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Bioactive Constituents, Metabolites, and Functions

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c9, t11, c15-CLNA and c9, t11, t15-CLNA from *Lactobacillus plantarum* ZS2058 Ameliorate DSS-Induced Colitis in Mice

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ABSTRACT: To investigate the specific functions of conjugated fatty acids (CFAs) produced by the probiotic bacterium, α-linolenic acid was isomerized by *Lactobacillus plantarum* ZS2058, and two different conjugated α-linolenic acid (CLNA) isomers were successfully isolated: c9, t11, c15-CLNA (CLNA1) and c9, t11, t15-CLNA (CLNA2). The effects and mechanism of CLNA crude extract and individual isomers on colitis were explored. CLNA significantly inhibited weight loss, the disease activity index, colon shortening. Additionally, CLNA alleviated histological damage, protected colonic mucous layer integrity and significantly upregulated the concentration of tight junction proteins (ZO-1, occludin, E-cadherin1 and claudin-3). CLNA significantly attenuated the level of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) while upregulating the expression of the colonic anti-inflammatory cytokine IL-10 and nuclear receptor PPARγ. Moreover, CLNA increased the activity of oxidative stress-related enzymes (SOD, GSH and CAT) and the myeloperoxidase activity was significantly decreased by CLNA. Meanwhile, the concentrations of CLNA in the liver and conjugated linoleic acid (CLA) in colonic content were significantly increased because of the treatment of CLNA. Furthermore, CLNA could rebalance the intestinal microbial composition of colitis mice, including increasing the α-diversity. CLNA1 and CLNA2 increased the abundance of *Ruminococcus* and *Prevotella,* respectively.

KEYWORDS: Conjugated linolenic acid (CLNA), Colitis, Intestinal barrier function, Oxidative stress, Gut microbiota

■ INTRODUCTION

Conjugated fatty acids (CFAs), which are defined as the positional and geometric isomers of unsaturated fatty acids, contain one or more nonmethylene interrupted double bonds in either cis or trans conformation.[1] One CFA that has been intensively investigated is conjugated linoleic acid (CLA), which exhibits a variety of health benefits, including anticancer,[2-3] antioxidation,[4] antiatherosclerotic,[5] antidiabetic,[6,7] and antiobesity effects.[8-9] In addition to CLA, the conjugated linolenic acid (CLNA) isomers, which are another type of CFA, have recently received enhancive attention due to their biological benefits being similar to those of CLA. CLNAs can be collected from plant oil or bacterial fermentation broth, and plant-derived CLNAs have been extensively studied, while bacteria-derived CLNAs not yet been studied. The typical plant-derived CLNAs contain c9, t11, c13-CLNA, c9, t11, t13-CLNA, t9, t11, c13-CLNA, c8, t10, c12-CLNA and t8, t10, c12-CLNA, while typical bacteria-derived CLNAs contain c9, t11, c15-CLNA and t9, t11, c15-CLNA. Although the cis-trans properties of the double bonds are different, they all have conjugated double bonds. Some conjugated double bonds with particular cis-trans properties, such as c9, t11, in fatty acids can enhance their functionality. In this paper, the isomers of c9, t11, c15-CLNA and t9, t11, c15-CLNA were first separated from the fermentation of *L. plantarum* ZS2058, and their function in a mice model was investigated.

IBD comprises two types of inflammatory conditions, crohn's disease (CD) and ulcerative colitis (UC). Although the mechanism of IBD has not been fully figured out, several factors are considered in IBD, including immunologic abnormalities, intestinal

barrier dysfunction, oxidative stress, and expansion of inflammatory mediators.[10-11] Some treatments alleviate IBD by inhibiting inflammation, such as aminosalicylates and corticosteroids, particularly hydrocortisone, budesonide, prednisone and antibiotics.[12-13] However, there may be some side effects when using anti-inflammatory drugs, such as headache, abdominal pain, diarrhea and nasopharyngitis.[14-16] Currently, there are some new therapies for IBD which are different from traditional medicine, such as prebiotics and some microbial metabolites like unsaturated fatty acids.

The effects of plant-derived conjugated linolenic acid on colitis have been widely studied. Tarek Boussetta et al. proved that punicic acid (c9, t11, c13-CLNA) can inhibit TNF-α-induced priming of NADPH oxidase, which is related to the p38MAPKinase/Ser345-p47phox-axis and MPO release.[17] C9, t11, t13-CLNA can improve DSS-induced colitis in mice by improving the level of peroxisome-activated receptor-γ (PPARγ).[18] The structures of c9, t11, c15-CLNA (CLNA1) and t9, t11, c15-CLNA (CLNA2) were exactly similar to plant-derived CLNA; therefore, they may have similar effects on colitis. To date, there was only in vitro experiments on *Lactobacillus*-derived CLNA, which have proved that it can inhibit the growth of SW480 cells and is more toxic than LNA and CLA.[19-20] No study has reported the efficacy of the two isomers in vivo, perhaps due to the difficulty of screening high-yield CLNA strains and the complexity of extracting and separating CLNA isomers. Therefore, one of the purposes of this study is to explore the function of CLNA1 and CLNA2 in colitis and provide a medical basis for the use of conjugated fatty acids as adjuvants in the treatment of colitis. In addition, different isomers usually showed different bioactivity

due to cis, trans configurations of their double bonds.[21] For example, all-trans conjugated fatty acids have more effective tumor suppressing activity.[22] Therefore, the other purpose of this study was to explore the difference between the two isomers in colitis.

■ MATERIAL AND METHODS

Microorganism Cultivation and Preparation of the CLNA Mixture. *L. plantarum* ZS2058 was subcultured twice in de Man, Rogosa and Sharpe (mMRS) medium at 37℃ for 24 h, inoculated into 2 L of mMRS with α-linolenic acid (Nu-check Prep, Elysian, MN) at a final concentration of 30 mg/ml, and cultured for 48 h. After culturing, the fermentation broth was placed in a pear-shaped separatory funnel with the ratio of fermentation broth:isopropanol:n-hexane = 3:2:3 (v/v/v), shaken thoroughly, and allowed to stand for 15 min. The solution was layered, and the upper transparent n-hexane layer was collected. The n-hexane layer was then evaporated on a rotary evaporator and redissolved in methanol (chromatography grade) to a concentration of 40 mg/mL.

Separation of CLNA and Purity Detection. The crude extracts were separated by RP-HPLC on an Ultimate® 5 μm C30 semipreparative column (10×250 mm) (Yuexu Technology, Shanghai, China) by a Waters 2545 RP-HPLC. The CLNA isomers were separated in methanol:water:formic acid (80:20:0.01, vol/vol) at a flow rate of 5 mL/min with an injection volume of 700 μL. The CLNA fragments were detected by a UV detector at absorbances of 205 nm and 233 nm, and fractions containing the single isomer were collected automatically when both absorbances were detected. The

113 methanol and water from the pooled fractions were removed by rotary evaporation
114 (37℃), and then the single CLNA isomer was re-extracted with methanol for storage.
115 The purities of the extracted CLNA1 (c9, t11, c15-CLNA) and CLNA2 (t9, t11, c15-
116 CLNA) were determined by GC-MS as previously described.[23]
117 **Animals and Diets.** Seven-week-old male C57 BL6/J mice were housed in the Animal
118 Housing Unit of Jiangnan University for one week to adapt. The mice were maintained
119 at a room temperature of 23-25℃, room humidity of 40-70% and a 12-h light/dark cycle.
120 The mice were kept in standard laboratory IVC cages, where sterile water and standard
121 laboratory chow were provided libitum.
122 The fatty acids were dissolved in skim milk and oral gavaged, and the composition
123 of fatty acids of every group were listed in Table 1. All the animals were fed with
124 standard laboratory chow; its component was listed in Table 2. The detailed
125 experimental protocols were as follows: 72 C57BL6/J male mice were randomly
126 divided into 9 groups: control (no DSS), DSS + vehicle, DSS + α-linolenic acid (ALA),
127 DSS + punicic acid (PA), DSS + CLNA mixture, vehicle + CLNA mixture (no DSS),
128 DSS + CLNA1, DSS + CLNA2, DSS + drug (mesalazine, 5-aminosalicylic acid). Fatty
129 acids, drugs or vehicle was given once a day by gavage from days 1 to 14. All the fatty
130 acids were emulsified in 10% skim milk, and the dose of every type of CLNA isomer
131 was 400 μg/d. 400 μg/d was the dose of pure isomer to ensure the effective contrast.
132 The CLNA mixture and PA (Jian HaiRui Co., Ltd, Jian, China) was quantified by the
133 purity. 5-aminosalicylic acid (Etiasa Pharmaceutical Co., Ltd., Saint-Cloud, Paris,
134 France) was dissolved in PBS, 5 mg/d. DSS (MP Biomedicals, LLC, Irvine, CA) was

dissolved in the water and was given from days 7 to 14. The animal experiment protocol has been approved by the Ethics Committee of Jiangnan University, China (JN. No.20181130c0900515[258]) and in compliance with the Directive of 2010/63/European Community.

Induction and Assessment of Colitis. The dextran sodium sulfate was dissolved in drinking water for mice at a concentration of 3% to induce the colitis.[24] Mice were allowed to drink freely and DSS water was replaced daily. During DSS treatment from days 7 to 14, the stool consistency, weight loss, and hematochezia were detected daily to determine the disease activity index (DAI)[25-26]. The occult blood in the feces was measured by an Occult Blood kit (Nanjing Jiancheng Co., Ltd., Nanjing, China). The standard for evaluation of the disease activity index were listed in the Table 3. And the mice were sacrificed at the day 15. The Carnoy's solution (ethanol:chloroform:acetic acid, 6:3:1, vol/vol/vol) was used to fix the colon segments at 4°C for 8 h. Then the colon tissue was embedded in paraffin, sectioned (5 mm) and stained with alcian blue or H&E staining. Pannoramic MIDI Digital Slide Scanner (3D Histech Co., Ltd., Budapest, Hungary) was used to scan and capture the images of the dyed sections. The severity of colonic histological damage in each mice was graded and the valuation system of pathological scores was based on a previously reported method.[27]

Biochemical Assays. The frozen colons stored at -80°C were weighed and homogenized in saline solution by a high flux tissue crushing instrument. Then the homogenate was centrifuged at 12,000 *g*, 4°C for 10 min. The activity of myeloperoxidase (MPO) in colon was detected by commercially available kits (Nanjing

157 Jiancheng Co., Ltd., Jiangsu, China) according to the manufacturer's instructions. The
158 activity of antioxidant enzymes (SOD, GSH, CAT) were assessed by enzyme-linked
159 immunosorbent assay (ELISA) kits (Nanjing SenBeiJia Biotechnology Co., Ltd.,
160 Jiangsu, China). The total protein concentration in tissue homogenate was measured by
161 the BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). In the result of
162 MPO and antioxidant enzymes, the activity were presented in the form of U/g colon
163 protein and U/mg colon protein respectively.

164 **Level of Cytokines in Colon Tissues.** The frozen colons stored at -80°C were weighed
165 and homogenized in potassium phosphate containing 1% phosphatase inhibitor and 1%
166 protease inhibitors (Beyotime Biotechnology, Shanghai, China). Then, the cytokines
167 IL-10, IL-1β, IL-6, and TNF-α were detected by the R&D ELISA Kit (R&D Systems,
168 Minneapolis, MN) according to the instructions. The total protein concentration in
169 tissue homogenate was measured by the BCA protein assay kit (Beyotime
170 Biotechnology, Shanghai, China), and the results were presented in the form of pg/mg
171 total colon protein.

172 **Tight Junction Protein Bioassays.** The concentrations of E-cadherin 1, occludin, ZO-
173 1, and claudin-3 in colon tissue homogenate were measured by commercially available
174 ELISA kits (Nanjing SenBeiJia Biotechnology Co., Ltd., Nanjing, Jiangsu, China). The
175 results were presented in the form of pg/ml colon homogenization buffer.

176 **Fatty Acid Analysis.** The liver and colonic content were weighed and homogenized in
177 potassium phosphate, then the fatty acids were extracted and methylated as previously
178 described. Then fatty acid methyl ester were dissolved in hexane and detected by gas

chromatography (GC)−mass spectrometry (the parameters of the instrument were as previously described).[28,29]

Mouse Fecal Genome Extraction and Analysis. A certain amount of mouse feces was weighed, and the genome was extracted with a Fast DNA Spin Kit for Feces (MP Biomedicals, LLC, Irvine, CA). After extraction, the V3−V4 region of the 16S ribosomal RNA (rRNA) gene was PCR-amplified using the primers 341F: 5′-CCTAYGGGRBGCASCAG-3′ and 806R: 5′-GGACTACNNGGGTATCTAAT-3′, nucleic acid electrophoresis was carried out using the PCR products, and the corresponding strip was purified by the QIA quick Gel Extraction Kit (Qiagen, Germany). Both the genome extraction kit and the DNA purification kit were performed according to the manufacturers' instructions. After sequencing, bioinformatics analysis of the 16S rRNA sequence data was carried out as previously described.[30]

Statistical Analysis. All data are presented as the mean ± SEM of each group (n=8). The statistical significance between DSS group and other groups were analyzed by SPSS 19.0 software (SPSS Inc., Chicago, IL) in the arithmetic ANOVA. A p value of <0.05 indicates a significant difference. Data analysis was performed and mapped by GraphPad Prism 7 (GraphPad software, Inc.) Microbiota-related analyses were conducted by QIIME and R 3.5.0. Linear discriminant analysis (LDA) effect size (LEfSe) was performed by Python 2.7 and R 3.5.0.

■ RESULTS

Preparation and Detection of c9, t11, c15-CLNA and c9, t11, t15-CLNA. The α-linolenic acid was isomerized by *L. plantarum* ZS2058, which can transform

approximately 60% of ALA into CLNA[31], then the CLNA mixtures were extracted from the fermentation. Fatty acids with conjugated double bonds are characterized by an absorption peak at 233 nm. Fragment ion peak signals for these two isomers are m/z 292 and 261. A total of 600 mg of c9, t11, c15-CLNA (CLNA1) and c9, t11, t15-CLNA (CLNA2) was collected by preparative liquid chromatography. The purities of CLNA1 and CLNA2 were 93.09% and 94.09%, respectively, and the chemical structures of the two isomers are shown in Figure 1C. The cis-trans property of the C_{15} double bond of CLNA1 and CLNA2 was different. The double bond positions in plant-derived CLNAs were universally located at C_9, C_{11}, C_{13}, while the *Lactobacillus*-derived CLNA in the current study was located at C_9, C_{11}, C_{15}.

CLNA1 and CLNA2 Alleviate DSS-induced Colitis in Mice. The changes in the body weight and disease activity index (DAI) were measured daily during DSS treatment, and the colon length was measured after the mice were euthanized. Four days after DSS administration, the weight of the mice began to drop dramatically, and the final weight was 8.3% lower than the initial weight. Feeding the mice with CLNA mixture, CLNA1 and CLNA2 showed 5.7%, 6.0% and 7.0% weight loss, respectively (**Figure 2A**), demonstrating that the weight loss of the other groups was relieved compared with that of the DSS group. With the treatment of DSS, the body weight loss and DAI of mice increasing daily (**Figure 2B**).

The DAI indicates the severity of colitis during modeling in mice. The DSS group presented the highest DAI, which manifested the most serious weight loss, diarrhea and gross bleeding. The DAI was considerably reduced in the other six groups, but ALA

showed a relatively weaker effect. At the end of the experiment on day 14, the DAI for the DSS group reached a mean of 9.38, while oral administration of CLNAs, PA and drug markedly decreased the DAI to approximately 5.25.

Colon length characterizes the degree of inflammation in colon tissue and is an important indicator in colitis models. DSS challenge led to colon length shortening by 22.9% compared to that of the control group, whereas punicic acids and CLNA2 resulted in colon shortening by 12.1% and 16.8% compared to the control group. This indicated that CLNA appeared to significantly recover the colon length. Pretreatment with CLNA1 (5.31±0.181) and CLNA mixture (5.18±0.120) appeared to partially recover the colon length, but not significantly (**Figure 2C**).

H&E staining was used to observe histopathological injury (**Figure 2D**). In normal mice, the colon mucosa is intact, the villi are neat, and the crypt structure is healthy. The goblet cells were abundant in the colon tissue of normal mice, and no inflammatory cell infiltration or mucosal erosion can be observed. However, in the mice with colitis, increased loss of submucosal structures, epithelial crypts and irregular crypts as well as more widespread bowel edema can be observed. These characteristics in DSS colon tissue mean that the colitis model was successfully established. The histological injury score (**Figure 2E**) was evaluated based on the H&E staining images, and the score showed quantifiable results of tissue damage. The score of the mice in DSS group (9.67 ± 0.25) was significantly higher than normal mice (0.00 ± 0.00) ($P < 0.0001$). Treatments with CLNA1, CLNA mixture and PA significantly reduced the inflammation score of the colon. This indicated that CLNA either from *Lactobacillus*

245 or plants can relieve inflammatory cellular infiltration, submucosal edema, loss and
246 hyperplasia of crypts, and severe epithelial structure damage. From the results of
247 histopathological score, CLNA1 showed the most effective protection on the injury of
248 colon tissue. Tissue damage was reduced by treatment with ALA, which indicated
249 weaker effects compared to CLNA1 and CLNA mixture. And the drug also showed a
250 significant remission of colon tissue, which showed that the positive control group
251 made sense.

252 **CLNA1 and CLNA2 Regulate Inflammatory Cytokine Level in Colonic Tissue.** To
253 evaluate the effects of CLNA on inflammatory cytokines, the level of proinflammatory
254 cytokines and anti-inflammatory cytokines in colonic tissue was analyzed. Treatment
255 with DSS significantly increased the level of the cytokines IL-1β (**Figure 3A**), TNF-α
256 (**Figure 3B**) and IL-6 (**Figure3D**), whereas oral administration of CLNAs markedly
257 inhibited the DSS-induced upregulation of cytokine concentrations. Notably, TNF-α,
258 the most significant proinflammatory cytokine, decreased by 1.45-fold by CLNA1,
259 1.82-fold by CLNA2 and 1.92-fold by drug in the colon compared to the DSS group.
260 DSS significantly reduced the expression of IL-10 (**Figure 3C**), whereas this change
261 was attenuated by CLNAs. PPAR-γ (**Figure 3E**) showed no significant change between
262 the control, CLNA control, DSS and drug groups. However, the other five groups
263 treated with various C18:3 fatty acids all showed an increase in the expression of
264 PPAR-γ, which indicated that this change was characteristic of fatty acids. In particular,
265 the effects of CLNA1 (77.53 ± 3.82) and CLNA mixture (70.247 ± 5.52) on PPAR-γ
266 were more obvious compared to the DSS group (56.99 ± 1.28).

CLNA1 and CLNA2 Protect the Colonic Mucous Layer and Epithelium Structure. To investigate the effects of CLNAs on the mucous layer and goblet cells, the protein of mucin2 (MUC2) was measured (**Figure 4B**). The concentration of MUC2 showed a significant reduce in the DSS-treated mice (278.04 ± 6.31), whereas CLNA1 (339.28±6.47), CLNA2 (343.78±5.92) and ALA (330.13±6.03) treatments maintained its content at normal levels compared with levels in the control group (334.90±11.78). Other groups also indicated a tendency of protection on the mucous layer compared to the DSS group, although there was no significant difference. In the DSS group, the colon tissue slice stained by alixin blue (**Figure 4A**) showed that the mucin produced by goblet cells were severely damaged. CLNA1, CLNA2 and CLNA mixture significantly protected against the destruction of the mucosal layer, which were stronger than ALA at the same dose.

To evaluate the effects of CLNAs on the epithelium structure, the expression of tight junction proteins, including ZO-1 (**Figure 4C**), occludin (**Figure 4D**), E-cadherin1 (**Figure 4E**), claudin-3 (**Figure 4F**), and in the colon was measured. Treatment with mesalazine, CLNA1, CLNA2, CLNA mixture and ALA significantly increased the concentrations of the four key TJ proteins compared with the DSS group. Moreover, the protective effects of CLNAs on E-cadherin 1 and claudin-3 were stronger than mesalazine. From the overall results of the four TJ proteins, the PA displayed a weaker protective effect on the intestinal barrier compared with CLNAs, which indicated that the CLNAs derived from *Lactobacillus* may be better than those from plants. There was no obvious difference between the CLNA1, CLNA2, and CLNA

mixture groups, so it is impossible to conclude which isomer is more potent.

CLNA1 and CLNA2 Regulated Oxidative Stress. Oxidative stress is the essential cause of cell and tissue damage. It can result in abnormal metabolism of oxygen free radicals and excessive activation of apoptosis. Oxygen free radicals could result in the release of inflammatory mediators.[32] And many papers have reported that plant-derived CLNA showed an antioxidant activity.[33] To investigate the influence of *Lactobacillus* derived CLNAs on oxidative stress, CAT activity (**Figure 5A**), SOD activity (**Figure 5B**) and GSH-PX (**Figure 5C**) activity in the colon were measured. All these enzymes play key roles in protecting cells from damage induced by inflammation and oxidative stress. There was no significant change between the control, DSS and CLNA control groups. However, increased levels of the three indexes were observed in the other treatment groups. Treatment with CLNA1 significantly increased SOD and GSH activity 1.29- and 1.48-fold compared with DSS treatment, respectively. In addition, CLNA2 could significantly increase the level of CAT 1.27-fold compared with DSS. The activity of SOD and GSH-PX in colon tissues were significantly increased by CLNA1, while CLNA2 significantly increased CAT activity, which indicated that CLNA alleviated colitis by inhibiting oxidative stress. In addition, CLNA1 showed a more remarkable influence on oxidative stress. These results were similar to those of studies on plant-derived CLNA. PA and α-ESA have been proven to reduce oxidative stress and lipid peroxidation and restore antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase.[34] Treatment with ALA or PA could induce a higher activity of CAT and SOD. For the drug control group, CAT activity

showed an obvious improvement compared to other groups. To evaluate the effects of CLNA on colonic inflammatory enzymes, the activity of MPO in colon, which can reflect neutrophil infiltration, was measured. All the treatments in this experiment can significantly decrease the activity of MPO induced by DSS. Only ALA group showed a relatively weak effect on MPO compared with other treatment groups (**Figure 5D**).

The Fatty Acid Composition in the Blood, Liver and Colonic Contents. To evaluate the distribution of orally administered CLNA in mice, the composition of fatty acids in blood, liver and colonic contents was analyzed. The composition of fatty acids in blood showed no significant difference among the nine groups (**Figure 6A**). No CLNA or CLA was detected in the blood, which may be due to the content being too low to detect or the conjugated fatty acids being metabolized at the point of blood collection. There was a considerable change in liver and colonic contents, which mainly focused on the proportion of CLA or CLNA in total fatty acids. In the liver, the percentages of CLNA in total fatty acids from the CLNA control and CLNA mixture groups were 3.04±0.17 and 1.48±0.23, respectively, which manifested an increase compared to the control (2.39±0.38) and DSS groups (0.70±0.12) (**Figure 6B**). Other five groups showed no significant differences on the content of CLNA in liver compare to DSS group. Interestingly, unlike the liver, there was no significant difference in the ratio of CLNA in colonic contents between the nine groups. However, the ratio of CLA in total fatty acids increased significantly in the CLNA treatment group. The CLNA Mix group (1.74±0.22) showed the most obvious enhancement in the CLA ratio, which was 3.28-fold compared with that of the DSS group (0.55±0.11). In addition, the CLNA control

group also showed an increase in the CLA percentage at 0.77±0.06 compared with the control group at 0.09±0.09. These results indicated that CLNA was eventually metabolized into CLA in the contents of the colon. (**Figure 6C**). Another interesting result indicated that the DSS group showed a higher CLA content than the control group, while the content of CLA in the CLNA mixture group was also higher than that in the CLNA control group. This may be because DSS-induced colitis can result in the production of CLA. In addition, ALA can also significantly increase the composition of CLA. Whereas PA group was similar to drug group without significant increase on the content of CLA.

CLNA1 and CLNA2 Influenced the Intestinal Microbiota. To determine the correlation between gut microbiota and the effects of CLNA treatment, the gut microbiota of mice treated with CLNAs, DSS, PA, ALA, and mesalazine and the control group were studied based on 16S rDNA amplicon sequencing. The microbial distribution of nine groups on the phylum level was analyzed (**Figure 7A**). Compared with the control group, the intestinal microbial structure of mice in the DSS group changed significantly. The predominant phyla in control group were Bacteroidetes (68.19%), Firmicutes (20.09%) and Verrucomicrobia (6.36%) (**Figure 7A**). However, DSS treatment dramatically altered the bacterial composition at the phylum level, and the relative abundance of Proteobacteria increased from 0.92% to 16.33%, while the relative abundances of Bacteroidetes and Verrucomicrobia decreased to 53.64% and 4.67%, respectively (**Figure 7A**). The proportion of Firmicutes showed no significant change. However, CLNA1 significantly increased the abundance of Firmicutes. And

355 there was a great improvement on the abundance of Deferribacteres in drug group
356 (5.18%) compared to that in DSS group (1.27%).

357 α-Diversity was evaluated by the Chao1 and Shannon indexes (**Figure 7B**). Chao1
358 reflects the community richness of species within a single sample, while the Shannon
359 index represents microbial diversity. The Chao1 and Shannon indexes in the DSS group
360 were much lower than those in the control group, and they were significantly increased
361 by drug treatment. In addition, the Chao1 of the CLNA1, CLNA mixture and ALA
362 groups was also significantly different from that of the DSS group.

363 The gut microbiota diversity among different groups was analyzed by the LDA
364 effect size (LEfSe) (**Figure 7C, 7D**). The LDA score histogram was drawn to identify
365 statistically significant biomarkers and reveal the dominant microorganisms in each
366 group. The proportion of major bacterial communities largely shifted within the
367 different treatment groups. Among them, *Turicibacter* was dominant in the DSS group;
368 Firmicutes was dominant in the CLNA control group; *Parabacteroides* and
369 Porphyromonadaceae were the dominant microbes in the CLNA mixture group; and
370 Clostridiales and Clostridia were dominant microbes in the CLNA1 group. *Bacteroides*
371 and Bacteroidaceae were the dominant microbes in the ALA group. Prevotellaceae and
372 *Provetella* were dominant microbes in the CLNA2 group. DSS significantly decreased
373 Bacteroidetes and increased Proteobacteria. However, there was no dominant
374 microorganisms in drug control, which meant the abundance of microorganisms in drug
375 control showed no significant superiority compared with other groups. Furthermore,
376 the correlations between the colonic CLA concentration, TJ proteins, differential

377 microorganisms, and inflammation markers were analyzed. Colon length, histological
378 scores, MPO, and DAI were the characteristic index in colitis and had higher weightings
379 in the network analysis (**Figure 7E**). The concentration of colonic CLA positively
380 correlated with *Desulfovibrio* and *Enterobacter* but negatively correlated with
381 *Coprococcus*.

382 ## ■ DISCUSSION

383 Probiotics have been claimed to possess functions such as suppressing inflammation,
384 protecting the intestinal barrier, and increasing the body's antioxidant capacity.[35-36] The
385 benefits of probiotics have been attributed to certain beneficial metabolites produced
386 by them. In this study, we used *Lactobacillus plantarum* ZS2058 to isomerize α-
387 linolenic acid and assess the function of the CLNA produced. The structure of
388 *Lactobacillus*-derived CLNA is different from that of plant-derived CLNA, and they
389 can be defined as a new material synthesized by bacteria. Until now, CLNA1 and
390 CLNA2 have not been comprehensively studied, and there have been no functional *in*
391 *vivo* studies on any *Lactobacillus*-derived single isomer of CLNA.

392 There was no abnormality in the control mice fed the CLNA mixture; therefore, it
393 was speculated that the CLNA mixture was safe for 14 days of gavage. Tom et al. added
394 30.33% CLNA (c9, t11, c15-CLNA + c9, t13, c15-CLNA) to the diet of neonatal pigs
395 and concluded that short-term intake of CLNA was safe.[37] In addition, the weight of
396 the mice after 14 days of gavage of the CLNA mixture was less than that of the control
397 mice, indicating that *Lactobacillus*-derived CLNA can lower body weight and weight
398 gain. This is similar to the effect of plant-derived CLNA, such as punicic acid or α-

399 ESA, on obese mice.[38-40] From the results of the composition of conjugated fatty acids
400 in the blood, liver and colonic contents, there was no obvious change in the blood.
401 Hiroyuki reported that the inhibition of colonic tumors by pomegranate seed oil (70%
402 c9, t11, c13-CLNA) was associated with an increased content of c9, t11-CLA in the
403 lipid fraction of the colonic mucosa and liver.[41] CLA has been thoroughly certified to
404 alleviate colon cancer or colitis, so the function of CLNA may partially be due to the
405 increase in CLA in mice. In our study, the CLNA significantly increased in the liver,
406 while the CLA significantly increased in the colonic contents of mice given CLNA. We
407 hypothesized that CLNAs may be converted to CLA in the gut of mice.

408 PPARs are receptors for endogenous lipid molecules representing promising new
409 targets for the treatment and prevention of IBD.[42] PPARγ can inhibit the activation and
410 nuclear import of NF-κB by the IkB-α pathway in which NF-κB plays a key role in the
411 regulation of the inflammatory response and pathogenesis of IBD.[43] PPAR-gamma is
412 also an important target during the conjugated linolenic acids work on some diseases
413 like IBD and obesity.[42] The expression of PPAR-γ in mice treated with CLNA1 and
414 CLNA mixture was significantly increased compared with the control group. This was
415 consistent with the plant-derived CLNA and CLA. The loss of functional PPARγ or
416 PPARδ impaired the anti-inflammatory effects of punicic acid, such as upregulated
417 Foxp3 expression in T-cells and suppressed TNF-α.[44] α-ESA was also identified as a
418 natural PPARγ agonist and found to be effective in ameliorating disease-associated
419 phenotypes in mice with DSS colitis.[45] Bassaganya Riera et al. used PPARγ knockout
420 mice to prove that CLA was able to reduce colitis by activating PPARγ.[46] CLA induced

apoptosis in HT-29 and Caco-2 cells by upregulating PPARγ.[47] CLA in the colon targeted myeloid cell PPARγ to suppress colitis.[48] Overall, the CLNA1 and CLNA mixture alleviated colitis by targeting PPARγ to reduce inflammation, while CLNA2 showed an ambiguous effect on PPARγ.

A physical barrier was formed by the epithelium and the mucosal layer to avoid the potentially toxic and noxious agents to disseminate into the underlying tissue, which induced the inflammation in the colon. The alteration in cytokine profiles, in turn, further trigger the decline in tight junctions. Therefore, a vicious cycle of mucosal barrier dysfunction and inflammation is established.[49-50] Treatments with *Lactobacillus*-derived CLNAs increased the concentration of MUC2, contributing to the integrity of the colonic mucous layer and goblet cells. Tight junction proteins connect enterocytes and play an important role in the integrity of the intestinal barrier.[51] The CLNA1, CLNA2 and CLNA mixture significantly upregulated TJ proteins (E-cadherin 1, ZO-1, claudin-3, and occludin) and CLNA1 showed a stronger role in protecting the intestinal barrier than PA. *Lactobacillus*-derived CLNA also manifested stronger regulation of the expression of relative cytokines than PA. As for the level of cytokine, PA just showed significance on IL-6, but *Lactobacillus* derived CLNA showed significance on IL-β, TNF-α and PPAR-gamma. So *Lactobacillus*-derived CLNA showed more effective influence against colitis based on the TJ protein and cytokine results. However, no obvious difference was observed in the expression of TJ proteins and cytokines between the CLNA1 and CLNA2 groups.

Interaction disorder between intestinal microbes has been confirmed as a critical

443 defect resulting in intestinal inflammation.[52] In the current study, DSS decreased
444 bacterial species richness and shifted the bacterial community composition. The
445 CLNA1 isomer significantly increased the α-diversity, and the CLNA control group
446 was more diverse than the control group. A higher abundance of the phylum
447 Verrucomicrobia was observed in the CLNA2 and ALA groups, which was more than
448 twice as abundant as the DSS group. Verrucomicrobia was considered to be associated
449 with the higher expression of MUC2.[53] In humans, there is an overall trend toward
450 lower biodiversity and decreased abundance of Firmicutes in people with IBD
451 compared with controls.[54] CLNA1 increased the abundance of the phylum Firmicutes
452 approximately 1.5 times compared with the DSS group. At the genus level, CLNA1
453 treatment significantly increased the abundance of *Parabacteroides* and *Dorea*.
454 CLNA2 increased the abundance of *Prevotella*, which had a positive association with
455 the control group.[55] Ce´line et al. reported that PUFA-derived bacterial metabolites,
456 including CLNA and CLA, were positively correlated with specific fecal bacteria
457 (*Bifidobacterium spp.*, *Eubacterium ventriosum* and *Lactobacillus spp.*).[56] The
458 abundance of *Bifidobacterium* was exactly improved in the CLNA control group.
459 *Bacteroides fragilis* strains can invade intestinal tissues and cause damage.[57] DSS
460 significantly increased the abundance of *Bacteroides* compared to the control and
461 CLNA control groups; however, no significant decrease was observed in the other
462 treatment groups. Thus, our results indicated that CLNA treatment partially prevented
463 the microbiota changes induced by DSS.

464 In the current study, c9, t11, c15-CLNA and c9, t11, t15-CLNA were proven to

alleviate colitis in mice and have different functions compared with other fatty acids. From the weight loss, colon length, DAI and pathological scores, CLNA1, CLNA mix and PA showed relatively stronger effects on colitis, while CLNA2 and ALA showed relatively week effects. For cytokines, mucin protein, tight junction proteins and antioxidant enzymes, CLNA1 and CLNA mixture showed a strong regulation on those indexes, while CLNA2, PA and ALA showed a relatively week regulation. The primary mechanisms of relieving DSS-induced colitis by the two isomers involved inhibiting proinflammatory factors, protecting mucosal barriers, and regulating oxidative stress and intestinal microbial damage. CLNA entered the bowel lumen and then increased the concentration of CLA, which could relieve colitis through all of the above aspects.[30] Furthermore, CLNA in the bowel lumen improved the activity of antioxidant-related enzymes, which could improve the intestinal barrier. At the same time, CLNA could directly enter into the mucus layer and epithelial cells to regulate MUC2 and TJ proteins. In addition, CLNA can enter the lamina propria of mice and regulate the levels of pro-inflammatory or anti-inflammatory cytokines to reduce the inflammatory response. CLNA could increase the α-diversity in colonic contents and regulate the bacterial flora. These results will assist us explore the mechanism of CLNAs to reduce colitis and investigated the mechanism of CLNA regulating other immune-related diseases, so as to guide the further development of CLNA research.

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664 ■ **FIGURE CAPTIONS**

665 **Figure 1. Preparation and purity detection of CLNA1 and CLNA2.**

666 (A) Separation of CLNA1 and CLNA2 by liquid chromatograph. (B) Mass spectrum of
667 CLNA1 and CLNA2. (C) Chemical structures of conjugated linolenic acid (CLNA)
668 isomers used in this study (CLNA1: c9, t11, c15-CLNA, CLNA2: t9, t11, c15-CLNA).

669

670 **Figure 2. Symptoms of DSS-induced colitis.**

671 (A) Body weight, (B) disease activity index (DAI), (C) colon length, (D) histological
672 examination (the scale bars are 200 μm). (E) colonic histological injury.
673 Data are presented as mean ± SEM (n =8 mice per group). *: $p < 0.05$, **: $p < 0.01$ and
674 ***: $p < 0.001$, ****: $P < 0.0001$ compared with DSS group.

675

676 **Figure 3. Effects of CLNA on inflammatory cytokines in colonic tissues.**

677 (A) IL-1β, (B) TNF-α, (C) IL-10, (D) IL-6, and (E) PPAR γ.
678 Data are presented as mean ± SEM (n =8 mice per group). *: $p < 0.05$, **: $p < 0.01$ and
679 ***: $p < 0.001$, ****: $p < 0.0001$ compared with DSS group.

680

681 **Figure 4. Effects of CLNA on the mucous layer.**

682 (A) Alcian blue staining (scale bar = 200 μm), (B) concentration of MUC2, (C)
683 expression of ZO-1 in colon, (D) expression of Occludin in colon, (E) expression of E-
684 cadherin1 in colon, (F) expression of Claudin-3 in colon.
685 Data are presented as mean ± SEM (n =8 mice per group). *: $p < 0.05$, **: $p < 0.01$ and

686 ***: $p < 0.001$, ****: $P < 0.0001$ compared with DSS group.

687

688 **Figure 5. Effects of CLNA on the activity of oxidative-stress-related enzymes in**
689 **the colon.**

690 (A) CAT, (B) GSH, (C) SOD, and (D) MPO.

691 Data are presented as mean ± SEM (n =8 mice per group).*: $p < 0.05$, **: $p < 0.01$ and
692 ***: $p < 0.001$, ****: $p < 0.0001$ compared with DSS group.

693

694 **Figure 6. Concentration of CLNA and CLA in the blood, liver and colonic content**.

695 (A) The composition of main fatty acids in blood. (B) The concentration of CLNA and
696 CLA in the colonic content. (C) The concentration of CLNA1 and CLNA2 in liver.

697 Data are presented as mean ± SEM (n =8 mice per group). *: $p < 0.05$, **: $p < 0.01$ and
698 ***: $p < 0.001$, ****: $p < 0.0001$ compared with DSS group.

699

700 **Figure 7. Evaluation of Illumina MiSeq sequencing data showing that CLNA could**
701 **modulate the overall structure of gut microbiota**.

702 (A) Microbial distribution at the phylum level. (B) α-Diversity indicated by the Chao1
703 index and Shannon index. (C) Cladogram. (D) Distribution histogram based on LDA,
704 with a log LDA score above 3.0. (E) Correlation analysis of significant taxa, colitis
705 indexes, tight junction proteins, antioxidant enzymes, cytokines in the colon and the
706 concentration of colonic CLA. Only significant correlations (*: $p < 0.05$, **: $p < 0.01$)
707 are displayed.

■ TABLEs

Table 1. The composition of fatty acids of every group.

Group	Composition					
CLNA1	93.09% CLNA1	6.91% ALA				
CLNA2	94.09% CLNA2	5.91%CLNA1				
CLNA Mix	1.18% C18:0	3.49% C18:1	0.48% C18:2	13.23% ALA	72.65% CLNA1	8.97% CLNA2
PA Mix	76.46% PA	2.14% C9:4	3.71% C16:0	3.15% C18:0	5.94% C18:1	8.60% C18:2

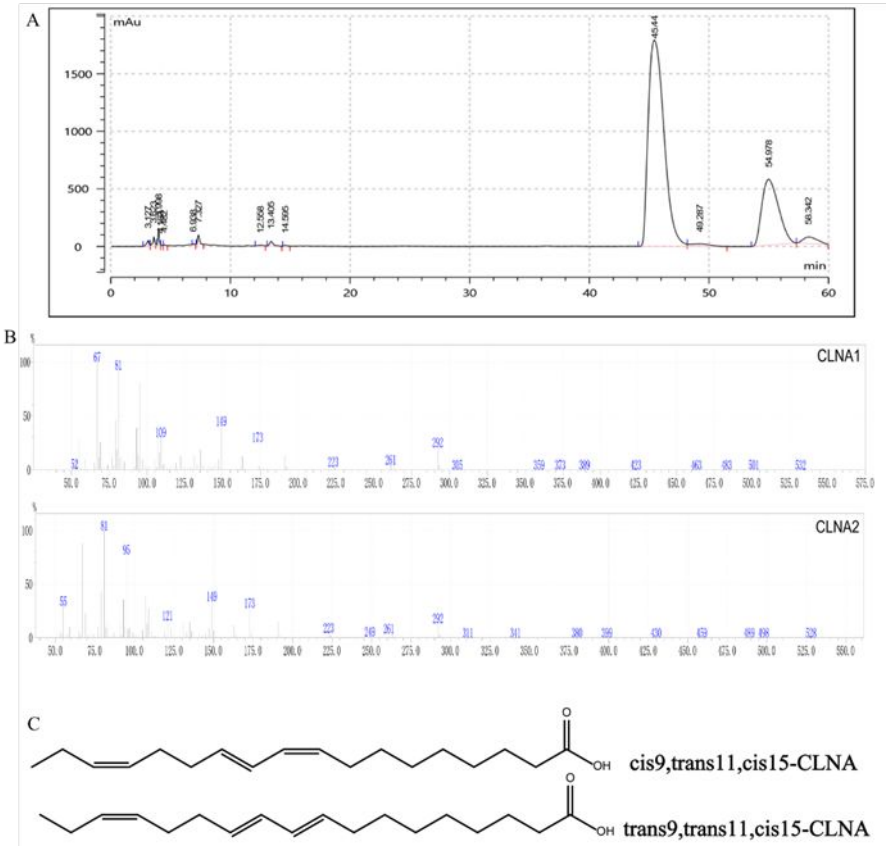
Table 2. The component of standard laboratory chow.

Component	Content (g/kg)
Water	98.0
Crude ash	51.4
Crude protein	190.1
Crude fat	49.6
Crude fiber	25.7
Calcium	11.3
Phosphorus	7.0

Table 3. The standard for evaluation of the disease activity index.

Weight Loss (%)	Occult Blood or Gross Bleeding	Stool Consistency	Score
0	Negative	Normal	0
1-5	Negative	Loose Stool	1
6-10	Hemoccult Positive	Loose Stool	2
11-15	Hemoccult Positive	Diarrhea	3
＞15	Gross Bleeding	Diarrhea	4

Normal stools = well formed pellets; loose stools = pasty stool that does not stick to the anus; and diarrhea = liquid stools that sticks to the anus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CLNA2
C
cis9,trans11,cis15-CLNA
trans9,trans11,cis15-CLNA
OH
O

Figure 1

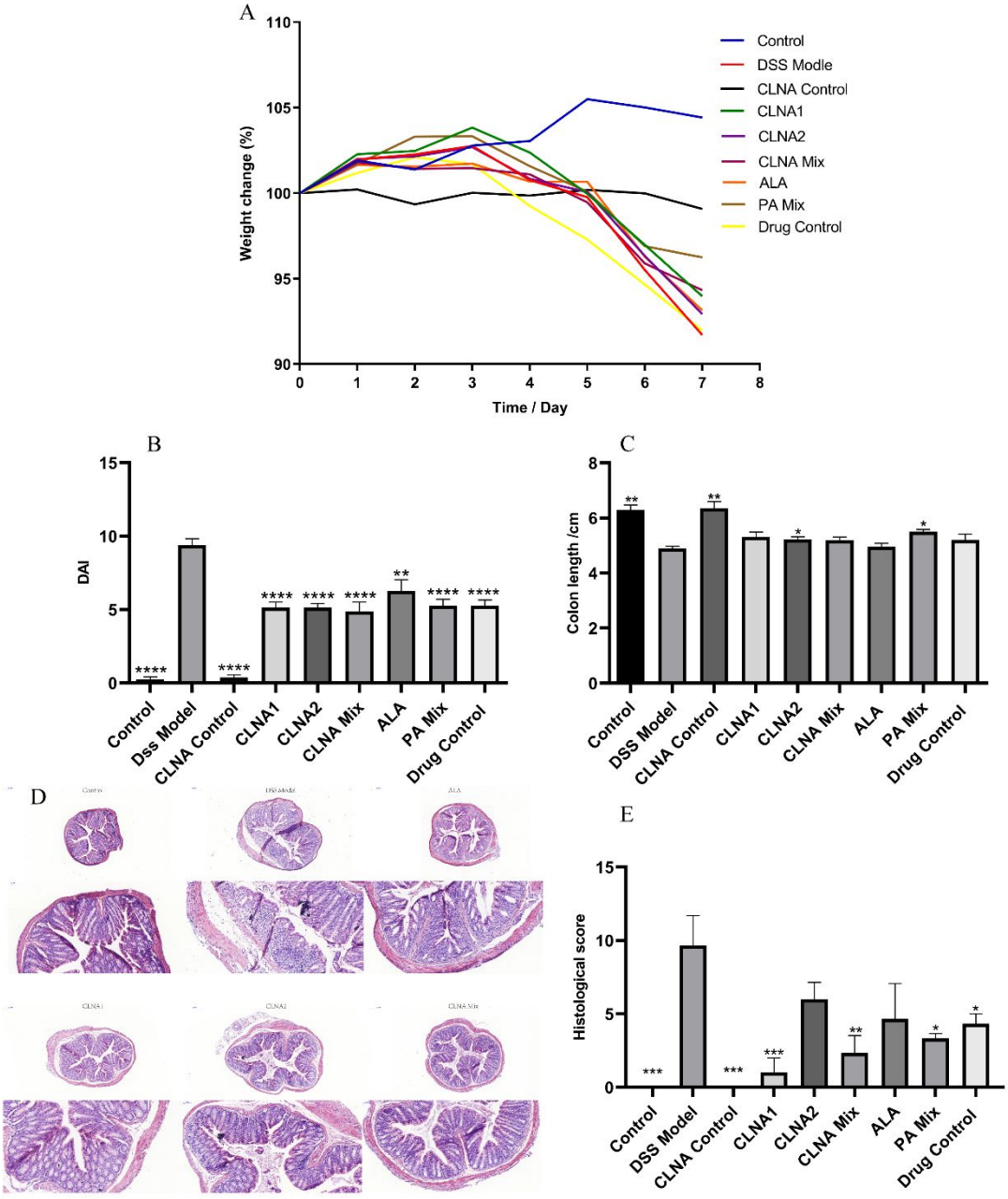

A
Weight change (%)
Time / Day
Control
DSS Modle
CLNA Control
CLNA1
CLNA2
CLNA Mix
ALA
PA Mix
Drug Control
B
DAI
C
Colon length /cm
D
E
Histological score

Figure 2

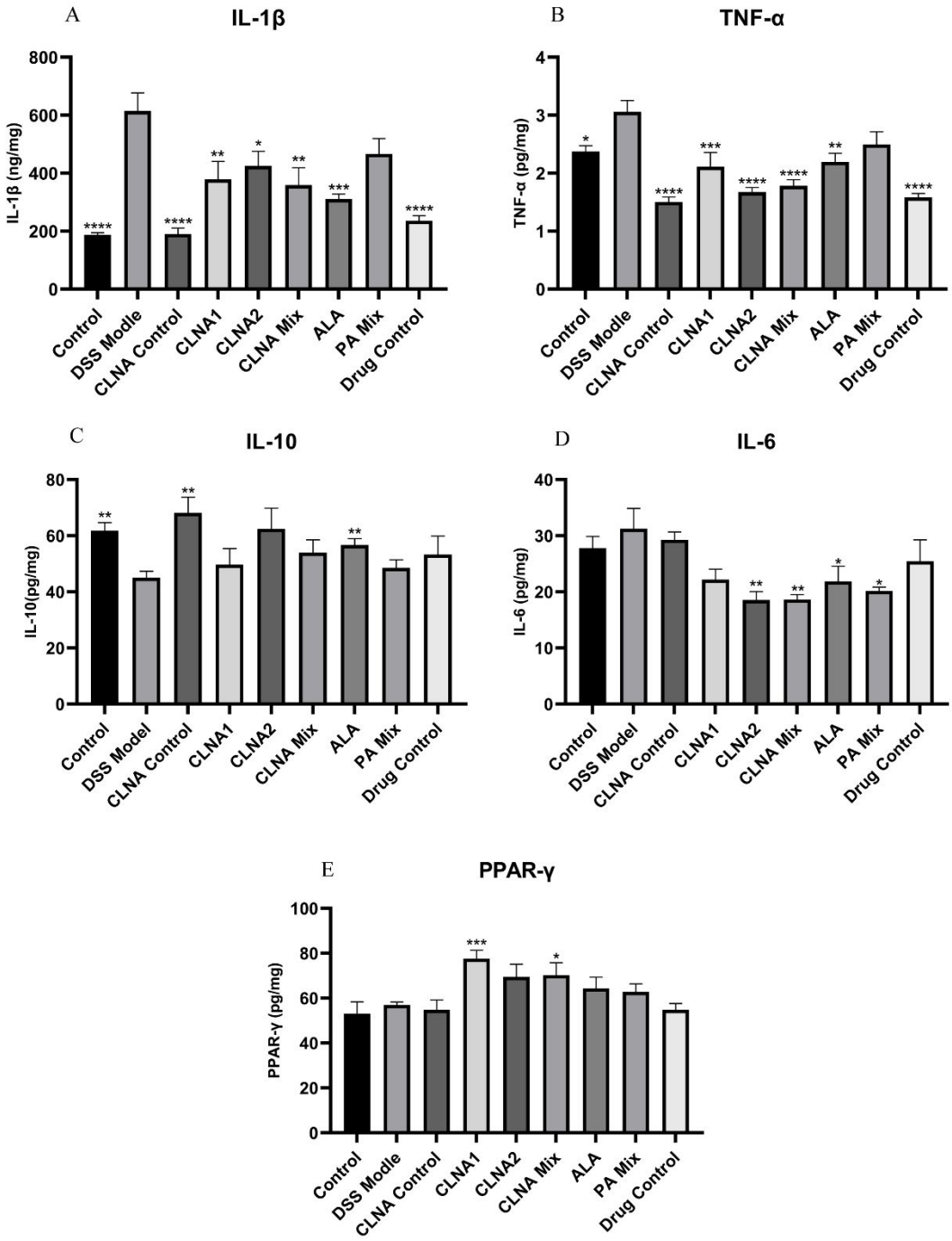

A
IL-1β
IL-1β (ng/mg)
800
600
400
200
0
Control
DSS Modle
CLNA Control
CLNA1
CLNA2
CLNA Mix
ALA
PA Mix
Drug Control
B
TNF-α
TNF-α (pg/mg)
4
3
2
1
0
C
IL-10
IL-10(pg/mg)
80
60
40
20
0
D
IL-6
IL-6 (pg/mg)
40
30
20
10
0
E
PPAR-γ
PPAR-γ (pg/mg)
100
80
60
40
20
0

Figure 3

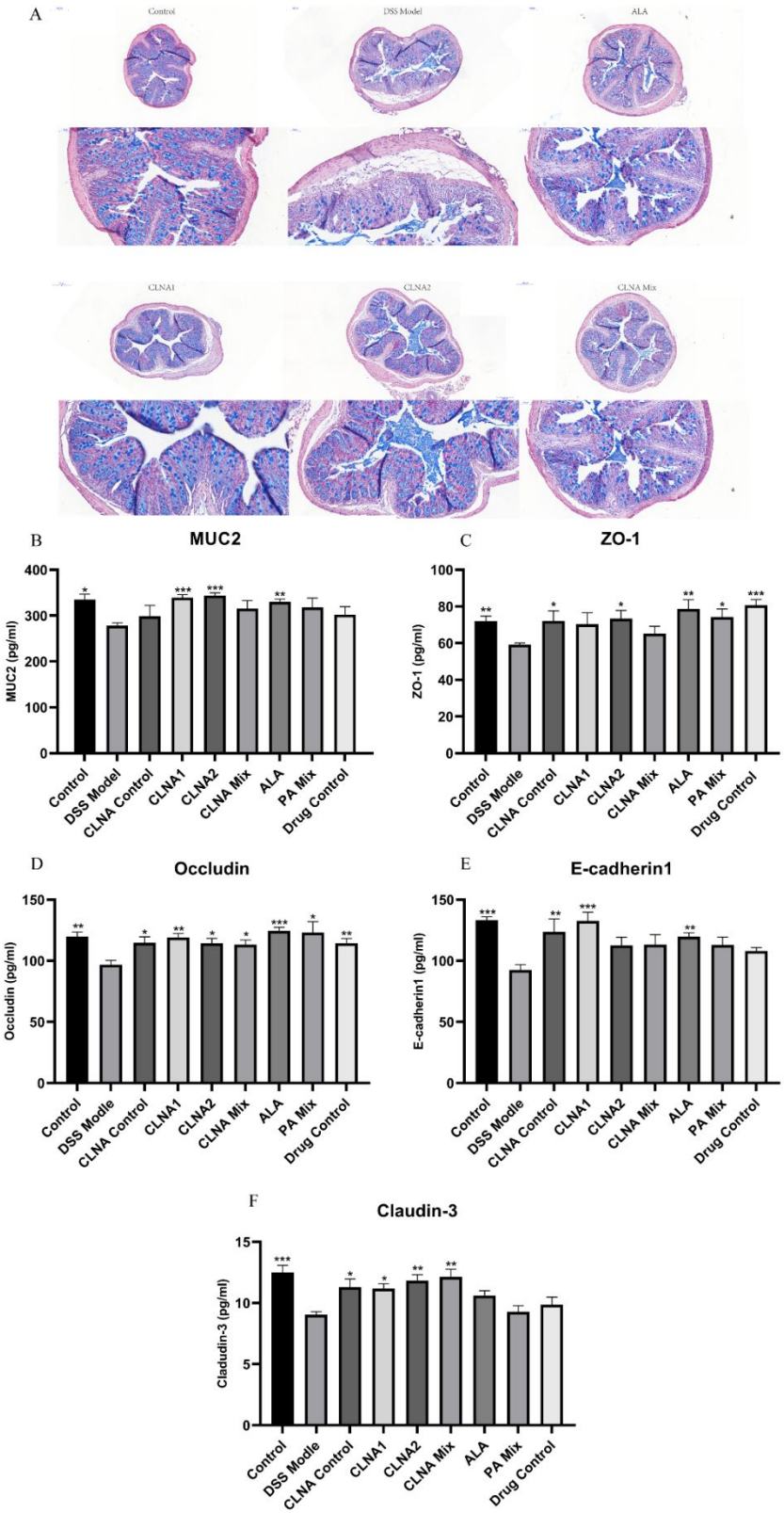

A
Control
DSS Model
ALA
CLNA1
CLNA2
CLNA Mix
B
MUC2
MUC2 (pg/ml)
C
ZO-1
ZO-1 (pg/ml)
D
Occludin
Occludin (pg/ml)
E
E-cadherin1
E-cadherin1 (pg/ml)
F
Claudin-3
Cladudin-3 (pg/ml)
Control
DSS Modle
CLNA Control
CLNA1
CLNA2
CLNA Mix
ALA
PA Mix
Drug Control

Figure 4

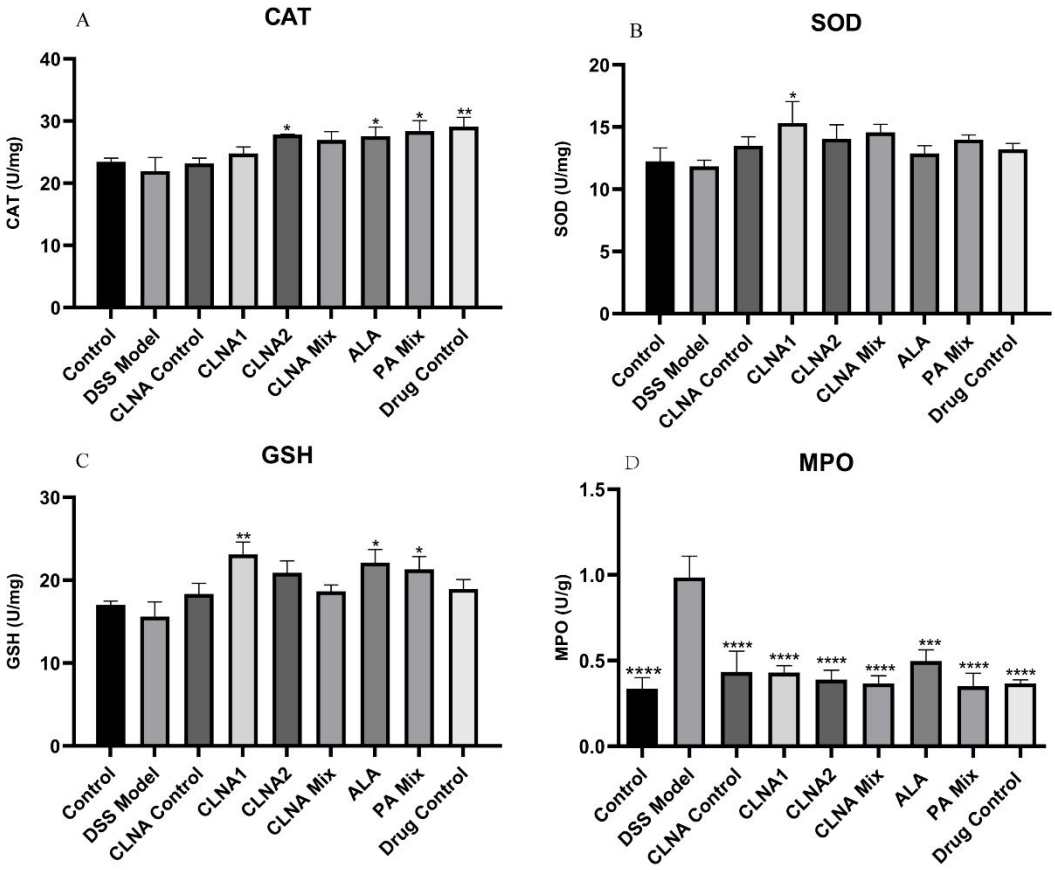

A
CAT
CAT (U/mg)
B
SOD
SOD (U/mg)
C
GSH
GSH (U/mg)
D
MPO
MPO (U/g)
Control
DSS Model
CLNA Control
CLNA1
CLNA2
CLNA Mix
ALA
PA Mix
Drug Control

Figure 5

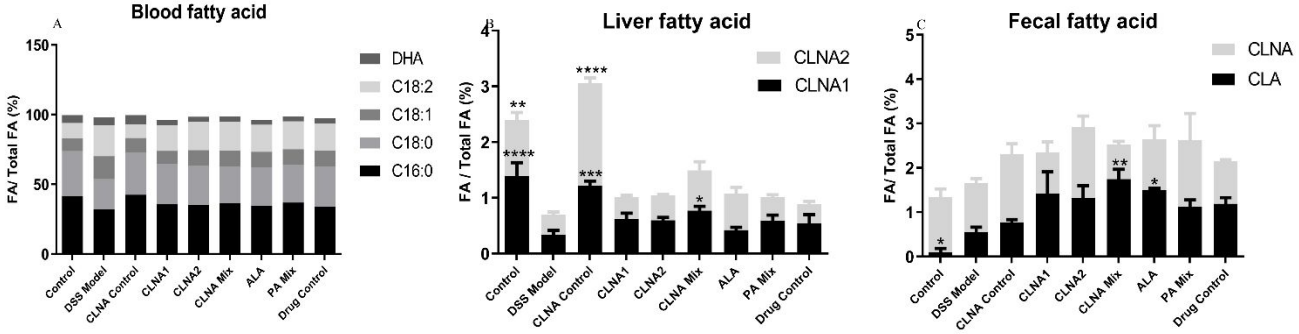

A
Blood fatty acid
FA/ Total FA (%)
150
100
50
0
DHA
C18:2
C18:1
C18:0
C16:0
Control
DSS Model
CLNA Control
CLNA1
CLNA2
CLNA Mix
ALA
PA Mix
Drug Control
B
Liver fatty acid
FA / Total FA (%)
4
3
2
1
0
CLNA2
CLNA1
**
****
****
***
*
C
Fecal fatty acid
FA/ Total FA (%)
5
4
3
2
1
0
CLNA
CLA
*
**
*

Figure 6

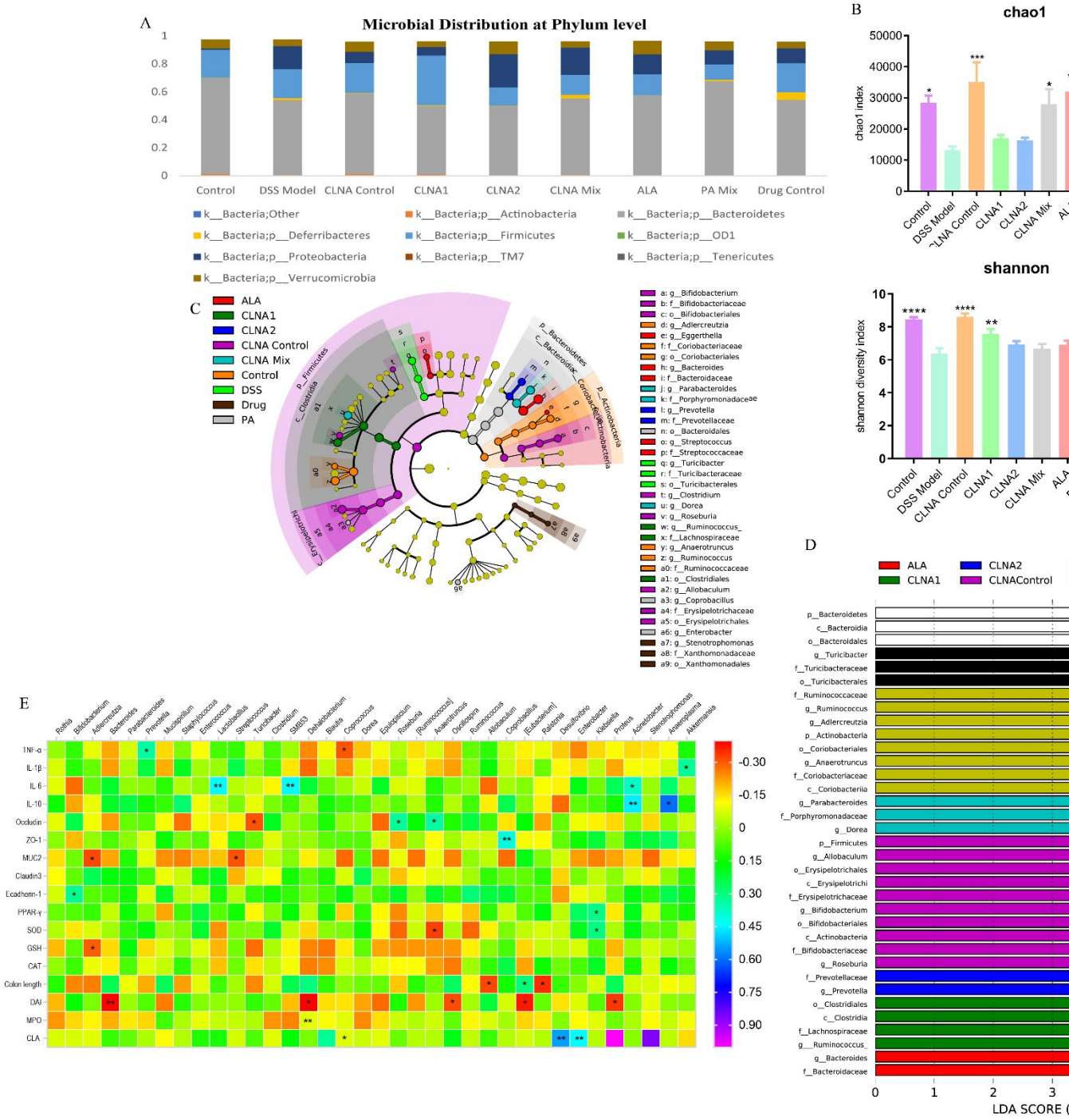
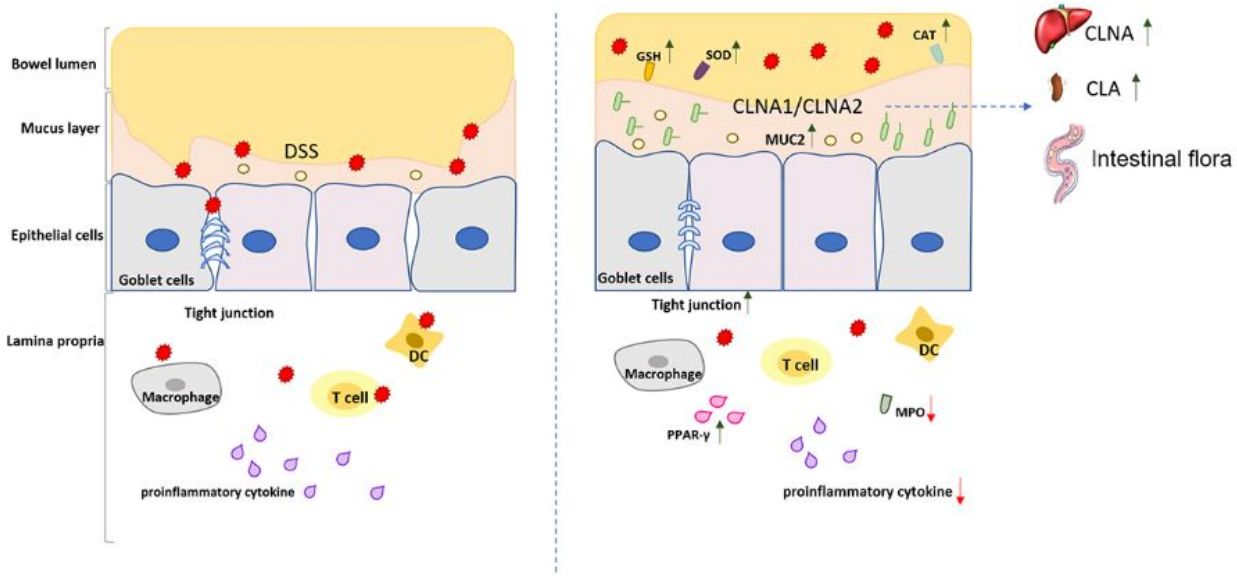

A
Microbial Distribution at Phylum level
Control
DSS Model
CLNA Control
CLNA1
CLNA2
CLNA Mix
ALA
PA Mix
Drug Control
k__Bacteria;Other
k__Bacteria;p__Actinobacteria
k__Bacteria;p__Bacteroidetes
k__Bacteria;p__Deferribacteres
k__Bacteria;p__Firmicutes
k__Bacteria;p__OD1
k__Bacteria;p__Proteobacteria
k__Bacteria;p__TM7
k__Bacteria;p__Tenericutes
k__Bacteria;p__Verrucomicrobia
B
chao1
chao1 index
shannon
shannon diversity index
C
ALA
CLNA1
CLNA2
CLNA Control
CLNA Mix
Control
DSS
Drug
PA
D
ALA
CLNA1
CLNA2
CLNAControl
LDA SCORE
E
TNF-α
IL-1β
IL-6
IL-10
Occludin
ZO-1
MUC2
Claudin3
Ecadherin-1
PPAR-γ
SOD
GSH
CAT
Colon length
DAI
MPO
CLA

Figure 7


Bowel lumen
Mucus layer
Epithelial cells
Lamina propria
DSS
Goblet cells
Tight junction
DC
Macrophage
T cell
proinflammatory cytokine
GSH
SOD
CAT
CLNA1/CLNA2
MUC2
Goblet cells
Tight junction
Macrophage
T cell
DC
MPO
PPAR-γ
proinflammatory cytokine
CLNA
CLA
Intestinal flora

TOC GRAPHIC