

Dihydroartemisinin Alleviates Diclofenac Sodium-Induced Small Intestinal Mucosal Injury by Regulating the MAPK/NF- κ B Signaling Pathway and Gut Microbiota Homeostasis

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ABSTRACT

Background: Dihydroartemisinin (DHA), a well-established antimalarial agent has been increasingly recognized for its antiviral and antitumor effects across multiple diseases. However, the potential effects of DHA on Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)-induced intestinal mucosal injury remain largely unexplored. The current work was designed to characterize the therapeutic actions and the underlying molecular mechanisms governing DHA against histopathological damage and gut microbiota dysbiosis in a rat model of Diclofenac Sodium (DS)-induced Small Intestinal Mucosal Injury (SIMI). **Materials and Methods:** A rat model of DS-induced SIMI was established, and the animals were treated with varying doses of DHA. Histological injury scores were evaluated by Hematoxylin and eosin (H&E) staining. Protein expression levels were determined using immunofluorescence, immunohistochemistry and Western blot analysis. 16S rRNA-based sequencing was applied to assess compositional variations of the gut microbiota. **Results:** Our findings demonstrated that DHA treatment increased ileal length, while reducing the pathological inflammation scores and the secretion of pro-inflammatory cytokines, including TNF- α , IL-1 β , IL-6 and CD68-positive macrophage infiltration. Furthermore, DHA attenuated intestinal epithelial cell apoptosis, promoted cell proliferation, and preserved intestinal mucosal barrier integrity, accompanied by downregulated expression of CHGA, LYZ and MYC, as well as inactivation of MAPK/NF- κ B signaling pathway. Moreover, DHA elevated the relative abundances of *Lachnospiraceae*, *NK4A136*, *Lactobacillus*, *Intestinimonas* and *Bacteroidetes*, while decreasing the abundance of *Desulfovibrionaceae* in SIMI models. **Conclusion:** DHA mitigates NSAID-induced SIMI by inhibiting the MAPK/NF- κ B signaling pathway and modulating gut microbiota homeostasis.

Keywords: Dihydroartemisinin, MAPK, Microbiota, Small intestinal mucosal injury.

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INTRODUCTION

Diclofenac Sodium (DS), a common NSAID, is primarily used for the management of osteoarthritis and other related conditions. However, accumulating clinical evidence has demonstrated that NSAIDs may induce gastric mucosal injury, bleeding or perforation. With the widespread application of capsule endoscopy, increasing attention has been paid to NSAIDs-induced

Small Intestinal Mucosal Injury (SIMI) (Ehrhard *et al.*, 2013; Endo *et al.*, 2015; McMahon & Monroe, 1992; Watanabe *et al.*, 2013). NSAIDs may also trigger widespread erosions and ulcerative lesions within the small-intestinal mucosa, along with complications such as bleeding and intestinal obstruction. It has been reported that NSAIDs enteropathy results in 16,500 deaths and 100,000 hospital admissions annually in the United States (Wolfe *et al.*, 1999) and distal small intestinal lesions can be detected in two-thirds of patients on prolonged NSAID treatment (Graham *et al.*, 2005; Maiden, 2009). Notably, 40% to 53% of patients develop lower gastrointestinal tract damage after two weeks of NSAID therapy (Fujimori *et al.*, 2009; Lee *et al.*, 2021).

The mechanical barrier of the intestinal mucosa is disrupted in NSAIDs-provoked lower gastrointestinal mucosal damage



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(Bjarnason *et al.*, 2018). NSAID-induced SIMI is characterized by increased intestinal mucosa permeability, which facilitates bacterial translocation across the intestinal epithelium and ultimately leads to intestinal mucosal damage (Ravikumar *et al.*, 2004). Nevertheless, the exact molecular mechanisms governing intestinal mucosal barrier impairment remain incompletely elucidated. Elucidating these regulatory mechanisms is critical for identifying effective preventive and therapeutic strategies against NSAIDs-induced SIMI.

Derived from artemisinin, dihydroartemisinin (DHA) represents its key biologically active metabolite (Keating, 2012; Tu, 2016). Beyond its well-documented antimalarial efficacy, DHA exerts a wide array of pharmacological functions, including strong anti-inflammatory and antitumor bioactivities. Additionally, DHA exerts immunomodulatory effects in the treatment of multiple sclerosis and rheumatoid arthritis (An *et al.*, 2017; Hou & Huang, 2016). The Mitogen-Activated Protein Kinase (MAPK)/NF- κ B signaling is a pivotal pathway governing inflammation-related pathogenesis across numerous disorders (Hollenbach *et al.*, 2004; Johnson & Bailey, 2003). Aberrant activation of the MAPK/NF- κ B cascade drives the synthesis and secretion of pro-inflammatory cytokines (Guan *et al.*, 1998), thereby contributing to the development and progression of NSAID-induced SIMI.

Our previous studies demonstrated that DHA prevents against Dextran Sodium Sulphate (DSS)-elicited colitis by dampening excessive activation within NLRP3 inflammasome and MAPK/NF- κ B signaling (Liang *et al.*, 2020). However, current therapeutic strategies for NSAID-induced SIMI remain limited, and the potential of DHA to repair SIMI remains unreported in prior publications. The current investigation reveals, for the first time, that DHA may exert protective effects against DS-induced SIMI via suppressing the MAPK/NF- κ B signaling pathway and modulating gut microbiota composition. These findings provide a novel theoretical basis and a promising therapeutic strategy for the clinical application of DHA in treating DS-induced SIMI.

MATERIALS AND METHODS

Chemicals

DHA (UV $\geq$ 98%) was purchased from Mansiter Biotechnology (Chengdu, China). DS was purchased from Temmler Werke GmbH (Munich, Germany).

Establishment of DS-induced SIMI rat model

Every experimental protocol was authorized by the Animal Ethics Committee of Shanghai Sixth People's Hospital, and DS-induced SIMI rat model was constructed by referring to our previously published paper (Chen *et al.*, 2024). Sprague Dawley (SD) rats (male, 2-month-old and weighing 200-220 g) were purchased from the Model Animal Research Center of Nanjing University, where they were raised in a specific-pathogen-free

environment. 6 rats were resided in individual ventilated cages under SPF conditions; a 12-hr light/dark cycle and controlled ambient temperature were sustained throughout housing. After a 7-day acclimatization period in the experimental facility, a total of 24 rats (weighing 200-220 g) were randomly allocated and blinded into four groups (each $n=6$): CTR group, DS group (7.5 mg/kg, intragastric administration i.g), DS + L.D.DHA group (low-dose DHA, 5 mg/kg, i.g), DS + H.D.DHA group (high-dose DHA group, 25 mg/kg, i.g). An equivalent dose of normal saline was intraperitoneally injected into control animals. Efforts were undertaken to minimize group-wise animal counts and lessen pain and distress among experimental rats. Pentobarbital Sodium was used to induce rapid anesthesia after intraperitoneal injection with a duration of 30-60 min, and cause relatively mild depression of respiration and circulation in rats, making them suitable for short-term procedures in model establishment. Rats were euthanized and subjected to CO₂ inhalation (progressive flow rate) and chest opening was used to confirm cardiac arrest. The exclusion criteria for the rats used for DS-induced SIMI models was listed as follows: before the enrollment, the rats should be excluded with abnormal health (diarrhea, bloody stools, weight deviation from mean $\pm 10\%$, infection/trauma/hair removal), the product line, age, weight do not meet the standard, the rats with immunodeficiency or mixed genetic background, abnormal drinking or feeding habits and uneven baseline grouping. During the model construction phase, the rats should be excluded with extremely low water intake or refusal to drink, weight drop of $\geq 20\%$, severe bloody stool/perianal rupture and mental exhaustion and inability to eat or drink. After successful model construction, the rats should be also excluded with no shortening of the colon and no ulceration/crypt injury/inflammatory infiltration. Based on these exclusion criteria, we enrolled the current cohort of rats and established the model. The animal study was approved by Ethics Committee in Animal Experiments of Shanghai Sixth People's Hospital (No. 2021-0038). Our animal research conforms to ARRIVE 2.0 guidelines (Sert *et al.*, 2020).

Small intestinal tissue processing in rats

All rats were anesthetized by intraperitoneal administration of 0.6% pentobarbital sodium. After adequate anesthetic depth was verified, each animal was fixed in the supine posture on a dissection platform. The abdominal cutaneous surface was sterilized, and a midline abdominal incision was made, followed by layered dissection of abdominal tissues. The intestinal tract was dissected segmentally, and the entire small intestine was excised and its length measured for each animal. A 4-cm segment of ileum, located 5 cm proximal to the ileocecal valve, was collected for injury scoring; it was rapidly rinsed with ice-cold PBS. A 5 cm segment of small intestine, taken 10 cm proximal to the ileocecal valve. The remaining small intestine was incised longitudinally to assess gross morphological damage. The above procedures described were performed as a terminal procedure under deep

anesthesia, from which the rats did not regain consciousness, in line with ARRIVE reporting guidelines.

Hematoxylin and Eosin (H&E) staining and histological score

Paraffin-embedded ileum tissues were stained with H&E (Su *et al.*, 2021). Histological damage was quantified using a validated histopathological scoring system (Chiu *et al.*, 1970). For each slide ($n=10$), five non-overlapping fields were randomly selected at 400 $\times$ magnification under a light microscope. H&E staining enables comprehensive evaluation of tissue architectural damage, inflammatory cell infiltration, and histopathological scoring.

Immunofluorescence (IF) analysis

IF analysis was carried out by referring to our previously published paper (Chen *et al.*, 2024). Primary antibodies against caspase-3 (ab184787, clone EPR18297, RRID:AB_2756763, 1:200, Cambridge, MA, USA), TNF- α (ab1793, clone 52B83, RRID:AB_302615, 1:200, Cambridge, MA, USA), IL-1 β (ab315084, clone EPR24895-116, RRID:AB_3105874, 1:200, Cambridge, MA, USA), IL-6 (ab259341, clone EPR23819-11, RRID:AB_2819721, 1:200, Cambridge, MA, USA), and CD68 (ab53444, clone FA-11, RRID:AB_869052, 1:200, Cambridge, MA, USA) were incubated with the slides overnight at 4°C. Slides were washed with PBS and incubated with anti-rabbit IgG (#34206ES60, Polyclonal, RRID: AB_2909605, 1:300, Yeason, China). Next, slides were washed with PBS and counterstained with DAPI dihydrochloride (Yeason, China) for 10 min. Images were captured using a laser scanning confocal microscope (Leica TCS SP5, Germany). For quantification, five non-overlapping fields were randomly selected per slide ($n = 10$) at 400 $\times$ magnification with ImageJ software.

Immunohistochemistry (IHC)

IHC analysis was performed by referring to our previously published paper (Chen *et al.*, 2024). Primary antibodies against CHGA (ab254557, clone EPR22537-249, RRID: AB_2819144, 1:200; Abcam, Cambridge, MA, USA), Muc2 (ab308191, clone HL1724, 1:200; Abcam, Cambridge, MA, USA), LYZ (ab223503, clone SP329, RRID: AB_2894542, 1:200; Abcam, Cambridge, MA, USA), MYC (#18583, clone E5Q6W, RRID:AB_2798764, 1:200; CST, Danvers, MA, USA), Ki67 (#9129, clone D3B5, RRID:AB_2687948, 1:200; CST, Danvers, MA, USA), p38 (#8690, clone D13E1, RRID:AB_10949192, 1:200; CST, Danvers, MA, USA), p-p38 (#9211, clone 12D8, RRID:AB_331641, 1:200; CST, Danvers, MA, USA), p65 (#3987, clone E498, RRID:AB_2228936, 1:200; CST, Danvers, MA, USA), and p-p65 (#3033, clone 93H1, RRID:AB_331284, 1:200; CST, Danvers, MA, USA) were applied.

Western blot analysis

Total protein was extracted from frozen rat ileum tissue. Western blot assays were conducted according to our previously reported

procedures (Chen *et al.*, 2024). Primary antibodies against CHGA (ab254557, clone EPR22537-249, RRID: AB_2819144, 1:1000; Abcam, Cambridge, MA, USA), MUC2 (ab308191, clone HL1724, RRID:AB_2890123, 1:1000; Abcam, Cambridge, MA, USA), LYZ (ab223503, clone SP329, RRID: AB_2894542, 1:1000; Abcam, Cambridge, MA, USA), and Ki67 (ab92742, clone EPR3610, RRID: AB_10562296, 1:1000; Abcam, Cambridge, MA, USA) were applied.

16S rRNA-seq analysis of gut microbiota

Fresh fecal samples were collected from individual rats in each group at the time of sacrifice. Samples were immediately placed into sterile, RNase-/DNase-free 2.0 mL microcentrifuge tubes, snap-frozen in liquid nitrogen, and stored at -80°C. All procedures were performed under aseptic conditions to avoid cross-contamination. Then, the V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified using the universal primer set: 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Sequencing was performed on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) using a 2 $\times$ 300 bp paired-end strategy. Quantitative Insights into Microbial Ecology (QIIME, v1.8.0) pipeline was used to process the sequencing data, as previously described (Maiden, 2009).

TUNEL assay

4- μ m paraffin sections of 4% paraformaldehyde-fixed rat small intestinal mucosa were deparaffinized, rehydrated, and incubated with 20 μ g/mL proteinase K for 20 min at room temperature; after PBS rinsing, sections were blocked with 3% hydrogen peroxide, equilibrated, and then incubated with fluorescein-dUTP TUNEL reaction mixture at 37°C for 1 h in a dark humidified chamber. Nuclei were counterstained with DAPI in the dark for 5-10 min, and slides were sealed with anti-fade mounting medium for fluorescence microscopic observation, where apoptotic cells appeared green and nuclei stained blue.

Statistical analysis

Data are given as Means $\pm$ SD. The normality assumption and variance homogeneity were validated before statistical inference. One-way ANOVA was used for multi-group comparisons, and Tukey's *post-hoc* test was applied for pairwise analyses. Statistical calculations were executed in GraphPad Prism, and $P < 0.05$ represented statistical significance.

RESULTS

DHA reduced pathological scores in the DS-induced SIMI rat model.

We found that DHA lowered pathological scores in the DS-induced SIMI rat model. Quantitative results revealed that small intestinal length was decreased in the DS group compared with the

CTR group ($p<0.001$). Conversely, small intestinal length was markedly increased in both the Low-Dose (L.D.) DHA ($p<0.001$) and High-Dose (H.D.) DHA groups ($p<0.001$) relative to the DS group (Figures 1A-1C). H&E staining demonstrated severe small intestinal injury in DS-induced SIMI models, characterized by extensive villus atrophy, disorganized glandular architecture, and marked inflammatory cell infiltration, compared with the CTR group ($p<0.01$). Notably, these small intestinal pathological lesions were significantly ameliorated in the L.D.DHA ($p<0.01$) and H.D. DHA group ($p<0.001$) relative to the DS group (Figures 1B and 1D).

DHA inhibited cell apoptosis in the DS-induced SIMI rat model

We found that DHA suppressed cell apoptosis in the DS-induced SIMI rat model. To evaluate this effect, IF staining for TUNEL and cleaved Caspase-3 was performed. The results showed that TUNEL-positive apoptotic cells were greatly augmented in the DS group when contrasted against the CTR group, but markedly reduced in the L.D.DHA and H.D. DHA group relative to the DS group (Figures 2A and 2B; $p<0.001$). Similarly, the IF intensity of Caspase-3 was dramatically elevated in the DS group ($p<0.001$), but significantly attenuated following treatment with either Low-Dose (L.D.) DHA ($p<0.001$) or High-Dose (H.D.) DHA

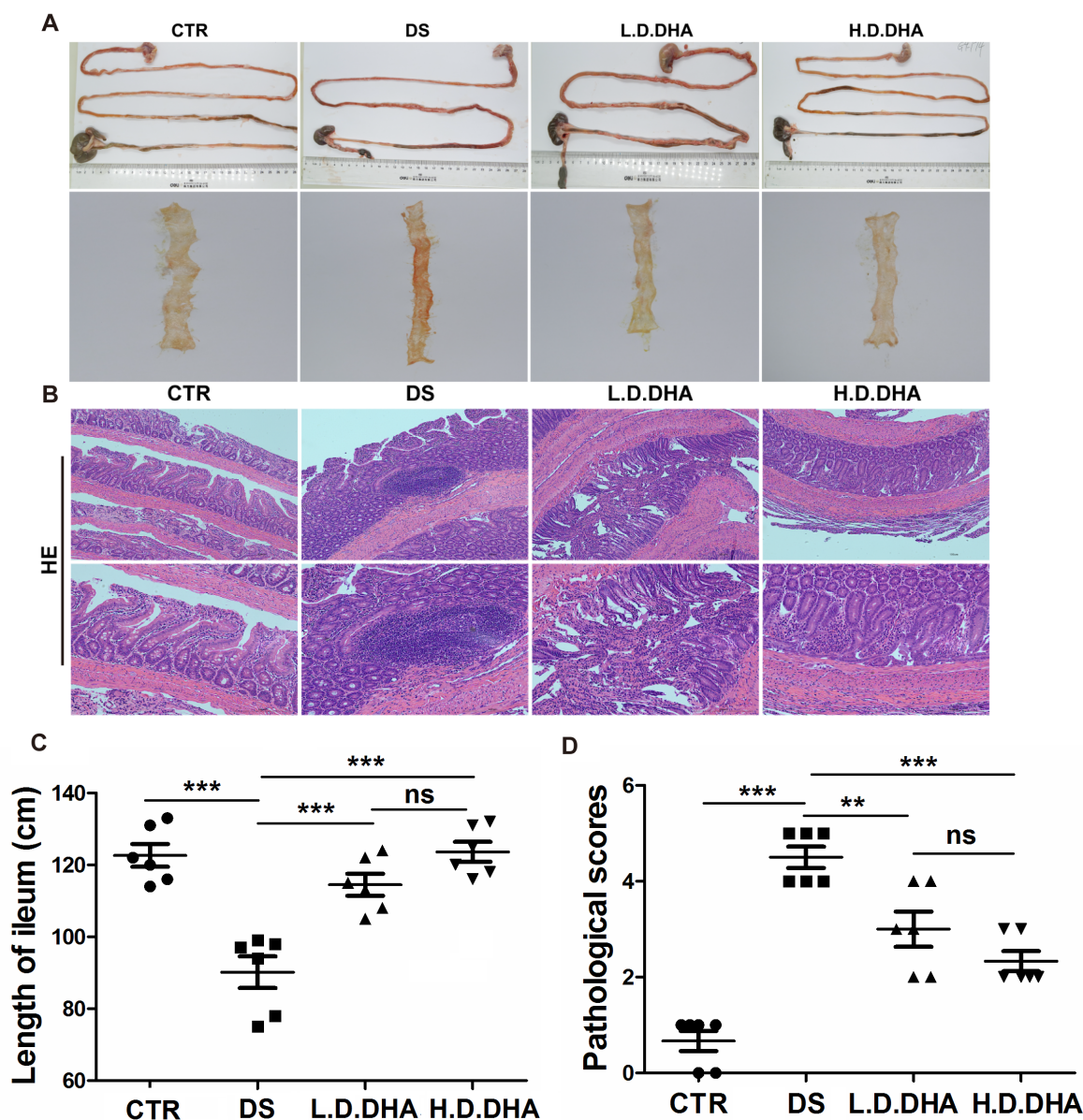


Figure 1: DHA decreased pathological inflammation scores in the DS-induced SIMI rat model. (A, C) Measure of the small bowel length and injury in each group ($n=6$). (B, D) H&E staining was used to evaluate the histopathological inflammation scores in each group ($400\times$, $n=6$). (D) The data are presented as the means $\pm$ SD of six individual rats, and one-way ANOVA for the statistical analysis between the four groups. ** $p<0.01$ or *** $p<0.001$ vs DS group; ns $p>0.05$ vs L.D.DHA group.

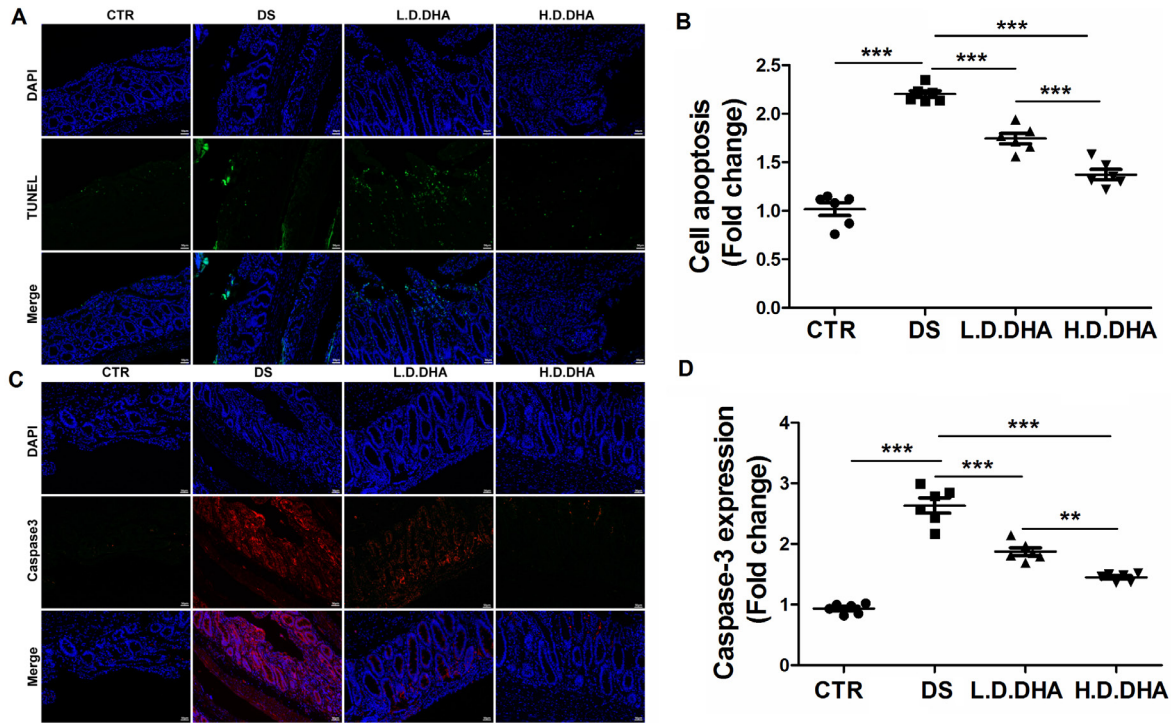


Figure 2: DHA inhibited cell apoptosis in the DS-induced SIMI rat model. (A, B) Representative immunofluorescence labeling for TUNEL assay using the frozen ileum tissue sections in each group (400×, n=6). (C, D) Representative immunofluorescence labeling for Caspase-3 in frozen ileum tissue section in each group (400×, n=6). The data are presented as the means ± SD of six individual rats and ANOVA for the statistical analysis between the four groups. ***p<0.001 vs DS group, **p<0.01 vs L.D.DHA group.

(p<0.001) in the DS-induced SIMI rat model (Figures 2C and 2D).

DHA reduced the release of inflammatory factors in the DS-induced SIMI rat model

We found that DHA suppressed the production and release of pro-inflammatory factors in the DS-induced SIMI rat model. To validate this effect, IF staining was performed to detect the expression and localization of key inflammatory mediators in ileal tissue sections. To validate this effect, IF staining was utilized to assess the expression and subcellular distribution of critical inflammatory mediators in ileal tissue sections. TNF-α, IL-6, IL-1β, and CD68 were visualized as red fluorescence, while nuclei were counterstained blue with DAPI. As opposed to the CTR-treated animals, the DS group presented remarkably elevated positive immunofluorescence intensity for TNF-α (Figure 3A), IL-6 (Figure 3B), IL-1β (Figure 3C), and CD68 (Figure 3D) (p<0.001). This pro-inflammatory phenotype was markedly reversed by treatment with both Low-Dose (L.D.) DHA (p<0.05) and High-Dose (H.D.) DHA (p<0.001) in the DS-induced SIMI rat model (Figures 3A-3D).

DHA preserved intestinal mucosal barrier integrity in the DS-induced SIMI rat model

We found that DHA maintained intestinal mucosal barrier function in the DS-induced SIMI rat model. To investigate this

protective effect, IHC and Western blot analyses were performed to determine the protein expression levels of Chromogranin A (CHGA), Mucin 2 (MUC2), Lysozyme (LYZ), Ki-67, and MYC in intestinal tissues. As illustrated in Figures 4A-4J, the protein levels of CHGA, LYZ, and MYC were upregulated in the DS group compared with the CTR group (p<0.05), but downregulated in both the Low-Dose (L.D.) DHA and High-Dose (H.D.) DHA groups relative to the DS group (p<0.05). Conversely, the protein levels of MUC2 and Ki-67 were reduced in the DS group (p<0.01), and this reduction was effectively reversed by treatment with either L.D. DHA (p<0.05) or H.D. DHA (p<0.001) in the DS-induced SIMI rat model.

DHA modulated gut microbiota composition in the DS-induced SIMI rat model

Alpha and beta diversity analyses were further carried out to characterize gut microbial alterations. Our results revealed that DHA remodelled gut microbial community architecture in rats with DS-evoked SIMI. Taxonomic assignment of 16S rRNA gene reads was conducted at both the phylum and genus taxonomic ranks. Microbial profiling revealed 13 phyla and 215 genera in the CTR group, compared with 14 phyla and 218 genera in intestinal tissues from the DS-induced SIMI group. Despite comparable taxonomic richness, marked compositional shifts were observed at the phylum level. Specifically, the dominant phyla *Firmicutes* and *Bacteroidetes* accounted for 71.89% and 22.02% of the total

microbiota in the CTR group, versus 67.20% and 24.74% in the DS group, respectively. Notably, rare phyla including *Elusimicrobia* were uniquely detected in the CTR group, whereas *Fusobacteria* was exclusively present in the DS group. Furthermore, relative to the DS group, DHA treatment significantly increased the relative abundance of *Bacteroidetes* while decreasing that of *Fusobacteria* in the DS-induced SIMI rat model (Figures 5A-5D).

At the genus level, the most abundant genera in the CTR group were *Muribaculaceae*, *Lachnospiraceae_NK4A136*, and *Lachnospiraceae* (accounting for 18.29%, 11.48%, and 5.55% of the total microbiota, respectively). In contrast, the dominant genera in the DS group were *Muribaculaceae*, *Lachnospiraceae_NK4A136* and *Lactobacillus* (representing 18.30%, 9.45%, and 7.72% of the total, respectively). Principal coordinates Analysis

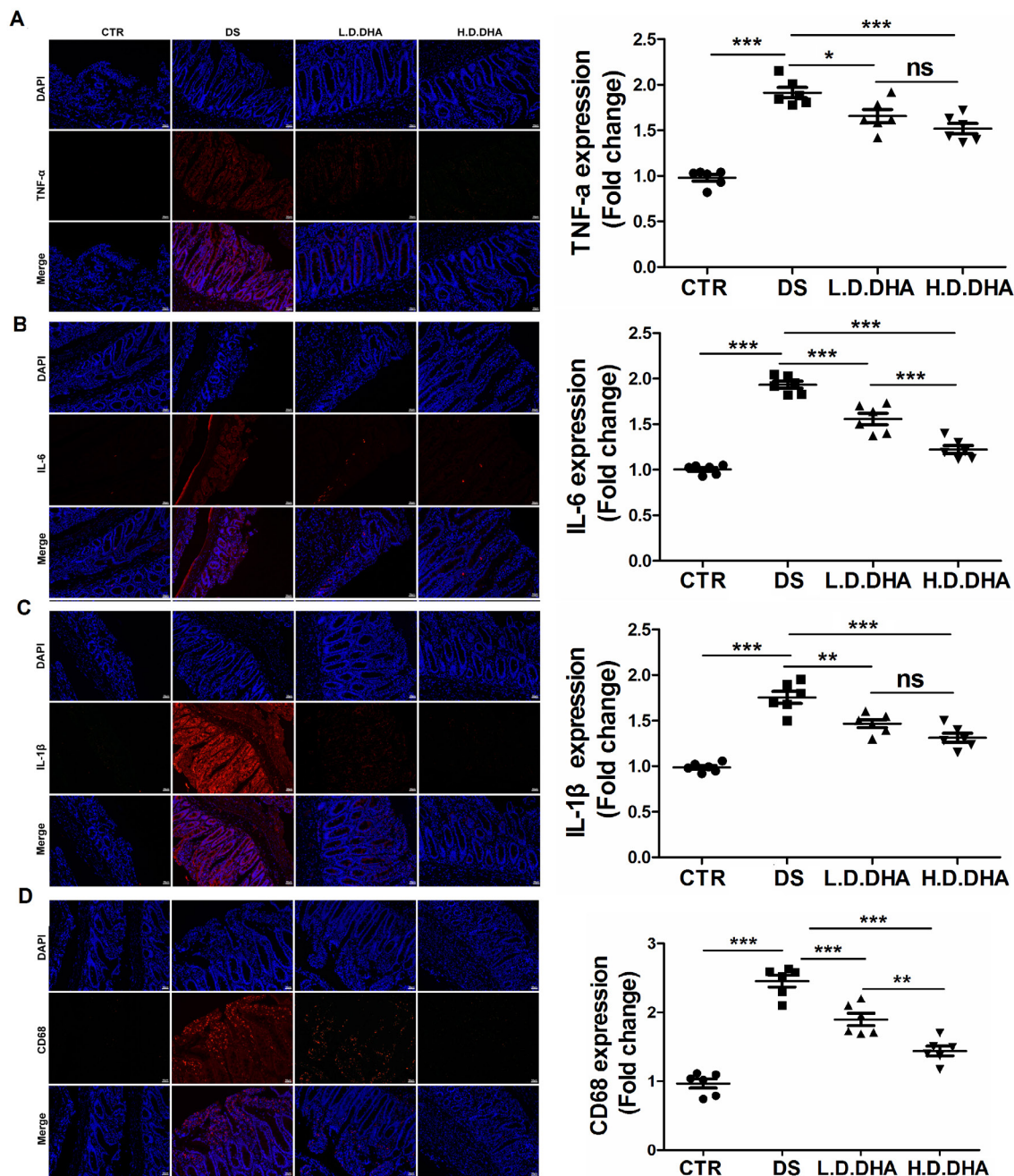


Figure 3: DHA reduced the release of inflammatory factors in the DS-induced SIMI rat model. (A) Representative immunofluorescence labeling for TNF-α in frozen ileum tissue section in each group (400×, n=6). (B) Representative immunofluorescence labeling for IL-6 in frozen ileum tissue section in each group (400×, n=6). (C) Representative immunofluorescence labeling for IL-1β in frozen ileum tissue section (400×, n=6). (D) Representative immunofluorescence labeling for CD-68 in frozen ileum tissue section in each group (400×, n=6). The data are presented as the means ± SD of six individual rats and ANOVA for the statistical analysis between the four groups. * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$ vs DS group. ns $p > 0.05$, ** $p < 0.01$ or *** $p < 0.001$ vs L.D.DHA group.

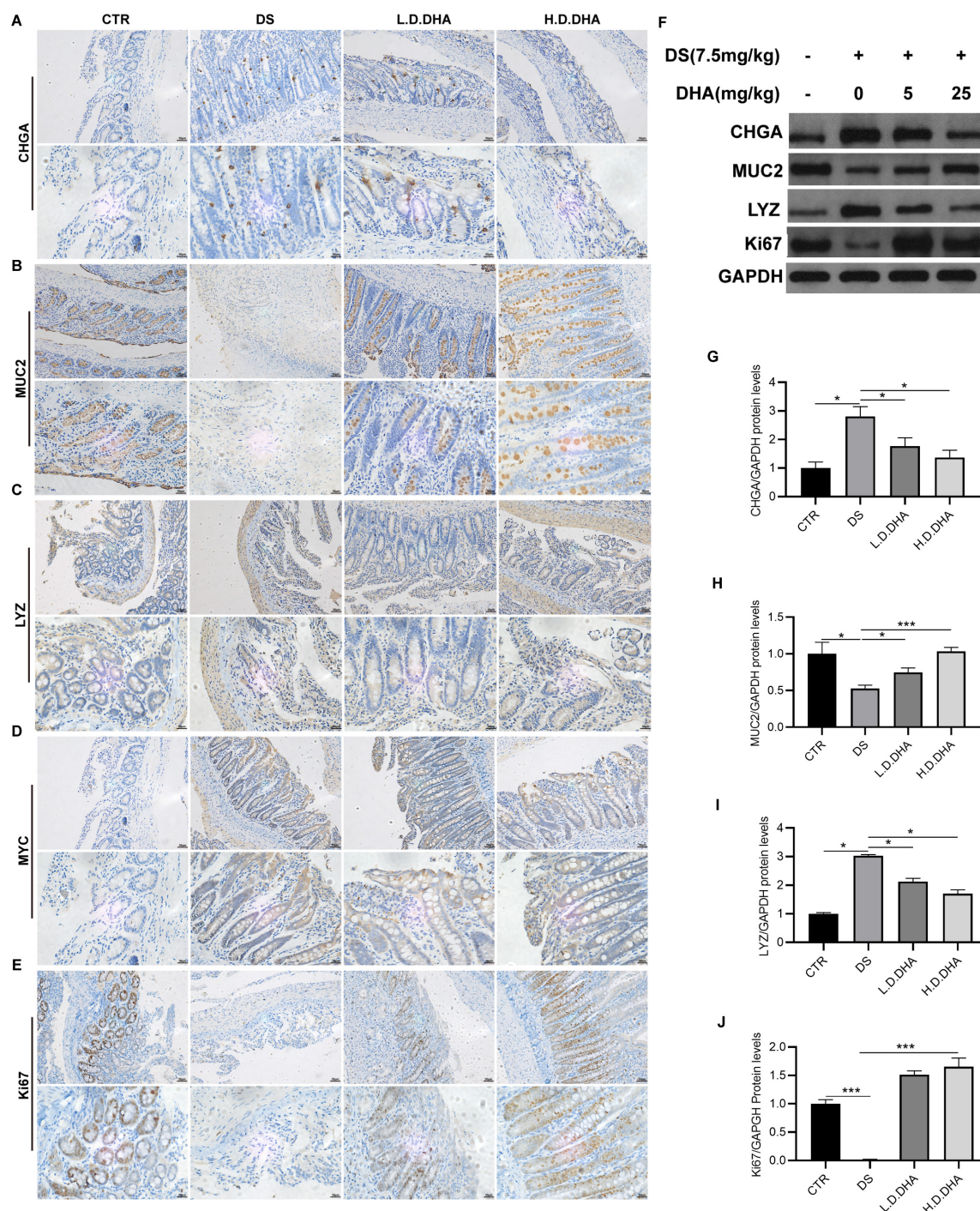


Figure 4: DHA maintained intestinal mucosal barrier in the DS-induced SIMI in rat model. (A-E) Representative photographs of IHC staining of CHGA, MUC2, LYZ, Ki67 and MYC (400x, n=6). (F-J) Western blot analysis of the protein levels of CHGA, MUC2, LYZ and Ki-67 in each group. The data are presented as the means $\pm$ SD of six individual rats, and ANOVA for the statistical analysis between the four groups. * $p < 0.05$ or *** $p < 0.001$ vs DS group.

(PCoA) revealed distinct separation of gut microbial communities among the CTR, DS, and DHA groups (Figure 5C). A total of 11 microbial biomarkers were identified with an LDA score > 3.0 , of which 6 were specific to the DS group. The taxon with the highest LDA score in the CTR group was *Clostridiales* Family XIV, while *Desulfovibrionaceae* exhibited the largest LDA value in the DS group. Specifically, unclassified *Clostridiales* Family

XIV and *Betaproteobacteriales* were enriched in the CTR group, whereas unclassified *Desulfovibrionaceae*, *Rikenellaceae*, *Alistipes*, *Enterobacteriaceae*, and *Enterobacteriales* were significantly more abundant in the DS group (Figure 5D). Furthermore, relative to the DS group, DHA treatment significantly increased the relative abundance of *Lachnospiraceae*_NK4A136, *Lactobacillus*, *Intestinimonas*, and *Bacteroidetes*, while decreasing that of

Desulfovibrionaceae in the DS-induced SIMI rat model (Figure 5B).

DHA suppressed MAPK/NF- κ B signaling activation in the DS-induced SIMI rat model

Our previous study established that the MAPK/NF- κ B signaling pathway mediates DS-induced SIMI in rats (Chen *et al.*, 2024). Here, we demonstrated that DHA inhibited the activation of the MAPK/NF- κ B pathway in this rat model. IHC analysis revealed that the protein levels of total p38, p-p38, total p65, and p-p65 were markedly elevated in the DS group compared with the control group ($p < 0.0001$). Conversely, treatment with Low-Dose (L.D.) DHA ($p < 0.001$) and High-Dose (H.D.) DHA ($p < 0.0001$) significantly attenuated the upregulation of these proteins relative to the DS group (Figures 6A-6D).

DISCUSSION

NSAIDs-induced SIMI exhibits increasing clinical prevalence and severity, underscoring the necessity for early intervention and prevention (Sostres *et al.*, 2013). Prior investigations show that commensal microbes contribute to SIMI pathogenesis following NSAID administration (Kent *et al.*, 1969; Robert & Asano, 1977; Uejima *et al.*, 1996). Paradoxically, specific microbial taxa may display the protective properties (Uejima *et al.*, 1996; Wallace

et al., 2011). *Lactobacillus* and *Bifidobacterium* can repress the proliferation of gram-negative bacteria (*Eubacterium limosum*, *Escherichia coli*) in ileal ulcer models (Robert & Asano, 1977). Adaptive microbial remodeling may mitigate the pathogenic processes in SIMI. But some studies report that nondigestible stachyose alleviates SIMI in mice via tuning intestinal exosomal miRNAs, independent of gut microbiota (Hao *et al.*, 2025). Our findings indicated that DHA modulated gut microbiota composition and alleviated DS-induced SIMI in the rat model. DHA increased the enrichment levels of *Bacteroidetes* but decreased those of *Fusobacteria* in DS-induced SIMI rat model. Supplementing *Bacteroidetes* combined with DHA may provide a new treatment strategy for SIMI in clinical practice.

Chromogranin-A (CHGA), a pro-hormone secreted by enterochromaffin (EC) cells in the gastrointestinal tract, modulates gut physiology. Elevated CHGA expression correlates with inflammation and autoimmunity (Maj *et al.*, 2025). In this study, we found that DHA administration reduced CHGA protein levels and attenuated inflammatory responses. MUC2 enhances gut homeostasis through dendritic cell modulation and suppression of inflammatory signaling (Shan *et al.*, 2013). We observed that DHA treatment significantly upregulated ileal MUC2 production. LYZ ameliorates intestinal epithelial injury and systemic inflammation, as demonstrated in acute pancreatitis

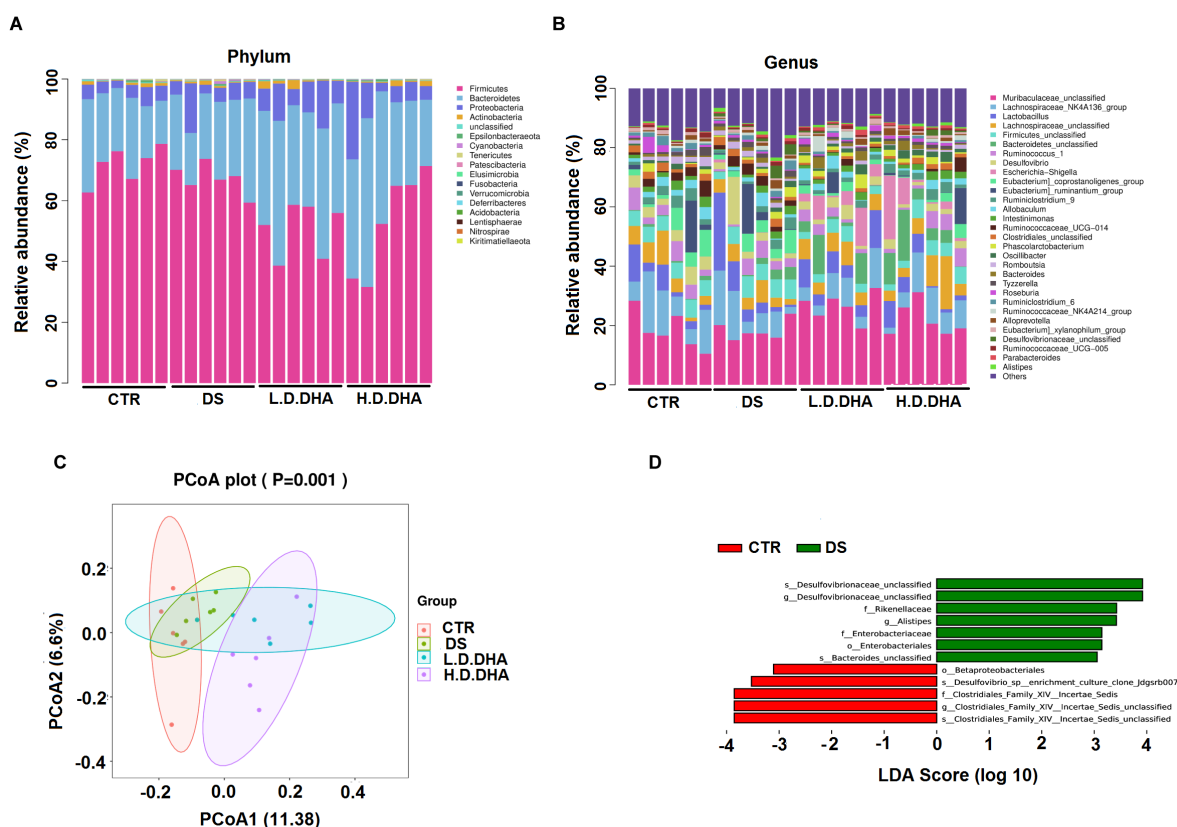


Figure 5: DHA altered the distribution of gut microbiota in the DS-induced SIMI rat model. (A) The composition and relative abundance of dominant microbes at the phylum level in each group. (B) The composition and relative abundance of dominant microbes at the genus level in each group. (C) Principal-coordinate analysis of the microbial communities in each group. (D) The functional characterization of the enriched microbes in each group.

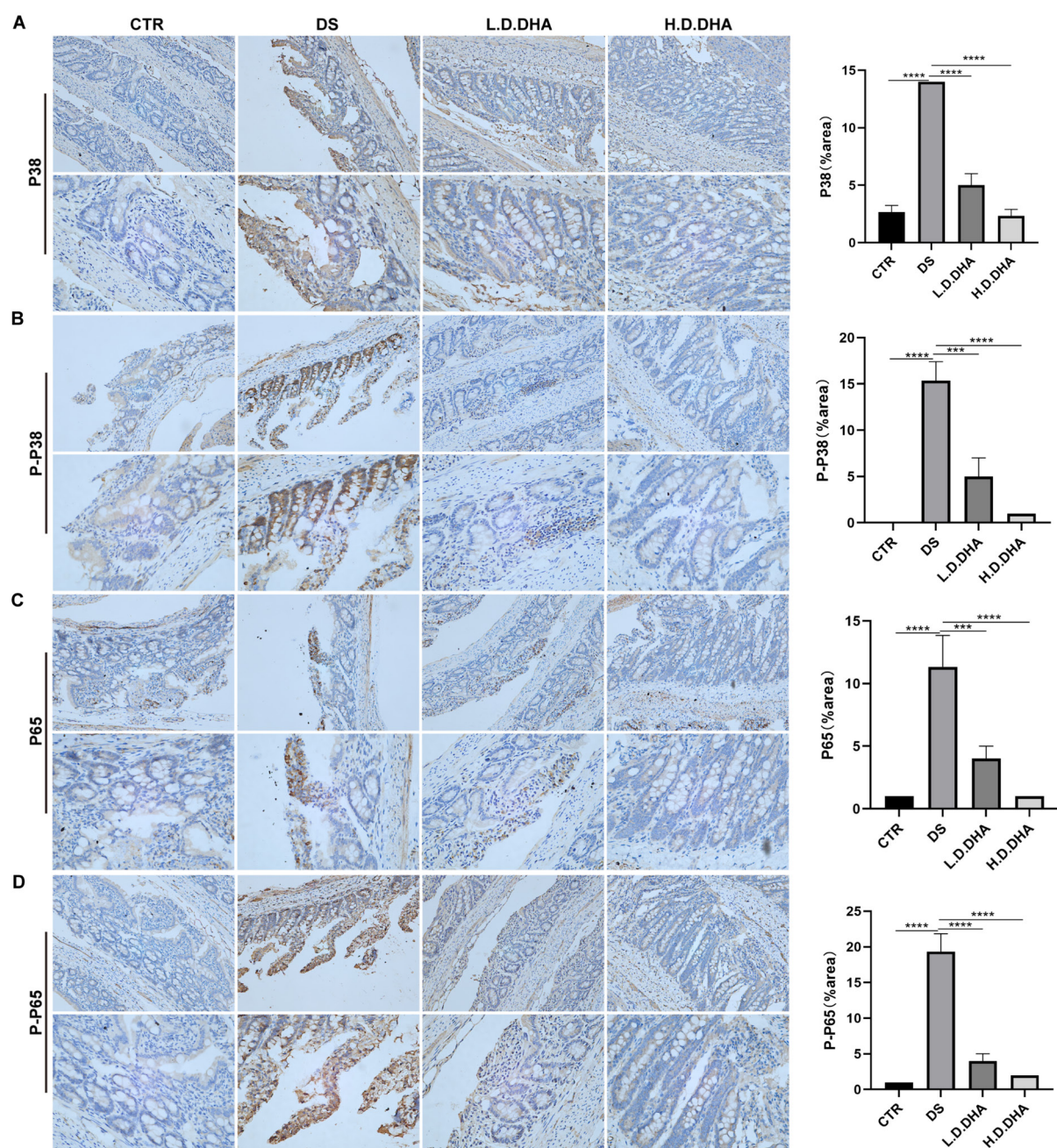


Figure 6: DHA suppressed the activation of MAPK/NF- κ B signaling in the DS-induced SIMI rat model. IHC analysis of the protein levels of (A) MAPK p38, (B) MAPK p-p38, (C) NF- κ B p65 and (D) NF- κ B p-p65 in the ileum tissues in each group (400 $\times$, $n=6$). The data are presented as the means $\pm$ SD of six individual rats, and ANOVA for the statistical analysis between the four groups. *** $p<0.001$ or **** $p<0.0001$ vs DS group.

models (Fu *et al.*, 2022). Our data indicated DHA treatment downregulated LYZ expression and reduced ileal inflammation. Reduced Ki-67 staining in intestinal crypt cells correlates with impaired mucosal repair (Cheng *et al.*, 2021). In this study, Ki-67 protein expression increased dose-dependently followed by DHA treatment. MYC regulates eukaryotic cell cycle progression through its encoded transcription factor, which controls cell proliferation, differentiation, and apoptosis. We found MYC protein expression exhibited dose-dependent reduction followed by DHA administration. Other studies show that Fexuprazan promoted mucosal barrier restoration by safeguarding mucin

synthesis and maintaining tight junction protein (ZO-1 and claudin-1) levels (Lee *et al.*, 2025), while we emphasized the protective effect of DHA via downregulation of CHGA, LYZ and MYC and upregulation of MUC2 and Ki-67 in SIMI models.

NF- κ B activation elicits proinflammatory cytokine release, while cytokines reciprocally activate NF- κ B, establishing an amplification loop for inflammatory signaling (Pan *et al.*, 2025). MAPK represents a canonical NF- κ B activation pathway, and MAPK/NF- κ B axis activation promoting TNF- α , IL-1 β , and IL-6 production (Niu *et al.*, 2025). DHA demonstrates

anti-inflammatory properties relevant to DS-induced injury (Jiang *et al.*, 2021), involved in monocyte/macrophage activation and pro-inflammatory mediator release (IL-1 β , TNF- α , IL-6, CD68). DHA also alleviates sepsis-associated encephalopathy via HIF1A/HMOX1 pathway (Gong *et al.*, 2025). But, in our study, MAPK/NF- κ B pathway was found to be inactivated, and the release of TNF- α , IL-1 β , IL-6 and CD68 was reduced in DHA-treated SIMI rats. Thus, DHA combatted cytokine release, reduced apoptosis of intestinal tissue cells, and prevented small bowel injury in rats.

Nevertheless, several caveats should be noted for this study. First, we exclusively examined MAPK/NF- κ B signaling activity during the protective process of DHA against DS-induced SIMI. Although this pathway is critically involved, we cannot exclude the potential contribution of other signaling cascades that may also participate in the pathogenesis and intervention of SIMI. Second, the protective effects of DHA were only verified in preclinical animal models; clinical trials in human patients with NSAID-induced intestinal injury are still lacking, which restricts the translational potential and generalizability of our findings. Third, the precise mechanism by which DHA-mediated alterations in gut microbiota contribute to intestinal protection remains not fully elucidated. Further studies combining microbiota transplantation and multi-omics analyses are warranted to clarify the causal relationship. Fourth, this study focused on a single molecule with an acute treatment regimen, lacking comparisons with standard clinical interventions such as probiotics or conventional anti-inflammatory agents. Issues including optimal dosage, pharmaceutical formulation, long-term safety profiles, and the translational relevance of rat models to human SIMI also need to be carefully addressed in future investigations. In addition, formal sample size calculation was not performed in the current study, and the relatively small number of animals may introduce potential statistical bias and affect the reliability and generalizability of the results (Zhang & Hartmann, 2023).

Based on our findings that DHA may protect against NSAID-induced SIMI via modulation of MAPK/NF- κ B pathway and gut microbiota, future research should clarify crosstalk between the MAPK/NF- κ B pathway and other cascades, and confirm the causal link between gut microbiota alterations and DHA's protective effect using FMT and multi-omics analyses. Next, phase I/II clinical trials are needed to validate DHA's safety and efficacy in humans, along with pharmacokinetic studies to optimize its formulation.

CONCLUSION

Our findings demonstrated that DHA may exert protective effects against DS-induced SIMI via the MAPK/NF- κ B signaling pathway and modulation of gut microbiota. These results not only elucidate the molecular mechanism underlying DHA-mediated intestinal protection but also highlight its potential as a

preventive or therapeutic agent against NSAID-related SIMI, thereby offering important implications for future mechanistic studies, preclinical development, and clinical management of NSAID-induced intestinal complications.

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ABBREVIATIONS

DHA: Dihydroartemisinin; **NSAIDs:** Non-Steroidal Anti-Inflammatory Drugs; **DS:** Diclofenac Sodium; **SIMI:** Small Intestinal Mucosal Injury; **H&E:** Hematoxylin and Eosin; **MAPK:** Mitogen-Activated Protein Kinase; **IBD:** Inflammatory Bowel Disease; **SD:** Sprague-Dawley; **CTR:** Control; **IF:** Immunofluorescence; **IHC:** Immunohistochemistry; **CHGA:** Chromogranin-A; **TUNEL:** Terminal deoxynucleotidyl transferase dUTP Nick End Labeling; **MUC2:** Mucin 2; **ANOVA:** Analysis Of Variance; **PCoA:** Principal Coordinates Analysis; **LefSe:** Linear discriminant analysis Effect Size; **LDA:** Linear Discriminant Analysis; **OUT:** Operational Taxonomic Unit; **QIIME:** Quantitative Insights Into Microbial Ecology; **DAB:** 3,3'-Diaminobenzidine.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTIONS

Experimental design was proposed by HC, RH and LY. The practical work was performed by CW, BS and CX. Data collection and analysis were conducted by JZ. All authors were responsible for reformatting, drafting, and preparing the manuscript. Lastly, RH was responsible for manuscript preparation and submission. All authors read and approved the final manuscript.

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ETHICAL APPROVAL

The animal study was reviewed and approved by the Ethics Committee in Animal Experiments of Shanghai Sixth People's Hospital (No. 2021-0038).

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