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Preliminary report

Astragalus polysaccharides attenuated inflammation and balanced the gut microflora in mice challenged with *Salmonella typhimurium*

Na Dong[a,1], Xinran Li[a,1], Chenyu Xue[a], Chensi Wang[a], Xinyao Xu[a], Chongpeng Bi[a], Anshan Shan[a,*], Deshan Li[b,c,**]

[a] Laboratory of Molecular Nutrition and Immunity, Institute of Animal Nutrition, Northeast Agricultural University, Harbin, PR China
[b] Bio-pharmaceutical Lab, College of Life Sciences, Northeast Agricultural University, Harbin, PR China
[c] Key Laboratory of Agricultural Biological Functional Gene, Harbin, Heilongjiang Province, PR China

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ABSTRACT

Salmonella typhimurium (*S. t.*) is one of the main pathogens that causes acute gastroenteritis. To evaluate the anti-inflammatory mechanism of *Astragalus polysaccharide* (APS) in vivo and its influence on the intestinal flora, BALB/c mice were infected with *S. t.* to establish a model of diarrhea. The disease activity index (DAI) scores showed that APS attenuated *S. t.*-induced weight loss and diarrhea in mice. APS significantly reduced the index of the liver and spleen as well as the ALT and AST levels in serum ($P < 0.05$). Hematoxylin and eosin (H&E) results indicated that APS significantly increased jejunum villus height and crypt depth and reduced the infiltration of inflammatory cells ($P < 0.05$). Additionally, APS increased the tight junction (TJ) proteins expression levels of ZO-1, Occludin and Claudin-1 in the jejunum. The results of 16S rDNA showed that APS significantly increased the number of *Lactobacillus and Bifidobacterium* spp. to normal levels (compared with the control group). In addition, APS significantly decreased the mRNA expression levels of the proinflammatory cytokines TNF-α, IL-1β, IL-6 and IL-17 in the jejunum ($P < 0.01$) as well as the proteins expression levels of COX-2 and iNOS ($P < 0.05$). Western blot confirmed that prefeeding with APS inhibited *S. t.*-induced expression of TLR4 and MyD88 in the jejunum and further inhibited nuclear factor-κB (NF-κB) activation, including the nuclear translocation of the p65 NF-κB subunit and the phosphorylation and degradation of IκB-α. This was the key to APS inhibition of the production of inflammatory factors and inflammatory mediators in the jejunum.

1. Introduction

Salmonella typhimurium (*S. typhimurium*, *S. t.*) is one major cause of foodborne diseases worldwide [1]. *S. typhimurium*, as an invasive bacterium, can cause acute gastroenteritis and primarily attacks the ileum, colon, and the entire gastrointestinal tract, which causes inflammation of the intestines and diarrhea [2]. Long-term survival of *S. t.* in the intestine will increase the risk of developing enteritis due to the virulence proteins of the bacteria [3]. *S. t* invades the host epithelial cell layer and migrates to the liver and spleen tissue, which causes liver and spleen damage [4]. At present, antibiotics are still the means of treating *Salmonella* invasion; high doses and long-term use of antibiotics can kill harmful bacteria, but beneficial bacteria (such as *Lactobacillus*, *Bifidobacterium*, etc.) that grow in the intestines will also be killed. Thus, treatment with long-term high-dose antibiotics will destroy the balance of the flora of the animal's intestines and further reduce the body's immunity. In addition, the long-term use of antibiotics can cause bacterial resistance. Therefore, there is an urgent need to find an effective medicament for reducing the intestinal damage caused by *S. t.*

Astragalus polysaccharide (APS) is extracted from *Astragalus Membranaceus* (AM) roots and is generally purified by dissolving in distilled water, dialysis and lyophilization [5]. APS has been indicated to have various biological activities, including immuno-modulatory [6], anti-inflammatory [7], antioxidant, anti-tumor and antiviral activities [8], among others. APS attenuated the pathological progression of rheumatoid arthritis (RA) by exerting pro-apoptotic and anti-inflammatory effects in IL-1β-stimulated fibroblast-like synoviocytes (FLSs) by regulating the PI3K/AKT/mTOR-autophagy pathway [9]. Furthermore, APS reduced the over expression of tumor necrosis factor (TNF)-α and IL-1β during experimental colitis induced by a hapten

* Corresponding author.
** Correspondence to: D. Li, Bio-pharmaceutical Lab, College of Life Sciences, Northeast Agricultural University, Harbin, PR China.
E-mail addresses: shanshimen1@163.com (A. Shan), deshanli@163.com (D. Li).
[1] These authors contributed equally to this work.

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[10]. APS supplementation induced a transgenerational endotoxin tolerance-like effect in the jejunum mucosa of broiler chickens [11]. APS modulated the immunity of host organism through activation of TLR4-mediated MyD88-dependent signaling pathway [12]. Although many studies have explored the anti-inflammatory action of APS, it still remains unclear whether APS can prevent or treat intestinal damage caused by harmful bacteria.

To investigate whether APS has a preventive effect on intestinal inflammation caused by invasive harmful bacteria, in this study, the expression of pro-inflammatory cytokines TNF-α and NO in *S. t.*-infected RAW264.7 cells was studied *in vitro*. Then, *in vivo* experiments with BALB/c mice were infected by *S. t.*, and a model of diarrhea was established. The effects of APS on intestinal barrier repair and its anti-inflammatory function and mechanism of action were further studied by using qRT-PCR and western blot. Moreover, the microbial community of mouse colon contents was further analyzed by 16S rDNA.

2. Materials and methods

2.1. Chemicals and antibodies

Astragalus polysaccharide (APS) and phosphate-buffered saline (PBS) solution were purchased from Solarbio (China); fetal bovine serum (FBS) and RPMI 1640 Cell culture medium were purchased from Gibco (USA); phenylmethanesulfonylfluoride (PMSF), SDS-PAGE gel preparation kit and an enhanced chemiluminescence (ECL) reagent were purchased from Beyotime (China); polyvinylidene fluoride (PVDF) membranes were purchased from Millipore (USA); SYBR Green Mix kit was purchased from Takara (Japan); iNOS, COX-2, TLR-4, MyD88, p-NF-κB p65, NF-κB p65, p-IκB-α and IκB-α were purchased from Cell Signaling Technology (USA); β-actin primary antibody was purchased from Beyotime (China); the secondary antibody HRP-labeled goat anti-rabbit IgG (H + L) was purchased from Beyotime (China).

2.2. Cell culture

The RAW264.7 cells were cultured in RPMI 1640 medium (HyClone) with 10% FBS and 1% P/S at 37 °C in a humidified atmosphere containing 5% CO_2 and were passaged every 1–2 days.

2.3. Determination of inflammatory factors TNF-α and NO

The RAW264.7 cells were plated at a density of 2×10^5 cells per well in 96-well plates and stimulated with *S. t.* (1×10^5 cfu mL^{-1}) in the absence or presence of APS (6.25–400 μg/mL) for 12 h at 37 °C. Untreated cells and LPS-only treated cells served as negative and positive controls, respectively. The isolated supernatant fraction was mixed with an equal volume of Griess reagent according to the manufacturer's protocol, and samples were incubated at room temperature for 10 min. Nitrite production was measured by determining the absorbance at 450 nm, and the concentrations were determined using a standard generated with $NaNO_2$. Using the same method, the levels of TNF-α were determined by ELISA kits in accordance with the manufacturer's instructions. TNF-α was measured by determining the absorbance at 540 nm.

2.4. Animal experiments

A total of 72 BALB/c mice (male, 4–5-week-old, 18–20 g) were obtained from the Liao Ning Chang Sheng Biotechnology Co., Ltd. All mice were housed in a sterile environment with controlled temperature and humidity with a 12 h light/dark cycle and were allowed free access to food and water. The mice were randomly divided into 6 groups of 12 mice each. Each group was given different treatments, as follows: 1) oral administration of sterile PBS from day 1 to day 10 (Control); 2) oral administration of sterile PBS from day 1 to day 6 followed by oral challenge with *S. t* (5.19×10^9 cfu mL^{-1}) on day 7 (*S. t.*); 3) oral administration of APS (100, 200, 400 mg/kg) from day 1 to day 10 and oral challenge with *S. t.* (5.19×10^9 cfu mL^{-1}) on the day 7. These mice were further divided into three groups according to different APS concentrations (*S. t.* + APS 100 mg/kg, *S. t.* + APS 200 mg/kg, *S. t.* + APS 400 mg/kg); 4) oral administration of APS from day 1 to day 10. The APS was dissolved in PBS. During this period, stool morphology and body weight of mice were recorded daily. After blood sampling, the mice were killed by cervical dislocation. Liver, spleen, jejunum and colon contents were collected. All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University (NEAU- [2011]-9).

Table 1
Evaluation of disease activity index (DAI).

DAI score	Weight loss (%)	Stool consistncy
0	None	Normal
1	1-5	-
2	5-10	Loose stools
3	10-20	-
4	> 20	Diarrhea

Table 2
Sequences of primers used for qRT-PCR.

Target	Sequence (5′–3′)	Accession
TNF-α	Forward: GATGCCAGCTATGAGCCACTTC Reverse: CTGCCATCACCACACTGTCCT	NM_001278601.1
IL-1β	Forward: GCACTGAGAGCATGATCCGAGAC Reverse: CGACCAGGAGGAAGGAGAAGAGG	BC011437.1
IL-6	Forward: GCTGCTGCTCCTGCTTCTAGTG Reverse: AGGTGAATTCCTTGCACGGTCTG	NM_012589.2
IL-17	Forward: ACTGGCTGTTGCCTTCTTGG Reverse: TGGGGTGGAAAGGTGTGGAA	FJ389361.2
ZO-1	Forward: AGCTGCCTCGAACCTCTACTCTAC Reverse: GCCTGGTGGTGGAACTTGCTC	D14340.1
Occludin	Forward: TGGCTATGGAGGCGGCTATGG Reverse: AAGGAAGCGATGAAGCAGAAGGC	BC138679.1
Claudin-1	Forward: ACAACGACACGCCGCTCATG Reverse: GGTGGTGATGCCGACAGAAGAC	BC089635.1
mGAPDH	Forward: TGT TCC TAC CCC CAA TGT GT Reverse: TGT GAG GGA GAT GCT CAG TG	HZ465752.

Table 3
Primers used for the absolute quantification of bacterial 16S rDNA copy numbers.

Target	Sequence (5′–3′)	References
Total bacteria	Forward: ACTCCTACGGGAGGCAGCAG Reverse: ATTACCGCGGCTGCTGG	Qian et al. [44]
Lactobacillus	Forward: AGCAGTAGGGAATCTTCCA Reverse: CACCGCTACACATGGAG	Qian et al. [44]
Bifidobacterium spp.	Forward: TCGCGTC(C/T)GGTGTGAAAG Reverse: CCACATCCAGC(A/G)TCCAC	Qian et al. [44]
Enterococcus spp.	Forward: CCCTTATTGTTAGTTGCCATCATT Reverse: ACTCGTTGTACTTCCCATTGT	Qian et al. [44]
Enterobacteriaceae family	Forward: CATTGACGTTACCCGCAGAAGAAGC Reverse: CTCTACGAGACTCAAGCTTGC	Qian et al. [44]

2.5. Evaluation of disease activity index (DAI)

The stool morphology and body weight of the mice were recorded daily. The disease activity index (DAI) is assessed by dividing the weight loss and stool consistency score by 2. Each score was determined as follows: change in body weight loss (0: none, 1: 1–5%, 2: 5–10%, 3:

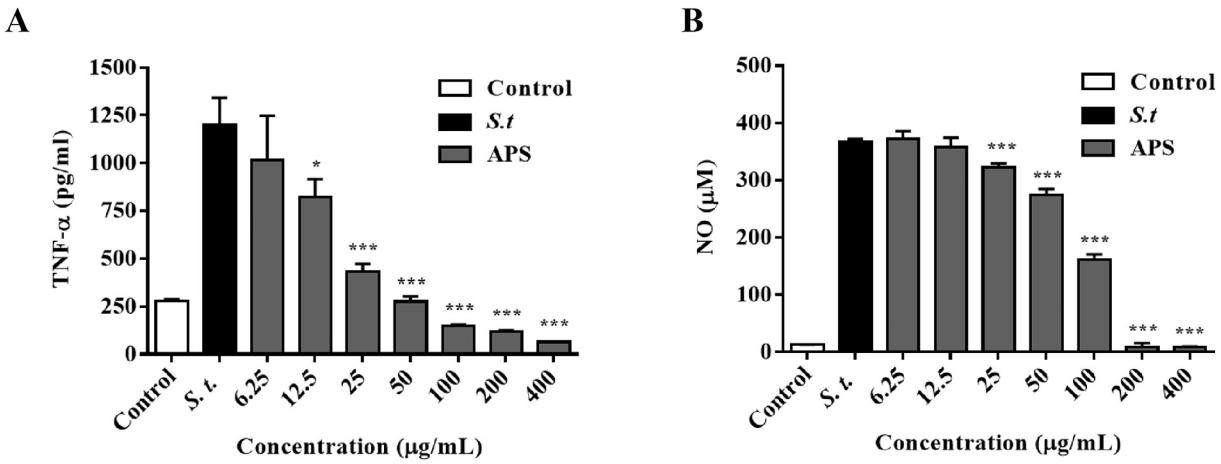

Fig. 1. The effect of APS on the *S. t.*-induced inflammatory factors TNF-α and NO. Amounts of (A) TNF-α and (B) NO in cell culture supernatants were determined by ELISA and Griess reagent, respectively. Values are the mean ± SD (n = 3) of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the *S. t.*-treated group.

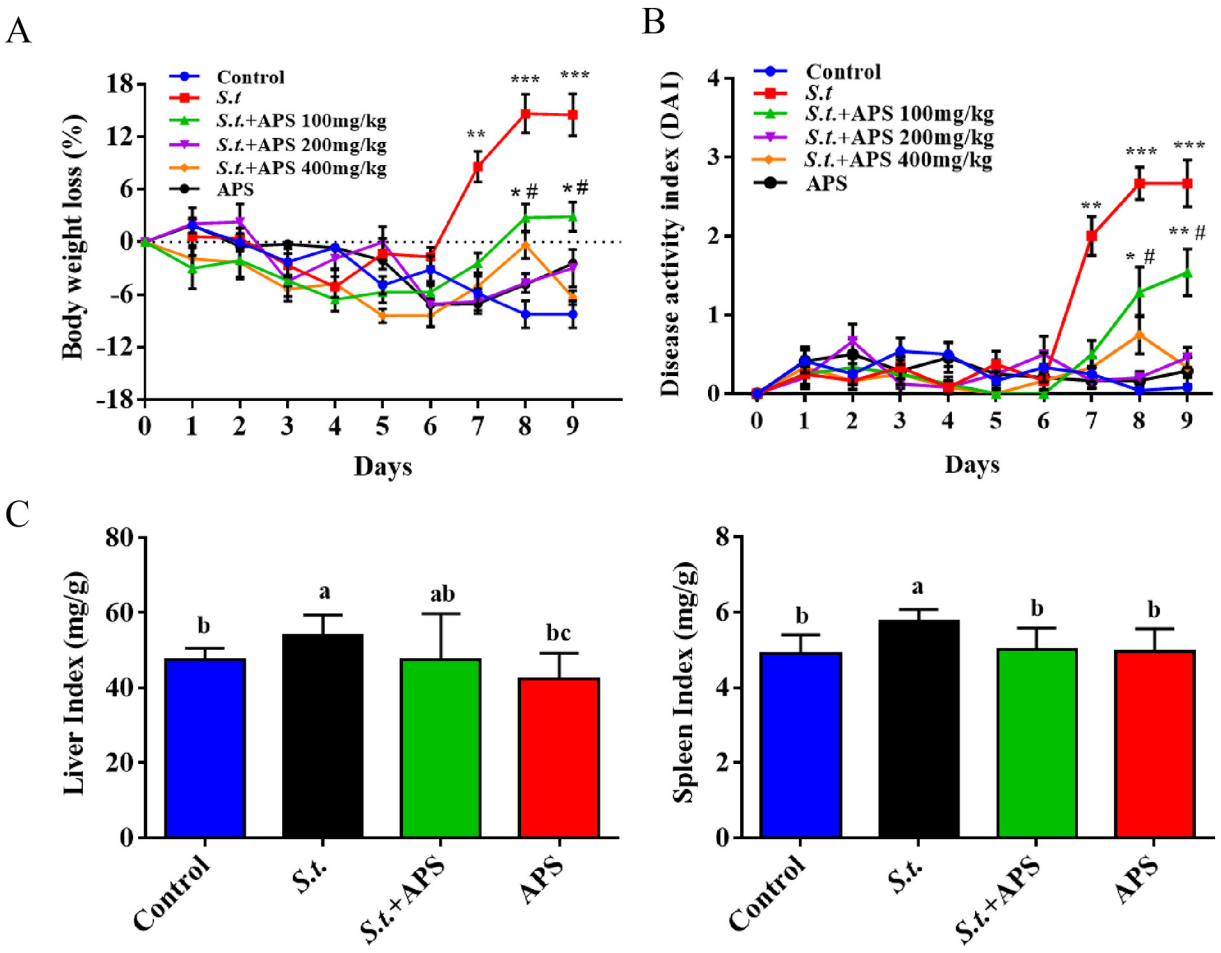

Fig. 2. APS attenuated the *S. t.*-induced diarrhea severity and weight loss of mice. (A) Body weight change (n = 18). (B) Changes in DAI levels; (C) immunity organs in infected BALB/c mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the vehicle-treated control group; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. the *S. t.*-treated group; n.s.: not significant. Different letters indicate that the change between each group is statistically significant ($P < 0.05$).

10–20%, 4: > 20%) and stool consistency (0: normal, 1 and 2: loose stool, 3 and 4: diarrhea). Body weight loss was calculated as the percent difference between the original body weight (day 0) and the body weight on any particular day (Table 1).

2.6. Blood sample analysis

Blood samples were collected from the orbital venous plexus 72 h after *S. t.* challenge. The blood samples were centrifuged at 1000*g* for 10 min at 4 °C. The resultant sera were obtained and stored at −20 °C. According to the manufacturer's instructions, the levels of alanine

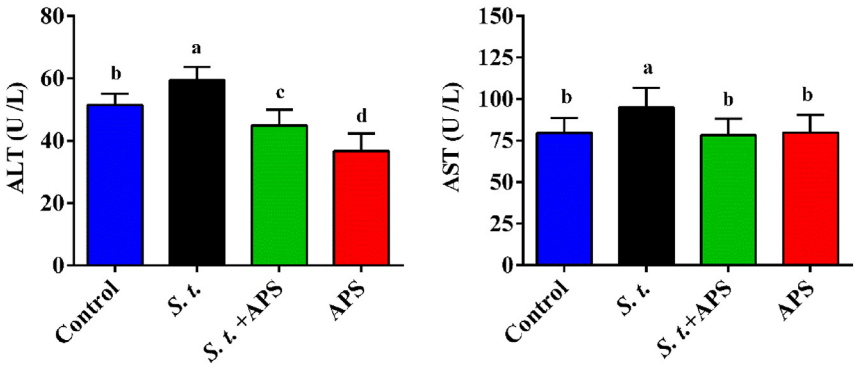

Fig. 3. Effects of APS on blood biochemical parameters of BALB/c mice (n = 6). At 72 h after *S. t.* challenge, blood samples were collected from the orbital venous plexus. The levels of ALT and AST were analyzed by using ELISA kits. Different letters indicate that the change between each group is statistically significant ($P < 0.05$).

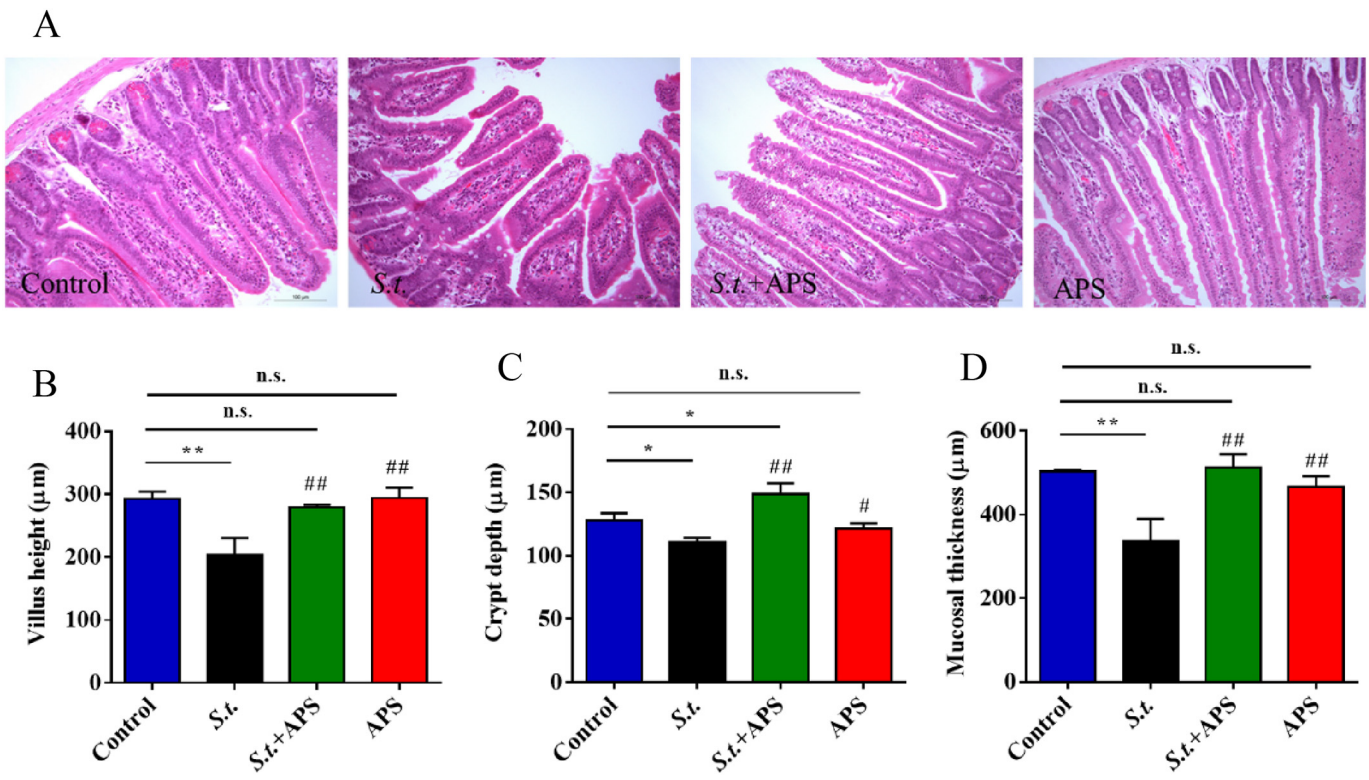

Fig. 4. Effects of APS on the jejunum inflammatory cytokines and intestinal morphology of *S. t.*-induced inflammation in mice. (A) H&E staining (original magnification 200×). (B) Villus height, (C) crypt depth. (D) Mucosal thickness. Values are the mean ± SD (n = 3) of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the vehicle-treated control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the *S. t.*-treated group; n.s.: not significant.

aminotransferase (ALT) and aspartate aminotransferase (AST) activity were analyzed by using ELISA kits (m1037606, ML-Biology, China).

2.7. H&E staining

The jejunum tissue of the same site was collected and fixed in 10% paraformaldehyde for at least 24 h at room temperature. After dehydration in different concentrations of alcohol, the tissue was embedded in paraffin and then sectioned and stained with H&E to evaluate the severity of intestinal inflammation, the extent of mucosal injury, and crypt damage. The stained slides were observed with a Motic 3000 photomicrography system (USA).

2.8. Determination of immunoglobulin by ELISA

According to the manufacturer's instructions, the ELISA kits (ML-Biology, China) method was used to determine s-IgA.

2.9. Real-time PCR

Total RNA was isolated from jejunum tissue by TRIzol (Invitrogen, CA, USA). Then, cDNA was generated with 1 μg of RNA as a template. Real-time PCR was performed using a 7500 Real-Time PCR system (Applied Biosystems) with the SYBR Green Mix kit (Takara, Japan). Primers in this study are listed in Table 2. The mRNA expression levels were normalized to β-actin using the ($2^{-\Delta\Delta CT}$) method. Each test was reproduced at least three times using three replicates.

2.10. Western blot analysis

We collected 0.1 g jejunum tissue sample, which was ground, homogenated and ultrasound processed and then treated with 1 mL RIPA containing 10 μL PMSF (Beyotime catalog no. P0013B). Tissue debris was removed by microcentrifugation (4 °C, 13,000 rpm, 40 min), which was followed by quick freezing of the supernatants. Protein concentrations were determined according to the Bradford method using a Bio-Rad Protein Kit. Protein was electroblotted onto a PVDF

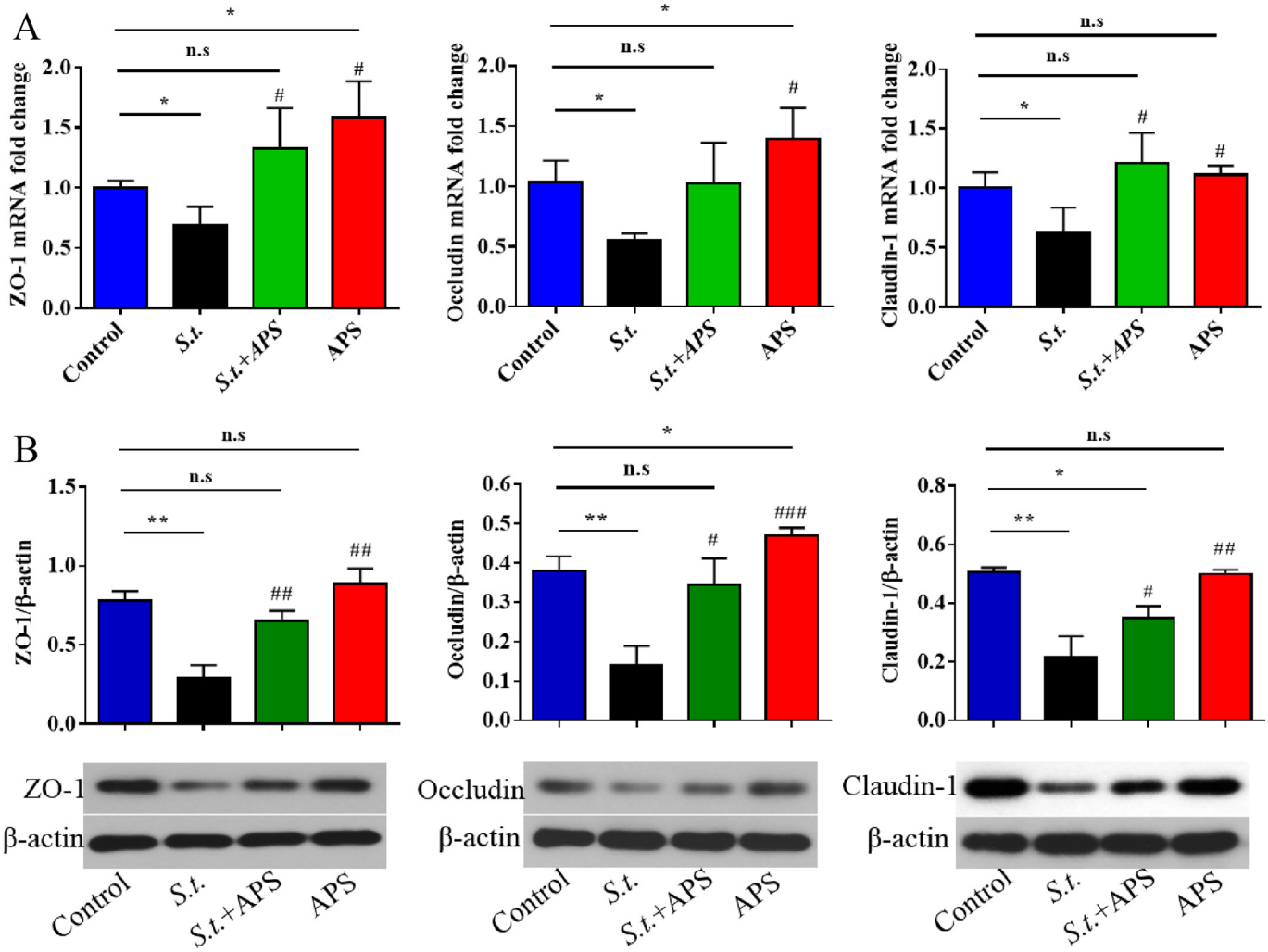

Fig. 5. Effects of APS on the expression of tight junction protein in the jejunum of *S. t.*-infected BALB/c mice. (A) qRT-PCR analysis of the mRNA expression level of ZO-1, Occludin, Claudin-1. (B) Western blot analysis of the expressions of ZO-1, Occludin and Claudin-1. Values are the mean ± SD (n = 3) of three independent experiments. $^{*}P < 0.05$ vs. the vehicle-treated control group; $^{\#}P < 0.05$ vs. the *S. t.*-treated group; n.s.: not significant.

membrane following separation on a 10% SDS–polyacrylamide gel electrophoresis. After blocking with 5% skim milk, the membrane was incubated with primary antibody (dilution, 1:1000) (Cell Signaling Technology, USA) or with anti-mouse β-actin monoclonal antibody (Beyotime, China). Next, the membranes were incubated with the corresponding HRP-conjugated secondary antibody (diluted, 1:1000) for 1 h at room temperature then were washed. The protein bands were observed by using a super enhanced chemiluminescence (ECL) plus detection system (P0018, Beyotime, China) and documented with X-ray film (Clinx Science Instruments Co., Ltd., Shanghai, China). At least three separate replicates were performed for each experiment.

2.11. Real-time PCR quantification for colon microflora

The sample of total DNA for colon microflora (n = 6) was extracted by using the E.Z.N.A.R Stool DNA kit (Omega Biotechnology, USA). The DNA concentration was measured by a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The primers used for the quantification of different bacteria and total bacteria are shown in Table 3. The PCR conditions were the same as described in the relative quantitative Real-time PCR.

2.12. Statistical analysis

The statistical analysis data were presented as the mean ± SD. The data were analyzed by the one-way analysis of variance (ANOVA) using Graph Pad Prism 6.2 software (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Effects of APS on inflammatory cytokines in S. t.-induced RAW264.7 cells

The anti-inflammatory effect of APS was studied by stimulating RAW264.7 cells with *S. t.* in the presence/absence of APS at various concentrations. The levels of tumor necrosis factor (TNF)-α and nitric oxide (NO) were chosen to evaluate the anti-inflammatory effect of APS. Fig. 1A shows that APS inhibited the production of the inflammatory factor TNF-α in a concentration-dependent manner and produced almost 100% inhibition of TNF-α production at a concentration of 50 μg/mL. A similar trend for the inhibition of TNF-α production was observed with the APS (Fig. 1B). APS significantly reduced the production of NO at a concentration of 25 μg/mL, and an APS concentration of 200 μg/mL reduced the NO concentration to the control level (no *S. t.* treatment).

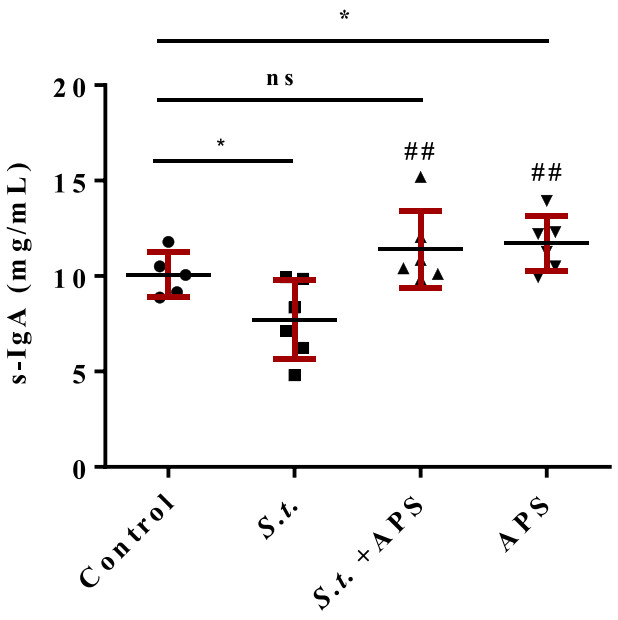

Fig. 6. Effect of APS on jejunum s-IgA levels in *S. t.* -infected BALB/c mice (n = 6). s-IgA levels were determined by ELISA. Values are the mean ± SD (n = 3) of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the vehicle-treated control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the *S. t.*-treated group; n.s.: not significant.

3.2. APS attenuated the diarrhea severity and weight loss of mice with S. t.-induced diarrhea

To analyze the effects of APS on jejunum inflammation, APS was orally administered to mice for 6 days (controls did not receive APS); then, mice were received *S. t.* orally for 3 days. As shown in Fig. 2A, the bodyweight of *S. t.*-induced mice decreased notably during the experimental period. The bodyweight changes of the *S. t.* + APS (200 mg/kg) and *S. t.* + APS (400 mg/kg) groups were not significantly different from those of the control group. In addition, the status of mouse feces was recorded daily and scored to generate a combinatorial DAI, which comprises stool consistency and body weight loss, that was used to assay the therapeutic effects of APS. We found that DAI was significantly increased in *S. t.*-induced mice (Fig. 2B). However, prefeeding with APS (100–400 mg/kg) followed by oral administration of *S. t.*, revealed that the DAI value was significantly reduced ($P < 0.05$), and the APS concentration of 200 mg/kg was the most effective. Next, the study used a concentration of 200 mg/kg APS for testing. APS prevented any increase of the liver and spleen weight caused by edema in the infected mice (Fig. 2C).

3.3. APS decreased histological S. t.-induced changes in the murine jejunum

To evaluate the therapeutic effects of APS on *S. t.*-induced intestinal inflammation, pathological examinations of jejunum sections were performed after H&E staining. Tissue sections from vehicle-treated control mice showed a histologically normal jejunum structure, whereas histological analysis of jejunum sections of mice with *S. t.*-induced colitis exhibited mucosal inflammation in the jejunum; as shown in Fig. 4A, the jejunal structure of the *S. t.* group showed strong edema rupture and infiltration of inflammatory cells. The integrity of the jejunum villi in the prefed APS group was significantly improved compared with the *S. t.* group. The length of the villus was not significantly different from that of the control group ($P > 0.05$) (Fig. 4B). There was a significant increase in crypt depth and mucosal thickness in the *S. t.* + APS group (Fig. 4C and D) ($P < 0.01$).

3.4. APS improved the expression of tight junction proteins in the murine jejunum

The expressions of tight junction (TJ) proteins (ZO-1, claudin-1, and occludin) were measured to investigate the protective effect of APS on the intestinal barrier. qRT-PCR and western blot (Fig. 5A and B) consistently showed that the level of TJ proteins were significantly reduced in the *S. t.*-alone treated group ($P < 0.05$). However, the level of TJ proteins were significantly increased in the *S. t.* +APS-treated group ($P < 0.05$) and were not significantly different from the control group. In addition, the expression levels of ZO-1 and Occludin were significantly increased in the APS-alone treated group ($P < 0.05$).

3.5. Effect of APS on s-IgA in jejunum

The effect of APS on s-IgA in the jejunum of mice was shown in Fig. 6. The *S. t.* treatment group significantly reduced the expression of A in the jejunum ($P < 0.05$). However, the expression of s-IgA in the jejunum of mice treated with APS and then in *S. t.* was not significantly different from that of the control group. The expression level of s-IgA in the jejunum of mice treated with APS alone was significantly increased ($P < 0.05$).

3.6. Microbial analysis in the colon

Fig. 7 showed that the number of *Lactobacillus* and *Bifidobacterium* spp. in the colon of the *S. t.* group was significantly decreased, while the number of *Enterobacteriaceae* and *Bacteroidetes* microbes significantly increased ($P < 0.05$). Compared with the *S. t.* group, the number of *Lactobacillus* was significantly increased in the *S. t* + APS group ($P < 0.05$), and the number of *Enterobacteriaceae* family microbes was significantly decreased ($P < 0.05$). The *S. t.* +APS group was not significantly different from the control group. *Lactobacillus* was significantly increased in the APS group ($P < 0.05$), and other strains were not significantly different.

3.7. APS suppressed the production of cytokines and the expression of COX-2 and iNOS in the jejunum of mice with S. t.-induced inflammation

Over expression of pro-inflammatory cytokines plays an important role in pathogenesis in intestinal inflammation [13]. Accordingly, we investigated the suppressive effect of APS on the *S. t.*-induced productions of pro-inflammatory cytokines in jejunum tissue. As shown in Fig. 8A, *S. t.* was found to significantly increase IL-6, IL-1β, TNF-α and IL-17 mRNA expression levels ($P < 0.01$). However, APS significantly reduced the mRNA expression levels of these inflammatory factors induced by *S. t.*, and there was no significant difference in the expression of these four proinflammatory cytokines' mRNA in the group fed APS alone. The protein expression levels of the pro-inflammatory enzymes COX-2 and iNOS were shown in Fig. 8B. *S. t.* significantly induced the expression on COX-2 and iNOS in the jejunum of mice. However, the expression levels of COX-2 and iNOS proteins were significantly down regulated in the *S. t.* +APS-treated group. This indicates that APS inhibits the expression of COX-2 and iNOS proteins.

3.8. APS attenuated the expression of NF-κB-related proteins in mice with S. t.-induced jejunum inflammation

As shown in Fig. 9, APS significantly inhibited TLR4 and MyD88 protein expression induced by *S. t.* In addition, *S. t.* induced the expression of phosphorylated p65 (p-p65) and IκB-α (p-IκB-α) proteins in jejunum tissues ($P < 0.05$), while the IκB-α protein expression level decreased ($P < 0.05$). APS significantly reduced the phosphorylation levels of p65 (p-p65) and IκB-α (p-IκB-α) proteins and showed that degradation of the IκB-α protein was alleviated.

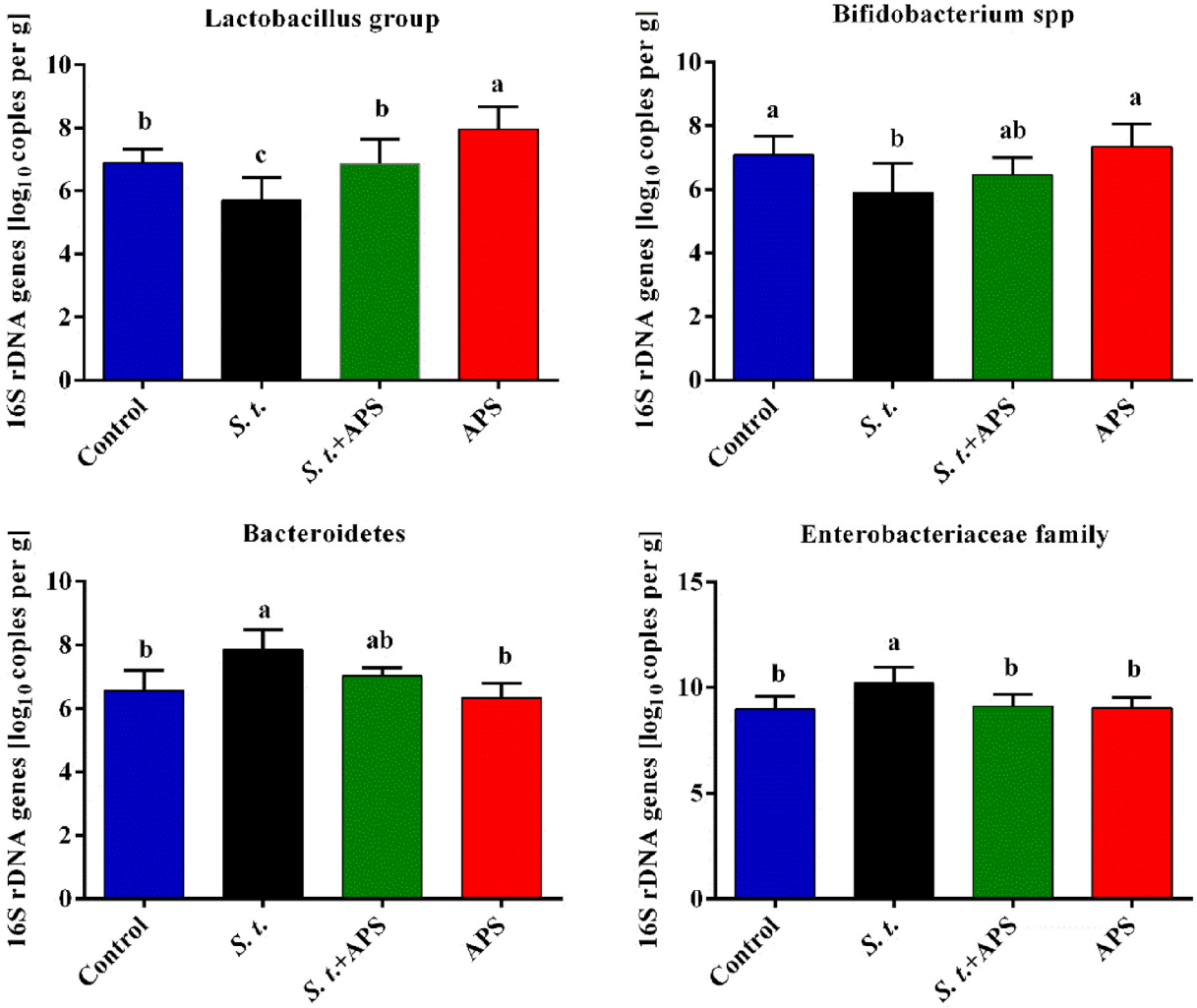

Fig. 7. The dose effect of APS on the composition of the mouse colonic microbiota. Different letters indicate the change between each group is statistically significant ($P < 0.05$).

4. Discussion

Bacterial infection is responsible for many serious and fatal diseases. The diagnosis of pathogenic bacteria is an essential need of clinical microbiology. Among all the food-borne pathogens, *Salmonella* is one of the most frequent causes of food borne infectious disease [14]. *Salmonella* invades the intestinal tract and disrupts the intestinal barrier function, leading to diarrheal enteritis. However, it also enters the body and attacks the liver and spleen, impairing the function of the liver and spleen [15]. It is urgent to seek an effective drug with few damaging side effects to treat intestinal inflammation and body damage caused by *Salmonella* invasion.

APS has been proven to have various biological activities, including antioxidant, radical scavenging and anti-inflammatory activities, as well as immune regulation and antiviral activities [16]. In this study, prefeeding with APS was able to alleviate the weight loss and diarrhea symptoms observed in *S. t.*-induced mice (Fig. 2A), and the DAI was not significantly different from the control group (Fig. 2B). This suggests that APS can prevent diarrheal intestinal inflammation caused by *S. t.* In addition, APS significantly reduced the liver and spleen indexes of the infected mice (Fig. 2C), which indicated that APS can relieve the organ edema of liver and spleen induced by *S. typhimurium*. ALT and AST levels in the serum are important indicators of liver function, and APS significantly reduced serum ALT and AST levels (Fig. 3), further indicating that APS reduces liver disease. The above data indicate that APS can effectively alleviate *S. t.*-induced diarrhea in mice and protect the liver and spleen.

The small intestine is required for nutrient absorption and serves critical roles in oral drug uptake and metabolism; it is also a target of many pathogens and toxins [17]. To prevent the body from being invaded by endogenous or exogenous microorganisms and their toxins, the intestinal epithelium has a function of separating the substances in the intestinal cavity and preventing the invasion of pathogenic antigens, which is called the intestinal barrier function. The maintenance of normal intestinal barrier function depends on the balance of the intestinal mucosal epithelial barrier, the intestinal flora and the intestinal immune system [18]. Therefore, maintaining intestinal epithelial barrier integrity is important to maintain defense against pathogen invasion and inflammation [19]. Tight junction (TJ) proteins, such as claudin-1, Occludin and ZO-1 are intercellular adhesion complexes that are essential to the barrier function of epithelia and endothelia. When pathogenic bacteria and viruses attack, they hijack TJ proteins to enter and infect cells and promote the production of inflammatory factors [20]. In this study, APS inhibited the decrease of Claudin-1, Occludin and ZO-1 caused by *S. t.* infection, and the APS-treated group alone showed significantly increased ZO-1 and claudin-1 proteins expression (compared with the control group) (Fig. 5). This indicates that APS can effectively protect the integrity of the jejunal TJ of mice, thereby preventing *S. t.* from penetrating the intestinal barrier and destroying intestinal epithelial cells. This effect is consistent with that observed by

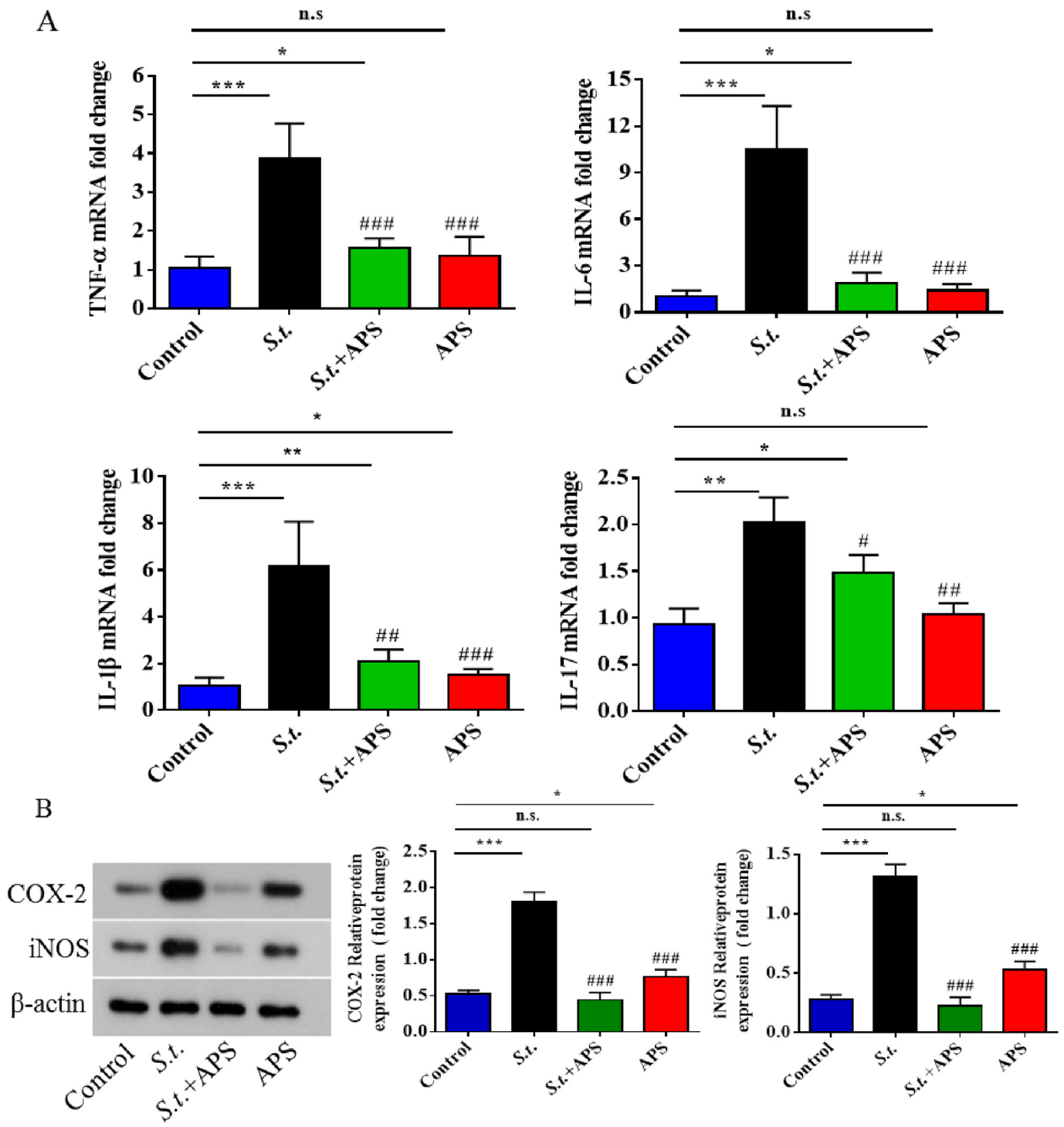

Fig. 8. Effect of APS on the production of pro-inflammatory cytokines and the proteins expression of iNOS and COX-2. (A) Effects of APS on expression of inflammatory in the jejunum of *S. t.*-infected BALB/c mice. (B) The expression of iNOS and COX-2 were determined by western blot analysis using specific antibodies. The expressions of iNOS and COX-2 were analyzed with ImageJ and normalized against -actin. Values are the mean ± SD (n = 3) of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the vehicle-treated control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. the *S. t.*-treated group; n.s: not significant.

Wang et al. [21], who found higher Occludin and ZO-1 mRNA expression in the jejunum of broiler chickens fed with APS than in those not fed with APS. Furthermore, APS also reduced pathological changes in the intestinal tissue and atrophy of the intestinal villus in *S. t.*-treated mice (Fig. 4A). The above studies indicated that APS had a positive effect on intestinal mucosal integrity.

The intestinal microflora is a large bacterial community that colonizes the intestine. Intestinal microbes play a dual role in the intestinal barrier function. On the one hand, they act as antigens for the intestinal mucosal barrier; on the other hand, intestinal microflora can provide nutrients for intestinal mucosal cells, maintain intestinal microecological balance, activate the intestinal immune system, and constitute intestinal barrier function [22]. Exogenous pathogens and toxins can impair the intestinal barrier function, and their mechanism of action is to destroy tightly linked proteins, resulting in decreased resistance of the intestinal mucosal epithelium. Examples include damage

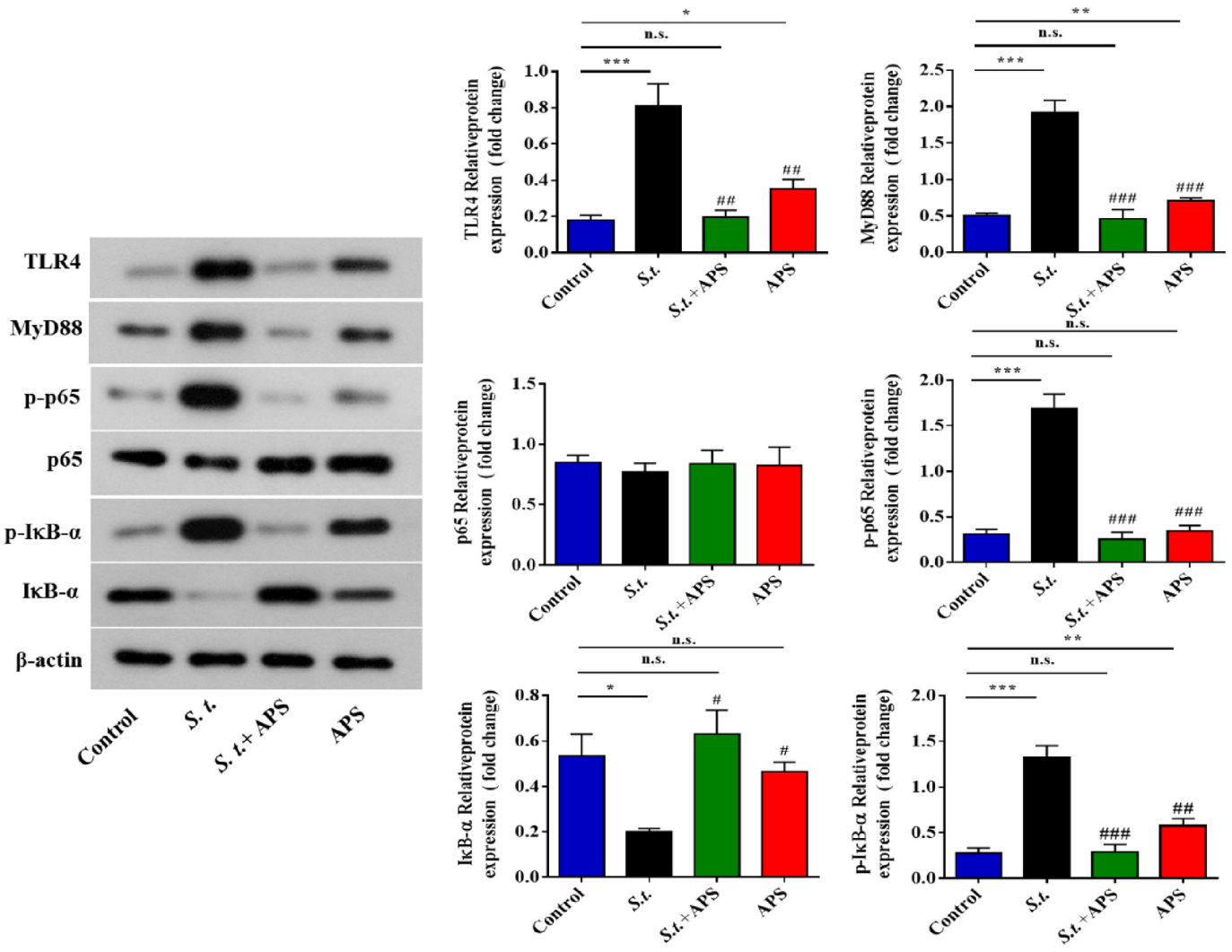

Fig. 9. Effect of APS on the nuclear translocation of NF-κB and the protein expression of NF-κB-dependent proteins in *S. t.*-infected mouse jejunum. The values are the mean ± SD (n = 3) of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.1$, $^{***}P < 0.001$ vs. the vehicle-treated control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. the *S. t.*-treated group; n.s: not significant.

caused by intestinal bleeding *Escherichia coli*, *S. typhimurium*, *Clostridium perfringens* and *Vibrio cholerae* [23]. Intestinal secretory immunoglobulin (Ig)A (s-IgA) can prevent attachment of microbial components to intestinal epithelial cells and can also promote the clearance of microorganisms, e.g. pathogens that have breached the epithelial barrier [24]. S-IgA represent the main element of the immune system involved in maintenance of gut flora homeostasis. The absence of normal s-IgA leads to a significant shift in anaerobe populations in the small intestine. Suzuki et al. showed that the lack of s-IgA causes a dramatic increase in the number of intestinal anaerobic commensal bacteria such as *Bacteroides* and *Clostridium* [25]. In the present study, s-IgA levels in the jejunum of mice in the APS-treated group were significantly elevated. Therefore, APS maintains a balance of intestinal flora that may be closely related to its inhibition of s-IgA reduction.

In addition, some parasitic bacteria in the intestine can also protect the intestinal barrier, including *Lactobacillus* and *Bifidobacterium* spp., which can resist and repel the invasion of exogenous pathogens and the invasion of intestinal mucosal cells. *Lactobacillus* and *Bifidobacterium* spp. can also create a biomembrane on intestinal mucosa to serve as a barrier against colonizing pathogens and improve gut immunity [26]. APS not only increased the abundance of *Lactobacillus* and *Bifidobacterium* but also decreased the number of *Bacteroidetes* and *Enterobacteriaceae* in the colon (compared with the *S. t.* group) (Fig. 7). This suggests that APS regulates and maintains the balance of the intestinal flora by increasing s-IgA and the number of probiotics to enhance local bacterial colonization and/or competitive rejection against pathogens. Therefore, the intestinal biocarrier function is protected.

Pro-inflammatory cytokines are crucial biomarkers that are released in intestinal mucosa upon hyperactivation of immune cells after induction of enteritis. It has been confirmed that selective blockade of these inflammatory mediators decreases neutrophil/macrophage migration, improving colitis progression [27]. In this experiment, APS significantly reduced the expression of the inflammatory factors TNF-a, IL-1β, IL-6 and IL-17 mRNA (Fig. 8A). Pro-inflammatory cytokines (including TNF-α, IL-6 and IL-1β) are elevated in intestinal disease and associated with the severity of inflammation. TNF-α and IL-6 are essential for pro-inflammatory cytokines that directly affect intestinal epithelial tissue. Excessive expression of TNF-α results in damage to the epithelial barrier, enhanced epithelial cells apoptosis and secretion of chemokines [28]. In addition, IL-1β is also a key factor in intestinal disease [29]. A previous study indicated that lower level of T cells with excessive IL-17 were important players in the pathogenesis and prognosis of IBD [30]. In addition, the inducible enzymes COX-2 and iNOS are inflammatory proteins predominantly expressed at sites of inflammation. COX-2 is unexpressed under normal conditions in most cells, but elevated levels of COX-2 are found under inflammatory

conditions; these two enzymes are thus largely responsible for causing inflammation [31]. APS significantly reduced the expression of COX-2 and iNOS proteins in jejunum induced by *S. t.* (Fig. 8B). which suggests that the inhibitory effects of APS on intestinal inflammation are related to the suppression of these pro-inflammatory mediators.

To further study the mechanism of APS exerting its anti-inflammatory action in the intestine, it is necessary to understand the ways in which APS inhibits the expression of pro-inflammatory mediators and pro-inflammatory cytokines. Recognition of *S. typhimurium* is largely mediated by Toll-like receptors (TLRs). TLRs target a range of ligands for *Salmonella*, including lipopolysaccharide (TLR4), lipoprotein (TLR2) and flagellin (TLR5) [32–36]. *S. typhimurium* 14028 is a Gram-negative bacterium, and lipopolysaccharides (LPS) are found in the cell wall of Gram-negative bacteria which can interact with the receptors of immune cells. LPS induce inflammation via the upregulation of different pro-inflammatory mediators, such as iNOS and COX-2 [37], and pro-inflammatory cytokines, including interleukin-1 (IL-1), IL-6 and TNF-α [38,39]. Adaptor molecules such as MyD88 are essential mediators of TLR4-induced NF-κB signaling [40,41]. Lv et al. research showed treatment of APS ameliorated the severity of colitis in a mouse model of DSS-induced colitis. This protective effect is likely mediated by inhibition of NF-κB activation in colonic tissues, which in turn downregulates the pro-inflammatory responses in colonic tissues. These results suggest that APS represents a potential therapeutic approach for treating ulcerative colitis [42]. In the present study, APS inhibited *S. t.*-induced expression of TLR4 and MyD88 proteins in the jejunum of mice (Fig. 9). This indicates that APS can inhibit the binding of the *S. t.* cell wall LPS to the TLR4 receptor protein and further inhibit the activation of NF-κB. The NF-κB p65 protein binds to the IκB protein and is present in the cytoplasm. When intestinal tissues are stimulated by external stimuli, nuclear factor-kappa B kinases (IKKs) can phosphorylate the IκB family of proteins [43]; this is followed by ubiquitination and degradation, which further phosphorylates p65 and releases it into the nucleus, where it promotes inflammation. APS inhibits the phosphorylation of p65 caused by *S. t.* while preventing the degradation of the IκB-α protein (Fig. 9), which effectively inhibits NF-κB activation. Based on the above data, APS inhibits the production of pro-inflammatory mediators and inflammatory factors by inhibiting the activation of the *S. t.*-induced TLR4/MyD88/NF-κB pathway.

5. Conclusions

In our study, APS relieved inflammation through inhibiting body weight loss and reducing the liver and spleen index of infected mice. APS increased the intestinal barrier function by repairing damaged structures of the jejunum villi, increasing the expression of tight junction proteins ZO-1, Occludin and Claudin-1, and regulating the number of *Lactobacillus*, *Bifidobacterial* and *Enterobacteriaceae* in the gut to balance the intestinal flora. Additionally, APS inhibited the production of the inflammatory factors TNF-α, IL-1β, IL-6 and IL-17 by inhibiting the activation of the TLR4/MyD88/NF-κB signaling pathway induced by *S. t.* Therefore, APS is expected to be an alternative to antibiotics for the treatment of intestinal inflammation caused by bacterial infections.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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