Aged Pu-Erh Tea Reduced Oxidative Stress-Mediated Inflammation in DSS-Induced Colitis Mice by Regulating Intestinal Microbes

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Research

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Abstract

Background: Colitis is associated with gut microbiological disorders and oxidative stress-mediated intestinal inflammation. Pu-erh tea has been used as a beverage for bioactive potential in antioxidation and anti-inflammation. With increase of storage, the change of its bioactive components creates the difference of health care function. However, there is no evidence to show whether the storage period of Pu-erh tea affects its anti-inflammatory and anti-oxidant capacity in the colitis, or even intestinal protection.

Results: In this study, 3.5% DSS-induced colitis mice were treated with 10 mg/kg bw/day extracts, aged 14 years (P2006) and unaged (P2020) Pu-erh tea respectively, for 1 week. It was found that Pu-erh tea, especially P2006, inhibited the intestinal oxidative stress-mediated inflammation signaling pathway (TLR4/MyD88/ROS/p38 MAPK/NF-κB p65), up-regulated the expression of intestinal tight junction proteins (Mucin-2, zonula occludens 1, occluding), promoted M2 polarization of macrophages, and in turn, improved the intestinal immune barrier, which stemmed from the reshaping of intestinal microbiota (e.g., increased *Lachnospiraceae_NK4A136_group* and *Akkermansia* levels).

Conclusions: These results indicate that the changes of intestinal microflora caused by aged Pu-erh are the key to alleviate DSS-induced colitis, and speculate that drinking aged Pu-erh tea (10 mg/kg bw/day in mice, a human equivalent dose of 7 g/60 kg bw /day) has a practical effect on alleviating and preventing the development of intestinal inflammation.

Background

Inflammatory bowel disease (IBD),including ulcerative colitis (UC) and Crohn's disease (CD) [1], is a non-specific, chronic inflammatory disorder of the colonic mucosa, which affecting the mucosa of the whole gastrointestinal tract and is significantly correlated with increased colon cancer-specific mortality [2]. Although the etiology and key drivers of persistent mucosal inflammation of IBD are unclear [3, 4], it has been reported that imbalance of gut microbiota and inflammatory factors are the main causes [5]. Metabolites produced by microorganisms mediate the immune system and stimulate non-specific immune response [6], resulting in increased release of inflammatory cell mediators and reactive oxygen species. Intestinal crypts, goblet cells and other epithelial mucosal barrier damage were aggravated, leding to the occurrence and development of IBD [7]. Moreover, oxidative stress is one of the most important factors in the pathophysiological process of IBD [8]. Now, the current mainstays of IBD treatment is generally drug therapy, which are vedolizumab (75%), anti-tumor necrosis factor (TNF)-α agents (62.5%), and ustekinumab (43.8%) [9]. However, These have significant side effects and may not be fully controlled despite ongoing IBD treatment. Thus, it is urgent to a safer and more effective treatments to alleviate IBD.

Recently, some researches indicate that tea has a postive effect in the adjuvant treatment of colitis, especially in regulating the gut microflora and microbial metabolites [10, 11], inhibiting inflammatory

factors [12], reducing tissue oxidative stress [13]. Tea, as the world's top six healthy beverages, contains more than 700 compounds such as polyphenols (25%~35%), polysaccharides (20%~25%), protein (25%~30%), 26 kinds of amino acids, more than 50 kinds of mineral elements and other functional components [14, 15]. Studies in mice have shown that it can alleviate and improve intestinal inflammation by reducing inflammatory factors such as Interleukin-1β (IL-1β), IL-6, TNF-α [16], and down-regulate cyclooxygenase-2 (Cox-2)-mediated I-κB kinase (IKK) and nuclear factor-κB (NF-κB) transcription [17]. As a dietary supplement, several studies indicated that the intake of tea increased the abundance of probiotics (e.g. *Bifidobacterium* and *Akkermansia*), and lowered the abundance of harmful bacteria (e.g. *Bacteroides*, *Parasutterella*, and *Desulfovibrio*) in the gut [18].

As a kind of post-fermented tea, our previous work showed that Pu-erh tea was the most effective one in the adjuvant therapy of dextran sulfate sodium (DSS)-induced IBD in mice [19]. In China, Pu-erh tea is known as the "drinkable antique". With increase of storage, the change of its bioactive components creates the difference of health care function in anti-cancer, weight loss and intervention of urinary metabolism [20, 21]. However, there is no evidence to show whether the Pu-erh tea storage period affects its anti-inflammatory and anti-oxidant capacity in colitis, or even intestinal protection. The adjuvant therapeutic mechanism of aged Pu-erh tea on IBD needs to be further studied.

In this study, the influence of Pu-erh tea aging on anti-inflammation and anti-oxidant capacity in colitis was determined by evaluating the variations of Pu-erh tea in different storage periods in the adjuvant treatment of colitis. To reveal whether the above effects originate from alterations in gut microbiome-relative abundance, the fecal microbiota transplantation (FMT) was carried out.

Methods

Chemicals and reagents

Dextran Sulfate Sodium Salt (DSS, molecular weight = 36 kDa to 50 kDa) was obtained from MP Biomedicals (Irvine, CA, USA). The release of cytokines, including IL-1β, IL-6, IL -10, IL-22, TNF-α, and Lipopolysaccharide (LPS) in serum were assayed using ELISA kits, according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). Commercial kits IL-4, total bile acid (TBA), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), myeloperoxidase (MPO), macrophage-stimulating factor (M-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Murine and tea sample

Mice of the C57BL/6 J (20 ± 2 g, 7 weeks, male) were purchased from the Center of Experimental Animals at Chongqing Medical University and kept under controlled temperature (22 to 24 °C) and light (12 / 12 h light-dark cycle) conditions in SPF grade laboratory. Animal experiments were carried out in the Institutional Animal Care and Use Committee of the Southwest University (permit number, 20190003). Two different years of Pu-erh tea (produced in 2006, produced in 2020, TAETEA GROUP, Yunnan, China)

were extracted with pure water (1:15, w/v) at 75 °C for 45 min. After filtration, and pure water (1:10, w/v) was added to the residue for the second extraction [19].The filtrates were mixed and freeze-dried, followed by storage at -80 °C for later use. The samples were respectively Pu-erh tea 2006 (P2006) and Pu-erh tea 2020 (P2020).

Experimental design

In the previous experiment, (Dextran Sulfate Sodium Salt, molecular weight = 36 kDa to 50 kDa) DSS-treated mice, which received normal diet and 3.5% (w/v) DSS in drinking water, were given intragastric administration with various concentrations of P2006 (1, 10, 50, 100, 200 mg/kg bw) to confirm the best concentration. The results showed that Pu-erh tea had a better protective effect on weight loss caused by DSS at the concentration of 10 mg/kg (Figure S1). Based on the previous laboratory research results [19], 10 mg/kg was selected as the dosage of this experiment.

7-week-old mice were acclimated for 7 days and supplied with a control chow diet administered, then they were randomly divided into five groups, ten mice per group, including Control group (Control) that received normal diet and water; DSS-treated group (DSS) that received normal diet and 3.5% (w/v) DSS in drinking water, Pu-erh tea infusion group that received normal diet and 3.5% (w/v) DSS in drinking water, and administrated with 200 ul of DTE (10 mg/kg bw) by daily gavage, according to the difference of tea storage year, it could be divided into DSS + 0.1% Tea (2006) and DSS + 0.1% Tea (2020) respectively. DSS-treated group and Pu-erh tea infusion group were received 3.5% (w/v) DSS for 7 days, then replaced DSS with normal water for 4 days before the end of the trial (Figure 1A).

Accurately recording the weight, food intake, and water consumption of mice every day, and calculated the energy intake and food efficiency ratio according to formulas. At the end of these experiments, mice were fasted overnight and anesthetized with 20% urethane, Then the blood samples were collected and stored at room temperature for half an hour before centrifugation (4 ^{o} C, 5000 rpm, 10 min) to obtain the serum sample. The tissues (liver, colonic tissue, intestinal contents) were collected and pre-treated for further analysis.

Part of the colon and liver were dissected and stained with hematoxylin-eosin (H&E) for pathological analysis; Immunohistochemical (IHC) was used to analyze the corresponding protein expression (Mucin-2 (MUC2), zonula occludens 1 (ZO-1), occluding, toll-like receptor 4 (TLR4), myeloid differentiation factor 88 (MyD88), p38 mitogen-activated protein kinase (p38MAPK), nuclear factor-κB (NF-κB) p65, Kelch-like ECH-associated protein 1 (Keap1), nuclear factor E2-related factor 2 (Nrf2), heme oxygenase 1 (HO-1), NADPH oxidase 2 (NOX2) and NADPH oxidase 4 (NOX4)), and immunofluorescence (IF) was used to analyze the corresponding protein expression of reactive oxygen species (ROS), M1 and M2 type macrophages. The relative expressions of all were calculated according to the following formula using Image-Pro Plus 6.0 software:

$$\text{relative expressions} = \frac{\text{integrated optical density}}{\text{tissue area}}$$

Gut microbiota analysis

Stool samples were snap-frozen in liquid nitrogen and stored at -80 °C until further analyzed. DNA was extracted from the fecal samples using the DNA isolation kit (Omega Bio-Tek, Norcross, GA). For each fecal sample, the V3-V4 region of the 16S rRNA gene was amplified by PCR using the forward primer (5'-ACTCCTACGGGAGGCAGCAG-3') and the reverse primer (5'- GGACTACHVGGGTWTCTAAT-3'). Then pooled the amplified fragments in equal amounts and performed high-throughput sequencing on an Illumina MiSeq Platform (Illumina, USA). The data were analyzed using the free online platform of Majorbio Cloud Platform (www.majorbio.com).

Fecal microbiota transplantation

Donor Mice. In the fecal microbiota transplantation experiment, 18 mice were divided into two groups, including Normal and Pu-erh tea groups. Feces of the donors were collected at the end of 4 weeks. Free distilled water for the Normal group, the Pu-erh tea group was given with a concentration of 0.25%, and the dosage was determined according to previous experiments [10, 22]. Four weeks later, three rats were selected from each group and killed. The heart, liver, spleen, lung, and kidney were taken for pathological analysis, and 16sRNA was used to determine intestinal microbes.

Recipient Mice. Twenty-four rats were divided into four groups, including Control, 3.5% (w/v) DSS, TFM (receive the fecal bacteria from mice of the Pu-erh tea group), and WFM (receive the fecal bacteria from mice of the Normal group). After a 7-day acclimation, the Control group was given normal drinking water, while the other treatment groups received 3.5% (w/v) DSS. After 7 days of treatment, replaced DSS with normal water for 4 days before the end of the trial, and the measurement indicators were the same as above.

The process of fecal microbiota transplantation refers to previous research methods [18]. The feces collected from the donor group were placed in sterile phosphate buffer solution (PBS) (feces : PBS = 1 : 10, w/w), centrifuged at 2000 g at 4^{o}C for 1 min, the supernatant was collected and centrifuged at 15000 g for 5 min. The deposited bacteria were resuspended in the same volume of PBS. Gavaging the obtained suspension to the recipient mice every day until the end of the experiment (10 mL/kg bw).

Statistical analysis.

All the test data were shown as means ± standard error of the mean (SEM). Data differences between the different groups were analyzed using SPSS 20.0 (IBM SPSS, USA). The difference of the data was significant at $P < 0.05$, particularly significant at $P < 0.01$, and extremely significant at $P < 0.0001$. The graphics were generated with GraphPad Prism 8.0 (GraphPad Software, San Diego, USA) and OriginPro, 2019b (OriginLab Corporation, Northampton, MA, USA).

Results

Pu-erh tea protected against colitis in the DSS-induced mice

A pre-experiment was carried out to determine the optimal concentration of aged Pu-erh by gavage based on previous research [19] (Figure S1A). The results showed that compared with other concentrations, 10 mg/kg Pu-erh tea effectively reduced the trend of weight loss during DSS treatment, and faster restored body weight during the stop of administration (Figure S1B). 10 mg/kg Pu-erh tea markedly reduced colon shortening induced by DSS when compared with other treatment groups (Figure S1C, D). High-concentration Pu-erh tea aggravated the degree of colitis in DSS-treated mice, and 4 mice died at a concentration of 200 mg/kg in our experiment (Figure S1E). Therefore, considering the survival status of mice and the recovery of colon, 10 mg/kg Pu-erh tea was chosen as the concentration in this experiment.

The bodyweight of DSS-fed mice was significantly lower than that of the control group, although it rose slightly after stopping the administration (Figure 1B). While Pu-erh tea consumption effectively reduced the trend of weight loss caused by DSS, and quickly restore the weight of mice following cessation of drug delivery. Consistent with the trend of body weight changes, Pu-erh tea increased the food intake and drinking water in mice with colitis (Figure S1F, G). In terms of pathological characterization of colitis, Pu-erh tea effectively inhibited the weight loss, increased colon length, improved colon tissue lesions, and decreased DAI score (Figure 1C-E). According to H&E staining, we observed that compared with the control group, the intestinal tract of DSS-treated mice was significantly damaged, showing goblet cell decreased, inflammatory cell infiltration, colon crypt, and intestinal villus structure damage (Figure 1F). However, Pu-erh tea significantly improved colon pathology and lower the histological scores of colitis. Additionally, the liver pathology (e.g. hepatocyte destructions, hepatic inflammation, and enlargement) caused by DSS was also ameliorated by Pu-erh tea (Figure 1G).

Pu-erh tea inhibited oxidative stress-mediated inflammation pathway transduction

Oxidative stress (OS) is related to the pathogenesis of a variety of acute and chronic inflammatory diseases, which is one of the important signals of IBD [23, 24]. Studies have shown that tea has a role in anti-oxidation and reducing inflammation. To explore whether Pu-erh tea improve colitis by down-regulating the activation of oxidation stress-mediated inflammation pathway, the expression of related proteins and enzymes in mice were measured. The results revealed that the expression of ROS activity was significantly increased in the intestine of DSS-treated mice, while Pu-erh tea significantly reduced its expression ($p < 0.001$) (Figure 2A, B). The expression of NOX2 and NOX4 also reduced by Pu-erh tea supplementation (Figure 2A, C, D), especially in P2006, which further confirmed the phenomenon of increased ROS in DSS-treated mice in the above experiment results. Besides, P2006 significantly reduced the MPO activity, while restored the GSH-Px and SOD activities in DSS-induced mice (Figure 2E).

Next, the expression of colonic inflammatory pathway proteins (TLR4, MyD88, p38MAPK, and NF-κB p65) in different treatment groups were measured. The results showed that Pu-erh tea significantly down-regulated the levels of these inflammatory pathway proteins, which were increased by DSS induction (Figure 3A). Similar to the trend of ROS, treatment with P2006 significantly reduced inflammation pathway protein expressions compared with P2020.

The increase of ROS stimulates the production of inflammatory cytokines in neutrophils and T cells, which are closely related to the occurrence and deterioration of colitis [6]. Besides, overexpression of inflammatory cytokines can induce apoptosis of intestinal mucosal cells, thereby aggravating the colitis response in mice. In Figure 3B, datas showed the Pu-erh tea significantly down-regulated the levels of major inflammatory cytokines including IL-1β, IL-6, TNF-α and LPS increased in serum of DSS-induced mice. As expected, the expression levels of anti-inflammatory cytokines in the serum of DSS-induced mice were restored to the control level after Pu-erh tea treatment. Among them, the levels of IL-4 and IL-22 of mice treated with P2006 exceeded the control level ($P < 0.05$).

Keap1 - Nrf2 - ARE is a key pathway in the anti-oxidative stress response [25, 26], which plays an important role in cellular defense through anti-inflammatory, antioxidant, autophagy, and other physiological functions (Figure 3C). DSS-induced colitis result in increased oxidation levels further stimulates the activation of anti-oxidative stress pathways. Our result show that, both protein expression of Nrf2 and HO-1 in the colon were decreased in the DSS-treated mice relative to their control and Pu-erh tea-treated colitis mice (Figure 3A). Additionally, compared with the control group, Pu-erh tea significantly enhanced the anti-oxidative stress response in the DSS-induced colitis mice by stimulating the dissociation of Keap1/Nrf2. Taken together, these results indicated that the occurrence of colitis was accompanied by oxidative stress. Pu-erh tea reduced inflammation by inhibiting the activation of oxidation-mediated inflammatory pathway, especially in 2006. OS promoted the development of colitis. H&E dyeing results (Fig 1F) further confirmed this conclusion. We therefore hypothesized that the protective effect of Pu-erh tea on intestinal may depend on the improvement of intestinal immune barrier function.

Pu-erh tea enhanced the intestinal mucosal barrier function of DSS-induced colitis mice

To verify this hypothesis, the intestinal tight junction proteins (ZO-1, MUC2, and occludin) expression was measured. Our results showed that the higher expression of ZO-1 and MUC2 in the colon were raised in Pu-erh tea treated groups mice, especially in P2006 ($p < 0.05$) (Figure 4A). Compared with DSS-treated mice, ZO-1 and MUC2 in the control group and the Pu-erh tea-treated group mice were more evenly expressed on the colon villi (Figure 4B, C). The expression of occludin in the colon of DSS-induced colitis mice was reduced, while Pu-erh tea supplementation nearly complete attenuation of the DSS-induced disorderly distribution and increase the expression of occludin in colonic epithelial cells (Figure 4D).

Pu-erh tea promotes macrophage polarization toward a M2 phenotype

Macrophages play a key role in innate immune response and occupy an important position in intestinal immunity, affecting the development of colitis through polar differentiation. We next investigated the expression level of M1 and M2 macrophages in the colon for Pu-erh tea treated DSS-induced colitis mice (Figure 4E-G). Both expressions of M2 macrophages and relative protein level of GM-CSF in the serum were increased in P2006 and P2020 groups relative to 3.5% DSS groups (Figure 4 H). On the contrary, the expression of M1 macrophages and M-CSF in the serum were decreased in Pu-erh tea treated mice, especially in the P2006 group (Figure 4 I).

Pu-erh tea significantly reshaped the gut microbiota in DSS-induced mice

The former studies have shown that gut microbes are closely related to the development of oxidative stress and inflammation. It is crucial in maintaining intestinal homeostasis. To identify the effect of Pu-erh tea on reshaping the gut microbiota with DSS-induced mice, we analyzed gut microbiota composition using 16S rRNA sequencing. The microbial community richness and diversity were increased in Pu-erh tea-treated DSS mice compared with saline-treated DSS mice (Figure 5A, B). The microbial community composition from different groups has obvious clustering, on the principal coordinate analysis (PCoA) plots based on Bray-Curtis (Figure 5C). The DSS-treated group was significantly different from the healthy group and the Pu-erh tea-treated group. However, the microbial community composition structure between the tea-treated groups was similar. The prominent microbiotas at the phylum level in the DSS-treated group were *Firmicutes* and *Bacteroidota*, while the abundance of *Firmicutes* was reduced and *Bacteroidota* was increased, tea intake reversed this trend caused by DSS (Figure 5E). Moreover, at the genus level, Pu-erh tea-treated group increased the abundance of *Lactobacillus* and reduced the abundance of *Bacteroides* caused by DSS (Figure 5D, F, G). The relative abundance of *Bifidobacterium* and *Lachnospiraceae* in the control group was significantly increased compared with the DSS-treated mice (Figure 5H). Pu-erh tea-treated DSS mice contained significantly higher proportions of *Turicibacter* and *Romboutsia* compared with the other groups. Pu-erh tea significantly increased the relative proportion of *Akkermansia* and *Lactobacillus*. Compared with the control, DSS-treated mice increased the abundance of *Parasutterella* and *Bacteroides*, which were associated with the progression of colitis [27, 28]. However, the abundances of these genera (especially *Bacteroides*) were reduced by the Pu-erh tea-treated mice ($p < 0.05$).

Much of the research have shown that microflora changes play an important role in the induction and treatment of colitis, and the interaction between microflora and intestinal immunity has been widely described. As a niche for microbial colonization, the integrity of the intestine affects the composition of the microflora. Upon colonization, the microbiota participates in and helps maintain the integrity of the epithelial barrier and shapes the mucosal immune system [5, 29]. In our experimental results, the administration of Pu-erh tea, especially given P2006, improving the microflora and intestinal immunity of DSS-induced colitis mice, which was consistent with the conclusion. This indicated that aged Pu-erh tea showed greater potential in adjuvant therapy of colitis compared with unaged Pu-erh tea. To determine whether aged Pu-erh tea may improve the status of DSS-induced colitis mice by reshaping intestinal microbes, we transferred the microbiota of long-term P2006-treated mice to DSS-induced colitis mice, followed by examination of inflammation-related traits (Figure 6A).

Pu-erh faecal transplants inhibited the development of intestinal oxidative stress-mediated inflammation

Analysis of the donor mice indicated that P2006 treatment did not affect the body weight and the histological differences in organs (heart, liver, spleen, lung, and kidney), demonstrating that 1 month of P2006 drinking will not cause toxicity (Figure S1L). Similar to the results shown above (Figure 1B; Figure S1 F, G), P2006 fecal transplantation group (TFM) increased body weight, food intake, and water drink

compared with fecal transfer from normal treatment mice (WFM; Figure 6B; Figure S1 I, J). TFM treatment decreased DSS-induced loss of goblet cells, protected intestinal villi, and avoided the shortening of the DSS-induced colon, compared with faecal transfer from WFM-fed mice (Figure 6C, Figure S2 B, C).

In order to determine roles of P2006 regulted gut microbiota in the regulations of the oxidative stress-mediated inflammation pathway, we measured the expression of ROS, oxidases (SOD, GSH-Px, MPO) and pathway related proteins. As a result, TFM group showed higher SOD and GSH-Px content compared to the WFM group, lower than their donors (Figure 6E). ROS and MPO expression were lower than WFM (Figure 6D, Figure S2 A). TFM dramatically elevated the expression of antioxidant pathway proteins such as NRF2 and HO-1, while reduced the expression of inflammatory pathway proteins TLR4, MyD88, p38MAPK, and NF-κB p65 (Figure 6F, G, Figure S3). Expression of pro-inflammatory cytokines was also reduced in serum of DSS-induced mice after transfer of faeces from P2006-treated mice (Figure 6H). In addition, the expression of anti-inflammatory cytokines increased following TFM. Concurrently, Keap1, NOX2, and NOX4 expression decreased, which related to ROS production, following TFM.

Immune barrier and macrophage expression analysis of our dataset demonstrated an increase of immune barrier protein expression and a reduction in canonical M1 inflammatory macrophages, following P2006 fecal microbiota transplantation, consistent with the effect of direct gavage of P2006. Compared with the group of WFM, P2006-mediated bacterial changes significantly increased the expression of MUC2, ZO-1, and occludin proteins in the mouse intestine, and improved the intestinal barrier function (Figure 6I, Figure S4 A). Furthermore, TFM also produced significant changes in M1 and M2 expression (Figure 6J, Figure S4B), which compared with WFT mice, one of the reasons was that microbes changed the expression of macrophage stimulating factors M-CSF and GM-CSF.

To confirm that Pu-erh modulates the gut microbiota, we determined the composition of intestinal bacteria in feces of 0.25% P2006-treated mice. Long-term drinking of P2006 increased the abundance of the intestinal flora in mice, which compared with the normal treatment group (Figure S5A, E, F). TFM increased the abundance of *Bifidobacterium*, *Lachnospiraceae*, *Akkermansia,* and *norank_f_Muribaculaceae*, which was consistent with the trend of mice fed with P2006, and the abundance was higher than WFM (Figure 7A; B, Figure S6). Intestinal type analysis revealed that mice with different treatments were divided into three archaeal intestinal types, and the dominant bacteria families were *norank_f_Muribaculaceae* (Control, TFM, and WFM) and *Bacteroides* (Model) (Figure 7C). Among these, *Dubosiella*, *Ileibacterium,* and *Desulfovibrio*, which were decreased by DSS feeding, were all reversed by WFM and TFM (Figure 7E, Figure S6). Of note, *Clostridium_sensu_stricto_1* and *Escherichia-Shigella* were all drastically increased by DSS (Figure S6). Interestingly, *Lachnospiraceae_NK4A136_group* and *Alloprevotella* were not detected in the DSS-treated group, while improved significantly after receiving fecal transplantation (Figure S6). These results indicated that microbiota transplantation changed the composition of the intestinal flora in DSS-induced colitis mice, which may be the cause of maintaining the intestinal environment and reducing intestinal inflammation.

Discussion

Previous studies have shown that with the increase of harmful bacteria and inflammatory factors, ROS cannot be removed in time, the surge of oxygen free radicals ($HO\cdot$, $O_2^{-}\cdot$) destroys the stability of the intestinal mucosal barrier and aggravates colitis [30]. Pu-erh tea lowered inflammatory cytokines and improved intestinal mucosal homeostasis by regulating intestinal microbes, which produced beneficial effects on colitis in DSS-induced model. Although a large number of reports have shown that tea play an important role in anti-oxidant and anti-inflammatory [31-33]. It has rarely reported whether the Pu-erh tea storage period affects its anti-inflammatory and anti-oxidant capacity in colitis, or even intestinal protection. Our study shows that aged Pu-erh tea (10 mg/kg bw/day) prevents and alleviates the activation of oxidative stress-mediated inflammatory signaling pathway transduction and the release of inflammatory factors in DSS-induced colitis mice by modulating the composition of the gut microbiota, maintaining intestinal barrier integrity, and improving the defense of immune system. Comprehensive analysis, compared with P2020, P2006 is more significant in results. These results confirmed other reported inflammatory pathways and inflammatory factors-lowering effects of Pu-erh tea in DSS-induced mice.

Compared with P2020, P2006 had better protective effects on the DSS-induced colitis mice. In this study, compared with P2020, the contents of caffeine and gallic acid in P2006 were significantly decreased, while catechin and tea polysaccharide were significantly increased (Figure S1K), which was consistent with the results of a recent study by Zhang and Luo et al [34, 35]. Polysaccharides have been shown to alleviate LPS-induced kidney damage and intestinal inflammation [36, 37]. By increasing the level of antioxidant enzymes, reducing the production of pro-inflammatory cells, and promoting the phagocytic function of the macrophage system, polysaccharide plays an important role in anti-oxidation and anti-inflammation. Additionally, catechin has radical scavenging activities (hydroxyl, peroxyl, superoxide), showing strong antioxidant ability [38, 39]. Catechin-enriched green tea and oolong tea significantly increased the antioxidant activity of mice, reducing lipid peroxidation and liver inflammation, and has the effect of improving mildly hypercholesterolemic subjects [40]. In conclusion, polysaccharide and catechin have the potential to alleviate intestinal oxidative stress-mediated inflammation. Based on composition changes, compared with P2020, the protective effect of P2006 on colitis may be related to the increased content of tea polysaccharide and catechin.

Inflammation is closely related to ROS production [41]. The anti-oxidation effects of Pu-erh tea have been demonstrated in the colitis experiment. In this study, Pu-erh tea reversed DSS-induced oxidative stress (increased ROS, NOX2 and NOX4 levels) of colitis, and up-regulated the expression of SOD and GSH-Px in colon and liver. Gastrointestinal mucosal damage induces the generation of ROS, which destroys the balance between oxidation system and antioxidant system. Nrf2/HO-1 plays an active role in resisting oxidative damage, protecting cells and tissues, and is one of the most important antioxidant mechanisms in cells. P2006 significantly up-regulated the expression of Nrf2/HO-1 protein and inhibited the expression of proteins in the oxidative stress-mediated inflammatory pathway. Interestingly, the FMT results are similar to direct drinking P2006, indicating that gut microbes affect the oxidation mechanism. The results of 16sRNA determination showed that compared with the DSS model group, long-term drinking of P2006

(donor group) or inoculation of P2006 (TFM) showed greater abundance of *Bifidobacterium* and *Lactobacillus*, while the number of *Escherichia* and *Clostridium* decreased. The composition of the gut microbes of inflammatory enteritis usually shows a decrease in the number of *Bifidobacteria*, *Lactobacilli*, etc., an increase in the number of *Staphylococcus*, *Clostridium*, and excessive proliferation of *Enterobacteriaceae* and *Bacteroidaceae* [42, 43]. Disturbance of the gut microbes stimulates ROS production within enterocytes. Once the production and clearance of ROS are unbalanced, it will cause OS, which damages a variety of macromolecular substances (such as lipids, proteins, nucleic acids), and participates in the occurrence of various gastrointestinal diseases [44, 45]. Moreover, molecular oxygen, such as hydrogen peroxide, produced by the degradation of ROS in the gut lumen, can promote the aerobic respiration of *Escherichia* and aggravate inflammation [46].

As reported, gut microflora reduced intestinal inflammation by regulating the release of related active factors (such as IL-10, IL-1β, etc.) in colon [47]. Our results supported this view by showing that the anti-inflammatory effects of P2006 can be transferred by fecal transplantation. P2006 significantly down-regulated the expression of LPS and its related receptor protein TLR, reducing TLR-mediated inflammatory pathways. Comparison of H&E staining between different treatment groups showed that the DSS model group had more significant inflammatory cell infiltration. This means that DSS-induced colitis mice, the imbalance of microorganisms increases the permeability of the intestine, and more inflammatory cytokines, including LPS, enter the enterohepatic circulation. Overactivation of TLR-mediated inflammatory pathways further aggravates inflammation [5]. Similarly, the intestinal immune barrier also plays an irreplaceable role in anti-colitis [48]. P2006 treatment and FMT experiment showed that, compared with the DSS model group, M2 macrophages significantly increased, alleviating intestinal inflammation through the production of IL-10 and other anti-inflammatory cytokines. Moreover, the expression of IL-4 increased, which provided the conditions for stimulating M1 to M2 type polarization. In contrast, the model group showed increased levels of GM-CSF and M1 type macrophages. We observed that both Pu-erh tea and FMT could inhibit colonic shortening and increase the expression of tight junction protein (MUC2, ZO-1, occludin) in colitis mice. Related studies have shown that the reduction of tight junction proteins shorten the distance between the bacteria to the IEC, and promote the invasion of harmful bacteria (*Bacteroidetes* and *Proteobacteria*) [49]. The weakened phagocytic capacity of macrophages, harmful bacteria were not cleared in time, which further exacerbated colon inflammation. These results suggested that diet-induced changes in the gut microbiome alter immune response. Compared with conventional dietary treatment, P2006 regulated microbiota groups could better assist intestinal immunity (e.g., reduction of *Bacteroides* and increase of *Akkermansia*).

Gut microbiome plays an important role in maintaining the body digestion, promoting the development of the immune system and the health of the host through the metabolism of substances. Our results showed that through correlation analysis, there was a significant correlation between intestinal flora and ROS production, activation of oxidation-mediated verification pathway and intestinal immune regulation (Figure 7F), which was consistent with previous results [50-52]. Among them, by fecal transfer from P2006-treated mice, the abundance of bacteria (*Bifidobacterium*, *norank_f_Muribaculaceae* and *Akkermansia*) increased that was positively correlated with antioxidant indexes and immune cells. The

abundance of *Bacteroides*, *Escherichia-shigella*, *Romboutsia* was downregulated, which positively correlated with the production of ROS and the activation of inflammatory pathways. In addition, *Lachnospiraceae_NK4A136_group* and *Alloprevotella* was significantly increased after gavage Pu-erh tea or fecal transplantation; *Clostridium_sensu_stricto_1* and *Escherichia-Shigella* were extremely abundant in the colitis model mice, and significantly reduced following gavage P2006 or fecal transplantation. As reported, *Lachnospiraceae_NK4A136_group* is a short-chain fatty acid-producing bacteria, which is related to butyric acid production [53], and plays an important role in regulating the balance of the intestinal environment and reducing the inflammation of the mouse colon and liver caused by HFD [54]. *Alloprevotella*, as a beneficial bacterium, improves intestinal flora imbalance in obese mice fed with high-fat and high-sugar diets [55]. Therefore, the improvement of abundance is beneficial to the alleviation of colitis. Moreover, *Clostridium_sensu_stricto_1* and *Escherichia-Shigella* have been reported to be associated with enteritis, intestinal mucositis and other diseases [56, 57], which were increased in the DSS-induced colitis mice studied here. Altogether, these results indicated that P2006 regulates the immune system and reduces the inflammation of colitis by affecting the composition of the gut microbiota.

Conclusions

These results indicate that the changes of intestinal microflora caused by P2006 extracts (10 mg/kg bw/day) are the key to alleviate DSS-induced colitis. P2006 inhibited the intestinal oxidative stress-mediated inflammation signaling pathway, promoted the expression of intestinal tight junction proteins, and improved the intestinal immune barrier, which stemmed from the reshaping of intestinal microbiota (Fig. 8). Thus, Aged Pu-erh tea should be a promising natural beverage in preventing the development of colitis. The equivalent dose of 7 g/60 kg/day may play a role in adjuvant therapy in patients with colitis. Notably, our results shown that the content of tea polysaccharide and catechin in P2006 was higher than the P2020, exploring their structure-activity relationship in colitis and the role of enterohepatic circulation will become our next research direction.

Declarations

Acknowledgments

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Author Contributions

Liang Zeng, Shanshan Hu, Shi Li, and Yan Liu designed the research. Shanshan Hu, Shi Li, and Yan Liu analyzed the data and wrote the paper. Shanshan Hu, Kang Sun, and Liyong Luo performed mouse feeding, cytokine, and enzyme activity assays. Shanshan Hu, Shi Li, Kang Sun and Yan Liu assisted in protein immunohistochemistry and immunofluorescence determination, and microbial detection.

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Availability of data and materials

The sequences generated in this study are stored in the National Center for Biotechnology Information (NCBI) and the project numbers are PRJNA726844 and PRJNA726847. Additionally, all other data is contained within the main manuscript and supplemental files.

Ethics approval and consent to participate

All experimental procedures were in accordance with the Guidelines for the Protection and Use of Laboratory Animals of China and approved by the Institutional Animal Care and Use Committee of the Southwest University (permit number, 20190003).

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

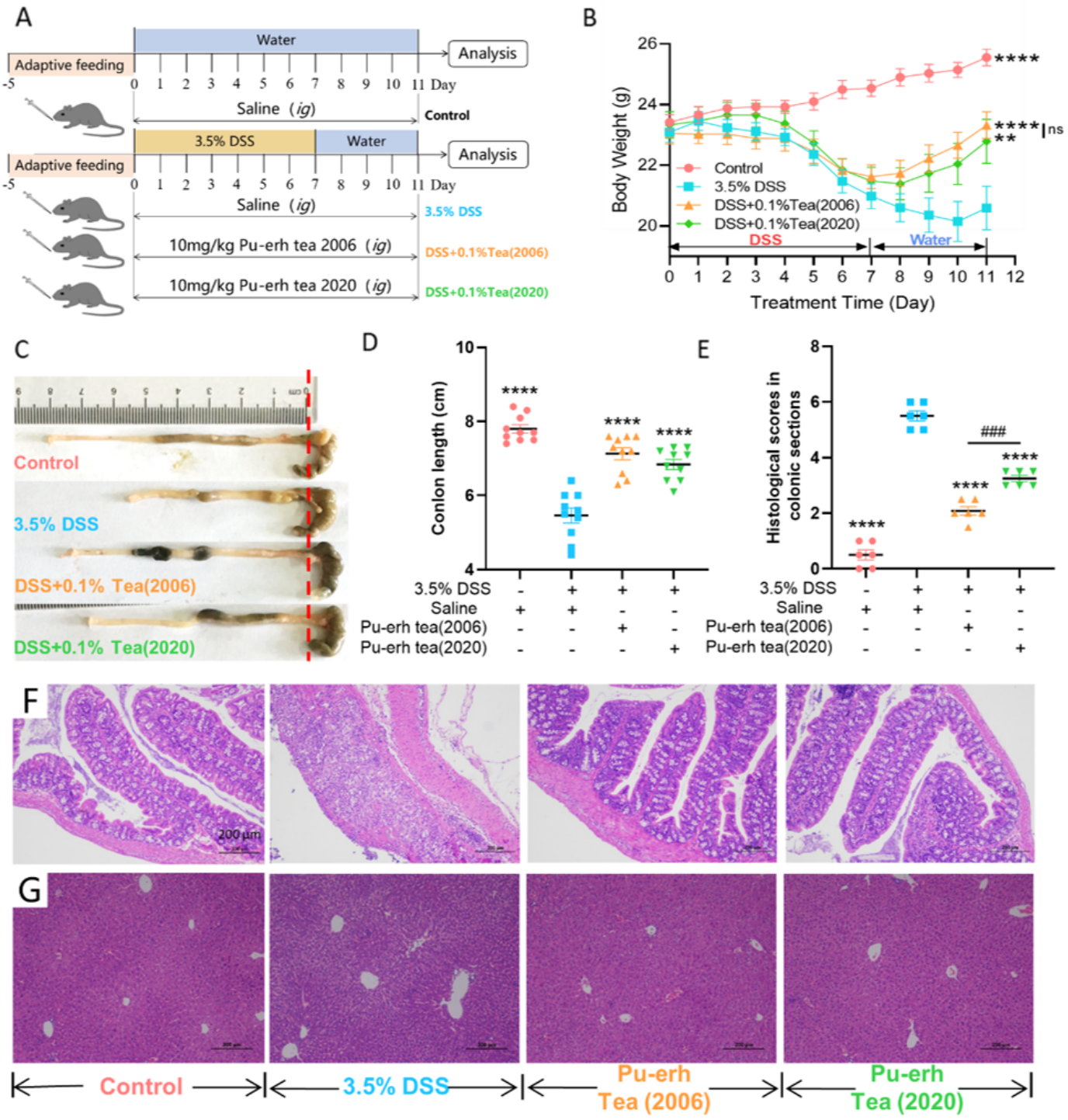

Figure 1

Pu-erh tea protected against colitis in the DSS-induced mice (A) Schematic diagram of the experimental design.(B) Body weighed (n=10), (C) representative colon image, (D) colon lengths (n=10), (E) and colonic histology score (n=6). (F and G) Representative H+E staining sections of colon and liver. Scale bar, 200 μm.Data are mean ± SEM.*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001 vs the DSS group. #p < 0.05,

##p < 0.01, ###p < 0.005, ####p < 0.001 vs the DSS + 0.1% Tea (2020) group, determined by Student's t test and one-way ANOVA with Bonferroni's multiple comparison test (B, D, E).

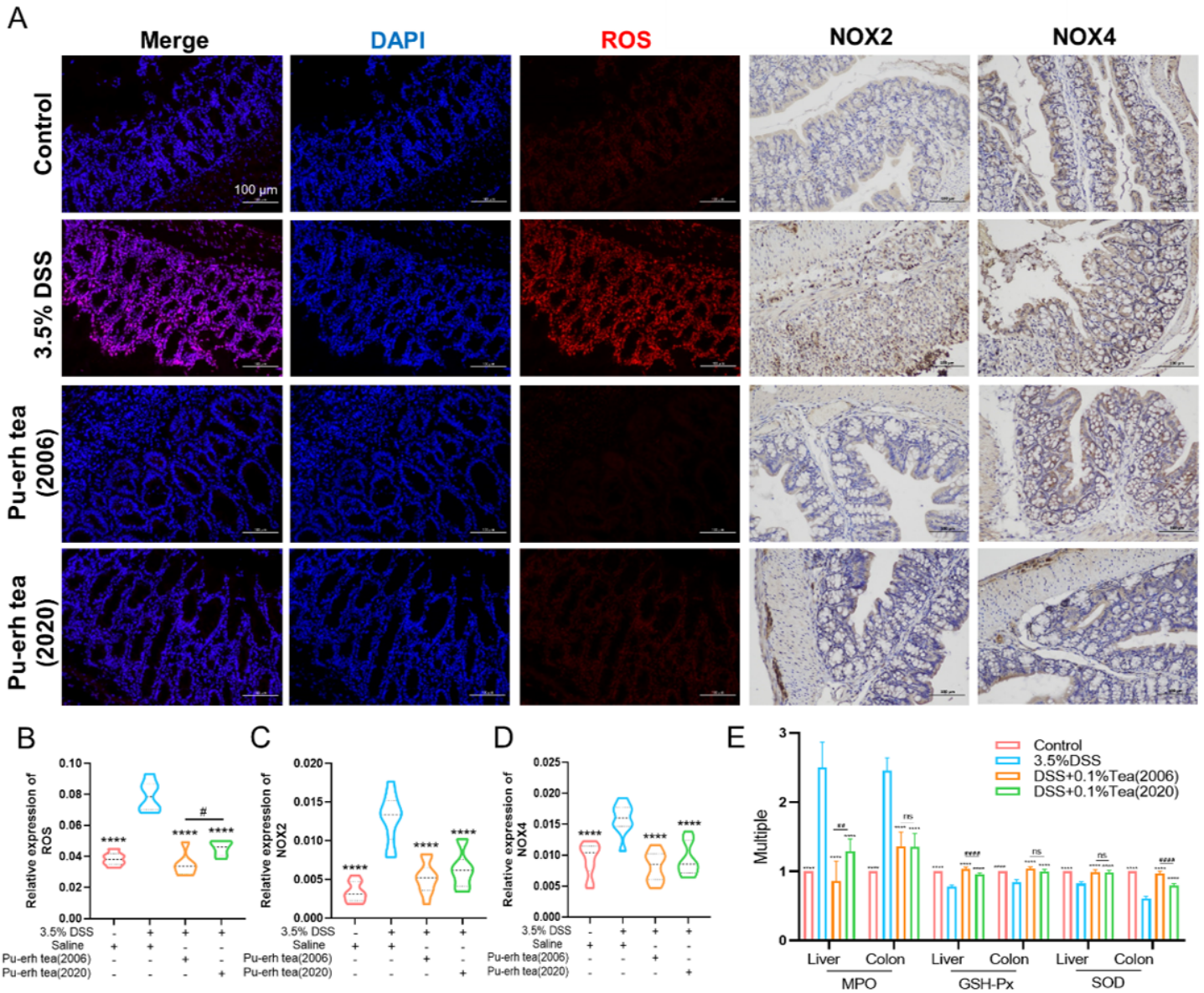

Figure 2

Pu-erh tea reduced the oxidative stress of DSS-induced colitis (A-D) Immunofluorescence analysis of reactive oxygen species (ROS); Immunohistochemical analysis of expressions of NADPH oxidase 2 (NOX2) and NADPH oxidase 4 (NOX4). Scale bar, 100 μm. The relative expressions of all were calculated according to the following formula using Image-Pro Plus 6.0 software (n=6). (E) Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and myeloperoxidase (MPO) level activities of colon and liver were measured, compared with those in control (n=5, two colon sections were randomly measured for each sample). Data are mean ± SEM.*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001 vs the DSS group.

#p < 0.05, ##p < 0.01, ###p < 0.005, ####p < 0.001 vs the DSS + 0.1% Tea (2020) group; ns, not significant as determined by Student's t test and one-way ANOVA with Bonferroni's multiple comparison test (B-E).

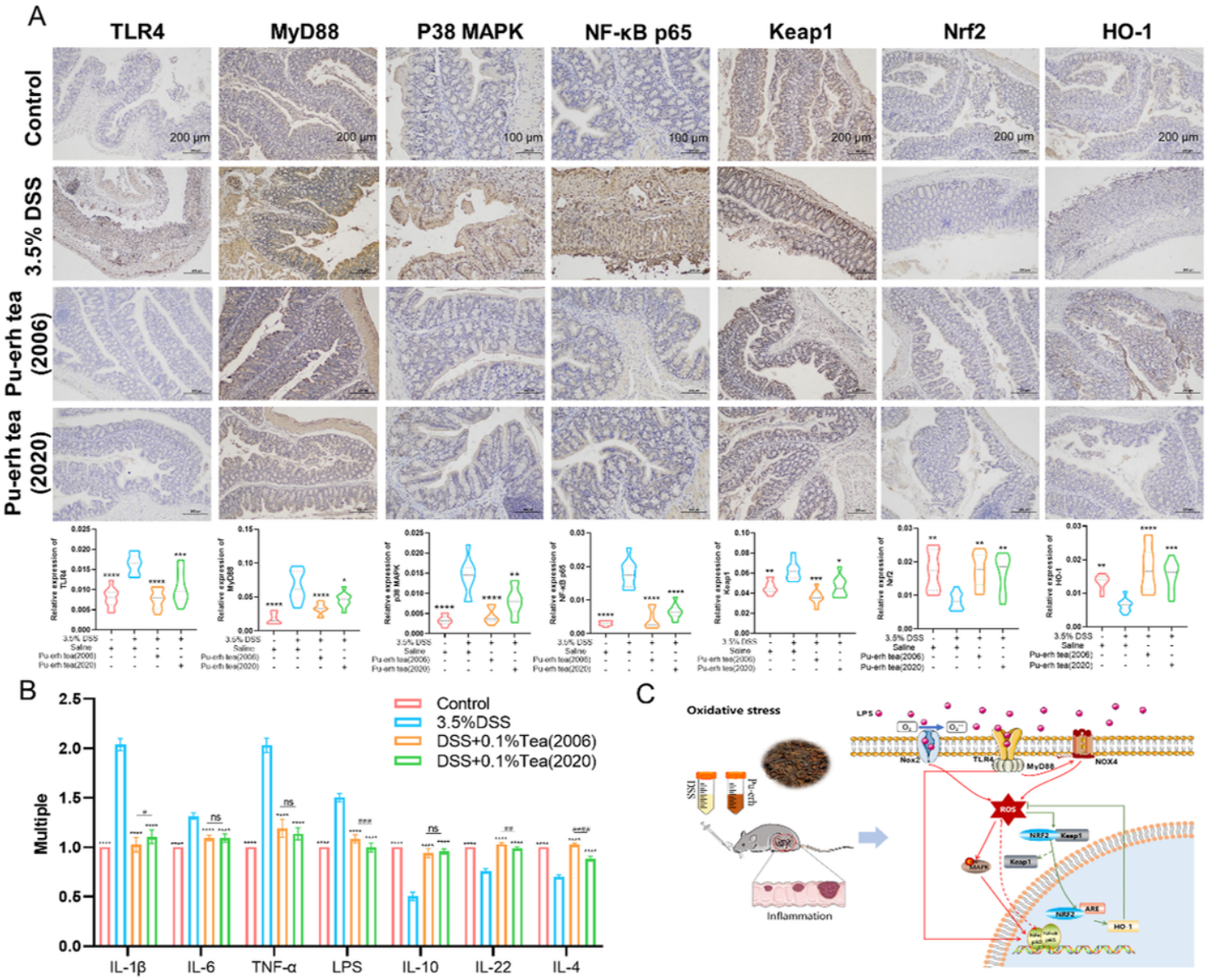

Figure 3

Pu-erh tea inhibited free oxygen-mediated inflammatory signaling pathway transduction (A) Immunohistochemical analysis of expressions of inflammatory signaling pathway proteins (TLR4/MyD88/P38 MAPK/NF-kB P65); Immunohistochemical analysis of expressions of antioxidant signaling pathway proteins (Keap1/Nrf2/HO-1). The relative expressions of all were calculated according to the following formula using Image-Pro Plus 6.0 software (n=5, two colon sections were randomly measured for each sample). (B) Inflammatory cytokines (IL-1β/IL-6/TNF-α/LPS) and anti-inflammatory cytokines (IL-10/IL-22/IL-4) measured by ELISA in the serum with the baseline of Control (n=5, two colon sections were randomly measured for each sample). (C) Schematic diagram of the Pu-erh tea on oxidative inflammation pathway. Data are mean ± SEM.*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001

vs the DSS group. #p < 0.05, ##p < 0.01, ###p < 0.005, ####p < 0.001 vs the DSS + 0.1% Tea (2020) group; ns, not significant as determined by Student's t test and one-way ANOVA with Bonferroni's multiple comparison test (A, B).

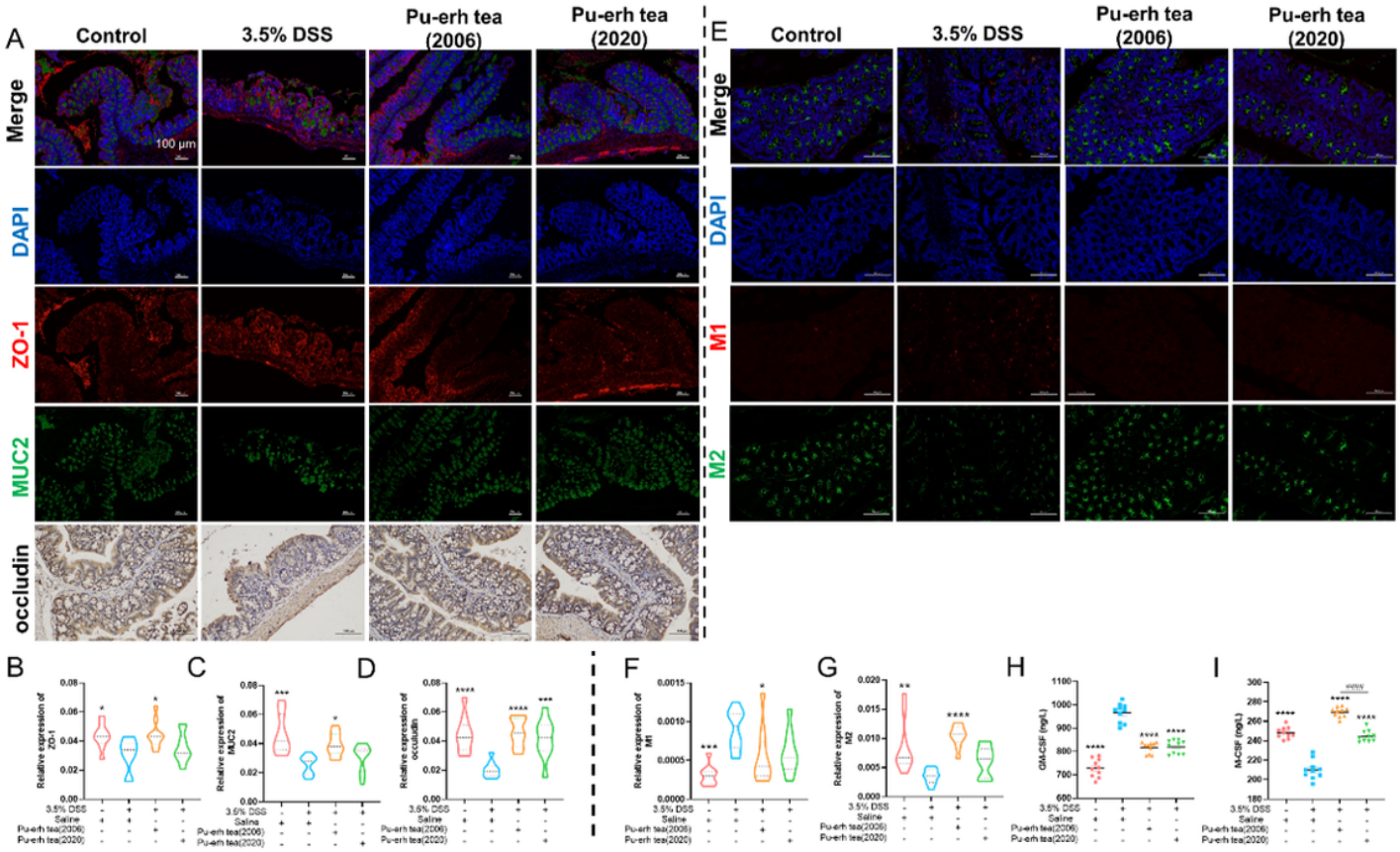

Figure 4

Pu-erh tea regulated anti-inflammatory immunity of DSS-induced colitis mice (A) Immunofluorescence analysis of Zonula occludens 1 (ZO-1) and mucoprotein 2 (MUC2) in colon; Immunohistochemical analysis of expressions of occludin. Scale bar, 100 μm. The relative expressions of all were calculated according to the following formula using Image-Pro Plus 6.0 software (B-D) (n=5, two colon sections were randomly measured for each sample). (E) Immunofluorescence analysis of M1 and M2 in colon. Scale bar, 100 μm. The relative expressions of all were calculated according to the following formula using Image-Pro Plus 6.0 software (F and G) (n=5, two colon sections were randomly measured for each sample). (H and I) Determination of the expression of macrophage colony stimulating factor (M-CSF and GM-CSF) in serum with ELISA (n=10). Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001 vs the DSS group. #p < 0.05, ##p < 0.01, ###p < 0.005, ####p < 0.001 vs the DSS + 0.1% Tea (2020) group; ns, not significant as determined by Student's t test and one-way ANOVA with Bonferroni's multiple comparison test (B-D, F-I).

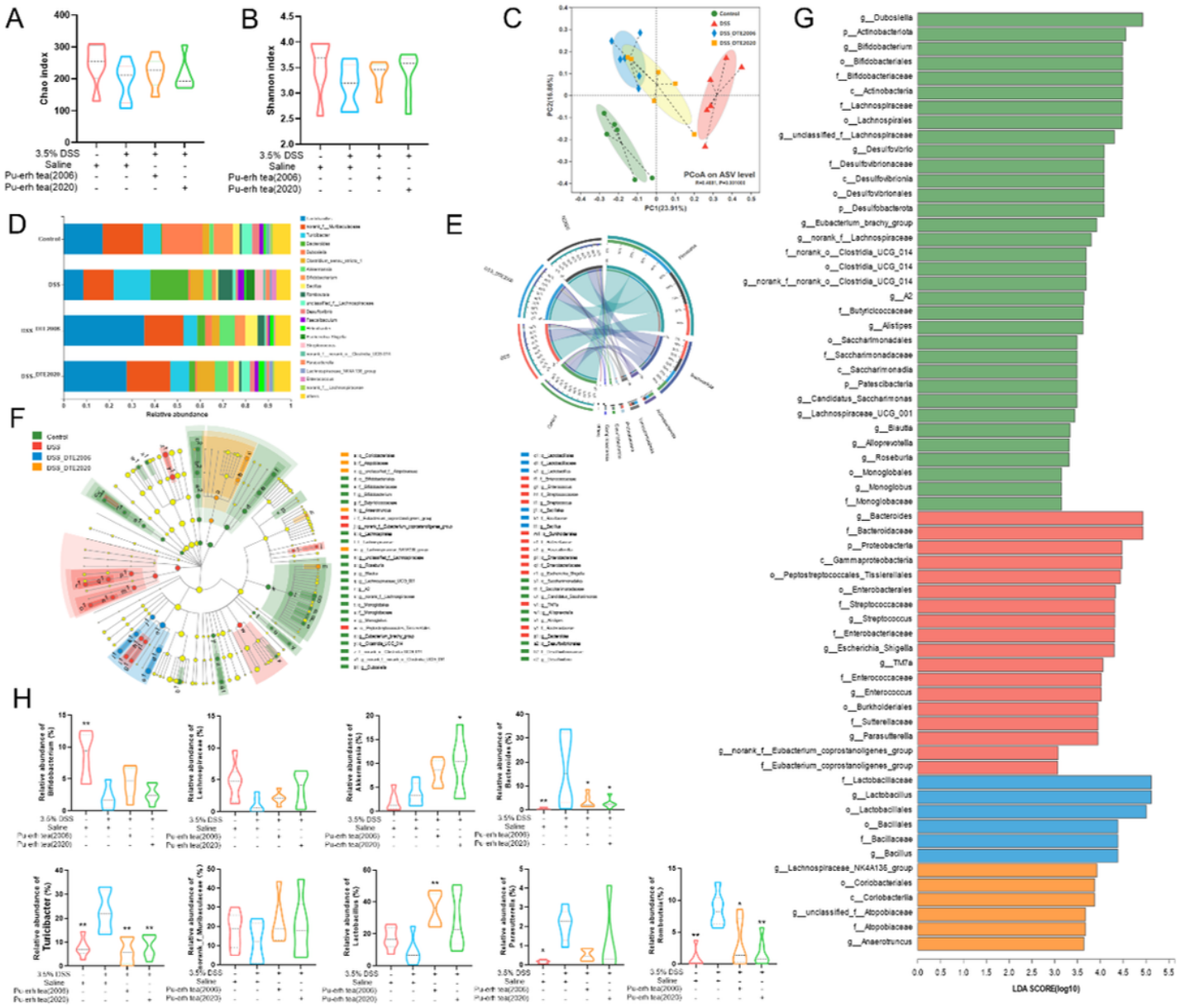

Figure 5

Pu-erh tea significantly reshaped the gut microbiota in DSS-induced mice (A and B) α - diversity is represented by the violin diagram of Chao index and Shannon index (n=6). (C) Principal co-ordinates analysis (PCOA) plot of the gut microbiota based on Bray–Curtis matrixes. (D and E) Gut microbiota composition at genus level (D) and phylum level (E) with relative abundance of more than 1%. (F and G) Linear discriminant analysis and its influencing factors. (H) Gut microbiota composition among experimental groups at the genus/species level (n=6). Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001 vs the DSS group. #p < 0.05, ##p < 0.01, ###p < 0.005, ####p < 0.001 vs the DSS + 0.1% Tea (2020) group; ns, not significant as determined by Student's t test and one-way ANOVA with Bonferroni's multiple comparison test (A, B, H).

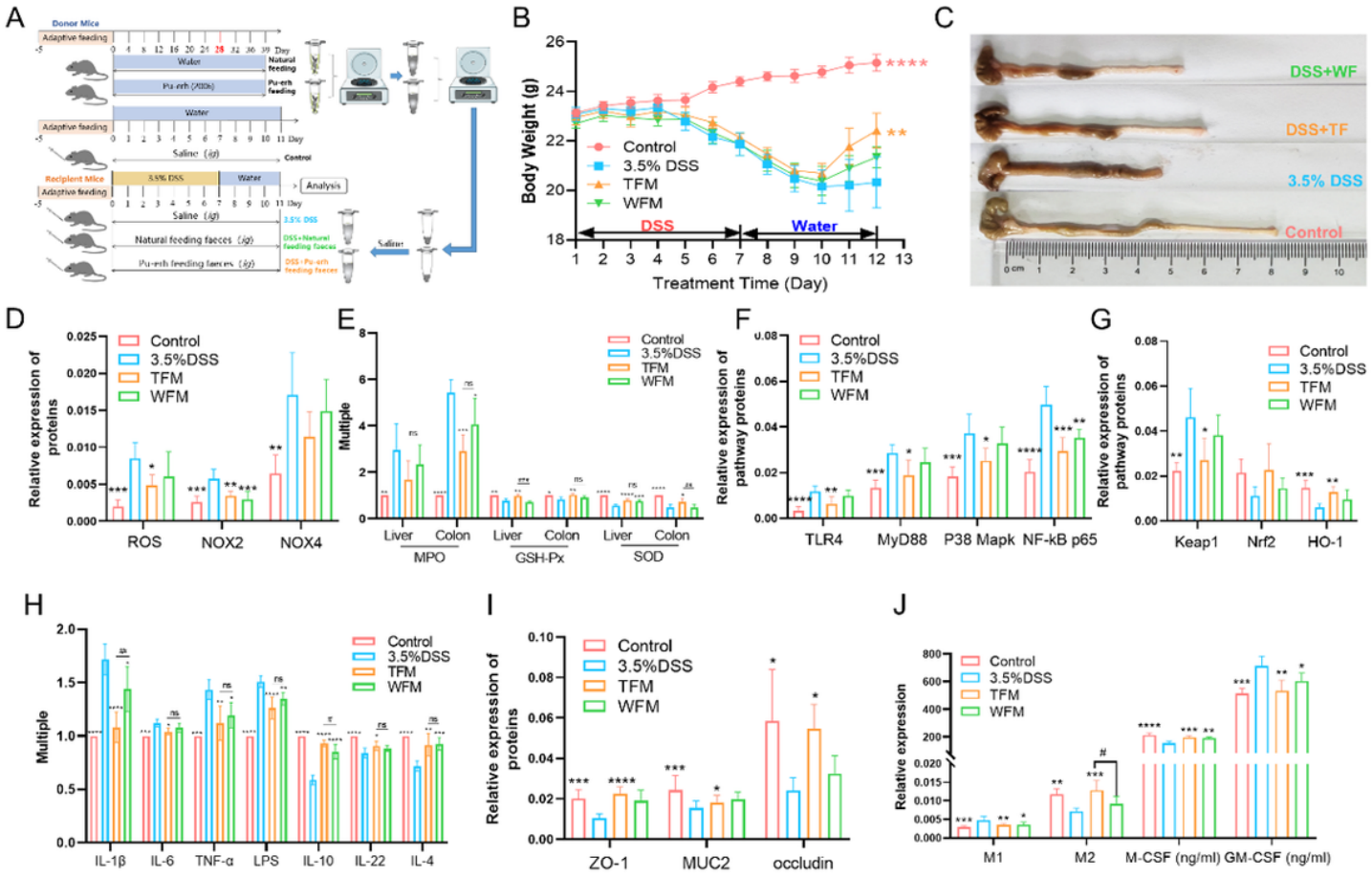

Figure 6

Pu-erh faecal transplants reduced inflammation (A) Schematic diagram of the fecal microbiota transplantation experimental design. (B) Body weighed (n=8), (C) representative colon image, (D) and the relative expressions of ROS, NOX2, and NOX4 (n=6). (E) GSH-Px, SOD and MPO level activities of colon and liver were measured at the end of experiment with the baseline of Control (n=6). (F and G) The relative expressions of inflammatory pathway proteins and antioxidant pathway proteins, were calculated using Image-Pro Plus 6.0 software (n=6). (H) Inflammatory cytokines (IL-1β/IL-6/TNF-α/LPS) and anti-inflammatory cytokines (IL-10/IL-22/IL-4) measured by ELISA in the serum with the baseline of Control (n=6). (I) The relative expressions of ZO-1, MUC2, and occludin, were calculated using Image-Pro Plus 6.0 software (n=6). (J) The relative expressions of M1, M2, were calculated using Image-Pro Plus 6.0 software. Determination of the expression of macrophage colony stimulating factor (M-CSF and GM-CSF) in serum with ELISA (n=6). Data are mean ± SEM.*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001 vs the DSS group. #p < 0.05, ##p < 0.01, ###p < 0.005, ####p < 0.001 vs the WFM group; ns, not significant as determined by Student's t test and one-way ANOVA with Bonferroni's multiple comparison test.

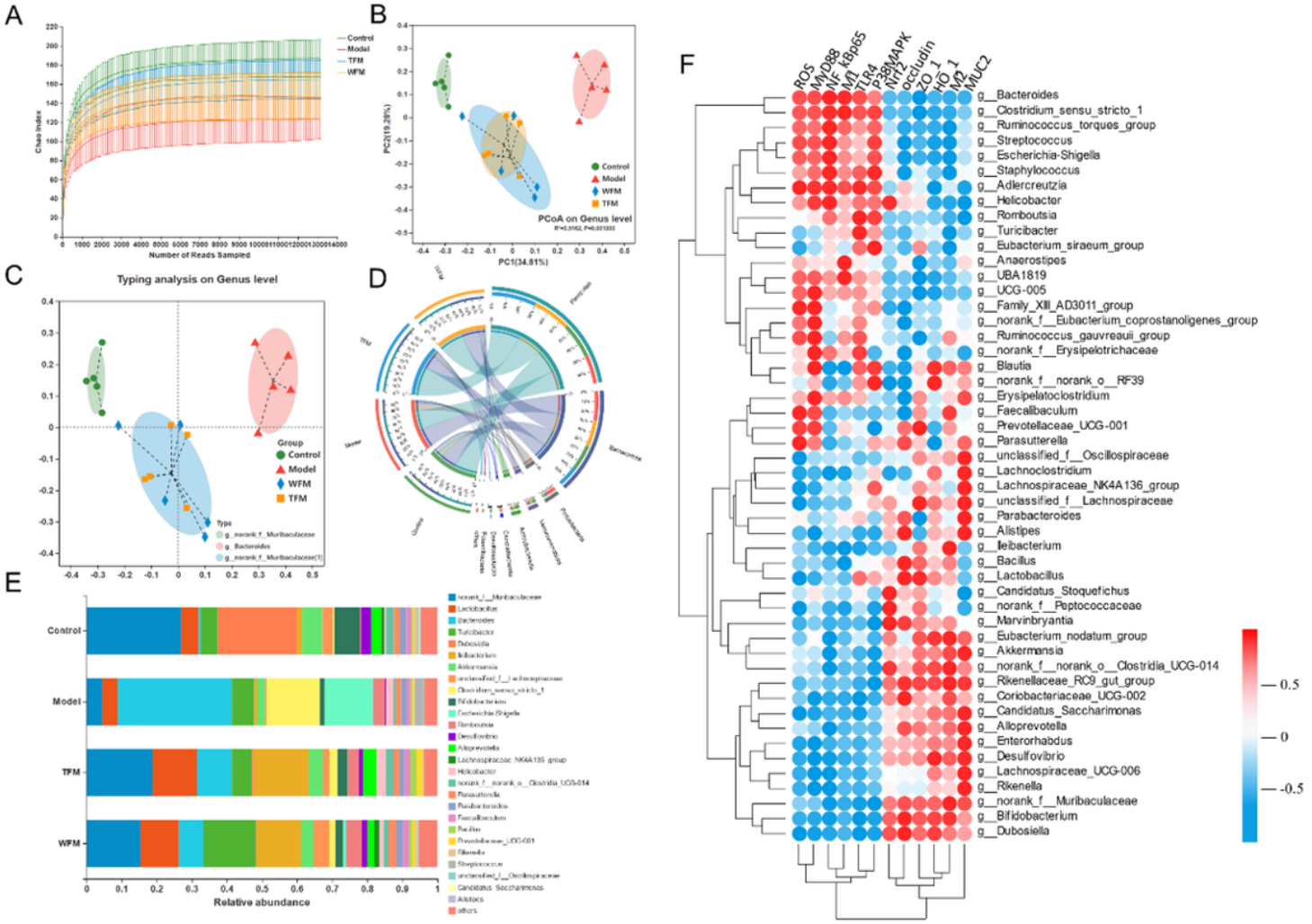

Figure 7

Pu-erh faecal transplants modulate gut microbiota composition (A) Evaluation of species α - diversity by Chao index (n=5). (B) Principal co-ordinates analysis (PCOA) plot of the gut microbiota based on Bray–Curtis matrixes (n=5). (C) Enterotypes analysis at genus level based on Bray–Curtis matrixes (n=5). (D and E) Gut microbiota composition at phylum level (D) and genus level (E) with relative abundance of more than 1%. (F) Relationship between oxidative inflammation, antioxidant pathway and gut microbiota (at genus level, abundance in the top 50).

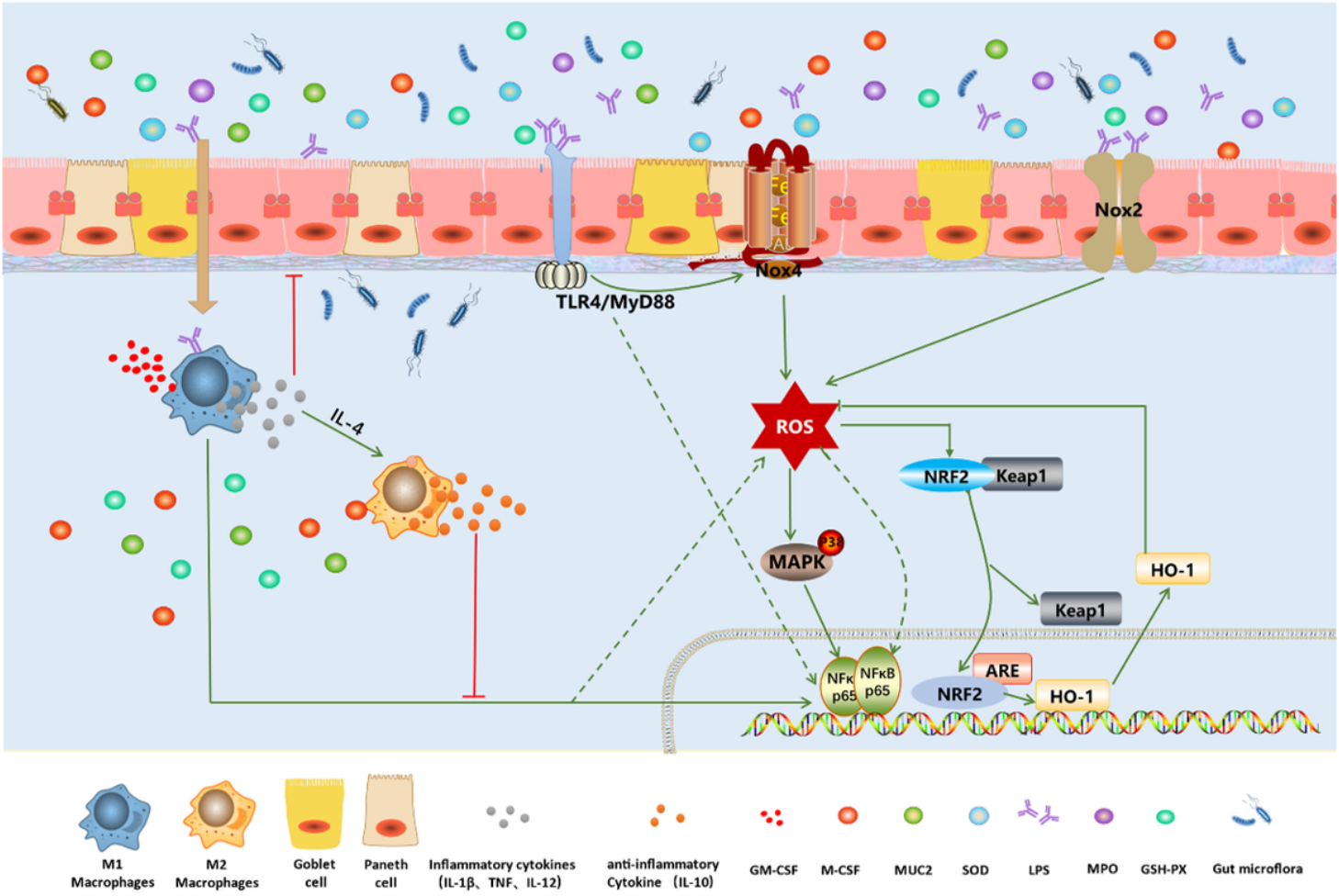

Figure 8

Full-text graphical Abstract Aged Pu-erh tea (2006) inhibited the intestinal oxidative stress-mediated inflammation signaling pathway, promoted the expression of intestinal tight junction proteins, and improved the intestinal immune barrier, which stemmed from the reshaping of intestinal microbiota.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryResults.docx