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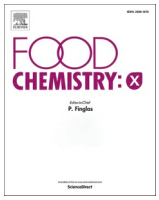

Aged oolong tea alleviates dextran sulfate sodium-induced colitis in mice by modulating the gut microbiota and its metabolites

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ABSTRACT

In this study, the mechanism of aged oolong tea (AOT) to alleviate colitis was investigated in terms of microbiome, metabolome, and fecal microbiota transplantation (FMT). AOT storage period could alleviate colitis in mice and there were some differences in AOT between storage periods, especially AOT-10. AOT improves UC by modulating oxidative stress and inflammatory factors and upregulating intestinal tight junction protein expression (Occludin, Claudin-1, ZO-1 and MUC2), which is associated with the recovery of gut microbiota. FMT and targeted metabolomics further demonstrate that the anti-inflammatory effects of AOT can reshape the gut microbiota through faecal bacterial transfer. Anti-inflammatory effects are exerted through the stimulation of metabolic pathways associated with amino acid, fatty acid and bile acid metabolites. Importantly, the study identified key bacteria (e.g., *Sutterella*, *Clostridiaceae_Clostridium*, *Mucispirillum*, *Oscillospira* and *Ruminococcus*) for the development and remission of inflammation. Conclusively, AOT may have great potential in the future adjuvant treatment of colitis.

Major compounds studied in this article.

Chemical compounds	PubChem CID	Formula	Source	Class
Glycine	750	C2H5NO2	Metabolome	Amino acids
Glutamine	5961	C5H10N2O3	Metabolome	Amino acids
Gamma-aminobutyric acid	119	C4H9NO2	Metabolome	Amino acids
Tyrosine	6057	C9H11NO3	Metabolome	Amino acids
Serine	5951	C3H7NO3	Metabolome	Amino acids
Linoleic acid	5,280,450	C18H32O2	Metabolome	Fatty acids
Myristic acid	11,005	C14H28O2	Metabolome	Fatty acids
Arachidonic acid	444,899	C20H32O2	Metabolome	Fatty acids
Lithocholic acid	9903	C24H40O3	Metabolome	Bile acids
Deoxycholic acid	222,528	C24H40O4	Metabolome	Bile acids

Introduction

Inflammatory bowel disease (IBD) includes ulcerative colitis (UC) and Crohn's disease (CD). The clinical manifestations of IBD are mainly mucosal ulceration, rectal bleeding, diarrhea, and weight loss, but can result in death (Turpin, Goethel, Bedrani, & Croitoru, 2018). UC, a major subtype of IBD, can easily develop into colorectal cancer (CRC) if not treated in a timely manner (Ekbom, Helmick, Zack, & Adami, 1990). However, the exact cause of UC is still unclear owing to several factors, including genetic inheritance, environment, the gut microbiota and immune response (Harris & Chang, 2018). It has been estimated that there are more than 10^{14} types of commensal bacteria in the human body performing multiple functions (e.g., nutrient absorption, host defense, energy supply, and immune development) (Perez-Muñoz, Arrieta, Ramer-Tait, & Walter, 2017). In recent years, several studies have shown that the gut microbiota is closely associated with intestinal inflammation (Harris & Chang, 2018). Finding effective treatments poses a great challenge due to the complex pathogenesis and the interaction of multiple environmental factors. Traditional treatments for IBD

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have resulted in significant undesirable side effects, antibiotic resistance or organ damage (Tung et al., 2006). In conclusion, a safe, effective approach without side effects, such as natural products derived from plants and foods, is extremely important to protect against colitis.

As an emerging treatment for IBD, fecal microbiota transplantation (FMT) involves the transfer of healthy fecal microbiota into patients to restore the balance of gut microbes and to treat certain diseases. FMT first appeared in ancient Chinese medical books (Zhang, Luo, Shi, Fan, & Ji, 2012). Additionally, modern medicine has proved that FMT has excellent efficacy in the treatment of recrudescent refractory and recurrent *Clostridium difficile* infections (RCDI) (Jalanka et al., 2016). Several studies have suggested that probiotics, polysaccharides, EGCG and other potent chemical components in tea can be used to treat a variety of diseases caused by microbiota imbalance (e.g., colitis, type 2 diabetes, obesity, and hyperlipidemia) through FMT techniques (Lu, Jing, Zhang, & Cao, 2022; Wu et al., 2021; Yuan et al., 2018). Therefore, FMT is increasingly being used to study the impact of drugs and diet on regulating the gut microbiota to alleviate or reverse disease.

Tea, as one of the world-renowned non-alcoholic beverages, has been shown to make positive and effective contributions as an adjuvant in the treatment of human diseases (Gong et al., 2022; Huang et al., 2021; Pan et al., 2013). Oolong tea, a famous traditional Chinese tea, is semi-fermented. In recent years, AOT has become increasingly popular in the consumer market, and there exists a commonly accepted belief among consumers in China that the longer tea is stored and oxidized, the better the flavor and greater the health benefits (Hong et al., 2021). Long-term use in folk medicine also shows that AOT regulates gastrointestinal discomfort, exhibits antioxidant and anti-inflammatory effects and relieves diarrhea. Many studies have reported that oolong tea polyphenol extracts and theasinensin A can induce apoptosis of human cancer cells (Guo et al., 2019; Wang et al., 2022). A recent study has suggested that AOT may inhibit obesity by inhibiting fatty acid synthesis and inflammation (Yuan et al., 2018). However, the role of the AOT storage period on DSS-induced colitis in mice has not been fully studied, particularly with regard to gut microbiota and metabolomic disorders in colitis.

In this study, the protective effects and mechanism of AOT at different storage periods on inflammation in DSS-induced colitis mice were shown. Furthermore, fecal microbiota transplantation, gut microbiome and metabolomic methods were used to study the potential action of AOT on gut microbiota and its metabolites, and revealed that its regulatory effects on gut microbiota homeostasis and metabolomic characteristics of the gut microbiota are important for the relief of colitis. The mechanisms by which AOT extracts alleviate colitis were elucidated from the microbial-microbial metabolites-host inflammation axis.

Materials and methods

Chemicals and reagents

DSS (Dextran Sulfate Sodium Salt, $M_W = 36–50$ kDa) was purchased from MP Biomedicals (Irvine, CA). LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and blood urea nitrogen (BUN) detection kits were provided by Ningbo Purui Bo Biological Technology Co., Ltd. (Ningbo, China). Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), myeloperoxidase (MPO), and malondialdehyde (MDA) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Animals

C57BL/6 mice (22±2 g, male, 7–8 weeks old, batch number is 20210002000820) were purchased from Wu's Animal Centre (Fuzhou, China) and housed in a standard animal room with constant temperature (23±2 °C) and humidity environment (70–75 %). Food and water were obtained ad libitum in a 12 h dark-light cycle. All mouse experiments were performed under the approval of the Animal Protection Review Committee of Fujian Agriculture and Forestry University (permit number, PZCASFAFU22040).

Tea samples and chemical characterization

Aged Wuyi Rock Tea, a type of oolong tea that is compliant with the international standard for oolong tea (ISO 20716:2022, *Oolong tea – Definition and basic requirements*), was provided by Liuhe (Wuyishan) Tea Co., Ltd. Four different years of AOT (produced in 2021, 2011, 2001, and 1991; Wuyishan, China) were crushed and then extracted (1:20, w/v) with pure water at 100 °C for 45 min. After filtration, the tea residue was extracted again for 45 min using the same operation, and the two tea extracts obtained were mixed, concentrated, freeze-dried, and then stored at −80 °C for later experimental use. The AOT extracts were filtered through a 0.22 μm membrane and then subjected to biochemical methods (Gao et al., 2022) and high-performance liquid chromatography (HPLC, Waters 2695, MA, USA) for the determination of the main active biochemical components. The conditions for a HPLC configured with a 2998 PDA detector are set as follows: mobile phase A: 2 % acetic acid, mobile phase B: acetonitrile, flow rate set to 1.0 mL/min; each injection volume was 10ul, wavelength: 280 nm, column temperature 35 °C. gradient elution: A, 100 %, 0 min; A, 68 %, 25 min; A, 100 %, 5 min. The chemical composition is shown in Table S1.

Experimental design

Initially, in Experiment 1, simulating the daily tea drinking habits of humans, we explored the effect of continuous daily consumption of AOT (storage periods of 0, 10, 20 and 30 years, respectively) from different years on the gut microbiota of mice (Fig. S1A). Next, in Experiment 2, further investigation of the effectiveness of AOT on DSS-induced colitis in mice was carried out (Fig. 2A). Six mice were selected as the Healthy group and were administered distilled water for 21 days. The DSS group was administered 3 % (w/v) DSS in distilled water. The AOT group, according to the difference in storage period, could be divided into DSS_T0, DSS_T10, DSS_T20, and DSS_T30, respectively. There groups were administered the same regime and dosage as those in Experiment 1 for 21 days and administered four different storage periods of AOT (200 mg/kg/d). In addition, we added Berberine Hydrochloride Tablets (BB) (5 mg/kg/d), a positive drug for colitis (Li et al., 2020), as a comparison, and this experimental group was defined as DSS_BB. However, the above experimental group was administered 3 % (w/v) DSS in drinking water from day 15 to day 21, except for the Healthy group. Finally, in Experiment 3, the association between the therapeutic benefits of AOT on colitis and the gut microbiota and its metabolites was explored, mainly through the fecal microbiota transplantation technique (Fig. 4A).

Characterization records and sample collection

During the experiment, changes in body weight and fecal condition were recorded for each group of mice, and the disease activity index (DAI) was calculated according to the method shown in Table S2. At the end of the experiment, all mice were anesthetized prior to cervical dislocation, blood was then collected from the eye, centrifuged and stored (at 4 °C and 5000 rpm for 10 min). The colon and liver were photographed and recorded for subsequent hematoxylin-eosin (H&E) staining and other projects, respectively.

Preparation of donor stool

Donor mice received the same treatment as the mice in Experiment 1. After the donor mice were gavaged for 14 days, fresh faeces were collected daily into sterile centrifuge tubes. The faeces of the donor mice were mixed with the corresponding volume of phosphate buffered saline (PBS), and then the mixture was homogenized after centrifugation (4 °C,

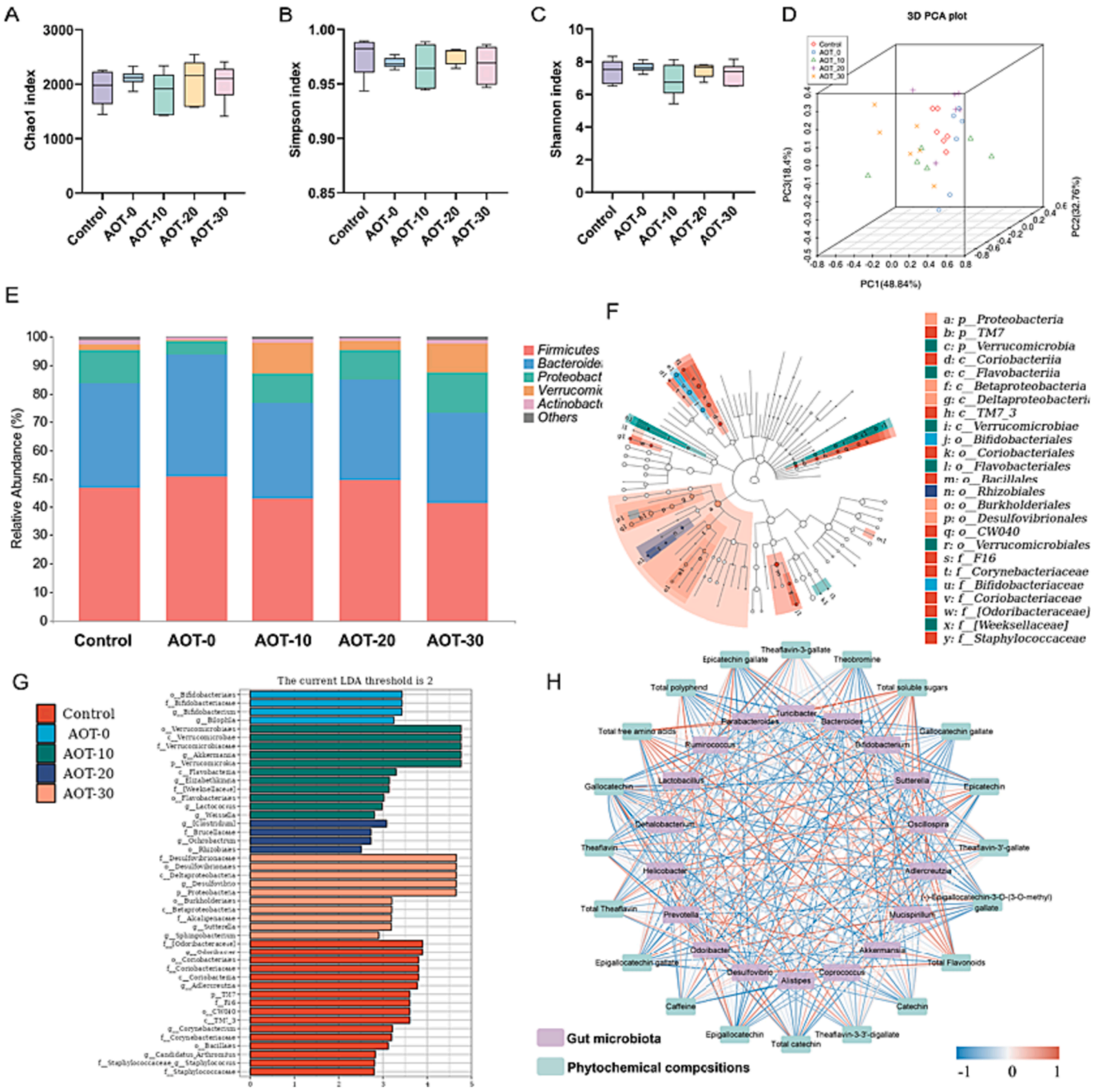

Fig. 1. AOT altered the gut microbial composition and diversity of the cecum contents samples from the mice. (A-C) α-diversity index. (D) Principle component analysis (PCA) plot of the gut microbiota. (E-G) Gut microbiota composition. (H). Visualisation of correlation networks based on partial correlations between phytochemicals and gut microbiota. Data are mean ± SD ($n = 6$). Different letters indicated statistically significant differences between the groups ($p < 0.05$).

1000 g, 3 min). The upper bacterial solution was collected, centrifuged again (4 °C, 12 000 g, 5 min,) and dissolved in an adapted volume of sterile PBS.

Detection of biochemical indices of serum samples

The preparation of mouse serum is as described previously. Biochemical indicators, including HDL-C, LDL-C, AST, ALT and BUN were assayed by kits provided by Ningbo Purui Bo Biological Technology Co., Ltd. (Ningbo, China).

Assessment of oxidative stress

The levels of GSH-Px, SOD, MDA and MPO in the liver and colon homogenates of mice in each experimental group were detected according to the detection method of the corresponding kit.

Real-time PCR

Total RNA of the colon tissues was extracted using an RNA-easy™ Animal RNA Isolation kit. cDNA was synthesized according to the instructions provided in the Kit (TransGen Biotech, Beijing, China). The relative expression of tight junction white-related genes (Occludin,

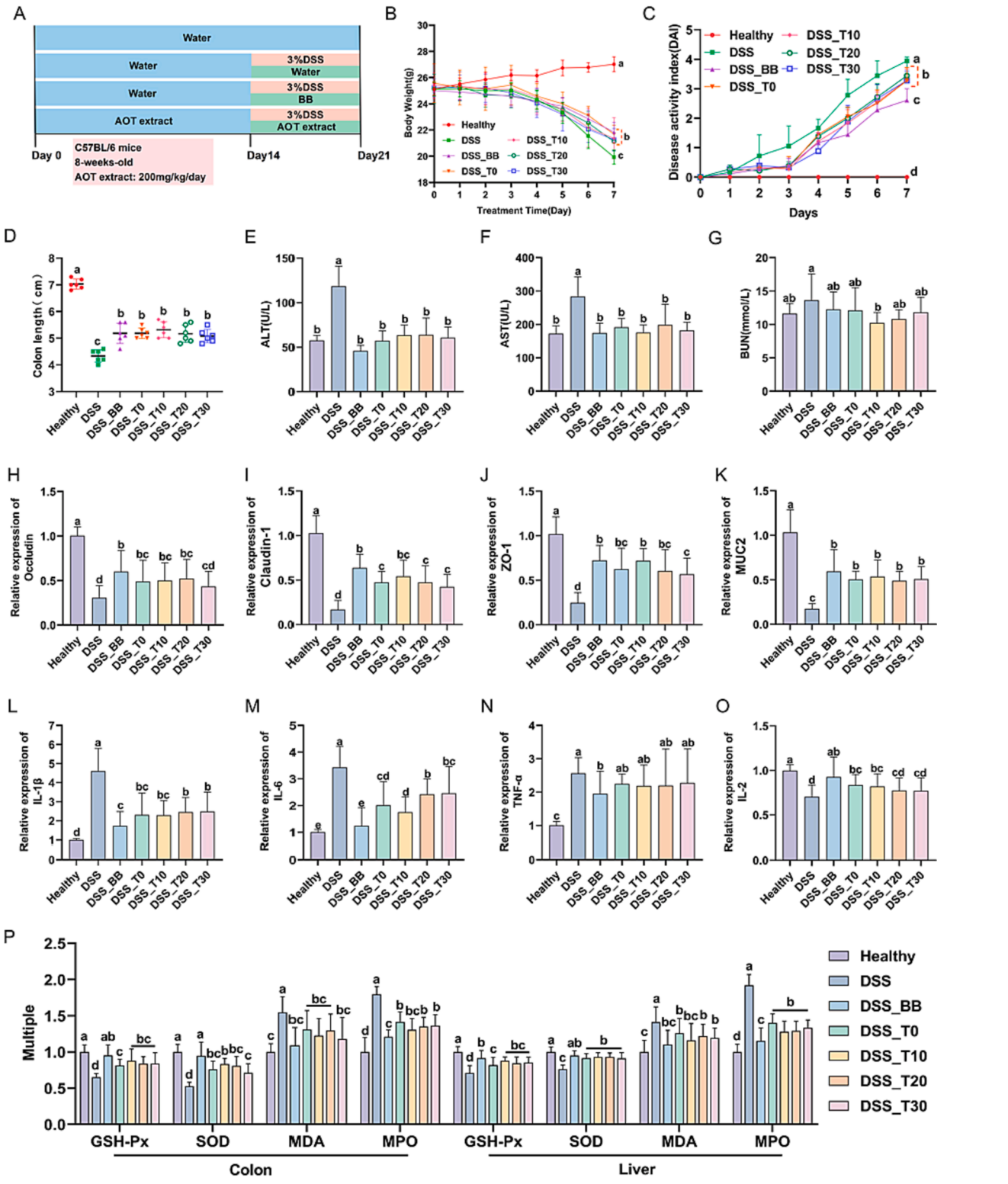

Fig. 2. AOT significantly modulates the inflammatory response in DSS-induced colitis mice. (A) Experimental design and timeline of the animal model and treatment. (B) Body weight. (C) Disease activity index. (D) Colon length. (E-G) Serum levels of ALT, AST, and BUN in DSS-induced colitis mice. (H-K) Colonic tissue mRNA levels of Occludin, Claudin-1, ZO-1, and MUC2. (L-O) Colonic tissue mRNA levels of IL-1β, IL-6, TNF-α and IL-2. (P) GSH-Px, SOD, MDA and MPO level activities of the colon tissue and liver tissue were measured compared with those in the Healthy group ($n = 6$ per group). Data are mean ± SD. Different letters indicated statistically significant differences between the groups ($p < 0.05$).

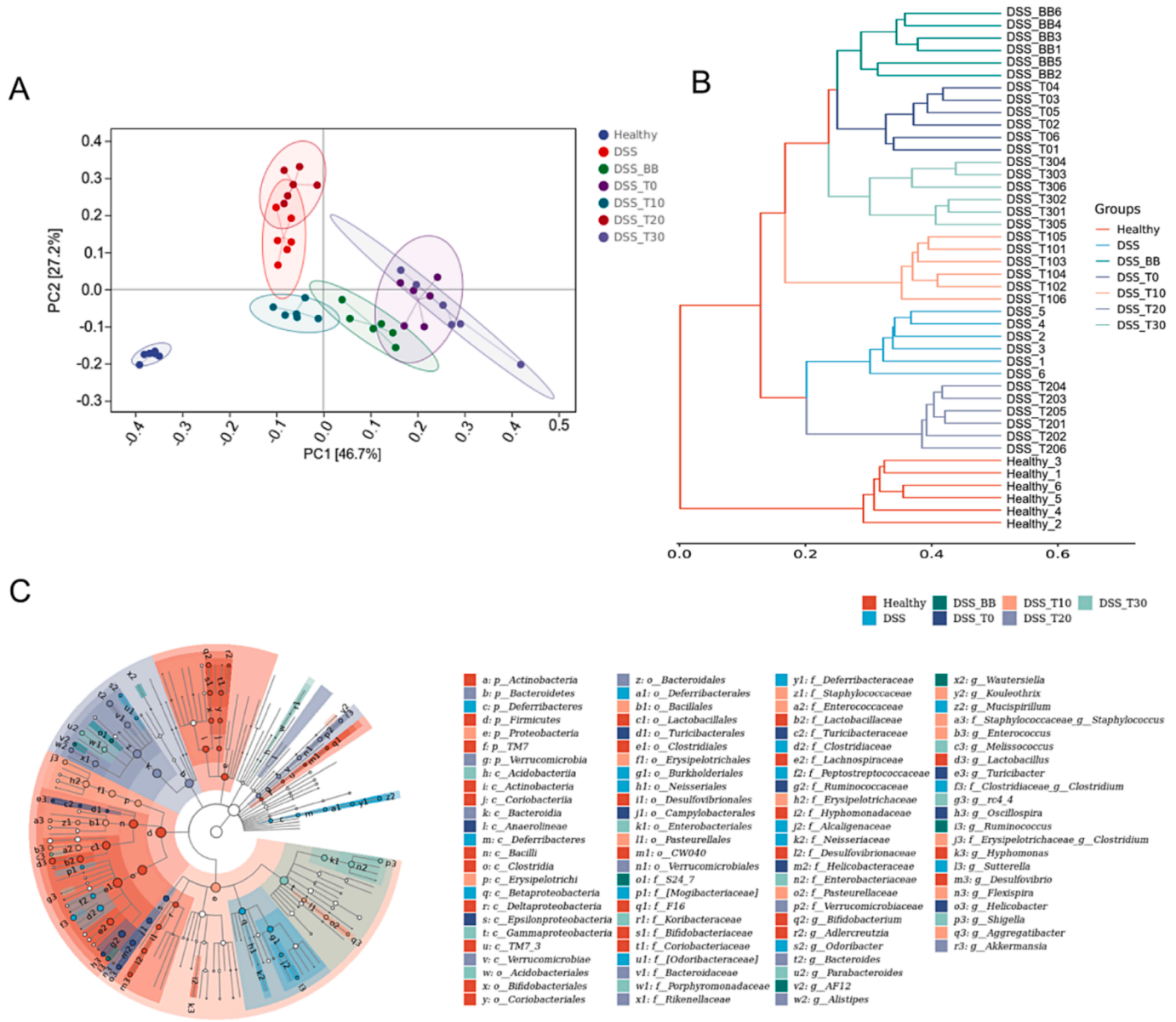

Fig. 3. AOT supplementation modulates the gut microbiota in DSS-induced colitis mice. (A) PCA analysis diagram. (B) Hierarchical clustering among seven groups based on the Bray − Curtis distance matrix. (C) Linear discriminant analysis.

Claudin-1, ZO-1 and MUC2) and inflammation-related genes (IL-1β, IL-2, IL-6, IL-10 and TNF-α) in colon tissues was calculated using the $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference. Specific primers for the relevant target genes for this experiment are shown in Table S3.

Gut microbiota analysis

Samples of cecum contents were collected from each group of mice, and DNA was extracted using the OMEGA Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA). After amplification of the samples in the highly variable V3-V4 region of the 16S rRNA gene, the PCR products were extracted and purified for quantification (338F (5′-ACTCCTACGG-GAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTA AT-3′)). Finally, sequencing of pooled libraries was performed with the Illumina NovaSeq platform using a paired-end sequencing strategy and the free online Personilbio cloud platform (https://www.genescloud.cn) for further processing.

Metabolomic profiling of cecal content samples

After the preparation of cecal content samples using a cold methanol/acetonitrile/water (2:2:1, v/v) extraction solvent, vortex mix. Ultrasonicate at low temperature for 30 min, let stand at −20 °C for 10 min, centrifuge at 14,000 g for 20 min at 4 °C, vacuum dry the supernatant, add 100 μL of aqueous acetonitrile and redissolve, take the supernatant for analysis. The prepared samples were separated on a UHPLC system with a HILIC (Waters UPLC BEH Amide column, 2.1 mm × 100 mm, 1.7 μm) and a C18 Waters UPLC BEH (C18-2.1x100 mm, 1.7 μm) column. The HILIC separation procedure was carried out by starting a gradient at a flow rate of 300 μL/min with an injection volume of 2 μL and a column temperature of 35 °C. The mobile phases were A: 90 % H2O + 2 mM ammonium formate + 10 % acetonitrile and B: 0.4 % formic acid in methanol, respectively. Mass spectrometry was performed on an AB 6500 QTRAP mass spectrometer (AB SCIEX) for mass spectrometry analysis. ESI positive/negative source condition parameter: Source temperature: 580℃; Ion Source Gas1 (GS1): 45; Ion Source Gas2 (GS2): 60; Curtain Gas (CUR): 35; IonSpray Voltage (IS): +4500 V/-

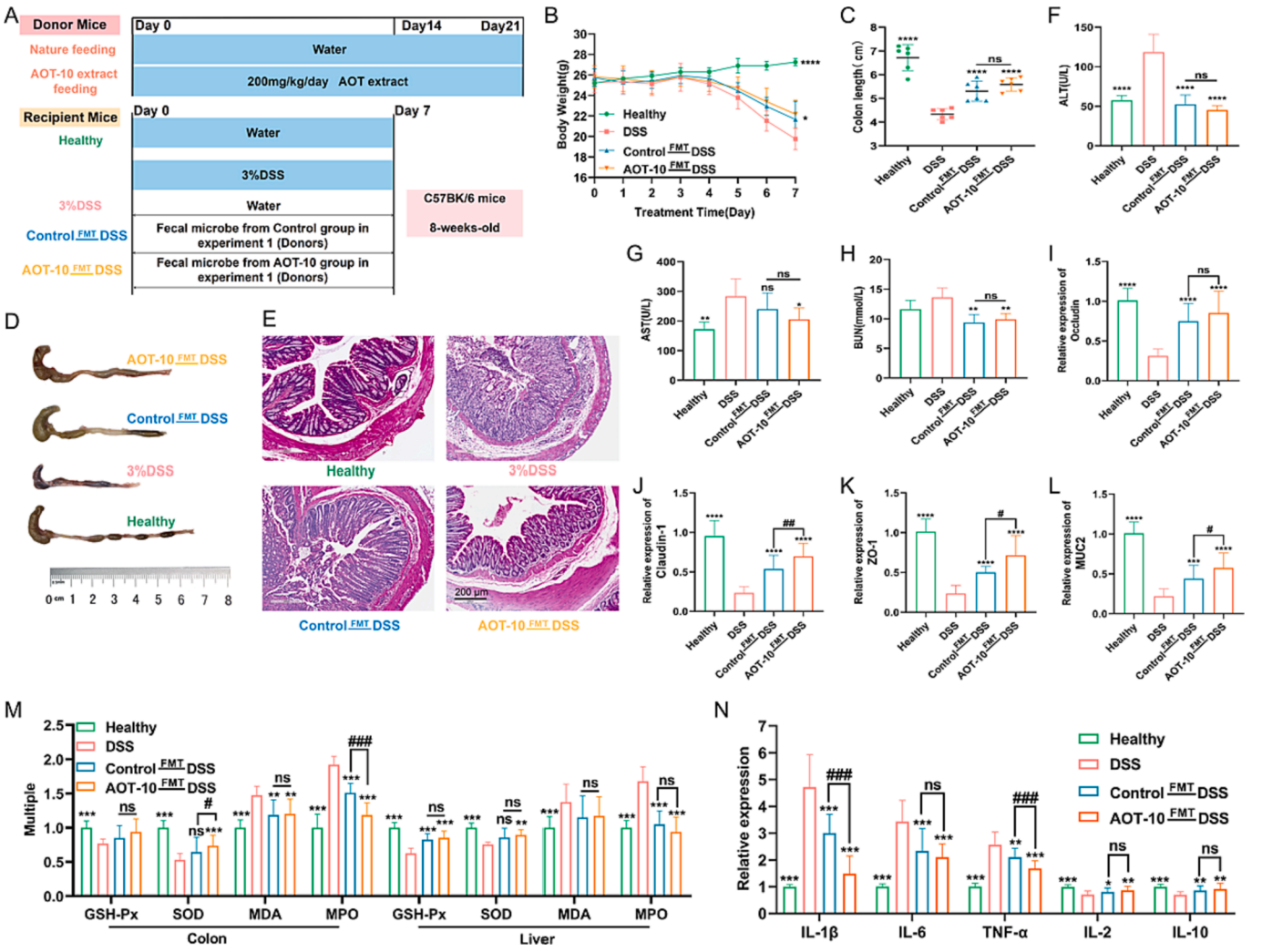

Fig. 4. FMT treatment ameliorates DSS-induced colitis in mice. (A) Schematic diagram of the FMT experimental design. (B) Body weight. (C) Colon length. (D) Representative image of colonic tissue. (E) Representative H&E staining sections of the colon (scale bars, 200 μm). Serum biological indicators of mice in each group during the FMT experimental period (F) ALT, (G) AST, and (H) BUN. (I-L) Colonic tissue mRNA levels of Occludin, Claudin-1, ZO-1, and MUC2. (M) GSH-Px, SOD, MDA and MPO level activities of the colon tissue and liver tissue were measured compared with those in the Healthy group. (N) Colonic tissue mRNA levels of IL-1β, IL-6, TNF-α, IL-2, and IL-10. ($n = 6$ per group). Data are mean ± SD. Data differences between two groups were compared using Student's *t*-test (two-tailed test), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ vs the DSS group. $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$, and $\#\#\#\#p < 0.0001$ vs the Control ^{FMT} DSS group.

4500 V. MultiQuant or Analyst was used to quantify the data measured on the test samples. Finally, data processing and the search for differential metabolites were performed as required for the experiment.

Statistical analysis

The results are represented as the mean ± standard deviation (SD). Bar graphs were created using GraphPad Prism 8.0 (GraphPad Software, San Diego, USA). An analysis of variance was performed on multiple data sets, and $p < 0.05$ was assessed as statistically significant and labeled with different lowercase letters. In the fecal microbiota transplantation experiment, the Student's *t*-test (two-tailed test) was used to compare data differences between two different treatment groups, and significance between groups was indicated by $\#/^{*}p < 0.05$, $\#\#/^{**}p < 0.01$, $\#\#\#/^{***}p < 0.001$, $\#\#\#\#/^{****}p < 0.0001$, or ns = not significant.

Results

Effects of long-term AOT intake on the physiological status and gut microbiota of mice

As shown in Fig. S1B and C, there was no significant difference in the length of the colons of the mice among each treatment group, which indicates that long-term drinking of AOT did not have a negative effect on the colon length of mice. Compared with the Control group, AOT did not significantly affect serum parameters, except for a remarkable decreased in LDL-C and BUN (Fig. S1D – H). Based on H&E staining (Fig. S1I and J), these groups did not show evidence of impaired histopathology, proving that the continuous consumption of AOT for 14 days is not toxic to mice.

There was no significant difference in the α-diversity index between the AOT-treated groups, compared with the control group (Fig. 1A – C). However, according to the results of PCA, a different microbiota profile was observed between the AOT-treated and control groups (Fig. 1D). The relative abundance of microbiota was regulated by AOT at the phylum, genus and species level. (Fig. 1E – G). According to the relative abundance it can be found that AOT showed an enrichment effect on

some of the beneficial bacteria (Fig. S2A – D) and there was some variation in the Firmicutes/Bacteroidetes (F/B) ratio (Fig. S2E). To further clarify the modulatory effect of tea extracts on the intestinal microbiota of mice, we performed a correlation analysis between the functional components of tea detected and the intestinal microbiota (Fig. 1H). Polyphenols are known to be therapeutically effective in IBD, and catechins were negatively correlated with the relative abundances of *Desulfovibrio*, *Bacteroides*, *Sutterella* and *Turicibacter*. Total flavonoids also increased the relative abundance of *Prevotella*. Total theaflavin and its four monomeric forms were negatively correlated with *Desulfovibrio* and *Sutterella*. Further analysis showed that during storage, catechins undergo oxidative polymerization reactions to produce polyphenols with anti-inflammatory and antioxidant effects, where polyphenols such as EGCG undergo oxidative polymerization reactions to form theaflavins, which were reflected in our phytochemical composition, and the theaflavins in the aged oolong tea (AOT-10) group were all higher than those in the other two storage periods of aged oolong tea (AOT-20 and AOT-30) and non-aged oolong tea (AOT-0). Thus, the above results suggest that the biological functions of tea chemical components have a potentially beneficial effect on the homeostasis of the gut microbiota. While inhibiting the associated harmful bacteria, they exhibit enrichment effects on some beneficial bacteria.

Effect of AOT on inflammation and intestinal barrier of DSS-induced colitis mice

Colon length, weight change and the DAI score are important indicators of the severity of intestinal inflammation. As shown in Fig. 2B, the DSS-treated mice showed significant weight loss from 5 to 7 days compared with that of the control group, whereas AOT alleviated this loss. In addition, AOT effectively decreased the DAI score (Fig. 2C) and recovered colon length (Fig. 2D and Fig. S3). In serum samples, the levels of AST and ALT were significantly higher in DSS-treated mice than those in the healthy group, whereas levels decreased significantly after treatment with BB and AOT, bringing them close to normal levels (Fig. 2E – G).

The mRNA expression levels of tight junction (TJ) proteins, including Occludin, Claudin-1, ZO-1, and MUC2 were detected in colon tissues by RT–PCR (Fig. 2H – K). The results showed that DSS treatment significantly downregulated the mRNA expression levels of the above genes compared with those of the healthy group, whereas restored their expressions to a certain extent. Taken together, these data presented suggest that AOT can promote the restoration of intestinal barrier function by regulating the expression of relevant genes, thus achieving the effect of promoting the restoration of intestinal barrier function.

Effect of AOT on oxidative inflammation and gut microbiota in mice with DSS-induced colitis

Further detection and analysis of inflammation-related genes revealed that the induction of DSS resulted in abnormal expression of inflammatory genes in mouse colonic tissue. However, after treatment, we found that the abnormal expression of inflammatory factors was reversed in the AOT and DSS_BB groups (Fig. 2L – O). Furthermore, AOT was administered to a DSS-induced C57BL/6 mouse model, and GSH-Px, MDA, SOD and MPO activities were measured in colon and liver tissues. The results showed that GSH-Px and SOD activities were significantly lower and MDA and MPO activities were significantly higher in the liver and colon tissues of DSS-induced mice, whereas all other groups were able to reverse the imbalance of the above four oxidative stress factors (Fig. 2P).

To determine the impact of AOT on the gut microbiota of DSS – induced colitis mice, 16S rRNA sequencing was used to analyze the differences in gut microbiota in each experimental group. The results of the PCA analysis are shown in Fig. 3A. DSS treatment led to a deviation in the microbiota compared with that of the healthy mice, whereas AOT treatment alleviated this trend. However, cluster tree analysis also suggested distinct microbiome profiles among these groups (Fig. 3B), which is consistent with the results of the PCA. These microbial alterations were also evidenced by a cladogram obtained via linear discriminant analysis effect size (LEfSe) (Fig. 3C). In our experimental results, long-term consumption of AOT and continuous administration during DSS inflammation not only reversed the tendency for enrichment of harmful bacteria, it also regulated the composition of the gut microbiota. The differences in microbiota further suggest that there are differences in the effects of different storage periods of AOT on the intestinal microbiota of mice with DSS-induced colitis. Specifically, DSS_T0 and DSS_T10 increased the abundance of *Oscillospira*, and DSS_T10 and DSS_T30 increased the abundance of *Ruminococcus* and *Coprococcus*. In addition, the PCA results showed that the AOT intervention caused a change in the structure of the gut microbiota of the mice towards the Control group, especially in the DSS_T10-treated group. However, it remains unclear whether the relief of colitis by AOT is accomplished by affecting the structure of the intestinal microbiota as well as its metabolic activity. The combined results of the above trials can show that the adverse effects associated with DSS were mitigated to some extent by the intervention of AOT stored for 10 to 30 years, but the effect of mitigation varied, with the DSS_T10 group outperforming the DSS_T20 and DSS_T30 groups.

Effect of FMT on pathological symptoms in DSS-induced colitis mice

Cumulative studies have shown that FMT is a novel therapy for UC that can alleviate gut barrier injury by altering the composition of intestinal microbiota (Cheng et al., 2018). In our previous study, AOT-10 was found to significantly enrich normal mice with *Akkermansia*. Furthermore, different storage periods of AOT, especially AOT-10, ameliorated both colonic inflammation and gut microbiota disorders in mice. In view of this, to further confirm whether the storage period of AOT could ameliorate DSS-induced colitis by remodeling the intestinal microbiota, we colonized the fecal microbiota from donor mice in the long-time AOT-10-treated and the Control group into DSS-induced colitis mice, respectively. The body weight, colon length, and pathological features were then measured. As expected, severe colitis was exhibited in the DSS group as indicated by significant weight loss, diarrhea, and overt blood in the stool from Day 5 (Fig. 4B). Compared with the DSS group, FMT treatment significantly improved the symptoms of colitis in mice. FMT treatment decreased DSS-induced shortening of the colon (Fig. 4C), whereas increased length of the colon was negatively correlated with colitis in mice. Representative images of the colon for each group are presented in Fig. 4D. Furthermore, the pathological features of the colon were evaluated by H&E staining (Fig. 4E and Fig. S4). We noticed no inflammation in the colon of the healthy group, whereas the intestinal tract of the DSS-treated group exhibited severe damage as evidenced by the pronounced decrease in goblet cells, the disruption of colon crypts and the infiltration of inflammatory cells. The abnormal elevation of serum indicators was restored after FMT treatment compared to the DSS group (Fig. 4F – H). In conclusion, we can confirm that FMT greatly improved the characterization of intestinal inflammation in UC mice.

Effect of FMT on colonic barrier function and inflammatory immunity in DSS-induced colitis mice

To further investigate whether FMT treatment has a positive effect on intestinal barrier integrity and oxidative stress levels in recipient mice, tight junction proteins (Occludin, Claudin-1, ZO-1 and MUC2) expression in the colonic tissue of each experimental mouse was measured by RT–PCR (Fig. 4I – L). The expression levels of Occludin, Claudin-1, ZO-1 and MUC2 were significantly higher in both the AOT-10 $\underline{^{FMT}}$ DSS and the Control $\underline{^{FMT}}$ DSS groups than those in the DSS group, whereas the expression levels of claudin-1, ZO-1 and MUC2 were

significantly higher in the AOT-10 $\underline{^{FMT}}$ DSS group than those in the Control $\underline{^{FMT}}$ DSS group. The abnormal expression of oxidative stress factors is an important signal in the pathogenesis of UC, which is mainly manifested as an imbalance of redox reactions in the body (Rana et al., 2014). The colon and liver results clearly demonstrated that DSS caused significant changes in oxidative stress capacity, resulting in a significant decrease in GSH-Px and SOD contents, and a significant increase in MDA and MPO contents, these trends were reversed by the AOT-10 $\underline{^{FMT}}$ DSS and Control $\underline{^{FMT}}$ DSS treatments (Fig. 4M). As can be seen, the AOT-10 $\underline{^{FMT}}$ DSS was more effective than the Control $\underline{^{FMT}}$ DSS treatment, which was mainly realized by the higher GSH and SOD contents and the lower MDA and MPO contents of the AOT-10 $\underline{^{FMT}}$ DSS group compared with levels of the Control $\underline{^{FMT}}$ DSS. Taken together, these results proved that AOT-10 $\underline{^{FMT}}$ DSS treatment can effectively reverse intestinal barrier damage and the imbalance in the oxidative stress response caused by DSS. The anti-inflammatory effect of FMT treatment on DSS-induced colitis mice was further investigated, and the relative expression of inflammatory factors at the mRNA level was similarly determined. The results revealed that the abnormal expression of inflammatory factors was alleviated by FMT, and the levels of IL-2 and IL-10 in mice treated with AOT-10 $\underline{^{FMT}}$ DSS even exceeded those of mice in the Control $\underline{^{FMT}}$ DSS group, although the differences were not significant (Fig. 4N).

Effect of FMT on the imbalanced gut microbiota of DSS-induced colitis mice

The results showed that the Control $\underline{^{FMT}}$ DSS and AOT-10 $\underline{^{FMT}}$ DSS treatment did not significantly change the Chao1, Simpson and Shannon index (Fig. 5A). As shown in Fig. 5B, a distinct microbiome profile was found among the four groups based on the PCA score plot. The relative abundance of Firmicutes decreased in the gut microbiota of DSS-treated mice compared with that of the healthy group. However, FMT treatment altered the above adverse alterations, showing demonstrated a microbiome similar to that of the healthy group. FMT treatment reduced the relative abundances of Proteobacteria, Deferribacteres, and Actinobacteria in comparison with DSS treatment (Fig. 5C). Furthermore, the F/B ratio was also increased (Fig. 5D).

As shown in Fig. 5E, harmful bacteria in particular, including the genera *Mucispirillum*, *Sutterella*, *Allobaculum* and *Clostridiaceae_Clostridium* were significantly increased in the DSS-treated group. An *Allobaculum* strain isolated from patients with IBD exacerbates colitis in mice (Rice et al., 2022). *Mucispirillum*, which inhibits the colonic mucosal layer, was identified by researchers as a bacterial marker for potential use in inflammatory colitis (Herp et al., 2019). *Sutterella* and *Clostridiaceae_Clostridium* are also harmful bacterial species directly associated with IBD (Monaghan et al., 2019). In our study, AOT-10 $\underline{^{FMT}}$ DSS significantly reversed the trend of increasing abundance of harmful microorganisms caused by DSS, and the abundance of these organisms in the AOT-10 $\underline{^{FMT}}$ DSS group was lower than that of the Control $\underline{^{FMT}}$ DSS. Additionally, FMT treatment significantly reversed the trend of reduced relative abundance of *Oscillospira*, *Ruminococcus* and *Coprococcus* in the DSS group.

To further clarify the efficacy of donor mouse gut microbiota transplantation on colitis in recipient mice, we investigated the relationships among oxidative stress indicators, inflammatory factors, tight junction protein-related genes and the gut microbiota at the genus and phylum levels. The correlation heatmap of the above indices is shown in Fig. S5. Beneficial bacteria (e.g., *Prevotella* and *Ruminococcus*) were significantly positively correlated with the relative expression levels of IL-2, IL-10, SOD, and tight junction proteins-related genes and negatively correlated with the relative expression levels of the proinflammatory factors and the activities of MPO and MDA. In addition, the potentially harmful bacteria *Sutterella* and *Allobaculum* were significantly positively correlated with the proinflammatory factors, MPO and MDA and negatively correlated with SOD and tight junction proteins. Collectively, these results again visualise that the balance of the gut microbiota is closely linked to inflammation in vivo. The gut microbiota of colitis mice is remodelled by fresh faecal bacteria from FMT donor mice and, importantly, that harmful bacteria closely associated with inflammation, particularly *Sutterella*, *Clostridiaceae_Clostridium*, *Mucispirillum*, etc., were significantly suppressed, perhaps an important mechanism for the relief of colitis by AOT.

FMT modulated the microbial metabolites in DSS-induced colitis mice

The microbial metabolites of each group were detected using UHPLC-ESI-QTAP MS. The PCA score plot (Fig. S6A) and partial least squares discrimination analysis (PLS-DA) (Fig. 6A) showed distinct clustering of microbial metabolites in the healthy group, DSS group and FMT-treated groups. We used 7-fold cross-validation and 200 response permutation tests to prevent overfitting during the modeling process, and the results showed good model robustness (Fig. S6B). In this research, a total of 220 known metabolites were identified in the cecum content of mice. The major classifications and percentages of these microbial metabolites are shown in Fig. 6B. All detected metabolites were visualized in the form of a heatmap to illustrate the significant differences between the four sets of data (Fig. 6C). There were 111 significantly upregulated and 53 significantly downregulated metabolites in the DSS group compared with the healthy group. Many of the obviously upregulated metabolites were restored to some extent, bringing them down and close to normal levels, after FMT treatment (Fig. 6D – E). These results suggested that fecal microbiota from donor mice treated with AOT-10 by long-term gavage can effectively restore the microbial metabolic profile of mice with DSS-induced colitis.

FMT regulated metabolic pathways related to colitis

For microbial metabolomics studies, understanding which metabolic pathways are affected by AOT-10 $\underline{^{FMT}}$ DSS that alleviates colitis should be a priority. The detected microbial metabolites were used for pathway analysis using KEGG topological analysis, and 24 enriched pathways were found among the four experimental groups. As shown in Fig. S7 and Fig. 6F, the dominant altered pathways were related to glycine, serine and threonine metabolism; biosynthesis of amino acids; protein digestion and absorption; fatty acid biosynthesis; the citrate cycle (TCA cycle); linoleic acid metabolism; central carbon metabolism in cancer; the glucagon signaling pathway and ABC transporters. We noted some interesting metabolites in the FMT treatment. A p-value of < 0.05 was used as a screening criterion for differential metabolites. An in-depth study of the different metabolites in each group revealed that the amino acid metabolites were more significantly affected during DSS-induced colitis. Disturbances or imbalances in the intestinal microbiota caused by intestinal inflammation and shortening of the colonic length can affect amino acid absorption, and our study verified this conclusion. The changes in several pathways associated with amino acid metabolites were also confirmed by KEGG enrichment analysis. The gamma-aminobutyric acid (PubChem CID: 119), glycine (PubChem CID: 750), isoleucine (PubChem CID: 6306), phenylalanine (PubChem CID: 6140), proline (PubChem CID: 145742), serine (PubChem CID: 5951), tyrosine (PubChem CID: 657) and valine (PubChem CID: 6287) were enriched in the DSS group, and these amino acid metabolites were reduced to near normal levels after FMT treatment. A consistent trend was observed for ornithine (PubChem CID: 6262), citrulline (PubChem CID: 9750) and glutamine (PubChem CID: 5961) in the arginine biosynthetic pathway. Unsurprisingly, both saturated and unsaturated fatty acids, including palmitic acid (PubChem CID: 985), linoleic acid (PubChem CID: 5280450), myristic acid (PubChem CID: 11005), oleic acid (PubChem CID: 445639), stearic acid (PubChem CID: 5281), and arachidonic acid (PubChem CID: 444899), were enriched in the DSS group. Higher levels of arachidonic and linoleic acids in the colonic mucosa of CD patients compared to those than in healthy subjects have been revealed in previous reports (Bühner et al., 1994), which is

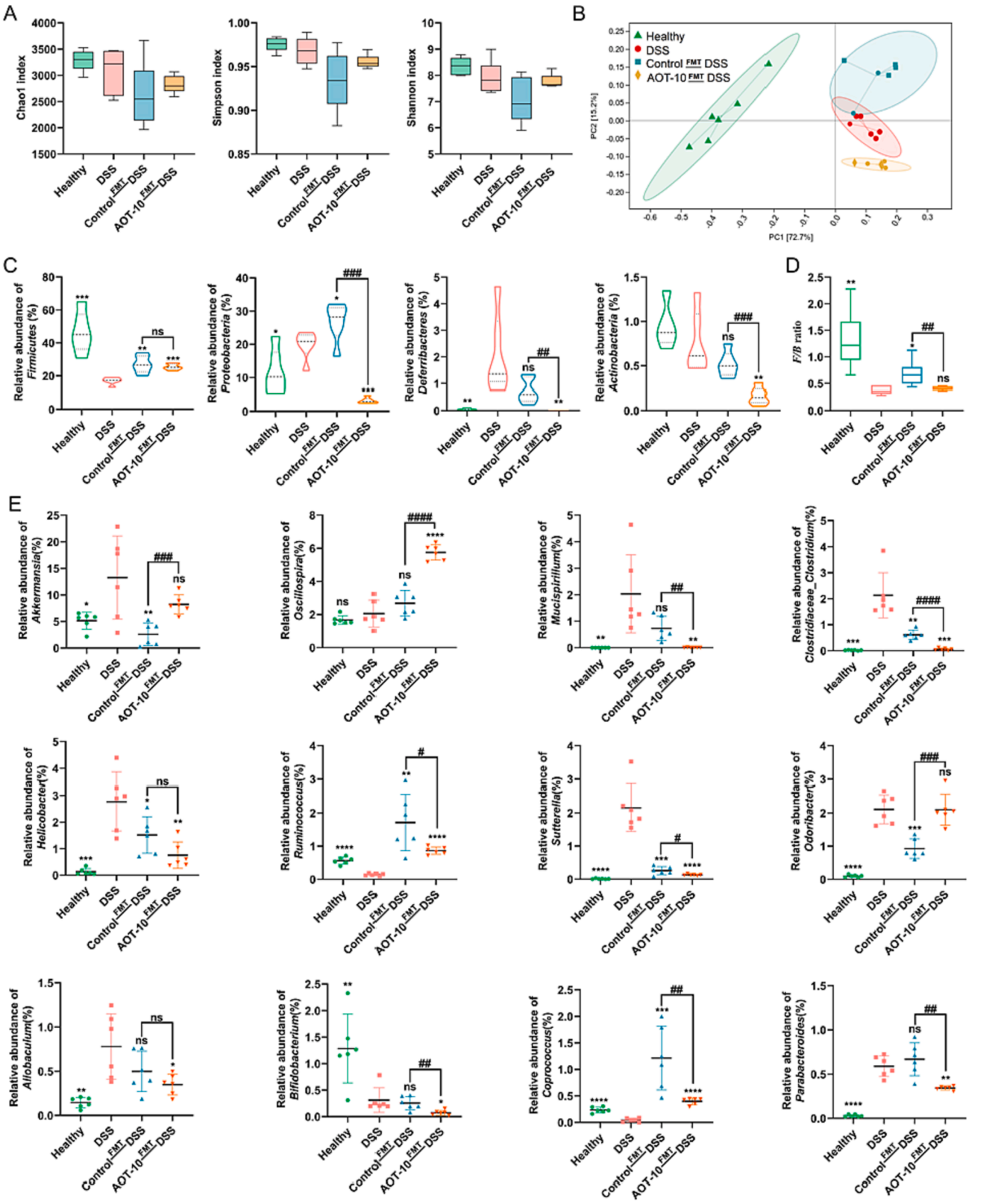

Fig. 5. FMT treatment modulates the composition of the intestinal microbiota. (A) α-diversity is represented by the box diagram of the Chao1 index, Simpson index, and Shannon index. (B) Principal component analysis. (C) The classification on the phylum level. (D) The *Firmicutes/Bacteroidetes* ratio of all groups. (E) Gut microbiota composition among experimental groups at the genus level. Data presented as means ± SD. Data differences between two groups were compared using Student's *t*-test (two-tailed test), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ vs the DSS group. $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$, and $\#\#\#\#p < 0.0001$ vs the Control $\underline{\text{FMT}}$ DSS group.

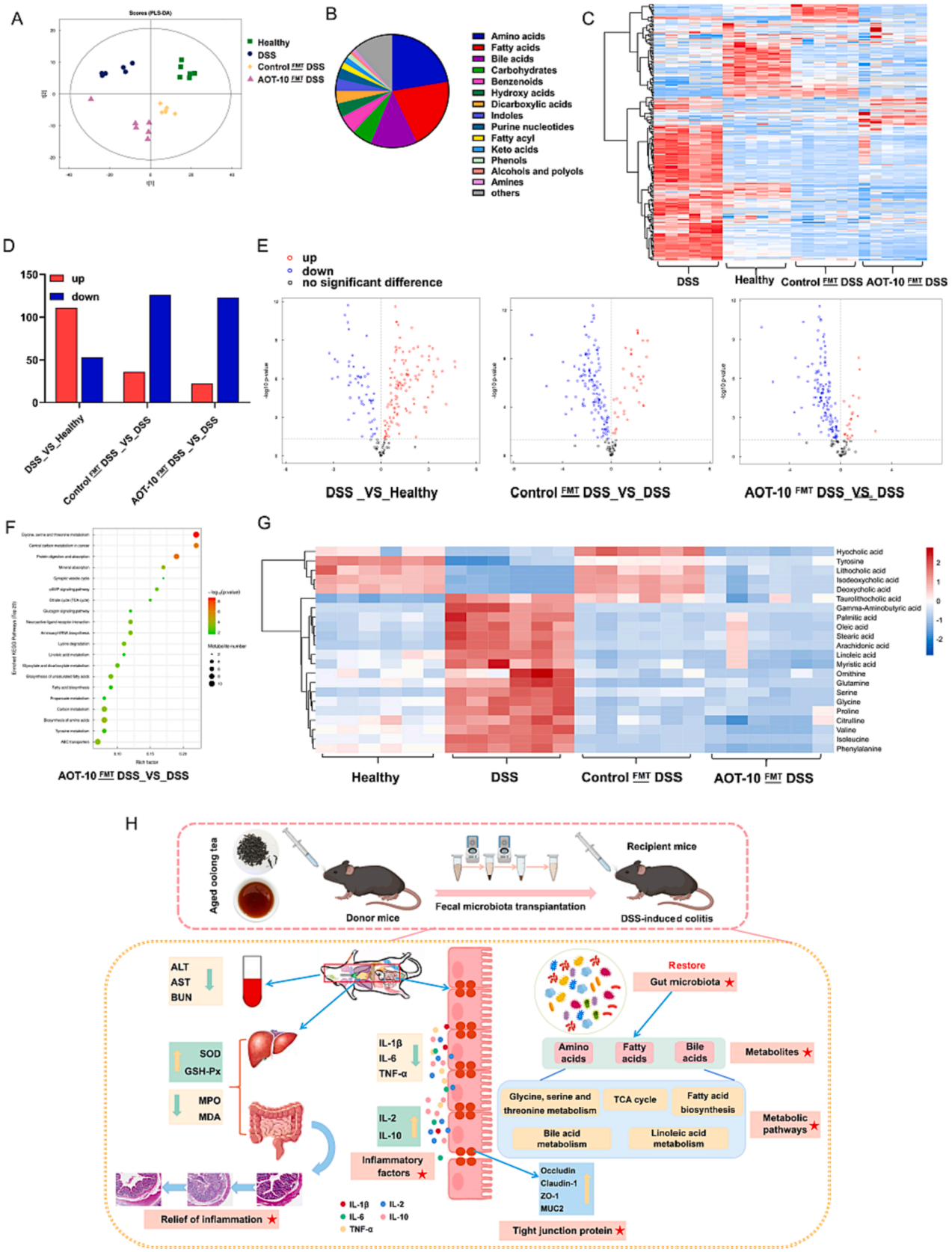

Fig. 6. FMT treatment modulates microbial metabolites in DSS-induced colitis mice. (A) PLS-DA score plots among four experimental groups. (B) Pie chart showing the percentage of each type of microbial metabolites. (C) Heatmap of contents of microbial metabolites after FMT treatment compared with the Healthy group. (D) Column chart showing the modulatory effect of FMT treatment on microbial metabolites of DSS-induced colitis mice. (E) volcano plot showing the differential variables between the groups. (F) KEGG enrichment pathway analysis. (G) Differential microbial metabolites in KEGG enrichment analysis. (H) Overview graph of AOT-10 for relief of colitis by FMT technology.

consistent with the results we obtained. In addition, metabolites associated with the primary bile acid biosynthetic pathway were regulated (Fig. 6G). Together, the level of metabolism of cecal contents in colitis mice was significantly modulated by fecal bacteria from AOT-10-treated donor mice.

To better explore the relationships between gut microbiota, differentially expressed microbial metabolites and inflammatory indicators, a correlation analysis of the above indicators was obtained, and the results are graphically represented in Fig. S8. First, the relationship between the gut microbiota and its metabolites was first investigated. The abundance of Firmcutes, *Coprococcus*, *Ruminococcus*, *Lactobacillus* and other beneficial bacteria were negatively correlated with the levels of amino acid metabolites and fatty acid metabolites and positively correlated with the levels of bile acid metabolites. Furthermore, the abundance of *Clostridiaceae_Clostridium*, *Mucispirillum*, *Helicobacter*, *Odoribacter*, and other intestinal microorganisms that promote the development of inflammation were positively correlated with the levels of amino acid metabolites and fatty acid metabolites and negatively correlated with the levels of bile acid metabolites. This finding is consistent with our previous results, which showed that the abundance of intestinal bacteria that promote inflammation was significantly increased, and the metabolites of amino acids and fatty acids in feces were significantly upregulated after DSS treatment. Regarding the relationship between differentially expressed metabolites and inflammatory indicators, almost all differential metabolites of amino acids and fatty acids were antagonistically associated with IL-2, IL-10 and SOD, but positively correlated with MPO activity and pro-inflammatory factors (IL-1β, IL-6, and TNF-α). Amino acid metabolites and fatty acid metabolites were negatively correlated with tight junction proteins, whereas the opposite was true for bile acid metabolites. As mentioned above, changes in the gut microbiota and its metabolites may further explain how AOT ameliorates DSS-induced colitis in mice.

Discussion

Currently, as a chronic intestinal inflammatory disease, UC cannot be cured (M. Li et al., 2021). Commonly used therapeutic drugs cause numerous side effects and adverse reactions in the human body. There is increasing interest in plant extracts and evidence for their use as a substitute for chemical drugs in the treatment of UC. Several types of tea extracts have been validated by FMT for their efficacy in alleviating colitis by remodeling the gut microbiota (Hu et al., 2021; Y. Liu et al., 2020). However, it remains unclear how the AOT-regulated gut microbiota plays a role in inflammation, and the underlying mechanism has been little studied. In this study, our results show that long-term intake of AOT produces prebiotic benefits in the intestinal microbiota of mice. In addition, FMT treatment further demonstrated that the gut microbiota and its metabolites were improved by AOT and play an extremely important role in the treatment of DSS-induced colitis.

Long-term intake of AOT exerts its effects in alleviating colitis by modulating the intestinal microbiota, alleviating inflammation-related gene expression, improving serum and oxidative stress abnormalities and intestinal barrier function, and the performance of AOT in terms of anti-inflammation varies between storage periods. The efficacy of AOT in terms of anti-inflammatory properties also varies between different storage periods. Tea has been shown to have potential as a dietary ingredient, with several studies demonstrating that daily moderate consumption of tea can potentially improve immunity and lower the risk of IBD (Ng et al., 2015). Intestinal bacteria break down substances in tea that cannot be directly absorbed into simpler compounds, which may regulate the structure and abundance of the gut microbiota (Chen Liu et al., 2023; Wu et al., 2021). Our findings suggested that a prebiotic effect of storing AOT for different periods on the gut microbiota compared with that of the normal group. The preventive effect of long-term administration of AOT on DSS-induced colitis was further validated using a simulation of approximately 5–8 g of tea per day for an adult (Nair & Jacob, 2016). The dysregulation of inflammatory cytokines and oxidative stress are two important signals of colon damage during the development of IBD (Hu et al., 2021). The intake of AOT was capable of increasing the levels of GSH-Px and SOD and decreasing the levels of MPO and MDA in the colon and liver tissues, which promoted the restoration of antioxidant status to near-normal levels. Numerous studies have shown that the active ingredients in tea can reduce the expression of inflammatory factors and enhance intestinal barrier function in the treatment of colitis (Hu et al., 2021; Wu et al., 2021). Based on previous reports, in this study we discovered the in vitro anti-inflammatory effects of AOT by establishing an LPS-induced inflammation model using RAW264.7 cells prior to experiments in mice (Fig. S9). The results of our in vitro cell assay and colitis study are consistent with previous studies of tea for the relief of colitis symptoms. AOT intake also altered the community structure of the intestinal microbiota in mice with DSS-induced colitis. Further comprehensive analysis of the results of inflammatory factors, tight junction proteins, oxidative stress factors and gut microbiota showed that AOT from different storage periods provided different degrees of relief in DSS-induced colitis mice. This is an intriguing finding, as the phytochemical makeup of AOT varies according to storage duration (Hong et al., 2021; Peng et al., 2022). The phytoactive components of AOT were analyzed at four different storage periods. The tea's chemical components experienced complex transformations during storage, and the total amount of catechins declined with increasing storage time, particularly polyphenols like EGCG, which is recognized as the primary anti-inflammatory and antioxidant. However, in the present study, the anti-inflammatory and antioxidant effects of AOT stored for a certain period of time did not decrease significantly. It is presumed that the oxidative polymerization of polyphenols occurred during storage, and the oxidative polymerization products also have good anti-inflammatory and antioxidant functions. In addition, the total theaflavins were significantly higher in the AOT-10 group than those in the other groups. The significant biological effects of the four theaflavin monomers on inflammation and maintenance of intestinal homeostasis in vivo were also demonstrated by Liu et al (Changwei Liu et al., 2022). Polysaccharides and flavonoids have been shown to alleviate colitis by inhibiting the expression of inflammatory factors and modulating the gut microbiota (F. Huang et al., 2016). For AOT stored for 20 to 30 years, the comparison revealed that the complex and diverse water-soluble polysaccharides and flavonoids in AOT tended to increase year by year as the storage time increased, which is consistent with the study of Hong (Hong et al., 2021) *et al.* and Peng (Peng et al., 2022) *et al.* Various flavonoids have been shown to regulate the integrity of the intestinal TJ barrier (Noda, Tanabe, & Suzuki, 2012). Therefore, we speculate that the release of more water-soluble sugar substances and flavonoid content during storage are important effective anti-inflammatory components of AOT-20 and AOT-30 in relieving colitis. However, compared with AOT-0 and AOT-10, the anti-inflammatory effects of AOT-20 and AOT-30 were slightly reduced, probably due to the dramatic transformation of polyphenolic substances such as catechins during prolonged storage, and the anti-inflammatory benefits brought by water-soluble polysaccharides and flavonoids could not fully offset the negative effects of reduced catechins. Taken together, the AOT-10 group was more effective in relieving colitis than AOT-20 and AOT-30.

FMT treatment with fresh faeces from AOT donor mice can reshape the gut microbiota of mice with colitis and thus exert anti-inflammatory effects. Numerous studies on the treatment of colitis with FMT have shown that the fecal microbiota provided by healthy volunteers can improve the manifestation of inflammation. (Tian et al., 2019). In our study, fresh fecal microbiota from control and AOT-10 donor mice were transplanted into colitis mice for 7 days. Interestingly, the results showed that the effects obtained with the FMT technique were better than those obtained by directly administering the AOT extract to mice suffering from colitis. We also found that AOT-10 $\overset{\text{FMT}}{\text{DSS}}$ alleviated various symptoms of colitis better than Control $\underline{\overset{\text{FMT}}{\text{DSS}}}$, including

restoring oxidative stress levels, regulating the expression of inflammatory cytokines, upregulating tight junction protein expression, and reversing the gut microbiota disorder caused by DSS. Specifically, the expression of pro-inflammatory cytokines was clearly reduced. The expression of relevant inflammatory factors mediated by the gut microbiota was also reflected in H&E-stained sections of colonic tissue, and the results showed that FMT significantly restored DSS-induced colitis, mass inflammatory cell infiltration, crypt structure destruction, and goblet cell reduction compared with the DSS model group. The interaction between the gut microbiota and the host intestinal immune system, and indeed the systemic immune system, is critical in the treatment of inflammation and the maintenance of intestinal homeostasis, and evidence suggests that long-term inflammation selects for bacterial groups in the gut that are suitable for survival under oxidative stress conditions, thereby misaligning the gut microbiota, which in turn exacerbates colitis symptoms by influencing the expression of inflammatory factors (Wang et al., 2019). LEfSe results further showed that *Clostridiaceae_Clostridium*, *Helicobacter*, *Parabacteroides*, *Sutterella*, and *Allobaculum* were mainly enriched in the DSS group, and they were the hallmark microorganisms in the development and progression of colitis. In our FMT experiments, inoculation with AOT-10 (i.e., fresh fecal bacteria from mice in the AOT-10 group) significantly decreased the abundance of these harmful microorganisms and increased the abundance of *Oscillospira*, *Ruminococcus* and *Coprococcus*, and the reversal effect was significantly better than that of Control $\overline{\text{FMT}}$ DSS. Notably, Studies have shown that *Ruminococcus* is involved in carbohydrate metabolism and *Oscillospira* is capable of producing short-chain fatty acids (SCFAs) such as butyrate, which are microbial markers of intestinal health and play an important role in stabilizing the intestinal barrier, reversing diarrhea, and suppressing immune responses (Konikoff & Gophna, 2016). The enrichment of beneficial bacteria has a role in maintaining the stability of the intestinal barrier, which further explains how AOT treatment ameliorates the development of colitis in mice. Subsequently, we conducted a correlation analysis between inflammatory related factors and the gut microbiota, and the results showed that beneficial bacteria such as Firmicutes, *Lactobacillus* and *Ruminococcus* were negatively correlated with proinflammatory factors and MPO and MDA activities but positively correlated with IL-2, IL-10 and SOD activity. These results suggest that AOT-induced changes in the composition and abundance of the gut microbiota alter the immune response induced by DSS (Hu et al., 2021). Significant inhibition of potentially harmful bacteria (e.g., *Sutterella*), and improvements in bacteria associated with protection of the intestinal barrier and modulation of inflammatory factors may be important potential targets for the relief of colitis.

Metabolites from the gut microbiota of mice were systematically analysed based on a metabolomics platform to explore the differences in metabolites following FMT treatment and to further understand the interconnections with the UC host microbiota. Alterations in the gut microbiota may alter associated metabolites and regulate metabolic pathways, with mutual adaptations between bacteria and metabolites working together to maintain immune homeostasis and colonic mucosal health (Smith et al., 2013). The results showed that DSS exposure significantly altered the gut microbiota metabolite profile, further inducing dysfunctional metabolic pathways. The significant increase in amino acid metabolite content after DSS treatment may be related to the shortened colonic length and disrupted intestinal epithelial tissue due to intestinal inflammation (Jansson et al., 2009). These results suggest that special consideration should be given to the amino acid balance in the colon in terms of relieving inflammation. Interestingly, our results showed the feces of mice in the DSS-treated group had significantly increased tryptophan levels in addition to being enriched for most amino acid metabolites. KEGG enrichment analysis revealed that these differential metabolites were mainly associated with glycine, serine and threonine metabolism, protein digestion and absorption, and biosynthesis of amino acids. After FMT treatment, the content of amino acid metabolites in the feces of DSS-induced colitis mice was significantly reduced to close to normal levels. In terms of fatty acid metabolites, FMT treatment reduced the levels of linoleic acid, stearic acid, and palmitic acid, indicating that FMT treatment upregulated linoleic acid metabolism and improved inflammation. Previous studies have shown that fecal levels of linoleic acid are higher in colorectal cancer patients than those in healthy subjects and that palmitic acid is also a signaling molecule in inflammatory conditions in the intestine (Ricchi et al., 2009). The composition of the intestinal microbiome can be regulated by bile acids (Fiorucci & Distrutti, 2015). In our study, fecal microbiota from donors significantly reversed the changes in bile acid metabolites in the cecum contents of mice with colitis. Previous studies have shown that the composition of the gut microbiome can be regulated by bile acids, as evidenced by correlation analysis of fecal microbiota and metabolites.

Conclusion

In summary, this study used FMT, microbiomics and metabolomics techniques to reveal the beneficial effects of aged oolong tea treatment in improving inflammation in the gut of colitis mice by restoring imbalances in the intestinal microbiota, altering the metabolites of the microbiota in the gut and stimulating metabolic pathways, which in turn improved barrier integrity and achieved suppression of inflammation in the gut of colitis mice (Fig. 6H). Concurrently, mice with inflamed colons that received fresh fecal microbiota from donor mice in the AOT-10 group exhibited lower levels of intestinal tissue inflammation than colitis mice that received fecal microbiota from donor mice in the Control group, further demonstrating that the anti-inflammatory effects exhibited by AOT can be transferred to colitis mice through the fecal microbiota of donor mice. On the microbial side, several key bacterial groups were identified by correlation analysis (e.g., *Sutterella*, *Clostridiaceae_Clostridium*, *Mucispirillum*, *Oscillospira* and *Ruminococcus*), which may be crucial for the treatment of colitis. Metabolomic analysis further showed that AOT had a better modulating effect on the metabolic abnormalities caused by DSS, including amino acid, fatty acid and bile acid metabolism. Therefore, aged oolong tea has great potential to become a natural beverage for the prevention and relief of colitis in the future and can play a role in the future treatment of colitis as an adjunctive therapy.

CRediT authorship contribution statement

Jun Wu: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Data curation, Conceptualization. **Xuming Deng:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Data curation, Conceptualization. **Yue Sun:** Writing – review & editing, Investigation. **Jing Li:** Writing – review & editing, Visualization. **Haomin Dai:** Writing – review & editing, Investigation. **Siyu Qi:** Investigation. **Yan Huang:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Weijiang Sun:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.101102.

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