A novel synbiotic protects against DSS-induced colitis in mice via anti-inflammatory and microbiota-balancing properties

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A novel synbiotic protects against DSS-induced colitis in mice via anti-inflammatory and microbiota-balancing properties

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Abstract: Inflammatory bowel disease (IBD) is a chronic immune-inflammatory disease. Gut microbes, intestinal immunity, and gut barrier function play a critical role in IBD. Growing evidence suggests that synbiotics may offer therapeutic benefits for individuals with colitis, suggesting an alternative therapy against colitis. With this in mind, we creatively prepared a new synbiotic combination consisting of a probiotic strain (*Lactobacillus reuteri*) along with one prebiotic Chitooligosaccharides (COS). The protective effect of the synbiotics on DSS-induced colitis and the underlying mechanisms were investigated. We demonstrated that the synbiotics ameliorated colitis in mice, which was evidenced by a significant reduction in body weight, shortening of the colon, and an increase in the DAI index. Notably, synbiotics reduced the intestinal inflammation and injury by synergistically decreasing inflammatory factors, inhibiting TLR4/Myd88/NF-κB/NLRP3 signaling, preventing macrophages infiltration, and enhancing the integrity of the intestinal barrier. Moreover, synbiotics selectively promoted the growth of beneficial bacteria (e.g., *Akkermansia*, *Lactobacilus*) but decreased the pathogenic bacteria (e.g. *Helicobacter*). BugBase's analysis supported its ameliorated role in reducing pathogenic bacteria.

Collectively, our findings revealed the novel synbiotic had a potential to treat colitis, which was associated with its anti-inflammatory and microbiota-balancing properties. This study will contribute to the development of functional synbiotic products for the treatment of IBD and will provide valuable insights into their mechanisms.

Keywords: Inflammatory bowel disease ; Intestinal inflammation ; Synbiotics ; Gut microbiota ; Gut barrier function

1. Introduction

Inflammatory bowel disease (IBD) is a persistent, idiopathic, chronic inflammation of the intestines of the cumulative ileum, rectum, and colon [1, 2]. Crohn's disease (CD) and ulcerative colitis (UC) are the main forms of IBD, but exhibit different pathophysiology. CD involves inflammation in the form of plaques from the mouth to the anus. UC is limited to the colon and causes persistent inflammation and ulceration in the colon [3]. In the past decade, IBD has emerged as a public health challenge worldwide with accelerating incidence in the newly industrialised countries of Asia, South America, and Africa, where societies have become more westernized [4]. Currently, it is difficult to completely treat IBD with long-term medications [5] and there are side effects and complications, and the possible immunosuppressive reaction may lead to infections, neurological diseases, malignant tumors and other adverse events [6], which further increase the complexity of the disease. Thus, novel therapies are needed.

Although the pathogenesis of IBD remains elusive, existing studies suggest that its pathogenesis may be related to a variety of factors, including immune imbalance, disruption of the intestinal mucosal barrier, and ecological imbalance of the bacterial community [7]. Growing research indicates that IBD is closely associated with gut flora dysbiosis [8]. When the ecosystem of gut microbiota is disrupted, the immune system is activated and inflammatory cells accumulate in the gut, leading to intestinal inflammation [9]. In addition, gut microbiota disorders have a significant impact on the composition and function of the gut flora, thereby underscoring its critical role in intestinal inflammation

[10]. Consequently, microbial therapy represents a pivotal approach to treat IBD. Concurrently, numerous studies have concentrated on probiotics, prebiotics, and synbiotics [11]with the objective of effectively alleviating IBD by regulating the intestinal flora [12].

Synbiotics are a combination of probiotics and prebiotics [13]. It can exert the physiological activity of probiotics and selectively increase the number of probiotics, thus regulating the intestinal flora [14]. Probiotics are a group of active microorganisms that can survive in the gastrointestinal tract and colonize the intestinal mucosa [15]. The majority of these microorganisms belong to the genera *Lactobacillus* and *Bifidobacterium* [16]. Prebiotics are defined as substances, predominantly sugars, that are utilized by the host flora and play a role in promotion of host health and homeostasis [17]. Clinical studies have demonstrated the efficacy of synbiotics in the treatment of various diseases, including the alleviation of IBD, the improvement of metabolic processes in individuals with type 2 diabetes, and the amelioration of non-alcoholic fatty liver disease[18].

With this in mind, we creatively prepared a novel synbiotic combination consisting of a probiotic strain (*L. reuteri,* belongs to bacteria of *Lactobacillus*, the most commonly used probiotics [19]), along with one prebiotic Chitooligosaccharides (COS) ，which shows potential in regulating gut microbiota and in enhancement of mucosal barrier function [20]. This study aimed to investigate the protective effect of the synbiotics on DSS-induced colitis in mice, and explored the underlying mechanisms, which will be helpful to products development of functional synbiotics to treat IBD.

2. Materials and methods

2.1. Preparation and administration of synbiotics

The new synbiotics were prepared in the Department of Microbiology and Immunology, School of Basic Medical Sciences, Shanxi Medical University (China, Shanxi). The probiotic *L. reuteri* LY2-2 was originally isolated from black donkey. *L. reuteri* LY2-2 was cultured in De Man, Rogosa and Sharpe (MRS) medium at 37 °C under anaerobic conditions using the Anaerogen

system (Oxoid, Basingstoke, UoK) for 48 h. Then, the grown cultures of *L. reuteri* LY2-2 was centrifuged at 6000 g for 10 min at 4 °C, washed three times with sterile PBS, and re-suspended in sterile PBS. COS, the oligomers of β-(1-4)-linked D-glucosamine, are compounds prepared from chitosan, a N-deacetylated derivative of chitin [21]. The prebiotic COS with deacetylation degrees greater than 95% and average MW below 1 kDa were kindly presented by Dalian Glycobio Co., Ltd (Dailian, China). For synbiotic treatment, it was suspended using a sterile PBS solution, including 1.0 $\times 10^9$ CFU *L. reuteri* LY2-2 and 250 mg·kg^{-1} COS for each mouse per day.

2.2. Animals and experimental design

Thirty male C57BL/6J mice (7-8 weeks old, 20 to 22 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed under specific pathogen-free (SPF) conditions with a room temperature of 23 ± 1 °C and a 12 h-light/dark cycle. All the animal experiments were conducted in accordance with the guidelines of the Ethic Committee of Shanxi Medical University (permit no. SYDL2023010).

After one week of acclimation, mice were randomized into the following four groups: control (Ctrl，n=8), colitis model (DSS, n=7), synbiotics (Syn, n=8) treatment group, and aminosalicylic acid (5-ASA, n=7) treatment group. Protective effect of the synbiotics was evaluated in DSS-induced colitis in mice for 15 days. Mice in the Syn group and 5-ASA group, synbiotics (0.2 mL) or 5-ASA (100 mg/kg) was orally gavaged once a day from day one until the termination of the experiment, respectively. From day 1 to day 15, mice in the Ctrl and DSS group were gavaged with PBS (0.2 mL). 3% DSS (MW: 36 000 to 50 000 Da; MP Biomedicals, Irvine, CA, USA) was added to the drinking water on days 7th to 15th to induce colitis in mice. All mice were sacrificed on the 15th day. Body weight (BW), spleen weight, rectal bleeding and stool consistency were monitored all throughout the study. At the end of the experiment, blood, spleen, colon, and fecal samples were collected. The experimental schedule was shown in Fig. 1a.

2.3. 16s rRNA gene sequencing

Fecal samples were freshly collected. DNA in the feces was extracted, followed by amplification of the V3–V4 hypervariable regions of the 16s rRNA gene. Sequencing was performed as described previously [22]. Next, the resulting sequences were quality-filtered, clustered, and taxonomically assigned on the basis of 97% similarity level against the Ribosomal Database Project (RDP) by usingthe QIIME software package (version 1.9.1; Knight Lab, San Diego, CA, USA). The remaining sequences were used for gut microbiota evaluation. Statistical analyses were performed, and bacterial abundance and diversity were calculated [22]. Pearson’s correlation analysis was performed to evaluate correlations between taxa and the factors related to colitis. BugBase was adopted to forecast bacterial composition based on the sequencing results. The data were presented as the mean ± SD, $p<0.05$ represented significant difference.

2.4. Serum and stool biochemical parameters

Serum LPS levels were measured using an ELISA kit (Abmart, China) according to the manufacturer's instructions. Fecal occult blood was detected by o-toluidine method (Solarbio, Beijing, China). The Disease Activity Index (DAI) score, which is a composite assessment of weight loss (%), fecal consistency and fecal bleeding was assessed in each group. Specific calculations are shown in Supplementary Information Table S1.

2.5. Histologic, immunohistochemical, and immunofluorescence analyses

At the end of the experiment, mice were euthanized, and selected distal colon tissues were fixed, dehydrated, soaked in xylene, paraffin-embedded, and sliced into 4-um sections. Paraffin sections were deparaffinized with xylene and then dehydrated with different concentrations of ethanol. Sections were subjected to Hematoxylin and eosin (H&E) staining, immunohistochemistry, immunofluorescence staining and Alisin blue staining (AB). H&E staining and AB staining were used to detect pathological changes and mucus in the colon

tissue, respectively.

Histologic Score. Images of H&E-stained colon tissue were taken using a research-grade upright light microscope (Leica DM6B) and scored according to the following composite scoring criteria (i) inflammation severity, (ii) depth of inflammation, (iii) crypt damage, (iv) percent involvement, as shown in Table S2.

Immunohistochemical staining was performed using the following primary antibodies, TNF-α (Bioss, China), IL-6 (Bioss, China), NLRP3 (Wanleibio, China), IL-1β (Bioss, China) and Caspase1 (Wanleibio, China). Primary antibodies for immunofluorescence were F4/80 (Abcam, USA), Myd88 (Wanleibio, China), NF-κB p65 (Beyotime Biotechnology, China), phospho-NF-κB p65 (Thermo Fisher Scientific, USA) and Occludin (Thermo Fisher Scientific, USA). Images were obtained under a microscope (AxioObser Z1, Germany) with a magnification of 200 and positive results were quantified using ImageJ software (Free software Foundation Inc., Boston, MA).

2.6. Flow cytometry analysis

Spleen cells of mice were collected, washed and adjusted to 1 x 10^6 cells/100 uL FACS buffer. For analysis of macrophages, Fc receptor blocking was carried out by incubating with the anti-mouse CD16/32 antibody (Elabscience, China), followed by staining with Percpcy5.5 anti-mouse F4/80 (eBioscience, USA). Samples were analyzed using a BD FACS Celesta flow cytometer.

2.7. Western blot analysis

Proteins were extracted from colon tissues. Primary antibodies were TLR4 (CST, USA), NF-κB p65 (Beyotime Biotechnology, China), phospho-NF-κB p65 (Thermo Fisher Scientific, USA), and β-actin (CWBIO, China). Protein bands were analyzed using ImageJ software (Free software Foundation Inc., USA).

2.8. Quantitative real-time PCR

Total RNA from tissues was extracted using TRIzol (Seven, China) and

reverse transcribed into cDNA using the reverse transcription kit (Mei5 Biotechnology, China). The levels of mRNA were measured by real-time PCR with SYBR green reagent (Mei5 Biotechnology) on a QuantStudio 3 system (Applied Biosystems, USA). The expression of individual genes was normalized to the mRNA level of β-actin. The gene specific PCR primers are as follows: TNF-α, F:GAGGTCAATCTGCCCAAGTA, R:GAGCCATAATCCCCTTTCTA. β-actin, F:GCTGTCCCTGTATGCCTCTG, R:TTTGATGTCACGCACGATTT. The relative amounts of the different mRNAs were quantified with the $2^{-\Delta\Delta Ct}$ method, and the fold change ratio was calculated and expressed as mean±SEM.

2.9. Statistical analysis

The numeric results are shown as the means±SEM. All data meet the assumptions of the tests (e.g., normal distribution). One-way analysis of variance (ANOVA) was used for comparison among the different groups. When ANOVA was significant, post hoc testing of differences between groups was performed using the least significant difference (LSD) test. The exact value of n within figures was indicated in figure legends. For every figure, statistical tests are justified as appropriate. Here, it should be noted that a P value of 0.05 was considered statistically significant.

3. Results

3.1. Synbiotics ameliorated DSS-induced colitis in mice

To verify the protective effect of synbiotics on colitis, DSS-induced colitis mice model was constructed and administrated with the synbiotics. 5-ASA was used as a drug control (Fig.1a). Throughout the experiment, we tracked the daily body weight, fecal occult blood, and fecal viscosity of the mice, which were used to capture the changes in the DAI of the mice. Compared with the Ctrl group, the DSS group showed a significant loss in body weight, increase in DAI index, shorter colon length, and obvious splenomegaly, however, synbiotics administration restored these changes induced by DSS (Fig. 1b-e). Additionally, the degree of weight loss and the degree of increase in DAI in the

Syn group was less pronounced than those in the ASA group, indicating that synbiotics had a more favorable ameliorative effect on DSS mice.

H&E results showed that the Ctrl mice had intact colonic structure with well-arranged glands at the bottom. After DSS treatment, the colonic tissues showed pathological changes such as epithelial cell erosions, disappearance of cup cells, severe infiltration of inflammatory cells, and obvious edema and thickening of the plasma membrane layer (Fig. 1f). These findings were consistent with the significantly increased colon histopathology score in the DSS group (Fig. 1g). However, the colonic structure was significantly improved after synbiotics administration, making it closer to the Ctrl group. This was corroborated by the lower histopathologic scores observed in the Syn group (Fig. 1g). These results demonstrated that synbiotics were effective in ameliorating colitis in mice.

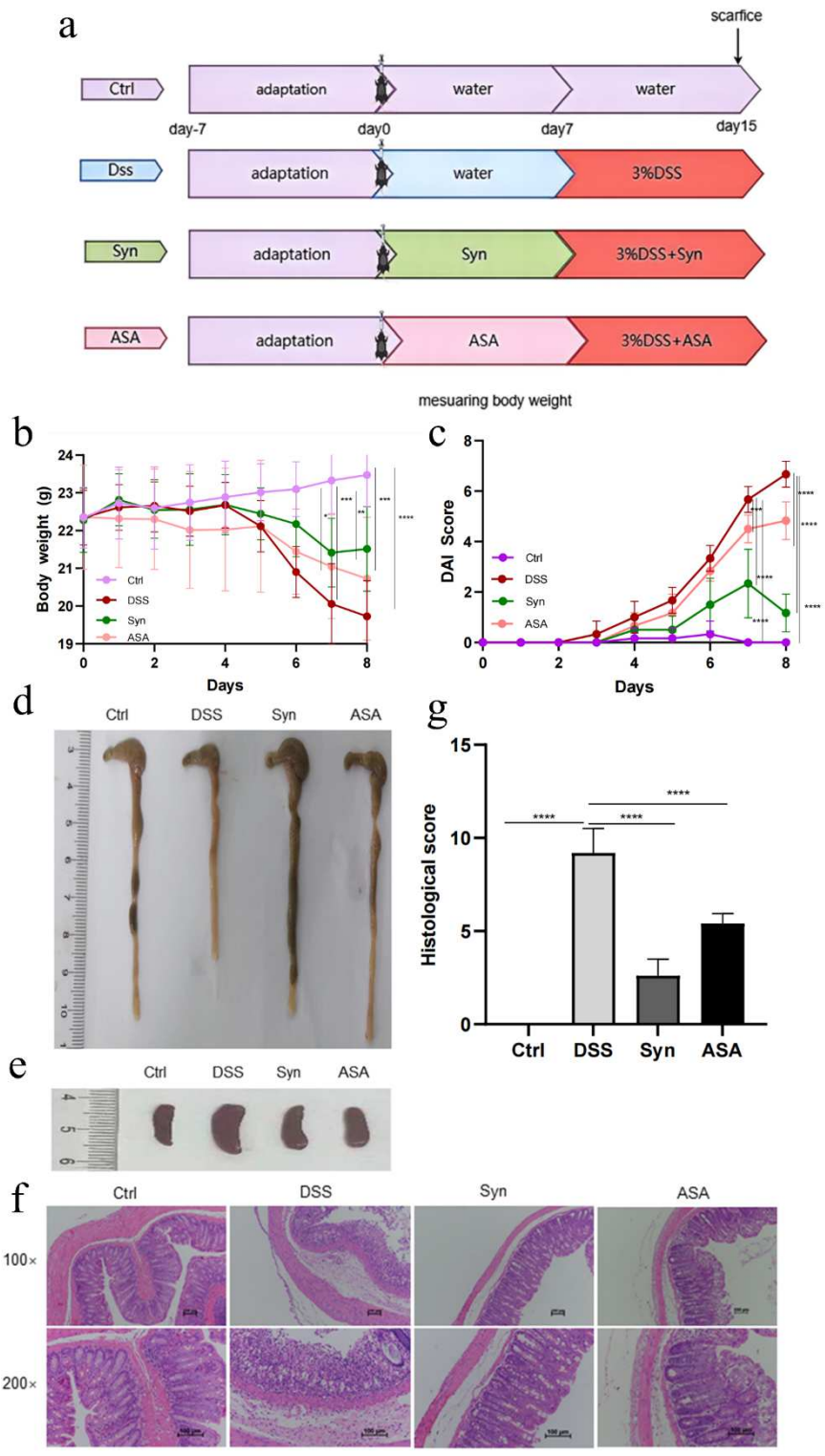

Fig.1 Synbiotics ameliorated DSS-induced colitis in mice. (**a**) Experimental design; (**b**) Mice body weight; (**c**) DAI score; (**d**) Representative picture of the colon; (**e**) Representative picture of the spleen; (**f**) H&E staining of the colon; **(g**) The histological score of H&E staining was determined. Values are presented as means ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

3.2. Synbiotics alleviated intestinal inflammation in colitis mice

3.2.1. Synbiotics reduced pro-inflammatory cytokines in colitis mice

To determine the role of synbiotics in colonic inflammation, we firstly analyzed the levels of pro-inflammatory factors in colonic tissues. As shown in Fig. 2a-c, compared with the Ctrl group, levels of IL-6 and TNF-α significantly increased by DSS, but suppressed by synbiotics. Similarly, at the transcriptional level, DSS promoted the expression of TNF-α, but synbiotics reduced the levles to a large extent (Fig. 2d). The findings showed that synbiotics significantly reduced pro-inflammatory cytokines in DSS-induced colitis mice, revealing a favorable anti-inflammatory effect.

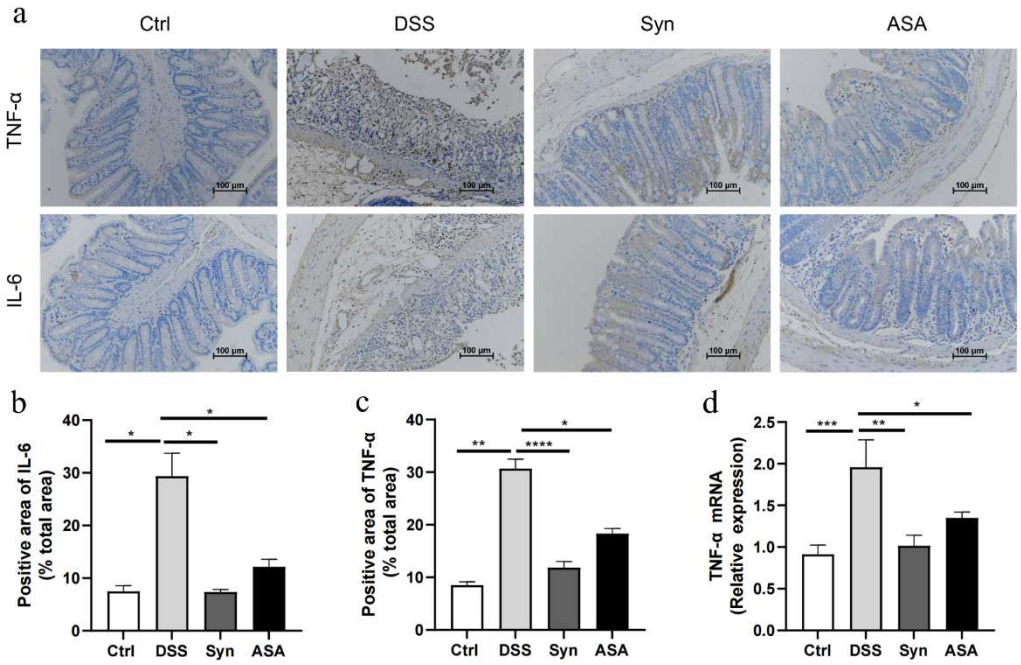

Fig.2 Synbiotics reduced pro-inflammatory cytokines in colitis mice. (**a**) Representative images of colon tissue IL-6 and TNF-α protein immumohistochemical staining; (**b** and **c**) Quantification analysis of (**a**); (**d**) Relative expression of gene TNF-α in colon tissue. Values are presented as means ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

3.2.2. Synbiotics intervention reduced macrophages infiltration in colitis mice

Macrophages are crucial for intestinal homeostasis and the pathology of IBD. In spleen of DSS-treated mice, macrophages increased but markedly

decreased after synbiotics administration (Fig. 3a, b) .To further investigated the effect of synbiotics on macrophages in colonic tissue, IF staining was performed. As expected, macrophages ($F4/80^{+}$) was higher in the colon of DSS-treated mice, however, synbiotics significantly reduced the macrophages accumulation (Fig. 3c). Taken together, the results suggested that synbiotics reduced macrophage infiltration, which supported its role in restricting inflammation in colitis mice.

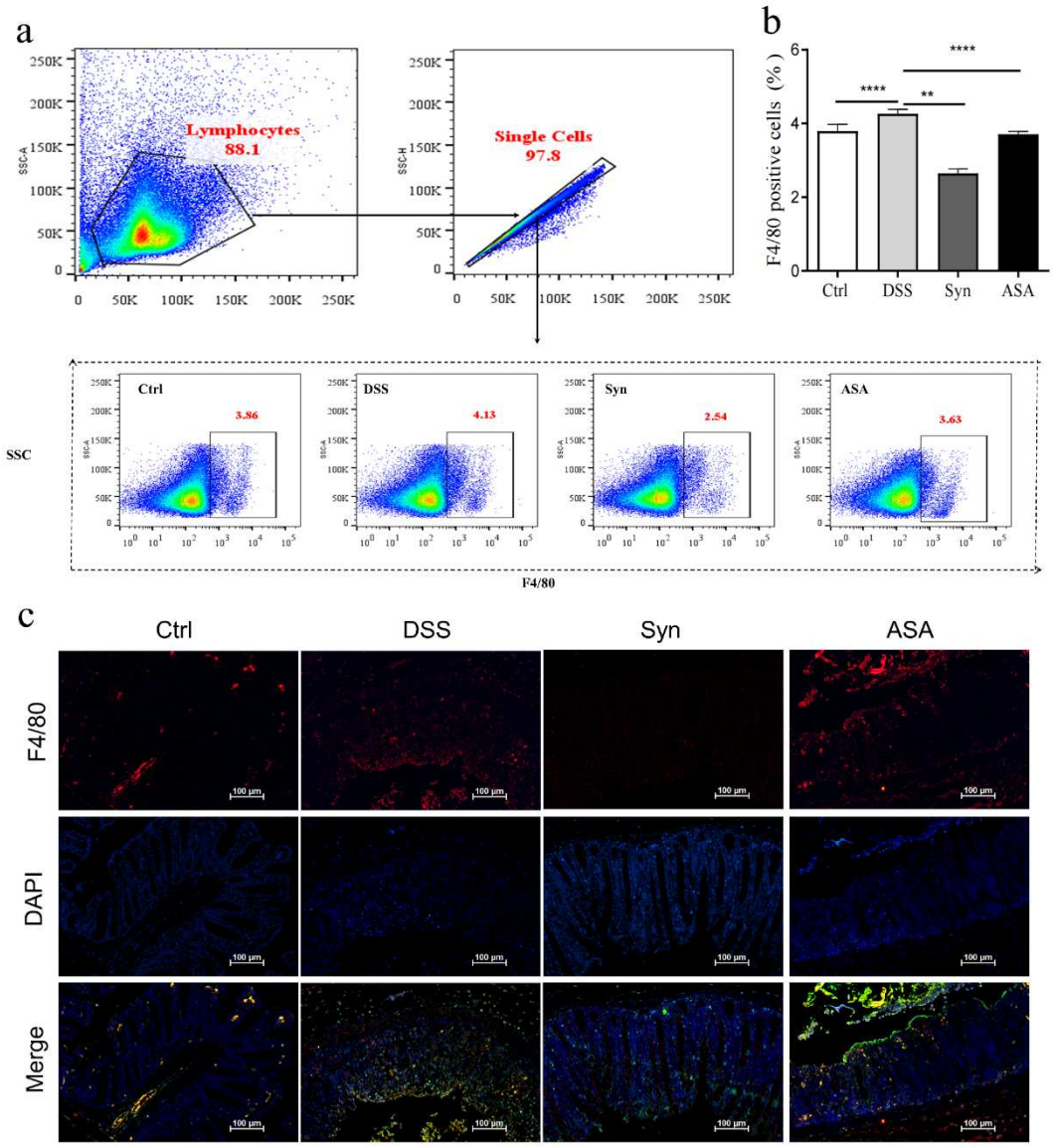

Fig.3 Synbiotics reduced macrophages infiltration in colitis mice. (**a, b**) Flow cytometry analysis of macrophage in spleen; (**c**) Immunofluorescence for F4/80 (red) protein in colon tissue, cell nucleus were stained with DAPI (blue), DAPI and F4/80 were merged in the last panels. 200× magnification

(scale bar, 100 μm). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

3.2.3. Synbiotics inactivated TLR4/Myd88/NF-κB/NLRP3 signaling pathway in colitis mice

NF-κB plays a crucial role in modifying the inflammatory response [23]. It has been shown that the increase of inflammatory cytokines is closely related to the activation of the NF-κB signaling pathway [24]. When the intestinal barrier function is impaired, LPS (lipopolysaccharide) invades, binds to TLR4, activates the NF-κB signaling pathway, and secretes inflammatory factors [25].Thereby, we detected the effect of synbiotics on TLR4/NF-κB signaling pathway. As shown in Fig. 4a, the protein expression of the signaling pathways was significantly higher in the DSS group compared with the Ctrl group, and the expression of TLR4, NF-κB, and P-P65 were upregulated 1.3, 2.8, and 1.7 times, respectively. Fortunately, synbiotics largely reduced the expression, almost reaching to the levels of the Ctrl group. Similar results were found in IF analyses of NF-κB and P-P65 (Fig. S1). Moreover, DSS induced increase in the expression of Myd88, and synbiotics largely reversed the changes (Fig. 4b). Therefore, synbiotics reduced intestinal inflammation by inhibiting NF-κB activity in colitis mice.

NF-κB activation upregulates NLRP3 protein expression, which promotes more IL-1β production and pyroptosis [26]. Thus, we investigated whether DSS-induced injury in colitis mice was associated with NLRP3 inflammasome. Immunohistochemical analysis revealed a significant increase in the expression of NLRP3, Caspase 1, and IL-1β proteins in the colonic tissues of the DSS-treated group in comparison to the control group (Fig. 4c). These increases were reduced by Syn administration (Fig. 4d-f), indicating that synbiotics reduced colon damage in the colitis mouse model by downregulating the expression of the NLRP3 inflammasome, signaling pathway components to inhibit pyroptosis.

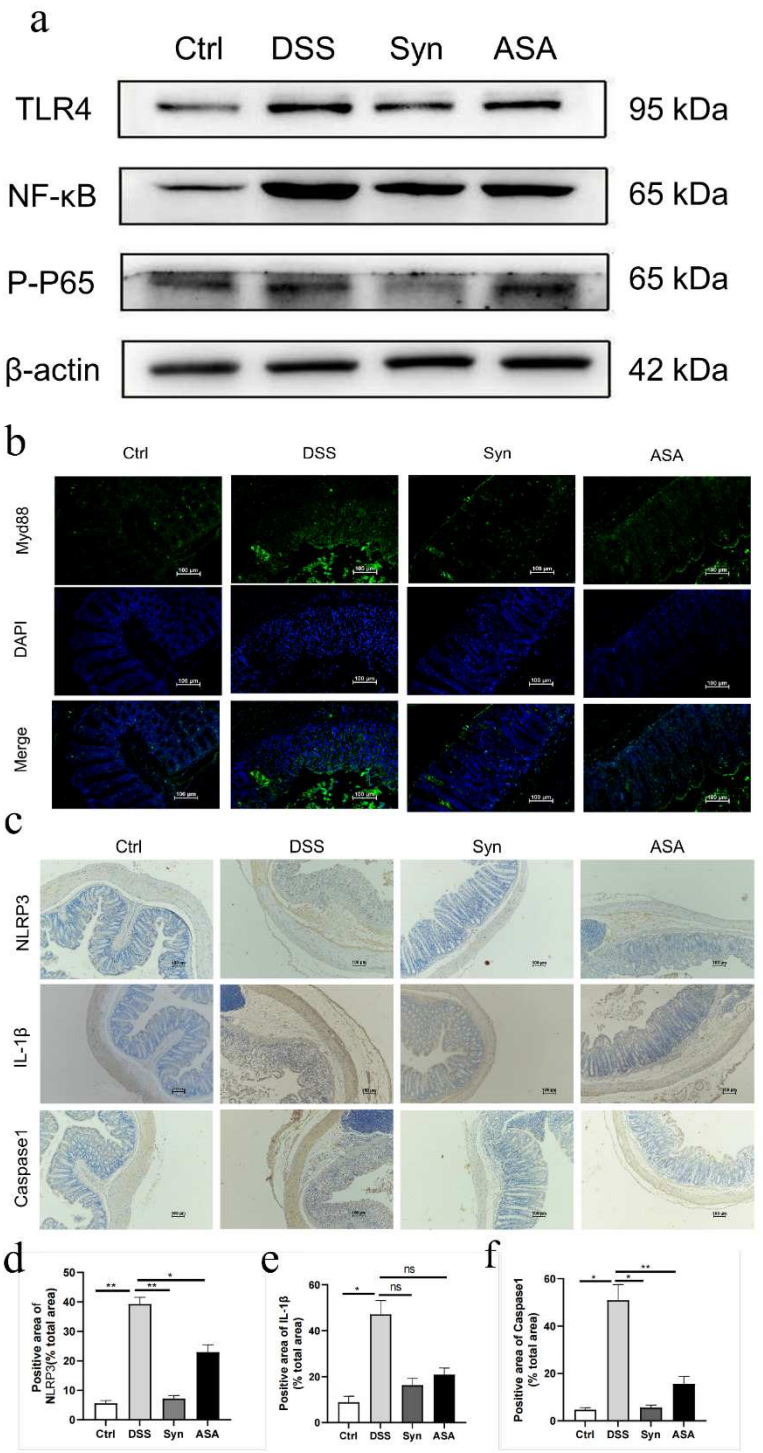

Fig.4 Synbiotics inactivated TLR4/Myd88/NF-κB/ NLRP3 signaling pathway in colitis mice. (**a**) The protein expressions of TLR4, NF-κB, and P-P65 were measured by Western blot; (**b**) Representative fluorescence images of Myd88 (green: immunofluorescence of Myd88; blue: immunofluorescence of DAPI); (**c**) Immunohistochemical expression detection of NLRP3, Caspase1, and IL-1β; (**d-f**) Quantification analysis of (**c**). Values are presented as means ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

3.3. Synbiotics reversed gut microbiota dysbiosis in colitis mice

Increasing evidence suggests that gut flora plays an important role in the pathogenesis of IBD[27]. Therfore, we investigated the changes in the gut microbiota by analyzing bacterial 16s rRNA sequencing in mouse feces.

3.3.1. Synbiotics altered gut microbiota richness and diversity

To assess the richness and evenness of the intestinal flora among the groups, we analyzed the α-diversity, which was expressed as Ace index and Shannon index, respectively. The results showed that the Ace and Shannon indices were decreased in the DSS group compared to the control group, however, there was no significant difference between the DSS and synbiotics groups (Fig. 5a), indicating that synbiotics did not seem to significantly alter the α-diversity. Venn diagrams allow for a better understanding of the richness or unique colonies shared between each group. As shown in Fig. 5b, we detected a total of 1641 OTUs in total abundance from all samples, and only 395 OTUs were shared between all groups. In addition, synbiotics pretreatment significantly increased OTUs in DSS mice. To further detect similarities between groups of microbial communities, we analyzed the β-diversity. All four groups were significantly separated (Fig.5c). A distinct difference in community composition could be found between the control and DSS groups, but the difference was reduced and was much closer to the control group after intervention with synbiotics (Fig. 5c). Non-metric multidimensional scaling (NMDS) analyses, which were consistent with PCoA analyses, confirmed that there were significant differences between the groups (Fig. 5d).These data suggested that synbiotics agent bacteria might optimized the richness and β-diversity of the intestinal flora in colitis mice.

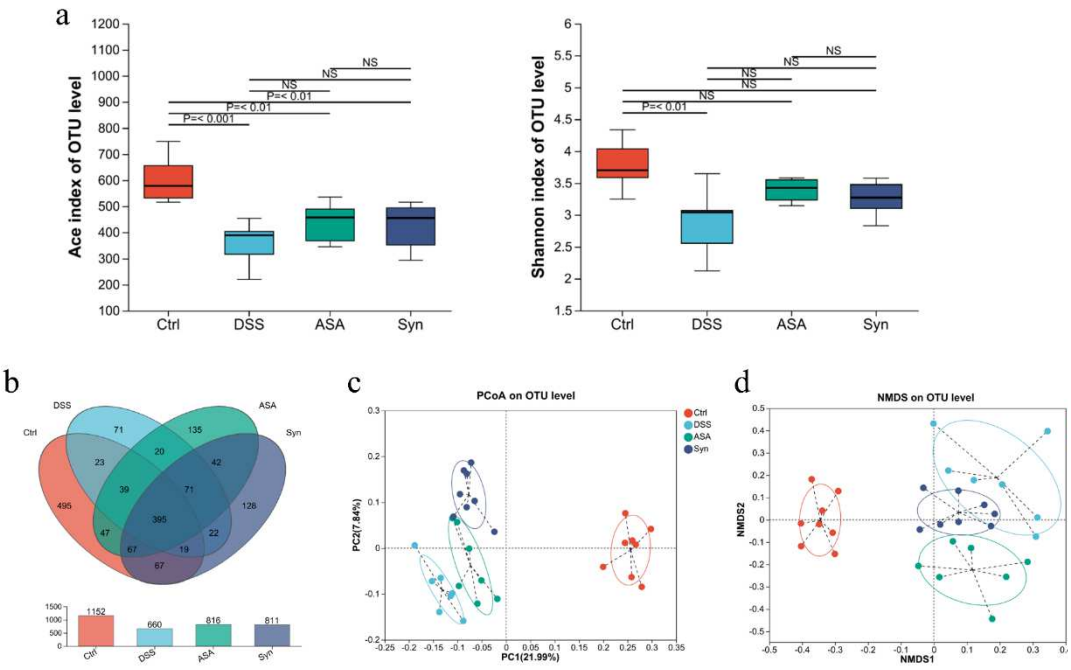

Fig.5 Synbiotics altered gut microbiota in DSS-induced mice. (**a**) α-diversity analysis: Ace index and Shannon index were used; (**b**) Venn diagram; （**c**） β-diversity was determined using weighted
UniFrac distance based PCoA; （**d**) NMDS analysis. Values are presented as means ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

3.3.2. Synbiotics changed gut microbiota composition

It is well established that intestinal dysbiosis exists in IBD patients [28, 29]. Herein, we investigated the composition of gut microorganisms from four aspects: phylum, family, genus, and species. The dominant bacteria were phylum *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* (Fig. 6a). Moreover, the abundance of phylum *Firmicutes* and *Proteobacteria* were increased in the DSS group compared to the control group, but the phylum *Bacteroidetes* was decreased (Fig. 6a). Interestingly, synbiotics treatment restored all these changes. At the family level, there was a significant decrease in the abundance of *Helicobacteraceae* and *Peptostreptococcaceae*, and increase in the abundance of *Muribaculacea* and *Akkermansiaceae* in the Syn group compared to the DSS group (Fig. 6b). At the genus level, the relative abundance of certain bacterial genera differed significantly between groups. For example,

the genera *Helicobacter* was significantly enriched in the DSS group, while genera norank_f_*Muribaculaceae*, *Akkermansia* and *Lactobacilus* was enriched in the synbiotics group (Fig. 6c). In addition, we also analyzed differences in the composition of the gut flora in species level. As shown in Fig. 6d, some major species were significantly affected by DSS. Synbiotics treatment remarkedly enhanced the growth of the Uncultured-bacterium-g-Norank-f-*muribaculaceae*, the *Lactobacilus-murinus* and *Akkermansia-muciniphila*, but significantly reduced Unclassified-g-*Helicobacter*.

To assess the characteristic marker genera of the intestinal flora in each group of mice, (LEfSe) was further analyzed. The LDA value greater than 4.0 was used as a screening criterion. Significant differences were shown in the abundance of taxa in the four groups (Fig. 7a). The main dominant groups in the NC group were *Muribaculaceae*, Norank-f-*muribaculaceae*, *Lactobacilus*, and Uncultured-bacterium-g-Norank-f-*muribaculaceae*. The bacteria that played a key role in the mice in the DSS group were family *Peptostreptococcaceae*, genera *turicibacter* and *Romboutsia*. The predominant group of bacteria in the synbiotics group was the beneficial bacterium *Akkermansia-muciniphila* (Fig. 7b). In short, the results indicated that synbiotics improved the composition of the intestinal flora of colitis mice.

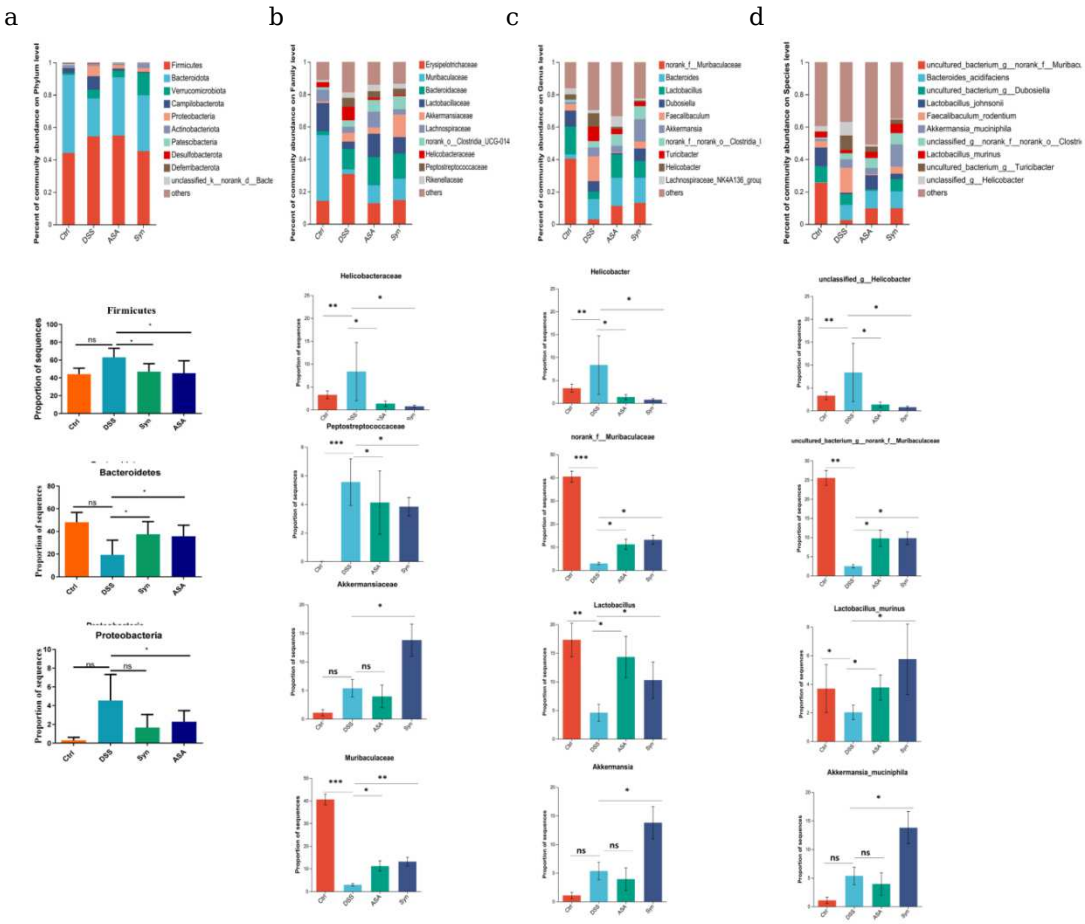

Fig.6 Synbiotics altered the composition of intestinal flora in DSS-induced mice. Characterization of the gut flora at the phylum (**a**), family (**b**), genus (**c**), and species (**d**) level. Data are expressed as means ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

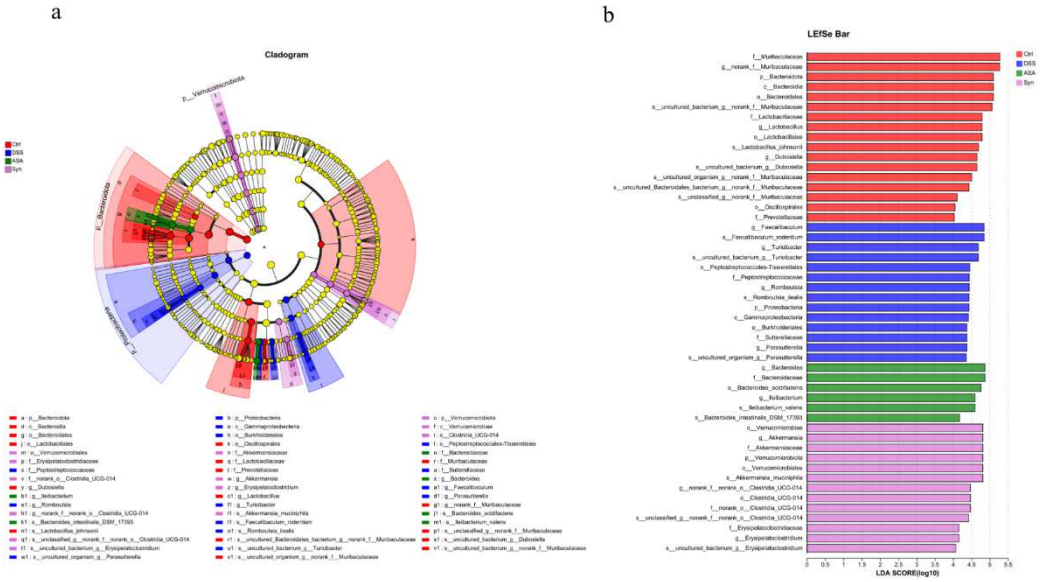

Fig.7 Synbiotics alters the gut microbiota biomarker in DSS mice. Identification of discriminant taxa among the four groups by LDA Effect Size (LeFSe) analysis. (**a**) Cladogram of the microbiota. Significant discriminant taxon nodes of the Ctrl, DSS, Syn, and ASA are represented by red, blue, green, and pink, respectively. Nondiscriminant taxon nodes are represented by yellow. Branch areas are shaded according to the highest ranked variety for that taxon; (**b**) The LDA score indicates the level of differentiation among the four groups. a threshold value of 4.0 was used as the cutoff level.

3.3.3. Synbiotics affected gut microbiota phenotype

BugBase was used to predict potential bacterial phenotypes for each group. We detected nine potential microbial phenotypes including aerobic, anaerobic, mobile element-containing, facultatively anaerobic, forming biofilm, Gram-negative, Gram-positive, potentially pathogenic, and tolerant (Fig. 8a). Our further analysis showed that the relative abundance of anaerobic and potentially pathogenic phenotypes was significantly higher in the DSS group compared to the control group, while the aerobic-containing and biofilm-forming phenotypes were significantly lower. Interestingly, the

synbiotics treatment significantly increased aerobic and biofilm-forming types, while significantly decreased the potentially pathogenic and anaerobic types (Fig. 8b). In addition, synbiotics treatment also decreased Gram-negativity, but the difference was not significant (Fig. 8a).

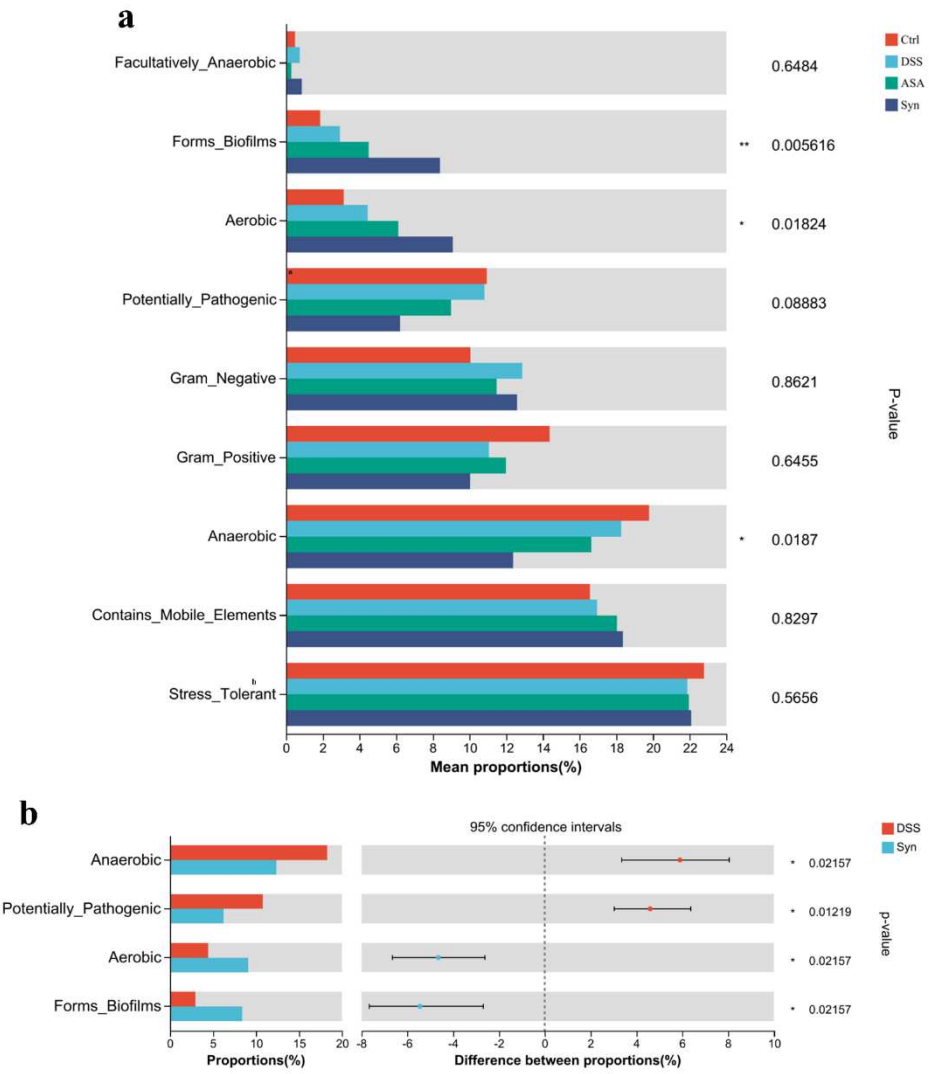

Fig.8 Synbiotics effects on gut microbiota phenotype basing BugBase including Aerobic, Anaerobic, Gram Positive, Gram Negative, Biofilm Forming, Pathogenic Potential, Mobile Element Containing. *p < 0.05; **p < 0.01; ***p <0.001; ****p < 0.0001.

3.4. Synbiotics improved intestinal damage and gut barrier function in colitis mice.

We explored whether the beneficial effects of synbiotics on colitis were associated with intestinal barrier function. Western blot and immunofluorescence analyses showed that the expression of the tight junction proteins Claudin-1 and Occludin was significantly reduced in the DSS group. Synbiotics treatment restored the levels of Claudin-1 and Occludin proteins (Fig. 9a, b). Cuprocytes can secrete mucin muc2, which further protects the intestinal barrier [30]. AB staining showed a great amount of mucin in blue color, while in the DSS group, the cuprocytes disappeared in a large number and the expression of mucin was significantly reduced (Fig. 9c). Fortunately, synbiotics significantly restored cuprocytes and acidic mucins. Of note, the levels of Claudin-1 and Occludin, and the expression of mucin were higher in the Syn group than those in ASA group, implying a better protective effect of synbiotics on DSS-induced colitis than ASA treatment. Enhanced intestinal barrier integrity will restrict translocation of LPS into circulation [31]. As expected, DSS induced higher levels of serum LPS compared to controls, whereas synbiotics treatment significantly lowered the expression levels (Fig. 9d), which further supported the positive role of synbiotics in gut barrier function in colitis mice.

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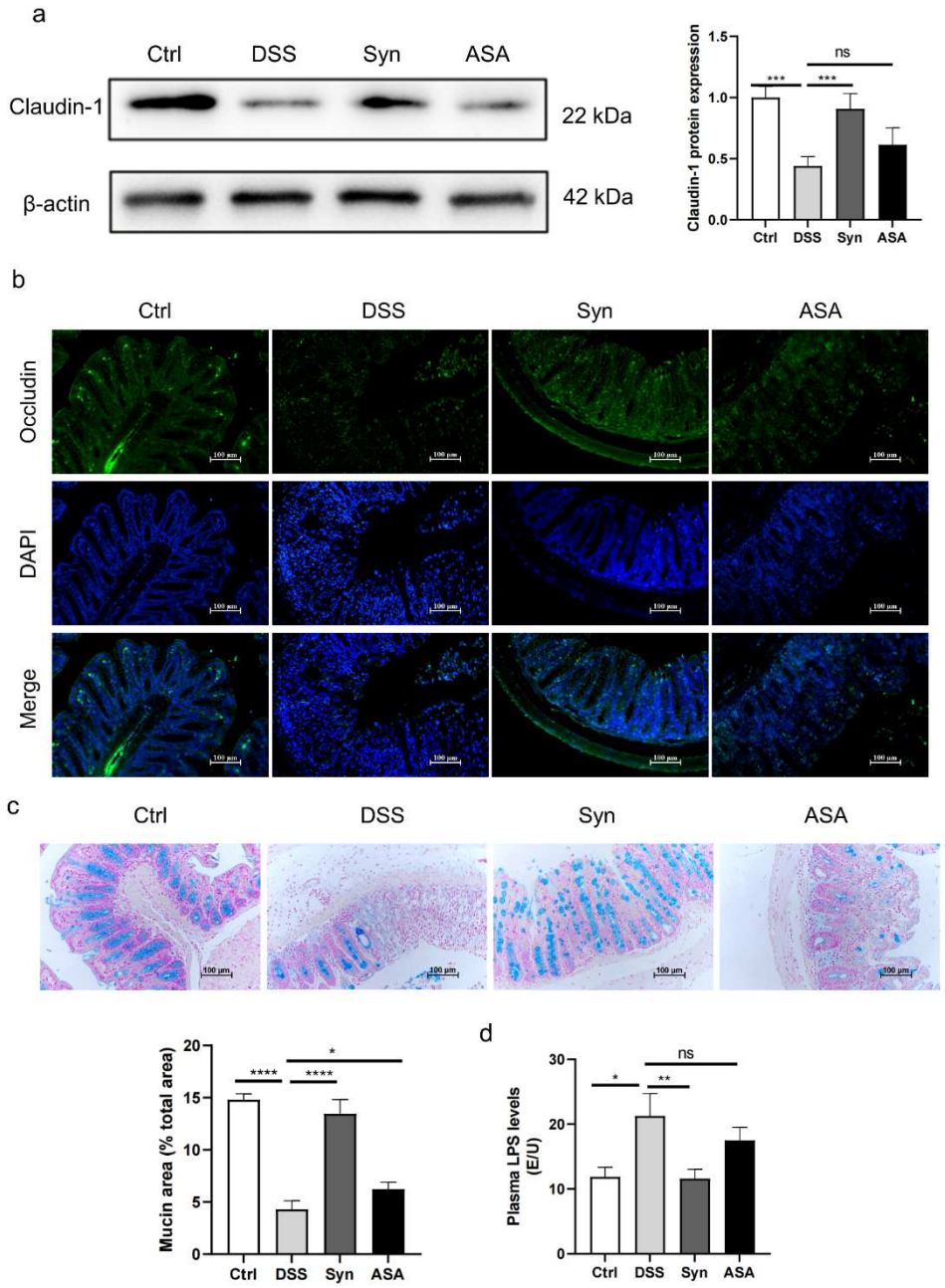

Fig.9 Synbiotics improved the intestinal permeability in colitis mice. (**a**) Western blot analysis of Claudin-1; (**b**) IF analysis of Occludin in colons. Scale bar, 100 um; (**c**) Alcian blue analysis of Mucin-2 in colons (Magnification of 200 ×); (**d**) Plasma LPS levels. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

3.5. Correlation analysis of gut microbiota and colitis-related parameters

The correlations and interactions between the dominant top 15 species and the IBD-related indices were elucidated by Spearman's correlation analysis. As shown in Fig. 10, uncultured_bacterium_g__norank_f__*Muribaculaceae*, uncultured_organism_g__norank_f__ *Muribculaceae*, *Lactobacillus johnsonii*, and uncultured_bacterium_g__ *Dubosiella* were significantly positively correlated with the body weight and gut barrier function, while negatively correlated with DAI, pro-inflammatory cytokine expression, LPS translocation, and TLR4/NF-κB related proteins expression. Thus, these bacterial species possibly promoted IBD and inflammation development. Conversely, *Bacteroides_acidifaciens*, uncultured_bacterium_g__*Turicibacter*, *Romboutsia_ilealis*, and uncultured_organism_g__*Parasutterella* were significantly positively correlated with DAI, pro-inflammatory cytokine expression, LPS translocation, and TLR4/NF-κB-related proteins expression, while negatively correlated with the body weight and gut barrier function. Thus,

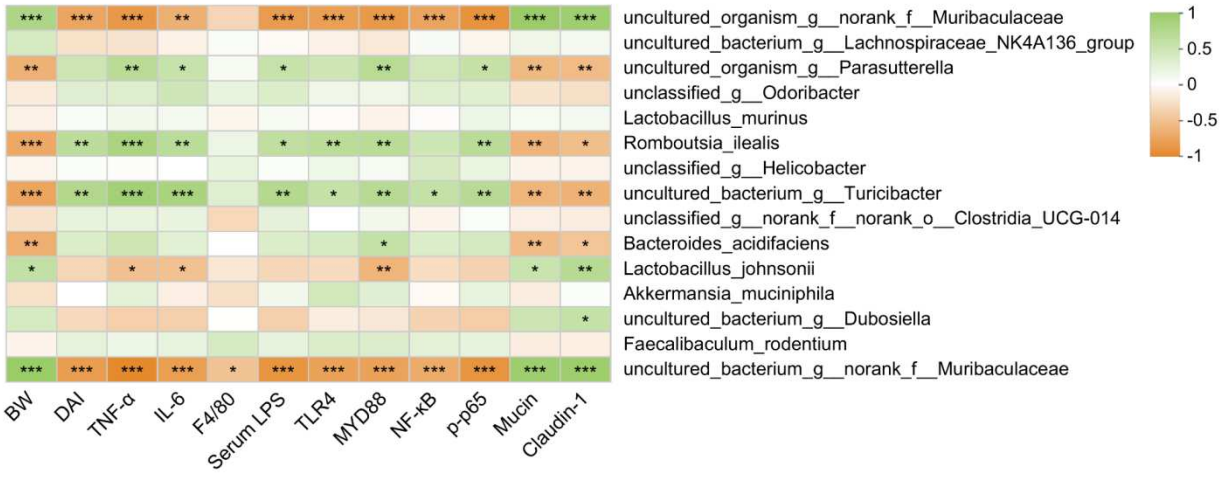

Fig.10 The relationship between IBD index, inflammatory response, LPS translocation, TLR4/NF-κB pathway, gut barrier function, and the top 15

genera were estimated by Spearman's correlation analysis. $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$.

4. Disscussion

We have previously isolated one *L. reuteri* strain (*L. reuteri* LY2-2) and identified its probiotic properties. Considering the alleviating effect of *L. reuteri* on colitis and the advantages of synbiotics, a novel synbiotic combination consisting of *L. reuteri* LY2-2 and COS was prepared. We hypothesized that supplementation with the new synbiotics could also ameliorate colitis, and the present study confirmed our hypothesis. The results demonstrated that synbiotics could alleviate symptoms of colitis such as reduced weight loss, DAI and reduced inflammatory cell infiltration. These data confirmed the positive role of the newly synbiotics in colitis, suggesting an alternative therapy against colitis, which will be helpful to products development of functional synbiotics to prevent and treat IBD. An in-depth analysis showed that the specific mechanisms of action of synbiotics might be attributed to its anti-inflammatory and microbiota-balancing properties.

It is well established that inflammation plays a pivotal role in the pathogenesis of IBD. The current evidence indicates that inflammation plays a role in multiple aspects of the IBD process [32]. It is involved in intestinal barrier disruption, and dysbiosis also stimulates the release of inflammatory factors from immune cells, which in turn promotes inflammation. Pro-inflammatory cytokines exacerbate intestinal inflammation [33]. Among them, TNF-α can promote the proliferation and differentiation of T cells and increase intestinal inflammation. IL-6 activates NF-κB to regulate DSS-induced colitis in mice. Consequently, the impact of synbiotics on inflammation was initially evaluated. When comparing various indicators of inflammation, we found that the synbiotics significantly reduced the levels of pro-inflammatory cytokines TNF-α and IL-6 in colonic tissues, which was in agreement with a previous study [34], indicating the potential of synbiotics to restrict intestinal

inflammation.

Most previous work has concluded that macrophages are major players in intestinal immune homeostasis, distinguishing between harmless antigens and potential pathogens to maintain homeostasis in the body [35]. Under normal conditions, macrophages have a role in protecting the gut from inflammatory damage. In patients with IBD, intestinal immunoregulation is compromised, leading to activation of chronic recurrent immune responses and generation of gastrointestinal inflammation [36]. Macrophages are therefore crucial for intestinal homeostasis and the pathology of IBD, and macrophages accumulate in large numbers in active IBD, recruiting pro-inflammatory factors (including TNF-α, IL-6, and nitric oxide) ,thus exacerbate intestinal inflammation [37]. Our study also identified increased accumulation of macrophages in the colonic tissues induced by DSS, while synbiotics application significantly reduced the pro-inflammatory macrophages infiltration, which might partially account for the anti-inflammatory effects of the synbiotics on colitis.

The dysbiosis of intestinal flora induces the activation of TLR4 by PAMPs, which initiates the Myd88-dependent signaling pathway to activate the NF-κB signaling pathway. This, in turn, initiates the expression of NLRP3 inflammasome, and ultimately leads to triggering of a series of inflammatory responses and pyroptosis [38]. Thus, the TLR4/Myd88/NF-κB signaling pathway is important for inflammatory responses mediated by the NLRP3 inflammasome [39]. Studies have shown that synbiotics can alleviate obesity by inhibiting the TLR4/NF-κB pathway and NLRP3 inflammasome [40]. Consequently, the present study sought to further elucidate the role of synbiotics on the TLR4/Myd88/NF-κB/NLRP3 pathway in colitis. The novel synbiotics in this study significantly inhibited the up-regulation of TLR4, Myd88, NF-κB, and NLRP3 inflammasome, suggesting that the signaling pathway was involved in the ameliorative effect of synbiotics on intestinal inflammation and intestinal injury.

The intestinal mucosal barrier is the first barrier between the intestinal tract and the external environment, and is extremely important in minimizing the attack of pathogens and the absorption of toxins [41]. Its structural basis is the intestinal epithelial cells and their tight junction proteins [42]. Under

normal conditions, the tight junctions between cells close the gaps between neighboring intestinal epithelial cells, preventing the free entry of toxic and harmful substances from the intestinal lumen, thus maintaining the intestinal barrier [43]. During IBD, the intestinal barrier is dysfunctional, resulting in an increase in LPS ectasia, which in turn activates immune cells, thereby exacerbating the symptoms of IBD. LPS binds to host TLR4, promotes NF-κB activation and the subsequent production of inflammatory factors, which collectively contribute to the promotion of inflammation. In this study, synbiotics were shown to attenuate DSS-induced impairment of colonic mucosal integrity, resulting in enhanced intestinal barrier function and reduced translocation of serum LPS, thereby providing further relief from colitis.

Dysbiosis of the intestinal flora has been largely considered as one of main factors to exacerbate DSS-induced chronic inflammation, thus treating colitis by regulating the intestinal flora has received increasing attention[44]. The gut microbiota is an important bridge between environmental factors and host health [45]. Synbiotics are mixtures of one or more probiotics and prebiotics that improve host welfare by improving the survival and colonization of microorganisms in the gastrointestinal tract, selectively stimulating the growth and/or activating the metabolism of one or a limited number of beneficial bacteria[46].

Our study suggested that the synbiotics optimized the composition and structure of gut microbiota of colitis mice. For example, the synbiotics application could altered the β-diversity of intestinal microbiota of DSS-treated mice, driving it much closer to the control mice. More importantly, synbiotics application significantly improved the gut flora dysbiosis of colitis mice, as evidenced by decrease of the abundance of harmful bacteria and increase of beneficial bacteria. Specifically, the disturbance of intestinal flora induced by DSS caused a significant increase in the phylum *Firmicutes* and *Proteobacterias* and a significant decrease in the phylum *Bacteroidetes*. Fortunately, supplementation with synbiotics reversed the negative changes caused by DSS, which was in agreement with the effects of some probiotics on the amount of *Proteobacteria* and F/B ratio caused by DSS [47, 48].

Proteobacterias produces endotoxins and is considered a major pathogen [49]. A high abundance of *Proteobacteria* may cause inflammation and lead to intestinal dysbiosis [50]. Moreover, *Helicobacter* generally disrupts the intestinal microenvironment, leading to disruption of intestinal homeostasis and promotion of intestinal inflammation, which in turn induces UC [51]. Previous study demonstrated that probiotics, such as *Lactobacillus*, could inhibit the growth of *Helicobacter* and relieve UC [52]. Similarly, we observed decreased abundance of *Helicobacter* in the synbiotic-treated group compared to that in the DSS group. In addition, beneficial bacteria such as Norank-f-*muribaculaceae*, *Lactobacilus*, and *Akkermansia* could also be enriched with intake of synbiotics in our study. These beneficial bacteria have been reported to reduce inflammation, inhibit harmful bacteria and oxidative stress, and ameliorate intestinal mucosal inflammation. Norank-f-*muribaculaceae* is an intestinal commensal bacterium that inhibits the growth of some pathogenic bacteria through competitive action [53]. It also modulates the host immune system, improves antimicrobial capacity, and reduces colonic inflammation. *Akkermansia*, a widely used probiotics, may exert an inhibitory effect on enteritis. The lower abundance of *Akkermansia* was associated with higher inflammation scores and lower levels of sulfated MUCs in the mucus layer [54, 55]. Given that, we concluded that synbiotics application could restore the intestinal homeostasis via balancing the composition of intestinal flora, decreasing the amount of potential pathogenic bacteria and augmenting the number of beneficial bacteria. The results of phenotype analysis that synbiotics significantly decreased the potentially pathogenic bacteria of DSS-treated mice further supported our speculation.

In summary, the present study showed that the new synbiotics had an ameliorative effect on colitis, which may be related to its anti-inflammatory and microbiota-balancing properties (Fig.11). In particular, synbiotics reduced pro-inflammatory factors, increased intestinal barrier function and restored the structure and composition of the intestinal flora. Our results support the theory of synbiotics supplementation in improving colitis, suggesting an alternative therapy against colitis, which will contribute to the development of functional synbiotic products for the treatment of IBD and will provide valuable insights

into their mechanisms.

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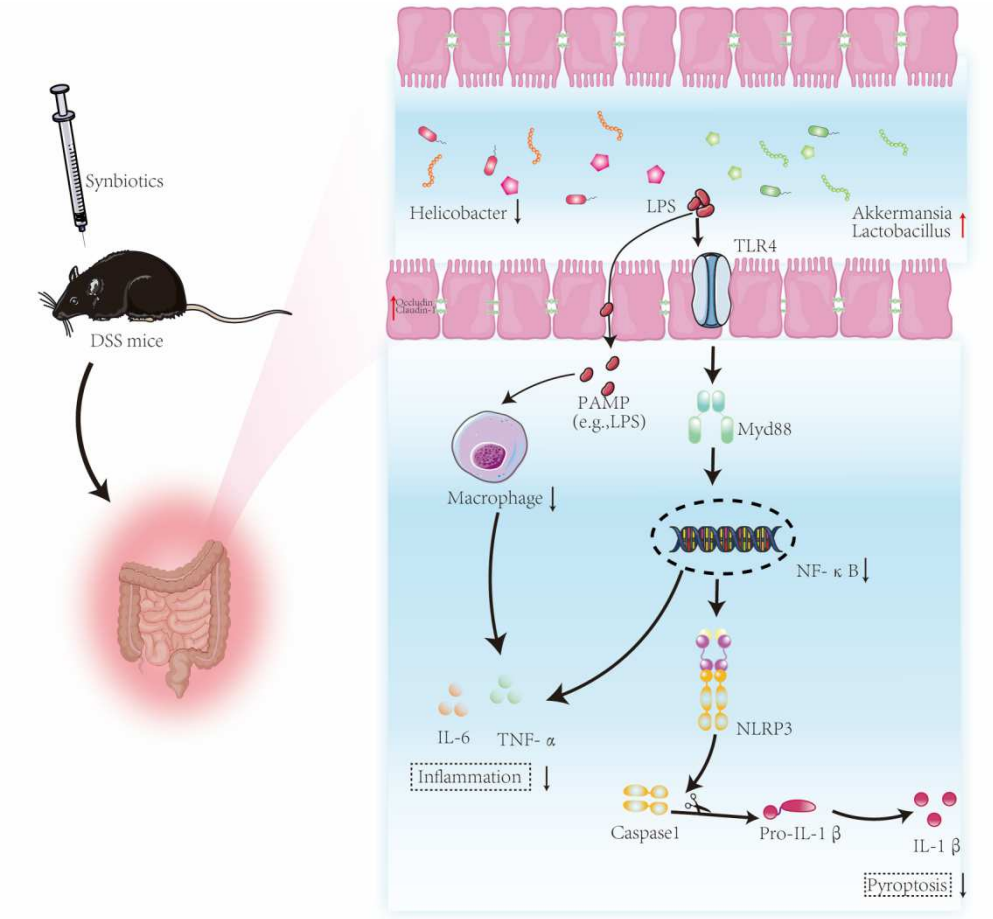

Fig. 11 Synbiotics induced an alteration in the gut microbiota to enrich probiotic bacteria and enhanced the gut barrier function. Meanwhile, synbiotics reduced the endotoxin from gut into circulation and the TLR4/Myd88/NF-κB/NLRP3 pyroptosis signaling pathway is suppressed. Ultimately, colitis was attenuated.

Abbreviations

IBD Inflammatory bowel disease
UC ulcerative colitis
CD Crohn's disease
5-ASA 5-aminosalicylic acid
IL-6 Interleukin-6
TNF-α Tumor necrosis facto
IL-1β Interleukin-1β
DAI Disease activity index
MRS Man, Rogosa and Sharpe
PCA Principal Component Analysis
NMDS Non-metric multidimensional scaling
LEfSe linear discriminant analysis (LDA) effect size
HE Hematoxylin and eosin

ANOVA one-way analysis of variance
RT-PCR Real-time polymerase chain reaction
TJ Tight junctions

Authors contributions

M.T., T.Z. and H.L. designed the experiments. Y.Y. and M.T. wrote the manuscript. Y.Y. , Y.Q. and G.Y. performed the experiments. Y.Y., Y.Q. and H.L. analyzed the data. G.L. and T.Z. analyzed gut microbiota. All authors contributed to manuscript revision, reading, and approval of the submitted version.

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Data availability

Data will be made available on request.

Declarations

Ethical approval

Animal experiments were approved and performed in accordance with the Ethic Committee of Shanxi Medical University (permit no. SYDL2023010). They were therefore performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Conflicts of 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.

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Figures

Figure 1

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Figure 8

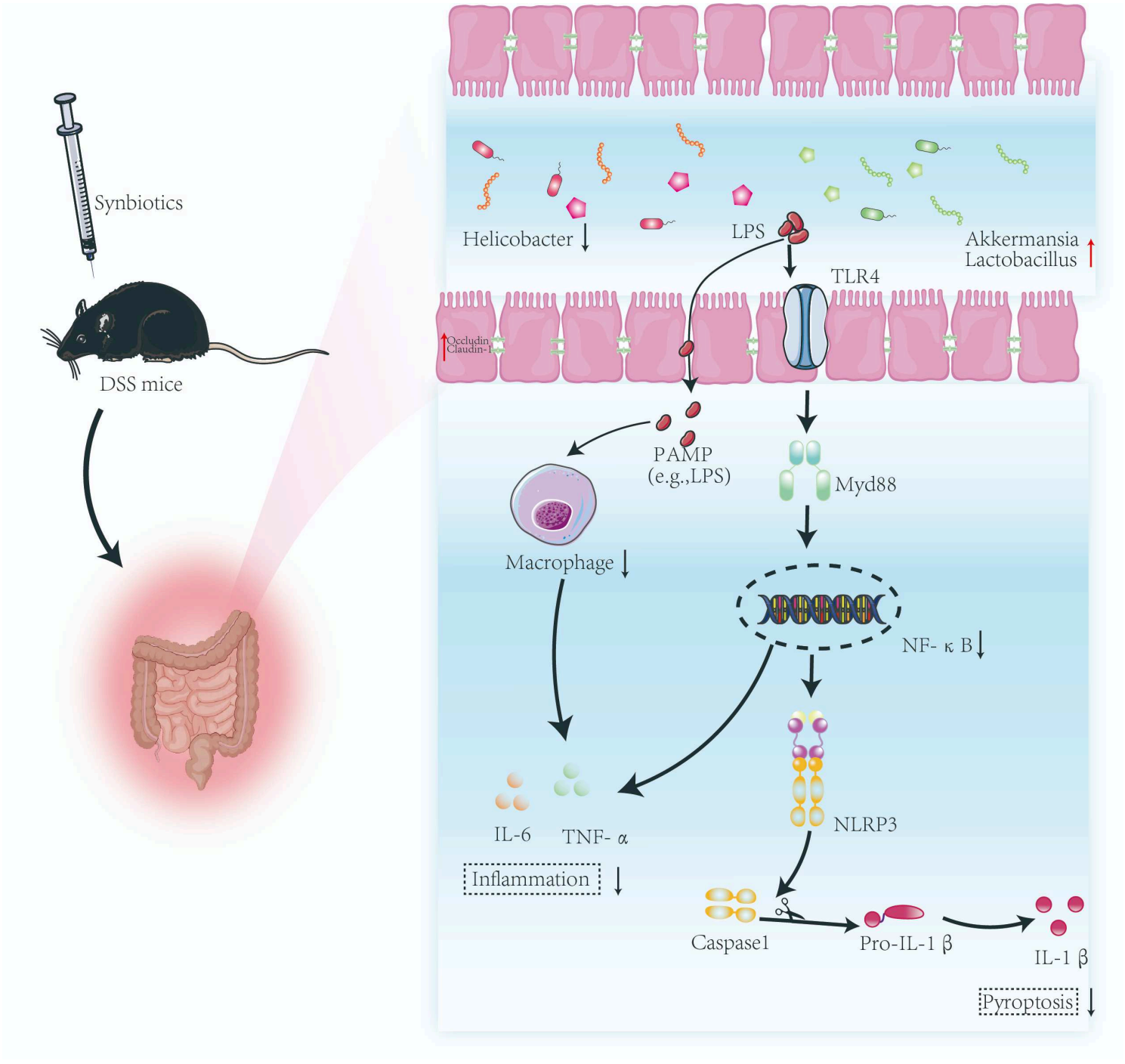

Figure 9

Figure 10

Figure 11

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