

Xinshuaifang improves oxidative stress injury and myocardial remodeling in acute heart failure: Association with the gut-heart axis

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Abstract: Background: Acute heart failure (AHF) is a leading cause of death from cardiovascular disease and effective treatments are lacking. **Objectives:** To investigate whether Xinshuaifang improves oxidative stress injury and myocardial remodeling in AHF mice by regulating the gut-heart axis. **Methods:** C57BL/6 mice aged 8 – 10 weeks and weighing 20–25 g were randomized into 7 groups (n=8/group): control, AHF model, AHF+Xinshuaifang, AHF+control FMT, AHF+intervention FMT, AHF+Xinshuaifang+antibiotics and antibiotics-only control. AHF was induced by isoproterenol injection (5 mg/kg, 7 days). Gut microbiota composition, oxidative stress markers (MDA, GSH, SOD, MPO), cardiac function (LVDD, LVDs, LVEF, NT-proBNP), myocardial fibrosis and tight junction protein expression (ZO-1, Occludin, Claudin-1, Zonulin) were assessed. **Results:** Compared with controls, model mice showed significantly reduced *Verrucomicrobia* and *Akkermansia* abundance ($P<0.01$), increased MDA and GSH levels, decreased SOD activity and MPO levels, reduced LVEF, elevated NT-proBNP ($P<0.01$), decreased ZO-1, Occludin and Claudin-1 expression and increased Zonulin expression. Xinshuaifang intervention significantly reversed all these changes ($P<0.01$): it increased beneficial bacteria, reduced oxidative stress, improved cardiac function, upregulated ZO-1/Occludin/Claudin-1 and downregulated Zonulin. The FMT intervention group showed similar protective effects, whereas the Xinshuaifang+antibiotics group showed attenuated protection. **Conclusion:** Xinshuaifang improves cardiac function and alleviates oxidative stress and fibrosis in AHF mice by regulating gut microbiota, upregulating tight junction proteins and restoring intestinal barrier function.

Keywords: Acute heart failure; Gut-heart axis; Heart failure prescription; Myocardial remodeling; Oxidative stress

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INTRODUCTION

Acute heart failure (AHF) originates from primary heart disease or is caused by the progression of chronic heart failure to the acute decompensated stage, which frequently leads to acute pulmonary edema, severe systemic congestion and malignant arrhythmias. It is one of the main causes of death in patients with cardiovascular disease (Burzo *et al.*, 2026). Epidemiological surveys (Bazmpani *et al.*, 2022) show that the re-hospitalization and mortality rates of AHF patients are 15% to 30%. Although significant pharmacological progress has been made in treating AHF patients with diuretics, positive inotropic agents, vasodilators and neurohormonal antagonists, patients still die during hospitalization or within 30 days after discharge (Ruocco *et al.*, 2026). Therefore, exploring more effective treatment strategies is crucial to improving patient prognosis.

In recent years, extensive research on the gut-heart axis and heart failure has been conducted, providing innovative diagnostic and therapeutic approaches for heart failure (Rui *et al.*, 2026). Under normal conditions, the intestinal barrier is intact, and short-chain fatty acids, such as butyrate, a metabolite of the flora, have anti-inflammatory effects and help maintain intestinal epithelial barrier integrity (Gao *et al.*, 2021). However, in the state of heart failure, due to weakened cardiac output and insufficient intestinal perfusion, the intestinal barrier function is severely

damaged, resulting in a significant increase in the translocation of intestinal microbiota, an increase in conditional pathogens and a decrease in beneficial bacteria (Kon *et al.*, 2022; Panganiban *et al.*, 2025). This change not only continuously increases intestinal permeability but also allows bacterial endotoxins to breach the intestinal barrier and enter the bloodstream, thereby triggering a systemic inflammatory response (Fujimoto *et al.*, 2021). This process also activates pathways such as Toll-like receptor 4 (TLR4)/NF- κ B pathway, exacerbating myocardial fibrosis and inflammation (Agoston-Coldea & Negru, 2024), forming a vicious cycle of "intestinal flora-inflammation-damage to cardiac function". Relevant studies (Murphy *et al.*, 2021) have shown that changes in intestinal microbiota composition can affect the pathogenesis of cardiovascular diseases such as heart failure (HF). Previous studies have indicated that the relative abundance of butyrate-producing bacteria in the intestines of HF patients decreased significantly and the levels of inflammatory factors IL-6 and CRP increased significantly from 2.3 ng/mL and 158 ng/mL to 2.9 ng/mL and 186 ng/mL, respectively (Modrego *et al.*, 2023); In rats with chronic heart failure, the levels of plasma lipopolysaccharide (LPS) and IL-6 increased significantly (Wang and Zhang, 2021) and these changes were closely related to the deterioration of cardiac function (Packer *et al.*, 2024). Therefore, targeting the intestinal flora and regulating the function of the "gut-heart axis" may become a new strategy to delay the progression of AHF. The main drugs used in Western medicine for the treatment of AHF

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are diuretics, cardiogenic agents, vasodilators and neuroendocrine inhibitors. Although these drugs can effectively improve the clinical symptoms of patients, long-term use may lead to adverse reactions and even worsen the condition. Therefore, identifying new therapeutic targets for heart failure remains a major challenge and a hotspot in the current cardiovascular research field. In this context, traditional Chinese medicine, with its advantages of holistic regulation and multi-target effects, offers new ideas for treating heart failure (Guo *et al.*, 2024).

Traditional Chinese medicine has a long history of preventing and treating heart failure, emphasizing the unity of man and nature, holistic concepts, and syndrome differentiation and treatment, and has significant advantages in improving cardiac function and clinical symptoms (Luo *et al.*, 2023). According to the theoretical system of traditional Chinese medicine, heart failure can be classified into "palpitations", "asthma", and "edema", and its pathogenesis is fundamentally characterized by a deficiency of both qi and yin (Jiang *et al.*, 2022). As an important school of modern Chinese medicine, the Menghe School of Medicine emphasizes overall regulation (Wang *et al.*, 2023a). Its empirical prescription "Xinshuaifang" adopts "benefiting heart qi, warming heart yang, activating blood circulation and promoting diuresis" as its basic therapeutic principle. While focusing on the warming yang and benefiting qi of herbs such as *aconite*, *cinnamon twig*, *ginseng*, and *astragalus*, it also combines *Ophiopogon japonicus*, *Polygonatum odoratum*, and stone to achieve yin-yang balance, which is consistent with the complex pathogenesis of heart failure (Fan *et al.*, 2023). Relevant studies have shown that Xinshuaifang can increase PPAR α mRNA and protein expression levels, decrease AMPK mRNA and protein expression levels, regulate myocardial energy metabolism substrates in CHF rats, and delay the onset and progression of heart failure (Liu *et al.*, 2023). Furthermore, intestinal fermentation deficiency and energy metabolism imbalance are important features in the pathophysiological process of heart failure. Previous studies have shown that various active ingredients of traditional Chinese medicine, such as *Astragalus polysaccharides*, *ginsenosides* and *berberine*, as well as compound formulas such as *Shenfu Decoction* and *Shengmai Powder*, can improve cardiac function and inhibit myocardial remodeling by regulating intestinal microbiota, promoting short-chain fatty acid production, enhancing intestinal barrier function and inhibiting the TLR4/NF- κ B and NLRP3 inflammasome pathways (Rui *et al.*, 2026). However, existing evidence remains largely fragmented and descriptive: while Xinshuaifang has been shown to regulate myocardial energy metabolism, whether it also acts via the gut-heart axis—specifically by modifying gut microbiota composition, reducing endotoxin translocation, or suppressing systemic inflammation—has not been systematically investigated. This represents a critical mechanistic gap, because the gut-

heart axis is now recognized as a key driver of the inflammatory response and ventricular remodeling in AHF. Without such evidence, the full therapeutic potential of Xinshuaifang remains underexplored. Based on this, the present experiment aims to investigate whether Xinshuaifang exerts cardioprotective effects by modulating the gut-heart axis pathway, specifically focusing on oxidative stress and ventricular remodeling in AHF patients, thereby providing new targets and strategies for AHF treatment.

MATERIALS AND METHODS

Experimental animals

In this study, clean-grade C57BL/6 male mice, aged 8-10 weeks and weighing 20-25 g, were purchased from Jiangsu Wukong Biotechnology Co., Ltd. (License Number: SCXK (Su) 2020-0003). All animals were housed under constant temperature ($22 \pm 2^{\circ}\text{C}$) and humidity ($50 \pm 10\%$) conditions, with a 12-hour light/12-hour dark cycle, and had free access to food and water. Environmental enrichment was provided in all cages, including nesting material (sterile cotton fluff), wooden chew sticks, and plastic tunnels, to improve animal welfare. All ISO subcutaneous injections were performed under mild isoflurane anesthesia (1.5%) to minimize injection pain. During the experimental period, mice were observed daily for food intake, water intake, activity levels, and fur condition, with monitoring according to humane endpoint criteria (described below). Humane endpoint criteria were predefined as follows: (1) body weight loss exceeding 20% of baseline; (2) persistent immobility or inability to access food and water independently; (3) severe dyspnea or cyanosis; (4) tumor-like mass formation or severe infection. Monitoring was performed twice daily (8:00 AM and 8:00 PM) by trained animal caretakers. After 7 days of adaptation feeding, the experiments were conducted as described previously (Storves *et al.*, 2025).

Sample size calculation and experimental design

This study is a randomized controlled animal experiment. The left ventricular ejection fraction (LVEF) was used as the primary outcome measure. Based on the results of the previous pre-experiment (control group LVEF = $78.5 \pm 5.2\%$, model group LVEF = $52.3 \pm 6.1\%$), using PASS 15.0 software (Whittle *et al.*, 2025). The set test power ($1 - \beta$) was 0.8, the significance level α was 0.05 (two-sided) and the effect size (Cohen's) d was 4.6. It was calculated that each group needed at least 6 mice. Given the possibility of deaths or modeling failures during the experiment (dropout rate approximately 25%), a final analytical sample size of $n = 8$ mice per group was included, totaling 56 mice across all 7 groups. All seven experimental groups (G1 – G7) had an identical sample size of $n = 8$.

The sample size calculation was applied uniformly to each of the seven experimental groups, including the antibiotic-only control group (G7) and the two FMT groups (G4 and G5). The rationale for including these specific groups is as

follows: (1) Antibiotic-only control group (G7): This group was designed to exclude the possibility that broad-spectrum antibiotics might directly affect cardiac function or induce oxidative stress independent of gut microbiota depletion. Based on a preliminary experiment ($n = 3$ mice per group), antibiotic treatment alone did not significantly alter LVEF compared with the control group (mean difference: 1.2%, 95% CI: -2.1% to 4.5%, $P = 0.43$), indicating no direct cardiotoxic effect. However, to definitively rule out a type II error, a formal sample size of 8 mice was calculated using the same LVEF-based parameters as for the other groups. (2) FMT groups (G4 and G5): These groups were designed to test the causal role of Xinshuaifang-reshaped gut microbiota via a "gain-of-function" approach. The expected effect size for FMT was derived from a separate pilot FMT experiment ($n = 3$ per group), where LVEF in recipients of Xinshuaifang-shaped microbiota improved from $52.1 \pm 5.8\%$ to $68.3 \pm 6.2\%$ (Cohen's $d = 2.6$). Using this effect size with $\alpha = 0.05$ and power = 0.8, the required sample size was calculated as 5 mice per group, rounded up to 8 to account for a 25% dropout rate and to maintain consistency with the sample sizes used in other treatment groups across the study. Thus, including all seven groups with the same sample size ($n = 8$) ensures balanced statistical power for pairwise comparisons and enables rigorous causal inference into the role of gut microbiota in mediating the cardioprotective effects of Xinshuaifang.

Randomization and blinding

Randomization and concealed allocation

The mice were allocated to each experimental group using a random number table. The random sequence was generated by an independent researcher who was not involved in the subsequent experiments. The allocation information was sealed in an opaque envelope and opened one by one before the intervention began. To control for potential confounding factors, all mouse cages were randomly arranged in the animal facility, and cage positions were rotated daily. Drug gavage, FMT procedures, and echocardiographic examinations were all performed at fixed times each day (9:00–11:00 AM) to control for circadian rhythm effects. All operators strictly followed standardized operating procedures, and the order of treatments for each group was randomized daily.

Blinding

This study employed a double-blind design. The animal caretakers were responsible for administering the drugs and performing fecal microbiota transplantation (FMT) procedures but were not involved in subsequent outcome assessments or data analysis. Cardiac ultrasound examinations, histopathological evaluations, qRT-PCR, and Western blot assays were conducted by operators unaware of the group assignments. During the data analysis stage, the data files remained coded by group until the end of the statistical analysis.

Establishment of acute heart failure (AHF) mouse model

As described previously (Su *et al.*, 2025), the AHF model was induced by subcutaneous injection of isoproterenol (ISO, Sigma-Aldrich, USA, catalog number I5627). Except for the control group, all other groups of mice were subcutaneously injected with ISO solution at a dose of 5 mg/kg every day for 7 consecutive days. This method consistently induced the AHF model, characterized by a significant decrease in LVEF and an increase in NT-proBNP levels. The control group mice received the same volume of sterile normal saline by injection. On the 7th day after modeling, the model was confirmed successful by detecting plasma NT-proBNP levels and cardiac ultrasound (model inclusion criteria: LVEF < 55% and NT-proBNP increase relative to the control group > 2 times).

Experimental grouping

The mice were randomly divided into the following 7 groups, with 8 mice in each group: Control group (G1), AHF model group (G2), AHF + Xinshuaifang group (G3), AHF + Control FMT group (G4), AHF + Intervention FMT group (G5), AHF + Xinshuaifang + Antibiotic group (G6) and Antibiotic Control group (G7). Among them, G1 and G7 did not undergo ISO induction; G1 was a blank control, and G7 received only antibiotic treatment to exclude the influence of the antibiotics themselves on cardiac function. Groups G2–G6 induced AHF via ISO. G3 and G6 received intragastric administration of Xinshuaifang. G4 transplanted the fecal microbiota of the AHF model group mice and G5 transplanted the fecal microbiota of the Xinshuaifang group mice. G6 and G7 simultaneously received broad-spectrum antibiotic treatment to deplete the intestinal microbiota.

Preparation and dosage of xinshuaifang

Xinshuaifang is formulated by combining the following fixed ingredients of traditional Chinese medicine: 20 g of red ginseng (*Panax ginseng* C.A.Mey.), 20 g of processed *Aconitum carmichaelii* Debeaux., 20 g of *Salvia miltiorrhiza* Bunge., 16 g of *Poria cocos* (Schw.) Wolf., 10 g of *Leonurus japonicus* Houtt., 5 g of *Ziziphus jujuba* Mill., 14 g of *Ziziphus jujuba* Mill. var. *spinosa* (Bunge) Hu ex H.F.Chow., 12 g of *Astragalus membranaceus* (Fisch.) Bunge., 12 g of *Santalum album* L., 12 g of *Saussurea costus* (Falc.) Lipsch., 12 g of *Amomum villosum* Lour., 12 g of *Angelica sinensis* (Oliv.) Diels., 12 g of *Paeonia lactiflora* Pall., 12 g of *Arctium lappa* L., 12 g of *Alisma plantago-aquatica* L. and 2 g of *Plantago asiatica* L. All herbal materials were purchased from a single batch (batch number: XSF-20240801) and authenticated by Changzhou Hospital of Traditional Chinese Medicine. Voucher specimens were deposited at the hospital herbarium (accession number: 20240801). The herbs were mixed with 450 mL of distilled water and simmered slowly until concentrated to approximately 100 mL. The decoction was filtered through a 0.45 μm membrane, freeze-dried to a powder (yield: 24.3% w/w), and stored at -80°C under

nitrogen protection to prevent degradation. To ensure batch-to-batch consistency and experimental reproducibility, three independently prepared batches of Xinshuaifang (batch numbers: XSF-20240801, XSF-20240815, XSF-20240901) were analyzed by HPLC. The chromatographic fingerprint similarity among the three batches was 0.96 ± 0.02 (range: 0.94–0.98). The contents of the main active ingredients were quantified as follows: tanshinone IIA was 1.82 ± 0.09 mg/g freeze-dried powder and ginsenoside Rg1 was 3.56 ± 0.15 mg/g freeze-dried powder, with inter-batch coefficients of variation of 4.9% and 4.2%, respectively. For dosage conversion, according to the principle of clinical equivalent dose conversion, the dosage for mice was calculated using the body surface area method (Liu *et al.*, 2025a). The weight of the mice was 20 g and the body surface area is approximately 0.007 m^2 (using the formula body surface area (m^2) = $0.007184 \times \text{weight (kg)}^{0.425} \times \text{height (cm)}^{0.725}$ and taking a height of 9 cm for calculation). Then, the equivalent dose for mice is: $118.0 \text{ g/m}^2 \times 0.007 \text{ m}^2 = 0.826 \text{ g}$ of raw drug / 20 g of mouse body weight. Converted to the dose per kilogram of body weight as follows: $0.826 \text{ g} / 0.02 \text{ kg} \times 1.25$ (safety factor) = 2.58 g of raw drug/kg body weight, rounded to 2.5 g of raw drug/kg body weight. Based on the above conversion results, the daily intragastric dose for mice is 2.5 g of raw drug/kg body weight and the daily intragastric volume for each group of mice is 10 mL/kg (dissolving the freeze-dried powder in sterile normal saline to prepare a solution of 0.25 g of raw drug/mL). The solution was administered once daily for 7 consecutive days. The control group mice received the same volume of sterile normal saline.

Antibiotic treatment

To deplete the gut microbiota, before FMT or during the period of traditional Chinese medicine intervention, vancomycin (Sigma-Aldrich, USA, item number V2002), gentamicin (Sigma-Aldrich, USA, item number G1264) and ampicillin (Sigma-Aldrich, USA, item number A9518) were added to the mice's drinking water at concentrations of 0.5 and 1 g/L respectively, allowing the mice to drink freely for 7 consecutive days. After antibiotic treatment was completed, fecal samples were collected for 16S rRNA sequencing to verify the efficacy of microbiota depletion. The criterion for successful microbiota depletion was that the Chao1 index was reduced by more than 90% compared to the control group, and the relative abundance of the *Proteobacteria* phylum was greater than 80%. To exclude the influence of the antibiotics on cardiac function, an independent antibiotic control group (G7) was established. In this group, the mice only received antibiotic treatment and did not undergo ISO modeling or traditional Chinese medicine intervention.

Fecal microbiota transplantation (FMT)

Source of FMT donors

The donors for the control FMT group (G4) were the mice in the AHF model group (G2); the donors for the

intervention FMT group (G5) were the feces of mice in the AHF + Xinshuaifang group (G3). In this study, all FMT donors were from mice and no clinical patient feces were used to avoid the influence of species differences on the results.

FMT procedure

The FMT procedure followed the method in reference (Li *et al.*, 2021). The donor feces were collected in an anaerobic workstation (Whitley A35, UK), homogenized with sterile PBS (1:10, w/v), filtered through a 100 μm cell sieve immediately after homogenization and either used immediately or stored at -80°C . Each recipient mouse was intragastrically administered (containing approximately 1×10^9 CFU) daily for 7 consecutive days. Mice in the control group and those not receiving FMT were intragastrically administered the same volume of sterile PBS.

Experimental timeline

The experimental timeline was as follows: Days 1-7 were the adaptation feeding period. From Day 1 to Day 7, mice in groups G2 to G7 are injected subcutaneously with ISO (5 mg/kg) daily to induce the AHF model; simultaneously, groups G3 and G6 are given Xinshuaifang by gavage daily, groups G4 and G5 receive FMT daily and groups G6 and G7 are given antibiotic solutions daily. On Day 8, cardiac ultrasound, sample collection (blood, heart, feces), and subsequent tests are conducted.

Oxidative stress indicator detection

Take the heart tissue from mice, homogenize it on ice, centrifuge, and then collect the supernatant. The levels of malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD) and myeloperoxidase (MPO) are detected according to the instructions of the commercial reagent kits. All samples are set with duplicate wells. The MDA and SOD reagent kits are purchased from Nanjing Jiancheng Biotechnology Research Institute (item numbers A003-1 and A001-3), the GSH reagent kit is purchased from Biyuntian Biotechnology Co., Ltd. (item number S0053) and the MPO reagent kit is purchased from Shanghai Enlink Biotechnology Co., Ltd. (item number ML-E-3168).

Cardiac function indicator detection

Collect blood from the tail vein of mice, centrifuge to separate plasma and detect the NT-proBNP level using an ELISA reagent kit (Shanghai Enlink Biotechnology Co., Ltd., item number ML-E-202205703). Each sample is set up with duplicate wells, and the concentration is calculated from the standard curve.

Cardiac ultrasound examination

Use the high-frequency animal ultrasound imaging system (VisualSonics Vevo F2, Canada) to perform cardiac ultrasound examination on mice. After anesthetizing the mice with 1.5% isoflurane (Shenzhen Ruiwode Life Science Technology Co., Ltd., item number R510-22),

remove the hair from the chest and measure the left ventricular end-diastolic inner diameter (LVDs), left ventricular end-systolic inner diameter (LVd) and left ventricular ejection fraction (LVEF) in the two-dimensional M-mode ultrasound mode. Each indicator is measured continuously over 3 cardiac cycles, and the average value is taken. All operations and analyses are completed by the same technician who is unaware of the group assignment.

Gut microbiome analysis

DNA extraction and sequencing

Collect fecal samples from each group of mice and store them at -80°C. Use the QIAamp PowerFecal Pro DNA Kit (Qiagen, Germany, item number 51804) to extract total DNA from the feces. Perform PCR amplification of the V3-V4 region of the 16S rRNA gene using the primer sequences shown in table 1. After library construction, conduct paired-end 250 bp sequencing on the Illumina NovaSeq 6000 platform (USA).

Bioinformatics analysis

The original sequences are filtered for low quality using Trimmomatic (version 0.39). Use the QIIME2 software (version 2022.2) for DADA2 denoising, feature table construction and classification annotation (reference database: SILVA v138). In terms of sequencing depth, an average of 85,000 valid sequences were obtained from each sample. The normalization method is to level all samples to 40,000 sequences/sample before conducting α -diversity (Chao1, Shannon, Simpson) and β -diversity (Bray-Curtis distance, Principal Coordinate Analysis, PCoA) analyses. Differentially abundant microbiota are identified using LEfSe analysis (linear discriminant analysis LDA threshold greater than 2.0) and Wilcoxon rank sum test ($P < 0.05$, FDR correction).

Histopathological analysis of cardiac tissue

After the mice were sacrificed, the cardiac tissues were collected and fixed in 4% paraformaldehyde (Shanghai Youbiao Technology Co., Ltd., item number YB1001) for 48 hours. The tissues were then dehydrated with gradient ethanol and embedded using a paraffin embedding machine (Jinhua Jili Medical Equipment Co., Ltd., model LP-500) and sectioned using a tissue sectioning machine (Shenyang Hengsong Technology Co., Ltd., model HS-TP-G) (thickness 5 μ m). HE staining and Masson staining (Beijing Solleva Biotechnology Co., Ltd., item number G1340) were performed to observe the myocardial structure, inflammatory infiltration and degree of myocardial fibrosis. In the Masson staining, collagen fibers were blue and myocardial fibers were red. The percentage of fibrotic area was measured using ImageJ (v1.53c, National Institutes of Health, USA), and the average of 3 non-consecutive sections/samples was calculated. Image acquisition was completed under a fluorescence microscope (Shanghai Aono Optics Co., Ltd., model AN-620).

qRT-PCR detection

Cardiac tissues were taken and total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, USA, item number 15596026). The RNA concentration and purity ($A_{260}/A_{280} > 1.8$) were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). 1 μ g of total RNA was used and reverse transcription was performed using the HiScript III RT SuperMix reverse transcription kit (Nanjing Novonta Biotechnology Co., Ltd., item number R323-01). qRT-PCR reactions were conducted using ChamQ Universal SYBR qPCR Master Mix (Nanjing Novonta Biotechnology Co., Ltd., item number Q711-02) on a PCR analyzer (Shenzhen Liangyi Technology Co., Ltd., model HM-P16). The reaction conditions were: 95°C for 3 minutes of pre-denaturation; 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds for 40 cycles; and verification of amplification specificity using melting curve analysis. β -actin was used as the internal reference and the relative expression of the target gene was calculated using the $2^{-\Delta\Delta C_t}$ method. Three technical replicates were used for each sample. The primer sequences for the target gene are shown in table 1. The experimental steps refer to the reference (Zhang *et al.*, 2025a).

Western blot detection

Total protein from the cardiac tissues was extracted using RIPA lysis buffer (Shanghai Luxun Biotechnology Co., Ltd., item number RC21237) and proteinase inhibitors (Hangzhou Anluze Biotechnology Co., Ltd., item number ALZ230039) and phosphatase inhibitors (Shanghai Xitang Biotechnology Co., Ltd., item number G0362) were added. Protein concentration was determined using the BCA protein quantification kit (Birny Tian Biotechnology Co., Ltd., item number P0012). 30 μ g of total protein was used and after 10% SDS-PAGE electrophoresis (Bio-Rad Mini-PROTEAN Tetra system, USA, 80 V constant voltage, 120 minutes), it was wet transferred to a PVDF membrane (Merck Millipore, Germany, item number IPVH00010, 300 mA, 90 minutes). Incubate with 5% skimmed milk for 2 hours. Then, add ZO-1 primary antibody (Abcam, UK, item number ab221547, dilution ratio 1:1000), Occludin primary antibody (Abcam, UK, item number ab216327, dilution ratio 1:1000) and Claudin-1 primary antibody (Abcam, UK, item number ab15098, dilution ratio 1:1000). Incubate at 4°C overnight. The next day, wash the membrane 3 times with TBST for 10 minutes each time, add HRP-labeled secondary antibody (Abcam, UK, item number ab205718, dilution ratio 1:5000) and incubate at room temperature for 1 hour. After washing the membrane, use ECL chemiluminescent solution (Thermo Fisher Scientific, USA, item number 34580) for development. Analyze the gray value of the bands using ImageJ software (v1.53c) and calculate the relative protein expression using β -actin primary antibody (Abcam, UK, item number ab8226, dilution ratio 1:5000) as the internal reference.

Table 1: Primer sequences for RT-qPCR.

Gene	Forward primer	Reverse primer
ZO-1	TGCTAATGCCTCGGAAAGAGATGAC	GCTGTGGAGACTGCGTGGGAATG
Occludin	TGGCTATGGAGGCGGCTATGG	ACTAAGGAAGCGATGAAGCAGAAGG
Claudin-1	GCTGGGTTTCATCCTGGCTTCTC	CCTGAGCGGTCACGATGTTGTC
Zonulin	GCTGTTGTCACTCTCCTGCTCTG	CGGCAGCGATAGCGAACCAAG
MMP3	GACGATGATGAACGATGGACAGAG	GCCTTGGCTGAGTGGTAGAGTC
Fibronectin	CGAAGTCAGTGTCTATGCTCTCAAG	GGTCTCTGTAGCGTCCGTCAC
β -actin	CTGGCACCACACCTTCTACAATGAG	AGAGGCATACAGGGACAGCACAG

Statistical analysis

Data were processed using SPSS 27.0 and GraphPad Prism 16.0 software. The data were presented as mean $\pm$ standard deviation. One-way analysis of variance (ANOVA) was used for comparisons among multiple groups and pairwise comparisons were conducted using Tukey's post hoc test. The Levene method was used for homogeneity of variance test and Welch's ANOVA and Games-Howell test were used when the variances were not homogeneous. $P < 0.05$ was considered the significance level. Kruskal-Wallis test was used to compare the differences in α diversity among intestinal microbiome groups and PERMANOVA (999 permutation tests) was used for β diversity. The different bacterial groups were identified by LEfSe analysis ($LDA > 2.0$, $P < 0.05$) and DESeq2 ($|\log_2FC| > 1$, $FDR < 0.1$) in combination.

RESULTS**Adverse events and mortality**

During the entire experimental period, no mouse deaths occurred. All mice survived to the end of the experiment, and no serious adverse events (such as persistent dyspnea, severe arrhythmias, or behavioral abnormalities) were observed in any group.

The composition of the intestinal microbiota

16S rDNA sequencing analysis showed no significant differences in the Chao1, Simpson and Shannon indices among the groups (Fig. 1A-C). The principal coordinate analysis (PCoA) diagram indicated that the intestinal microbial community of the Xinshuaifang group (G3) clustered more closely with the control group (G1), while the model group (G2) was significantly separated from the control group (Fig. 1D).

At the phylum level, the relative abundance of the phylum *Verrucomicrobia* in the model group (G2) was significantly lower than that in the control group (G1); the relative abundance of the *Verrucomicrobia* phylum in the Xinshuaifang group (G3) significantly increased (Fig. 1E). At the genus level, the relative abundance of the *Akkermansia* genus in the model group (G2) was significantly lower than that in the control group (G1); the relative abundance of this genus in the Xinshuaifang group (G3) significantly increased (Fig. 1F, H). LEfSe analysis revealed significant differences in the genera such as *Alistipes*, *Turicibacter*, *Lachnospiraceae* and *Akkermansia* among the groups (Fig. 1G).

Cardiac muscle oxidative stress indicators

Compared with the control group (G1), the content of MDA and GSH in the heart tissue of the model group (G2) was significantly increased ($P < 0.05$), while the activity of SOD and the level of MPO were significantly decreased ($P < 0.05$) (Fig. 2).

Compared with the model group (G2), the content of MDA and GSH in the mice of the Xinshuaifang group (G3), the AHF + intervention FMT group (G5) and the AHF + Xinshuaifang + antibiotic group (G6) was significantly decreased ($P < 0.05$) and the activity of SOD and the level of MPO were significantly increased ($P < 0.05$). The magnitude of change was the greatest. The indicators of the control FMT group (G4) and the antibiotic control group (G7) showed no significant difference compared with the model group.

Cardiac function and myocardial fibrosis

Compared with the control group (G1), the LVDd and LVDs of the model group (G2) mice significantly increased, while LVEF significantly decreased and the plasma NT-proBNP level significantly increased ($P < 0.05$) (Fig. 3A-D).

Compared with the model group (G2), the LVDd, LVDs and NT-proBNP levels of the Xinshuaifang group (G3), the AHF + intervention FMT group (G5) and the AHF + Xinshuaifang + antibiotic group (G6) mice were all significantly lower and LVEF significantly increased ($P < 0.05$). The change amplitude was the greatest in the Xinshuaifang group (G3). The cardiac function indicators of the control FMT group (G4) and the antibiotic control group (G7) did not differ significantly from those of the model group.

The results of HE and Masson staining (Fig. 3E) showed that the myocardial fibers in the control group mice were neatly arranged, with no obvious inflammatory cell infiltration or collagen deposition. The myocardial fibers of the model group mice were disordered, with inflammatory cell infiltration and increased myocardial fibrosis, and the percentage of collagen fiber area was significantly increased ($P < 0.05$). The myocardial tissue structure of the Xinshuaifang group and the intervention FMT group improved, with reduced inflammatory infiltration and significantly reduced fibrosis area ($P < 0.05$).

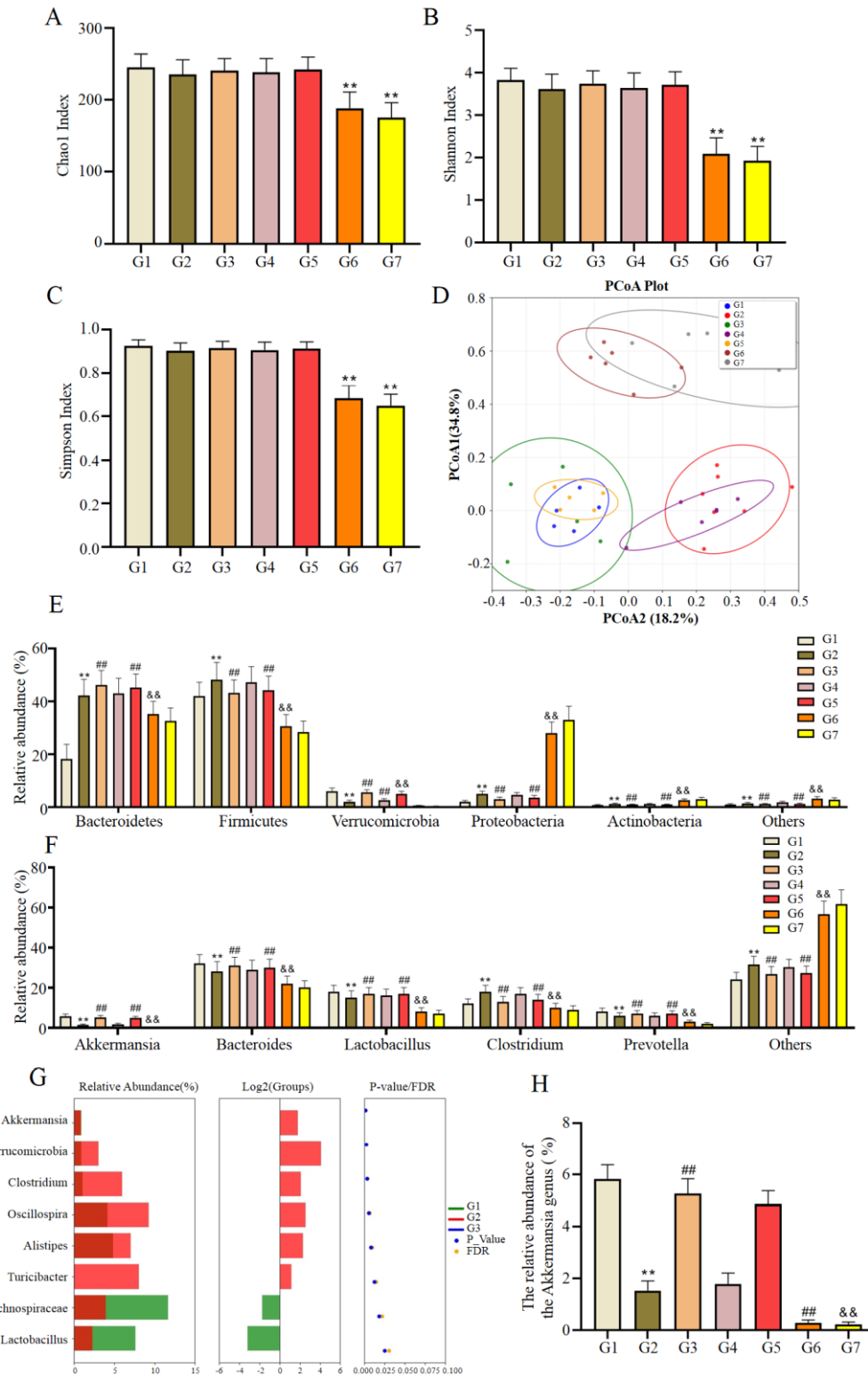


Fig. 1: The composition of the intestinal microbiota

Note: (A) Chao1 Index; (B) Shannon Index; (C) Simpson Index; (D) Principal Coordinates Analysis (PCoA) Plot; (E) Microbial composition at the phylum level; (F) Microbial composition at the genus level; (G) LefSe Analysis Plot; (H) Relative abundance of *Akkermansia* species.

**P < 0.01 compared with the G1, ###P < 0.01 compared with the G2, &&P < 0.01 compared with the G3.

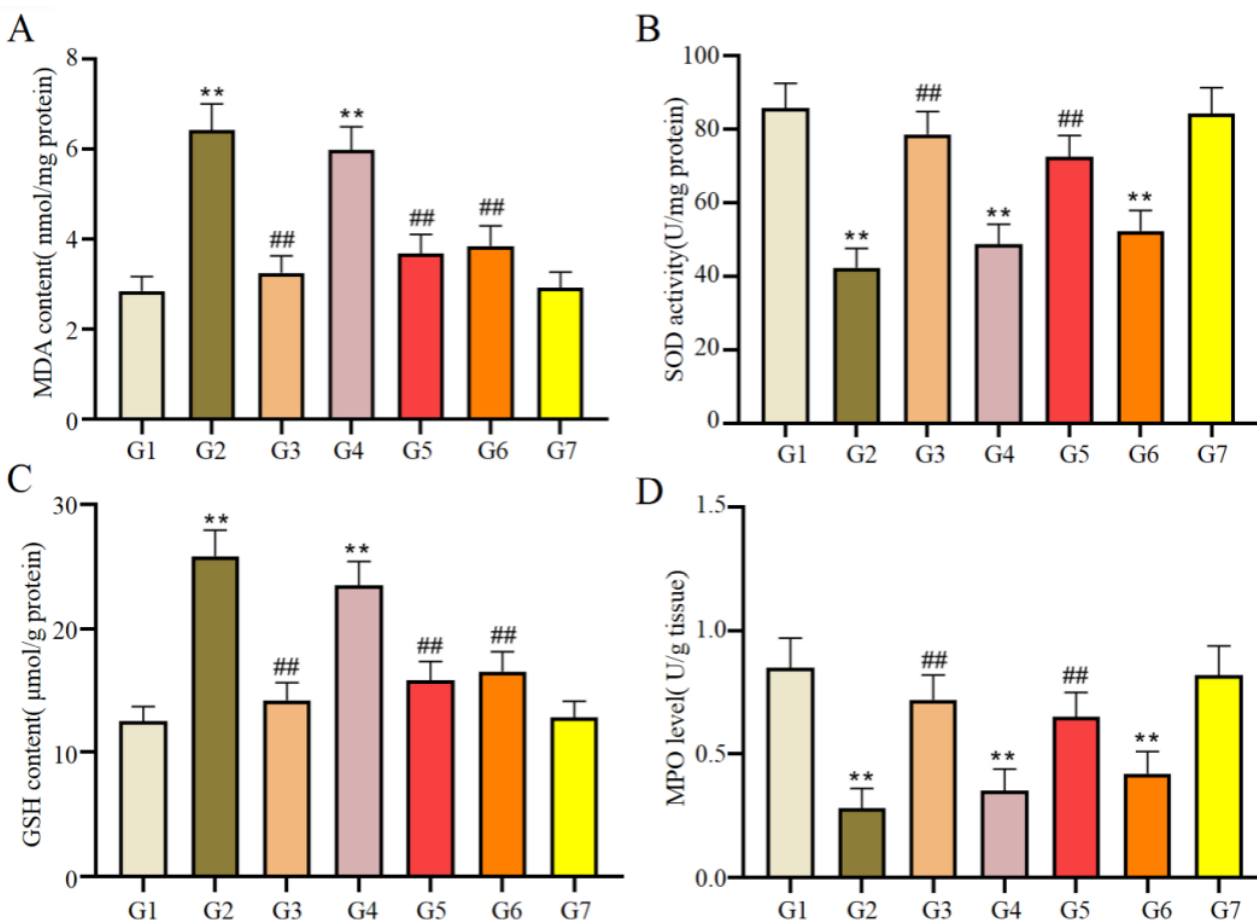


Fig. 2: Cardiac muscle oxidative stress indicators

Note: (A) MDA; (B) SOD; (C) GSH; (D) MPO.

** $P < 0.01$ compared with the G1, *** $P < 0.001$ compared with the G1, ## $P < 0.01$ compared with the G2.

Expression of tight junction proteins

Compared with the control group (G1), the mRNA and protein expression levels of ZO-1, Occludin and Claudin-1 in the model group (G2) were significantly decreased ($P < 0.05$), while the mRNA and protein expression levels of Zonulin were significantly increased ($P < 0.05$) (Fig. 4).

Compared with the model group (G2), the expression levels of ZO-1, Occludin and Claudin-1 in the Xinshuaifang group (G3), the AHF + intervention FMT group (G5) and the AHF + Xinshuaifang + antibiotic group (G6) were all significantly increased ($P < 0.05$), while the mRNA and protein expression levels of Zonulin were significantly decreased ($P < 0.05$). The change in the Xinshuaifang group (G3) was the greatest. The expression levels of tight junction proteins in the control FMT group (G4) and the antibiotic control group (G7) were not significantly different from those in the model group.

DISCUSSION

Xinshuaifang is composed of red ginseng, processed aconite root, ginseng, *Salvia miltiorrhiza*, *Poria cocos* and

Ophiopogon japonicus. Studies have shown that *Salvia miltiorrhiza* has the effect of promoting the proliferation of cardiac muscle cells in rats with myocardial infarction (Cao *et al.*, 2026); *Ginseng* and *ophiopogon japonicus* have been shown to reverse myocardial hypertrophy (Li *et al.*, 2021); and *poria cocos* can promote urine output in HF patients (Su *et al.*, 2023) and may exert protective and therapeutic effects in patients with cardiovascular disease. In terms of oxidative stress, the model group showed significantly increased MDA and GSH levels and significantly decreased SOD activity and MPO levels in cardiac tissue, indicating significant oxidative stress injury in AHF mice. After Xinshuaifang intervention, the above indicators were significantly improved: MDA and GSH levels decreased, while SOD activity and MPO levels increased. Notably, mice receiving fecal microbiota transplantation from Xinshuaifang-treated donors also showed similar improvements in oxidative stress, whereas those receiving transplantation from model group donors did not. Furthermore, after Xinshuaifang was combined with antibiotics to deplete the gut microbiota, a protective effect against oxidative stress was still observed, but it was less pronounced than that of Xinshuaifang alone.

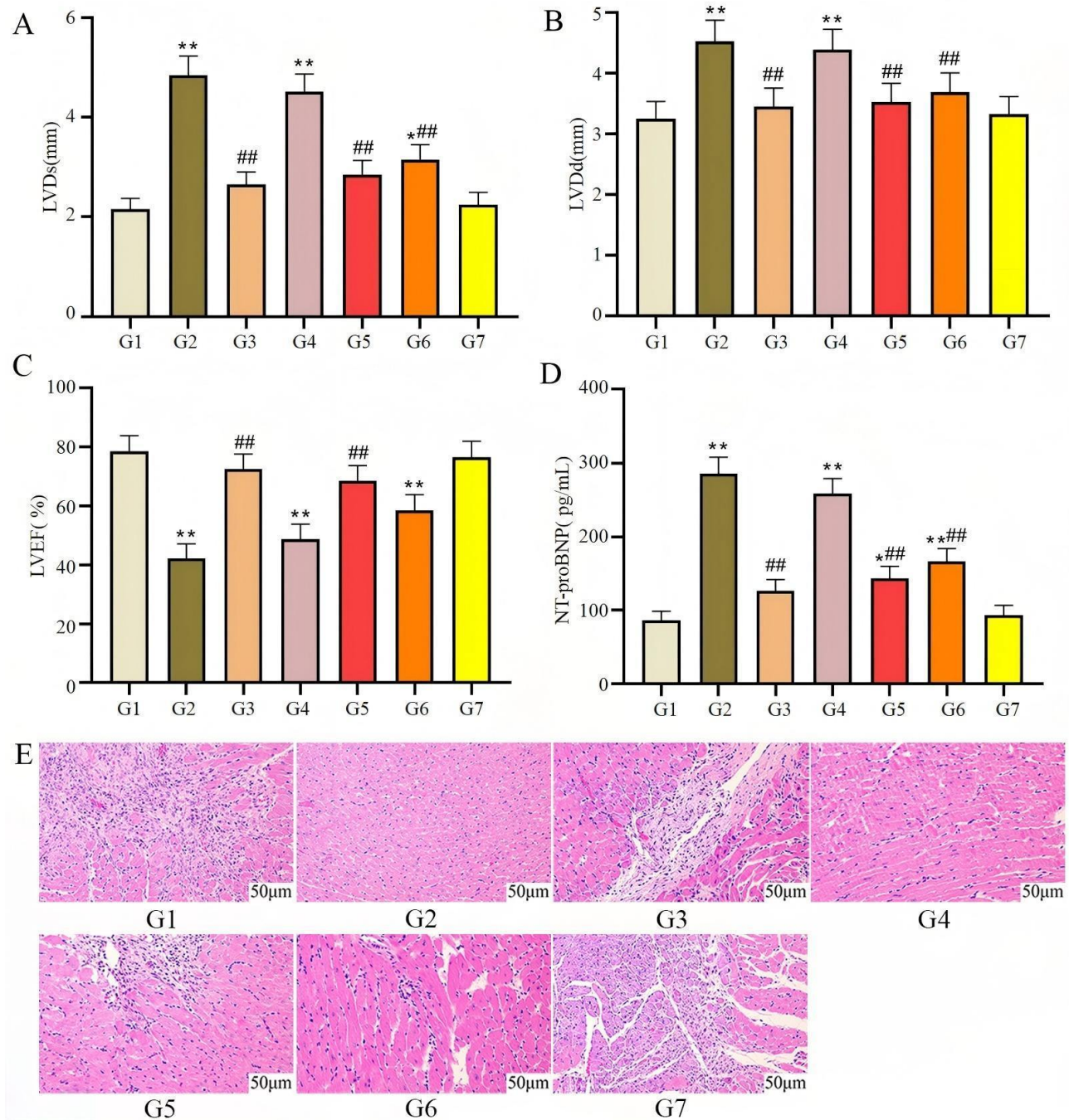


Fig. 3: Cardiac function and myocardial fibrosis.

Note: (A) LVDs; (B) LVDd; (C) LVEF; (D) NT-proBNP; (E) H and E staining.

* $P < 0.05$ compared with the G1, ** $P < 0.01$ compared with the G1, ### $P < 0.01$ compared with the G2.

These results suggest that the effect of Xinshuaifang on reducing oxidative stress in AHF mice is partially dependent on the gut microbiota but may also involve other mechanisms, and the specific mechanisms remain to be further studied. It has been reported that probiotics can improve intestinal barrier function, enhance intestinal microbial diversity, and reduce inflammation and oxidative stress, thereby reducing cardiovascular disease risk to a certain extent (Lopes et al., 2026; Pavlidou et al., 2022).

However, whether Xinshuaifang can improve intestinal microbiota and contribute to myocardial functional remodeling in AHF is worthy of further investigation. In this study, 16S rDNA sequencing analysis showed no significant differences in the Chao1, Simpson, and Shannon indices among the groups, suggesting that Xinshuaifang intervention did not significantly alter the α -diversity of the gut microbiota in AHF mice.

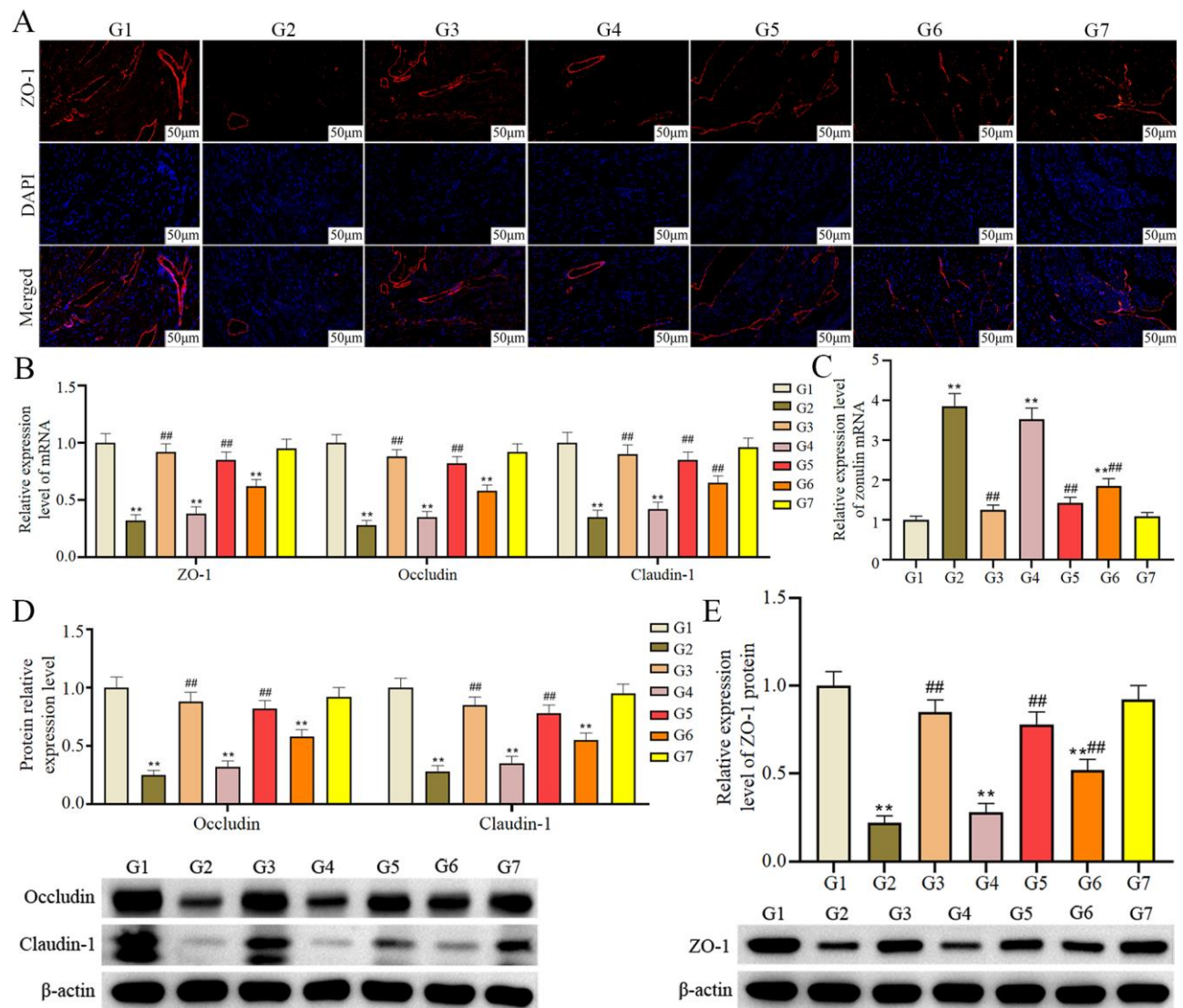


Fig. 4: Expression of tight junction proteins.

Note: (A) Immunofluorescence detection; (B) mRNA expression of ZO-1, occludin and claudin-1; (C) mRNA expression of zonulin; (D) protein expression of occludin and claudin-1; (E) protein expression of ZO-1.

**P < 0.01 compared with the G1, ##P < 0.01 compared with the G2.

However, PCoA analysis showed that the β -diversity of the gut microbiota in the Xinshuaifang group was more similar to that of the control group, while the model group was clearly separated from the control group, suggesting that Xinshuaifang can restore the dysregulated gut microbiota structure of AHF mice toward normal. Further analysis revealed that the relative abundance of *Verrucomicrobia* and its representative genus, *Akkermansia*, in the model group was significantly lower than in the control group, whereas after Xinshuaifang intervention, the relative abundance of both taxonomic levels significantly rebounded. LEfSe analysis further confirmed significant differences in *Akkermansia* and other genera among the groups. These results indicate that Xinshuaifang can partially restore the gut microbiota structure in AHF mice, especially by enriching protective *Verrucomicrobia* and

Akkermansia. Based on the above observations and existing literature, this study hypothesizes that the Qi-tonifying agent *Astragalus membranaceus* in the Xinshuai formula can alleviate intestinal ischemia and hypoxia caused by heart failure by enhancing myocardial contractility and improving systemic and local intestinal blood circulation. This, in turn, reduces calcium overload and restores mitochondrial function through the PKC ϵ -Namt-Sirt5 axis, thereby providing sufficient oxygen and glucose to intestinal epithelial cells and promoting mucin synthesis (Feng *et al.*, 2025; Liu *et al.*, 2025b). Furthermore, after fermentation by the microbial community, the metabolites of *Radix Ophiopogonis* in the formula can serve as precursors for monosaccharides, oligopeptides, amino acids, and short-chain fatty acids, directly providing energy metabolism substrates for mucin

glycosylation (Baker et al., 2024; Cante et al., 2024). Through the above two potential mechanisms, Xinshuaifang may restore the intestinal mucin-secreting capacity of AHF mice, thereby creating a microenvironment conducive to the growth of beneficial bacteria, such as *Verrucomicrobia* and *Akkermansia*. However, it should be emphasized that these mechanistic hypotheses remain speculative, as the present study did not directly measure short-chain fatty acid levels, mucin production, or intestinal oxygenation. Future studies are needed to test these hypotheses directly.

Research on Jiashen Decoction found, through integrated metabolomics analysis, that this formula enriched *Lactobacillus* and *Lachnospiraceae*, thereby regulating gut microbiota to improve heart failure (Cui et al., 2023). In contrast, the present study found that Xinshuaifang significantly increased *Verrucomicrobia* and *Akkermansia*, suggesting that different formulas may exert their effects by enriching different beneficial bacteria. Research on Zhilong Huoxue Tongyu capsule revealed, through metabolomics, that its effect is related to myocardial energy metabolism, involving the citric acid cycle, butyrate metabolism and fatty acid biosynthesis pathways (Chen et al., 2025); whereas the present study focused on the gut-heart axis, finding that Xinshuaifang exerts its effects by regulating gut microbiota and repairing the intestinal barrier. This difference reflects that traditional Chinese medicine formulas may exert cardioprotective effects through multiple pathways and targets. However, the present study did not perform metabolomics analysis, and the intervention period was short, failing to reach the threshold for significant improvement in overall diversity; thus, no significant change in α -diversity was observed. Future research needs to integrate metabolomics analysis, extend the intervention period, use germ-free animal models to verify causal relationships and promote the chemical standardization of Xinshuaifang.

Relevant studies have shown that antibiotic experiments can verify the mediating role of the gut microbiota in the cardioprotective effects of traditional Chinese medicine formulas (Zhong et al., 2024). On this basis, the present study adopted a "bidirectional validation" strategy to evaluate the causal role of gut microbiota in the efficacy of Xinshuaifang: on the one hand, through fecal microbiota transplantation (FMT) from the "gain" perspective and on the other hand, through antibiotic intervention from the "loss" perspective. The FMT experiment found that AHF mice receiving fecal microbiota transplantation from Xinshuaifang-treated donors (Group G5) were significantly superior to mice receiving fecal microbiota transplantation from model group donors (Group G4) in terms of oxidative stress, cardiac function and tight junction protein expression, indicating that the gut microbiota reshaped by Xinshuaifang is sufficient to transmit the cardioprotective effect. The antibiotic

experiment found that broad-spectrum antibiotic pretreatment significantly weakened the protective effect of Xinshuaifang (Group G6 vs. Group G3), supporting the conclusion that Xinshuaifang's efficacy depends on the gut microbiota. The two experiments mutually supported each other from both positive and negative directions, strongly supporting the causal inference that "gut microbiota mediates the cardioprotective effect of Xinshuaifang." However, it should be noted that the FMT experiment could not determine which specific genera played a key role, nor could it detect the degree of colonization of the donor microbiota in the recipients. The antibiotic experiment had the potential for nonspecific depletion and off-target effects. Yet, some indicators in Group G6 continued to improve, suggesting that Xinshuaifang may have direct cardioprotective effects independent of the gut microbiota. Future research needs to use germ-free mouse models, specific bacterial colonization experiments, and combined metabolomics analysis to further rigorously verify the causal relationship.

Cardiac function test results showed that the model group had significantly increased LVDd, LVDs and NT-proBNP levels and significantly decreased LVEF, indicating successful establishment of the AHF model. After Xinshuaifang intervention, the above cardiac function indicators were significantly improved. Intervention FMT also improved cardiac function in AHF mice, while control FMT had no effect. Histopathological staining further confirmed that Xinshuaifang and intervention FMT reduced myocardial fiber disarray, decreased inflammatory cell infiltration and collagen deposition and reduced the area of myocardial fibrosis. These results indicate that Xinshuaifang can improve cardiac function and reduce myocardial fibrosis in AHF mice and this effect can be transmitted through fecal microbiota transplantation, suggesting that the gut microbiota plays a key mediating role. The reason for this is that when AHF occurs, the cardiac output of mice decreases and blood is preferentially supplied to important organs such as the heart and brain, which in turn reduces the blood supply to the digestive tract, leading to intestinal ischemia and intestinal wall edema, further damaging the tight junction proteins between intestinal epithelial cells, resulting in a significant increase in intestinal permeability, making it easier for the bacterial endotoxin component lipopolysaccharide (LPS) to enter the blood from the intestinal cavity (Deleemans et al., 2022; Liao et al., 2022). LPS entering the blood triggers systemic low-grade chronic inflammation in AHF mice, releasing a large number of pro-inflammatory cytokines, thereby further aggravating heart damage and forming a vicious cycle (Zhang et al., 2025b; Zhang et al., 2021). These findings suggest that active ingredients in Xinshuaifang (e.g., ginsenosides) may be fermented by beneficial bacteria such as *Verrucomicrobia* to produce short-chain fatty acids (Xu et al., 2025), mainly including acetic acid, propionic acid and butyric acid. These short-

chain fatty acids may activate signaling pathways such as the AMPK/mTOR pathway to upregulate the expression of tight junction proteins, thereby effectively reducing intestinal permeability and may stimulate MUC2 protein to enhance mucosal barrier function, thus inhibiting LPS absorption and the initiation of systemic inflammation and oxidative stress from the source (Wang *et al.*, 2022; Yang *et al.*, 2025). At the same time, the drug's active ingredient, Danshen, can protect mice from intestinal barrier damage by downregulating the GSDMD-mediated pyroptosis pathway, thereby creating a favorable environment for beneficial bacteria (Zhang *et al.*, 2025c). However, the present study did not directly measure short-chain fatty acid (SCFA) levels in feces or plasma, nor did it assess the activation status of any proposed signaling pathways. Therefore, the mechanistic links between changes in the microbiota, tight junction protein expression, and cardiac protection remain speculative and require direct experimental validation. Furthermore, this study used a control group of mice treated with Xinshuaifang (Xinshuaifang) to eliminate intestinal microbiota after broad-spectrum antibiotic treatment. The results showed no significant changes in oxidative stress markers or cardiac function indicators in these mice, further confirming that Xinshuaifang primarily exerts therapeutic effects by regulating the "gut-heart axis."

To investigate changes in intestinal barrier function, this study assessed tight junction protein expression. The results showed that, compared with the control group, the model group had significantly decreased mRNA and protein expression levels of ZO-1, Occludin, and Claudin-1, and significantly increased Zonulin expression (an indicator of intestinal permeability), suggesting intestinal barrier damage in AHF mice. After Xinshuaifang intervention, the expression of ZO-1, Occludin, and Claudin-1 significantly rebounded, and Zonulin expression significantly decreased, indicating the restoration of intestinal barrier integrity. Intervention FMT also upregulated tight junction protein expression and decreased Zonulin levels, while control FMT had no such effect. These results suggest that Xinshuaifang repairs intestinal barrier function by regulating gut microbiota and upregulating tight junction protein expression, thereby potentially reducing endotoxin translocation and the systemic inflammation and oxidative stress it induces. Based on the results of this study and previous literature, this may be because SCFAs produced after Xinshuaifang regulates the microbiota directly promote the transcriptional activity of tight junction protein genes such as Occludin and ZO-1 through epigenetic regulation, thereby upregulating their mRNA and protein expression, increasing intestinal immunoglobulins and digestive enzyme activity and maintaining the normal structure and function of intestinal villi, ultimately reducing the risk of harmful substances entering the bloodstream and promoting myocardial remodeling in AHF mice (Kuo *et al.*, 2021; Wang *et al.*, 2023b). In the study (Peng *et al.*,

2024), it was also found that, by administering Chinese medicine to rheumatoid arthritis mice, Occludin and Claudin-1 expression increased and Zonulin expression decreased, thereby restoring intestinal barrier damage in the mouse colon, further verifying the results of this study.

In this study, Xinshuaifang not only improved cardiac function and reduced myocardial oxidative stress but also increased the abundance of beneficial gut microbiota, including *Verrucomicrobia* and *Akkermansia*. Fecal microbiota transplantation from Xinshuaifang-treated donors reproduced most of the protective effects in recipient mice, while antibiotic depletion of gut microbiota eliminated the therapeutic benefits. Furthermore, both Xinshuaifang and FMT restored the expression of intestinal tight junction proteins and reduced zonulin levels, suggesting improved intestinal barrier integrity. Together, these pieces of evidence indicate that the gut-heart axis is a functional therapeutic target for Xinshuaifang in the treatment of AHF. From a translational perspective, gut microbiota composition, particularly the abundance of *Akkermansia*, may serve as a potential pharmacodynamic biomarker in future clinical trials of Xinshuaifang. Fecal microbiota transplantation or probiotic/prebiotic strategies that mimic the microbiota changes induced by Xinshuaifang could also be explored as adjunctive therapies for AHF. However, direct clinical extrapolation requires caution given the simplified gut ecosystem of laboratory mice compared to the complex human microbiota.

The limitations of this study include the following. First, the animal sample size was small, which may limit statistical power and the generalizability of the conclusions. Second, the intervention period was short, preventing assessment of long-term efficacy or safety. Third, the Xinshuaifang used in this study was not validated for consistency of active ingredients between batches beyond basic HPLC fingerprinting and the lack of comprehensive chemical standardization may affect the reproducibility of the results. Fourth and most importantly, the proposed mechanisms involving short-chain fatty acid production and downstream signaling pathways remain speculative because the present study did not directly measure SCFA levels in feces or plasma, nor did it assess pathway activation (e.g., AMPK/mTOR, which was mentioned in the introduction but not tested here). Fifth, this study failed to directly associate specific microbiota changes with functional outcomes such as SCFA production or tight junction protein expression. Sixth, it was not clearly documented whether blinding was implemented for all outcome assessments, so detection bias may exist. Therefore, the mechanistic links between changes in the microbiota, intestinal barrier integrity, and cardiac protection, while supported by correlational evidence and bidirectional validation (FMT and antibiotics), require direct experimental confirmation.

Future research needs to use larger sample sizes, longer treatment periods, chemically standardized herbal extracts and direct measurements of SCFAs and pathway activation to validate the conclusions of this study and advance the clinical translation of gut-heart axis therapy.

CONCLUSION

In conclusion, Xinshuaifang improved cardiac function, alleviated myocardial oxidative stress and fibrosis in AHF mice, and modulated gut microbiota composition. FMT and antibiotic experiments supported a causal role of the gut microbiota in mediating these effects and Xinshuaifang upregulated ZO-1/Occludin expression, suggesting improved intestinal barrier integrity. However, this study did not directly measure SCFA levels in feces or plasma, so the proposed SCFA-mediated mechanism remains speculative. The mechanistic link between changes in the microbiota and regulation of the gut-heart axis remains to be validated. These findings provide a preliminary experimental basis for further research, but direct clinical translation will require additional validation, including metabolite measurement and larger-scale studies.

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Author's contribution

Kun Wang: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Visualization; Gaochao Wang: Conceptualization, Resources, Supervision, Writing – review & editing, Funding acquisition. All authors participated in data interpretation and manuscript revision and agreed to the submission and publication of the final version.

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Data availability statement

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Ethical approval

This experiment was approved by the Animal Ethics Committee of Changzhou Hospital of Traditional Chinese Medicine (Approval Number: QJ20255045) and all operations followed the "Guidelines for the Care and Use of Laboratory Animals". This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

Conflict of interest

There are no conflicts to declare.

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