<table>
<thead>
<tr>
<th>Title</th>
<th>Orally administered CLA ameliorates DSS-induced colitis in mice via intestinal barrier improvement, oxidative stress reduction, inflammatory cytokine and gut microbiota modulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Authors</td>
<td>Chen, Yang; Yang, Bo; Ross, R. Paul; Jin, Yan; Stanton, Catherine; Zhao, Jianxin; Zhang, Hao; Chen, Wei</td>
</tr>
<tr>
<td>Publication date</td>
<td>2019-11-06</td>
</tr>
<tr>
<td>Type of publication</td>
<td>Article (peer-reviewed)</td>
</tr>
<tr>
<td>Link to publisher’s version</td>
<td>10.1021/acs.jafc.9b05744</td>
</tr>
<tr>
<td>Rights</td>
<td>© 2019, American Chemical Society. This document is the Accepted Manuscript version of a Published Work that appeared in final form in Journal of Agricultural and Food Chemistry, © American Chemical Society, after peer review and technical editing by the publisher. To access the final edited and published work see <a href="https://pubs.acs.org/doi/abs/10.1021/acs.jafc.9b05744">https://pubs.acs.org/doi/abs/10.1021/acs.jafc.9b05744</a></td>
</tr>
<tr>
<td>Download date</td>
<td>2024-08-06 03:57:00</td>
</tr>
<tr>
<td>Item downloaded from</td>
<td><a href="https://hdl.handle.net/10468/9500">https://hdl.handle.net/10468/9500</a></td>
</tr>
</tbody>
</table>
Bioactive Constituents, Metabolites, and Functions

Orally Administered CLA Ameliorates DSS-induced Colitis in Mice via Intestinal Barrier Improvement, Oxidative Stress Reduction, Inflammatory Cytokine and Gut Microbiota Modulation

Yang Chen, Bo Yang, R. Paul Ross, Yan Jin, Catherine Stanton, Jianxin Zhao, Hao Zhang, and Wei Chen

J. Agric. Food Chem., Just Accepted Manuscript • DOI: 10.1021/acs.jafc.9b05744 • Publication Date (Web): 06 Nov 2019

Downloaded from pubs.acs.org on November 14, 2019

Just Accepted

“Just Accepted” manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides “Just Accepted” as a service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. “Just Accepted” manuscripts appear in full in PDF format accompanied by an HTML abstract. “Just Accepted” manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are citable by the Digital Object Identifier (DOI®). “Just Accepted” is an optional service offered to authors. Therefore, the “Just Accepted” Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these “Just Accepted” manuscripts.
Orally Administered CLA Ameliorates DSS-induced Colitis in Mice via
Intestinal Barrier Improvement, Oxidative Stress Reduction, Inflammatory Cytokine and Gut Microbiota Modulation

Yang Chen†,‡, Bo Yang†,‡,ζ,*, R. Paul Rossζ,ξ, Yan Jinψ, Catherine Stantonζ,θ,ξ,*, Jianxin Zhao†,‡,ζ, Hao Zhang†,‡,ζ,Φ, and Wei Chen†,‡,δ,ε

†State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu, China
‡School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu, China
δNational Engineering Research Center for Functional Food, Jiangnan University, Wuxi, Jiangsu, China
ζInternational Joint Research Center for Probiotics & Gut Health, Jiangnan University, Wuxi, China
ΘTeagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland
ξAPC Microbiome Ireland, University College Cork, Cork, Ireland
ΨBeijing Innovation Center of Food Nutrition and Human Health, Beijing Technology and Business University (BTBU), Beijing, China
ΦWuxi Translational Medicine Research Center and Jiangsu Translational Medicine Research Institute Wuxi Branch
ΨDepartment of Gastroenterology, The Affiliated Wuxi Second People's Hospital of Nanjing Medical University, Wuxi, China

*Correspondence: Bo Yang & Catherine Stanton
School of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, China.
E-mail: bo.yang@jiangnan.edu.cn & catherine.stanton@teagasc.ie

ABSTRACT

Dietary supplementation with CLA has been reported to alleviate the effect of colitis in mice, but the mechanisms involved need further exploration. The study aimed to investigate how orally administered CLA alleviates DSS-induced colitis in mice. CLA was administered at five different doses: 40 mg/d, 20 mg/d, 10 mg/d, 5 mg/d and 2.5 mg/d. Doses of CLA at 10 mg/d and higher alleviated colitis symptoms and reduced inflammation induced by DSS, in which 40 mg/d, 20 mg/d and 10 mg/d CLA significantly increased the concentration of MUC2 and goblet cells, but neither 5 mg/d CLA nor 2.5 mg/d CLA had any effects. Meanwhile, 40 mg/d CLA and 20 mg/d CLA treatments significantly up-regulated the concentration of tight junction proteins (ZO-1, occludin and claudin-3) and ameliorated epithelial apoptosis caused by DSS. Moreover, oxidative stress-related enzymes (SOD, GSH-PX, CAT) and inflammatory cytokines (TNF-α, IL-10, IL-6) were modulated by 40 mg/d CLA and 20 mg/d CLA. Furthermore, 40 mg/d CLA rebalanced the gut microbiota damaged by DSS, including reducing Bacteroides and increasing Bifidobacterium and Odoribacter. In conclusion, CLA supplementation alleviated DSS-induced colitis in a dose-dependent manner by modulating inflammatory cytokines and oxidation stress, maintaining the mucosal barrier and reverting microbiota changes.
KEYWORDS: conjugated linoleic acid, colitis, intestinal barrier function, oxidative stress, gut microbiota

INTRODUCTION

Conjugated linoleic acid (CLA) was the positional and geometric isomers of linoleic acid. Twenty eight CLA isomers have been identified from milk, dairy and ruminant meat. The predominant isomer in dietary sources is cis9, trans11-CLA (c9, t11-CLA) which constitutes up to 90% of total CLA and is associated with positive health benefits. Trans10, cis12-CLA (t10, c12-CLA) is another common isomer which accounts for 1-10% of total CLA from diet and is associated with anti-obesity effects. CLA has demonstrated potent immunomodulatory effects that are exhibited in an isomer specific manner. These effects have been demonstrated in a wide range of inflammatory based disorders including inflammatory bowel disease (IBD), atherosclerosis and diabetes.

IBD comprises Crohn’s disease (CD) and ulcerative colitis (UC); their main characteristic is intestinal mucosal inflammation, and patients may have frequent recurrences and severe clinical forms. Though the etiology of IBD is not fully understood, several factors, including intestinal barrier dysfunction, immunologic abnormalities, expansion of inflammatory mediators and oxidative stress are involved in the pathogenesis of IBD. 5-aminosalicylic acid (5-ASA), corticosteroids, particularly prednisone, hydrocortisone, and budesonide, have yielded positive
results in IBD treatment by inhibiting inflammation. However, prolonged use of this type of drug may result in other diseases such as hypertension, diabetes and osteoporosis. New therapies for IBD which differ from traditional pharmacological treatments are being investigated and include prebiotics and some microbial metabolites such as unsaturated fatty acids.

It is worth noting that CLA has been shown to relieve IBD symptoms in animal models. Feeding DSS-challenged C57BL/6J mice and their PPARγ-knock-out derivatives 1% CLA-supplemented diets proved that CLA was able to reduce colitis by activating PPAR-γ. In C57BL/6J colitis mice, CLA supplementation (100 mg/kg/day) prevented colonic shortening, significantly reduced the disease activity index and NF-kB expression, and caused an increase of PPAR-γ and trefoil factor family 3 (TFF3) expression. Evans et al., found that administration of a CLA-supplemented diet (1 g CLA/100 g diet) to C57BL/6J colitis mice improved disease activity, decreased expression of tumor necrosis factor-α (TNF-α), and prevented colitis in part through a PPAR-γ-dependent mechanism. Furthermore, a diet supplemented with 1% CLA in C57BL/6J colitis mice reduced mucosal damage and inflammatory mediator infiltration suggesting a PPAR-γ-dependent mechanism mediated by macrophages. These studies differed in terms of CLA concentrations administered to mice. Furthermore, the exact amount of CLA ingested by the mice and the concentration of CLA entering the intestinal tract of mice remains unclear. Moreover, it is important to fully comprehend the mechanisms by which CLA relieves colitis.

Against this background, the aim of the current study were to identify biologically...
effective concentrations of orally administered CLA in mice and to uncover new potential pathways by which CLA alleviates colitis. The results from this study elucidate the relationships between the oral dose of CLA, the CLA content reaching the colon and colitis remission effect and provides more mechanisms by which CLA relieves colitis.

MATERIALS AND METHODS

Animals and Experimental Design

Male C57BL6/J mice (n = 64), 8-week-old and weighing 22-24 g, were raised at room temperature (25°C±2°C) and photoperiod (12 h/12 h light/dark period) in the barrier facility of Animal Center of Jiangnan University. Then, the 64 mice were divided into 8 groups (n=8/group) and fed standard chow and sterile water.

Table 1 showed the experimental procedures. Briefly, 2.5% (w/v) DSS (molecular weight 36,000-50,000, MP Biomedicals, LLC, Irvine, CA, USA) was added to the drinking water to induce colitis; the control group was fed with 200 μL skim milk (13% w/v) daily; the medically treated group, termed mesalazine group received 200 μL 10 mg/mL mesalazine (Etiasa pharmaceutical Co., Ltd., Saint-Cloud, Paris, France), while five CLA groups orally received 200 μL of different concentrations of CLA (emulsified with 13% w/v skim milk). CLA (50:50 mixture of c9, t11 and t10, c12 CLA isomers, purity: >99%, Nu-Check-Prep, Elysian, MN, USA) was emulsified with 13% w/v skim milk at different concentrations. The orally administered CLA concentrations were 40, 20, 10, 5 and 2.5 mg/d, respectively. The protocol for present study was approved by
the Ethics Committee of Jiangnan University, China (JN.No20180615c0560730[109]) and complied with the Directive of 2010/63/ European Community.

Assessment of Colitis

During DSS treatment, the changes of body weight and the disease activity index (DAI) of mice were measured everyday as the method of previous literature. Colon length was measured after dissecting the mice. Colon tissues were collected, dehydrated, embedded, sliced, and stained with Haematoxylin and Eosin (H&E) as the previous method. The valuation system of pathological score was referred to previous reported.

Biochemical Assays

The freshly excised colon was rinsed, homogenized in tissue lysis buffer, and then centrifuged at 10,000 × g at 4 °C for 15 min. The change of myeloperoxidase (MPO) activity, cyclooxygenase 2 (COX-2) activity and inducible nitric oxide synthase (iNOS) activity in the colon were assessed by commercially available ELISA kits (Nanjing Senbeijia Biotechnology Co., Ltd., Nanjing, Jiangsu, China) according to the manufacturer’s instructions. The change of the colonic malonic dialdehyde (MDA), superoxide dismutase (SOD) activity, catalase (CAT) activity and glutathione peroxidase (GSH-PX) activity were assessed by the corresponding Kit (Nanjing Jiancheng Co., Ltd., Nanjing, Jiangsu, China). The protein concentration was measured by the BCA method using the BCA Protein Assay Kit (Beyotime Biotechnology,
Shanghai, China). The activities of MPO, COX-2, iNOS in the colon were presented as pictograms U/g colon protein, while that of SOD, CAT and GSH-PX were presented as pictograms U/mg colon protein.

**Alcian Blue and Periodic Acid-schiff (PAS) Staining**

Distribution of mucin in the colon was investigated by alcian blue staining as the method of Steedman.\(^{37}\) The number of goblet cells was investigated by PAS staining as previously described.\(^{38}\)

**The Level of Cytokines in Colon Tissue**

The concentrations of IL-4, TNF-α, IL-1β, IL-10 and IL-17 were measured by commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) in the supernatants of freshly isolated pieces of colon tissue homogenized with potassium-phosphate buffer (1% protease inhibitor cocktail) and centrifuged at 10,000 × g at 4 °C for 15 min. The protein concentration was measured by the BCA method using the BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China) and the results were expressed as pg/mg protein of colon.

**Measurement of Tight Junction Proteins**

The concentrations of E-cadherin 1, occludin, ZO-1 and claudin-3 were measured by commercially available ELISA kits (Nanjing Senbeijia Biotechnology Co., Ltd., Nanjing, Jiangsu, China) in the supernatants of freshly isolated pieces of colon tissue
homogenized with potassium-phosphate buffer (1% protease inhibitor cocktail) and centrifuged at 10,000 × g at 4 °C for 15 min. The protein concentration was measured by the BCA method using the BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China) and the results were expressed as pg/mg protein of colon.

Fatty Acid Analysis

The extraction and methylation of fatty acid in blood, liver and colon were performed as previous described. Then, fatty acid were recovered with hexane and measured by GC-MS (the parameters of the instrument was described as previously described). The temperature programming of the gas chromatography was described as the method of Yang et al.

Measurement of Transmission Electron Microscopy (TEM)

The integrity of the tight junction (TJ) proteins of epithelial cells was assessed by measuring TEM. Colon tissues were collected, immobilized, dehydrated, embedded, sliced, and stained as the previous method. Then, the sections were detected under HITACHI H8100 TEM (Hitachi, Tokyo, Japan) with an accelerating voltage of 200 kV.

DNA Extraction, PCR Amplification, Sequencing and Bioinformatics Analysis

Faecal samples from mice were collected to assess the changes in the composition of intestinal flora. Metagenomic DNA of the fecal samples was extracted by using a FastDNA Spin Kit for Feces (MP Biomedicals, LLC, Irvine, CA, USA). The V3-V4
region of the 16S rRNA gene was PCR amplified using primers (341F: 5′-CCTAYGGGRBGCASCAG-3′; 806R: 5′-GGACTACNNGGGTATCTAAT-3′) as the method of Yang et al.35 After sequencing, bioinformatics analysis of the 16S rRNA sequence data was conducted as previously described.42

182 **Statistical Analysis**

183 GraphPad Prism 7 and SPSS 22.0 were used to analyze date. P value of < 0.05 was considered to indicate statistical significance. Microbiota-related analyses were conducted by QIIME and R 3.5.0. Linear Discriminant Analysis Effect Size (LEfSe) were performed by python 2.7 and R 3.5.0. Network diagram of sample and OTU and bacterial-interaction patterns of the validation cohort were performed by Cytoscape 3.6.0.

190 **RESULTS**

191 **CLA Improved the Colitis Symptoms**

192 The changes of body weight and DAI were measured daily during DSS treatment. Body weight of mice dropped significantly (Figure 1A) while the DAI rose continuously (Figure 1B) due to DSS treatment. DSS treatment resulted in 12.3% weight loss at the end of the trial compared with the animals’ initial weight (Figure 1A). However, the body weight of the mice in control group showed no significant difference. Compared with the DSS group, the weight loss of all the five CLA groups showed no significant difference.
Treatment with different concentrations of CLA had different effects on DAI. The DAI increased to 10.50 ± 0.38 in the DSS group. The alleviating effects of mesalazine (DAI=7.13 ± 0.34, P < 0.01), 40 mg/d CLA (6.13 ± 0.61, P < 0.01), 20 mg/d CLA (7.12 ± 0.64, P < 0.01) and 10 mg/d CLA (7.37 ± 0.72, P < 0.05) on colitis were significant when compared with the DAI of the DSS group (Figure 1B). 5 mg/d CLA and 2.5 mg/d CLA treatment led to an insignificant decrease in the DAI (8.87 ± 0.48, 9.25 ± 0.31) compared with the DSS treatment.

The colon length in the control group was 7.11 ± 0.18 cm (Figure 1C). The colons was normal reddish and the feces was granular in the control group. In comparison, the colon length of DSS-treated mice was 5.8 ± 0.12 cm, which showed dark red colons, swollen, bleeding intestinal wall (Figure 1D). DSS treatment led to a 16.4% reduction of colon length compared with the control group, whereas mesalazine, 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA treatments prevented the colon shortening process by 5.6%, 5.4%, 5.5% and 9.5%, respectively (Figure 1C). Thus, mesalazine, 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA treatment could significantly prevent colon shortening, but 5 mg/d CLA and 2.5 mg/d CLA could not, consistent with the results of DAI.

CLA Recovered the Damage in Colonic Tissue Caused by DSS and Regulated Inflammatory Enzymes

H&E staining was used to evaluate the histopathological injury. The colons of normal mice had intact mucous membranes and neat villi with healthy crypt structure (Figure 2A). The colon tissue of normal mice was enriched in goblet cells without inflammatory
cell infiltration or mucosal erosion. However, the mice in the DSS group showed intestinal mucosa and submucosal edema, severe inflammatory cell infiltration, crypt loss and epithelial injury.

The colon injury score of the DSS-treated mice (13.75 ± 0.25) was significantly higher than that of the normal mice (1.38 ± 0.38) (P < 0.0001) (Figure 2B). 40 mg/d CLA and mesalazine treatment significantly improved the inflammation of colon, and tissue damage was reduced to different extents in the 20 mg/d CLA and 10 mg/d CLA groups. Among all the groups, 40 mg/d CLA and mesalazine treatment showed more effects of protecting colon: the crypts were intact and no significant disappearance for the goblet cells. Furthermore, 40 mg/d CLA and mesalazine treatment showed the least edema, and the least extent of inflammatory cell infiltration in the submucosa and serosa. The colon tissue injury scores of the mice treated with 40 mg/d CLA and mesalazine were 3.13 ± 0.29 and 4.00 ± 0.42, respectively, similar to that of the control group. Fossae deformation, partial loss of mucosal epithelial cells, structural damage of the muscular layer were found in the mice of 20 mg/d CLA and 10 mg/d CLA groups. However, 5 mg/d CLA and 2.5 mg/d CLA treatments did not protect against the colon tissue damage. The tissue injury score in 5 mg/d CLA and 2.5 mg/d CLA was 12.25 ± 0.45 and 13.38 ± 0.38, close to DSS group.

In order to evaluate the effect of CLA on colonic inflammatory enzymes, MPO, COX-2, and iNOS activities were measured. Mesalazine, 40 mg/d CLA, 20 mg/d CLA, 10 mg/d CLA, 5 mg/d CLA and 2.5 mg/d CLA treatment decreased the MPO activity induced by DSS from 4.83 ± 0.21 to 3.26 ± 0.16, 3.48 ± 0.26, 3.66 ± 0.29, 3.41 ± 0.54,
4.05 ± 0.27 and 3.99 ± 0.32 U/g, respectively (Figure 2C). Apart from 5 mg/d CLA and 2.5 mg/d CLA groups, the MPO activity of mice in the other groups all showed significant differences when compared with the DSS group, which was consistent with the results of DAI and colon length. The DSS treatment resulted in highest COX-2 activity (27.25 ± 1.934 U/g protein), while mesalazine, 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA treatment significantly decreased COX-2 activity (22.58 ± 1.194, 22.65 ± 1.039, 22.41 ± 0.964 U/g protein) (Figure 2D). Moreover, the iNOS activities of the mice of all the seven groups were lower than that of the DSS group, although the differences were not significant (Figure 2E).

CLA Protected the Intestinal Barrier

To evaluate the influence of CLA on the mucous layer and goblet cells, the concentration of mucin2 (MUC2) and goblet cell numbers were measured. The results of alcian blue and PAS staining showed that the goblet cells were severely damaged, and large amounts of MUC2 disappeared in DSS group (Figure 3A and 3B). Mesalazine, 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA treatments significantly protected the reduction of goblet cells and the destruction of mucosal layer.

The content of MUC2 was analyzed by ELISA. The concentration of MUC2 was significantly reduced in the mice of DSS treatment (66.74 ± 1.52 pg/mg protein), whereas 40 mg/d CLA and mesalazine treatment could maintain its content (80.53 ± 3.783 and 78.50 ± 1.946 pg/mg protein, respectively) at normal levels compared with control (79.98 ± 1.48 pg/mg protein) (Figure 3C). Moreover, 20 mg/d CLA and 10 mg/d
CLA treatments significantly increased the content of MUC2 compared with the DSS group, while MUC2 in the 5 mg/d CLA and 2.5 mg/d CLA groups were similar to that of the DSS group. Remarkably, mice challenged with DSS suffered a loss of mucus-producing goblet cells compared with untreated mice. However, 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA treatments significantly relieved the loss of goblet cells at a reasonable level (p < 0.01) (Figure 3D). The concentration of MUC2 verified the above mentioned phenomenon (Figure 3C and 3D).

To evaluate the effect of CLA on the epithelium structure, TJ proteins and epithelial apoptosis were measured. Mesalazine, 40 mg/d CLA, 20 mg/d CLA, 10 mg/d CLA and 5 mg/d CLA treatments increased the concentrations of ZO-1 by 20.7%, 50.7%, 51.9%, 33.6% and 33.2%, respectively, compared with DSS treatment (Figure 4A). Moreover, DSS treatment decreased the concentration of E-cadherin 1, occludin and claudin-3 compared with the control (Figure 4B, 4C, 4D). By contrast, 40 mg/d CLA and 20 mg/d CLA significantly increased the concentration of occludin and claudin-3. The concentrations of occludin and claudin-3 were up-regulated to certain levels in 10 mg/d CLA, 5 mg/d CLA and 2.5 mg/d CLA groups, but were not significantly different to DSS (Figure 4B and 4C). However, E-Cadherin1 levels in all the CLA groups were significantly higher than that in the DSS group (Figure 4D).

To further verify that CLA can affect the tight junction of intestinal epithelial tissues, TEM was used to observe the tight junction of intestinal epithelial tissues. In the control mice, intestinal barrier was intact, the microvilli (Mv) of the epithelial cells were neatly arranged, and TJ, adheres junction (AJ), desmosome (De) were integrate. However,
fractured a widened or fractured TJ, AJ and De, curated microvilli was observed in the DSS treated mice. These were improved significantly following 40 mg/d CLA treatment (Figure 4E), which showed that CLA can improve the TJ and AJ of intestinal epithelial tissues.

Furthermore, Hoechst 33258, a special fluorescent dye, was used to stain for the colon tissues. Hoechst 33258 could differentiate between apoptotic and normal cells by using a fluorescence microscope. The nuclei of normal cells show diffuse homogeneous blue fluorescence; however, apoptotic cells present with strong blue fluorescence. As expected, the control treatment did not induce apoptosis of cells, but when treated with DSS, typical morphological changes were observed, as the image displays, with nuclear fragmentation, chromosomal condensation and cell shrinkage (Figure 4F). 40 mg/d CLA, 20 mg/d CLA and mesalazine treatments could prevent apoptotic cells, while a large number of apoptotic cells appeared in the mice in 5 mg/d CLA and 2.5 mg/d CLA groups (Figure 4F).

**CLA Regulated Oxidative Stress**

In order to evaluate the influence of CLA on oxidative stress, MDA level, CAT activity, GSH-PX activity and SOD activity of colon were measured. 40 mg/d CLA and 20 mg/d CLA treatment significantly increased SOD activity with a 1.22- and 1.20-fold compared with DSS treatment, respectively (Figure 5A). However, there was no significant difference among all the CLA groups and other groups for MDA (Figure 5B). In addition, mesalazine, 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA treatments
exhibited significant increments on GSH-PX activity (17.5%, 25.5%, 24.3% and 18.7%) 
\( p < 0.05 \) compared with DSS treatment (Figure 5C). DSS and 20 mg/d CLA treatment showed the lowest (35.65 ± 1.11 U/mg protein) and the highest (45.1 ± 2.12 U/mg protein) CAT activity, respectively. Furthermore, mesalazine, 40 mg/d CLA, 10 mg/d CLA and 2.5 mg/d CLA treatments showed significant increments on CAT activity compared with DSS treatment \( (p < 0.05) \) (Figure 5D).

**CLA Regulated Inflammatory Cytokines**

In order to evaluate the influence of CLA on inflammatory factors, TNF-α, IL-1β, IL-10 and IL-6 concentrations were analyzed by ELISA. Inflammatory factors in the colon of DSS-treated mice were significantly higher than those in the normal mice, with significant increases in TNF-α, IL-1β and IL-6 (Figure 6). Notably, IL-1β, the most significant pro-inflammatory cytokine, increased by 2.42-fold in the colon (Figure 6A). CLA-feeding decreased the concentrations of colonic TNF-α and IL-6 (Figure 6B and 6C). Compared with the DSS treatment group, 40 mg/d CLA and 20 mg/d CLA treatment significantly decreased the concentration of TNF-α in the colon (Figure 6B). In mesalazine, 40 mg/d CLA and 20 mg/d CLA groups, the concentration of IL-6 in the colon was significantly lower than in the DSS group; however, no significant differences were observed for 10 mg/d CLA, 5 mg/d CLA, 2.5 mg/d CLA and DSS groups (Figure 6C). Mesalazine treatment significantly decreased the concentration of IL-1β, while the mice of all the CLA groups showed no significant reduction compared to that in the DSS group (Figure 6A). Moreover, the concentration of the anti-
inflammatory cytokine, IL-10, in mesalazine, 40 mg/d CLA and 20 mg/d CLA groups increased to 52.7%, 57.1% and 33.6% compared with DSS group (35.26 ± 0.93 pg/mg protein), respectively (Figure 6D). Interestingly, 40 mg/d CLA, 20 mg/d CLA, 10 mg/d CLA and 5 mg/d CLA treatment significantly increased PPAR-γ concentration, while mesalazine treatment did not (Figure 6E). Overall, 40 mg/d CLA exerted the most significant inflammatory modulation effect, followed by mesalazine and 20 mg/d CLA.

Correlation between Tight Junction Proteins, Antioxidant Enzymes and Cytokines Regulated by CLA and Colitis Indices in Mice

In order to evaluate the relationship between tight junction proteins, antioxidant enzymes and cytokines regulated by CLA and colitis indices, the spearman correlation was analyzed. Different concentrations of CLA had different effects on the content of tight junction proteins, antioxidant enzymes and cytokines in mice colon tissue. The concentrations of ZO-1, occludin and E-Cadherin1 showed extremely negative correlations with DAI and tissue histological scores, while claudin-3 had no significant correlation with the colon length, MPO, DAI and tissue histological score (Figure 7A). Moreover, ZO-1 showed highly positive correlation with colon length, while occludin showed highly negative correlation with MPO.

In addition, the activity of GSH-PX and CAT displayed a high negative correlation with DAI and tissue histological scores. However, the concentration of MDA was the positive correlation with histological score and DAI. TNF-α and IL-6 showed high degree correlation with DAI and histological scores, but did not display correlation with
the colitis index MPO. Furthermore, IL-10 only showed negative correlation with histological score. IL-1β did not display correlation with any of the four colitis indices because CLA did not regulate it. It is notable that PPAR-γ displayed a high degree of correlation with colon length, DAI and tissue histological scores (Figure 7A).

The Effect of Orally Administered CLA on the Concentration of CLA in the Colon, Blood and Liver

In order to evaluate the distribution of orally administered CLA in mice, the CLA levels in colonic contents, blood and liver were analyzed. The concentrations of CLA in colonic contents from 40 mg/d CLA, 20 mg/d CLA, 10 mg/d CLA, 5 mg/d CLA and 2.5 mg/d CLA groups were 1.347 ± 0.153, 0.743 ± 0.057, 0.614 ± 0.11, 0.443 ± 0.076, 0.226 ± 0.092 g/100g FAME, respectively, which were higher than that in the control group as well as mesalazine and DSS groups (Figure 7B). Similar to that observed in the colon, CLA concentrations in blood and liver from the CLA groups showed a decreasing trend from 40 mg/d CLA to 2.5 mg/d CLA in line with the decreasing concentrations of CLA administered to the mice (Figure 7C and 7D). The concentrations of CLA in colonic, blood and liver samples from control, mesalazine and DSS treatment groups were not statistically different from each other (Figure 7B, 7C and 7D).

In order to evaluate the effect of the concentration of CLA in the colon on inflammatory markers of colitis, the interdependent quantitative relationships between the colonic CLA concentration and inflammatory markers of colitis were analyzed via
unary linear regression. The concentration of colonic CLA displayed extremely negative correlations with DAI and tissue histological scores ($p < 0.0001$). Moreover, colonic CLA showed a high positive correlation with colon length, while a highly negative correlation correlated with MPO ($p < 0.05$). The results show that the amount of CLA reaching the colon directly correlated with the oral dose of CLA, and the content of CLA in the colon significantly and positively correlated with the relief effect of colitis.

Modulation of Intestinal Microbiota by CLA

In order to evaluate the effect of CLA on the intestinal microbiota, the gut microbiota of mice treated with CLA at 40 mg/d, mesalazine group, DSS and control groups were investigated based on 16S rRNA-amplicon sequencing. Mice with chronic stress (DSS group) showed dramatic alteration of the gut microbial structure compared with the control. In the control group, the dominant phyla were Actinobacteria (19.27%), Bacteroidetes (32.16%), Firmicutes (36.41%) and Verrucomicrobia (5.75%) (Figure 8A). However, DSS treatment significantly changed the composition of bacteria at the phylum level, and the relative abundance of Bacteroidetes increased to 68.83% and the relative abundances of Actinobacteria, Firmicutes and Verrucomicrobia decreased to 3.76%, 16.70% and 2.06%, respectively (Figure 8A). Even though the relative abundance of Bacteroidetes in the CLA and mesalazine groups was higher than that in the control group, it was significantly decreased compared with the DSS group. Moreover, CLA treatment significantly increased the relative abundance of Firmicutes,
compared with DSS treatment. Alpha diversity was evaluated by Chao1 and Shannon index. After CLA treatment, Shannon index increased and was significantly different compared with microbiota from the DSS group, but Chao1 index showed no statistical differences compared with DSS treatment (Figure 8B and 8C). Beta diversity was reflected by principal coordinates analysis (PCoA) of weighted UniFrac distance. The results showed that the gut microbiota of DSS treatment mice was significantly different from the mice in the control group, and administration of CLA could remit the shift of gut microbiota induced by DSS treatment (Figure 8D). OTUs of all four groups were evaluated to identify the unique and shared genus. Different groups had their own distinct OTUs which were not shared with native controls. There were six, eight, eight and twenty-five distinct OTUs in control, DSS, mesalazine and CLA groups, while others OTUs were shared among those groups (Figure 8E).

The gut microbiota diversity among different groups was analyzed by LEfSe (LDA Effect Size). The LDA score histogram was drawn to identify statistically significant biomarkers and reveal the dominant microorganisms in each group (Figure 9A and 9B). Dominant communities of five, seven taxa and six taxa were found in the DSS, mesalazine and CLA groups, respectively. Among them, *Bacteroides* and Bacteroidaceae were the dominant in the DSS group; S24_7 and Verrucomicrobia were the dominant in the mesalazine group; while Proteobacteria, Odoribacteraceae and *Odoribacter* were the dominant microbes in the CLA group (Figure 9B). Relative abundance of selected taxa showed that the abundance of S24-7, *Bifidobacterium*, *Lactobacillus* and *Akkermansia* significantly decreased in DSS treatment mice, but the
abundance of *Bacteroides* significantly increased (Figure 9C). Compared with the DSS group, CLA treatment mice showed an increased abundance of *Bifidobacterium* and *Odoribacter* (*p < 0.05*) and a significantly decreased abundance of *Bacteroides* (Figure 9C).

The effect of CLA treatment on bacterial interaction patterns were further analyzed. In DSS group, Clostridiaceae (OTU82) and Peptostreptococcaceae (OTU101) were the core microbes. Clostridiaceae correlated positively with Coriobacteriaceae (OTU20), *Enterobacter* (OTU172), *Blautia* (OTU93), *Eubacterium* (OTU124) and *Paraprevotella* (OTU39) (Figure 9D). Moreover, Peptostreptococcaceae had a positive correlation with *Staphylococcus* (OTU58), *Clostridium* (OTU122) and *Trabulsiella* (OTU180). In CLA treatment group, it was found that Enterobacteriaceae (OTU170) and *Anaeroplasma* (OTU194) were the core microbe. Enterobacteriaceae showed a positive correlation with *Pseudomonas* (OTU190), but negative correlation with *Anaeroplasma* (OTU194), *Ruminococcus* (OTU110), *Oscillospira* (OTU191), *Bifidobacterium* (OTU19), which was also the core microbe (Figure 9D). Thus, CLA treatment changed the core gut microtioa and their interaction pattern.

Furthermore, the correlations among colonic CLA concentration, TJ proteins, differential microorganisms and inflammation markers were analyzed. Colon length, histological score, MPO and DAI were the most important indicators for colitis, and showed the bigger weightiness in the network analysis (Figure 9E). Colonic CLA concentration positively correlated with colon length, IL-10 and TJ proteins (occluding, ZO-1 and E-Cadherin1), in contrast, negatively correlated with *Bacteroides*,...
histological score, DAI, MPO and pro-inflammatory cytokines (TNF-α, IL-1β).

Interestingly, the relative abundance of *Bacteroides* were positively correlated with histological scores and DAI; however, it was negatively correlated with IL-10 and TJ proteins (occluding and ZO-1), indicating *Bacteroides* has a negative effect on colitis. It can be found that TJ proteins (occluding, ZO-1 and E-Cadherin1) had a negatively correlation with histological score, MPO and DAI. Furthermore, TNF-α and IL-10 showed an important correlation with the indicator of colitis. Notably, there was no significant correlation between the differential microorganisms and antioxidant related enzymes.

**DISCUSSION**

Salicylate, steroids, immunosuppressants, and anti-tnf-alpha drugs are traditionally used to treat patients with IBD. However, different response rates and potential side effects appeared in these therapies. Thus, exploring novel therapeutic and preventive approaches for IBD is important and has attracted increasing interest.43-47 A number of studies have demonstrated that CLA can ameliorate experimental IBD in mice and pigs.29-32, 48, 49

In this study, some clinical symptoms in mice were significantly alleviated by administering CLA to mice for 7 days prior to DSS treatment and continuing CLA administration for another 7 days simultaneously with DSS treatment. In particular, treatments 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA alleviated colon shortening, diarrhea and hematochezia. In addition, 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA
treatments significantly mitigated intestinal mucosa and submucosal edema, inflammatory cell infiltration, crypt loss and epithelial injury resulting from DSS challenge. Thus, 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA treatments significantly decreased DAI, histological injury score, and increased colon length, but 5 mg/d CLA and 2.5 mg/d CLA treatments did not elicit these effects, which indicates that CLA relieves colitis in a dose-dependent manner.

The mucosal barrier, the first line of protection of the intestinal tract, is mainly composed of mucous layer and epithelial cell layer. It prevents intestinal bacteria toxins and other exogenous substances from invading the intestinal tissues and, subsequently, prevents intestinal mucosal injury. 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA treatments increased the concentration of MUC2, which could maintain the integrity of the colonic mucous layer and protect goblet cells. Moreover, the goblet cell numbers of the mice treated with 40 mg/d CLA, 20 mg/d CLA and 10 mg/d CLA were higher than that of DSS treated mice, which was consistent with the results of MUC2. TJ proteins of enterocytes predominantly regulate the integrity of the intestinal barrier and play an important role in IBD. In the present study, 40 mg/d CLA and 20 mg/d CLA treatment up-regulated TJ proteins (E-cadherin 1, ZO-1, claudin-3 and occluding). Thus, we conclude that to a certain extent high CLA concentrations can maintain the integrity of epithelium structure. A previous study by Wang and colleagues showed that 1% CLA added in the diet up-regulated the mRNA levels of MUC2, E-cadherin 1, claudin-3, ZO-1 and occludin in DSS-induced colitis mice. Therefore, oral administration of CLA (40 or 20 mg/d) up-regulates the concentration of these TJ proteins, which indicates
that appropriate concentrations of CLA can improve the intestinal barrier function. Furthermore, 10 mg/d CLA and 5 mg/d CLA treatments increased the concentration of E-cadherin 1 and ZO-1 ($p < 0.05$) compared with DSS treatment, while 2.5 mg/d CLA (2.5 mg/d) only increased the concentration of E-cadherin 1. Therefore, the regulation of CLA on TJ proteins occurred in a dose-dependent manner.

Oxidative stress can lead to abnormal oxygen free radical metabolism and excessive activation of apoptosis. Moreover, the oxygen free radicals lead to increase release of inflammatory mediators (such as cytokines and chemokines), which not only cause epithelial cell damage, but also aggravates oxidative stress, leading to the destruction of the intestinal mechanical barrier. Chinnadurai and colleagues found that feeding high CLA (19.54 mg/g fat) enriched ghee to rats during the pubescent period resulted in an increase in CAT and SOD enzyme activities both in blood and liver. Large yellow croaker fed with soybean oil-based diets plus 0.83% CLA significantly increased the activity of CAT and total antioxidant capacity (T-AOC) in liver. Moreover, SOD, CAT and GSH-PX can ameliorate the peroxidation reactions in colitis. In the current study, CLA (40 and 20 mg/d) significantly increased the activity of CAT, SOD and GSH-PX in colon tissue, which indicated that CLA alleviated colitis by inhibiting oxidative stress. Moreover, 10 mg/d CLA treatment increased the activities of CAT and GSH-PX in the colon, while only CAT activity was increased by 5 mg/d CLA and 2.5 mg/d CLA. Thus, CLA regulated oxidative stress related enzymes in a dose-dependent manner.

IBD may be associated with uncontrolled, highly activated inflammation of the
intestinal mucosa. Studies have shown that the anti-inflammatory effect of CLA was mainly achieved by regulating the expression and activity of PPARγ. PPARγ is one of the three subtypes of PPARs (PPARα, PPARβ/δ and PPARγ), in which PPAR-γ belongs to the nuclear receptor superfamily. PPARγ can inhibit the activation and nuclear import of NF-κB through the IkB-α pathway, in which NF-κB plays a key role in the regulation of the inflammatory response and pathogenesis of IBD. Hontecillas et al. found that CLA can inhibit inflammation of the colon in a colitis model caused by pathogenic bacteria, and increase the expression of PPAR in the colon. Since then, Bassaganya-Riera et al. used PPARγ knockout mice to prove that CLA was able to reduce colitis by activating PPARγ. In colon cancer cell lines HT-29 and Caco-2, CLA induced cell apoptosis by up-regulation of PPARγ. In the current study, 40 mg/d CLA, 20 mg/d CLA, 10 mg/d CLA, 5 mg/d CLA and mesalazine increased the concentration of PPARγ, which was consistent with previous research. NF-κB pathway can be activated by TNF-α, which was produced by macrophages; at the same time, NF-κB could promote the secretion of TNF-α, IL-6 and IL-1β. In RAW264.7, a macrophage cell-line from mice, CLA reduced the mRNA expression levels of INF-γ, COX2, TNF-α, IL-1 and IL-6 genes through the PPARγ pathway. Moreover, CLA significantly down-regulated the expression of TNF-α, IL-1β, IL-6 and up-regulated IL-10. In our current study, CLA (40 or 20 mg/d) decreased the concentration of TNF-α and IL-6 while increasing that of IL-10 in the colon, which may be due to the activation of PPARγ and the inhibition of NF-κB, thus resulting in a lower secretion of pro-inflammatory cytokines (TNF-α and IL-6). However, these pro-
inflammatory cytokines were not up-regulated by 10 mg/d CLA, 5 mg/d CLA mg/d
CLA treatments. Thus, inflammatory cytokines were regulated by CLA in a dose-
dependent manner.

It is known for that in healthy individuals a symbiotic relationship exists between the
gut microbiota and host, and the gut microbiota is closely related to the pathogenesis of
IBD. With PCoA analysis, it was found that the gut microbiota of DSS-treated mice
was dramatically different from that of the control group, but CLA (40 mg/d) treatment
mitigated gut microbiota shift induced by DSS challenge. At the phylum level, the
relative abundance of Bacteroidetes in the DSS treatment group was 2.14 times more
than that in the control group, while that in the CLA (40 mg/d) administered group
significantly decreased compared with the DSS group. Moreover, CLA treatment
increased the relative abundance of Verrucomicrobia, which was considered to be
associated with the higher expression of MUC2. At the genus level, CLA treatment
increased the abundance of Bifidobacterium and Odoribacter and significantly
decreased the abundance of Bacteroides compared with DSS treatment. Bifidobacterium was reported to remit colitis. Moreover, Odoribacter was reduced as
a result of DSS treatment, which could ameliorate ulcerative colitis, and may increase
host inflammation by reducing production of short chain fatty acids. Most commonly,
Odoribacter is known for of its ability to produce butyrate, which can improve the
intestinal barrier and relieve colitis. Notably, the current results showed that
Odoribacter had a positive correlation with occludin and negative correlation with IL-
6. Some Bacteroides fragilis strains can invade intestinal tissue and cause damage.
addition, certain *B. vulgatus* and *B. ovatus* have been found to affect the development of IBD.\(^{70-72}\) In the present study, the results showed that *Bacteroides* were negatively correlated with TJ proteins (occluding and ZO-1), IL-10 and positively correlated with inflammatory markers of colitis (DAI and histological score), which was consistent with previous results.\(^{70-72}\)

Sokol et al.,\(^{73}\) reported a skewed microbial interaction pattern in IBD patients and found that the concomitant analysis of microbiota showed a dense and homogenous correlation network in healthy subjects, but an unbalanced network in IBD patients. In the present study, unbalanced microbiota was found in DSS treatment group, in which the core microbes were Clostridiaceae, Peptostreptococcaceae, *Staphylococcus*, *Trabulsiella* and *Paraprevotella*, and correlated positively with Coriobacteriaceae, *Enterobacter*, *Eubacterium*. Additionally, Clostridiaceae, Peptostreptococcaceae, *Staphylococcus*, *Trabulsiella*, *Enterobacter* and *Paraprevotella* have been confirmed to aggravate colitis.\(^{74, 75}\) However, CLA treatment could improve the unbalanced microbiota. In CLA treatment group, Enterobacteriaceae, one of the core microbes, showed a positive correlation with *Pseudomonas*, but negative correlation with *Anaeroplasma*, *Ruminococcus*, *Oscillospira* and *Bifidobacterium*. Interestingly, *Ruminococcus*, *Oscillospira* and *Bifidobacterium* were reported to improve colitis.\(^{64, 74}\, 75\) Meanwhile, *Oscillospira* could produce butyrate to improve the intestinal barrier and relieve colitis,\(^{67, 68, 74}\) and *Ruminococcus* was negatively correlated with CD.\(^{75}\) Thus, our results indicated that CLA (40 mg/d) treatment partially prevented the microbiota changes induced by DSS.
Thus, orally administrated CLA resulted in some CLA entering the colon, where it acts as an anti-inflammatory agent. Interestingly, the present study found a significant positive correlation between CLA content in the colon and the relief effect of enteritis. When the oral dose of CLA exceeded 10 mg/d, 0.613 mg/mL CLA reached the colon, which has a relief effect on colitis; however, when the oral dose of CLA was 5 mg/d, 0.443 mg/mL CLA reached the colon, showing no significant improvement in colitis. This suggests that CLA does have a dose-dependent relationship in relieving colitis. In view of the efficacy of CLA for mice, clinical trials investigating the efficacy of CLA in UC patients need to be conducted in the future. According to the effective dose of the current study and the dose conversion relationship between animals and humans, the oral dose of CLA in future clinical trials of UC patients should be more than 42 mg/kg body weight.

The primary mechanisms that CLA significantly ameliorated DSS-induced colitis involved in inhibiting pro-inflammatory factors, maintaining mucosal barriers, regulating oxidative stress and intestinal microbial damage. From all those results, it can be found that CLA entered into the bowel lumen then decreased the abundance of *Bacteroides* and increased the abundance of *Bifidobacterium*, which could impact the concentration of AJ proteins and inflammatory cytokines. Furthermore, CLA that in the bowel lumen could improve antioxidant related enzymes, which could increase AJ proteins and improve intestinal barrier. Simultaneously, CLA could directly penetrate into the mucus layer and epithelial cells to regulate MUC2 and AJ proteins. Additionally, CLA could enter into the lamina propria in the mice treated DSS, then
reduced inflammation and regulated the cytokines. Thus, CLA could not only indirectly improve intestinal barrier and regulate inflammatory factors through the regulation of bacterial flora and oxidative stress, but also could directly regulate mucin and TJ protein as well as inflammatory factors, which could directly improve related indexes of colitis. These results will help us understand the mechanisms by which CLA alleviates colitis and regulates other immune-related diseases, and hence guide further development of CLA products.

AUTHOR INFORMATION

Corresponding Author

*Telephone: 86-510-85912155, +353-25-42606; E-mail: bo.yang@jiangnan.edu.cn & catherine.stanton@teagasc.ie

Funding

This research was supported by the National Natural Science Foundation of China (Nos. 31801521, 31722041), the Fundamental Research Funds for the Central Universities (No. JUSRP51702A), National First-Class Discipline Program of Food Science and Technology (JUFSTR20180102), Postgraduate Research & Practice Innovation Program of Jiangsu Province (KYCX19_1829), Wuxi Young Talent Foundation (QNRC075) and the Jiangsu Province “Collaborative Innovation Center for Food Safety and Quality Control”.

Notes

The authors declare no competing financial interest.
REFERENCES


(6) Choi, J. S.; Jung, M. H.; Park, H. S.; Song, J. Effect of conjugated linoleic acid isomers on insulin resistance and mRNA levels of genes regulating energy


13) Toomey, S.; Harhen, B.; Roche, H. M.; Fitzgerald, D.; Belton, O. Profound


Coloproct. 2003, 23 (3), 172-182.


(32) Bassaganya-Riera, J.; Viladomiu, M.; Pedragosa, M.; De Simone, C.; Carbo, A.; Shaykhutdinov, R.; Storr, M. Probiotic bacteria produce conjugated linoleic acid locally in the gut that targets macrophage PPAR γ to suppress colitis. Plos One


(54) Zuo, R.; Ai, Q.; Mai, K.; Xu, W. Effects of conjugated linoleic acid on growth,
non-specific immunity, antioxidant capacity, lipid deposition and related gene
expression in juvenile large yellow croaker (Larmichthys crocea) fed soyabean oil-

(55) Naito, Y.; Takagi, T.; Yoshikawa, T. Molecular fingerprints of neutrophil-
dependent oxidative stress in inflammatory bowel disease. **J. Gastroentero** 2007, 42 (10), 787-798.

(56) Yuan, G.; Chen, X.; Li, D. Modulation of peroxisome proliferator-activated
receptor gamma (PPAR γ) by conjugated fatty acid in obesity and inflammatory

Malainer, C.; Schuster, D. Natural product agonists of peroxisome proliferator-

Wu, G. D. A novel therapy for colitis utilizing PPAR-γ ligands to inhibit the

(59) Ewaschuk, J. B.; Walker, J. W.; Diaz, H.; Madsen, K. L. Bioproduction of
conjugated linoleic acid by probiotic bacteria occurs in vitro and in vivo in mice. **J.
Nutr.** 2006, 136 (6), 1483-1487.

(60) Tak, P. P.; Firestein, G. S. NF-κB: a key role in inflammatory diseases. **J. Clin.

(61) Yu, Y.; Correll, P. H.; Heuvel, J. V. Conjugated linoleic acid decreases production


(67) Chen, G.; Ran, X.; Li, B.; Li, Y.; He, D.; Huang, B.; Wang, W. Sodium butyrate inhibits inflammation and maintains epithelium barrier integrity in a TNBS-


(73) Sokol, H.; Leducq, V.; Aschard, H.; Pham, H. P.; Jegou, S.; Landman, C.; Cosnes,


### Table 1  Animal model experimental design.

<table>
<thead>
<tr>
<th>Day</th>
<th>Control 13% wt/v skim milk 200 μL once a day (day1-day14)</th>
<th>DSS 13% wt/v skim milk 200 μL once a day (day1-day7)</th>
<th>Medicine 10 mg/mL mesalazine 200 μL once a day (day1-day7)</th>
<th>CLA-1 200 mg/mL CLA 200 μL once a day (day1-day7)</th>
<th>CLA-2 100 mg/mL CLA 200 μL once a day (day1-day7)</th>
<th>CLA-3 50 mg/mL CLA 200 μL once a day (day1-day7)</th>
<th>CLA-4 25 mg/mL CLA 200 μL once a day (day1-day7)</th>
<th>CLA-5 12.5 mg/mL CLA 200 μL once a day (day1-day7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13% wt/v skim milk 200 μL once a day (day1-day14)</td>
<td>2.5% (w/v) DSS in water+13% wt/v skim milk 200 μL once a day (day1-day7)</td>
<td>2.5% (w/v) DSS in water+10 mg/mL mesalazine 200 μL once a day (day1-day7)</td>
<td>2.5% (w/v) DSS in water+200 mg/mL CLA 200 μL once a day (day1-day7)</td>
<td>2.5% (w/v) DSS in water+100 mg/mL CLA 200 μL once a day (day1-day7)</td>
<td>2.5% (w/v) DSS in water+50 mg/mL CLA 200 μL once a day (day1-day7)</td>
<td>2.5% (w/v) DSS in water+25 mg/mL CLA 200 μL once a day (day1-day7)</td>
<td>2.5% (w/v) DSS in water+12.5 mg/mL CLA 200 μL once a day (day1-day7)</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1 Symptoms of DSS-induced colitis. (A) Body weight, (B) Disease activity index (DAI), (C) Colon length, (D) Macroscopic pictures of colons. *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001. All data are presented as mean ± SEM (n=8 mice per group).
Fig. 1B

189x173mm (300 x 300 DPI)
Fig. 1D

100x244mm (300 x 300 DPI)
Fig. 2 Effect of CLA on the histological injury and enzyme activities in colon of colitis. (A) Histological examination, Scale bars, 200 and 50 μm, (B) Colonic histological injury, (C) MPO, (D) COX-2, (E) iNOS. *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001. All data are presented as mean ± SEM (n=8 mice per group).
Fig. 2B

197x188mm (300 x 300 DPI)
Fig. 2C

MPO activity (U/g)

Control, DSS, Mesalazine, 40 mg/d CLA, 20 mg/d CLA, 10 mg/d CLA, 5 mg/d CLA, 2.5 mg/d CLA

195x186mm (300 x 300 DPI)
Fig. 2D

195x189mm (300 x 300 DPI)
Fig. 2E

194x186mm (300 x 300 DPI)
Fig. 3 Effects of CLA on the mucous layer. (A) Alcin blue staining, Scale bar = 200 μm (B) Histological sections of the colon (stained with PAS), Scale bars, 20 μm, (C) Concentration of MUC2, (D) The number of goblet cells. *: p < 0.05, **: p < 0.01, ***: p < 0.001. All data are presented as mean ± SEM (n=8 mice per group).

267x204mm (300 x 300 DPI)
Fig. 3B

199x76mm (300 x 300 DPI)
Fig. 3C

MUC2 (pg/mg protein)

Control, DSS, Mesalazine, 40 mg/d CLA, 20 mg/d CLA, 10 mg/d CLA, 5 mg/d CLA, 2.5 mg/d CLA

194x184mm (300 x 300 DPI)
Fig. 4 Effects of CLA on TJ proteins in colon and apoptosis of colonic epithelial cells. (A) ZO-1, (B) Occludin, (C) Claudin-3, (D) E-Cadherin1, (E) TEM of intestinal epithelial tissues, tight junction (TJ), adheres junction (AJ), desmosome (De), microvilli (Mv). Scale bar = 1 μm, (F) apoptosis of colonic epithelial cells. Scale bar = 200 μm. *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001. All data are presented as mean ± SEM (n=8 mice per group).
Fig. 4B

Ocludin (pg/mg protein)

Control  DSS  Mesalazine  40 mg/d CLA  20 mg/d CLA  10 mg/d CLA  5 mg/d CLA  2.5 mg/d CLA

199x188mm (300 x 300 DPI)
Fig. 4C

Claudin-3 (pg/mg protein)

Control, DSS, Mesalazine, 40 mg/d CLA, 20 mg/d CLA, 10 mg/d CLA, 5 mg/d CLA, 2.5 mg/d CLA

199x188mm (300 x 300 DPI)
Fig. 4E
Fig. 4F
Fig. 5. Effects of CLA on the activity of oxidative stress-related enzymes in colon. (A) SOD, (B) MDA, (C) GSH-PX, (D) CAT. *: p < 0.05, **: p < 0.01, ***: p < 0.001. All data are presented as mean ± SEM (n=8 mice per group).
Fig. 5B

MDA (nmol/g protein)

Control, DSS, Mesalazine, 40 mg/d CLA, 20 mg/d CLA, 10 mg/d CLA, 5 mg/d CLA, 2.5 mg/d CLA

199x182mm (300 x 300 DPI)
Fig. 5C

199x185mm (300 x 300 DPI)
Fig. 5D
Fig. 6. Effects of CLA on inflammatory cytokines in colonic tissue. (A) IL-1β, (B) TNF-α, (C) IL-6, (D) IL-10 and (E) PPARγ. *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001. All data are presented as mean ± SEM (n=8 mice per group).
Fig. 6B

203x182mm (300 x 300 DPI)
Fig. 6C

IL-6 (pg/mg protein)

Control  DSS  Mesalazine  40 mg/d CLA  20 mg/d CLA  10 mg/d CLA  5 mg/d CLA  2.5 mg/d CLA

203x183mm (300 x 300 DPI)
Fig. 6D

203x183mm (300 x 300 DPI)
Fig. 6E

203x184mm (300 x 300 DPI)
Fig. 7. CLA concentration in different tissues and correlation of CLA and colitis indices. (A) Correlation analysis of colitis indices and different parameters. (B-D) CLA concentration in the colonic, blood and liver. (E-H) The interdependent quantitative relationships between the colonic CLA concentration and inflammatory markers, DAI, histological scores, colon length and MPO. *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001. All data are presented as mean ± SEM (n=8 mice per group).
Fig. 7B

197x189mm (300 x 300 DPI)
Fig. 7C

197x186mm (300 x 300 DPI)
Fig. 7D

CLA in liver (g/100g FAME)

Control  DSS  40 mg/d CLA  20 mg/d CLA  10 mg/d CLA  5 mg/d CLA  2.5 mg/d CLA

197x188mm (300 x 300 DPI)
Fig. 7E

$R^2 = 0.3698$

$p < 0.0001$
Fig. 7F

$R^2 = 0.7708$

$p < 0.0001$

Histological score vs. CLA in colon (g/100g FAME)
Fig. 7H

$R^2 = 0.113$

$p < 0.05$

MPO vs. CLA in colon (g/100g FAME)

207x167mm (300 x 300 DPI)
Fig. 8 Evaluation of illumina MiSeq sequencing data showing that CLA could modulate the overall structure of gut microbiota. (A) Microbial distribution at the phylum level, (B) Alpha diversity indicated by Chao1 index, (C) Shannon index, (D) PCoA, with extended functionality for labeling groups, with normal probability ellipsoids for different groups, (E) Network diagram of sample and OTU. *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001. All data are presented as mean ± SEM (n=8 mice per group).
Fig. 8B

Chao1 index

Control, CLA, Medicine, DSS

194x170mm (300 x 300 DPI)
Fig. 8C

194x172mm (300 x 300 DPI)
Fig. 8D

250x202mm (300 x 300 DPI)
Fig. 9: Effects of CLA on dominant microorganisms. (A) Cladogram. (B) Distribution histogram based on LDA, with a log LDA score above 3.0. Significant taxa are labeled and annotated with tags in the right panel. (C) Relative abundance of S24-7, Bifidobacterium, Lactobacillus, Akkermansia, Bacteroides, and Odoribacter. (D) Effect of CLA treatment on bacterial-interaction patterns of the validation cohort. Bacterial abundances were analyzed using Spearman’s test. Only significant correlations (p-value < 0.05, |R2| > 0.6) are displayed with an edge. The edge colors indicate positive (red) or negative (green) correlations, which depended on Spearman’s correlation coefficient. The node size represents the weight. (E) Correlation analysis of the concentration of colonic CLA, significant taxa, colitis indices, tight junction proteins, antioxidant enzymes and cytokine in colon. Only significant correlations (p-value < 0.05, |r2| > 0.6) are displayed with an edge. The edge colors indicate positive (red) or negative (green) correlations, which depended on Spearman’s correlation coefficient. The node size represents the weight. n=8 mice per group.
Fig. 9B

152x172mm (300 x 300 DPI)
Fig. 9C

125x113mm (300 x 300 DPI)
Figure 9E

209x213mm (300 x 300 DPI)
CLA alleviates DSS-induced colitis in mice from four aspects: intestinal microorganisms, oxidation stress, intestinal barrier, inflammatory cytokines. 40 mg/d, 20 mg/d and 10 mg/d CLA significantly increased the concentration of MUC2 and goblet cells. Meanwhile, 40 mg/d CLA and 20 mg/d CLA treatments significantly up-regulated the concentration of tight junction proteins (ZO-1, occludin and Claudin-3) and ameliorated epithelial apoptosis caused by DSS. Moreover, oxidative stress-related enzymes (SOD, GSH-PX, CAT) and inflammatory cytokines (TNF-α, IL-10, IL-6) were modulated by 40 mg/d CLA and 20 mg/d CLA. Furthermore, 40 mg/d CLA rebalanced the gut microbiota damaged by DSS, including reducing Bacteroides and increasing Bifidobacterium and Odoribacter.