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ORIGINAL RESEARCH

Enteric-Coated Aspirin Induces Small Intestinal Injury via the Nrf2/Gpx4 Pathway: A Promising Model for Chronic Enteropathy

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Background: Aspirin is widely used to prevent and treat cardiovascular diseases. The most common side effect is gastrointestinal damage. In recent years, aspirin-associated enteropathy has received increasing attention. This study aimed to establish a chronic model of aspirin-associated enteropathy, investigate the effect of enteric-coated aspirin on the intestinal flora, and explore the specific molecular mechanism of small intestinal damage.

Methods: C57BL/6J mice were given aspirin for 45 days to induce chronic small intestinal injury. The intestinal mucosal injury was observed macroscopically and microscopically. Intestinal mucus levels were assessed by PAS staining. The intestinal permeability was measured by FD4. The oxidative stress levels of the small intestine were detected by immunofluorescence and immunohistochemistry. The mRNA and protein levels of inflammatory factors, tight junctions, and antioxidant defense-related genes were measured by qRT-PCR and Western Blot. The MPO activity, SOD activity and MDA content in serum were measured. The mitochondrial morphology and paracellular space were observed under transmission electron microscopy. The fecal samples were analyzed by high-throughput sequencing of 16S rRNA V3-V4 amplicons.

Results: Aspirin induced weight loss, reduced food intake and increased faecal occult blood in mice. Aspirin led to a shortened small intestine, macroscopic and microscopic damage to the intestinal mucosa, and local inflammation. Aspirin disrupted the intestinal barriers and increased the permeability of the small intestine. Aspirin destroyed mitochondrial structure and damaged antioxidant capacity, and aspirin may induce oxidative stress through Nrf2/Gpx4 signaling pathway. Intestinal flora analysis showed that aspirin could induce changes in the abundance of Akkermansia and Lactobacillus.

Conclusion: Long-term administration of enteric-coated aspirin successfully established a chronic small intestinal injury model in mice. It increased oxidative stress in the small intestine by disrupting mitochondrial structure and impairing antioxidant capacity. This damaged the intestinal mucosal barrier, increased intestinal permeability, and triggered gut microbial dysbiosis and inflammation.

Keywords: aspirin, oxidative stress, ferroptosis, mucosal barrier disruption, enteritis

Introduction

Aspirin is an anti-platelet aggregation drug widely and chronically used to treat cardiovascular and cerebrovascular diseases.[1] Approximately 18.9% of the Chinese on long-term oral aspirin had recurrent lower gastrointestinal bleeding within 5 years.[2] Aspirin-associated enteropathy manifests as mucosal erythema, erosion, ulceration, and ring constriction circle formation, which can be complicated by bleeding and obstruction.[3] Using enteric-coated aspirin as an independent risk factor adds to the damage to the small intestinal mucosa.[4] The lower gastrointestinal side effects of aspirin have become an urgent clinical problem.

Previous studies on NSAID-associated enteropathy have primarily focused on indomethacin and diclofenac.[5] The conventional oral application of aspirin results in rapid absorption in the stomach and duodenum, with damage occurring mainly in the upper gastrointestinal tract, without causing severe damage to the small intestine.[6] However, capsule

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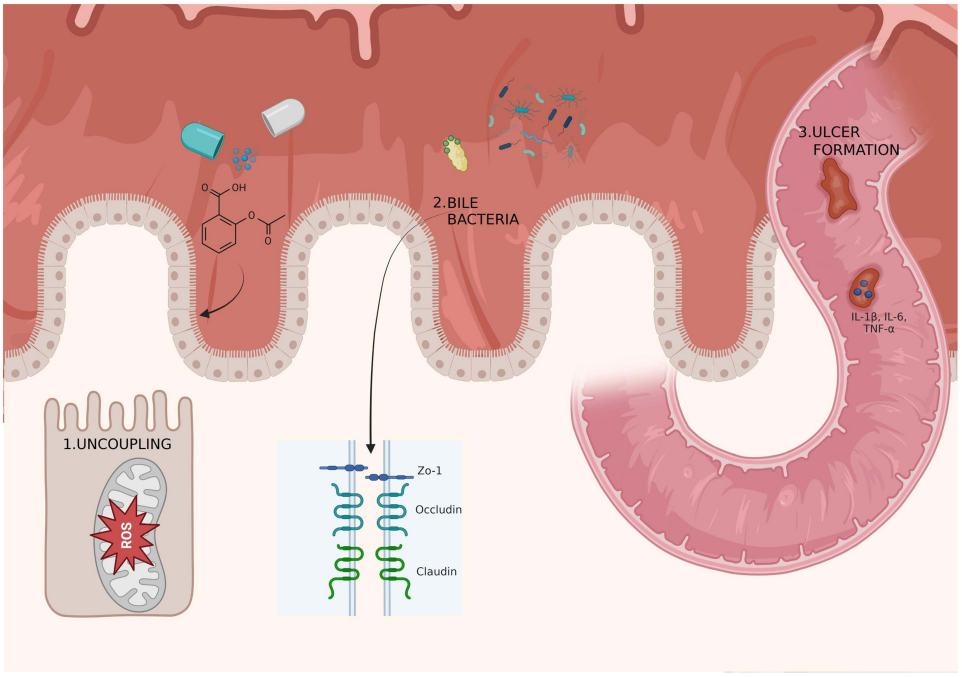
Graphical Abstract



endoscopy does reveal small bowel erosion and ulceration in chronic low-dose aspirin users.[4] We are establishing a novel model of aspirin-associated enteropathy to induce chronic small intestinal mucosal damage through long-term administration, simulating prolonged drug use in humans.

There is a very classical three-hit theory about NSAID-induced intestinal damage. First, direct contact between NSAIDs and intestinal mucosa damages the mitochondria of intestinal epithelial cells, generates large amounts of oxygen radicals, and reduces ATP. Then, the tight junctions between cells are disrupted and intestinal permeability increases. Eventually, bile, proteolytic enzymes, and bacteria in the intestinal lumen penetrate the mucosal barrier, resulting in local inflammation.[7] We will follow this theory step by step to verify whether the aspirin-induced enteropathy model is successfully established.

Mitochondrial oxidative phosphorylation uncoupling and oxidative stress are the essential pathological features in NSAID-associated enteropathy.[8] NSAIDs uncouple or inhibit oxidative phosphorylation to induce drug toxicity in the small intestine.[9] They cause mitochondrial damage by uncoupling oxidative phosphorylation, increasing reactive oxygen species (ROS), and decreasing ATP production, thereby decreasing cell viability.[10,11] NSAIDs have been shown to damage mitochondrial ultrastructure in vivo and in vitro.[6] This impairment of mitochondrial structure and function is thought to be necessary for the development of NSAID-associated enteropathy.

Increased epithelial permeability is recognized as an early event in aspirin-induced small intestinal mucosal damage. Aspirin elevates reactive oxygen species (ROS), particularly mitochondrial superoxide anion levels, which lead to cysteine oxidation and the downregulation of the tight junction ZO-1, ultimately compromising epithelial cell integrity.[12] Furthermore, secondary factors such as bile acids, bacteria, food components, or other unidentified agents may exacerbate mucosal damage.[13] These findings suggest that aspirin increases intestinal permeability via superoxide-mediated tight junction disruption, triggering localized intestinal inflammation and providing a key pathological mechanism underlying the development of enteropathy.

Ferroptosis is different from apoptosis, necrosis and other well-characterized types of regulated cell death. It is characterized by iron-dependent lipid peroxidation reactions.[14] Ferroptosis plays a crucial role in various pathological

conditions, including cancer and inflammatory diseases. Preliminary studies have suggested that aspirin may contribute to ferroptosis in tumors through multiple mechanisms.[15,16] Recent reports have also underscored the association between ferroptosis and digestive diseases, such as intestinal ischemia-reperfusion injury and inflammatory bowel disease.[17] However, the potential link between aspirin-associated enteropathy and ferroptosis remains to be explored.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor, and many ferroptosis-related genes are transcriptionally regulated by Nrf2. It plays a crucial role in the cellular response to oxidative stress.[18] Glutathione peroxidase 4 (GPX4) is an important downstream target gene of Nrf2. GPX4 is a selenoprotein that uniquely reduces hydroperoxides in membrane lipids. This ability to reduce phospholipid hydroperoxides, thereby inhibiting lipoxygenase-mediated lipid peroxidation, underscores its critical role in preventing ferroptosis.[19] An essential role for GPX4 in controlling intestinal homeostasis in intestinal epithelial cells has been demonstrated.[20] However, the role of Nrf2/GPX4 in aspirin-associated enteropathy remains poorly understood.

Based on the above background, our study will follow the three-hit hypothesis to verify whether the model is successfully established at three levels: organism injury and local inflammation, intestinal barrier disruption and intestinal permeability alteration, and mitochondrial damage and oxidative stress. This project will further explore the effect of enteric-coated aspirin on intestinal flora and the molecular mechanism of aspirin-related enteropathy. We hope to provide reliable theoretical evidence and possible molecular targets for treating aspirin-related enteropathy.

Materials and Methods

Ethics Statement

The C57BL/6J mice (male, 6–8 weeks old, 24.20±0.86g) in this study were obtained from Tongji Hospital Experimental Animal Center. Under the national guidelines for the care and use of laboratory animals, our study was authorized by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Animal Ethics No. TJH-202109006. No accidental death of mice occurred during the whole experiment. All mice were injected intraperitoneally with 1% sodium pentobarbital before execution to ease the pain.

Chronic Injury to the Small Intestine Induced by Long-Term Administration of Aspirin

We chose enteric-coated aspirin (Yongxin Pharmaceutical Industry (Kunshan) Co., Ltd., batch number: 10N035), and administered it directly via gavage in microgranules, without grinding the drug into powder or dissolving it in any vehicle. All mice were divided into three groups after one week of acclimatization, the control group was given the enteric-coated component of the drug, polyacrylate resin II, the low-dose group was given aspirin 150 mg/kg/d, and the high-dose group was given aspirin 300 mg/kg/d, all administered continuously for 45 days.

Gross Pathology and Histopathology

Body weight, food intake, activity, and stool condition of the three groups of mice were observed and recorded daily. Following euthanasia, the entire gastrointestinal tract was extracted from the mice, and the lengths of the small intestine, colon, and total intestine were measured and documented. The small intestine was incised longitudinally along the contralateral side of the mesentery to observe the damage on the inner surface of the intestinal lumen. We measured the long and short axes of mucosal damage with digital precision calipers, multiplied the long and short axes and summed them for each small intestine as the lesion index. All animal tissues were fixed in 10% formalin and routinely processed into paraffin sections within 24 hours. The specimens were stained with hematoxylin and eosin and observed with a white light microscope. The ileum of each mouse was evaluated in a range of 10 villous crypt units. The average of the scores in each range was used as the final score of the specimen, and the intestinal mucosal damage was calculated using Chiu's score.[21] The damage grading system is as follows: Grade 0 represents normal mucosal villi. Grade 1 is characterized by the development of subepithelial Gruenhagen's space, usually at the apex of the villus, often accompanied by capillary congestion. Grade 2 involves the extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria. Grade 3 shows massive epithelial lifting down the sides of the villi, with a few tips possibly being denuded. Grade 4 is marked by denuded villi with exposed lamina propria and dilated capillaries, and an increase in

cellularity of the lamina propria may be observed. Finally, Grade 5 indicates digestion and disintegration of the lamina propria, along with hemorrhage and ulceration.

In vivo Intestinal Permeability Test

FITC-dextran 4 (FD4) refers to a fluorescently labeled dextran with a molecular weight of 4 kDa. It cannot naturally cross the intact intestinal epithelial barrier and is commonly used to assess changes in intestinal barrier function or permeability. An incision along the midline of the abdomen exposed the abdominal cavity. The target intestinal segment was spread in a fan shape outside the abdomen without damaging the mesenteric vessels by positioning it 3 cm and 8 cm from the ileocecal region to the side of the mouth. The segment was cut at 2.5 cm proximal to the ileocecal region. The intestine was flushed two to three times with PBS after a warm water bath (37°C), and the intestinal fluid was drained from the broken segment as much as possible. A silk ligature was taken at 3 cm and 8 cm proximal to the blind end, and 250 μL FD4 solution (MKBio, Shanghai, China) (0.25 mg/10 g) was injected into the 5 cm long target intestinal segment. After 30 min of light avoidance, the right atrium was punctured to extract blood; the target intestinal segment was cut off, and its contents were taken. The blood and intestinal fluid were centrifuged (3500 rpm, 10 min), and the supernatant of the blood and intestinal fluid specimens were taken. The FD4 fluorescence value (excitation wavelength 485nm, emission wavelength 528 nm) was measured by a multifunctional enzyme marker (BioTek, USA), and the FD4 blood/intestinal fluid fluorescence value was used to show the permeability.

Periodic Acid-Schiff Staining

We performed PAS staining to observe changes in the intestinal mucus layer and goblet cells. Fresh small intestinal tissues were immediately fixed in Carnoy's solution (Servicebio, Wuhan, China) for 3 h. The fixed small intestinal tissues were embedded in paraffin and sectioned. The sections were dewaxed and rehydrated, acidified with periodate, washed with pure water, stained with Schiff's solution (Servicebio, Wuhan, China) under light-proof conditions, rinsed in running water for 5 min, dehydrated, and sealed. The number of goblet cells and changes in the mucus layer was analyzed using a white light microscope (Olympus, Japan).

Immunohistochemistry

Tissue sections were dewaxed and hydrated, antigen repaired using EDTA Antigen Repair Solution (BIOSSCI, Hubei, China), endogenous peroxidase blocked by 3% H2O2, blocked using goat serum (BOSTER, Wuhan, China) for 1 h at room temperature, then incubated overnight at 4°C using primary antibody against 4-HNE (#bs-6313R, Bioss, China), followed by 1 h at room temperature using a secondary antibody (#AS014, Abclonal, China), dropwise with DAB working solution (BIOSSCI, Hubei, China) for colour development. Nuclei were then stained with hematoxylin and dehydrated before being observed and photographed under a white light microscope (Olympus, Japan).

Immunofluorescence

Paraffin sections were dewaxed and hydrated, and antigens were retrieved in EDTA antigen repair solution (BIOSSCI, Hubei, China) and then cooled to room temperature. 3% H2O2 blocked endogenous peroxidase, and samples were closed with goat serum (BOSTER, Wuhan, China) at room temperature for 1 h. Tissue sections were incubated overnight at 4°C with ZO-1 specific primary antibody (#61-7300, Thermo Fisher, USA). Tissue sections were washed three times with TBST buffer, incubated with fluorescent secondary antibody (#AS053, Abclonal, China) for 1 hour, and stained with DAPI for cell nuclei. Samples were photographed using a fluorescence microscope (Olympus, Japan).

Determination of Oxidative Stress Levels

Blood samples were centrifuged at 3500 rpm for 10 min, and the supernatant was used for the subsequent assay. The determination of SOD (Njjcbio, Nanjing, China) often accompanied the MDA (Njjcbio, Nanjing, China) measurement, and the levels of SOD and MDA were determined according to the method of the assay kit.

Electron Microscopic Observation of Cell Interstitial and Mitochondrial Morphology

Small intestine samples were immersed in 2.5% glutaraldehyde at 4 °C. Small intestine samples were rinsed 3 times by PBS, then fixed with osmium acid for 30 min, rinsed once more by PBS, dehydrated with a gradient ethanol solution, 1:1 acetone plus epoxy resin at 40°C for 2 h, epoxy resin at 40°C for 3.5 h. After embedding the sections, all small intestine samples were observed and photographed by transmission electron microscopy (HITACHI, HT7800, Japan).

Quantitative Real-Time PCR (qRT-PCR)

RNA was extracted from small intestinal tissues using Trizol lysate (Takara, Japan) and reverse transcribed into cDNA using 5× ABScript III RT Mix (Abclonal, Wuhan, China). For qRT-PCR analysis, the reaction mixture contained a cDNA template, upstream and downstream primers (diluted to 10 μM) and SYBR qPCR Master Mix (Vazyme, Nanjing, China). The reaction conditions of the PCR instrument were set: pre-denaturation phase, 95°C, time 30s, cycle number 1; cyclic reaction phase, 95°C, time 10s, 60°C, time 30s, cycle number 40; melting curve phase, 95°C, time 15s, 60°C, time 1 min, 95°C, time 15s, cycle number 1. 2-ΔΔCt was used to calculate the relative expression, and the primer sequences involved are detailed in Table 1.

Western Blot

Small intestine tissue was lysed using RIPA lysis buffer (Servicebio, Wuhan, China), and the samples were added to the loading buffer (NCMBio, Suzhou, China) and incubated for 10 minutes at 100°C. The sample proteins were separated by electrophoresis through a 10% protein gel, transferred to PVDF membranes (Millipore, USA) and incubated for 1.5 hours at room temperature using 5% skim milk. The samples were incubated with anti-ZO-1 (#61-7300, Thermo Fisher, USA) Anti-Occludin (#ab216327, Abcam, UK), Anti-Claudin-1 (13,050-1-AP, Proteintech, China), Anti-Nrf2 (#WL02135, Wanleibio, China), Anti-Slc7a11 (#A2413, ABclonal, China), Anti-Gpx4 (#A11243, ABclonal, China), Anti-HO-1 (#WL02400, Wanleibio, China), anti-β-actin (#60008-1-Ig, Proteintech, China) primary antibody overnight at 4°C, followed by incubation of the samples with HRP-labeled secondary antibody (#AS014/ #AS003, Abclonal, China). Protein samples were visualized using an ultrasensitive chemiluminescence detection kit (Epizyme, Shanghai, China) and photographed using an automated exposure machine (Tanon, Shanghai, China).

Table 1 Primer sequences used for this study

Targeted Genes	Forward Primer Sequences (5′-3′)	Reverse Primer Sequences (3′-5′)
cox1	GATTGTACTCGCACGGGCTAC	GGATAAGGTTGGACCGCACT
cox2	TGCACTATGGTTACAAAAGCTGG	TCAGGAAGCTCCTTATTTCCCTT
IL-1	TGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGATT
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
TNF-α	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG
ZO-1	TTTCAGAGTGGGGAAACCTCC	CACTCTTCCTTAGCTGCTGAAC
Occludin	TTGAAAGTCCACCTCCTTACAGA	CCGGATAAAAAGAGTACGCTGG
Claudin-1	AGCACCGGGCAGATACAGT	GCCAATTACCATCAAGGCTCG
CAT	AGCGACCAGATGAAGCAGTG	TCCGCTCTCTGTCAAAGTGTG
Sod2	CAGACCTGCCTTACGACTATGG	CTCGGTGGCGTTGAGATTGTT
Gpx1	AGTCCACCGTGTATGCCTTCT	GAGACGCGACATTCTCAATGA
Gpx4	TGTGCATCCCGCGATGATT	CCCTGTACTTATCCAGGCAGA
Nrf2	TAGATGACCATGAGTCGCTTGC	GCCAAACTTGCTCCATGTCC
Slc7a11	AGGGCATACTCCAGAACACG	GGACCAAAGACCTCCAGAATG
HO-1	AGGTACACATCCAAGCCGAGA	CATCACCAGCTTAAAGCCTTCT
β-actin	GTGACGTTGACATCCGTAAAGA	GCCGGACTCATCGTACTCC

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16S rRNA

After perianal disinfection, mice were induced to defecate by abdominal massage. Then, 4–6 capsules of faeces were collected with sterile forceps, stored in sterile cryopreservation tubes, placed in liquid nitrogen, and transferred to a −80°C refrigerator for storage after 30 minutes for subsequent 16S rRNA analysis (Anachro, Wuhan, China).

Statistical Analysis

All data were analyzed by GraphPad Prism 8 and expressed as mean ± standard deviation. Comparisons between group means were assessed by two-tailed Student's *t*-test or one-way analysis of variance (ANOVA). P values < 0.05 were considered statistically significant.

Results

Long-Term Aspirin Administration Caused Macroscopic Damage to the Small Intestine in Mice

The treatment of animals was carried out according to the timeline shown below (Figure 1A). On day 45 of animal modeling, we assessed aspirin-induced intestinal bleeding using a fecal occult blood (FOB) kit (Figure 1B). In either the low-dose or high-dose group, FOB levels were significantly higher in mice administered aspirin than in mice in the control group. Weakly positive FOB was detected in the absence of aspirin administration. The mechanical damage to the oesophagus by gavage could account for this. Long-term aspirin administration resulted in weight loss in a dose-dependent manner (Figure 1C). Similarly, the daily food intake of mice was reduced in a dose-dependent manner (Figure 1D). At the gross histological level, a significant reduction in intestinal length was observed after 45 days of aspirin administration (Figure 1E). We measured the length of different segments of the intestine, showing that long-term aspirin administration significantly shortened the length of the small intestine. Nevertheless, there was no significant effect on the length of the colon (Figure 1F). Long-term aspirin administration (150 mg/kg/d, 300 mg/kg/d) under non-fasting conditions caused severe damage in the small intestine, with visible scattered erosions and ulcers (Figure 1G) and lesion index of intestinal mucosa increased dose-dependent. There was a negative correlation (Figure 1H) between the two values of lesion index and small intestine length in mice ($y = -1.221*x + 45.17$, $r^2 = 0.5357$; $P = 0.0019$), where y is the lesion index (mm^2) and x is the length of the small intestine (cm).

Long-Term Aspirin Administration Induced Microscopic Injury to the Small Intestine in Mice

Under the white light microscope, the control group showed normal intestinal mucosa and villi morphology. In contrast, the small intestinal villi in the low-dose group were significantly shortened, the tips of the villi were visibly exfoliated, and the intestinal mucosa in the high-dose group was severely damaged and showed local ulcer foci (Figure 2A). We assessed intestinal mucosal damage with Chiu's score, which increased in a dose-dependent manner (Figure 2B). According to the scale, we applied Image J software to measure the length of small intestinal villi and the depth of the intestinal crypts in different subgroups, and long-term aspirin administration resulted in significant shortening of the intestinal villi (Figure 2C). Compared with the control group, the depth of the intestinal crypts increased significantly in the low-dose group, while the depth of the crypts tended to decrease in the high-dose group (Figure 2D). We also performed HE staining of the stomach, colon, and liver from different groups of mice. Long-term administration of enteric-coated aspirin had no significant effect on mice's stomach, colon or liver (Figure S1A). In both the low and high-dose groups, cox1 and cox2 were significantly downregulated, and we verified that aspirin exerted its pharmacological effect of cyclooxygenase inhibition (Figure 2E). In the small intestine, mRNA levels of pro-inflammatory cytokines IL-1, IL-6 and TNF-α were significantly increased in the administered group (Figure 2F). MPO activity in mice serum, a marker of neutrophil activation, was significantly increased only in the high-dose group after aspirin treatment (Figure 2G).

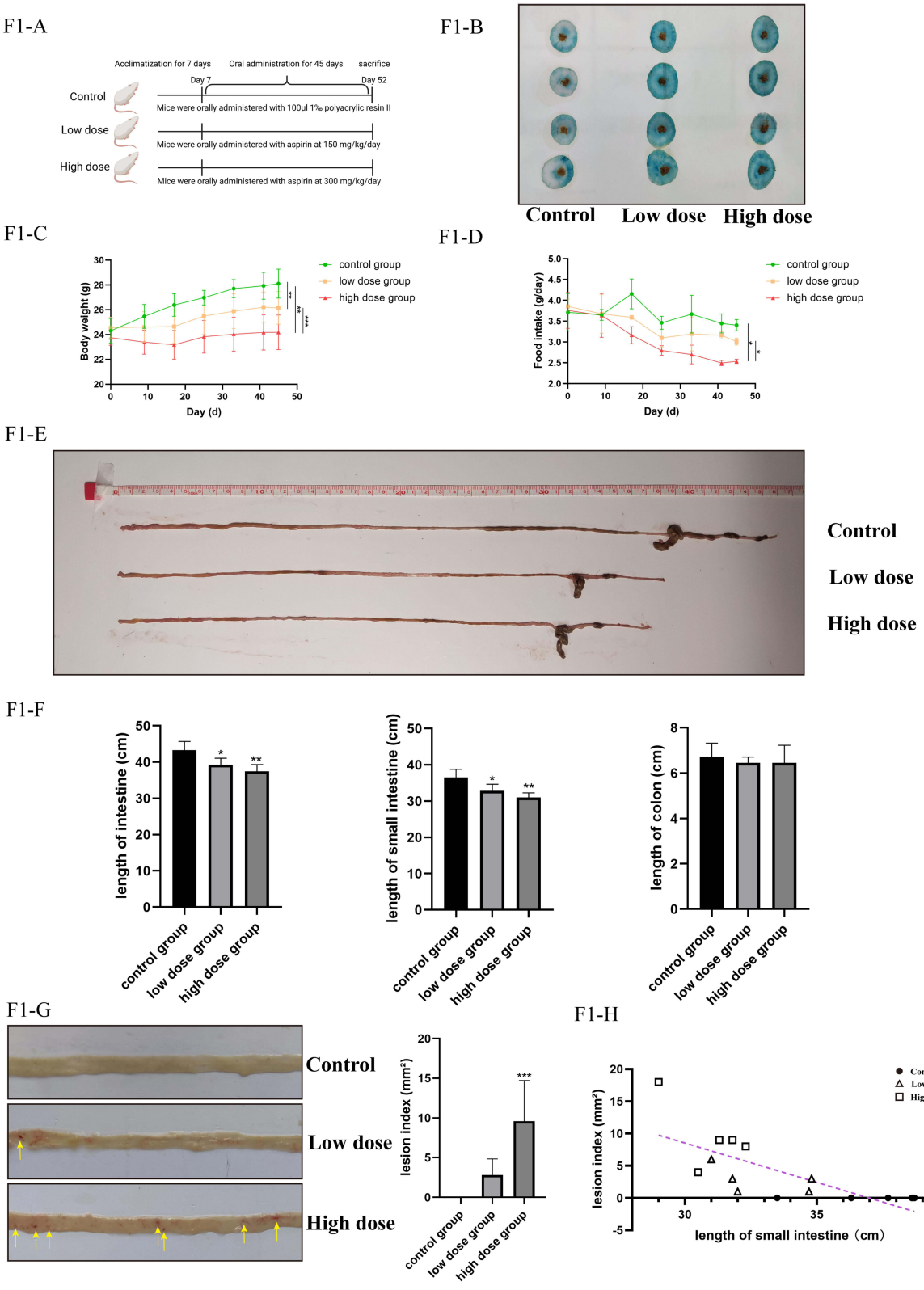

Figure 1 Long-term aspirin administration caused macroscopic damage to the small intestine in mice. (**A**) A timeline of experimental treatments for different groups. n = 7 per group. (**B**) Fecal occult blood (FOB) in aspirin-administered mice. n = 4 per group (All samples in the experiment exhibited the same or very similar trends, and the results were as expected. Therefore, it is likely that increasing the sample size would not significantly alter the conclusion). (**C**) The trend in body weight of three groups of mice over time. n = 7 per group. (**D**) The trend of daily food intake over time in three groups of mice. n = 7 per group. (**E**) Length of the intestine on day 45 of gavage. n = 5 per group. (**F**) Comparison of the length of different intestinal segments. n = 5 per group. (**G**) Photographs of aspirin-induced mucosal lesions in the small intestine. The yellow arrows indicate the mucosal ulcers of the small intestine. Mucosal damage in each mouse was quantified by lesion index. n = 5 per group. (**H**) Relationship between the length of small intestine and lesion index in mice administered with aspirin. n = 5 per group. Significance **P*<0.05, ***P*<0.01, ****P*<0.001. Each repeat was performed as a separate, independent observation.

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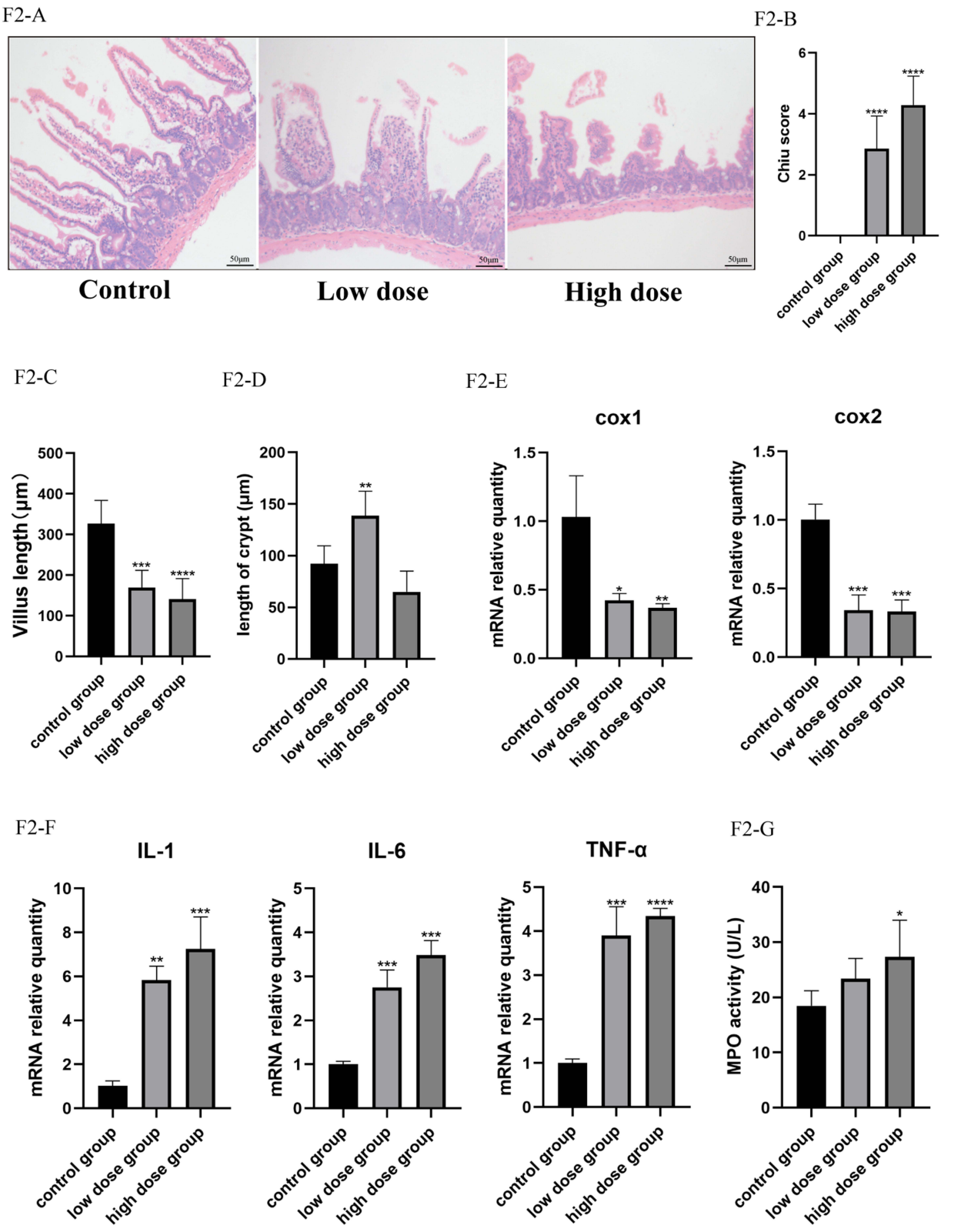

Figure 2 Long-term aspirin administration induced microscopic injury to the small intestine in mice. (**A**) HE staining of paraffin sections of small intestine. n = 5 per group. The magnification is 200X, and the field of view diameter is 1.1 mm. (**B**) Microscopic injury of intestinal mucosa in the three groups was assessed by Chiu's score. n = 5 per group. (**C**) Length of small intestinal villus in different groups. n = 5 per group. (**D**) The depth of the intestinal crypts in different groups. n = 5 per group. (**E**) The expression of cox1 and cox2 in the small intestine was assessed by qRT-PCR. n = 6 per group. (**F**) The expression of small intestinal inflammatory factors IL-1, IL-6 and TNF-α was assessed by qRT-PCR. n = 6 per group. (**G**) Effect of enteric-coated aspirin on MPO activities in mice serum. n = 5 per group (Two serum samples in the experimental group experienced hemolysis, which affected absorbance measurements; therefore, the corresponding results were excluded from the analysis). Significance **P*<0.05, ***P*<0.01, ****P*<0.001, *****P* <0.0001. Each repeat was conducted as a separate, independent experiment.

Chronic Aspirin Application Disrupted the Intestinal Mucosal Barrier and Increased Intestinal Permeability

The mRNA and protein levels of ZO-1, Occludin and claudin-1 were significantly reduced after aspirin administration (Figure 3A and B). We documented the procedure for conducting permeability experiments in live animals and obtained preliminary results: aspirin-treated mice exhibited a trend toward increased gut permeability compared to those treated with a vehicle (Figure 3C). Immunofluorescence assay revealed that aspirin stimulation decreased the staining of ZO-1 (Figure 3D). We also performed Periodic Acid-Schiff staining (PAS) to evaluate intestinal mucus levels, and the luminal surfaces of enterocytes, as well as the cytoplasm of goblet cells (GCs), were stained purplish red due to the presence of mucin. Chronic aspirin stimulation reduced the number of GCs and affected mucin secretion, disrupting the intestinal mucosal barrier (Figure 3E). Compared to the control group, we observed irregular widening of the paracellular pathways in the aspirin group. Partial impairment of the intestinal epithelium was also observed in the high-dose group (Figure 3F).

Long-Term Aspirin Administration Damaged Mitochondria, Reduced the Expression of Genes Related to Antioxidant Defense System, and Induced Lipid Peroxidation in Intestinal Tissues

We found that enteric-coated aspirin application downregulated the expression of a series of genes related to antioxidant defence systems in the small intestine (Figure 4A). We detected higher levels of ROS in the small intestine of aspirin-treated mice (Figure 4B). Compared with the control group, SOD activity was significantly downregulated in the serum of both the low-dose and high-dose groups (Figure 4C). The assay of SOD often cooperated with the determination of MDA, and we found that the MDA content in the serum of mice in the high-dose group was significantly upregulated (Figure 4D). In addition, immunohistochemical results suggested that aspirin increased the intensity of the oxidative stress marker 4-HNE staining in the ileum, especially at the tip of the intestinal villi (Figure 4E). However, there was no increase in the intensity of 4-HNE staining in the liver (Figure S1B). Compared to normal mitochondria with discrete cristae in the control group, the aspirin-administered group showed disruption of mitochondrial cristae and massive mitochondrial vacuolization (Figure 4F).

Enteric-Coated Aspirin May Induce Oxidative Stress Through the Nrf2/Gpx4 Signaling Pathway

Among the antioxidant defence systems, we focused on the Gpx4 gene, which was most significantly downregulated by aspirin. Reviewing the relevant literature, we learned that Gpx4 is a crucial gene in the ferroptosis pathway. GSH depletion, GPX4 inactivation, and lipid peroxidation are all manifested in the pathological progression of intestinal diseases such as inflammatory bowel disease. We subsequently found that applying enteric-coated aspirin decreased the mRNA expression of Nrf2, Slc7a11 and HO-1 (Figure 5A). At the protein level, we also confirmed the downregulation of a series of molecules of the Nrf2/Gpx4 signalling pathway (Figure 5B).

OTU Analysis and Diversity Analysis of Intestinal Bacteria

Based on the unweighted UniFrac distance matrix, the samples were clustered using the UPGMA method, and the results showed that the samples in the control and high-dose groups were well clustered (Figure 6A). Five samples from each group were sequenced to obtain OTU data presented as Venn diagrams. After bioinformatic analysis, sequences were grouped into OTUs with a > 97% similarity threshold, and finally, 2513 OTUs were identified, of which 755 OTUs were detected in all three groups (Figure 6B). We used histograms to analyze differences in the relative abundance of OTUs among the three groups at the phylum, order, family, and genus levels. After enteric-coated aspirin treatment, at the phylum level, the proportion of the Verrucomicrobiota increased, and the proportion of the Firmicutes decreased; At the class level, the proportion of Verrucomicrobiae increased, and the proportion of Bacilli decreased; At the order level, the proportion of Verrucomicrobiales increased, and the proportion of Lactobacillales decreased; At the family level, the proportion of Akkermansiaceae increased, and that of Lactobacillaceae decreased; At the genus level, the proportion of

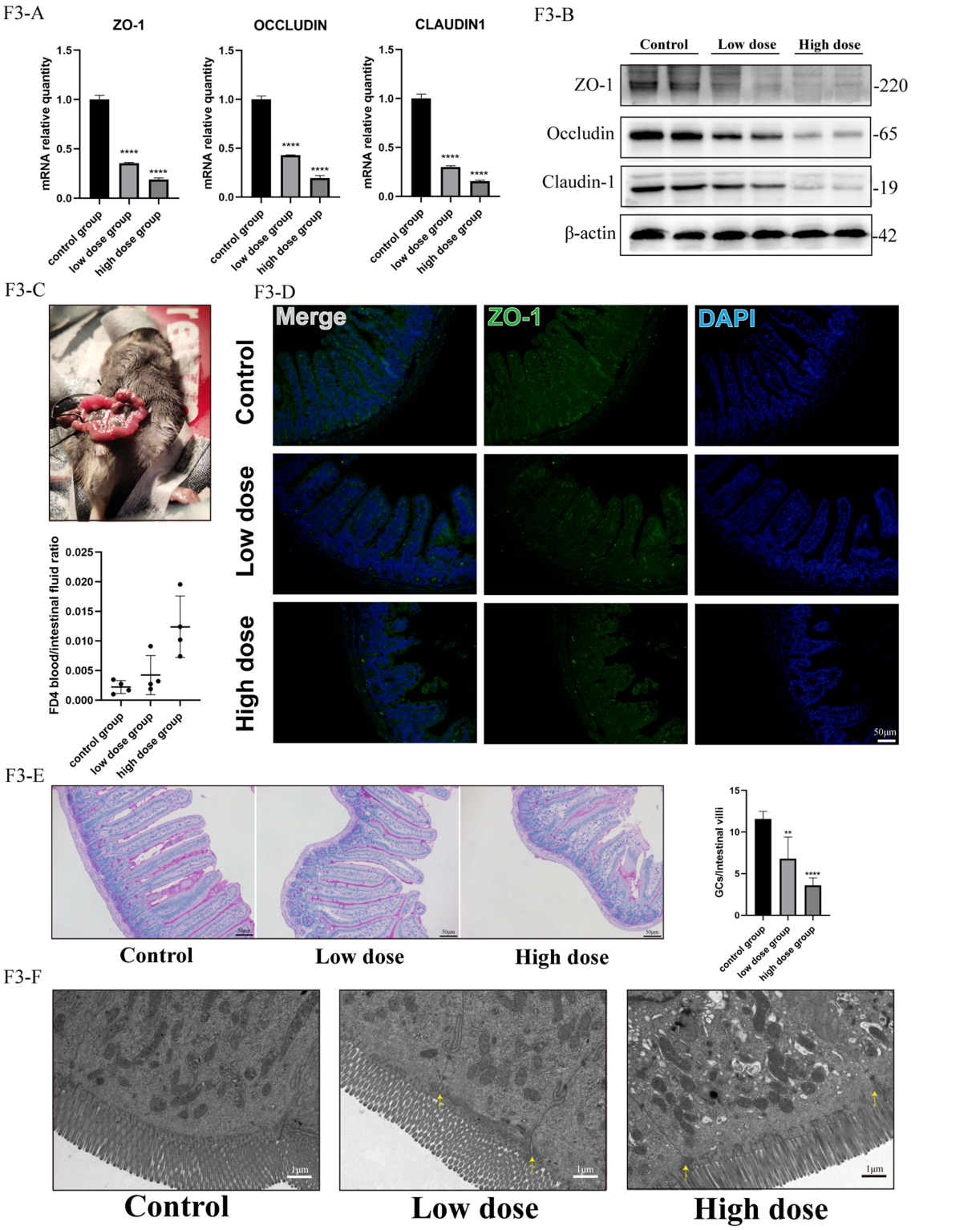

Figure 3 Chronic aspirin application disrupted the intestinal mucosal barrier and increased intestinal permeability. (**A**) The expression of ZO-1, Occludin and claudin-1 were assessed by qRT-PCR. n = 6 per group. (**B**) The expression of tight junction proteins was assessed by Western blot. n = 6 per group. (**C**) In vivo intestinal permeability assay, intestinal permeability was measured by the ratio of FD4 in blood to intestinal fluid. n = 4 per group (This experiment was conducted in live animals, which involves high technical demands and is time-consuming. Some mice died during the surgical procedure). (**D**) The expression of the tight junction protein ZO-1 on intestinal villus was shown by immunofluorescence. n = 5 per group. The magnification is 200X, and the field of view diameter is 1.1 mm. (**E**) PAS staining of ileum in each group, counting of GCs on each intestinal villus. n = 5 per group. The magnification is 200X, and the field of view diameter is 1.1 mm. (**F**) Electron transmission micrographs of mouse small intestine. The yellow arrows indicate the abnormally widened paracellular pathways. n = 2 per group (Due to limitations imposed by the laboratory's funding cycle and equipment restrictions, only a limited number of samples can be processed at this time). The magnification is 5.0kX. Significance ***P*<0.01, *****P* <0.0001. Each repeat was conducted as a separate, independent experiment.

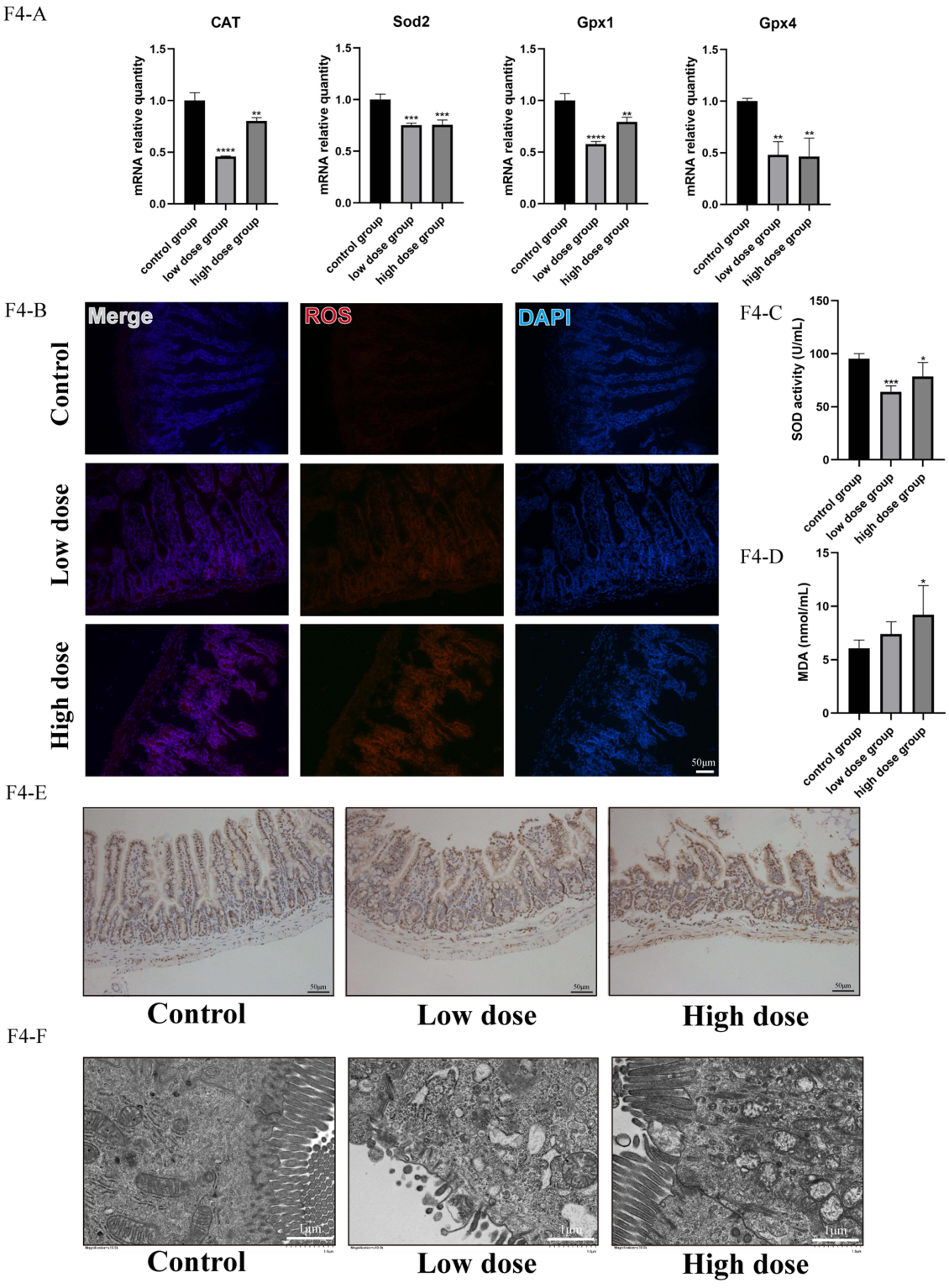

Figure 4 Long-term aspirin administration damaged mitochondria, reduced the expression of genes related to antioxidant defense system, and induced lipid peroxidation in intestinal tissues. (**A**) The expression of CAT, sod2, Gpx1 and Gpx4 were assessed by qRT-PCR. n = 6 per group. (**B**) The expression of ROS in frozen sections of small intestinal tissues were detected by immunofluorescence. n = 5 per group. The magnification is 200X, and the field of view diameter is 1.1 mm. (**C**) Measurement of SOD activity in mice serum with superoxide dismutase assay kit. n = 5 per group (Two serum samples in the experimental group experienced hemolysis, which affected absorbance measurements; therefore, the corresponding results were excluded from the analysis). (**D**) Measurement of MDA in mice serum with malondialdehyde assay kit. n = 5 per group (Two serum samples in the experimental group experienced hemolysis, which affected absorbance measurements; therefore, the corresponding results were excluded from the analysis). (**E**) Immunohistochemical staining of 4-hydroxynonenal (4-HNE) revealed the degree of lipid peroxidation of intestinal tissues in each group. n = 5 per group. The magnification is 200X, and the field of view diameter is 1.1 mm. (**F**) Representative electron transmission micrographs of mitochondria in intestinal epithelium. n = 2 per group (Due to limitations imposed by the laboratory's funding cycle and equipment restrictions, only a limited number of samples can be processed at this time). The magnification is 10.0kX. Significance **P*<0.05, ***P*<0.01, ****P*<0.001, *****P* <0.0001. Each repeat was performed as a separate, independent experiment.

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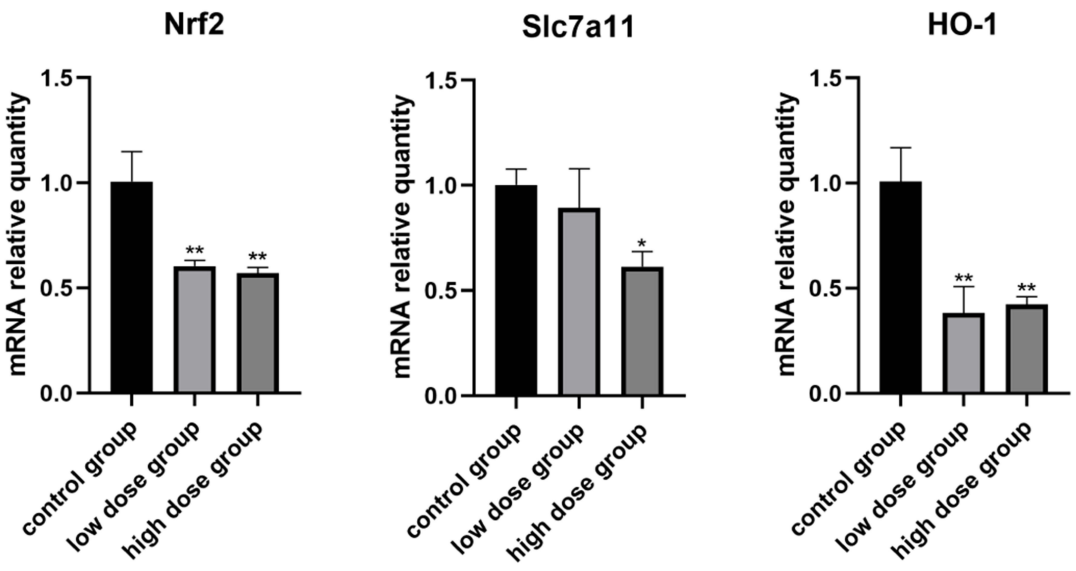
F5-A



F5-B

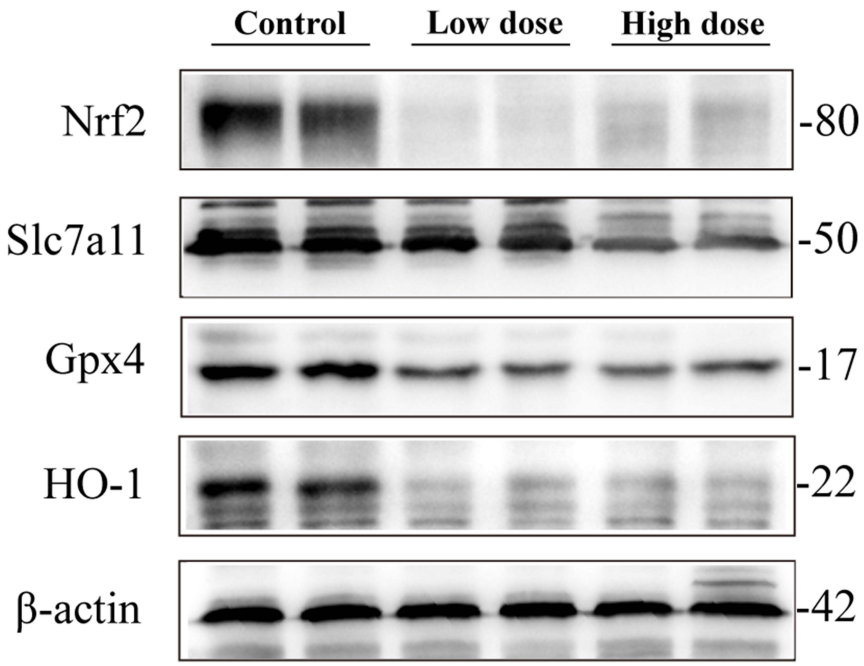


Figure 5 Enteric-coated aspirin may induce oxidative stress through the Nrf2/Gpx4 signaling pathway. (**A**) The expression of Nrf2, Slc7a11 and HO-1 was assessed by qRT-PCR. The number of sample size is n = 6 per group. (**B**) Protein levels of Nrf2 and its downstream targets Slc7A11, HO-1 and GPX4 were detected by Western blot. The number of sample size is n = 6 per group. Significance **P*<0.05, ***P*<0.01. Each repeat was performed as a separate, independent experiment.

Akkermansia increased, and that of Lactobacillus decreased significantly (Figure 6C). According to the α-diversity index Chao1 and observed_species of the three groups, the differences were significant when compared (Figure 6D). The 95%-confidence interval for the control and high-dose groups did not overlap based on PCoA or NMDS, indicating that the two have very different intestinal bacterial compositions (Figure 6E). PLS-DA analysis sorts the samples in a new low-dimensional coordinate system by finding the maximum covariance of the species abundance matrix and the given grouping information. As shown in the figure, samples from the control, low-dose and high-dose groups are effectively separated (Figure 6F). The ANOSIM showed that the differences between groups were more significant than those within groups (Table 2).

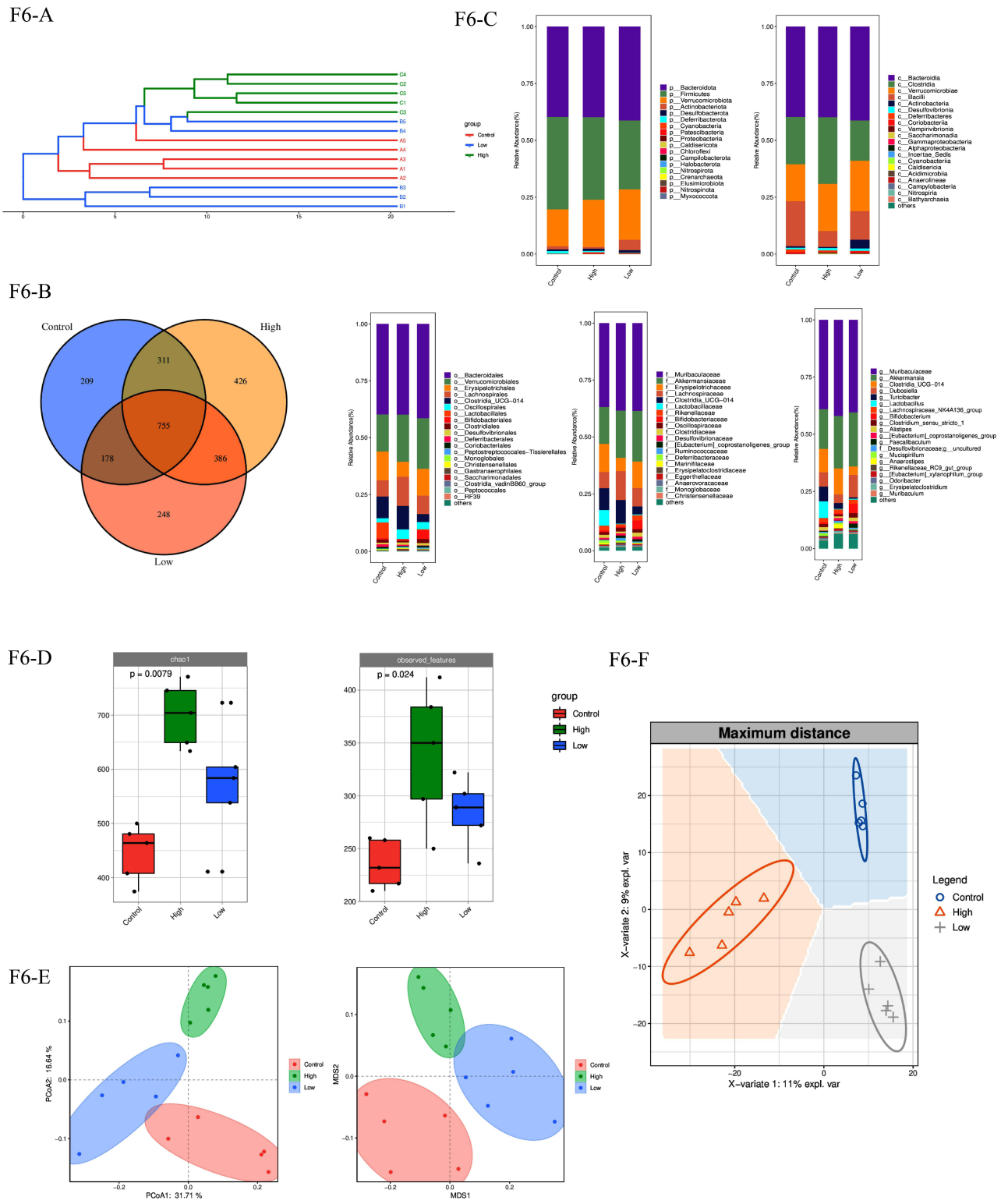


Figure 6 OTU analysis and diversity analysis of intestinal bacteria. (**A**) Cluster analysis of different groups of samples. (**B**) Venn diagram of the distribution of the three OTU groups. (**C**) Analysis of the relative abundance of intestinal flora at the level of phylum, order, family and genus. (**D**) Alpha diversity analysis. (**E**) Beta diversity analysis. (**F**) OTU-based PLS-DA Analysis. The number of sample size is n = 5 per group. Each repeat was performed as a separate, independent experiment.

OTU Analysis and Diversity Analysis of Intestinal Bacteria

We performed a cluster analysis of the similarity in the abundance of intestinal flora between samples and presented it in a heat map (Figure 7A). The abundance of Lactobacillus was significantly lower in the aspirin-administered group than in

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Table 2 ANOSIM results

Group	R statistic	P value	Number of Permutations
Control-Low	0.588	0.013	999
Control-High	0.632	0.008	999
Low-High	0.676	0.008	999
All	0.627	0.001	999

the control group. Among the three groups, the abundances of Atopobiaceae, Bacillaceae, Bacillus and Bacillales in the control group significantly differed from the other two groups. The abundances of Lachnospiraceae, Coriobacteriaceae, Prevotellaceae, and Family_XIII_AD3011_group in the low-dose group significantly differed from those observed in the other two groups. The abundance of Erysipelatoclostridium and Erysipelatoclostridiaceae in the high-dose group significantly differed from the other two groups (Figure 7B). Through Spearman network analysis, the nodes represent OTUs in the sample, and the node size is proportional to its abundance. The relative abundance proportion of different groups in that node is shown in a pie chart (Figure 7C). Based on the PCoA, the 95% confidence ellipses were utterly separated between the control and high-dose groups, indicating a significant difference in the functional enrichment of samples from the two groups (Figure 7D). Differential analysis of KEGG function using STAMP software showed that biological processes such as secondary bile acid biosynthesis, D-glutamine and D-glutamate metabolism, amino and nucleotide sugar metabolism, taurine and hypotaurine metabolism, DNA replication, and homologous recombination were reduced in the high-dose group compared with the control group; biological processes such as C5-branched dibasic acid metabolism, amino acid biosyntheses such as valine, and pantothenic acid and coenzyme A biosynthesis were increased (Figure 7E).

Discussion

We have introduced an innovative approach to drug administration in experimental animals. When aspirin comes into direct contact with the intestinal mucosa, it can uncouple mitochondrial oxidative phosphorylation. In contrast, with conventional oral aspirin administration, this uncoupling does not occur in the small intestine.[6] Since we do not dissolve the drug in any vehicle but directly administer the drug microgranules to the mice via gavage, the enteric-coated microgranules of aspirin are coated with polyacrylic acid resin II, which exhibits excellent acid stability. This coating effectively prevents premature release or degradation of the drug in the acidic gastric environment, ensuring that it safely passes through the stomach and releases its active ingredients only when it reaches the alkaline environment of the small intestine. Our approach is similar to intra-luminal drug administration in the small intestine.

The classical NSAID-associated enteropathy modelling drugs, such as indomethacin and diclofenac, are usually administered at 7.5 to 10 mg/kg/d.[5,22] The doses administered in our study were much higher than those described above. First, we found in our preliminary experiments that lower doses of aspirin did not cause damage to the intestinal mucosa. Second, it has been reported that aspirin needs to be administered at doses more than 10 times greater than those used in the above studies for small bowel injuries to occur consistently.[23] Further, aspirin is not excreted via the biliary tract, and the lack of enterohepatic circulation is one of the reasons why it requires high doses. In addition, the acid dissociation constant (pKa) of NSAIDs can explain the dose we used. The higher the pKa value, the lower the drug concentration required for uncoupling mitochondrial oxidative phosphorylation.[24] Aspirin has a relatively low pKa value compared to other NSAIDs, thus a higher concentration is needed.

We observed that enteric-coated aspirin significantly reduced the body weight of mice. Food intake is essential in NSAID-induced small intestinal injury,[25] as small intestinal injury can only be seen in experimental animals in a non-fasted state.[26] Our study found that enteric-coated aspirin, in turn, affects food intake and increases occult gastrointestinal bleeding. NSAIDs are known to cause occult bleeding and protein loss from the small intestine.[27] The reduction in food intake further decreases protein intake and absorption, potentially exacerbating an already suboptimal health condition.

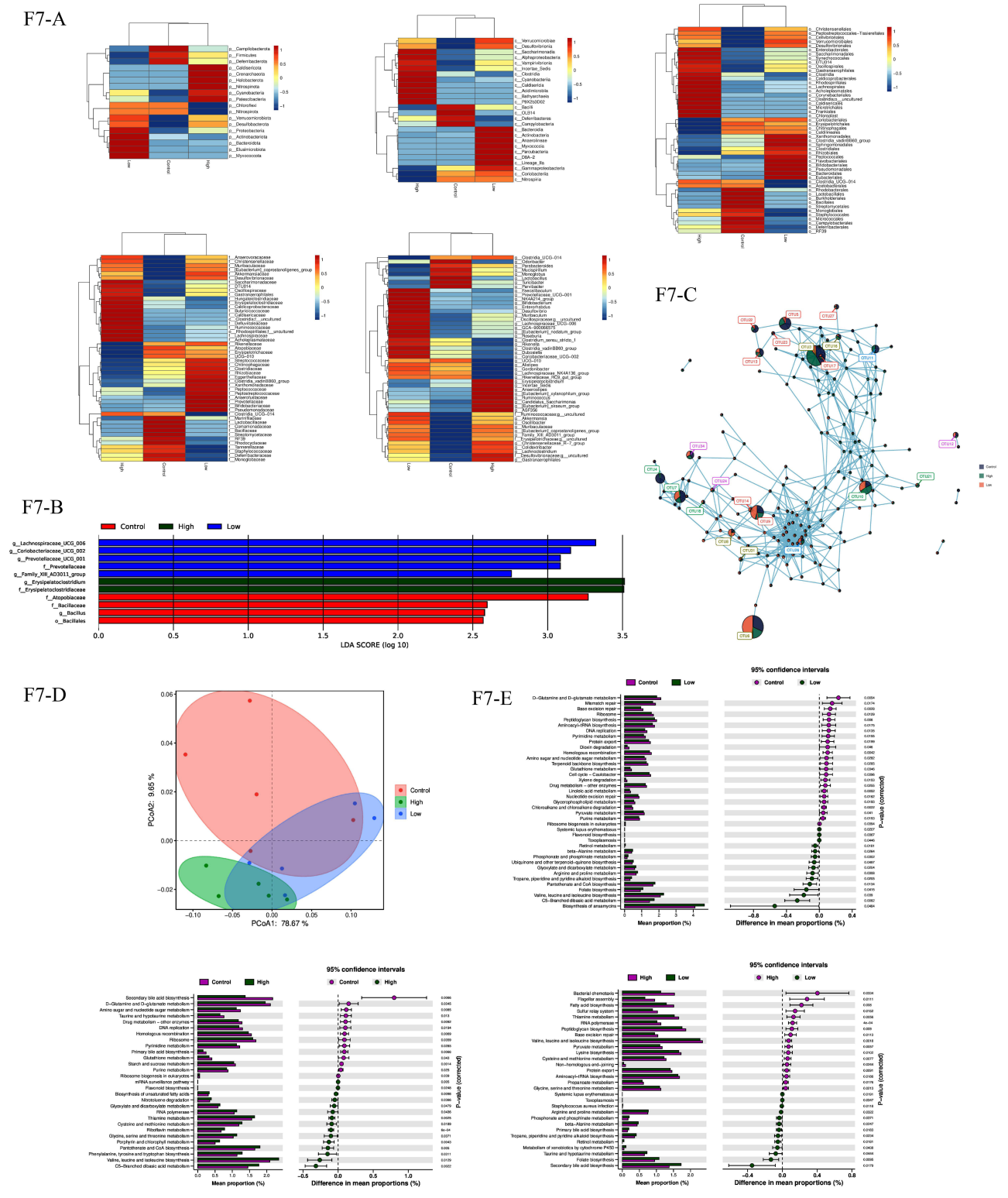

Figure 7 Heat map analysis and functional enrichment analysis of intestinal flora. (**A**) Heat map of bacterial abundance of the three groups. (**B**) Histogram of LDA effect values for marker species. (**C**) Seed network diagram of dominant species with grouped abundance pie charts. (**D**) Analysis of functional differences in samples. (**E**) Histogram of KEGG functional enrichment variance analysis. The number of sample size is n = 5 per group. Each repeat was performed as a separate, independent experiment.

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The enteric-coated aspirin significantly shortened the length of the small intestine, with no significant effect on the colon. NSAIDs primarily damage the distal small intestine,[27] which supports our findings. After high-dose aspirin administration, we observed scattered foci of erosion and ulceration on the luminal surface. It has been reported that aspirin alone cannot cause small intestinal inflammation or ulceration in rats,[10] which is inconsistent with our results. Their experiments took intraperitoneal injections of aspirin for animal modelling, and it lacks critical local effects since it is not in direct contact with the intestinal mucosa. We directly gavaged enteric-coated aspirin to prevent rapid drug absorption in the stomach and duodenum, successfully inducing inflammation and ulceration in the small intestine. We further used the method of Tanigawa et al[28] to calculate the lesion index of each mouse and found that the severity of lesions was drug-dose-dependent. Moreover, the lesion index negatively correlated with the length of the corresponding small intestine, consistent with the previous findings.[26] One study reported that in response to the effect of NSAID on small intestinal epithelial cells, the proliferation of crypt cells was enhanced to compensate for the damage.[29] In our study, this compensatory effect could be observed in the low-dose group, with an increase in intestinal crypt depth compared to the control group; We, however, observed a trend towards shortening of the intestinal crypt in the high-dose group, which could be a structural abnormality due to recurrent mucosal repair under chronic inflammation. In the present study, enteric-coated aspirin did not significantly affect the stomach. It has been reported that enteric-coated aspirin may be safer for gastric mucosa than conventional aspirin in the case of long-term use,[30] and the enteric-coated drug shifts the gastrointestinal damage farther away from the Treitz ligament.

Studies have reported that although the primary pharmacological effect of NSAID is the inhibition of cox enzymes, increased cox2 expression is a representative marker of NSAID enteropathy.[31] The increase is contrary to our findings, and since the modelling drug used in the literature is indomethacin, the variety and agent form of the drug may have caused the difference. We also observed elevated levels of MPO, IL-1, IL-6, and TNF-α, in agreement with similar studies reported.[32]

Disruption of intestinal tight junctions is an essential pathogenic mechanism leading to increased penetration of epithelial cells to harmful luminal molecules.[33] We found that enteric-coated aspirin significantly downregulated the tight junctions. Our findings are consistent with another NSAID-associated enteropathy study.[34] We also confirmed that irregular widening of the intestinal epithelial cell bypass occurs after long-term aspirin administration, which agrees with previous studies.[35,36] The mucus layer plays a vital role in lubricating and cushioning the intestinal barrier, and the goblet cells directly determine the mucus quality.[37] Then we found that enteric-coated aspirin significantly reduced the number of goblet cells, reducing mucin secretion and disrupting the intestinal mucus barrier. Irsogladine maleate, a mucosal protective agent, was reported to stimulate small intestinal mucus secretion, thereby inhibiting bacterial invasion and reducing NSAID-induced small intestinal injury,[38] suggesting a promising future for treating NSAID-associated enteropathy. Increased intestinal permeability is closely related to the severity of NSAID-induced enteropathy.[39] The intestinal permeability to macromolecules was assessed using the method described by Kao et al,[40] and compared to oral gavage of FD4, we injected FD4 directly into the ileum to evaluate the permeability of this segment alone. One limitation of this experiment is the insufficient sample size, which prevented us from performing statistical analysis. As a result, we could only reach a preliminary conclusion that high-dose aspirin may lead to increased ileal permeability.

Many studies have shown a strong association between inflammatory bowel disease and the secondary metabolites of lipid peroxidation, MDA, and 4-HNE.[20,41] The level of SOD activity indirectly reflects the body's ability to scavenge oxygen free radicals, which is impaired by enteric-coated aspirin; the level of MDA content indirectly reflects the severity of the body's attack by free radicals, which is elevated after drug administration. Further, we observed an increased degree of lipid peroxidation in the small intestine, with the most severe degree in the apical cells of the villi. Our findings are supported by studies reporting that well-differentiated villus tip enterocytes are more susceptible to the toxic effects of NSAID than intermediate cells and crypt cells.[29] Since the oxidative status in the intestine is related to the expression of hepatic redox-related enzymes,[8] we also examined the degree of lipid peroxidation in the liver, and the results suggested that enteric-coated aspirin had no significant effect on hepatic lipid peroxidation. Moreover, under electron transmission microscopy, we observed disruption of the mitochondrial cristae and massive mitochondria showing vacuolization. Severe mitochondrial damage in intestinal epithelium was also consistently observed in previous studies.[6] We observed downregulation of a series of antioxidant defense system-related genes, with enteric-coated aspirin inducing the most pronounced downregulation in Gpx4. The downregulation of Gpx4 may be caused by dietary long-

chain fatty acids, immune mediators, bile, and microbial metabolites.[20] The Gpx4-GSH axis has been reported to be the primary antioxidant defense system that protects cells from ferroptosis.[42] The cystine–glutamate transporter Slc7a11 is a significant source of cysteine for GSH synthesis.[43] In addition, Nrf2 is a crucial antioxidant transcription factor that regulates the expression of Slc7a11, Gpx4, and HO-1.[44–46] As expected, enteric-coated aspirin significantly down-regulated the Nrf2/Gpx4 signaling pathway. Studies have reported that the antioxidant L-carnitine is very effective against NSAID-induced small intestinal injury.[8] The natural product, the essential oil of Citrus limon, has a significant protective effect against aspirin-induced intestinal injury, possibly due to its antioxidant activity.[47] These provide us with a new idea for treating NSAID-related intestinal ulcers.

We observed that enteric-coated aspirin induced significant alterations in the composition of the intestinal microbiota. We further analyzed the changes in bacterial abundance at the phylum, order, family and genus levels, and the results suggested that the abundance of Akkermansia increased and that of Lactobacillus decreased after aspirin administration. Previous studies on the intestinal flora of NSAID-associated enteropathy support our findings to some extent. The relative abundance of Akkermansia was positively correlated with histopathological changes and the degree of inflammation.[48] Studies have reported a decrease in the abundance of Firmicutes and a significant reduction in the abundance of Lactobacillus in elderly individuals taking NSAIDs.[49] In a healthy gut, commensal non-pathogenic bacteria interact with the host mucosa to regulate the function of the intestinal mucosal barrier. In this context, the deficiency of Lactobacillus may contribute to the development of NSAID enteropathy.[50] Probiotics that regulate intestinal homeostasis have potential therapeutic effects on NSAID-induced intestinal damage. Lactobacillus gasseri OLL2716 may help to reduce aspirin-induced small bowel injury and alleviate gastrointestinal symptoms.[51] Lactobacillus plantarum TIFN101 upregulates genes associated with maintaining T and B cell functions and antigen presentation, potentially preventing NSAID-induced immune stress.[52] Further KEGG functional enrichment analysis suggested that enteric-coated aspirin altered various cellular biological processes such as secondary bile acid synthesis. Aspirin affects intestinal health and immune function by influencing the production of gut microbiota metabolites. Research shows that aspirin inhibits the growth of Parabacteroides goldsteinii and reduces its bile acid metabolite, 7-keto-lithocholic acid (7-keto-LCA), which acts as an FXR antagonist to promote intestinal epithelial repair and maintain gut homeostasis. By lowering 7-keto-LCA levels, aspirin weakens this protective effect, compromising gut barrier function. This underscores the crucial role of the microbiome-bile acid axis in gut health.[53] Studies have shown that aspirin can increase the levels of certain short-chain fatty acids (SCFAs) in the intestine, including propionic acid, valeric acid, isovaleric acid, and isobutyric acid.[54] SCFAs primarily exhibit anti-inflammatory effects in the gut, particularly in maintaining intestinal health and regulating immune balance. This response may represent a protective feedback mechanism. The gut microbiota can also modulate the efficacy of aspirin. L sphaericus causes aspirin degradation in the gut, thereby reducing aspirin bioavailability.[55] The gut microbiota can mediate the interactions between aspirin and other drugs, potentially affecting the pharmacological effects of aspirin and causing significant adverse reactions.[56]

Conclusion

We successfully constructed a chronic model of aspirin-associated enteropathy. We validated it at macroscopic and microscopic damage, intestinal mucosal barrier disruption, mitochondrial oxidative phosphorylation uncoupling and oxidative stress. We also found that enteric-coated aspirin may induce oxidative stress through the Nrf2/Gpx4 signaling pathway. Long-term aspirin administration did alter the composition and function of the intestinal microecology. Building upon the findings of this study, it would be valuable for future research to explore other molecular pathways involved in oxidative stress and intestinal barrier disruption, beyond the Nrf2/Gpx4 axis. Moreover, integrating gut microbiome and metabolomic analyses could help uncover systemic effects of aspirin on intestinal health. Comparative studies across diverse animal models and human populations could provide deeper insights into the generalizability of the mechanism. For diagnosis, identifying reliable biomarkers for early detection and monitoring of aspirin-induced intestinal damage could also enhance clinical relevance. Additionally, expanding research into natural compounds with antioxidant properties and their underlying mechanisms may offer new therapeutic options. Further investigation into specific probiotic strains or combinations that effectively alleviate intestinal injury and restore microbial balance is crucial. Ultimately, longitudinal clinical studies are necessary to confirm the safety and efficacy of these therapies in human populations, bridging animal model findings with clinical practice.

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Author Contributions

All authors made substantial contributions to the work reported, including in the conception, study design, execution, data acquisition, analysis, and interpretation. They participated in drafting, revising, and critically reviewing the article, gave final approval of the version to be published, agreed on the journal to which the article was submitted, and are accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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