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Synergy effects of Ganoderic acid A and Ganoderma lucidum polysaccharides on enhancing protection against DSS-induced colitis via gut microbiota rebalancing and inflammation suppression

Peiyi Wang^{a, b #}, Ken Cheng^{a #}, Dongcheng Shi^a, Peilong Sun^a, Wenling Tang^c, Ming Cai^{a *}

^a Key Laboratory of Food Macromolecular Resources Processing Technology Research (China National Light Industry), College of Food Science and Technology, Zhejiang University of Technology, Hangzhou, Zhejiang 310014, China

^b Engineering Research Center of Bio-Process, Ministry of Education, School of Food and Biological Engineering, Hefei University of Technology, Hefei, Anhui 230009, China

^c Lishui Central Hospital, Lishui, Zhejiang 323020, China

ABSTRACT: Inflammatory bowel disease (IBD) is characterized by persistent intestinal inflammation and gut microbiota dysbiosis. The study investigated the protective effects of *Ganoderma lucidum* polysaccharide (GLP) and ganoderic acid A (GAA) administered individually and in combination on dextran sulfate sodium (DSS)-induced colitis in mice. Disease activity index (DAI), colon length, histopathology, serum inflammatory cytokines (TNF- α , IL-1 β , IL-6), TLR4/NF- κ B pathway proteins, gut microbiota composition, and short-chain fatty acids (SCFAs) were evaluated. Combination therapy of GLP and GAA (GL-PA) significantly ameliorated body weight loss, reduced DAI scores, and restored colon length compared to individual treatments ($P < 0.05$). Histopathological analysis revealed preserved crypt architecture and reduced inflammatory infiltration. GL-PA markedly suppressed serum cytokine levels and inhibited TLR4/NF- κ B activation more effectively than GLP or GAA alone ($P < 0.05$). Microbiota analysis showed GL-PA restored gut diversity, increased Firmicutes/Bacteroidetes ratio, promoted beneficial bacteria (*Lactobacillus*, *Turicibacter*) while suppressing pathogenic bacteria (*Bacteroides*, *Escherichia-Shigella*, and *Desulfovibrionaceae*). GL-PA significantly enhanced SCFAs production, particularly propionate and butyrate ($P < 0.01$). Correlation analysis revealed associations between harmful bacteria and inflammatory markers, while beneficial bacteria correlated with SCFAs levels. The combination exhibits synergistic protective effects through dual mechanisms: inflammatory pathway suppression and gut microbiota homeostasis restoration. GL-PA represents a promising natural therapeutic strategy for IBD treatment.

Keywords: Natural products; Anti-inflammatory; NF- κ B signaling pathway; Gut microbiota; Short chain fatty acids (SCFAs); Synergistic effect

1. Introduction

Inflammatory bowel disease (IBD) is a chronic relapsing intestinal inflammatory disorder with an etiology that is not yet fully understood. It primarily encompasses ulcerative colitis (UC) and Crohn's disease (CD) [1]. Over the past few decades, the incidence of IBD has continued to rise globally, with a particularly

These authors contributed equally to this work.

*Corresponding author
caiming@zjut.edu.cn

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rapid increase observed in emerging developing countries in Asia, South America, and Africa [2]. IBD not only significantly impairs patients' quality of life but also imposes a substantial burden on healthcare systems. Its progressive nature often leads to recurrent hospitalizations, malnutrition, anemia, and colorectal cancer, resulting in significant economic and psychological pressures on both patients and their families [3]. The pathogenesis of IBD is complex, involving multiple interactive mechanisms, including genetic susceptibility, dysbiosis of the gut microbiota, immune system dysfunction, and environmental factors [4]. Current clinical treatments mainly rely on aminosalicylates, corticosteroids, immunosuppressants, and biologics. Although these therapies have demonstrated some efficacy in alleviating symptoms and slowing disease progression, they are limited still by suboptimal efficacy, high relapse rates, and frequent adverse reactions [5-6].

Natural products, in contrast, typically exhibit low toxicity and side effects, with good patient tolerability. Their long-standing traditional use and remarkable therapeutic effects have led to their widespread application in the treatment of various refractory intestinal diseases [7]. Moreover, natural products have played a crucial role in drug discovery and development, accounting for over 50% of globally approved small-molecule drugs, highlighting their immense pharmacological potential and translational value [8]. Importantly, natural products possess complex and multifaceted mechanisms of action against IBD, often simultaneously exerting anti-inflammatory, antioxidant, immune-modulatory, and gut microbiota-reshaping effects [9-11]. Therefore, further exploration of the synergistic effects or mechanisms of action of natural bioactive compounds or their combinations in IBD treatment may provide new insights for the development of safer and more effective therapeutic strategies.

Ganoderma lucidum polysaccharides (GLP) and ganoderic acid A (GAA) are important bioactive components of the medicinal and edible homology mushroom *Ganoderma lucidum*. Their pharmacological effects on immune regulation and anti-inflammation have been widely confirmed. Previous studies have shown that GLP mainly exerts its immune-regulatory function by activating macrophages, modulating T cell differentiation, and promoting interactions among immune cells [12-13]. In intestinal inflammation models, GLP can significantly inhibit the production of pro-inflammatory mediators, promote the repair of intestinal barrier function, and regulate the disturbed gut microbiota structure [12, 14-15]. On the other hand, GAA, as the most abundant triterpenoid compound in *Ganoderma lucidum*, has been proven to possess multiple beneficial activities, including anti-inflammation, anti-oxidation, and anti-tumor effects [16-17]. From the perspective of traditional Chinese medicine, the synergistic action of different bioactive components in *Ganoderma lucidum* is crucial [18]. However, current research, both in vivo and in vitro, has primarily focused on GLP, while studies on GAA remain relatively scarce. IBD, a complex inflammatory disorder involving multiple factors, requires a multi-target and multi-level intervention strategy to achieve optimal therapeutic effects. GLP and GAA, as two valuable natural products, hold promise for providing a synergistic effect against IBD. However, systematic research on whether they possess complementary or synergistic mechanisms is still lacking.

In our preliminary study using LPS-induced RAW264.7 macrophage cells, we found that compared with administration alone, combined treatment with GLP and GAA significantly downregulated the expression of

TNF- α , IL1- β , and IL-6, enhanced IL-10 secretion, and inhibited the generation of reactive oxygen species (ROS) and mitochondrial membrane potential loss. Western blot results further confirmed that this combined treatment could synergistically inhibit the expression of TLR4, p-I κ B α , and p-NF- κ B p65 in the TLR4/NF- κ B signaling pathway, thereby blocking the activation of the TLR4/NF- κ B pathway [19]. These cellular findings suggest that GLP and GAA may have complementary effects in inflammation regulation. However, their comprehensive anti-inflammatory effects in *in vivo* models, as well as their impact on gut microbiota homeostasis and metabolites, remain to be elucidated.

Therefore, based on the dextran sulfate sodium (DSS)-induced colitis mouse model, this study systematically evaluated the effects of combined intervention with GLP extracts and GAA on alleviating colitis. The synergistic mechanisms were explored from multiple dimensions, including symptom improvement, levels of inflammatory cytokines, the TLR4/NF- κ B signaling pathway, gut microbiota structure, and SCFA metabolites. This study aims to reveal the comprehensive regulatory potential of this natural product combination in the “inflammation-intestinal microbiota” interaction network and provide theoretical support for the development of natural product-based formulations and interventions for intestinal diseases.

2. Materials and methods

2.1 Chemicals and reagents

DSS and sulfasalazine (SASP) were purchased from Meilun Biotechnology Co., Ltd. (Dalian, China). Sodium carboxymethyl cellulose (CMC-Na) and short-chain fatty acids (SCFAs) standards, as well as the internal standard crotonic acid for analysis, were obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). GAA (purity $\geq 98\%$) was purchased from Alfa Biotechnology Co., Ltd. (Chengdu, China). ELISA kits for the detection of inflammatory cytokines (TNF- α , IL-6, IL-1 β) were sourced from Jingmei Biotechnology Co., Ltd. (Jiangsu, China). Occult blood test kits for evaluating bleeding status were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The bicinchoninic acid (BCA) protein assay kit were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). Primary antibodies for Western blot analysis were provided by Cell Signaling Technology (MA, USA), and secondary antibodies were purchased from Abcam (Cambridge, UK). All other reagents used were of analytical grade or higher.

2.2 Preparation of GLP extracts

Ganoderma lucidum powder was provided by Zhejiang WisePlus Health Co., Ltd. (Lishui, China). The extraction of GLP was performed according to the previously reported method [20], with minor modifications. Briefly, 200 g of *Ganoderma lucidum* powder was defatted with 95% ethanol for 24 h and then dried. The defatted powder was subsequently extracted twice in a water bath at 95 °C using a solid-to-liquid ratio of 1:15 (w/v), with each extraction lasting for 2 h. The combined filtrates were concentrated by rotary evaporation at 65 °C. Ethanol was added to the concentrated solution to a final concentration of 40% to precipitate impurities.

Subsequently, the ethanol concentration was increased to 80% to precipitate polysaccharides. The resulting precipitate was dialyzed against water for 48 h using a dialysis bag with a molecular weight cutoff of 3000 Da and then lyophilized to obtain the GLP extracts.

2.3 Design of animal experiments

In this study, a DSS-induced acute colitis mouse model was employed to evaluate the anti-inflammatory effects of GLP extracts and GAA administered separately and in combination. The experiments were conducted using 6-week-old male SPF-grade C57BL/6J mice (weighing 20 ± 2 g), which were purchased from the Experimental Animal Center of Hangzhou Medical College. The mice were housed in a pathogen-free barrier environment with a constant temperature (22 ± 2 °C) and relative humidity ($60 \pm 5\%$), under a 12 h light/dark cycle. During the experimental period, the mice were provided with AIN-93 standardized diet and sterile water. All animal experiments strictly adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the experimental protocol was approved by the Animal Ethics Committee of Zhejiang University of Technology, with the approval number MGS20240618070. Prior to the start of the experiment, the mice were acclimated to the environment for 7 days.

After the acclimatization period, 60 mice were randomly divided into six groups, with 10 mice in each group: the normal control group (NC), the model control group (MC), the positive control group (SASP), the GLP extracts group (GLP), the GAA group (GAA), and the combined intervention group of GLP extracts and GAA (GL-PA). Except for the NC group, all other groups were administered a 3% (w/v) DSS solution via drinking water for 7 consecutive days to induce acute colitis, followed by a 3-day recovery period with normal drinking water to simulate the natural repair process. During the induction of colitis and the recovery period, mice in each intervention group were gavaged daily with the corresponding treatments as follows: the NC and MC groups received 0.5% (w/v) CMC-Na solution; the SASP group received SASP (50 mg/kg); the GLP group received the GLP extracts (200 mg/kg); the GAA group received GAA (50 mg/kg); and the GL-PA group received a combination of the GLP extracts (200 mg/kg) and GAA (50 mg/kg). The dose of GLP was selected based on previously reported optimal doses that demonstrated protective effects in DSS-induced colitis models [21]. In addition, our preliminary *in vitro* studies revealed that significant synergistic anti-inflammatory effects were achieved when GAA and GLP were combined at a mass concentration ratio of 1:4 [19]. On this basis, we adopted the combination of GLP $200 \text{ mg} \cdot \text{kg}^{-1}$ and GAA $50 \text{ mg} \cdot \text{kg}^{-1}$ for the *in vivo* experiments in order to focus on mechanistic interactions under a biologically relevant and evidence-based dosing scheme. The detailed experimental scheme is shown in Fig. 1. All treatments were prepared in 0.5% (w/v) CMC-Na solution, and the gavage volume was calculated based on $0.1 \text{ mL}/10 \text{ g}$ body weight (BW). Gavage was performed at a fixed time each day.

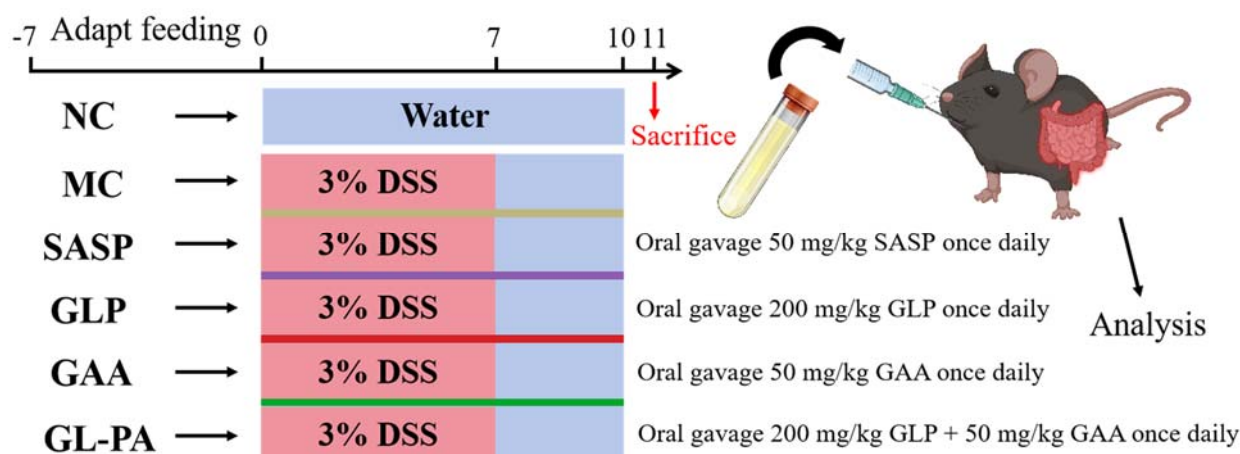


Fig. 1 Schematic diagram of colitis modeling and grouping protocol.

Throughout the experimental period, the body weight, food intake, water consumption, fecal morphology, and bleeding status of the mice in each group were continuously monitored and recorded. Fresh fecal samples were collected from the mice between days 7 and 10 of the experiment and stored at -80°C for subsequent analysis of gut microbiota and metabolites. On day 10, at the end of the experiment, the mice were anesthetized, and blood was collected via cardiac puncture, followed by euthanasia. Serum and colonic tissues were collected, with the distal colon reserved for histological analysis and the remaining colonic tissues and contents stored at -80°C for further biochemical analysis.

2.4 Evaluation of disease activity index (DAI)

In this study, the DAI scoring criteria were appropriately revised based on previous literature [22-23] standards and combined with the actual experimental conditions. The specific scoring details are shown in Table 1. Three indicators were monitored and recorded daily for each mouse. The scores for each indicator were summed and averaged to obtain the DAI score for that day. DAI scoring was performed by observers blinded to the treatment assignments to ensure unbiased assessment. This method can objectively reflect the inflammatory process and the effectiveness of interventions, providing a reliable basis for evaluating the construction of the colitis model and the therapeutic effects of the treatments.

Table 1 DAI scoring criteria.

Weight loss (%)	Stool consistency	Occult blood	Score
0	Normal	Normal	0
1-5	Loose (not adhering to anus)	Normal	1
5-10	Loose (adhering to anus)	Positive	2
10-15	Diarrhea	Positive	3
15-20	Severe diarrhea	Gross bleeding	4

2.5 Colonic histopathology analysis

The histopathological analysis of colonic tissues was performed using hematoxylin and eosin (H&E) staining to observe changes in the intestinal mucosal structure and to assess the degree of tissue damage. The specific method was referenced from our previous study [7]. The histopathological scoring criteria are shown in Table 2. The cumulative scores of each indicator were used to quantify the extent of colonic tissue damage,

thereby evaluating the inflammatory status and the therapeutic effects of the interventions. Histopathological evaluation was performed by observers blinded to the treatment assignments to ensure unbiased assessment.

Table 2 Histopathological scoring criteria for colonic tissue.

Inflammation severity	Inflammation extent	Crypt damage	Score
None	None	None	0
Mild	Mucosa	Basal 1/3 damaged	1
Moderate	Submucosa	Basal 2/3 damaged	2
Severe	Transmural	Complete crypt loss	4

2.6 Determination of inflammatory cytokines

The levels of tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) in mouse serum were measured according to the manufacturer's instructions of the commercial ELISA kits.

2.7 Expression levels of proteins related to the TLR4/NF- κ B signaling pathway

To evaluate the activation of the TLR4/NF- κ B inflammatory signaling pathway, the expression levels of proteins such as TLR4, I κ B- α , p-I κ B- α , NF- κ B p65, and p-NF- κ B p65 in colonic tissues were detected using Western blot analysis. The detailed method was referenced from our previous study [7]. Briefly, colonic tissues were homogenized and lysed with RIPA buffer to extract total proteins. After protein quantification using the BCA method, the samples were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked and then incubated with primary antibodies followed by HRP-conjugated secondary antibodies. The proteins were visualized using ECL detection and imaged with a chemiluminescent imaging system. The band densities were analyzed using ImageJ software (version 1.41, NIH, USA) to assess the expression and activation status of the signaling pathway-related proteins.

2.8 Determination of SCFAs in mouse colonic contents

The content of SCFAs in mouse colonic contents was determined using gas chromatography (GC). Precisely weighed 0.1 g of colonic contents from each group of mice was homogenized in 1 mL of PBS. A 500 μ L aliquot of the supernatant was then mixed with 100 μ L of crotonic acid internal standard solution (210 mmol/L) and 100 μ L of 25% metaphosphoric acid solution. The mixture was vortexed for 5 min and then stored at -20 °C overnight. After thawing the next day, the sample was centrifuged and filtered through a 0.45 μ m aqueous phase filter.

The analysis was performed on an Agilent 7820A GC equipped with a DB-FFAP column (0.32 mm $\times$ 30 m $\times$ 0.5 μ m), using crotonic acid as the internal standard. The GC conditions were as follows: the injection port temperature was set at 250 °C, with an injection volume of 1 μ L and a split ratio of 5:1. The carrier gas was high-purity nitrogen, and a constant linear velocity temperature program was used. The initial temperature was held at 75 °C for 0.5 min, then increased to 180 °C at a rate of 20 °C/min and held for 1 min, followed by an increase to 220 °C at a rate of 40 °C/min and held for 1 min. The total analysis time was approximately 8

min. The detector used was a flame ionization detector (FID) with a detection temperature of 250 °C. The auxiliary gases were nitrogen at 30 mL/min, hydrogen at 40 mL/min, and air at 400 mL/min.

2.9 Analysis of gut microbiota composition

The composition of the gut microbiota was analyzed using high-throughput sequencing of the 16S rDNA to assess the impact of different interventions on the structure of the gut microbial community. Colonic content samples from each group of mice were collected, and total genomic DNA was extracted according to the instructions of the FastPure Stool DNA Isolation Kit. The integrity of the DNA was checked by 1% agarose gel electrophoresis, and its concentration and purity were measured using a NanoDrop 2000 UV-Vis spectrophotometer.

Using the extracted DNA as the template, the V3–V4 region of the bacterial 16S rRNA gene was amplified with barcoded primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The PCR amplification program included initial denaturation at 95 °C for 3 min, followed by 27 cycles consisting of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s, with a final extension at 72 °C for 10 min. The PCR products were purified, quantified using a Qubit 4.0 fluorometer, and pooled in equimolar amounts for sequencing on the Illumina PE300/PE250 platform. The sequencing data were analyzed using the Majorbio Cloud platform for bioinformatics analysis.

2.10 Statistical analysis

All experimental data were analyzed using IBM SPSS Statistics 25 software. One-way analysis of variance (ANOVA) was used for intergroup comparisons. When significant differences were detected, post hoc tests such as LSD or Tukey's test were performed to identify specific differences between groups. Data visualization was completed using Origin 2021 and SPSS 27 software. Each experimental group included at least three replicates. All results are presented as mean $\pm$ standard deviation (SD). A p-value less than 0.05 was considered statistically significant.

3. Results

3.1 Composition analysis of GLP extracts

The yield of GLP extracts was $2.06 \pm 0.12\%$. The total sugar content of GLP extracts was determined to be $51.61 \pm 1.44\%$ based on the phenol-sulfuric acid method using a glucose standard curve ($y = 0.0085x + 0.0583$, $R^2 = 0.9982$). The reducing sugar content of GLP extracts was calculated to be $5.09 \pm 1.11\%$ using the dinitrosalicylic acid (DNS) method with a glucose standard curve ($y = 0.9785x - 0.0151$, $R^2 = 0.9992$). The protein content of GLP extracts was found to be $2.02 \pm 0.45\%$ according to the Coomassie Brilliant Blue method using a bovine serum albumin standard curve ($y = 0.0029x + 0.2984$, $R^2 = 0.9958$). The uronic acid content of GLP extracts was $11.19 \pm 1.11\%$ as determined by the orcinol method with a galacturonic acid standard curve ($y = 3.7513x + 0.1252$, $R^2 = 0.9932$). The total triterpenoid content of GLP extracts was 0.59

$\pm 0.04\%$ based on the vanillin-acetic acid-perchloric acid method using an ursolic acid standard curve ($y = 0.0631x - 0.065$, $R^2 = 0.9973$).

3.2 Effects of combined therapy on basic indicators in DSS-induced colitis mice

In the DSS-induced colitis model, mice typically exhibit typical clinical symptoms such as weight loss, decreased appetite, diarrhea, and bloody stools [24]. To evaluate the therapeutic effects, this study recorded the daily changes in body weight and food intake of the mice. The results showed that starting from day 5 of the experiment, the body weight of mice in the MC group significantly decreased, and food intake continuously declined, with inflammatory symptoms progressively worsening, indicating that DSS successfully induced acute colitis. Compared with the MC group, the interventions with GLP, GAA, and GL-PA all alleviated the trends of weight loss and decreased food intake to varying degrees, with the GL-PA group showing the most significant improvement (Fig. 2D, E).

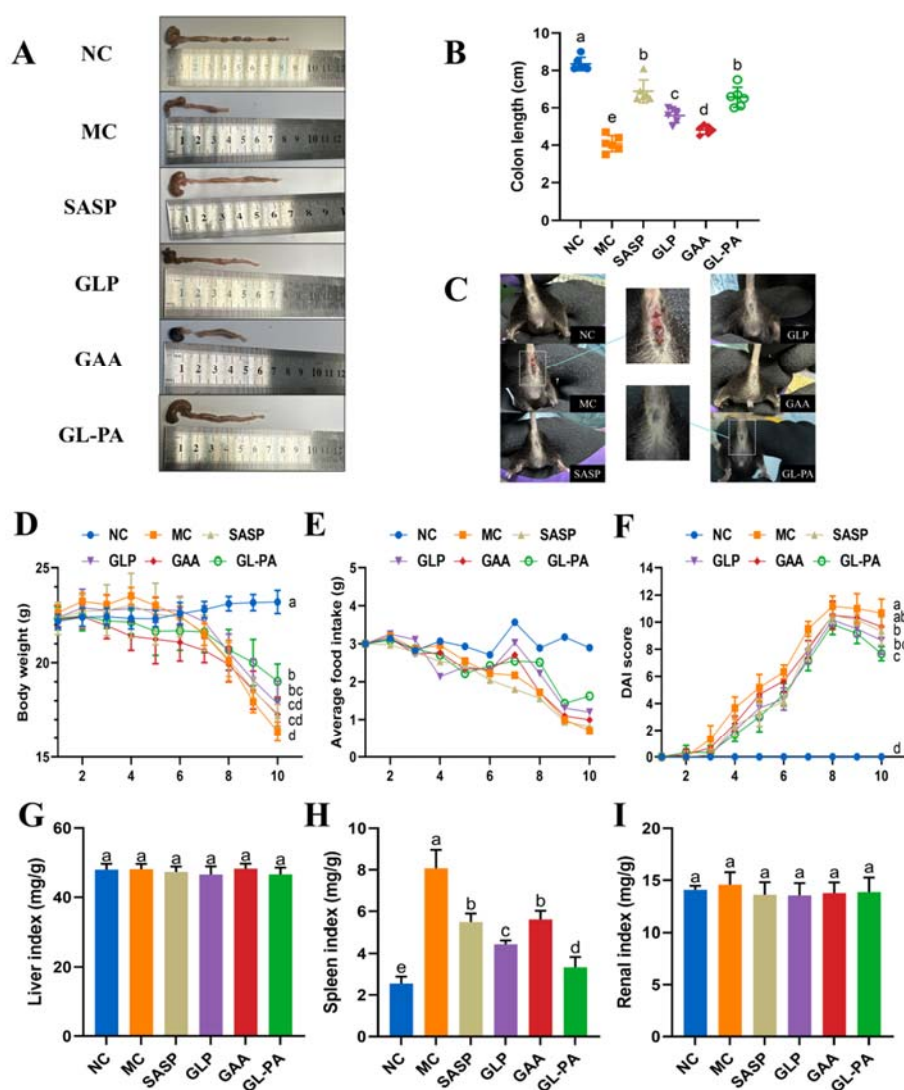


Fig. 2 Effects of interventions on basic physiological indices in DSS-induced colitis mouse model. (A) Appearance of the colon. (B) Colon length. (C) Occult blood status. (D) Changes in body weight. (E) Changes in food intake. (F) Disease Activity Index (DAI) score. (G) Liver index. (H) Spleen index. (I) Kidney index. Data were presented as mean $\pm$ SD ($n = 6$). Data points marked with different lowercase letters indicate significant differences between groups ($P < 0.05$).

As shown in Fig. 2C and F, mice in the MC group exhibited obvious rectal bleeding, and the DAI score was significantly increased ($P < 0.01$), further confirming the activation of the inflammatory state. After drug intervention, the bleeding was significantly reduced, and the DAI scores of all groups decreased. Compared with the MC group, although the GAA group showed a clear trend of improvement, there was no statistical difference. In contrast, the DAI scores of the SASP, GLP, and GL-PA groups significantly decreased ($P < 0.05$), with the GL-PA group showing the best effect among the intervention groups, indicating that the combined intervention had a stronger alleviating capacity.

Colon length is a commonly used indicator to reflect the severity of colitis [25]. As shown in Fig. 2A and B, the colons of mice in the MC group were significantly shortened, accompanied by redness and contraction. Therapeutic interventions alleviated this phenomenon to varying degrees, with the GL-PA group showing significant recovery of colon length ($P < 0.01$), approaching the level of the SASP group, indicating a more pronounced anti-inflammatory effect.

Additionally, organ index analysis revealed that the spleen index of mice in the MC group was significantly higher than that of the NC group ($P < 0.01$) (Fig. 2H), suggesting an elevated systemic inflammatory level [26]. After intervention, the spleen index of all groups decreased significantly, with the GL-PA group showing the largest reduction, which was better than that of GLP and GAA alone. No significant differences were observed in the liver and kidney indices ($P > 0.05$), indicating that the interventions did not induce obvious organ toxicity.

3.3 Histopathological evaluation of colon

H&E staining was performed on colonic tissues to assess the degree of structural damage and inflammatory status. As shown in Fig. 3A, the colonic tissues of mice in the NC group exhibited intact structure, with well-arranged mucosal epithelial cells, clear crypt architecture, abundant goblet cells, and no inflammatory cell infiltration. In contrast, the colonic tissues of mice in the MC group displayed severe pathological changes, characterized by epithelial cell shedding, crypt destruction, and prominent inflammatory cell infiltration, confirming the successful establishment of the DSS-induced colitis model. After therapeutic interventions, the tissue structure of each group was improved to varying degrees. The SASP and GL-PA groups showed better recovery of crypt architecture and a significant reduction in inflammatory infiltration, with tissue morphology approaching normal levels. Although the GLP and GAA groups exhibited some degree of repair, inflammatory cell infiltration was still observed in certain areas.

Histopathological scoring indicated that the MC group had a significantly higher score than the NC group ($P < 0.01$) (Fig. 3B). The scores of all intervention groups were significantly reduced ($P < 0.05$). Notably, the histopathological score of the GL-PA group was significantly lower than that of the GLP and GAA groups and was comparable to that of the SASP group, suggesting that the GL-PA intervention had a robust effect in ameliorating colonic tissue damage.

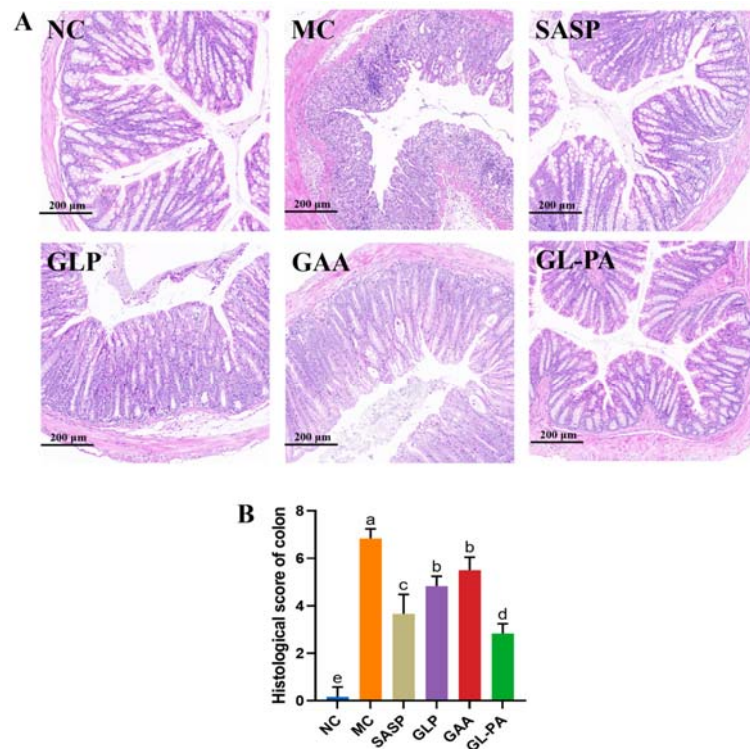


Fig. 3 H&E staining images of colon (A) and histopathological scoring (B). Scale bars are shown in the figure. Data were presented as mean $\pm$ SD (n = 6). Data points marked with different lowercase letters indicate significant differences between groups ($P < 0.05$).

3.4 Effects of combined therapy on inflammatory factors and TLR4/NF- κ B inflammatory signal pathway

To assess the systemic inflammatory status induced by DSS, the levels of TNF- α , IL-1 β , and IL-6 in mouse serum were measured. As shown in Fig. 4, compared with the NC group, the levels of these three pro-inflammatory cytokines were significantly elevated in the MC group ($P < 0.05$), indicating that DSS successfully triggered a significant inflammatory response. In all intervention groups, the levels of these inflammatory cytokines were significantly reduced compared with the MC group. Notably, the GL-PA group showed a more pronounced anti-inflammatory effect than the single intervention groups of GLP and GAA, as evidenced by the greater reduction in TNF- α , IL-1 β , and IL-6 levels.

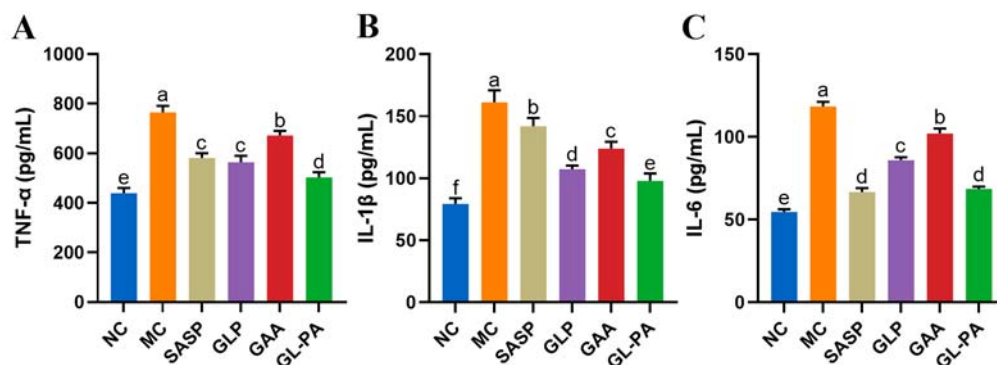


Fig. 4 Comparison of inflammatory cytokine levels in serum among different groups of mice. (A) TNF- α levels. (B) IL-1 β levels. (C) IL-6 levels. Data were presented as mean $\pm$ SD (n = 6). Data points marked with different lowercase letters indicate significant differences between groups ($P < 0.05$).

Furthermore, the expression of proteins related to the TLR4/NF- κ B signaling pathway in colonic tissues was examined by Western blot (Fig. 5). The results showed that the protein expression levels of TLR4, p-I κ B, and p-NF- κ B p65 in the MC group were significantly higher than those in the NC group ($P < 0.05$), indicating that this signaling pathway was activated under inflammatory conditions. After intervention, the expression or phosphorylation levels of these proteins were significantly inhibited in all groups ($P < 0.05$). The GL-PA group exhibited a more pronounced inhibitory effect on TLR4, p-I κ B, and p-NF- κ B p65 compared with the GLP and GAA groups.

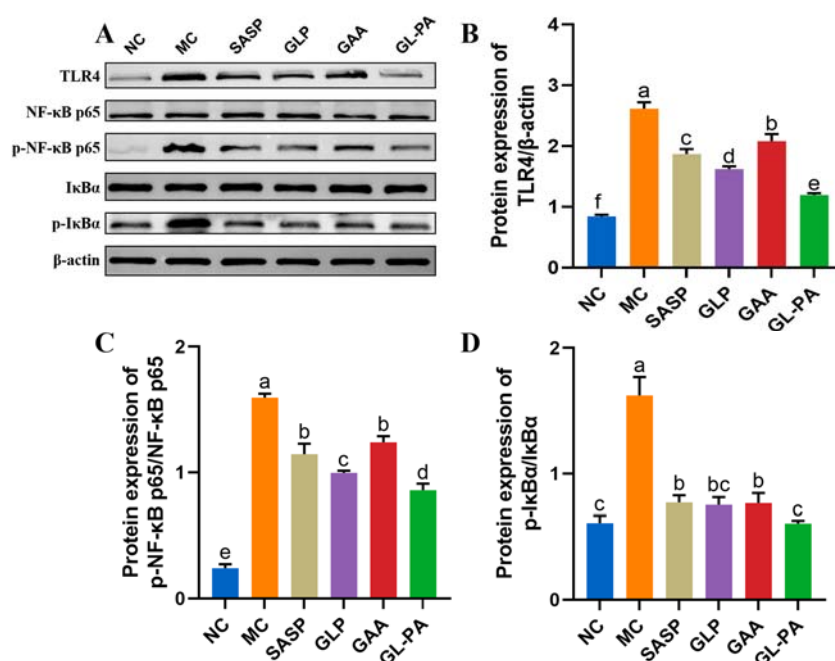


Fig. 5 Expression levels of proteins related to the TLR4/NF- κ B signaling pathway in mouse colonic tissues. (A) Representative immunoblot images of the related proteins. Expression levels of (B) TLR4, (C) p-NF- κ B p65, and (D) p-I κ B α . Data were presented as mean $\pm$ SD ($n = 3$). Data points marked with different lowercase letters indicate significant differences between groups ($P < 0.05$).

3.5 Effect of combined therapy on gut microbiota in mice with colitis

3.5.1 Analysis of α -diversity and β -diversity

To evaluate the effects of DSS and therapeutic interventions on the gut microbiota structure in mice, α -diversity analysis was first performed, with results shown in Fig. 6A-D. Compared with the NC group, the Ace index, Chao1 index, and Shannon index of the MC group were significantly decreased ($P < 0.05$), while the Simpson index was significantly increased ($P < 0.05$). These findings indicate that DSS treatment significantly reduced the species richness and evenness of the gut microbiota. Interestingly, when compared with the MC group, the GAA group showed no significant differences in these indices. In contrast, the GLP and GL-PA groups improved the Ace and Chao1 indices and significantly decreased the Simpson index ($P < 0.05$), suggesting that these interventions could maintain microbiota diversity and structural stability to some extent.

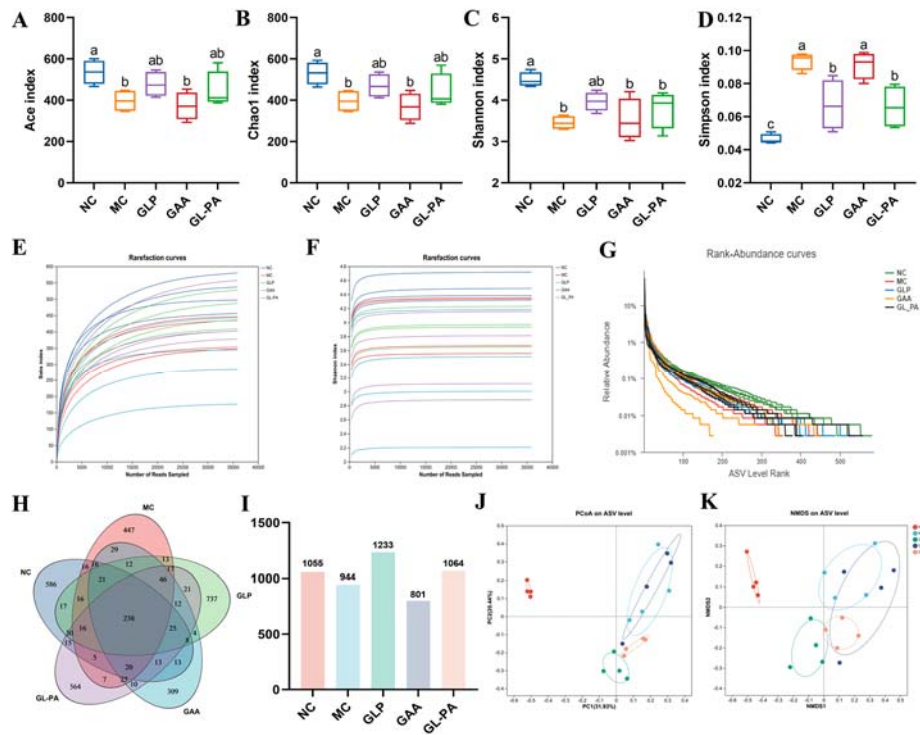


Fig. 6 Analysis of the alpha-diversity of the gut microbiota in each group of mice. (A) Ace index. (B) Chao1 index. (C) Shannon index. (D) Simpson index. Dilution curves (E), Shannon index curves (F) and grade abundance curves (G) of gut microbiota in mice. (H) Venn diagram based on ASV. (I) ASVs numbers. Analysis of the Beta-diversity of the intestinal flora in each group of mice. (J) PCoA. (K) NMDS. Data were presented as mean $\pm$ SD ($n = 4$). Data points marked with different lowercase letters indicate significant differences between groups ($P < 0.05$).

β -diversity analysis was further conducted using principal coordinates analysis (PCoA) and non-metric multidimensional scaling (NMDS) to assess differences in microbial community composition among samples (Fig. 6G-H). The PCoA results showed distinct separation of microbial communities among different groups, with a significant difference between the NC and MC groups. The GLP, GAA, and GL-PA groups exhibited reduced distances from the MC group, indicating a trend toward restoration of microbiota structure toward a normal state. The NMDS analysis results were consistent with those of PCoA, further demonstrating that DSS significantly disrupted the gut microbiota structure, and that microbial community composition was improved to varying degrees after therapeutic interventions.

3.5.2 Composition of gut microbiota at the phylum level

To assess the effects of different interventions on the gut microbiota composition in DSS-induced mice, the microbial communities in the colonic contents were analyzed at the phylum level, with results shown in Fig. 7. The dominant phyla included Firmicutes, Bacteroidetes, Verrucomicrobia, Proteobacteria, Desulfobacterota, and Campilobacterota. Compared with the NC group, the relative abundance of Firmicutes was significantly decreased in the MC group, while the relative abundances of the other five phyla were significantly increased ($P < 0.05$), indicating that DSS treatment significantly disrupted the gut microbiota structure. The interventions to varying degrees reversed these changes. The GL-PA group showed significant effects in increasing the relative abundance of Firmicutes and decreasing the relative abundances of

Bacteroidetes and Desulfobacterota, outperforming the single treatment groups of GLP and GAA ($P < 0.05$). In terms of inhibiting Proteobacteria, the GLP group performed better than other treatment groups ($P < 0.05$). Additionally, analysis of the Firmicutes-to-Bacteroidetes (F/B) ratio revealed that the MC group had a significantly lower ratio than the NC group. The GL-PA group significantly increased this ratio ($P < 0.01$), while no significant differences were observed in the single treatment groups. This finding suggests that the combined intervention has a stronger effect in modulating the gut microbiota structure.

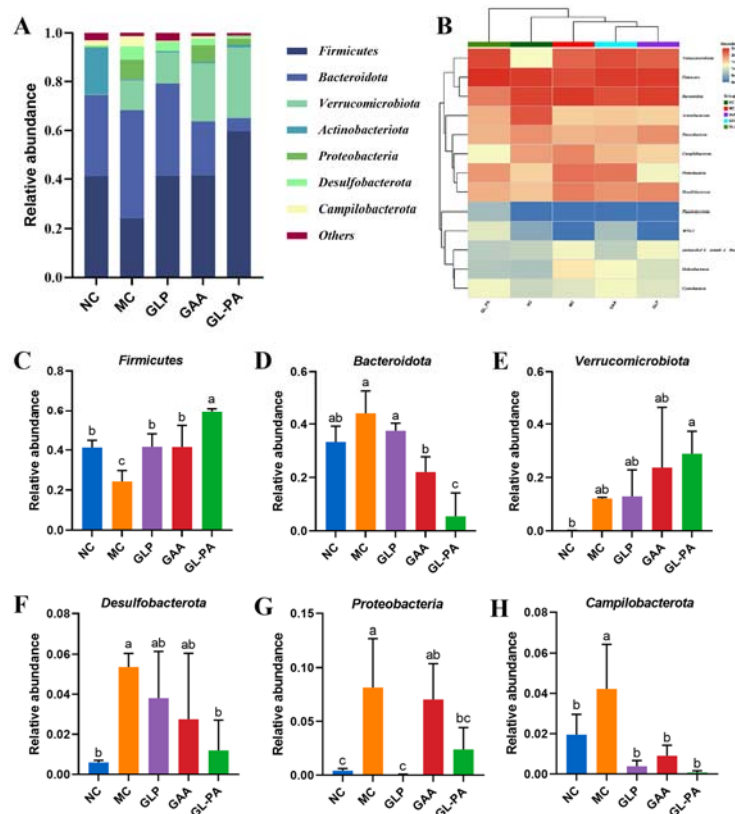


Fig. 7 Relative abundance of gut microbiota at the phylum level. (A) Microbial community structure of phylum level. (B) Heatmap. (C-H) Representative members. Data were presented as mean $\pm$ SD ($n = 4$). Data points marked with different lowercase letters indicate significant differences between groups ($P < 0.05$).

3.5.3 Composition of gut microbiota at the genus level

Further analysis of the gut microbiota composition at the genus level was conducted for each group, with results presented in Fig. 8. Compared with the NC group, the MC group exhibited significant changes in microbial composition, characterized by a marked decrease in the relative abundances of *Lachnospiraceae_NK4A136_group*, *Lactobacillus*, *Turicibacter*, and *Clostridia_UCG-014* (Fig. 8B–K). The GL-PA intervention significantly increased the relative abundances of *Lachnospiraceae_NK4A136_group*, *Lactobacillus*, and *Turicibacter* ($P < 0.05$). However, its effect on restoring the abundance of *Clostridia_UCG-014* was less pronounced than that of the GLP and GAA single treatments ($P < 0.01$), indicating certain intergroup differences.

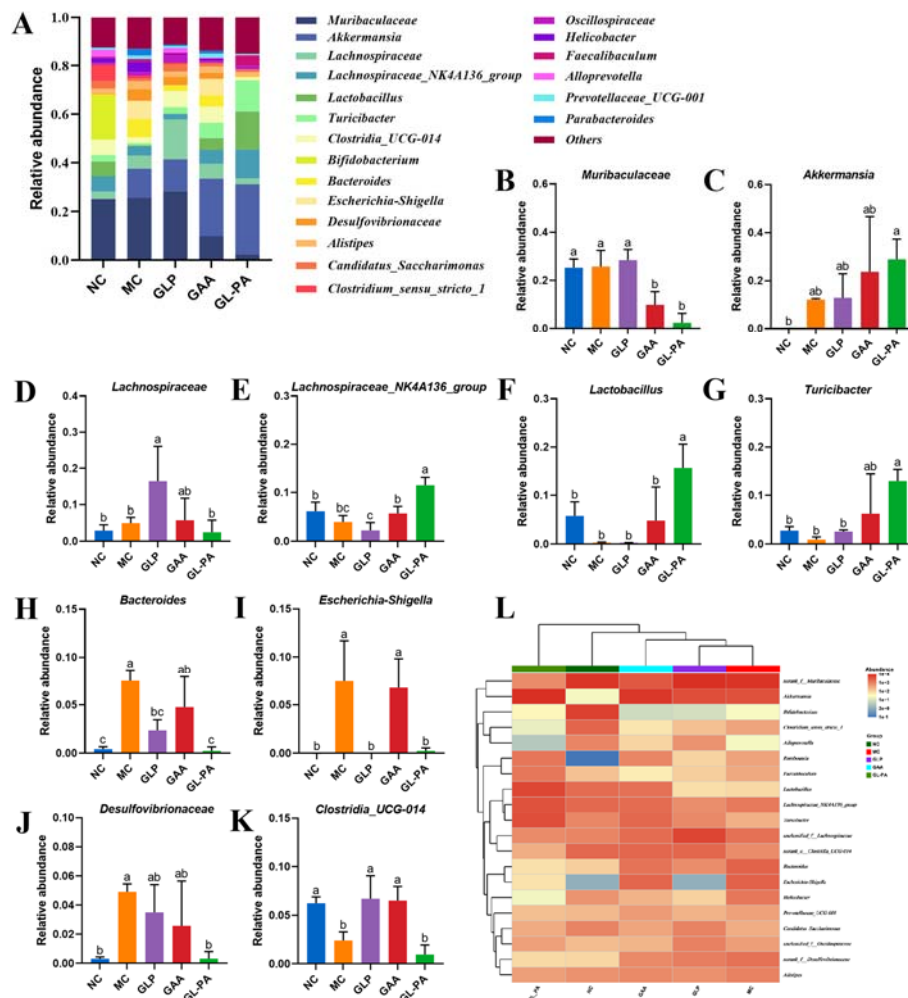


Fig. 8 Relative abundance of gut microbiota at the genus level. (A) Microbial community structure of genus level. (B-K) Representative members. (L) Heatmap. Data were presented as mean $\pm$ SD ($n = 4$). Data points marked with different lowercase letters indicate significant differences between groups ($P < 0.05$).

In addition, the relative abundances of *Akkermansia*, *Bacteroides*, *Escherichia-Shigella*, and *Desulfovibrionaceae* were significantly higher in the MC group ($P < 0.05$). After intervention, the GL-PA group significantly inhibited the abnormal enrichment of *Bacteroides*, *Escherichia-Shigella*, and *Desulfovibrionaceae*, with a better recovery trend than the single intervention groups. The relative abundance of *Akkermansia* was further increased in the GL-PA group. Notably, GLP alone significantly enhanced the relative abundance of *Lachnospiraceae*, demonstrating its unique advantage in modulating microbial community structure.

3.5.4 linear discriminant analysis effect size (LEfSe) analysis

To identify characteristic differences in the gut microbiota among the groups different, we employed LEfSe analysis to compare the microbial biomarkers across the NC, MC, GLP, and GL-PA groups at various taxonomic levels. The results are shown in Fig. 9A. At the phylum level, Bacteroidota was significantly enriched in the MC group, while Firmicutes was significantly enriched in the GL-PA group. At the family level, Clostridiaceae was enriched in the NC group, Enterobacteriaceae and Bacteroidaceae in the MC group, Muribaculaceae in the GLP group, and Lactobacillaceae and Peptostreptococcaceae in the GL-PA group. At

the genus level, the biomarkers enriched in each group were as follows: *Clostridium_sensu_stricto_1* and *Alloprevotella* in the NC group, *Escherichia-Shigella*, *Bacteroides*, and *Helicobacter* in the MC group, *norank_Muribaculaceae* in the GLP group, and *Lactobacillus* and *Romboutsia* in the GL-PA group. Fig. 9B further illustrates the taxonomic differences among the groups at various classification levels. The results indicate significant differences in microbial composition between the MC and GL-PA groups. After GL-PA intervention, the types and abundance levels of certain characteristic microbial taxa shifted closer to those of the NC group, suggesting a trend toward restoration of the microbiota structure.

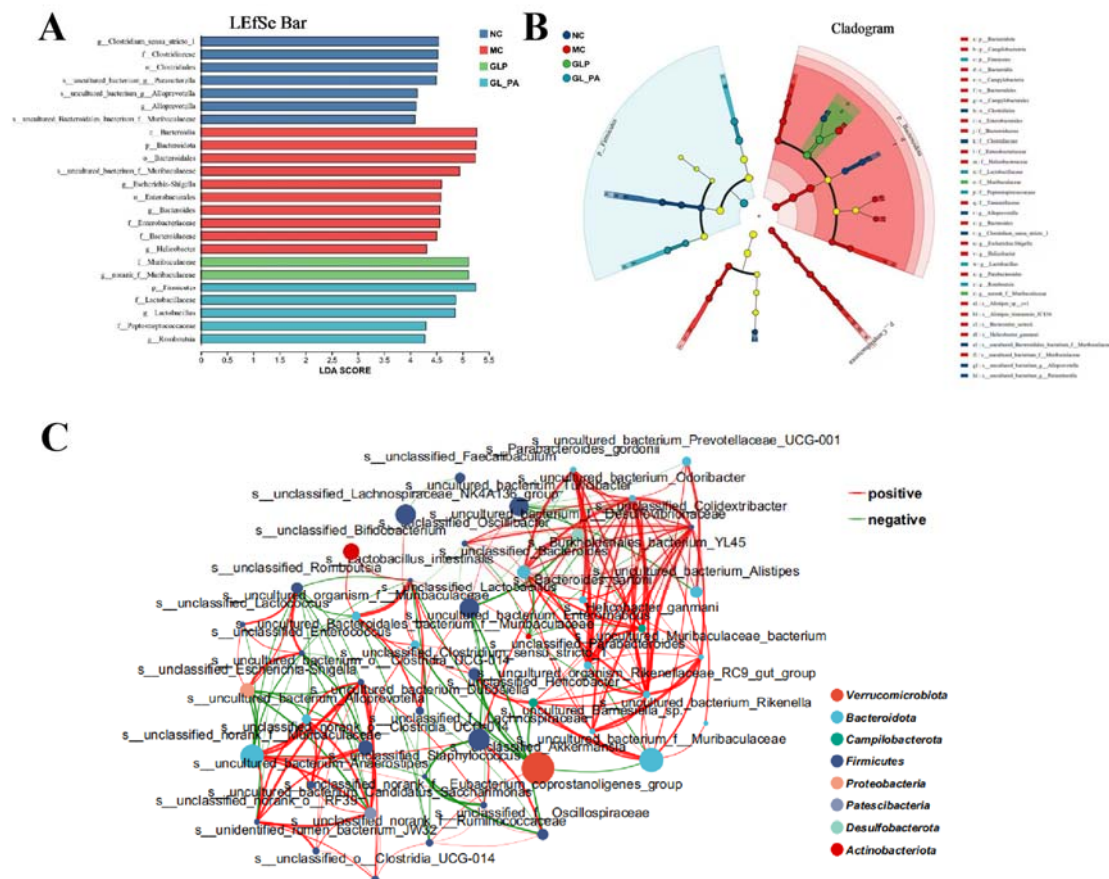


Fig. 9 LefSe analysis of the gut microbiota in mice model of DSS-induced colitis (A) LDA scores of the differentially abundant taxa. (B) Taxonomic cladogram obtained from LefSe analysis of 16S sequences. (C) Correlation network diagram among gut microbiota.

3.6 Effect of combined therapy on SCFAs level

To assess the effects of different interventions on gut metabolites, the levels of SCFAs in the colonic contents of mice from each group were measured, including acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate. The results are shown in Fig 10. Compared with the NC group, the MC group exhibited a significant decrease in the levels of all individual SCFAs and total SCFAs ($P < 0.05$), indicating that DSS-induced colitis led to a decline in SCFA metabolic capacity. After therapeutic interventions, the levels of SCFAs in all groups increased to varying degrees. Among them, the GL-PA group showed the most significant recovery in the levels of propionate, butyrate, isobutyrate, valerate, isovalerate, and total SCFAs ($P < 0.01$), which was markedly better than that observed in the single intervention groups.

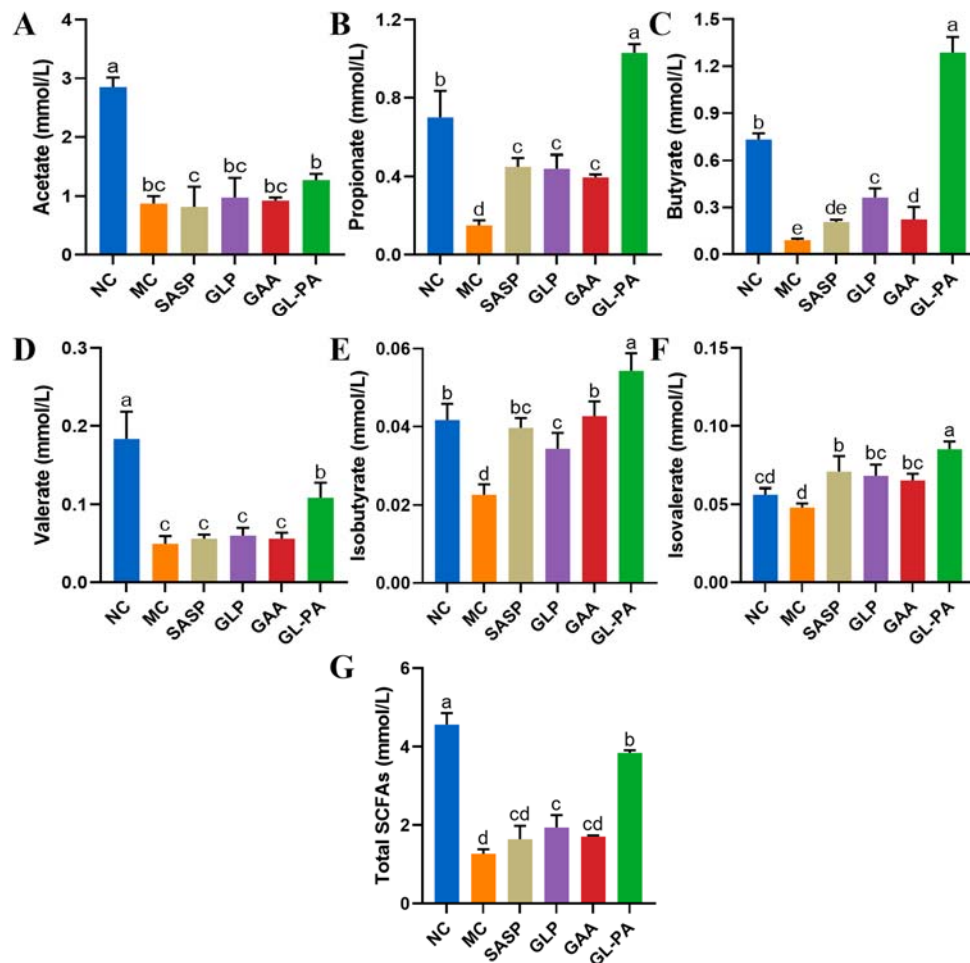


Fig. 10 SCFAs concentration in the intestinal contents of mice. (A) Acetate. (B) Propionate. (C) Butyrate. (D) Valerate. (E) Isobutyrate. (F) Isovalerate. (G) Total SCFAs. Data were presented as mean $\pm$ SD ($n = 6$). Data points marked with different lowercase letters indicate significant differences between groups ($P < 0.05$).

3.7 Correlation analysis between gut microbiota and other indexes

To explore the associations between gut microbiota and colitis-related indicators in this research model, Pearson correlation analysis was performed based on the relative abundance data at the genus level. The results are shown in Fig. 11. The analysis revealed that the spleen index, DAI score, levels of inflammatory cytokines, and the expression of proteins related to the TLR4/NF- κ B signaling pathway were significantly positively correlated with *Bacteroides*, *Escherichia-Shigella*, *Desulfovibrionaceae*, *Alistipes*, and *Parabacteroides*. In contrast, the levels of SCFAs were negatively correlated with *Bacteroides*, *Desulfovibrionaceae*, and *Alistipes*. Additionally, the DAI score was significantly negatively correlated with *Bifidobacterium*, while SCFAs were positively correlated with *Lactobacillus* and *Bifidobacterium*.

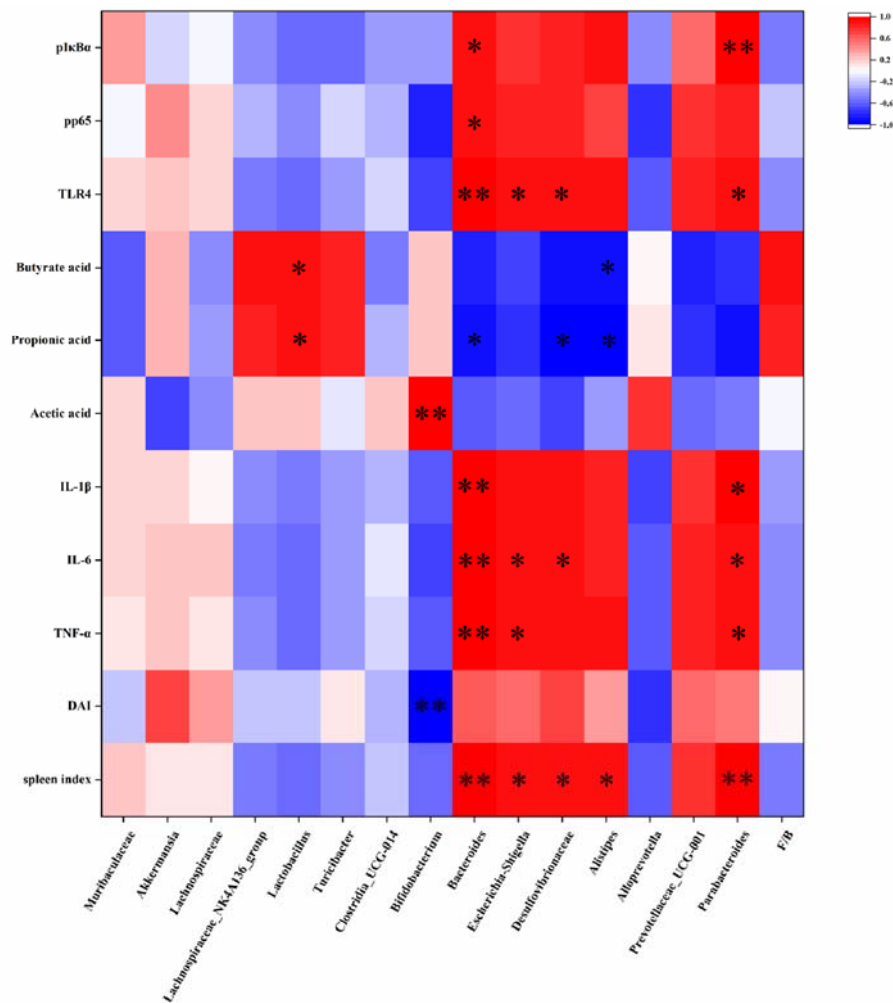


Fig. 11 Pearson's rank correlation heat map between the relative abundance of gut microbiota at the genus level and colitis-related indices.

4. Discussion

The combined intervention of GLP extracts and GAA demonstrated a significant synergistic effect in alleviating DSS-induced colitis, particularly outperforming single treatments in terms of inhibiting inflammatory responses and reshaping the gut microbiota. Notably, the GL-PA group not only reduced the expression of typical pro-inflammatory factors in the serum but also significantly inhibited the expression of key proteins in the TLR4/NF- κ B signaling pathway in the colon. This was accompanied by an increase in the abundance of beneficial bacteria and the restoration of SCFA levels. These multifaceted changes suggest that the combined intervention may exert an enhanced effect through the interaction of “inflammation-gut microbiota-metabolism.”

The TLR4/NF- κ B signaling pathway occupies a central position in the pathogenesis of IBD and serves as a key molecular bridge connecting microbial recognition and amplification of inflammatory responses. TLR4, as a pattern recognition receptor (PRR), acts as a sentinel in intestinal epithelial cells, detecting pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) to initiate the characteristic inflammatory cascade of IBD [27-28].

Upon activation of TLR4, downstream signaling leads to phosphorylation of I κ B and subsequent nuclear translocation of NF- κ B, which coordinates the transcriptional upregulation of pro-inflammatory mediators such as TNF- α , IL-1 β , and IL-6 [333]. Therefore, targeting this signaling pathway may be an ideal therapeutic strategy for IBD. The results of this study show that GL-PA combination therapy exhibits superior regulatory effects on the TLR4/NF- κ B pathway compared to monotherapy, suggesting the existence of potential synergistic effect. The anti-inflammatory potential of GLP is well-known and is primarily attributed to its direct impact on inflammation levels, modulation of the gut microbiota, and enhancement of cellular immune responses [29]. Specifically, it has been demonstrated to modulate immune responses by binding to pathogen PPRs [13]. Similarly, terpenoid compounds have shown significant efficacy in alleviating inflammation in experimental colitis by affecting the expression levels of NF- κ B signaling pathway-related proteins in colonic tissues [30–32]. Our findings are consistent with previous studies on the anti-inflammatory effects of GLP and GAA. The results of this study indicate that the GL-PA group significantly outperformed the monotherapy groups in reducing levels of pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6, suggesting that combination therapy more effectively suppresses the terminal inflammatory response as reflected by these key downstream cytokines. Based on these previous studies, we speculate that the enhanced efficacy of combination therapy may be partly attributed to the dual blockade of the inflammatory cascade by the two compounds. Notably, overactivation of the TLR4/NF- κ B pathway not only directly drives intestinal inflammation but is also closely associated with gut microbiota dysbiosis. Pro-inflammatory mediators released during inflammatory states can alter the intestinal microenvironment, affecting microbial colonization and growth. Conversely, dysbiosis of intestinal microenvironment can further activate the TLR4 pathway through the production of endotoxins, creating a vicious cycle of “colonic inflammation-gut microbiota dysbiosis” [33–34]. Therefore, the effective inhibition of the TLR4/NF- κ B pathway by GL-PA combination therapy not only helps control inflammatory responses but may also disrupt this vicious cycle by influencing the gut microbiota, thereby laying a molecular foundation for the improvement of intestinal microenvironment.

Dysbiosis of the gut microbiota is a crucial driving factor in the development and progression of IBD, and restoring microbial balance is one of the key therapeutic strategies. Typically, in DSS-induced colitis mouse models, a significant decrease in the relative abundance of Firmicutes and a significant increase in the relative abundance of Bacteroidetes, Proteobacteria, and Desulfovibrionaceae are observed [22, 35], which is consistent with our study results. As a dominant gut microbial phylum, the decline in the abundance of Firmicutes is often closely related to intestinal inflammation and impaired barrier function [36], while a decrease in the Firmicutes-to-Bacteroidetes (F/B) ratio is considered an important indicator of gut microbiota dysbiosis [37]. Our study results show that GL-PA combination therapy significantly restored the relative abundance of Firmicutes, thereby improving the imbalance in the F/B ratio. These changes were particularly pronounced in the combination therapy group compared to the groups treated with GLP or GAA alone. This suggests that GLP and GAA may exert enhanced therapeutic effects on colitis through synergistic regulation of the abundance of Firmicutes and Bacteroidetes. Additionally, we observed an increase in the abundance of

Verrucomicrobia in the colitis model. Nagalingam et al. [38] found that certain bacteria within the Verrucomicrobia phylum can depolymerize and utilize DSS, thus enabling them to grow more effectively in a DSS-rich environment. This may explain the increased relative abundance of Verrucomicrobia in all groups except the NC group.

Analysis at the genus level further revealed the precise regulatory potential of the combined treatment on specific functional microbial groups. After GL-PA intervention, the relative abundance of beneficial genera such as *Lactobacillus*, *Lachnospiraceae_NK4A136_group*, and *Turicibacter* was significantly increased. *Lactobacillus*, as a classic probiotic, can inhibit the growth of pathogenic bacteria through the production of lactic acid and bacteriocins, while modulating the host immune response and enhancing intestinal barrier function [39]. *Lachnospiraceae_NK4A136_group*, belonging to the family Lachnospiraceae, is an important butyrate-producing bacterium in the gut. Restoration of its abundance is beneficial for maintaining the energy metabolism of colonic epithelial cells and an anti-inflammatory environment [40]. *Turicibacter* is closely related to the development of the host immune system, and its proliferation is conducive to maintaining intestinal immune homeostasis and protecting the intestinal barrier [41]. It has also been shown that an increase in its relative abundance can alleviate symptoms of DSS-induced colitis in mice [42], which is similar to the results observed in the GL-PA-treated group. Additionally, *Turicibacter* is associated with lipid metabolism and has been reported to alter the host bile acid and lipid metabolic profiles [43]. Therefore, it is speculated that the alleviation of colitis by GL-PA treatment may also be related to these effects, which warrants further investigation. It is worth noting that a common characteristic of the aforementioned beneficial microbial groups is their strong ability to ferment carbohydrates, converting dietary fiber and other indigestible polysaccharides into short-chain fatty acids, particularly acetic acid, propionic acid, and butyric acid. Moreover, the abnormal enrichment of potential pathogenic bacteria such as *Escherichia-Shigella*, *Bacteroides*, and *Desulfovibrionaceae* was effectively inhibited by the combined treatment. These microbial groups often proliferate excessively under inflammatory conditions and exacerbate intestinal damage through the production of endotoxins and pro-inflammatory metabolites [44-45].

The improvement in microbial community structure further promoted the production of functional metabolites. In the GL-PA group, the total amount of SCFAs and the content of each component were significantly increased, especially the restoration of butyrate and propionate levels, which is of great significance for maintaining the integrity of the intestinal epithelial barrier and regulating immune homeostasis. Butyrate, as the primary energy source for colonic epithelial cells, not only promotes epithelial cell proliferation and differentiation but also exerts anti-inflammatory effects by activating GPR43/GPR109A receptors [46-47]. Propionate mainly improves gastrointestinal inflammatory diseases and colorectal cancer through mediating immune and metabolic signaling [48]. Our study found that compared with the groups treated with GLP and GAA alone, the GL-PA combination treatment more significantly enhanced the levels of propionate and butyrate. A previous study on the improvement of UC by ganoderic acid indicated that the anti-UC potential of ganoderic acid is closely related to its ability to improve microbial metabolites [15].

However, the group treated with GAA alone did not show a significant advantage in SCFA production. Nevertheless, it has been reported that GAA can promote tryptophan metabolism, particularly the production of 3-IAld^[49]. In summary, these results suggest that the regulation of microbial metabolic activity by a single component may have certain limitations. GLP, as a fermentable polysaccharide [50], theoretically can serve as a substrate for probiotics to generate SCFAs. However, in the DSS model, the abundance of probiotics was significantly reduced, and the microbial community structure was severely disrupted, which may lead to insufficient fermentation efficiency. Although GAA has certain anti-inflammatory and gut microbiota-regulating capabilities, its triterpenoid structure does not directly participate in the generation of SCFAs. In contrast, the combination of GLP and GAA may exert synergistic effects on two levels: “providing substrates” and “regulating microbial communities.” GLP provides a carbon source basis for SCFA production, while GAA improves the microbial microenvironment and inhibits the expansion of harmful bacteria, thereby indirectly promoting the recovery of probiotics and enhancing the metabolic capacity of the microbial community. This synergistic mechanism may be the basis for the significant increase in SCFA levels by combination therapy. In addition to significantly increasing the abundance of beneficial bacteria such as *Lactobacillus*, *Lachnospiraceae_NK4A136_group*, and *Turicibacter*, the GL-PA group also showed enrichment of *Lactobacillus* and *Romboutsia* in the LEfSe analysis, both of which are important SCFA-producing bacteria. This further confirms the potential association between microbial community optimization and enhanced metabolic capacity.

Although the present findings strongly support pharmacodynamic synergy between GAA and GLP, it is important to consider whether pharmacokinetic factors could also contribute to the observed effects^[51]. Previous pharmacokinetic studies have shown that GAA possesses limited oral bioavailability and undergoes rapid hepatic metabolism^[52-54], while polysaccharides like GLP and their derived oligosaccharides are largely non-absorbable macromolecules that primarily exert physiological effects in the intestinal lumen through the transformation of intestinal flora^[55-56]. Therefore, it is unlikely that their combination enhances systemic exposure to either compound. Rather, the synergistic effects observed in this study are more plausibly attributed to pharmacodynamic interactions—namely, the concurrent modulation of inflammatory signaling and gut microbiota restoration. Consistent with this interpretation, the GL-PA treatment group exhibited enhanced regulation of both TLR4/NF- κ B signaling and microbial composition without evidence of systemic toxicity or exaggerated exposure. Future studies incorporating pharmacokinetic–pharmacodynamic (PK-PD) modeling or metabolic tracking will be valuable to further substantiate this mechanism.

Overall, the protective effect of the combined treatment with GAA and GLP on DSS-induced colitis reflects the complex regulatory network between the gut and inflammatory immunity. On one hand, the combined treatment directly inhibits the activation of the TLR4/NF- κ B signaling pathway, significantly reducing the release of pro-inflammatory cytokines and thereby alleviating both local intestinal and systemic inflammatory responses. On the other hand, the therapeutic intervention reshapes the gut microbiota structure, increasing the relative abundance of beneficial bacteria while suppressing the overproliferation of potential

pathogenic bacteria. More importantly, the optimized microbial community promotes the production of SCFAs, particularly the restoration of key metabolites such as propionate and butyrate. Correlation analysis further confirmed the close relationship between microbial composition and inflammatory markers, suggesting that dysbiosis of the gut microbiota and inflammatory responses form a vicious cycle. The combined treatment is able to simultaneously and more effectively counteract multiple links in this cycle. Therefore, the synergistic action of GAA and GLP is not only reflected in the direct intervention of inflammatory pathways but also in the multidimensional regulatory network of gut microbiota-metabolites-inflammation, achieving a systemic protective effect against colitis. Although our current study did not directly measure intestinal permeability or tight junction proteins, the observed restoration of mucosal architecture, suppression of inflammatory cytokines, and enrichment of SCFA-producing bacteria collectively suggest potential improvement of the intestinal barrier. Future studies will include direct assessments of ZO-1, Occludin, DAO, and zonulin to further validate this mechanism.

5. Conclusion and prospect

This study demonstrated that the combined administration of GLP and GAA synergistically alleviates DSS-induced colitis by suppressing the TLR4/NF- κ B inflammatory pathway, restoring gut microbiota structure, and enhancing SCFA production. Compared with single treatments, the GL-PA combination provided more pronounced improvements in clinical symptoms, histopathology, cytokine levels, microbial composition, and metabolic function, highlighting its potential as a multi-target natural therapeutic strategy for IBD. Future studies should explore a wider range of dose combinations and adopt quantitative synergy models to better define optimal therapeutic ratios. Antibiotic-depletion or fecal microbiota transplantation models will be essential to clarify the causal role of gut microbiota. Long-term and relapsing colitis models, along with validation in human IBD cohorts, will help assess durability and translational relevance. In addition, mechanistic studies integrating molecular docking, dynamics simulations, and biochemical assays are needed to elucidate the molecular basis underpinning the synergistic actions of GLP and GAA. Together, these efforts will extend the present proof-of-concept findings and advance the development of multi-component natural compound therapies for IBD.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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