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*Original Article*

# Chebulagic acid ameliorates DSS-induced colitis in mice by improving oxidative stress, inflammation and the gut microbiota

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**Abstract:** Objectives: Chebulagic acid (CA), isolated from the fruits of *Terminalia chebula Retz*, has a number of pharmacological activities, but its effect on ulcerative colitis (UC) has not been reported. Here, we explored the protective effect of CA against dextran sulfate sodium (DSS)-induced acute colitis and elucidated the potential mechanisms. Methods: The mouse model of DSS-induced acute colitis was employed to evaluate the effect of CA on UC. The expression of pro-inflammatory cytokines and tight junction proteins were evaluated by quantitative real-time PCR (qRT-PCR). Western blotting was used to explore the potential signal pathway. The gut microbiota was analyzed by 16S rDNA amplicon sequencing. Results: The data showed that CA significantly mitigated colitis severity, as manifested by the suppression of weight loss, shortening of colon, disease activity index (DAI) and histopathological score. CA increased superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) activity and reduced malondialdehyde (MDA) content in the colon of colitis mice through inhibiting the mitogen-activated protein kinase (MAPK) pathway and the activating nuclear respiratoty factor 2 (NRF2)/heme oxygenase-1 (HO-1) pathway. Meanwhile, myeloperoxidase (MPO) activity and proinflammatory cytokines levels of the CA group were markedly decreased due to suppression of the nuclear factor kappa-B (NF-κB) signaling pathway. Moreover, CA could upregulate the expression of tight junction proteins and reduced apoptosis. Furthermore, CA remodeled the gut microbiota through suppressing the growth of harmful bacteria (*Clostridium_sensu_stricto_1*, *Streptococcus* and *Escherichia_Shigella*) and promoting the growth of beneficial bacteria (*Faecalibacterium*, *Dubosiella* and *Muribaculaceae*). Conclusions: This study revealed that CA treatment could ameliorate DSS-induced acute colitis mainly via reducing oxidative stress and inflammation, maintaining the integrity of the intestinal barrier and modulating diversity and abundance of gut microbiota; thus, CA may become a promising novel drug candidate for initial and maintenance therapy of UC.

**Keywords:** Ulcerative colitis, chebulagic acid, inflammation, oxidative stress, apoptosis, gut microbiota

## Introduction

Ulcerative colitis (UC) is a common type of inflammatory bowel disease (IBD) marked by persistent, unexplained inflammation in the colon and rectum, following a pattern of recurring flare-ups and periods of remission [1, 2]. It is common knowledge that UC usually presents with abdominalgia, diarrhea, and hematochezia and elevates the likelihood of developing colorectal cancer, which leads to high morbidity and impaired quality of life [3, 4]. Over the last ten years, the global prevalence of UC has increased significantly, especially in newly industrialized countries, which has caused a substantial socioeconomic burden [5, 6]. It is important to increase our comprehension of the pathophysiological mechanism of UC and develop novel effective therapies.

Despite the fact that the etiology and pathophysiology of UC remain ill-defined, numerous previous studies have shown that UC results from a mix of various variables including immune system abnormalities, genetic alterations, intestinal barrier dysfunction, environmental factors, oxidative stress damage, and gut dysbacteriosis [2]. Conventional medications for UC treatment including 5-aminosalicylic acid, glucocorticoids and immunosuppres-

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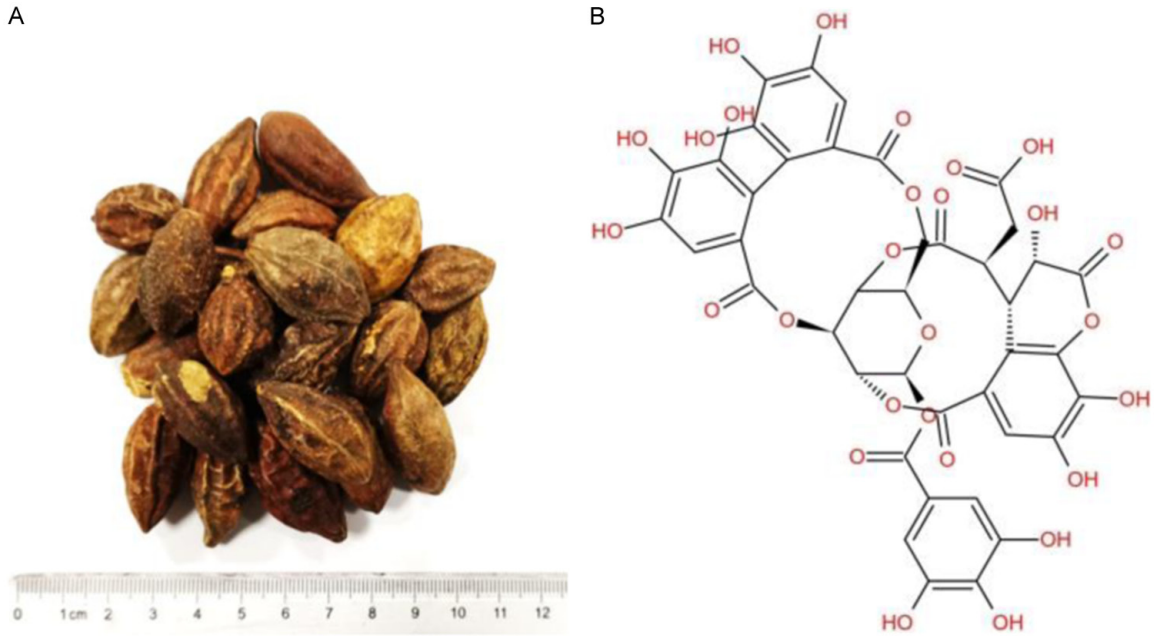


**Figure 1.** A: The fruits of Terminalia chebula Retz. B: The chemical structure of CA.

sants (azathioprine and 6-mercaptopurine) are effective in most mild to moderate UC patients because of their anti-inflammatory and immunosuppressive properties [7, 8]. However, a high proportion of UC patients remain refractory or intolerant to these therapeutic agents. With advances in molecular targeting therapies, new biologics and small molecules have been developed for the treatment of UC. Despite the fact that they have been proven to be effective, novel drugs are expensive and have obvious limitations including the lack of response and adverse side effects [9]. The disorder of gut microbiota is the significant pathogenic factors of UC, and probiotics supplementation and fecal microbiota transplantation have emerged as viable alternative treatments for UC in recent years. However, the effectiveness and safety of these therapies for UC is still controversial [10]. Hence, it is urgent to explore novel, effective and safe agents that reduce excessive inflammation and oxidative stress and remodel the gut microbiota for treating UC.

*Terminalia chebula Retz*, a very popular medicinal and edible medicine in South Asia and Southeast Asia, is widely used to treat a wide spectrum of diseases, such as chronic diarrhea, hemorrhoids, gastroenteritis, chronic cough, asthma, diabetes, tumors, and cardiovascular disease [11]. The reported studies have uncovered that the fruits of *Terminalia chebula Retz* (**Figure 1A**) are rich in phenolcarboxylic acids, tannic acid and bioflavonoids, which have shown various pharmacological activities [12, 13]. Chebulagic acid (CA, **Figure 1B**), an important hydrolyzable tannin isolated from its ripe fruits, is known as a cyclooxygenase (COX)-Lipoxygenase (LOX) dual inhibitor and has antioxidant, anti-tumor, anti-inflammatory and antimicrobial effects [14]. Research has verified that CA demonstrates antioxidant and anti-inflammatory properties via inhibiting the NF-κB and MAPK pathways. In addition to antibacterial and antifungal activity, CA has also shown antiviral activity against a variety of viruses such as influenza and Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) [15]. However, up to now there has been limited research exploring the potential preventive benefits of CA on UC in both animals and humans, and the precise regulatory mechanisms are yet unknown.

This study examined the potential effects of CA on oxidative stress, inflammation, intestinal barrier function, and intestinal microbiota in mouse models with DSS-induced acute colitis using several experimental methods. CA dose-dependently mitigated colitis severity by reducing clinical symptoms, pathological damage, oxidative stress, and inflammatory markers.

Remarkably, the beneficial effect owes to the regulation of MAPK, NRF2/HO-1, and NF-κB pathways and intestinal microflora.

## Materials and methods

### *Materials*

Chebulagic acid (HPLC ≥ 98%, LOT: AB1261-0020) was supplied by Chengdu Alfa Biotechnology Co., Ltd. (Chengdu, China), whereas mesalazine tablets (LOT: H20171358) were supplied by Dr. Falk Pharma GmbH (Freiburg, Germany). The colitis-inducing agent dextran sulfate sodium (LOT: 0216011080) was obtained from MP Biomedicals (Shanghai) Co., Ltd. (Shanghai, China). Fecal occult blood (FOB) detection kits were provided by Baso Diagnostics Inc. (Zhuhai, China). Isoflurane was provided by RWD Life Technology Co., Ltd. (Shenzhen, China). The specific sources of reagents, kits and antibodies are detailed in the corresponding sections.

### *Animals*

C57BL/6 male mice (23 ± 2 g), aged between 8 and 10 weeks, came from the Animal Research Center of Wuhan University (Wuhan, China). The animals were kept in a specific pathogen-free (SPF) facility with controlled environmental conditions, including a 12-hour light/dark cycle, temperatures of 22-24°C, and humidity levels of 55-65%. The mice had unlimited access to water and a conventional food. The animal tests were carried out in accordance with the guiding principles of the Animal Care and Ethics Committee of General Hospital of Central Theater Command China (Certification No.: 2022203, 28 March 2022).

### *Establishment of experimental colitis model and treatment*

The experimental schedule is shown in **Figure 2A**. Using a random numbers table, forty mice were divided into five groups (n = 8) after 7-day adaptation: control group, DSS group, low-dose CA group (LCA), high-dose CA group (HCA) and mesalazine group. The control group received a normal diet and was gavaged with saline every day. The DSS group was fed with 3% DSS solution freely and gavaged with saline every day. Mice in the LCA and HCA groups received 20 and 100 mg/kg CA once a day by gastric lavage, starting 7 days before drinking 3% DSS solution. The Mesalazine group was fed with 3% DSS solution freely and orally gavaged with 100 mg/kg mesalazine once a day. All mice were anesthetized with isoflurane on the 8th day, and the samples of cecal contents were collected and frozen in liquid nitrogen for subsequent detection. Colon length was measured and photographed. Blood samples and segments of the colon were gathered and stored in a -80°C freezer for further analysis. The remaining colon segments were fixed with 4% formalin.

### *Disease activity index score*

During modeling and drug treatment, the stool consistency, changes of weight loss and colorectal hemorrhage were recorded every day. Based on previous study [16], the mean value of these three parameters was calculated as disease activity index (DAI, **Table 1**).

### *Histology*

The fixed colon segments were embedded in paraffin and sliced (5 μm). After dewaxing, dehydration and staining with hematoxylin-eosin, the slices were photographed with 3DHISTECH's image analysis platform. As previously reported [16], histopathological damage was blindly scored for the severity of inflammation, the degree of polymorphonuclear neutrophil (PMN) infiltration, crypt structure damage, and the range of lesions (**Table 2**).

### *Determination of the oxidative stress markers*

The colonic levels of Superoxide Dismutase (SOD, #A001-3-2), Glutathione Peroxidase (GSH-PX, #A005-1-2) activity, and Malondialdehyde (MDA, #A003-1-2) content were measured using commercial assay kits (Nanjing Jiancheng, Nanjing, China) following the specified technique.

### *Myeloperoxidase (MPO) activity measurement*

The colonic tissue samples were weighed and homogenized with cold phosphate buffered saline (w/v, 1:9) on ice using a hand-held tissue homogenizer and then centrifuged at 12,000 rpm for 15 min. The supernatant was gathered and the activity of colonic MPO (#A044-1-1) was determined using commercially available kits (Nanjing Jiancheng, Nanjing, China).

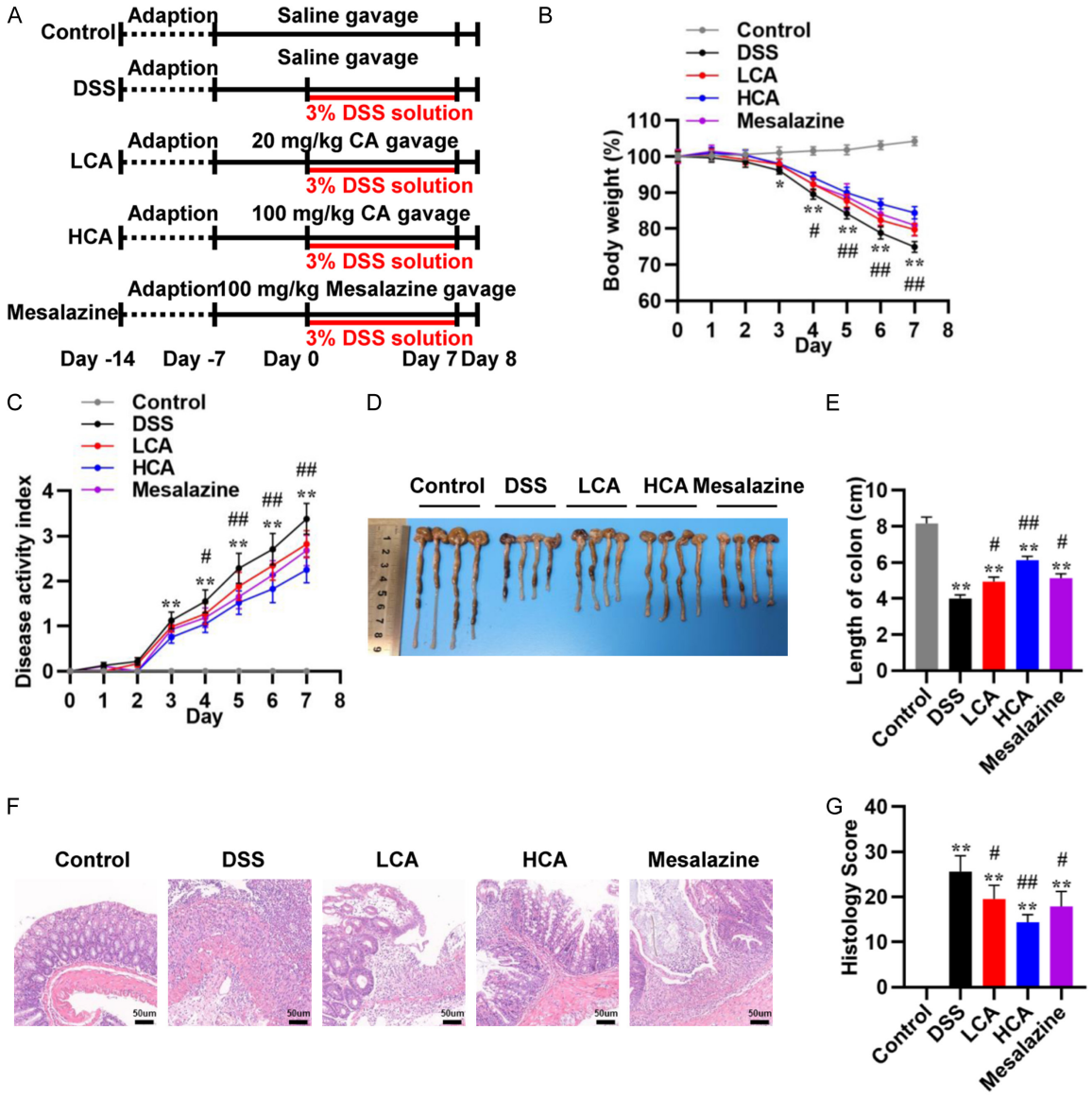


**Figure 2.** CA ameliorates DSS-induced acute colitis. A: Schedule of the experimental design. B: The percentage of body weight loss. C: Disease activity index (DAI) score. D: Representative images of the colon. E: Length of the colon. F: Representative HE staining of colon tissues, scale bar: 100 μm. G: Histology score. Values are expressed as the mean ± SD (n = 6-8 samples/group). *$P < 0.05$, **$P < 0.01$ versus the control group; #$P < 0.05$, ##$P < 0.01$ versus the DSS group.

**Table 1.** The disease activity index (DAI) scoring system

| Weight loss | Stool consistency | Fecal occult blood | Score |
|---|---|---|---|
| < 1% | Hard | Negative | 0 |
| 1-5% | - | - | 1 |
| 5-10% | Soft | Positive | 2 |
| 10-20% | - | - | 3 |
| > 20% | Diarrhea | Gross bleeding | 4 |

*Inflammatory cytokines measurement*

The levels of tumor necrosis factor-alpha (TNF-α, #MU30030), interleukin-6 (IL-6, #MU30044) and Interleukin-1 beta (IL-1β, #MU30369) in serum and colonic tissue were tested by using enzyme-linked immunosorbent assay kits (Bioswamp, Wuhan, China). Briefly, according to the protocol, all

**Table 2.** Histology scoring system

| Inflammation Severity | Polymorphonuclear neutrophil (PMN) infiltration/high-power field (HPF) | Injury depth | Tissue involvement (percentage factor) | Score |
|---|---|---|---|---|
| None | < 5 | None | 0-25% (× 1) | 0 |
| Slight | 5-20 | Mucosa | 26-50% (× 2) | 1 |
| Moderate | 21-60 | Submucosa And Mucosa | 51-75% (× 3) | 2 |
| Severe | 61-100 | Transmural | 76-100% (× 4) | 3 |
| - | > 100 | - | - | 4 |

reagents and samples were prewarmed to room temperature and prepared in clean test tubes. We incubated the standard and samples in an assay plate for 60 minutes at 37°C after adding HRP-Conjugate reagent. After four plate washings, we added Substrates A and B, and incubated for 15 minutes at 37°C. Afterward, the stop solution was included and measurements were taken within a 15-minute timeframe.

*Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling assay (TUNEL) assay*

Sections were stained with a commercial TUNEL assay kits (Roche, Germany) following the protocol as previously described [17], and then photographed by 3DHISTECH's image analysis platform. The positive cells labeled with green fluorescence were blindly counted in 10 HPF/section (×200).

*Quantitative real-time PCR (qRT-PCR)*

Total RNA was extracted from colon tissues using a commercial kit (Takara Company, Japan) according to protocol. The qRT-PCR test was performed in accordance with earlier instructions [17]. GAPDH was used as an internal reference gene. All primer sequences are as follows: TNF-α, forward: ACGTGGAACTGGCAGAAGAG, reverse: CTCCTCCACTTGGTGGTTTG; IL-1β, forward: TTGAGGGACCCCAAAAGATG, reverse: TGGACAGCCCAGGTCAAAG; IL-6, forward: CGACGGCCTTCCCTACTT, reverse: TGGGAGTGGTATCCTCTGTGAA; ZO-1, forward: CTCTCCTGTACCTCTTGAGCC, reverse: CAGAAATCGTGCTGATGTGCC; Occludin, forward: CCGGCCGCCAAGGTTC, reverse: GCTGATGTCACTGGTCACCTA; GAPDH, forward: CGTCCCGTAGACAAAATGGT, reverse: TTGATGGCAACAATCTCCAC.

*Western blotting*

Lysis buffer with broad-spectrum protease and phosphatase inhibitors was used for extracting total proteins, and nuclear proteins were extracted from the colon samples by means of a commercial nuclear protein extraction kit (Beyotime Biotechnology, China). Western blotting was conducted as previously reported [17]. The primary antibodies and dilutions are as follows: Histone H3 (CST, #4499, 1:1000), Occludin (Abcam, #ab216327, 1:1000), ERK1/2 (CST, #4695, 1:2000), β-actin (Beyotime, #AF0003, 1:5000), P38 (CST, #8690, 1:2000), ZO-1 (Abcam, #ab276131, 1:1000), p-ERK1/2 (CST, #4370, 1:2000), Bcl2 (Beyotime, #AF6285, 1:1000), p-P38 (CST, #4511, 1:2000), Bax (Beyotime, #AF0057, 1:1000), JNK (CST, #4668, 1:2000), Cleaved caspase-3 (CST, #9661, 1:1000), p-JNK (CST, #9251, 1:1000), NRF2 (CST, #12721, 1:1000), P65 (CST, #8242, 1:2000), HO-1 (CST, #43966, 1:1000), and p-P65 (CST, #3033, 1:1000). The secondary antibodies (A0208, 1:2000; A0216, 1:2000) were purchased from Beyotime Biotechnology.

*16S rDNA amplicon sequencing*

16S rDNA sequencing was performed as previously reported [18]. Total genomic DNA was extracted from mouse fecal samples using (MP Biomedicals, USA). The DNA samples pyrosequencing and further data analysis were completed by Applied Protein Technology Co., Ltd. (Shanghai, China).

*Statistical analysis*

The study data is displayed as the mean ± standard deviation (SD). The SPSS 25.0 statistical software (NY, USA) was used to data analysis. Significance was determined using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. STAMP software was used to verify the disparities in individual taxonomic abundances between the two groups. The quantitative analysis of biomarkers within various groups was conducted using LefSe.

ANOSIM and ADONIS were used to determine the differences in the microbial communities between the two groups using the Bray-Curtis dissimilarity distance matrices. Graphs were generated in GraphPad Prism 9.0 software. $P < 0.05$ indicated statistically significant differences.

## Results

*CA ameliorates DSS-induced acute colitis*

For exploring the potential impact of CA on colitis, male mice with acute colitis caused by DSS were given 20 mg/kg of CA (LCA group), 100 mg/kg CA (HCA group) and 100 mg/kg mesalazine (positive control group) by gavage. No mice died during the entire experiment. Weight loss and DAI scores were recorded every day throughout the experiment. As illustrated in **Figure 2B**, the percentage of body weight loss in the DSS group consistently increased from day 3 to day 7, whereas the HCA group showed a significant improvement on day 4. On day 7, the DSS group had 25.09% weight loss, and the weights of the LCA, HCA and mesalazine groups were reduced by 20% to 15% and 19%, respectively ($P < 0.01$). The DAI scores on day 7 were considerably higher in the DSS group compared to the control group. Treatment with CA at dosages of 20 and 100 mg/kg significantly reduced the DAI score ($P < 0.01$) compared to the DSS group (**Figure 2C**), indicating its effectiveness. A similar pattern of amelioration by CA was seen for colon length (**Figure 2D**, **2E**), and compared with the DSS group, the two CA groups and mesalazine groups observed a significant increase in colon length on day 7 ($P < 0.01$). In addition, HE staining was utilized to assess histopathological injury. The colonic histopathology in the DSS group showed excessive intestinal inflammatory response and epithelial barrier damage, while the two CA groups and the mesalazine groups had some protection, as indicated by reduced intestinal inflammation and maintenance of epithelial barrier integrity (**Figure 2F**). Furthermore, the histology score of the DSS group was 25.63 ± 3.50, which was higher than that of the other groups, whereas histology scores of the LCA, HCA and mesalazine groups markedly decreased to 19.50 ± 3.07 ($P < 0.05$), 14.38 ± 1.69 ($P < 0.01$), and 17.88 ± 3.31 ($P < 0.05$), respectively (**Figure 2G**). The data suggested that CA treatment dose-dependently alleviated the symptoms of colitis in mice.

*CA reduces colonic oxidative stress damage*

Oxidative stress is a significant pathogenic element of UC. The main oxidative stress markers were found in order to illustrate the antioxidative impact of CA in colitis produced by DSS. As shown in **Figure 3**, the colon tissue of the DSS group had lower levels of antioxidant enzymes (GSH-PX and SOD) and higher levels of MDA when compared to the control group ($P < 0.01$). Compared to the DSS group, LCA, HCA, and mesalazine therapy dramatically decreased MDA (**Figure 3A**) levels in colon tissue ($P < 0.01$) and considerably enhanced the levels of SOD (**Figure 3B**) and GSH-PX (**Figure 3C**) ($P < 0.05$, $P < 0.01$, $P < 0.05$, respectively).

To further research the antioxidative molecular mechanism of CA, MAPK (the main oxidative stress signaling pathways) relevant proteins were examined. As illustrated in **Figure 3D**, the ERK, P38 and JNK protein levels among the five groups had no significant differences. However, the p-P38 (1.63-fold of control mice, $P < 0.01$), p-ERK (4.66-fold of control mice, $P < 0.01$), and p-JNK (1.51-fold of control mice, $P < 0.01$) levels in the DSS group were greater than in the control group. HCA and mesalazine treatment significantly reduced the levels of p-P38 ($P < 0.01$), p-ERK ($P < 0.01$), and p-JNK ($P < 0.01$) compared with those in the DSS group. However, the p-P38 level between the LCA and DSS groups showed no obvious difference.

NRF2 and HO-1 are considered cytoprotective factors against oxidative stress [19]. As seen in **Figure 3E**, the nuclear NRF2 and HO-1 protein levels in the DSS group were significantly lower than those of the control group ($P < 0.05$ and $P < 0.01$, respectively); meanwhile, these reductions were reversed in the LCA, HCA and mesalazine groups, and levels of NRF2 and HO-1 in the HCA group sharply increased ($P < 0.01$). These findings implied that CA treatment might have the potential to mitigate colonic oxidative stress injury by dose-dependently inhibiting the MAPK pathway and activating the NRF2/HO-1 pathway.

*CA inhibits inflammatory responses*

Inflammatory processes are crucial in the development of UC [20]. To investigate the impact of CA on inflammatory reactions, proinflammatory cytokines (TNFα, IL-1β and IL-6) were detected in the serum and colon. In the

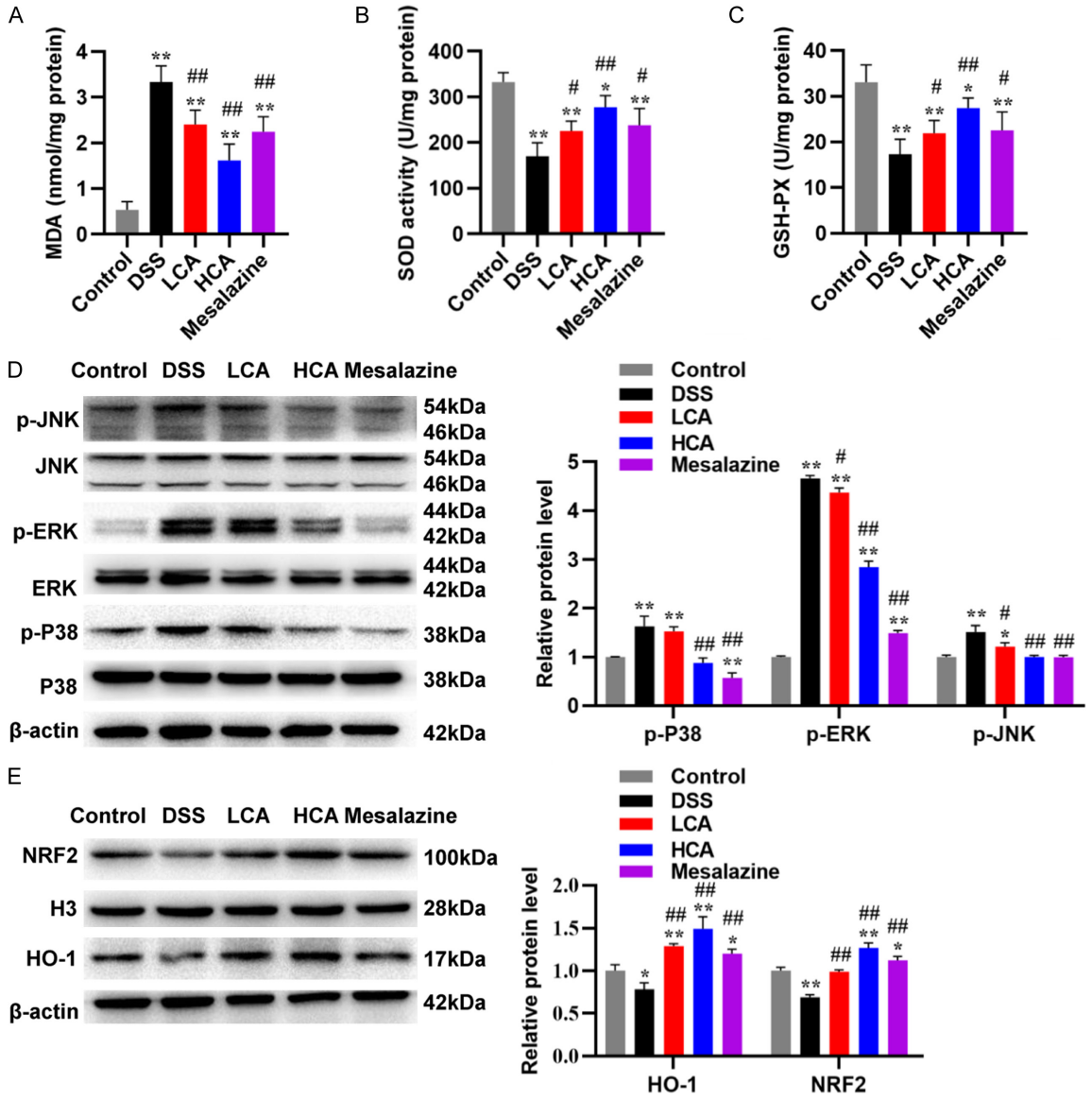


**Figure 3.** CA reduces colonic oxidative stress damage. A: MDA content assay of colon tissue. B: SOD activity of colon tissue. C: GSH-PX activity of colon tissue. D: Western blotting bands (left) and quantitative analysis of phospho-JNK (p-JNK), p-P38 and p-ERK expression levels (right) in colon tissue, representative of three experiments. E: Western blotting bands (left) and quantitative analysis of HO-1 and nuclear NRF2 expression levels (right) in colon tissue, representative of three experiments. Values are expressed as the mean ± SD (n = 6-8 samples/group). *$P < 0.05$, **$P < 0.01$ versus the control group; #$P < 0.05$, ##$P < 0.01$ versus the DSS group.

DSS group, the levels of three proinflammatory cytokines in the serum were higher than those in the control group (**Figure 4A-C**, $P < 0.01$). However, CA and mesalazine markedly reduced proinflammatory cytokine production, and the reduction of these three cytokines in the HCA group was more significant than those in the LCA group ($P < 0.01$). Consistent findings were noted regarding the mRNA expression levels of the three cytokines in colon tissue (**Figure 4D-F**). MPO activity, a crucial marker of neutrophil infiltration, was notably higher in the DSS group than in the control group ($P < 0.01$). However, the MPO activity in the HCA group was lower than that of the DSS group ($P < 0.01$), which was in accord with the colonic histopathology manifestation (**Figure 4G**).

In order to further elucidate the mechanism underlying CA's anti-inflammatory effect, NF-κB

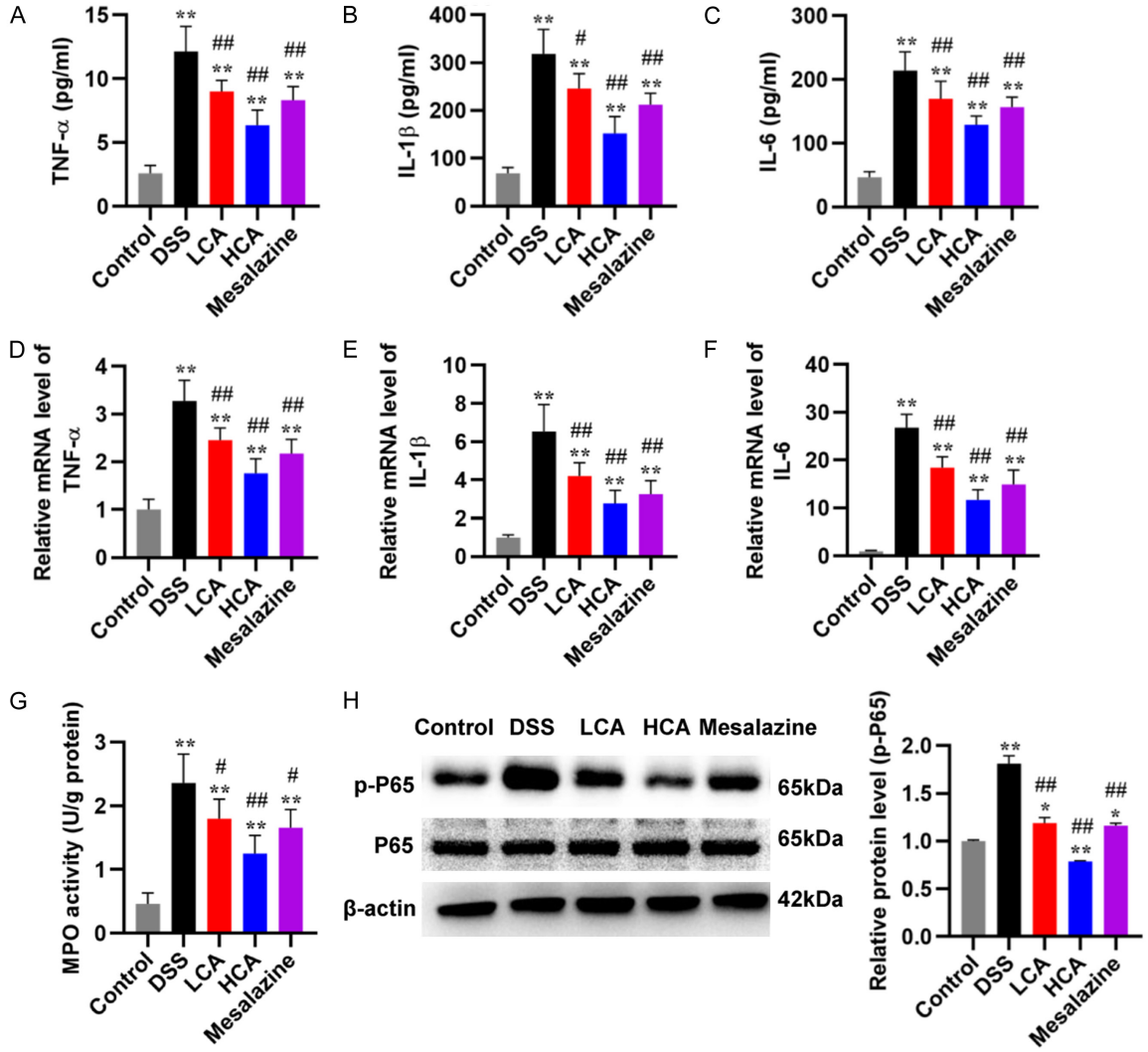


**Figure 4.** CA inhibits inflammatory responses. A-C: Serum TNF-α, IL-1β and IL-6 level analysis by ELISA. D-F: mRNA expression levels of TNF-α, IL-1β and IL-6 in colon tissue. G: MPO activity in colon tissue. H: Western blotting bands (left) and quantitative analysis of p-P65 expression levels (right) in colon tissue, representative of three experiments. Values are expressed as the mean ± SD (n = 6-8 samples/group). *$P$ < 0.05, **$P$ < 0.01 versus the control group; #$P$ < 0.05, ##$P$ < 0.01 versus the DSS group.

protein was measured in colon tissue. The protein expression of P65 among all groups had no significant difference (**Figure 4H**). Nonetheless, the p-P65 protein level (1.81-fold of control mice, $P$ < 0.01) in the DSS group significantly increased. Interestingly, upregulation of p-P65 protein expression level was suppressed in the two CA groups and the mesalazine group ($P$ < 0.01), but showed no difference between the LCA and mesalazine groups. These findings showed that CA treatment could reduce the production of inflammatory cytokines, at least in part, via the NF-κB pathway.

*CA improves the intestinal epithelial barrier*

The disruption of the epithelial barrier is the main pathological characteristic of human UC [21]. ZO-1 and Occludin are fundamental indicators of intestinal epithelial cells, serving as the primary tight junction proteins crucial for preserving the integrity of the epithelial barrier [22]. **Figure 5A**, **5B** illustrates that the mRNA expression of ZO-1 and Occludin in the DSS group was significantly lower than that of the control group ($P$ < 0.01). However, LCA, HCA and mesalazine treatment remarkably increa-

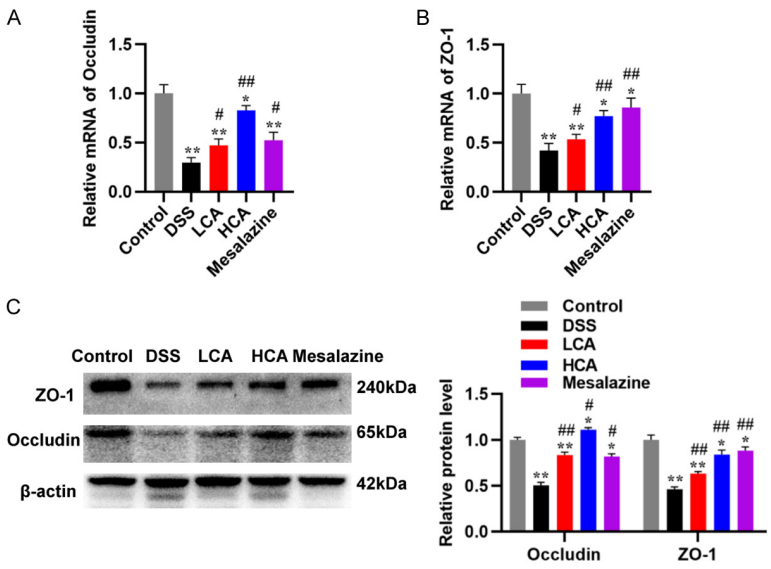


**Figure 5.** CA improves intestinal epithelial barrier. A: mRNA expression of ZO-1 in colon tissue. B: mRNA expression of occludin in colon tissue. C: Western blotting bands (left) and quantitative analysis of ZO-1 and Occludin expression levels (right) in colon tissue. Representative of three experiments. Values are expressed as the mean ± SD (n = 6-8 samples/group). *$P < 0.05$, **$P < 0.01$ versus the control group; #$P < 0.05$, ##$P < 0.01$ versus the DSS group.

sed the mRNA expression of ZO-1 ($P < 0.05$, $P < 0.01$, $P < 0.05$, respectively) and Occludin ($P < 0.05$, $P < 0.01$, $P < 0.01$, respectively). There was no difference in mRNA expression of ZO-1 between the HCA and mesalazine groups. Similar as was seen in RT-PCR, Western blotting showed that the levels of ZO-1 (46% of the control group, $P < 0.01$) and Occludin (51% of the control group, $P < 0.01$) proteins were significantly lower than in the control group. However, CA and mesalazine upregulated the protein level of ZO-1 and Occludin in colon tissues (**Figure 5C**). Interestingly, ZO-1 and Occludin protein levels in the HCA group were 1.82-fold ($P < 0.05$) and 2.20-fold ($P < 0.01$) higher in the DSS group, respectively, than in the control group. The findings demonstrated that CA increased the mRNA and protein levels of tight junction proteins, hence improving the function of the epithelial barrier.

*CA alleviates DSS-induced apoptosis in intestinal epithelial cells*

Apoptosis of intestinal epithelial cells contributes to the progression of UC [23]. To further determine the anti-apoptotic effect of CA, TUNEL assays and immunoblot analysis were performed to measure apoptosis in colon tissues. As previously reported, DSS treatment resulted in apoptosis of intestinal epithelial cells. There was a significant increase in the number of TUNEL-positive cells in the DSS group compared to the control group (**Figure 6A**, $P < 0.01$). The amount of TUNEL-positive cells was decreased by the LCA, HCA, and mesalazine treatments ($P < 0.05$, $P < 0.01$, $P < 0.01$, respectively). Correspondingly, the protein levels of pro-apoptotic proteins (Bax and cleaved caspase-3) in the DSS group were 3.14-fold ($P < 0.01$) and 4.41-fold ($P < 0.01$) higher than those in the control group, while the protein level of Bcl2 was reduced to 67% that of control group ($P < 0.01$). Nevertheless, CA and mesalazine treatments reversed this tendency. In comparison to the DSS group, the HCA group showed a 71% ($P < 0.01$) decrease in Bax and a 47% ($P < 0.01$) decrease in cleaved caspase-3 protein levels, whereas the Bcl2 protein level increased to 1.86 times that of the control group ($P < 0.01$) (**Figure 6B**). These data demonstrated that CA could inhibit DSS-induced apoptosis in intestinal epithelial cells through the Bax/Bcl2 and cleaved caspase-3 pathways.

*CA remodels the composition and abundance of gut microbiota*

Given that the disruption of gut microbiota is intricately linked to the onset and advancement of ulcerative colitis (UC) [24], 16S rDNA amplification sequencing was performed to investigate the effect of CA on gut flora. There were 874 operational taxonomic units (OTUs) found in all samples; of which, 207 OTUs were shared by all groups (**Figure 7A**). The amount of unique OTUs identified in the control group, DSS group, and HCA group was 232, 191, and 77, respectively. Moreover, the α-diversity of the gut microbiota was assessed by community richness (Chao1 and ACE index) and communi-

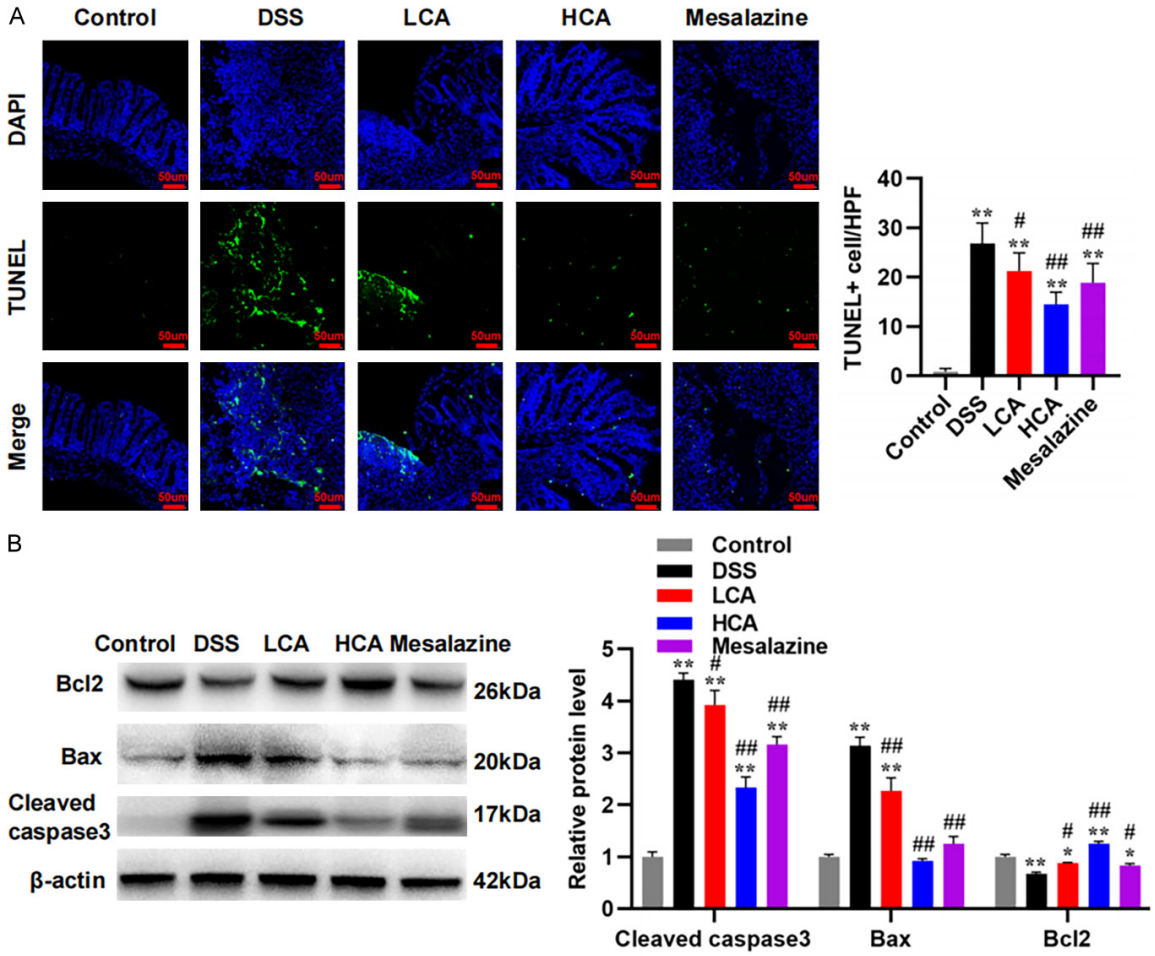


**Figure 6.** CA alleviates DSS-induced apoptosis in intestinal epithelial cells. A: Immunofluorescence images from the TUNEL assay in colon tissue (left); scale bar: 50 μm. The results were scored semiquantitatively by averaging the number of positively stained cells (mean ± SD)/field (right). B: Western blotting bands (left) and quantitative analysis of apoptosis-related proteins (Bcl-2, Bax and cleaved caspase-3) in colon tissue. Representative of three experiments. Values are expressed as the mean ± SD (n = 6-8 samples/group). *$P < 0.05$, **$P < 0.01$ versus the control group; #$P < 0.05$, ##$P < 0.01$ versus the DSS group.

ty diversity (Simpson and Shannon index). The Chao1 and ACE indexes among the three groups had no obvious difference (**Figure 7B**). However, the Simpson and Shannon indexes were much lower in the DSS group versus the control group ($P < 0.01$), and these decreases were partially reversed by HCA administration (compared to control group, $P < 0.05$). The β-diversity of the gut microbiota was assessed by PCoA based on the weighted UniFrac metric. As shown in **Figure 7C**, the community structure of the DSS group was distinct from that of the control group, while the HCA group was separate from the DSS group and similar to the control group. Remarkably, the outcomes of the ANOSIM study agreed with those of the PCoA analysis. As shown in **Figure 7D**, the R value of the three groups was 0.742 ($P < 0.01$), indicating a substantial difference in their community structure.

To further explore the change in gut microbiota, we analyzed alterations in specific bacterial populations at different levels (phylum, genus). As shown in **Figure 8A**, **8B**, *Firmicutes* and *Proteobacteria* were the two dominant phyla in all samples at the phylum level. The proportion of *Firmicutes* decreased from 35% in the control group to 14% in the DSS group and recovered to 38% in the HCA group, while the proportion of *Proteobacteria* increased from 1.6% in the control group to 66% in the DSS group and fell back to 32% in the HCA group. In addition, LefSe analysis was performed to determine the

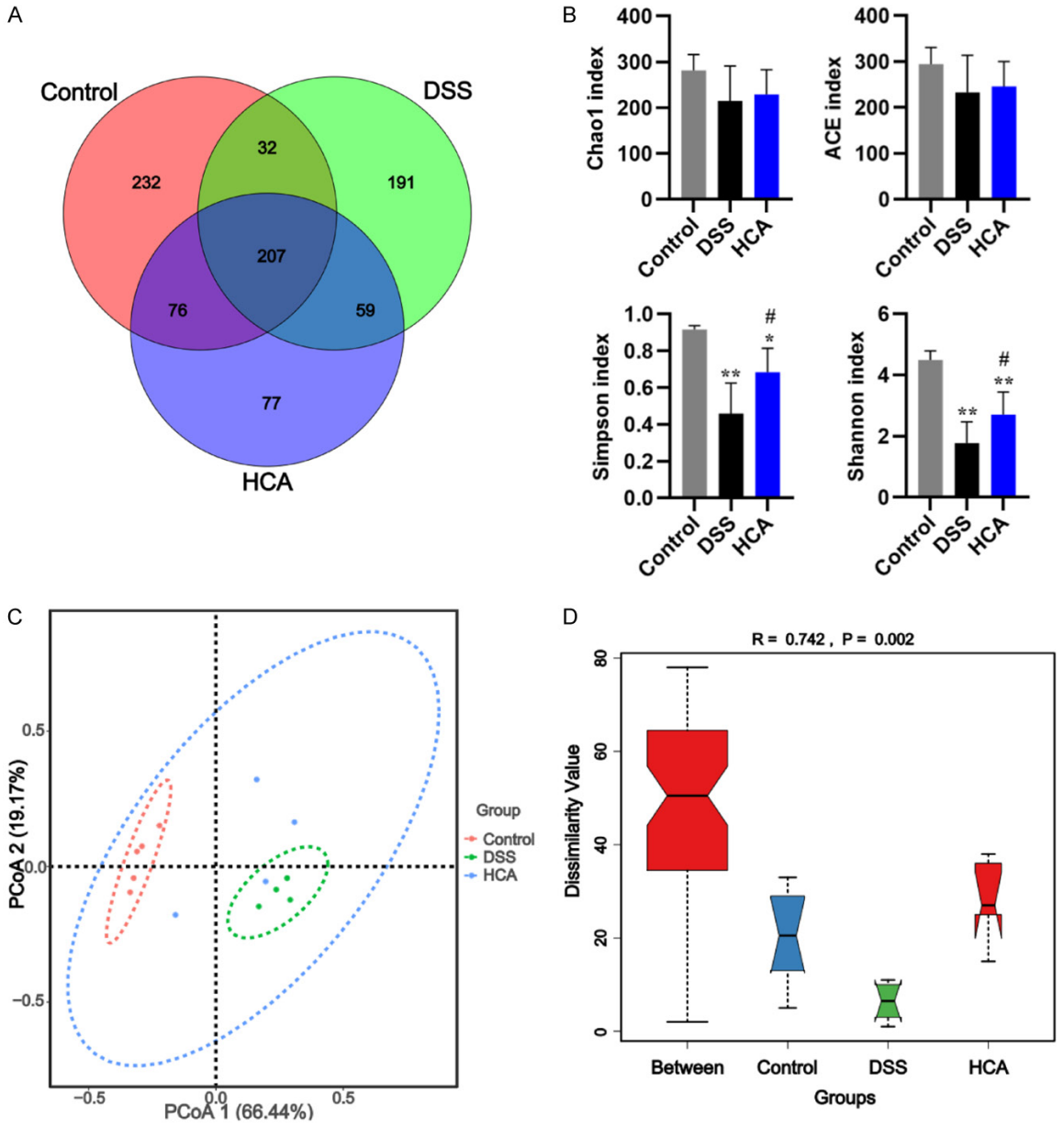


**Figure 7.** CA restores microbial diversity. A: Venn diagram. B: α-diversity was assessed by the Chao1, ACE, Simpson and Shannon indexes. C: β-diversity was assessed by weighted principal co-ordinates analysis (PCoA). D: Analysis of similarities (ANOSIM). Values are expressed as the mean ± SD (n = 3-4 samples/group). **P* < 0.05, ***P* < 0.01 versus the control group; #*P* < 0.05, ##*P* < 0.01 versus the DSS group.

differential flora. The cladogram of the three groups is shown in **Figure 8C**, and there were more than 20 biomarkers at the genus level. In the DSS group, the levels of *Escherichia_Shigella*, *Streptococcus*, and *Clostridium_sensu_stricto_1* were significantly higher compared to the control group. However, HCA therapy decreased the presence of these microorganisms. Compared to control group, the proportions of *Faecalibaculum*, *Muribaculaceae* and *Dubosiella* in the DSS group were lower, but these decreases were reversed by HCA treatment (**Figure 8D**). However, 16S rDNA sequencing analysis results indicated CA treatment remodeled the gut microbiota of DSS-induced colitis mice.

## Discussion

With a dramatic rise in the worldwide incidence and prevalence, UC has become one of the main public health challenges. Currently, a vari-

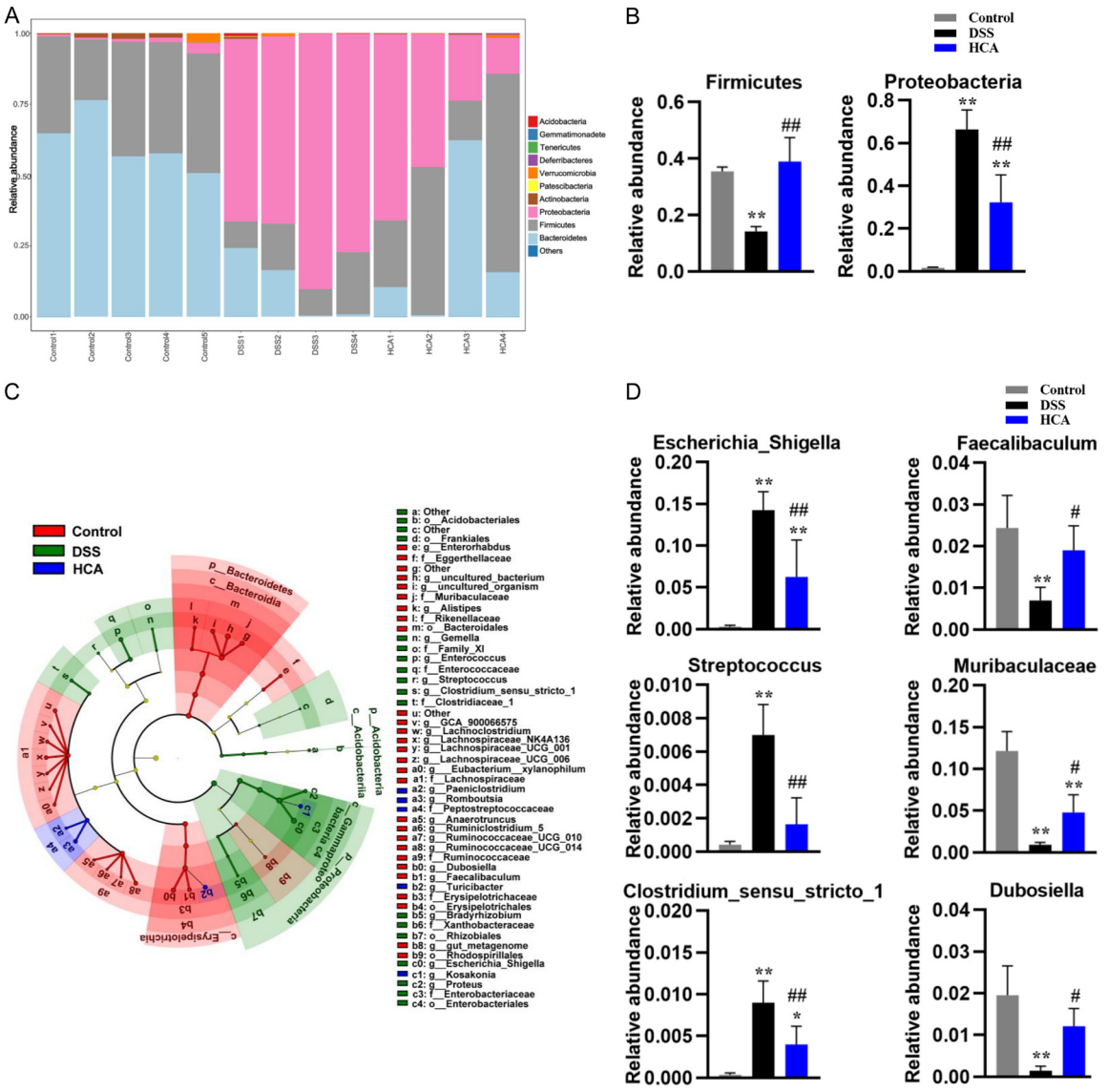


**Figure 8.** CA remodels specific gut bacterial composition. A: Histogram of the relative abundance of the top 10 microbial communities at the phylum level. B: Relative abundance (%) of *Firmicutes* and *Proteobacteria*. C: Linear discriminant analysis Effect Size (LEfSe) analysis (cladogram). D: Relative abundance (%) of *Faecalibacterium*, *Dubosiella, Muribaculaceae, Escherichia_Shigella*, *Streptococcus, and Clostridium_sensu_stricto_1*. Values are expressed as the mean ± SD (n = 3-4 samples/group). **P* < 0.05, ***P* < 0.01 versus the control group; #*P* < 0.05, ##*P* < 0.01 versus the DSS group.

ety of treatments for UC are available, but none of them can fully meet the needs of UC patients [25]. Traditional Chinese medicine has been commonly utilized as an alternative therapy for individuals with ulcerative colitis due to its effectiveness and safety, despite incomplete understanding of its causes [26, 27]. *Terminalia chebula Retz*, known as king of medicines in Tibetan medicine, is commonly utilized for treating diarrhea in China [28]. The reported studies have revealed that the extract and active component of *Terminalia chebula Retz* display therapeutic effects against colitis [29]. CA, one of the predominant components of *Terminalia chebula Ret* fruits, exerts good anti-inflammatory and antioxidant effects. However, limited research has explored the therapeutic impact of CA on UC. Mesalazine, also known as 5-aminosalicylic acid, is currently recognized as a first-line therapeutic agent for the treatment of UC, which has a good effect on inhibiting intestinal inflammation with severe adverse

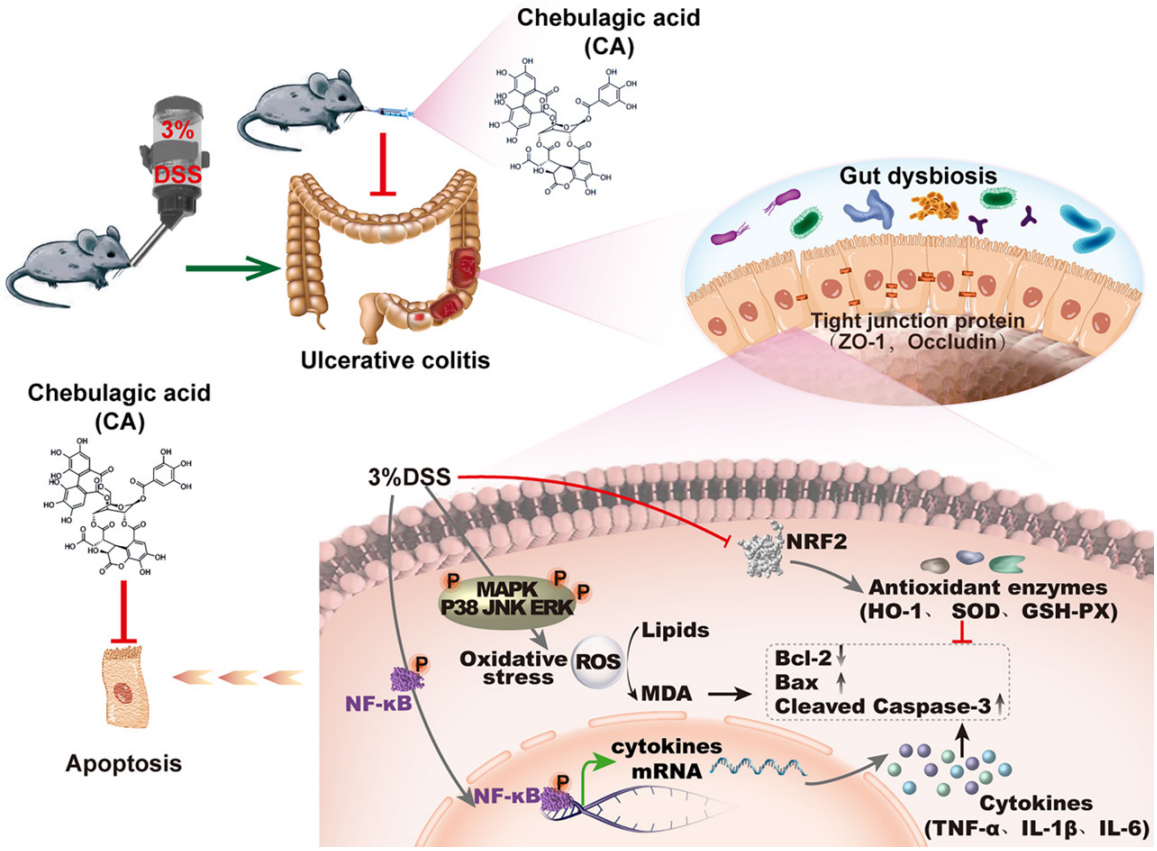


**Figure 9.** Proposed mechanistic model of CA effectively ameliorating DSS-induced colitis.

reactions being relatively rare. Mesalazine was frequently selected as a positive control in the drug treatment of colitis, which can provide a clear reference standard for comparing the effect of other drugs under testing. A recent study confirmed that mesalazine could restore the diversity of the gut microbiota and short chain fatty acids (SCFAs) [30]. In this work, these findings indicated that CA treatment could ameliorate DSS-induced colitis in animals through reducing oxidative stress and inflammation, improving intestinal barrier function and remodeling the gut microbiota, which was similar to the role of mesalazine. Furthermore, our results indicated that the beneficial effects were partially mediated through the activation of NRF2/HO-1 and the inhibition of the MAPK and NF-κB signaling pathways (**Figure 9**).

In this study, a mouse colitis model was successfully established using 3% DSS for 7 days, and no mouse deaths were observed during the experiment, which may be related to the mouse strain and characteristics as well as environmental factors. As we know, oxidative stress and inflammation are significant pathogenic components in UC, caused by various sources [31]. Oxidative stress is indicated by an imbalance between antioxidants and reactive oxygen species (ROS). Many previous studies have reported the overproduction of ROS and the reduction in antioxidant components in an animal model of UC [32]. Excessive ROS can harm intestinal cells and trigger inflammation, resulting in the production of several proinflammatory cytokines that may exacerbate oxidative stress and cell damage. Therefore, antioxidant and anti-inflammatory therapies are the main approaches for the treatment of UC. Consistent with Deng et al. [33], our study observed a significant increase in MDA content and MPO activity, a sharp decrease in SOD and GSH-PX activity in colon tissue, and significantly elevated levels of proinflammatory cytokines in DSS-induced colitis mice. On the contrary, CA treatment dose-dependently reduced MDA content, inflammatory cytokine levels, and MPO

activity. The MAPK pathway, which includes ERK, P38 and JNK and is an essential signal transduction pathway for multiple stimulus factors, plays an essential regulatory role in cell proliferation, apoptosis, oxidative stress, inflammation and other processes [34]. In particular, the MAPK pathway can regulate the transcriptional activity of NF-κB, which is an important transcription factor that regulates inflammation [35]. Many studies have shown that MAPK and NF-κB pathway activation aggravates the inflammatory response and oxidative stress damage, while inhibition of the MAPK and NF-κB pathways can be beneficial to UC [33, 36]. In this study, CA treatment dose-dependently reduced the phosphorylation of ERK, P38, JNK and P65, which is consistent with previous research in LPS-stimulated endothelial cells and RAW 264.7 macrophages [37]. What's more, previous study had revealed that CA could inhibit P38, ERK and P65 phosphorylation in TNF-α-induced retinal capillary endothelial cells [38]. Collectively, these findings demonstrated that CA treatment could attenuate oxidative stress and the inflammatory response by inhibiting the MAPK and NF-κB pathways in DSS-induced colitis mice.

Notably, CA treatment not only reduced oxidative stress and inhibited the inflammatory response but also increased the antioxidant capacity by restoring the expression of antioxidant enzymes (SOD, HO-1 and GSH-PX) in colon tissue. Previous studies have suggested that SOD and GSH-PX can reduce oxidative stress damage and inflammation and are considered potential drugs for UC treatment [39]. NRF2, a transcription factor belonging to the CNC family, is crucial in controlling the expression of molecules that fight inflammation and provide antioxidant protection. NRF2 exists in the cytoplasm under normal conditions, while after ROS stimulus, NRF2 moves to the nucleus and attaches to antioxidant response elements (AREs), leading to upregulation of antioxidant enzymes such SOD, HO-1, and GSH-PX. Many studies have found that traditional Chinese medicine alleviates DSS-induced colitis via activation of the NRF2/HO-1 pathway [40, 41]. In this study, CA treatment dose-dependently increased the protein level of nuclear NRF2, along with upregulating the expression of the downstream antioxidant enzyme HO-1. Ferroptosis is a type of cell death that is caused by lipid peroxides and dependent on iron. Much evidence suggests that inhibition of ferroptosis can ameliorate DSS-induced colitis [42, 43]. Numerous proteins related to lipid peroxidation and ferroptosis are regulated by NRF2 at the transcriptional level. Therefore, NRF2 has been identified as a core regulator in mitigating lipid peroxidation and ferroptosis [44]. CA, a COX-LOX dual inhibitor, was recently identified as a novel ferroptosis inhibitor [14, 45]. Therefore, the findings of this study indicated that CA could ameliorate acute colitis by activating the NRF2/HO-1 pathway.

Another remarkable finding in this study was that CA treatment could dose-dependently upregulate the ZO-1 and Occludin mRNA and protein expression, and inhibit the intestinal epithelial cells apoptosis. Breakdown of the intestinal barrier is one of the main characteristics of UC, which leads to the penetration of toxins and bacteria into the damaged mucosa [3, 46]. In addition, intestinal barrier dysfunction triggers an inflammatory response that contributes to the initiation and development of UC. It is well known that the intestinal barrier consists of tight junctions and intestinal epithelial cells. ZO-1 and Occludin are common tight junction proteins that are crucial for controlling the permeability of the intestinal barrier [47]. Our results confirmed that CA could maintain intestinal barrier integrity by increasing the expression of tight junction proteins. The balance between the proliferation and death of intestinal epithelial cells is essential for preserving the integrity of the intestinal barrier. Numerous studies have indicated that excessive apoptosis of epithelial cells caused by ROS and other factors is one of the major causes of UC [48, 49]. It has been found that the apoptosis of epithelial cells is overactivated in colitis mice and UC patients [50] due to the activation of the MAPK and NF-κB pathways that regulate apoptosis-related proteins. Recent investigations have shown that certain natural herbal medications can alleviate DSS-induced colitis by preventing epithelial cell death [51, 52]. Our results showed that CA treatment significantly inhibited epithelial cell apoptosis by upregulating the antiapoptotic protein Bcl-2 and downregulating the proapoptotic proteins caspase-3 and Bax, indicating that CA can improve intestinal barrier integrity by suppressing excessive apoptosis.

The gut microbiota, a complex community of microorganisms that includes bacteria, fungi and viruses, is essential for maintaining the intestinal barrier's integrity and regulating the inflammatory immune response and metabolism. Growing evidence confirms that disturbance of gut microbiota, also termed dysbiosis, contributes to the initiation and progression of UC [53]. Remodeling the gut microbiota is considered an attractive treatment for UC, so probiotics, prebiotics, antibiotics and FMT are commonly used in clinical practice. A great number of studies suggest that traditional Chinese medicines and their active ingredients can alleviate colitis by modulating the intestinal flora [26, 54]. Previous studies have reported that CA has strong antibacterial and antiviral properties; hence, it is speculated that CA may change the composition of the intestinal flora [13]. In this study, the gut microbiota in acute colitis mice showed a decrease in α-diversity and β-diversity, which is in line with previous studies [55]. However, CA treatment significantly increased the Simpson and Shannon indexes, indicating that α-diversity was restored by CA administration. The β-diversity analysis showed notable differences among the three groups, while CA treatment remodeled the composition of the gut microbiota. Furthermore, community composition analysis revealed that DSS-induced colitis mice exhibited a reduced abundance of *Firmicutes* and an enhanced abundance of *Proteobacteria* at the phylum level, which is in accordance with previous studies [55, 56]. Although a lower abundance of *Bacteroidetes* was detected in the DSS group, the difference among the three groups was not statistically significant. Intriguingly, our present study confirmed that CA treatment could revert the *Firmicutes/Proteobacteria* ratio in acute colitis mice back to that of control mice. *Firmicutes* comprise a diverse group of bacteria and are a major component of the gut microbiota, most of which are gram-positive bacteria, including *Lactobacillus*, *Enterococcus*, *Clostridium_sensu_stricto_1*, *Streptococcus*, *Faecalibacterium* and *Dubosiella*. Previous studies showed that UC patients displayed a reduced abundance of *Firmicutes*, and a gut microbiota low in *Firmicutes* increased susceptibility to colitis. Due to their short-chain fatty acids (SCFAs) metabolites, *Faecalibacterium* and *Dubosiella,* which are considered anti-inflammatory bacteria, have been found to be decreased in DSS-induced colitis mice and UC patients. *Streptococcus* and *Clostridium_sensu_stricto_1* are harmful bacteria and positively correlated with intestinal inflammatory response [57]. *Escherichia_Shigella*, a gram-negative bacterium belonging to *Proteobacteria* phylum that includes many kinds of pathogenic bacteria, has been proved to increase the risk of colitis. *Muribaculaceae* and its metabolites alleviate enterocyte damage and inflammation by blocking the NF-κB pathway [58]. In this study, a lower abundance of *Faecalibacterium*, *Dubosiella* and *Muribaculaceae* and a higher abundance of *Escherichia_Shigella*, *Streptococcus* and *Clostridium_sensu_stricto_1* were observed at the genus level in the DSS group. Notably, CA treatment partially reversed the alterations of gut microbiota in acute colitis mice by suppressing the growth of harmful bacteria and increasing that of beneficial bacteria.

This study, is subject to several limitations. First, many studies in recent years have focused on metabolites of gut microbiota such as SCFAs that are crucial in the development and progression of UC, yet the effect of CA on metabolites needs to be further explored. Second, immune disorder is an important feature of UC, whether CA affects microbiota-immune crosstalk in UC is unclear. Finally, the therapeutic effect of CA on colitis in mice needs to be verified in clinical trials.

In conclusion, the current study found that CA ameliorates DSS-induced colitis by reducing oxidative stress, inflammation, and intestinal epithelial cell apoptosis and improving intestinal barrier function. The beneficial effects are mediated, at least partly, via suppression of the MAPK/NF-κB pathways and activation of the NRF2/HO-1 pathway, which finally increase the expression of tight junction proteins and attenuate apoptosis in the colon. Additionally, CA therapy alters the gut microbiota by inhibiting the proliferation of dangerous bacteria and promoting the growth of beneficial bacteria. These findings indicate that CA may be a promising natural product-derived drug for the treatment of UC.

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## Disclosure of conflict of interest

None.

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