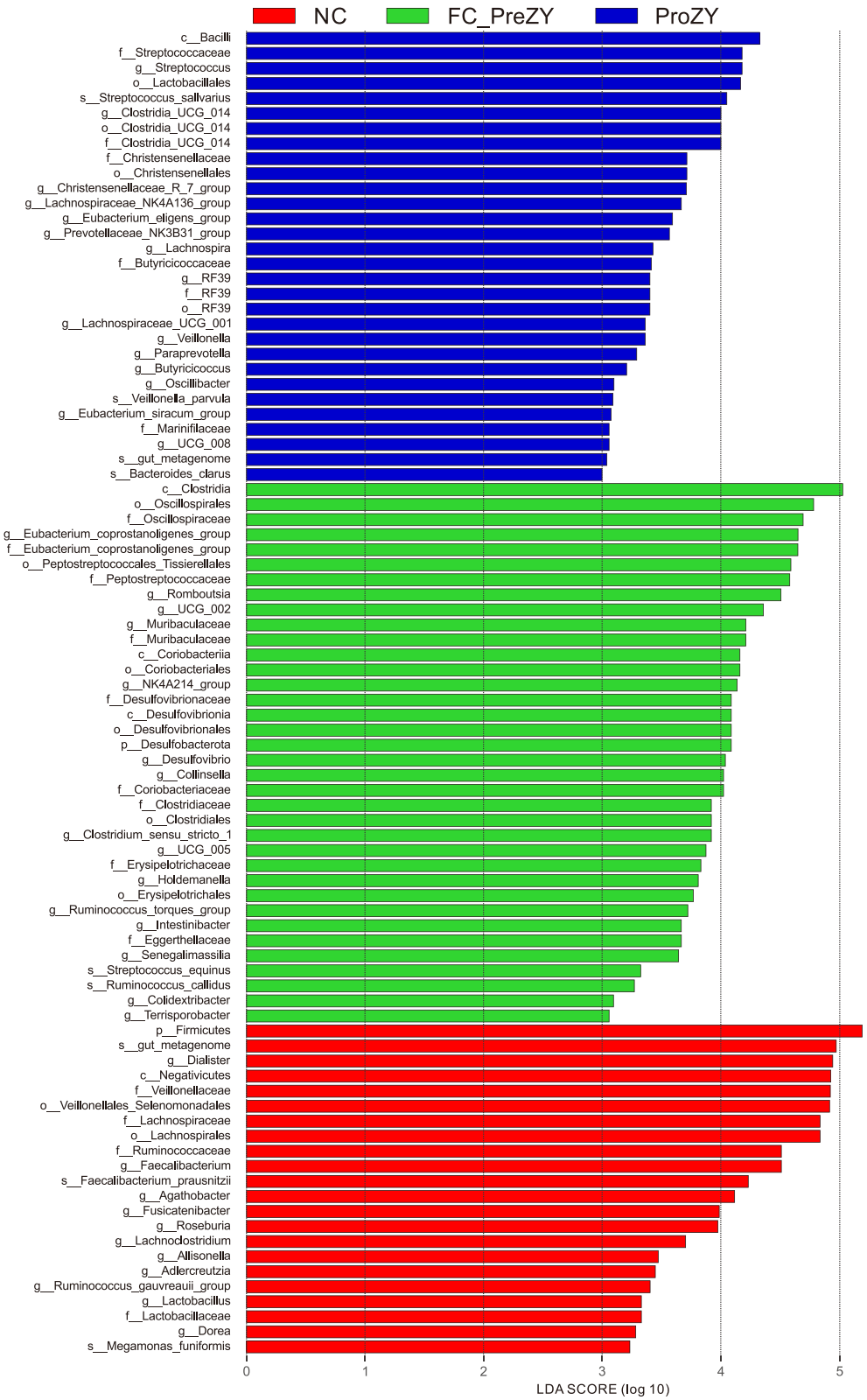

Figure 4 Analysis of differential flora at different taxonomic levels, "p_", phylum; "c_", class; "o_", order; "f_", family; "g_", genus; "s_", species.

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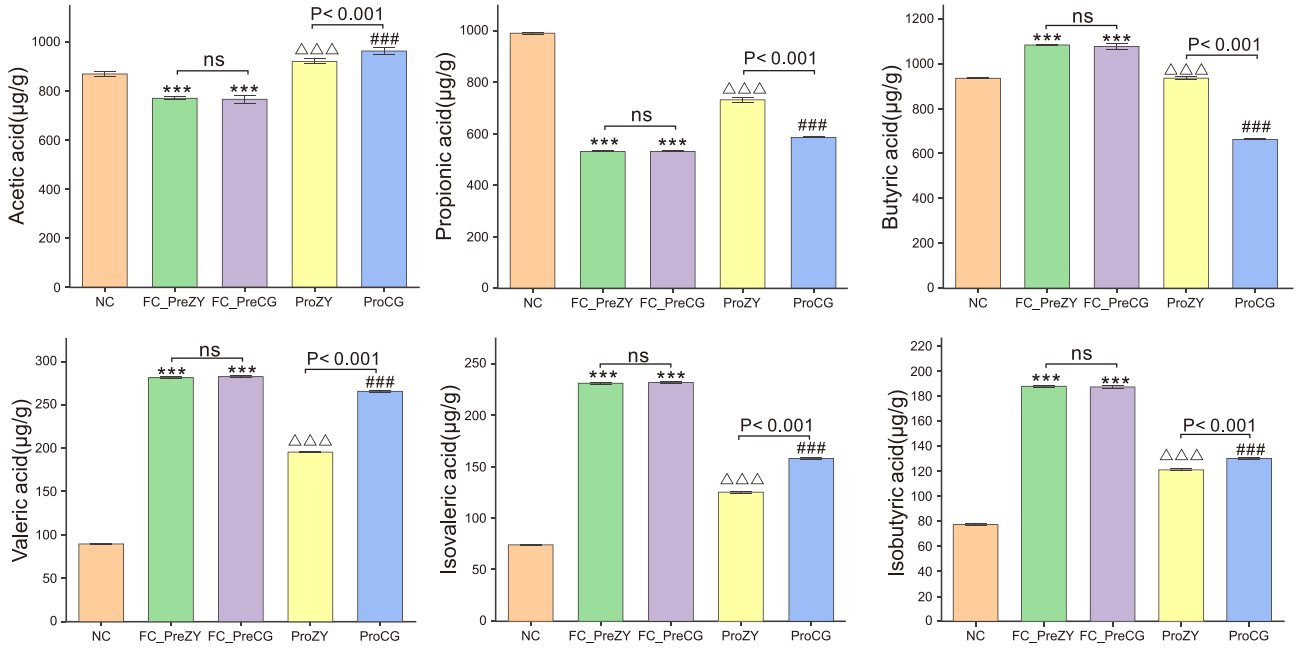

Figure 5 A comparative analysis of SCFA levels between the study groups. ***P<0.001 vs. NC, $^{\triangle\triangle\triangle}$ P<0.001 vs. FC_PreZY, $^{\#\#\#}$P<0.001 vs. FC_PreCG, ns: No significant difference.

capabilities in terpenoid backbone biosynthesis, lysine degradation, prodigiosin biosynthesis, and C5-branched dibasic acid metabolism.

Comprehensive Analysis of the Mechanism of FC Treatment with ZTD

In order to gain a more comprehensive understanding of the mechanism of action of the ZTD in the treatment of FC, we conducted Pearson correlation analyses with SCFAs and TLR4/NF-κB signalling pathway-related indicators for the flora with high relative abundance and significant therapeutic significance in each group. The results demonstrated significant correlations between flora and flora, between flora and SCFAs, and between flora and TLR4/NF-κB signalling pathway indicators. p__Firmicutes was negatively correlated with p__Bacteroidota. Lactobacillus spp, Bifidobacterium spp, and s__Faecalibacterium_prausnitzii positively correlated with acetic and propionic acid levels, and negatively correlated with butyric, isobutyric, valeric, and isovaleric acids, TLR4, NF-κB, TNF-α, and IL-6 levels (Figure 8). ZTD may treat FC through a variety of pathways, including the regulation of intestinal flora, the modulation of SCFAs production, and the modulation of inflammatory factor levels. Interestingly, correlation analyses suggest that these different pathways may be closely linked.

Discussion

The development of FC is closely related to the intestinal flora,[25–27] and our results showed that the structural composition of the intestinal flora in FC patients differed significantly from that of the healthy population, and ZTD could significantly reduce these differences. At the phylum level, ZTD significantly reduced the relative abundance of Bacteroidota and Desulfobacterota in FC patients. It has been noted that the level of Bacteroidota abundance in the intestines of FC patients was approximately twice that of healthy subjects, and the increased relative abundance of Bacteroidota may be one of the reasons for the slower passage of intestinal contents through the intestines.[28,29] Recently, it was reported that Desulfobacterota was higher in the faeces of constipated patients compared to healthy subjects,[30] and Desulfobacterota reduces sulphate in the colon to produce high concentrations of hydrogen sulphide, which would inhibit intestinal peristalsis and exacerbate FC symptoms.[31] At the genus level, Fusicatenibacter induces IL-10 production in the intestinal mucosa, which exerts anti-inflammatory effects.[32] Our results also suggest that Fusicatenibacter abundance is positively correlated with the production of acetic acid and propionic acid, a relationship that is favourable for further

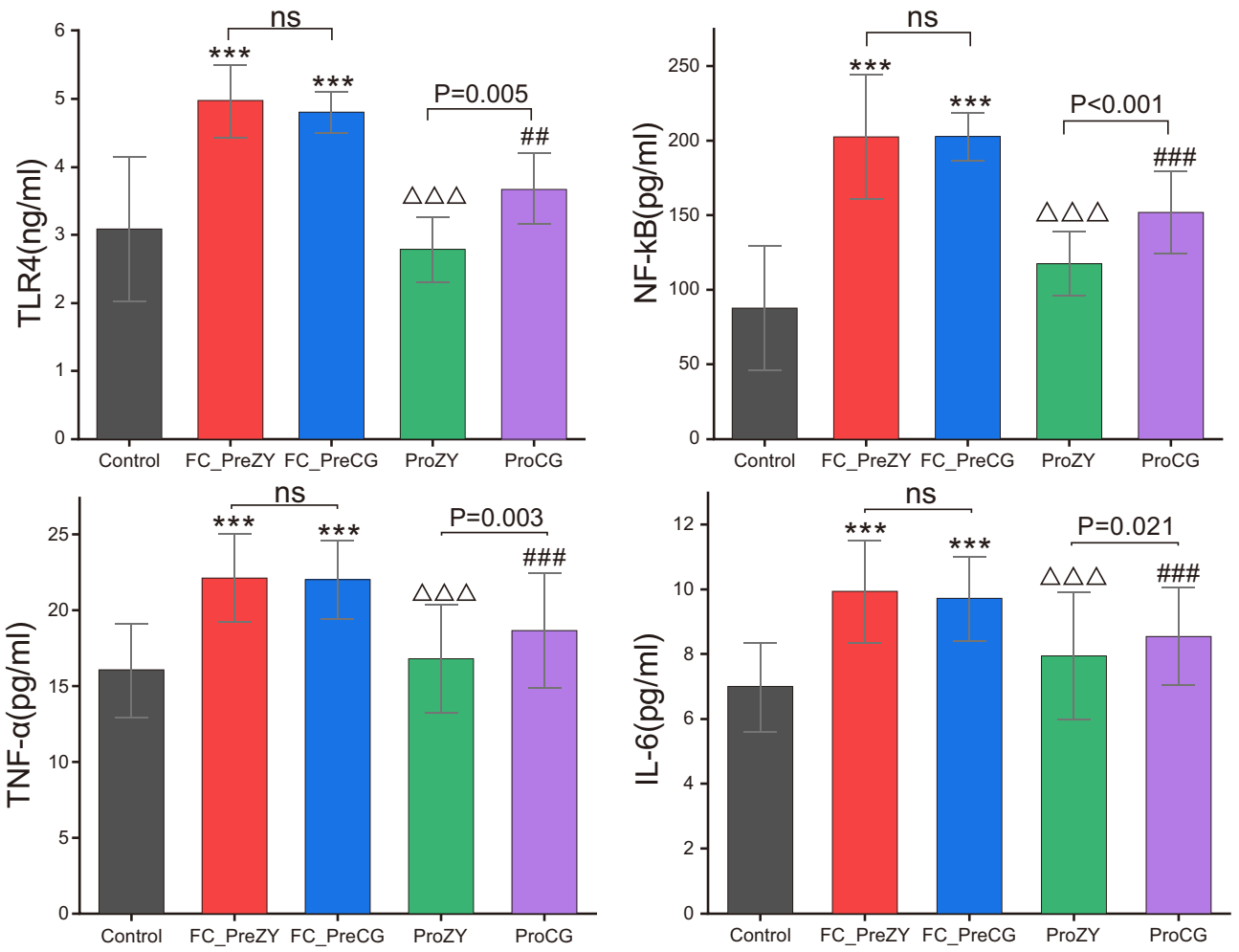

Figure 6 Comparison of serum TLR4, NF-κB, TNF-α and IL-6 levels between groups. ***P<0.001 vs. NC, $^{\triangle\triangle\triangle}$ P<0.001 vs. FC_PreZY, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. FC_PreCG, ns: No significant difference.

combating intestinal inflammation and maintaining intestinal mucosal integrity.[33] Faecalibacterium and Bacteroides can have a stabilising effect on the intestinal environment by regulating the immune system through Toll-like receptors.[23,24] Bifidobacterium spp. and Lactobacillus spp. are well-known beneficial intestinal flora, which can ferment oligosaccharides to produce lactic acid and acetic acid, thus lowering the pH value in the intestinal lumen and promoting intestinal peristalsis to facilitate the expulsion of stools, and the two genera can increase fecal water content by increasing the content of short-chain fatty acids in the lumen of the intestinal cavity of constipated mice.[34,35]

SCFAs are metabolites of intestinal flora and play an important role in maintaining the intestinal mucosal barrier, regulating intestinal immune responses, and promoting intestinal motility and absorption.[36,37] Acetic acid, propionic acid and butyric acid are the main SCFAs in the colon, and acetic acid can promote the production of immunoglobulin A (IgA) in the colon and affect the binding ability of IgA to specific intestinal bacteria, which may strengthen the immune barrier function of the intestinal mucosa by regulating the growth and colonisation of these bacteria in the colon.[38] Propionic acid induces low-frequency, high-amplitude contractions in the rat colon via G protein-coupled receptors in the intestinal mucosal epithelium, which play an important role in the propulsion of colonic contents.[39] Elevated concentrations of isovaleric acid cause relaxation of colonic smooth muscle and inhibit intestinal peristalsis.[40] Butyric acid may inhibit colonic mucin secretion, stimulate colonic water and electrolyte absorption making faeces dry and hard, and may also inhibit colonic smooth muscle contraction causing FC.[41,42] In addition our correlation analysis showed positive correlation between acetic acid and propionic acid levels and the abundance of Bifidobacterium spp. and

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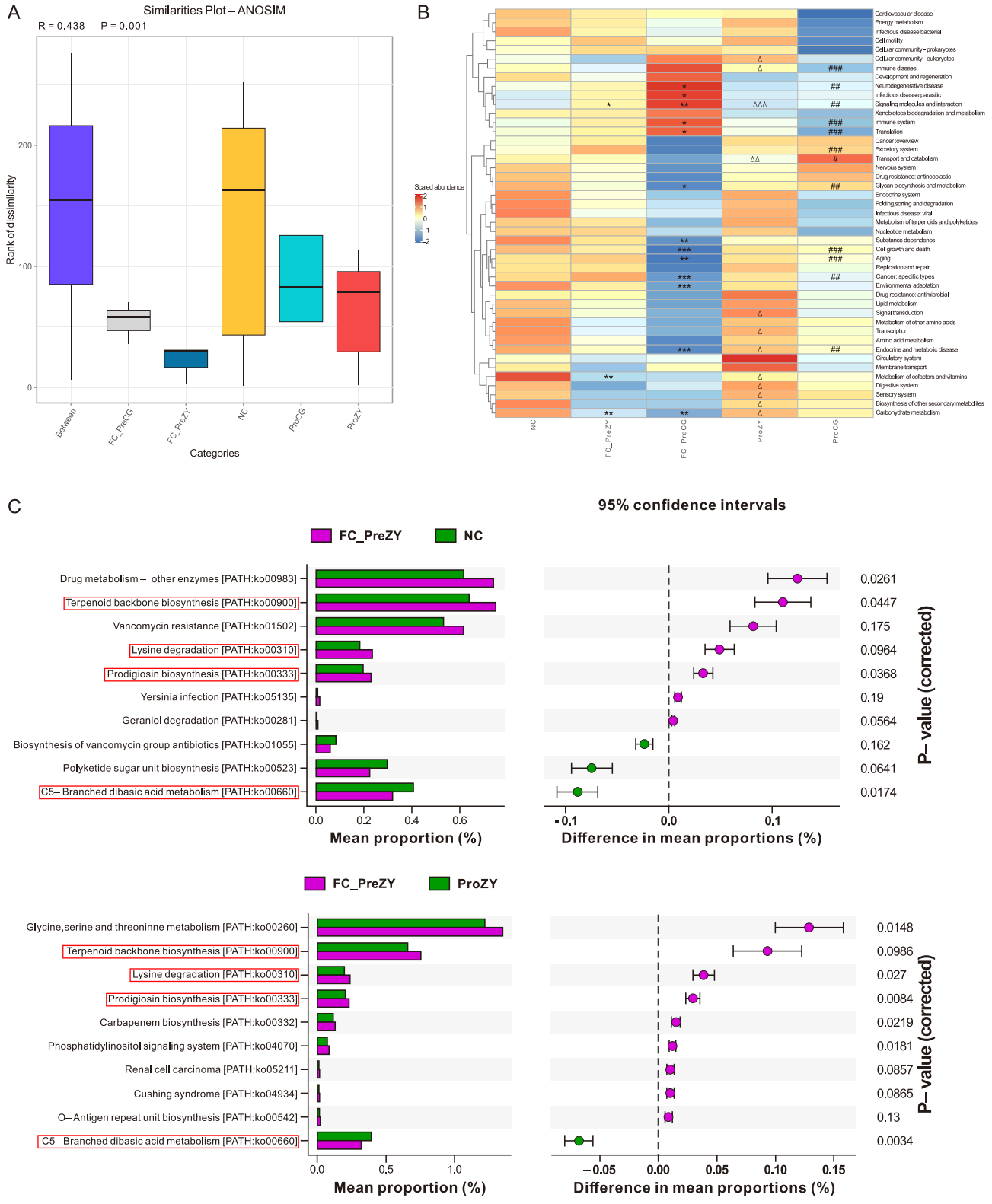

Figure 7 Functional analysis of the gut microbiota. **(A)**. Functional abundance based Anosim analysis; **(B)**. Comparison of functional abundance of the gut microbiota at Level 2, *P<0.05, **P<0.01, ***P<0.001 vs. NC, $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01, $^{\Delta\Delta\Delta}$ P<0.001 vs. FC_PreZY, $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. FC_PreCG; **(C)**. Effects of Level 3 ZTD on the function of intestinal microbiota in FC patients.

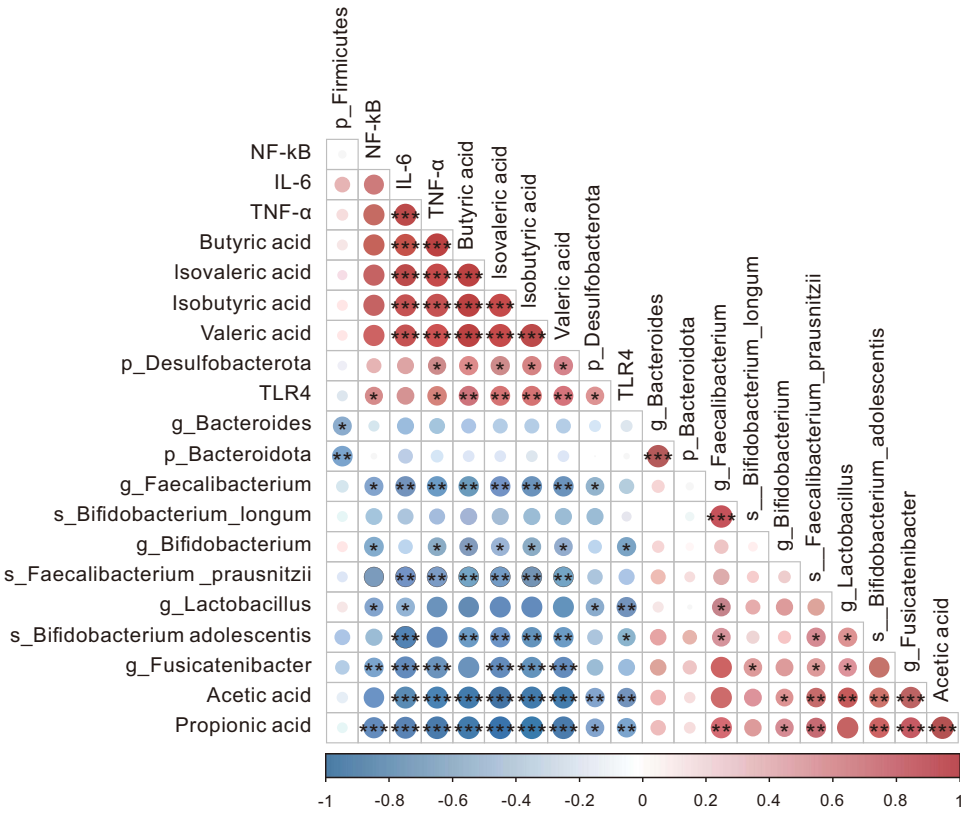

Figure 8 Correlation analysis. *P<0.05, **P<0.01, ***P<0.001.

Lactobacillus spp. One study[5] found that after 4 weeks of oral administration of Lactobacillus spp. to the subjects, there was an increase in Bifidobacterium spp. and other beneficial bacteria, and a decrease in other potentially pathogenic bacteria, and a mean of significant increase of acetic acid and propionic acid salts appeared in all age groups. Therefore, we hypothesised that the ability of the ZTD to effectively elevate the levels of acetic acid and propionic acid in FC patients may be related to the significant up-regulation of the relative abundance of Lactobacillus spp. and Bifidobacterium spp. and other beneficial bacterial genera, and that additionally the increase in these beneficial bacterial genera may inhibit the proliferation of pathogenic bacteria.

The results indicate that ZTD has a significant impact on reducing serum levels of TLR4, NF-κB, TNF-α and IL-6 in FC patients. TLR4 is responsible for recognising the gram-negative bacterial lipopolysaccharide (LPS), which binds to TLR4 on the surface of the immune cell, and the signalling bridging protein Myeloid Differentiation Factor 88 (MyD88). Subsequently, MyD88 binds to the cytoplasmic structural domain of TLR4, releasing downstream NF-κB. TLR4 and NFκB are expressed on intestinal smooth muscle, inducing the production of inflammatory mediators such as TNF-α and IL-6, as well as reactive oxygen species (ROS). It is hypothesised that these contribute to the reduction of smooth muscle contractility.[43,44] Interestingly, the role of intestinal flora is important in this process. In this study, we found that the ZTD significantly increased the relative abundance of s__Faecalibacterium_prausnitzii in FC patients, and the bacterium was negatively correlated with TLR4/NF-κB signalling pathway indicators. It has been shown that s__Faecalibacterium_prausnitzii activates TLR2 and its associated signalling pathways, and that its activated anti-inflammatory TLR2 signalling protects the organism from TLR4-mediated pro-inflammatory signalling induced by intestinal pathogenic bacteria.[23,45,46] In addition the bacterium also produces SCFAs such as propionic acid, and our correlation analysis showed that propionic acid was also negatively correlated with TLR4/NF-κB signalling pathway indicators, and one study[47] found that propionic acid inhibits histone deacetylase and reduces NF-κB activity, which reduces the release of the inflammatory factors TNF-α, IL-6, and IL-8, and improves the structure and function of the intestinal mucosal barrier. It is also worth our attention that this strain also produces an anti-inflammatory protein, MAM,

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which directly inhibits LPS-induced NF-κB activation.[48] Therefore, we speculate that s__Faecalibacterium_prausnitzii plays a very important role in the inhibition of TLR4/NF-κB signalling pathway transduction by ZTD.

It is essential for gut microbes to sustain active metabolic functions and physiological activities in order to ensure their survival within the host gut.[49] Our findings revealed a notable reduction in the functional abundance of carbohydrate metabolism, metabolism of cofactors and vitamins, and C5-branched dibasic acid metabolism in the gut microbes of FC patients. This suggests that the capacity of gut microbes in FC patients to engage in these metabolic processes may be impaired. Carbohydrate metabolism is closely related to the production of SCFAs.[11] Metabolism of cofactors and vitamins involves the production and metabolism of vitamin B. Vitamin B1 of bacterial origin can be absorbed by human colon cells, and neurotransmitters such as acetylcholine, dopamine, and serotonin play a key role in coordinating the contraction and relaxation process of intestinal muscles, while vitamin B1 can regulate the release and activity of these neurotransmitters, thus improving bowel function and reducing constipation symptoms.[50,51] Changes in the metabolic function of intestinal microorganisms in patients with FC may affect the normal production of metabolites, such as SCFAs and vitamins, which can be an important reason for the constipation. It is reassuring that the ZTD effectively improved the imbalance of these metabolic functions, which may reveal another mechanism for its treatment of FC.

The present study employed a combined multi-omics approach to investigate the mechanism of action of ZTD in the treatment of FC. In particular, given the multiple roles of strain s__Faecalibacterium_prausnitzii in the production of SCFAs and the inhibition of the release of pro-inflammatory factors, we hypothesise that this bacterium may be a key factor in the effective treatment of FC with ZTD. Further in-depth research on this bacterium will be conducted in the future. In addition, in order to more widely apply ZTD to clinical practice, it is necessary to design and implement multi-center, randomized controlled clinical trials in future studies to systematically evaluate the efficacy of ZTD in patients with functional constipation, and to compare it with existing standard therapies to confirm its advantages.

Conclusion

Our results suggest that the ZTD can significantly regulate the composition of the intestinal flora in FC patients, significantly upregulate the content of SCFAs such as fecal acetic acid and propionic acid, and inhibit the transmission of the TLR4/NF-κB pathway, which may be an important mechanism for its effective treatment of FC. Considering the clinical efficacy and the mechanism of action, the ZTD provides a safe and effective intervention strategy for patients with FC.

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Disclosure

The authors report no conflicts of interest in this work.

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