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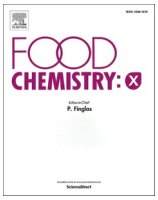

Regulatory effect of lactulose on intestinal flora and serum metabolites in colitis mice: *In vitro* and *in vivo* evaluation

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ABSTRACT

Lactulose is a common component in foods. However, the effect of lactulose on intestinal flora and overall metabolic levels remains unclear. Therefore, this study aims to explore the regulative role of lactulose on intestinal flora and serum metabolites via *in vitro* simulated colonic fermentation model and *in vivo* colitis mouse model. The results showed that lactulose significantly enriched beneficial bacteria including *Dubosiella* and *Bifidobacterium*, and reduced pathogenic bacteria such as *Fusobacterium*. Moreover, lactulose significantly inhibited dextran sodium sulfate-induced body weight loss, colon shortening, colonic inflammatory infiltration, and pro-inflammatory cytokines IL-6, TNF-α, IL-17, and IL-1β. Lactulose significantly affected serum metabolome in colitis mice and total 24 metabolites representing a high inter-group difference were obtained. Correlation analysis revealed that the changes in serum metabolites were closely associated with the role of intestinal flora, and thus affected phenotypic indicators. Our study provides a reference for nutritional characteristics and application scenarios of dietary lactulose.

1. Introduction

Lactulose is a semisynthetic and indigestible isomerization product that consists of two molecules fructose and galactose (Aït-Aissa & Aïder, 2014), which has received much attention due to its diverse applications in food and pharmaceutical industries (Wang, Wang, Lyu, Hua, Goddard, & Yang, 2022). Nowadays, lactulose has been used in alleviating the effect of a high-salt diet on hypertension (Zhang et al., 2019), improving cognitive functions in patients with cirrhosis (Prasad, Dhiman, Duseja, Chawla, Sharma, & Agarwal, 2007), and relieving systemic endotoxemia and related inflammation (Koutelidakis et al., 2003). Besides, lactulose also shows a potential role in alleviating intestinal inflammation (Hiraishi et al., 2022; Chen et al., 2013). It is worth noting that lactulose is not digested in the small intestine and travels untouched to the colon, where it acts as an energy source for bacteria that break down carbohydrate, and is fermented by gut microbiota to produce metabolites (Majid, Afshin, & Hossein, 2018). Previous studies have shown that lactulose can stimulate the enrichment of probiotics, especially *Bifidobacterium* and *Lactobacillus* in healthy colon (Majid et al., 2018). However, how lactulose affects the intestinal flora in individuals with intestinal inflammation remains unknown.

Ulcerative colitis (UC) is a relapsing and remitting intestinal disease with a high incidence around the world (Segal, LeBlanc, & Hart, 2021). The occurrence of UC is accompanied by mucosal inflammation and often induces rectal bleeding, diarrhea, tenesmus and abdominal pain, which takes a huge mental and physical burden on patients. At present, the main drugs used to treat UC include aminosalicylic acids, glucocorticoids and immunosuppressants (Murray, Nguyen, Parker, Feagan,

Abbreviations: UC, ulcerative colitis; DSS, dextran sulfate sodium; SCFAs, short-chain fatty acids; GMM, gut microbiota medium; H&E, hematoxylin and eosin; PBS, phosphate-buffered saline; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; IL-17, interleukin-17; IL-1β, interleukin-1β; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; QC, quality control; PCA, principal component analysis; OTU, operational taxonomic unit; PCoA, principal coordinate analysis; NMDS, non-metric multidimensional scaling; LEfSe, linear discriminant analysis Effect Size; PLS-DA, partial least squares discriminant analysis; VIP, variable importance in projection; KEGG, Kyoto Encylopaedia of Genes and Genomes.

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& MacDonald, 2020; Rubin, Ananthakrishnan, Siegel, Sauer, & Long, 2019). These drugs have somewhat relieved the colitis. However, long-term use will lead to reduced efficacy, increased adverse reactions and other problems (Le Berre, Roda, Nedeljkovic Protic, Danese, & Peyrin-Biroulet, 2020; Rubin et al., 2019). For example, the result of a multi-center survey found that some UC patients showed intolerance to mesalamine, and its frequency showed an upward trend (Hiraoka et al., 2021). Besides, studies have shown that prolonged exposure to prednisolone (2.5–5.0 mg/daily) might increase the risk of hip and vertebral fractures (Haugeberg, Uhlig, Falch, Halse, & Kvien, 2000; Van Staa, Leufkens, & Cooper, 2001). Considering the side-effect of these drug strategies on host health, accumulating studies focus on developing effective and safe strategies for alleviating UC.

Based on previous studies, the occurrence and prevalence of UC are related to some complex factors, including geography, age, sex, genetic factors and environmental factors, etc. (Ordás, Eckmann, Talamini, Baumgart, & Sandborn, 2012). Understanding the etiology and pathogenesis of UC has a positive effect on the development of strategies to alleviate UC. Notably, recent studies have shown the potential tight relationship between UC progress and gut microbiota (Wang et al., 2023). For example, compared with healthy human, the abundance of *Escherichia coli*, *Clostridium*, and *Bacteroides* was significantly enriched, whereas the abundance of *Bifidobacteria* was reduced in the UC patients (Zhong et al., 2019). On this basis, some animal experiments have also demonstrated that some species of gut microbes (such as *Duncaniella muricolitica* and *Alistipes okayasuensis*) could exert main effects in the dextran sulphate sodium (DSS) model (Forster et al., 2022). These findings reinforce that intestinal flora plays an important role in influencing intestinal inflammation. Hence, lactulose may be used as an effective strategy for UC treatment by regulating intestinal flora. Previous studies have shown that lactulose could suppress dextran sodium sulfate (DSS)-induced UC and azoxymethane (AOM)/DSS-induced inflammatory tumorigenesis (Hafer, Krämer, Duncker, Krüger, Manns, & Bischoff, 2007; Chen et al., 2013; Hiraishi et al., 2022). However, few studies have clarified what special species and how much species play a role when using lactulose for DSS-induced UC. Also, the potential mechanisms of lactulose in alleviating UC and the regulation of metabolites remain unclear. We hypothesize that lactulose affects intestinal flora and serum metabolic levels, and thus improves gut health.

In this study, we first evaluated the regulatory ability of lactulose on intestinal flora by *in vitro* fermentation. On this basis, DSS was used to construct a UC mouse model, and lactulose gavage was used to evaluate its regulatory effect on intestinal microflora and serum metabolites in colitis mice. To further understand the potential probiotic role of lactulose, the effects of lactulose on body weight, colon length, inflammatory indicators and intestinal structure pathology in mice were also tested. Our study will provide a theoretical foundation for developing lactulose as a new strategy for the modulation of intestinal microenvironment and the alleviation of intestinal inflammation.

2. Materials and methods

2.1. In vitro simulated colonic fermentation

Lactulose (BR, 85%) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Healthy volunteers (20–30 years old) were recruited, and should meet the following conditions before the experiment: 1) Not received any antibiotics for at least 6 months; 2) No probiotic-related food intake for 1 month; 3) No digestive diseases, such as diarrhea, indigestion, etc. In addition, a questionnaire survey was conducted before collecting fecal samples to ensure that all volunteers included in the study met the above requirements. Written informed consent for the use of their stool samples was authorized by the volunteer himself or her legal guardian. This study has been carried out in accordance with The Code of Ethics of the World Medical Association, and respects and preserves all privacy of the volunteers. The procedure of collecting feces does not cause any harm to the volunteers and no human experiment was involved.

To simulate the fermentation of lactulose in the colon, *in vitro* fermentation was completed under strictly anaerobic conditions, and the experimental procedure was conducted according to the previous report (Bai et al., 2021; Feng et al., 2020) with some modifications. The typical gut microbiota medium (GMM) (Bai et al., 2021; Mao et al., 2015) was used as the fermentation system. The stool samples were mixed and then homogenized at 1:15 (w/v) with the preprepared GMM liquid medium, and filtered through 4 layers of cheesecloth. The filtrate obtained was used as the inoculum. The *in vitro* fermentation experiment was divided into two groups: 1) Control group (CON): the GMM medium without addition of any carbohydrate (three replicates). 2) Lactulose group (RG): the lactulose was dissolved in GMM medium (1:100 w/v) in 15 mL test tubes (three replicates).

All mixtures were maintained overnight at 4 °C for hydrating. The mixture was incubated in an anaerobic chamber at 37 °C for 24 h, and the fermentation was immediately terminated by introducing liquid nitrogen. The fermentation solution was centrifuged at 10,000×*g* (4 °C) for 10 min, and the precipitation was taken for microflora analysis.

2.2. Animal experiments

Male C57BL/6J mice (20–24 g, SPF) were obtained from Hunan Slake Jingda Experimental Animal Co., Ltd. After 1 week of adaptive feeding, all mice were randomly divided into three groups (n = 5 per group): 1) Control group (CON): gavage with 200 μL sterile normal saline for 7 days. 2) DSS group (DSS): 3% DSS (w/v, 36–50 kDa, MP Biomedicals Ltd., Santa Ana, USA) was added in sterile water, and 200 μL sterile normal saline was intragastric administration for 7 days. 3) Intervention group (DSS + RG): 3% (w/v) DSS was added in sterile water, and lactulose was given by gavage daily (1000 mg/kg body weight) to estimate its roles in DSS-induced UC (Khuituan et al., 2019; Pan, Chen, Wu, Tang, & Zhao, 2009; Torello, Souza-Queiroz, & Queiroz, 2012).

Body weight and blood in feces were recorded daily during the intervention. The stool samples were collected before sacrifice for microflora analysis. After the experiment, all mice were anesthetized by intraperitoneal injection of 1% pentobarbital sodium (dose of 45 mg/kg) and sacrificed for cervical dislocation. The blood samples of mice were collected and centrifuged at 3000×*g* for 30 min to collect the serum for further analysis (Chen et al., 2021; Hickman, 2022). The animal experiment was approved by the Committee of Ethics of Southwest University, China (IACUC-20230221-06) and was completed according to the guidelines of the European Community (EU Directive 2010/63/EU) for animal experiments.

2.3. Histology examination

After the animal experiment, 0.5 cm fresh and clean colon tissues were collected and immediately put into a solution containing 4% paraformaldehyde for fixation. Colon tissues were then paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E). The pathological section description of colon tissues was referred to the previous report (Shinde et al., 2019).

2.4. Inflammatory cytokines examination

After the animal experiment, 0.1 g of colon tissue was collected and stored in liquid nitrogen for extracting the total protein (Liu et al., 2020). After that, colon tissue and ice-cooled phosphate-buffered saline (PBS) (1:9 w/v) were added to the mechanical homogenization, and then centrifuged (4℃, 3000×*g* for 5 min) to collect the supernatant. The contents of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interleukin-17 (IL-17) and interleukin-1β (IL-1β) in colon tissues were determined by enzyme-linked immunosorbent assay (ELISA) (R&D

System China Co., Ltd., Shanghai, China).

2.5. Analysis of the gut microbiota

Based on a previous study (Wang et al., 2017), the genome of fermentation solution precipitates and feces samples were extracted using the Fast DNA ® Spin Kit for Feces (MP Biomedicals Ltd., United States). The V3-V4 regions of the 16S rDNA were amplified by polymerase chain reaction (PCR). PCR amplicons were resolved and purified by 1% Agarose gel and Biospin GE kit respectively. Then, the purified product was used for analyzing and comparing the composition of fermentation sample and gut microbiota between different treatment groups. Data was analyzed and charts were made using the online linear discriminant analysis Effect Size (LEfSe) analysis tool (https://www.microbiomeanalyst.ca/).

2.6. Analysis of short-chain fatty acids (SCFAs)

According to the previous report by Mao et al. (Mao, Li, Ai, Zhao, Zhang, & Chen, 2016), we have analyzed the content of SCFAs in fecal samples as follows. The feces were freeze-dried and weighed. The dried feces were treated with resuspension, acidification and extraction. After that, the concentration of SCFAs was detected by gas chromatography-mass spectrometry with Rtx-Wax column. The tested SCFAs include acetate, propionate, butyrate, valerate, isobutyrate, and isovalerate.

2.7. Non-targeted metabolome analysis

100 μL serum was extracted into a 1.5 mL enzyme-free centrifuge tube, and 400 μL methanol solution pre-cooled at −80 °C was added to precipitate proteins. All samples were gently shaken for 30 s and placed at −20 °C condition for 1 h. The samples were then centrifuged at 12,000×*g* at 4 °C for 15 min. Then, 400 μL of supernatant was taken and evaporated at 4 °C until dry. The preparation of the quality control (QC) sample, specific detection process, and analysis method referred to the previous reports (He et al., 2019; Zhu et al., 2021). The data analysis and chart plotting were completed by using the online MetaboAnalyst analysis tool.

2.8. Statistical analysis

Spearman's correlation analysis was adopted to evaluate the correlation between different intestinal flora and different metabolites between groups. Data analysis was done and charts were made using GraphPad Prism 8.0.2 and R package. The results between different groups were considered statistically significant when $p < 0.05$.

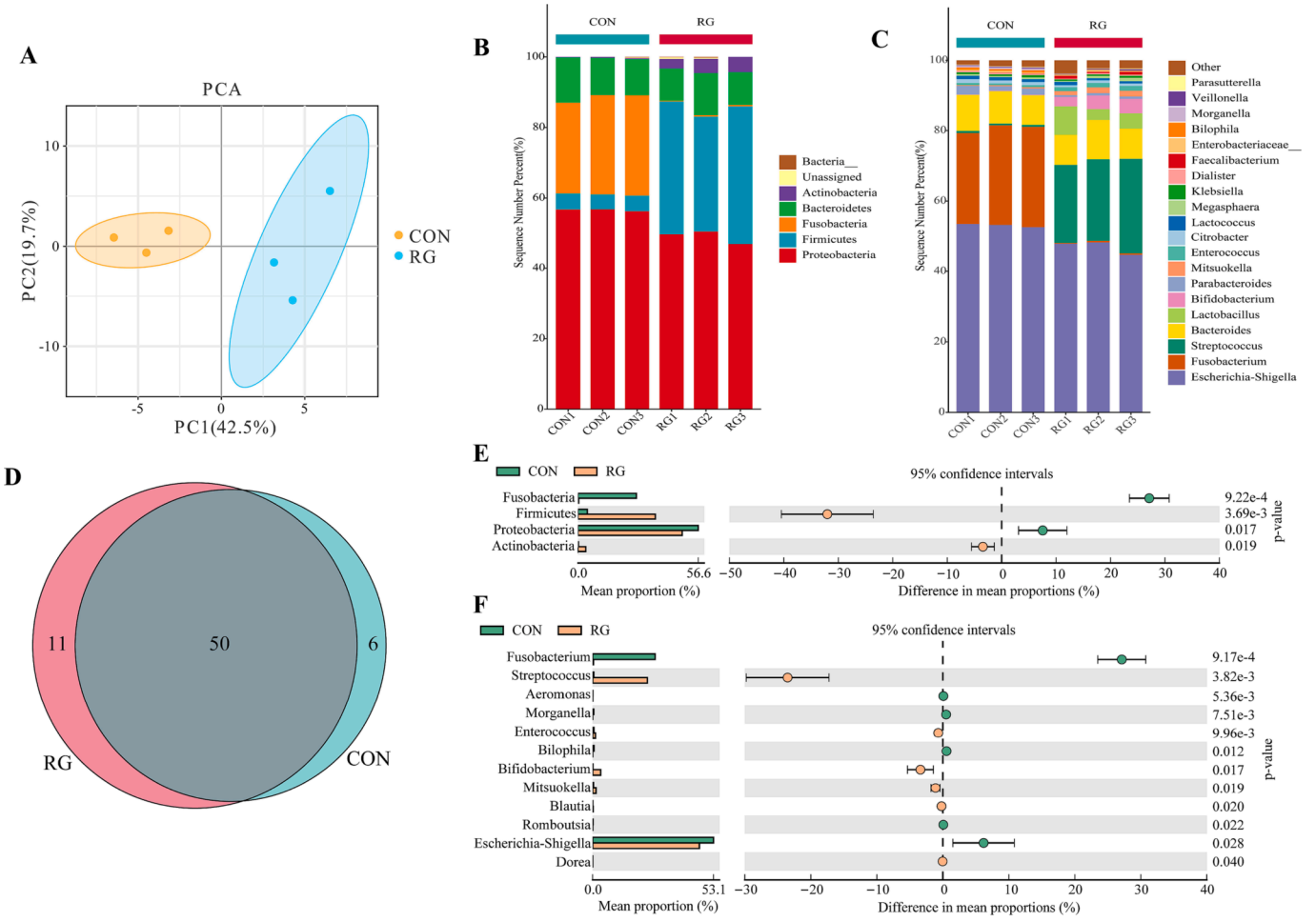

Fig. 1. Effects of lactulose on the structure and composition of intestinal flora *in vitro* experiments. (A) Principal component analysis (PCA) on the OTU levels, bar plot analysis at the phylum level (B) and the genus level (C), (D) Venn diagram based on OTU levels, and difference analysis of bacterial phylum (E) and bacterial genes (F) between groups.

3. Results

3.1. Lactulose fermentation changed gut microbial structure and composition

Three healthy volunteers that meet the requirements were included in the experiment and their fecal samples were used for *in vitro* fermentation experiments. The microbial structure and composition were analyzed to visualize the effect of lactulose on intestinal flora. Principal component analysis (PCA) showed that lactulose could significantly regulate the microflora structure of fermented samples (Fig. 1A). The intestinal microbiota composition at the phylum level and genus level was shown in Fig. 1B and 1C. From the figures, lactulose fermentation significantly changed the structure and composition of human gut microbiota simultaneously at the phylum level and genus level (Fig. 1B and 1C). The unique operational taxonomic unit (OTU) and shared OTU numbers were displayed in the Venn diagram (Fig. 1D). At the phylum level, lactulose significantly up-regulated the relative abundance of Firmicutes and Actinobacteria, and down-regulated the relative abundance of Proteobacteria and Fusobacteria compared with the blank group (Fig. 1E). At the genus level, lactulose could significantly up-regulate the relative abundance of *Streptococcus* and *Bifidobacterium*, and down-regulate the relative abundance of *Fusobacterium*, *Escherichia-Shigella* (Fig. 1F). These findings show that lactulose significantly changed gut microbial structure and composition from human feces *in vitro* fermentation system, and colonic lactulose fermentation significantly enriched the beneficial bacteria and inhibited the harmful bacteria.

3.2. Lactulose uptake affected body weight and colon length in colitis mice

Compared with control group, DSS induced significant weight loss and shortened colon length in mice. Compared with DSS group, lactulose administration in UC mice significantly alleviated the harm caused by DSS, including the up-regulation of body weight (Fig. 2A) and colon length (Fig. 2B and 2C). The result of H&E staining is shown in Fig. 2D. In the control group, intestinal mucosa, villi, goblet cells and crypts were intact, and no significant pathological changes or inflammatory reactions were found. In DSS group, submucosal edema, crypt damage, goblet cell disappearance and inflammatory cell infiltration were observed. Notably, lactulose-treated mice showed significant relief of colon damage, including a significant reduction of crypt and cup cells. This suggests that 1000 mg/kg dose of lactulose significantly restored the clinical features in colitis mice.

3.3. Lactulose regulated pro-inflammatory cytokine levels

The effect of lactulose on inflammatory status was examined and shown in Fig. 3. As shown in Fig. 3, DSS treatment boosted IL-6 levels and significantly promoted the content of pro-inflammatory factor TNF-α, IL-17, and IL-1β in the colon tissues of mice. By contrast, lactulose administration obviously reduced the levels of pro-inflammatory factors IL-6, TNF-α, IL-17, and IL-1β, compared to the DSS group. In general, lactulose showed a certain inhibitory effect on the colonic inflammatory status in colitis mice.

3.4. Lactulose did not affect SCFA concentration in mice feces

Dietary carbohydrates are often catabolized by intestinal flora in the

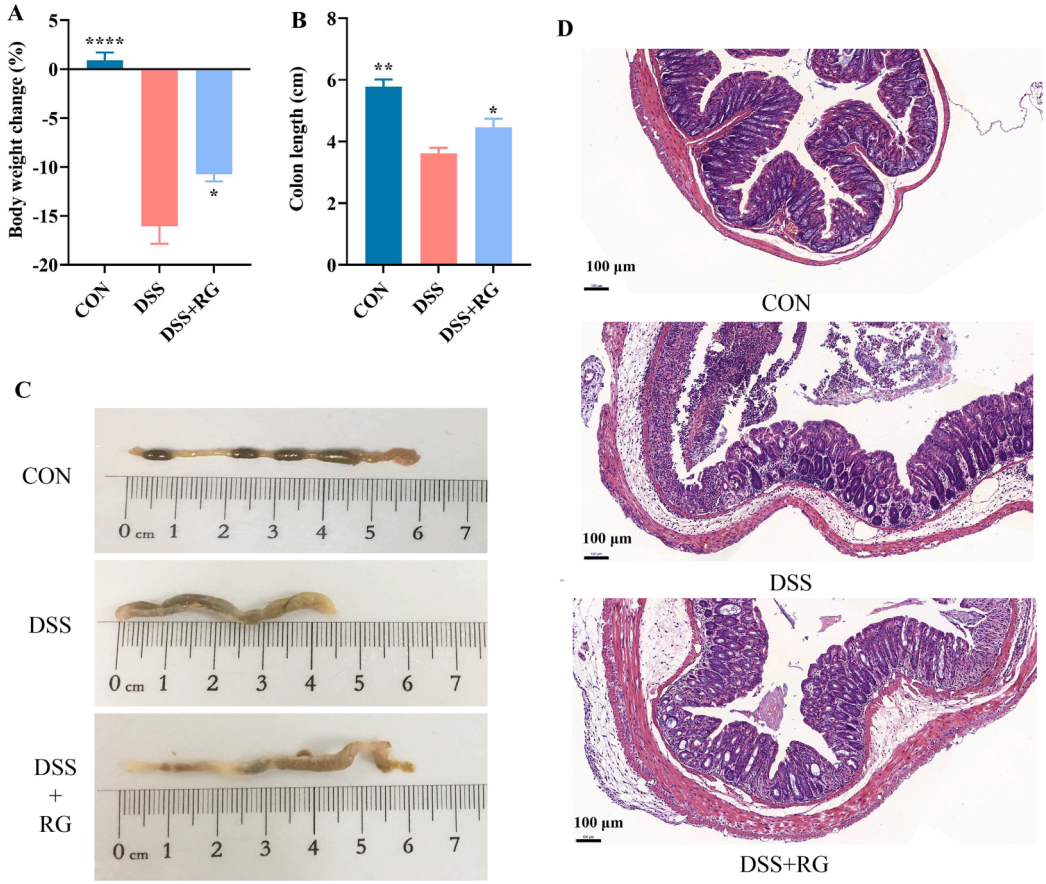

Fig. 2. Effects of lactulose on the physiological indices of UC mice. (A) Body weight change (%), (B) colon length, (C) image of colon, and (D) the H&E staining of the colon. The significant difference was indicated by comparing to DSS group.

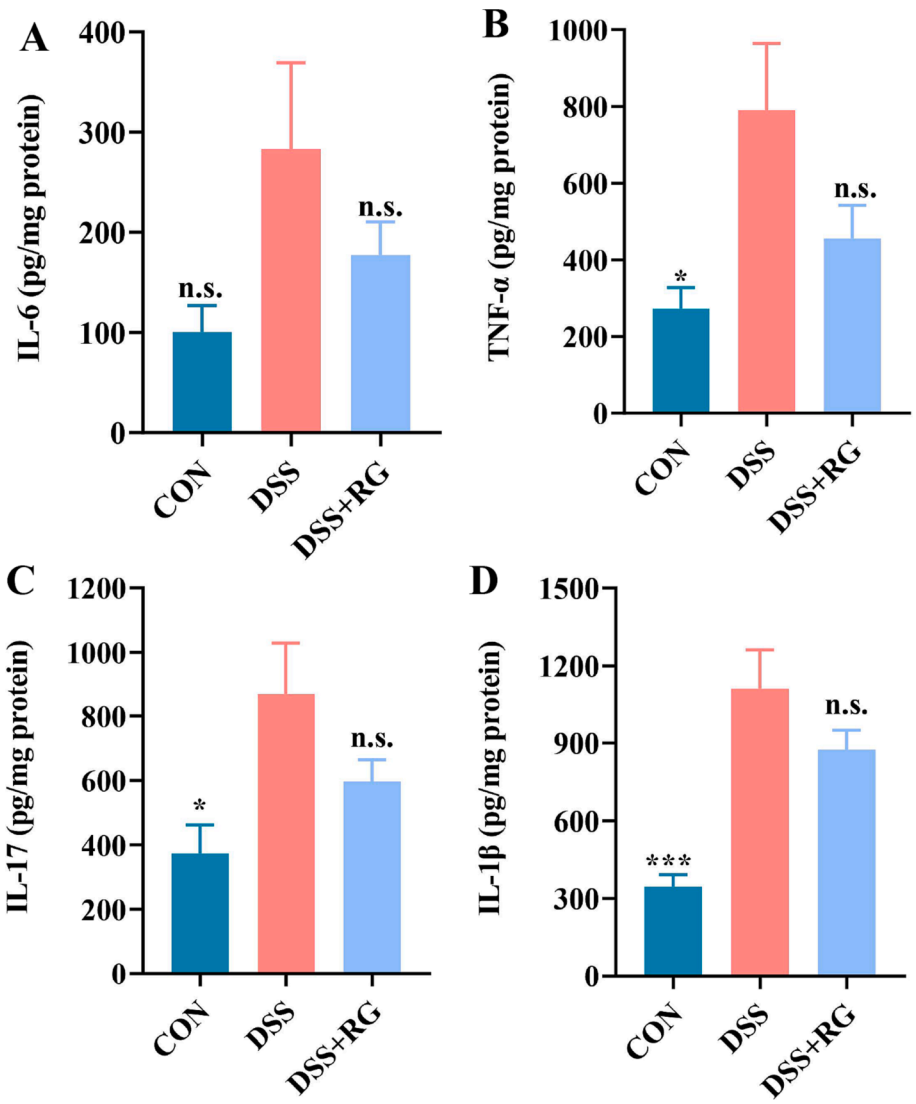

Fig. 3. Effects of lactulose on inflammatory cytokine contents in colon tissues of UC mice. The contents of IL-6 (A), TNF-α (B), IL-17 (C), and IL-1β (D). The significant difference was indicated by comparison with DSS group.

large intestine to produce SCFAs. Given that lactulose is non-digestible in the small intestine, the SCFA concentration was determined in the present study to investigate the association between colitis alleviation by lactulose and SCFA production. The result showed that DSS treatment significantly increased the contents of acetate, propionate, and butyrate (Fig. S1). Notably, lactulose treatment did not cause a significant effect on the contents of acetate, propionate, butyrate, valerate, isobutyrate, and isovalerate compared with DSS treatment group, which suggested that lactulose had no significant role in regulating the concentration of all SCFAs mainly existing in the colon. These results indicate that lactulose did not alleviate colonic inflammation via regulating intestinal SCFA production in colitis mice.

3.5. Lactulose improved the composition and structure of gut microbiota in mice

Due to the important role of intestinal flora in degrading dietary non-digestible carbohydrates, feces samples were collected and used to analyze the effect of lactulose on the composition and structure of gut microbiota. The results are shown in Fig. 4. Non-metric multidimensional scaling (NMDS) (Fig. 4A) and principal coordinate analysis (PCoA) (Fig. 4B) suggested that the structure of intestinal flora was significantly different between different treatment groups. The percentage and relative abundance of intestinal flora at the phylum level and genus level are respectively shown in Fig. 4C and 4D, which suggested that the relative abundance of intestinal flora in different groups of samples was significantly differential. Compared with control group, the α-diversity (including Simpson, Shannon, and Fisher indices) was slightly changed with no significance, which indicated that lactulose did not affect the overall bacterial community diversity in UC mice (Fig. 4E). The Venn diagrams showed unique and shared OTU data (Fig. 4H) that 54 OTUs were shared by three groups, among which the unique OTU numbers of the normal control group, the DSS group and the intervention group were 16, 4 and 3 OTUs respectively. Notably, 9 OTUs in the lactulose intervention group were shared with the control group (Fig. 4H).

Statistical analysis and LEfSe analysis showed that the administration of lactulose significantly changed the intestinal microbiota composition at the phylum level and genus level. At the phylum level, the ratio of Firmicutes/Bacteroidetes was not significantly changed by lactulose treatment (Fig. 4G). DSS treatment reduced the relative abundance of Bacteroidetes and Tenericutes, and elevated the relative abundance of Deferribacteres and Verrucomicrobia. By contrast, lactulose could restore these changes induced by DSS (Fig. 4F). The results of

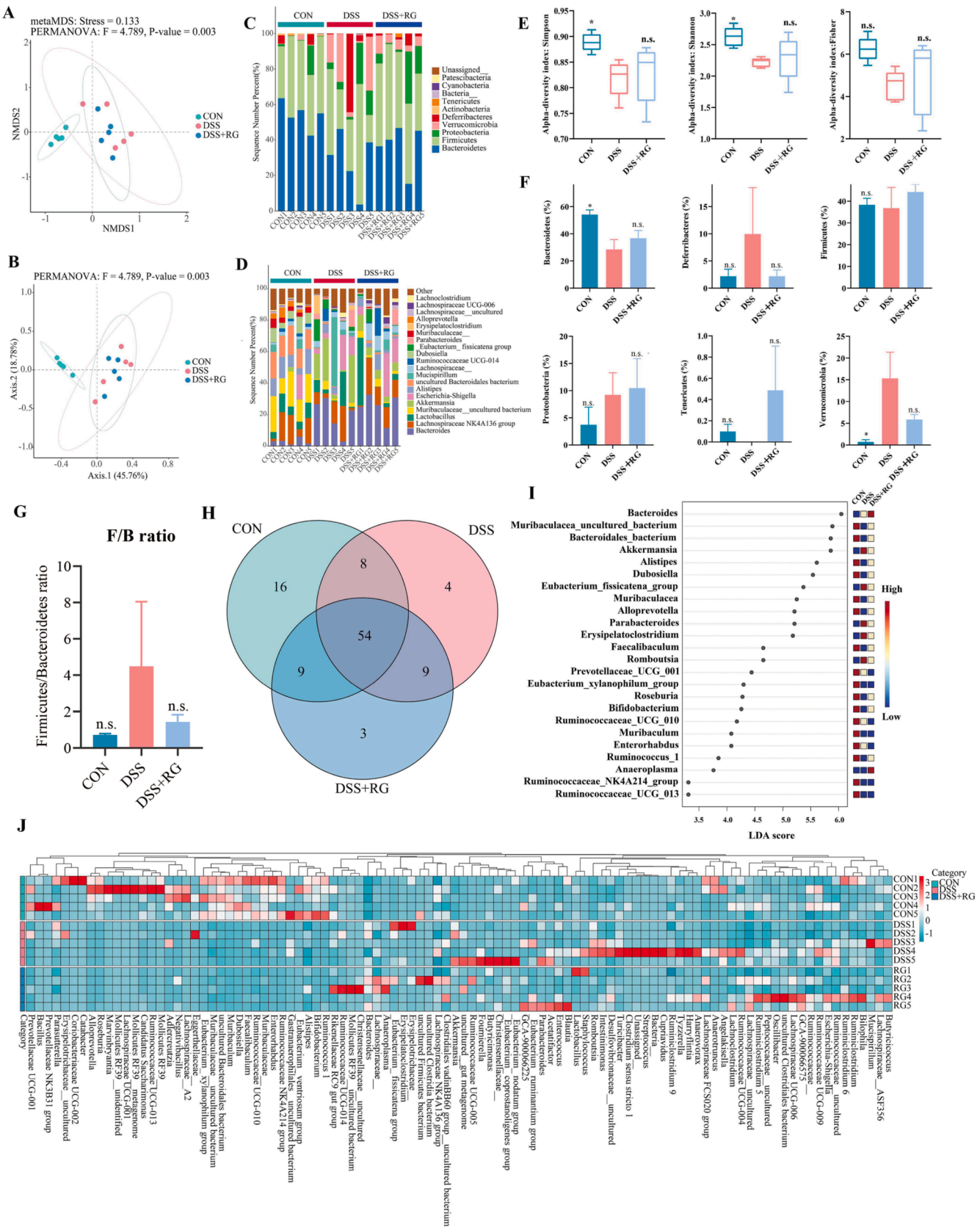

Fig. 4. Effects of lactulose on the structure and composition of mice gut microbiota. (A) Non-metric multidimensional scaling (NMDS), (B) principal coordinate analysis (PCoA), the bar diagram of microbiota composition at the phylum level (C) and genus level (D), (E) α-diversity of community, (F) the differences at the phylum level, (G) the ratio of Firmicutes/Bacteroidetes (F/B), (H) Venn diagram based on OTU level, (I) LEfSe analysis at the genus level, and (J) the heatmap of fecal microbiota in individual sample. The significant difference was indicated by comparing to DSS group.

LEfSe analysis of gut microbiota showed that, at the genus level, the DSS administration significantly enriched *Bacteroides*, *Akkermansia*, *Eubacterium_fissicatena_group*, *Parabacteroides*, *Erysipelatoclostridium*, and *Romboutsia*, and significantly reduced the abundance of *Alistipes*, *Dubosiella*, *Muribaculacea*, *Alloprevotella*, *Facalibaculum*, *Roseburia*, and *Bifidobacterium*, etc. Oral supplementation of lactulose could restore these effects of DSS on the intestinal flora (Fig. 4I). The results of the heatmap further showed significant differences in the intestinal flora at the genus level among the three groups (Fig. 4J). Above findings prove that lactulose altered intestinal microbiota composition at the OTU level in UC mice, and lactulose significantly inhibited the pernicious bacteria and enriched the beneficial bacteria, which is consistent with the result of *in vitro* experiment.

3.6. Lactulose significantly changed the serum metabolome

The combined data set from the negative ion and positive ion models was imported into the online MetaboAnalyst tool for multivariate statistical analysis. The sample distribution trend of PCA score maps and partial least squares discriminant analysis (PLS-DA) model based on metabolic data sets showed significant differences in serum metabolites between treatment groups (Fig. 5A and 5B). The dendrogram based on metabolite distribution showed a similar trend of samples' distribution to the PCA and PLS-DA models, and lactulose treatment group obviously displayed a closer distance from the control group compared with DSS group, which suggested lactulose supplementation restored the serum metabolite profiles close to the control group (Fig. 5D).

The variable importance in projection (VIP) values based on PLS-DA model of multivariate statistical analysis were obtained and metabolites with VIP $>$ 1 were selected as possible biomarkers between groups (Fig. 5C). Besides, the differences between sample groups were also assessed by using univariate analysis method and the metabolites significantly different between groups were shown in Fig. S2. Finally, these metabolites with VIP $>$ 1 of multivariate statistical analysis (Fig. 5C) and $p < 0.05$ of univariate analysis (Fig. S2) were regarded as differential biomarkers between groups. A total of 24 variables representing characteristic biomarkers with high inter-group confidence were obtained and the distribution of these metabolites was shown in Fig. 5E. The corresponding metabolic pathways enriched by KEGG topological analysis mainly included purine metabolism, phenylalanine metabolism, and pyruvate metabolism, etc., among which purine metabolism was significantly enriched (Fig. 5F). Taken together, these data suggest that lactulose altered the serum metabolic profile of colitis mice.

3.7. Correlation analysis

To investigate correlations between intestinal flora and metabolic changes in the serum influenced by lactulose, Spearman's correlation analysis was performed and the result was displayed in Fig. 6. As shown in Fig. 6A, *Bacteroides* and *Parabacteroides* were a positive correlation with 1-linoleoyl glycerol, morphine-d3, α-eleostearic acid, decarbamoyl-neosaxitoxin, inosine, leucylproline, hypoxanthine, and xanthine, and negative correlation with corticosterone, 3,5-di-*tert*-butyl-4-hydroxybenzyl alcohol, myristic acid, phenylacetylglycine, *trans*-3-indoleacrylic acid, acrylic acid, and 2-oxindole. Interestingly, *Enterorhabdus*, Prevotellaceae UCG-001, and Ruminococcaceae UCG-010 could also significantly regulate these substances, but the effect of was opposite to *Bacteroides* and *Parabacteroides*. Besides, *Romboutsia*, *Erysipelatoclostridium*, *Akkermansia* and *Eubacterium fissicatena* group were a positive correlation with the concentration of 2-arachidonoyl glycerol, adenosine, and (+/-)-CP 47,497-C7-hydroxy metabolite, but negative correlation with testosterone, cinnamoylglycine, isoquinoline, and methyl indole-3-acetate (Fig. 6A). The correlation network diagram is shown in Fig. 6B.

To investigate the association between serum metabolites influenced by lactulose and intestinal inflammatory indicators, Spearman's correlation analysis was conducted and the result was shown in Fig. 6C. From the figure, the levels of isoquinoline, methyl indole-3-acetate, cinnamoylglycine, testosterone in the serum were positively related to body weight change and colon length, but negatively related to IL-17, TNF-α, IL-6 and IL-1β. By contrast, serum metabolites including adenosine, (+/-)-CP 47,497-C7-hydroxy metabolite, 2-arachidonoyl glycerol exhibited the exact opposite trend. It is worthy that body weight change and colon length of colitis mice showed a negative correlation with cytokine levels IL-17, TNF-α, IL-6 and IL-1β, which is consistent with the above results.

4. Discussion

The integrity of intestinal mucosal barrier plays an important role in resisting the invasion of external toxins, pathogenic bacteria and other harmful substances (Paone & Cani, 2020). The maintenance and protection of intestinal mucosal barrier function is an important means to maintain intestinal health. Notably, the influence of external factors on intestinal barrier is mainly mediated by intestinal flora (Paone et al., 2020). Therefore, specific regulation of intestinal flora structure to protect intestinal health is a commonly used means at present. Among these, prebiotics is a typical and effective strategy that has been widely demonstrated to be able to protect intestinal health by regulating gut microbiota and metabolites.

Lactulose is widely regarded as a prebiotic. In this study, we first explored the regulatory effect of lactulose on intestinal microflora through *in vitro* fermentation experiment. The results showed that lactulose could significantly change the structure and composition of intestinal microflora. Accumulating studies have demonstrated the roles of *Bifidobacterium* species in alleviating host disease, especially intestinal inflammatory disease (Fan et al., 2021; Sun et al., 2020; Wang, Yin, Chen, & Davis, 2018). Besides, the abundance of *Escherichia-Shigella* is closely related to the occurrence and development of UC (Ma et al., 2022). Notably, in this study, lactulose could significantly enrich the potential probiotics (especially *Bifidobacterium*), and reduce the potentially pathogenic bacteria abundance (such as *Escherichia-Shigella*). Based on these results, lactulose might improve intestinal inflammation by regulating intestinal flora. Thus, a mouse model of UC was constructed to explore the effect of lactulose on regulating gut microbiota and UC. It was found that DSS induced significant intestinal inflammation and injury in UC mice, including decreased body weight and colon length. A large number of early reports have proved that the occurrence of UC could cause drastic changes in the inflammatory factors in the intestinal tissues, including up-regulation of the pro-inflammatory factors and down-regulation of the anti-inflammatory factors (Liu et al., 2020; Wang et al., 2021). Thus, we have also detected changes in the content of inflammatory cytokines in the colon tissues of mice. The results showed that lactulose could down-regulate the DSS-induced pro-inflammatory cytokines IL-6, TNF-α, IL-17, and IL-1β. Besides, the results of intestinal biopsy also proved the protective effect of lactulose on intestinal tissue. These physiological indicators demonstrate that lactulose can effectively reduce the severity of UC.

The protective effect of prebiotics on host health is largely attributed to the regulation of intestinal flora and metabolites. In order to further explore the main mechanism of lactulose in alleviating UC, we also tested the structural composition of intestinal flora and serum metabolites of UC mice. We found that the ratio of Firmicutes/Bacteroidetes was not significantly changed by lactulose treatment, which might be explained by the inter-group difference. Although the Firmicutes/Bacteroidetes ratio is important, gut health does not necessarily depend on the Firmicutes/Bacteroidetes ratio. And according to current evidence, it is difficult to associate the Firmicutes/Bacteroidetes ratio with a determined health status (Magne et al., 2020). By analyzing the effects of lactulose on the structure and composition of intestinal flora in colitis mice, we found that *Bacteroides* was one of the dominant bacterial groups in the intestinal tract. One of its typical characteristics is its

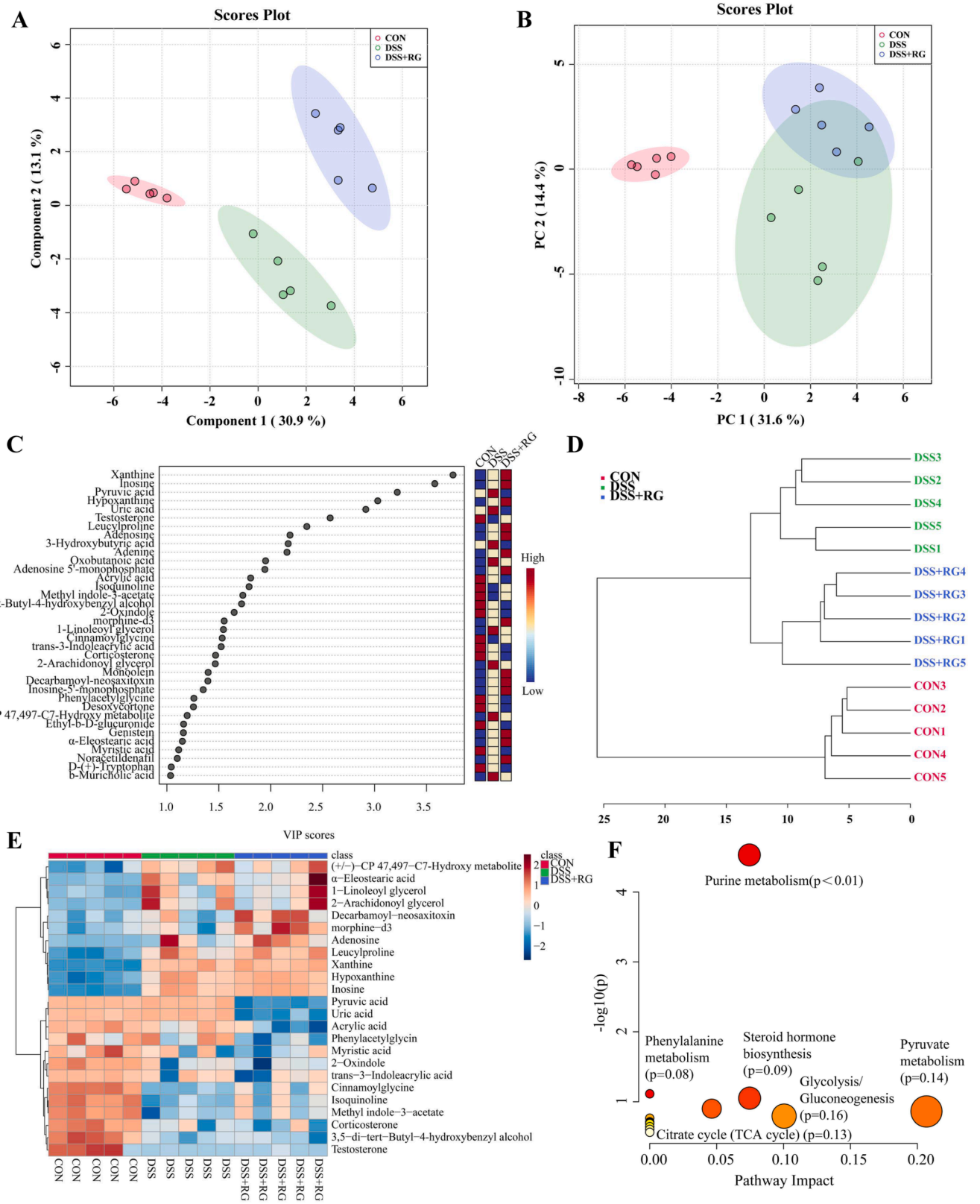

Fig. 5. Effects of lactulose on serum metabolome of UC mice. (A) The PCA score plots, (B) the PLS-DA score plots, (C) VIP analysis of the metabolites based on loading plots, (D) the clustering tree of metabolites among different groups, (E) a heatmap of significantly differential metabolites among groups, (F) KEGG pathways enriched by metabolites.

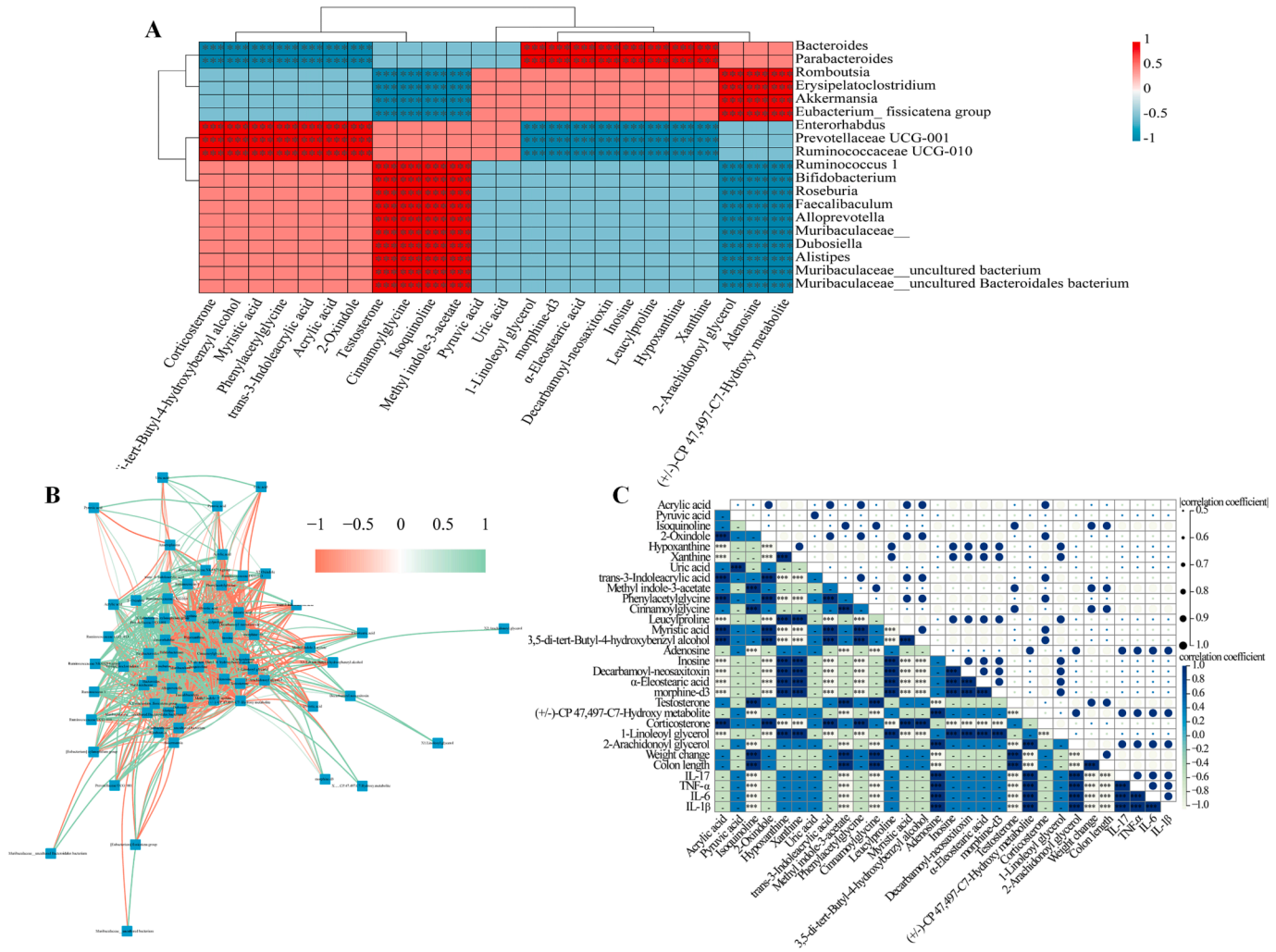

Fig. 6. Correlation analysis between different indicators. (A) The correlation analysis between the predominantly differential intestinal bacteria and metabolites, (B) the correlation network between the predominantly differential intestinal bacteria and metabolites, and (C) Spearman's correlation analysis between serum metabolites and phenotypic indicators.

strong metabolic capacity for dietary fiber and polysaccharide (Pereira et al., 2021; Schwalm & Groisman, 2017). Lactulose intake could significantly enrich *Bacteroides* in the intestinal tract of colitis mice, indicating the process of its utilization by intestinal flora. *Faecalibaculum* (Zagato et al., 2020) and *Roseburia* (Shen et al., 2022) are gut microbes that are closely associated with gut diseases. In the early stages of colorectal cancer, the abundance of *Faecalibaculum rodentium* is significantly reduced in the small intestine and colon. And targeted supplements *F. rodentium* can inhibit tumor cell proliferation by producing SCFAs (Zagato et al., 2020). *Roseburia intestinalis* inhibits Crohn's disease development by increasing the differentiation of anti-inflammatory Tregs (Shen et al., 2022). Similar to previous *in vitro* results, lactulose showed significant enrichment of *Bifidobacterium* in this study. *Bifidobacterium*, one of the most studied probiotics, has been widely shown to have a significant role in alleviating inflammatory bowel disease (Fan et al., 2021; Duranti et al., 2016; Engevik et al., 2021). Meanwhile, lactulose inhibited *Akkermansia* enrichment induced by DSS. Although some reports suggest that *Akkermansia* has the potential to relieve disease, in certain cases (especially damage to the intestinal mucosa), *Akkermansia* may adversely affect the host intestine. For example, the degradation of the mucous layer by *Akkermansia* promotes adhesion and invasion of the epithelium by *Escherichia coli*, thus exacerbating the severity of intestinal inflammation (Sugihara et al., 2022). *Lactobacillus* is one of the common normal flora in the Firmicute phylum. We found that the relative abundance of *Lactobacillus* was obviously raised by lactulose fermentation, but there was no significant difference between groups by using both *in vitro* system and mice model. This is consistent with the previous studies, which showed that lactulose treatment increased the relative abundance of *Bifidobacterium* and *Lactobacillus* but the change mainly occurred in *Bifidobacterium* (Tolonen et al., 2022; Vanhoutte, De Preter, De Brandt, Verbeke, Swings, & Huys, 2006). *Streptococcus* is considered to be likely associated with the occurrence and development of colitis (Heidarian, Noormohammadi, Aghdaei, & Alebouyeh, 2017). We found that DSS treatment increased the relative abundance of *Streptococcus* and lactulose supplementation reduced the relative abundance of *Streptococcus* in DSS-induced colitis mice, which is consistent with the previous study (Heidarian, Noormohammadi, Aghdaei, & Alebouyeh, 2017). These results emphasize that the anti-inflammatory role of lactulose may be attributed to its ability in enriching probiotics and inhibiting potential pathogens.

Notably, lactulose did not show a significant regulatory effect on intestinal SCFAs, especially butyrate in this study. Butyrate is beneficial for maintaining intestinal health and systemic health level through the mediation of various signaling pathways (Bach Knudsen et al., 2018). However, the improvements in gut health are not butyrate-dependent. For example, a clinical trial investigated the effect of fecal microbiota transplantation on UC and observed that change from baseline in stool concentrations of butyrate and other SCFAs between treatment groups

was not associated with any observed treatment effect (Costello et al., 2019). Butyrate is mainly produced by gut bacteria from the Firmicutes phylum (Hallert, Björck, Nyman, Pousette, Grännö, & Svensson, 2003; Gasaly, Hermoso, & Gotteland, 2021). However, the phylum Firmicute includes many various genera. In the present study, we found that lactulose increased the relative abundance of Firmicutes phylum including *Roseburia*, and *Ruminococcus*_1, while reduced the relative abundance of *Eubacterium_fissicatena_group*, *Erysipelatoclostridium*, and *Ruminococcaceae*_UCG_010, which also belong to Firmicutes. The production of butyrate is a result of the synergistic effect of all gut bacteria. Therefore, this might explain why the content of butyrate was not significantly increased by lactulose. Above findings demonstrated that SCFAs were not the key metabolites of lactulose in alleviating UC.

The occurrence of intestinal inflammation is accompanied by impairment of intestinal barrier function (Cui et al., 2021; Wang, Zhang, Guo, & Li, 2019), and then affects the changes of blood indexes (Xie et al., 2021). Thus, changes in serum metabolite could be a potential biological indicator of intestinal health (Uchiyama et al., 2017). In this study, lactulose intake significantly increased blood levels of xanthine, inosine, hypoxanthine, leucylproline, adenosine, adenine, oxobutanoic acid, etc. Some of these substances have been shown to have significant effects on relieving UC. For example, the content of xanthine is negatively correlated with intestinal inflammation in mice/humans. Oral xanthine can promote the survival and proliferation of intestinal epithelial cells and reduce the severity of colitis by activating aromatics receptors (AhR) and increasing the level of oxidative phosphorylation (Michaudel et al., 2023). Inosine can act on colon epithelial cells through the proliferator-activated receptor pathway to protect the colon (Li, Feng, Tian, Ji, Hu, & Chen, 2021). In contrast, lactulose also decreased blood levels of pyruvic acid, uric acid, 3-hydroxybutyric acid, oxobutanoic acid, acrylic acid, 1-linoleoyl glycerol, etc. Among these, 3-hydroxybutyric acid can promote the growth of probiotics, improve colon length, and thus reduce colon damage and intestinal inflammation (Yan et al., 2021). These changes in serum metabolites indicate the ameliorative effect of lactulose on colitis. In particular, lactulose remarkedly reduced uric acid levels in the serum and affected purine metabolism, which indicates that lactulose has the potential as an effective component for lowering blood uric acid level.

The results of correlation analysis showed that intestinal flora was closely related to serum metabolites while the changes in serum metabolites were significantly correlated with phenotypic indicators of colitis mice. These results further clarify the important role of intestinal flora in alleviating intestinal inflammation. In this study, lactulose played a role in regulating the abundance of *Bacteroides*, *Akkermansia Eubacterium fissicatena* group, *Parabacteroides*, *Alloprevotella*, *Facalibaculum*, *Roseburia*, *Bifidobacterium* and other gut microbes. After that, the metabolites of these gut microbiota entered the bloodstream through the intestinal barrier and thus affected serum metabolic indicators. Serum metabolites significantly modulated pro-inflammatory cytokines levels and further affected intestinal inflammation via systemic blood circulation (illustrated in Graphical Abstract). These results showed that lactulose might improve serum indexes in mice by regulating intestinal flora, and thus alleviating the severity of colonic inflammation in mice. Serum metabolites not only function as phenotypic indicators but also have the potential as a biomarker for intestinal health.

5. Conclusion

In conclusion, we found that lactulose could inhibit the relative abundance of potentially pathogenic bacteria, increase the relative abundance of potential probiotics, regulate the content of serum metabolites, and ultimately alleviate the severity of colonic inflammation. These results demonstrate that lactulose may be an effective strategy for relieving UC. However, the clinical application effect and safety of lactulose should be further explored in the future.

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CRediT authorship contribution statement

Junying Bai: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Botao Wang:** Data curation, Investigation, Methodology, Project administration, Writing – review & editing. **Xiang Tan:** Funding acquisition, Writing – review & editing. **Linhua Huang:** Funding acquisition, Writing – review & editing. **Shuangli Xiong:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100821.

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