Drug Design, Development and Therapy

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ORIGINAL RESEARCH

# Efficacy and Mechanism of Qianshan Huoxue Gao in Acute Coronary Syndrome via Regulation of Intestinal Flora and Metabolites

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**Purpose:** To study the efficacy of Qianshan Huoxue Gao (QS) in treating acute coronary syndrome (ACS) and to explore the mechanism of action from the perspective of intestinal flora regulation.

**Methods:** Male Sprague–Dawley rats were divided into control, model, QS, and atorvastatin groups; except for the control group, rats underwent ligation of the left anterior descending branch of the coronary artery. Following treatment for 28 days, cardiac function was evaluated using an echocardiographic assay; ELISAs for serum creatine kinase isoenzyme (CK-MB), cardiac troponin I (cTnI), high-sensitivity C-reactive protein (hs-CRP), interleukin (IL)-2 (IL-2), IL-6, and tumor necrosis factor-α (TNF-α); assessment of cardiac enzymes and inflammatory response; hematoxylin and eosin (HE) staining for histopathological changes in the heart, skin, and viscera; 16S rRNA gene sequencing for intestinal flora diversity and structural differences analysis; and we further investigated intestinal contents using metabolomics.

**Results:** Compared with controls, CK-MB and cTnI were increased ($P<0.01$); ejection factor and fractional shortening were decreased ($P<0.01$); left ventricular internal end-diastolic dimension and left ventricular internal end-systolic dimension were increased ($P<0.01$); and IL-2, IL-6, TNF-α, and hs-CRP were increased in the model group. Myocardial damage and inflammation were also observed by HE staining. QS improved these indexes, similar to the atorvastatin group; therefore, QS could effectively treat ACS. QS modulates the structure and abundance of the intestinal flora in ACS model rats, among which *Bacteroides, Lactobacillus*, and *Rikenellaceae_RC9_gut_group* are associated with cardiovascular disease. Metabolomics revealed that the intestinal metabolite content changed in ACS, with ethanolamine (EA) being the most relevant metabolite for ACS treatment by QS. EA was significantly positively correlated with *Eubacterium xylanophilum group, Ruminococcus, unclassified f__Oscillospiraceae, Intestinimonas, Eubacterium siraeum group, Lachnospiraceae NK4A136 group*, and *norank f__Desulfovibrionaceae*.

**Conclusion:** QS can effectively treat ACS and can restore regulation of the intestinal flora. EA may be the primary metabolite of QS, exerting a therapeutic effect in ACS.

**Keywords:** traditional Chinese medicine, acute coronary syndrome, 16S rRNA, ethanolamine, topical paste

## Introduction

In addition to Chinese oral medicine, there are a variety of treatments, such as Chinese Herbal Compress and Acupuncture, that can reduce or avoid the side effects of drugs taken internally, which is one of the many advantages of Chinese medicine and meridians.[1,2] Qianshan Huoxue Gao (QS) is a black ointment for external use by spreading the Chinese medicine on a cloth using modern extraction technology. QS can activate blood stasis, relax tendons, and relieve swelling and pain.[3–5] It is prepared from 20 herbs, including Eupolyphaga Steleophaga (Tubiechong), Rhei Radix et Rhizoma (Dahuang), Notoginseng Radix et Rhizoma (Sanqi), Corydalis rhizome (Yanhusuo), Draconis sanguis (Xuejie), Dipsaci radix (Xuduan), Phellodendri chinensis cortex (Huangbai), Olibanum (Ruxiang), Myrrha (Moyao), Catechu

Received: 8 November 2022
Accepted: 14 February 2023
Published: 22 February 2023



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(Ercha), Asari radix et rhizome (Xixin), Homalomenae rhizome (Qiannianjian), Cremastrae pseudobulbus pleiones pseudobulbus (Shancigu), Alismatis rhizome (Zexie), Cornu Naemorhedi (Shanyangjiao), Aucklandiae radix (Muxiang), Bletillae rhizoma (Baiji), Angelicae dahuricae radix (Baizhi), Cinnamomi ramulus (Guizhi), and Notopterygii rhizoma et radix (Qianghuo).

Modern pharmacological studies have found that the herbs in QS have a variety of anti-atherosclerosis (AS) mechanisms. The active ingredients of the Tubiechong, Sanqi, Xuduan, Zexie, Huangbai, and Shancigu can reduce triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and total cholesterol (TC) levels, and increase high-density lipoprotein cholesterol (HDL-C) levels.[6–11] Among them, Chuanxianoside VI can improve the ability of cells to scavenge free radicals and reduce the secretion of inflammatory factors.[12] Colchicine is contained in Shancigu, which inhibits inflammatory factors such as interleukin (IL)-6 to exert anti-inflammatory effects and suppress AS.[13] Dahuang lowers lipids, stabilizes plaques, regulates foam cell formation and macrophage polarization, and inhibits inflammatory responses mediated by various cytokines.[14] Xuejie reduces tumor necrosis factor (TNF)-α and IL-6 in rats with myocardial ischemia-reperfusion injury.[15] Boswellic acid, the active ingredient of Ruxiang, has a wide range of anti-inflammatory effects.[16] Moyao inhibits platelet aggregation and activation.[17] Guizhi upregulates p27 expression to prevent atherosclerosis.[18] Yanhusuo exerts anti-inflammatory effects by inhibiting the nuclear factor kappa B (NF-κB) pathway and reducing TNF-α, IL-6, and IL-1β.[19] Muxiang can inhibit the expression of IL-1β, and down-regulate intercellular adhesion molecule-1 and P-selectin, achieving anti-inflammatory effects.[20] Some studies have found that QS can be used to treat carotid plaques as it reduces patients' left plaque Crouse score, bilateral intima-media thickness, right peak systolic velocity and end diastolic velocity, and high-sensitivity C-reactive protein (hs-CRP), and relieve headaches and other symptoms, which is superior to using atorvastatin alone, and has no significant adverse effects.[21]

In our previous study, we found that QS slowed down the progression of AS; it reduced TC, TG, and LDL-C in the serum of ApoE/- mice ($P<0.05$), and significantly increased adiponectin (ADPN) expression ($P<0.05$). It is suggested that QS may slow the development of AS by regulating ADPN in terms of lipid metabolism and anti-inflammation. AS can involve multiple systems throughout the body, especially the arterial vascular beds of vital organs, and can lead to the development of acute coronary syndrome (ACS). ACS is an acute ischemic syndrome of the heart caused by thrombosis due to rupture or erosion of unstable atheromatous plaques in the coronary arteries, a group of clinical symptoms caused by acute myocardial ischemia.[22] As a severe type of coronary heart disease, ACS is characterized by high incidence, rapid progression, high death rate, and poor prognosis, and the death rate is increasing year by year.[23] Therefore, we further investigated the effect of QS on ACS.

Intestinal flora is one of the factors affecting the inflammatory state in the blood vessels, and restoring homeostasis of the intestinal flora is one of the options available to combat cardiovascular disease, including ACS.[24,25] Therefore, in this study, we used ligation of the left anterior descending branch of the coronary artery in Sprague–Dawley (SD) rats as a model of ACS, and used intestinal microbiology and metabolomics to lay the theoretical foundation to explore the efficacy and mechanism of QS in the treatment of ACS. The results of this study provide evidence for the topical treatment of ACS with traditional Chinese medicine.

# Materials and Methods

## Materials

### Equipment

Equipment included an animal ventilator (HX-200, Chengdu Taimeng Technology Co, Chengdu, China), bio-signal and pressure measurement system (RM6240BD, Chengdu Instrument Factory, Chengdu, China), small animal ultrasound imaging system (P6-VET, Jiangsu Dawei Medical Co., Jiangsu, China), gas chromatography time-of-flight mass spectrometry (GC-TOF/MS) system (Pegasus HT, Leco Corp., St. Joseph, MO, USA), Agilent 7890B gas chromatography (Gerstel, Muehlheim, Germany), Rxi-5 ms capillary column (30 m × 250 μm i.d., 0.25-μm film thickness; Restek Corporation, Bellefonte, PA, USA), and repeater Xstream electronic pipette (Eppendorf, Hamburg, Germany).

### Pharmaceuticals and Experimental Reagents

QS (Lot No. 20170504) was provided by Beijing Xiusheng Pharmaceutical Co. Atorvastatin calcium tablets (Pfizer Pharmaceuticals Ltd., Lot No. S56741), IL-2 (Lot No. R20210328), IL-6 (Lot No. R20210328), TNF-α (Lot No. R20210328), creatine kinase isozyme cardiac type (CK-MB) (Lot No. R20210327), and cardiac troponin I (cTnI) (Lot No. R20210327) kits were purchased from the Nanjing Jiancheng Institute of Biological Engineering. Sodium pentobarbital (2%), lidocaine, sodium chloride (0.9%), sodium penicillin, methoxyamine HCl, fatty acid methyl ester (C7-C30, FAMEs) standards, pyridine, and anhydrous sodium sulfate were obtained from Sigma-Aldrich (St. Louis, MO, USA). MSTFA (N-methyl-N-(trimethylsilyl)trifluoroacetamide) with 1% (vol/vol) trimethylchlorosilane (MSTFA, with 1% TMCS), methanol (Optima LC-MS), acetonitrile (Optima LC-MS), hexane, dichloromethane, chloroform, and acetone were purchased from Thermo-Fisher Scientific (FairLawn, NJ, USA). Ultrapure water was produced by a Mill-Q Reference system equipped with an LC-MS Pak filter (Millipore, Billerica, MA).

### Animals

Animals included 24 4-week-old male ApoE/- mice, body weight 18–22 g; ten 4-week-old male C57BL/6J mice; and 40 specific-pathogen free (SPF)-grade males SD rats, body weight 200–220 g. The animals were purchased from the China Academy of Food and Drug Administration, license number SCXK (Beijing) 2017-0005. They were housed in an SPF-grade laboratory at the Institute of Basic Theory of Traditional Chinese Medicine, Chinese Academy of Traditional Chinese Medicine, under the following conditions: temperature, 20–25 °C; humidity, 40%-60%; and a 12-h light/dark cycle. Rat chow was purchased from Beijing Co-operative Feed Co., Ltd. with production license number: Beijing Feeding Certificate (2018) 06073. All procedures were in accordance with the National Institute of Health Guide for the Use and Care of Laboratory Animals and were approved by the Animal Ethics Committee of the Institute of Basic Theory for Chinese Medicine (CACMS) (ethics approval No. 20200710b1600201).

## Method

### Effect of QS on AS

Twenty-four 4-week-old male ApoE/- mice, body weight 18–22 g, were randomly divided into four groups: the model group, the QS-low group (2.5 g/kg of topical application of QS, equivalent to 26 times ACD), the QS-high group (6 g/kg of topical application of QS, equivalent to 60 times ACD), and the atorvastatin group. Ten 4-week-old male C57BL/6J mice served as the control group. The hair was removed from the back of the mice in the QS-low and QS-high groups with an area of 2×4 cm. The QS was cut into 0.5×0.5 cm and 1.0×1.0 cm for low and high dose groups, and then applied to the skin. The atorvastatin group underwent gavage with 2.6 mg/mL according to 0.2 mL/10 g body weight. The control and model groups were given distilled water according to the same gavage volume. The body weight was recorded every 2 weeks, and the health status was also observed. Two weeks later, the mice were fasted without water overnight, and blood was taken from the eyes after anesthesia with ether and centrifuged at 6000 rpm to extract the serum. TC was determined by the COD-PAP method, TG by the GPO-PAP enzymatic method, HDL-C and LDL-C by a direct method, and ADPN was determined by an ELISA. The aortic vessels were extracted, and the skin of the treated area was taken in the QS-low and QS-high groups; the tissues were preserved in 4% paraformaldehyde solution. After optimal cutting temperature (OCT) embedding, the aortic vessels were frozen and sectioned for Oil Red O and hematoxylin and eosin (HE) staining. Skin tissues were paraffin-embedded and HE-stained.

### Effects of QS on ACS Model Rats

#### Grouping and Treatments

Establishment of the ACS model was performed by ligation of the left anterior descending branch of the coronary artery in SD rats.[26] Thirty male rats with successful modeling and survival were randomly divided into the model group, QS group, and atorvastatin group, with ten rats in each group. Atorvastatin was given at a dose of 10 mg/kg/day by gavage; saline gavage was given to the control group and the model group. QS (6 g/kg of QS, equivalent to 60 times ACD) was applied to the skin of the hair-removed area of the rats. It lasted for 28 days.

### General Observations

The changes in body weight, activity, hair luster, anorexia, and diarrhea of SD rats were observed and recorded. The body weight growth rate of rats in each group was calculated with the following formula: body weight growth rate = (final weight – starting weight)/starting weight*100%.

### Cardiac Ultrasound

After 28 days of the treatment, echocardiographic tests were performed to evaluate the cardiac function of SD rats. The SD rats were placed in the supine position on a thermostatic heating plate, and the extremities were fixed with adhesive tape. The heart rate (HR), ejection factor (EF), fractional shortening (FS), left ventricular internal end-diastolic dimension (LVDD), and left ventricular internal end-systolic dimension (LVDS) were measured using a small animal ultrasound imaging system.

### Myocardial Damage and Inflammatory Factor Testing

After 28 days of the treatment, rats were fasted without water for 12 h, anesthetized with 3% sodium pentobarbital intraperitoneally, and blood was taken from the abdominal aorta, which was centrifuged for 10 min at 1000 r/min. The serum was collected and snap-frozen in liquid nitrogen and stored at –80 °C. CK-MB, cTnI, hs-CRP, IL-2, IL-6, and TNF-α were detected using ELISA kits.

### HE Staining of Myocardial Histomorphology and Safety in the QS Group

After 28 days of the treatment, the heart left ventricle, skin, lung, spleen, and kidney of each group were fixed in a 10% neutral formaldehyde solution for 1 day, dehydrated in a conventional gradient, embedded in paraffin, sectioned with a thickness of 5 μm, and stained with HE. The morphology of myocardial tissue, skin, and organs at the site of application of QS was observed under the microscope, and the films were taken. Evaluation criteria of cardiac tissue lesions were as follows: myocardial fiber degeneration necrosis, myocardial fibrosis, and interstitial inflammatory cell infiltration were observed and rated as 0–4 points according to the degree of lesions from mild to severe. Normal was scored 0, mild or minimal amount was scored 0.5, mild or small amount was scored 1, moderate or more was scored 2, severe or large amount was scored 3, and a very severe or large amount was scored 4. All scores were accumulated, and the mean score "X ± SD" was calculated for each group. An independent sample *t*-test analysis was performed.

### Analysis of Intestinal Flora Diversity and Structural Differences by 16S Sequencing

The rats were anesthetized, a section of the rat ileum was dissected, and the feces were collected from the ileum and stored at –80 °C. The microbial genomic DNA was extracted from the fecal samples using the PowerSoil DNA Isolation Kit according to the instructions, amplified by 16S rRNA gene PCR and sequenced, and then high-throughput sequenced on the Illumina HiSeq 2500 platform using pair-end double-end sequence splicing. The analysis software Uparse 7.0.1090, Usearch 7, Qiime 1.9.1, Mothur 1.30.2, and PICRUSt 1.1.0 were applied to cluster and analyze the obtained valid sequences, called the operational taxonomic unit (OTU) set, for taxonomic analysis.

Based on the results of the OTU analysis, information on species richness and evenness of each sample was obtained; sample microbial α-diversity, β-diversity, and LEfSe analysis were performed; sample grouping analysis was performed based on partial least squares-discriminant analysis (PLS-DA); a statistical analysis of community structure was performed based on taxonomic information; and statistical analysis was performed with CK-MB, cTnI, hs-CRP, IL-2, IL-6, and TNF-α. Other clinical factors were analyzed for correlations using Spearman rank correlation coefficients, and correlation Heatmap plots were drawn. Differences between groups were analyzed using a *t*-test.

### Metabolomic Analysis of Intestinal Contents

The untargeted metabolomics profiling was performed on the XploreMET platform (Metabo-Profile, Shanghai, China). Approximately 100 mg of fecal samples from the ileum of rats in each group were weighed, placed in a centrifuge tube, and frozen. The frozen samples were harvested and stored in an Eppendorf SafeLock microcentrifuge tube. Samples were mixed with 25 mg of pre-chilled zirconium oxide beads and 10 μL of internal standard. Each aliquot of 50 μL of 50% pre-chilled methanol was added for automated homogenization. After centrifugation at 14,000 g and 4 °C for 20 min, the

supernatant was carefully transferred to an autosampler vial. Each aliquot of 175 μL of pre-chilled methanol/chloroform (v/v=3/1) was added to the residue for the second extraction. After centrifugation at 14,000 g and 4 °C for 20 min, each 100 μL of the supernatant was carefully transferred to an autosampler vial. All the samples in autosampler vials were evaporated briefly to remove chloroform using a CentriVap vacuum concentrator and further lyophilized with a FreeZone freeze dryer equipped with a stopping tray dryer. The sample derivatization and injection were performed by a robotic multipurpose sample MPS2 with dual heads. Briefly, the dried sample was derivatized with 50 μL of methoxyamine (20 mg/mL in pyridine) at 30° C for 2 h, followed by the addition of 50 μL of MSTFA (1% TMCS) at 37.5 °C for another 1 h using the sample preparation head. In parallel, the derivatized samples were injected with a sample injection head after derivatization.

Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The temperature of the injection and transfer interface were both set to 270 °C. The source temperature was 220 °C. The measurements were made using electron impact ionization (70 eV) in the full scan mode (m/z 50–500).

Four types of quality control samples, ie, test mixtures, internal standards, retention indices, and pooled biological QC samples, are routinely used in our comprehensive metabolomics platform. Metabolite annotation was performed by comparing the retention indices and mass spectral data with those previously generated from reference standards of known structures present in the JiaLib metabolite database using ChromaTOF software.

### Statistical Analysis

The experimental data were expressed as mean ± standard deviation from six independent repetitions, Q-Q plots was used to assess the distribution of each group, and one-way ANOVA followed by the LSD test of variance homogeneity and Dunnett's T3 test in variance heterogeneity was used to determine significant differences of multiple groups. $P < 0.05$ was deemed to be statistical significance. GraphPad Prism 7(GraphPad Software, San Diego, CA) was used for data analysis.

## Results

### QS May Slow Down as by Regulating Serum ADPN Levels

The effects of QS on weight growth rate are shown in Figure 1A. There were no significant differences in each group; thus, QS did not significantly affect the growth of mice over 12 weeks at the experimental dose. The TC, TG, and LDL-C were significantly higher in the model group than in the control group (Figure 1B, C and E). TG levels in the atorvastatin and QS-high groups were significantly lower than the model group ($P<0.05$); LDL-C levels were also somewhat lower than the model group, although there was no significant difference ($P>0.05$). The ADPN level was significantly lower in the model group compared with the control group (Figure 1F), while the QS-low, QS-high, and atorvastatin groups all had significantly higher levels compared with the model group ($P<0.05$).

The results of the HE staining (Figure 1G) showed that the model group was in the atherosclerotic lipid streak stage as the intima was damaged and not smooth, and had undergone proliferation; the endothelium was disordered; a large number of foam cells gathered into lipid streaks; and the endothelium was bulged and deformed. The results of Oil Red O staining (Figure 1H) showed that there were a few foam cell lipid particles under the intima of the model group. The occurrence of aortic plaques in mice in the high- and low-dose QS groups and the atorvastatin group was better than that in the model group, which was in the early stage of intimal injury. Pathological examination of the skin at the administration site in the QS-low and QS-high (Figure 1T) groups did not reveal any obvious lesions, indicating that QS treatment for 12 weeks at the experimental dose was not irritating to the skin.

These results indicate that QS is effective in slowing down the occurrence of AS, probably by regulating ADPN levels, lipid metabolism, and inflammation. Therefore, we further investigated the effect of QS on ACS using the high dose.

### QS Ameliorates the Inflammatory Response and Myocardial Injury in ACS Model Rats

There were no significant differences in the activity, hair, diet, and diarrhea of the SD rats in each group, and there were no abnormalities such as redness, rash, blistering, and rupture of the skin in the QS group. The body weight growth rates

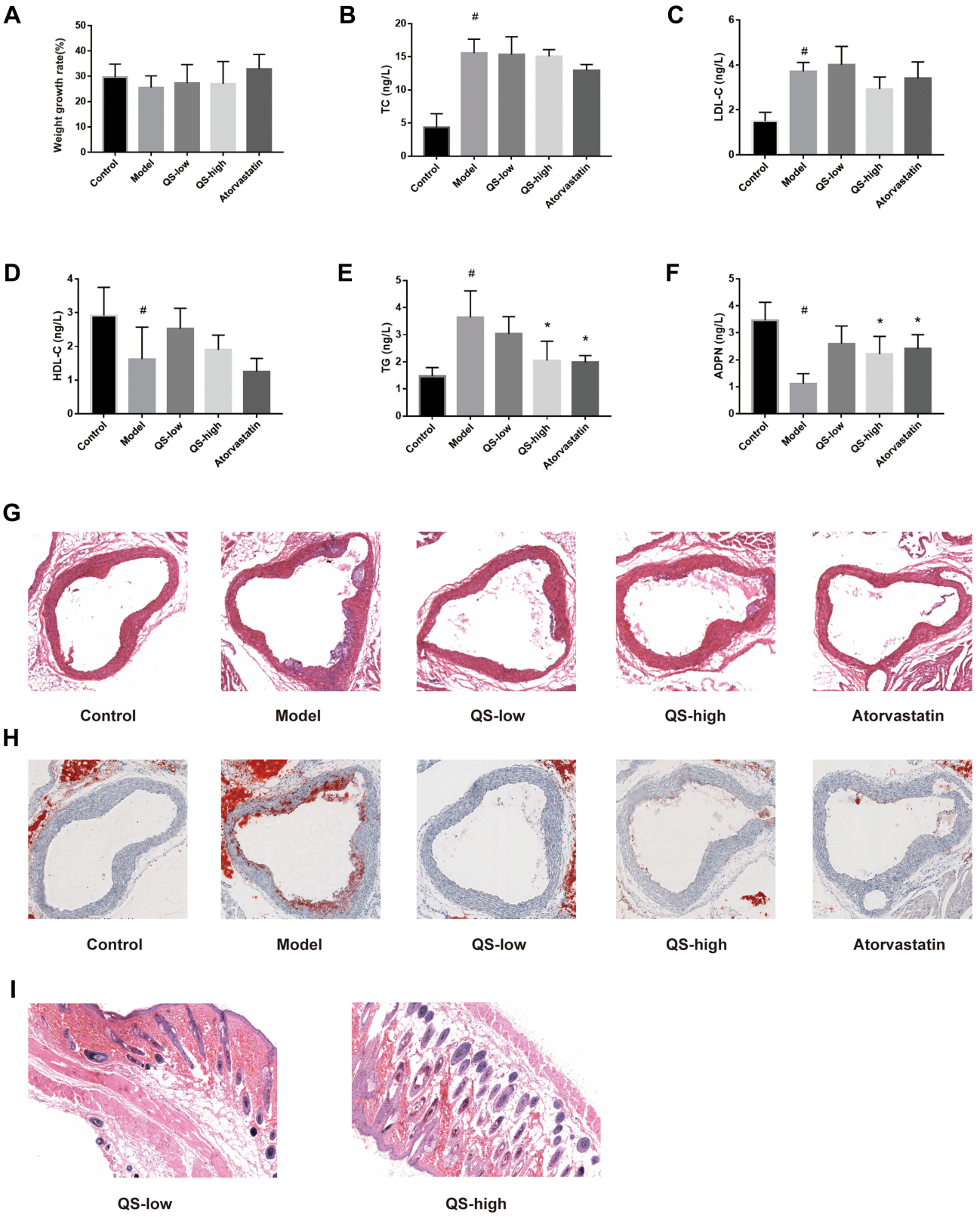


**Figure 1** Effect of Qianshan Huoxue Gao (QS) on atherosclerosis (AS) model rats. (**A**) Rate of body weight gain. (**B**) Total cholesterol (TC). (**C**) Low-density lipoprotein cholesterol (LDL-C). (**D**) High-density lipoproteins cholesterol (HDL-C). (**E**) Triglycerides (TG). (**F**) Adiponectin (ADPN). (**G**) Hematoxylin and eosin (HE) staining of aortic vessels in different groups. (**H**) Oil red O staining of aortic vessels in different groups. (**I**) HE staining of the skin in the QS groups. $^{\#}P<0.05$ compared with the control group; $^{*}P<0.05$ compared with the model group.

showed no significant differences in each group (Figure 2A). We tested several inflammatory indicators regarding ACS, and compared with the control group, IL-2, IL-6, hs-CRP, and TNF-α were significantly elevated in the model group (Figure 2B–E). IL-2, IL-6, TNF-α, and hs-CRP were improved in the QS group, similar to the atorvastatin group, indicating that QS may alleviate myocardial injury and the inflammatory response in ACS.

CK-MB and cTnI expression, and HE staining were used to illustrate the effect of QS in mitigating myocardial injury in ACS. Compared with the control group, CK-MB and cTnI were significantly elevated in the model group ($P<0.01$), and QS could reduce them to a certain degree (Figure 3A). The cardiac lesion scores and pathological results of each group are shown in Figure 3B and C. The myocardial tissue in the control group was neatly arranged and ordered, with no cell degeneration and clearly discernible transverse lines; however, the model group showed significant lesions ($P<0.05$), which were significantly improved in the QS group ($P<0.05$). Therefore, QS could mitigate myocardial injury in ACS.

Echocardiography was used to detect the effect of QS on cardiac systolic function. There was no significant difference in Heart rate among the groups. The cardiac function parameters in each group are shown in Figure 4A, and representative echocardiographic images are shown in Figure 4B. Compared with the control group, the EF and FS were significantly decreased and LVDD and LVDS were significantly increased in the model group, indicating that cardiac function was significantly impaired. Compared with the model group, EF and FS were increased and LVDD and LVDS were decreased in the QS and atorvastatin groups, indicating that QS could improve the heart contraction function.

From the above results, we found that QS could alleviate the inflammatory response and cardiac impairment induced in the ACS model. The next question was whether these changes are related to the intestinal flora or metabolites. Therefore, we further examined the changes in intestinal microorganisms and metabolites in the feces of SD rats in each group to explore the intestinal flora and metabolites that may be related to the treatment of ACS with QS.

## Effect of QS on the Intestinal Flora of ACS Models

We use 16s sequencing to analyze intestinal flora diversity and structural differences. The data presented in the study are deposited in the NCBI repository (https://www.ncbi.nlm.nih.gov/), accession number PRJNA916192. To investigate the

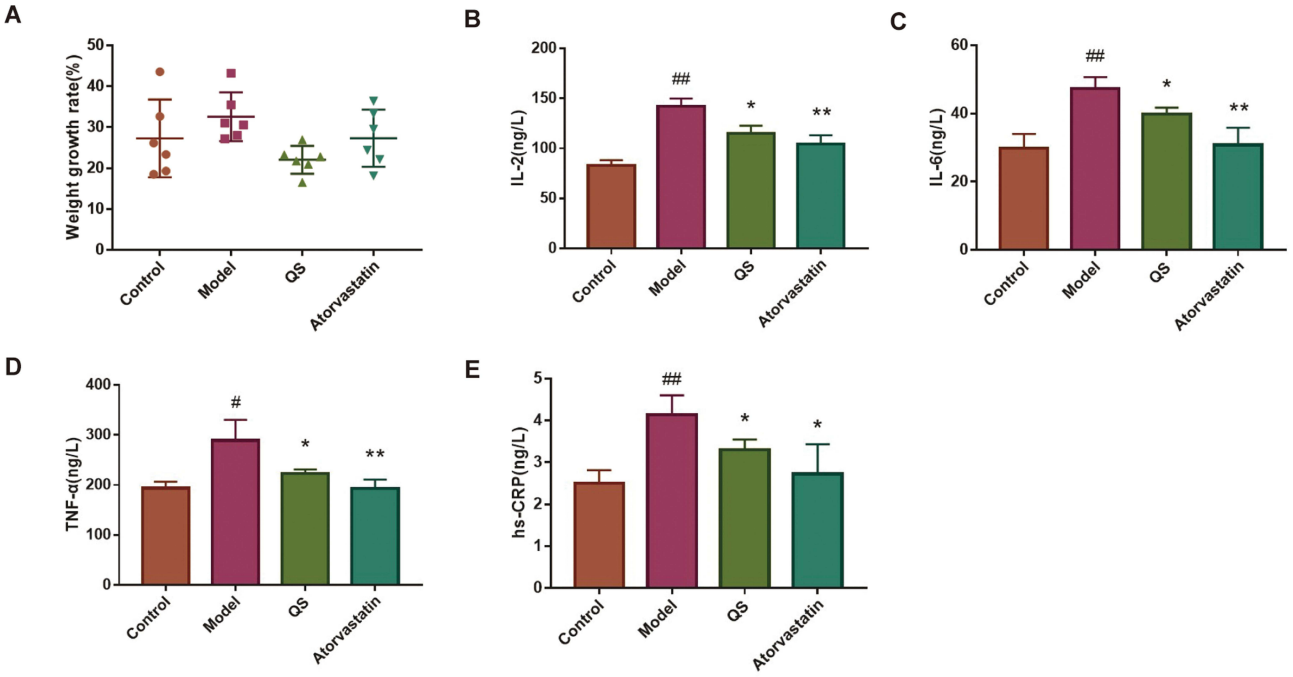


**Figure 2** Effect of Qianshan Huoxue Gao (QS) on acute coronary syndrome (ACS) model rats. (**A**) Rate of body weight gain. (**B**–**E**) ELISA assays for serum protein levels of the following: (**B**) interleukin-2 (IL-2), (**C**) IL-6 (**D**) tumor necrosis factor (TNF)- α, and (**E**) high-sensitivity C-reactive protein (hs-CRP). $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with the control group; $^{*}P<0.05$, $^{**}P<0.01$ compared with the model group.

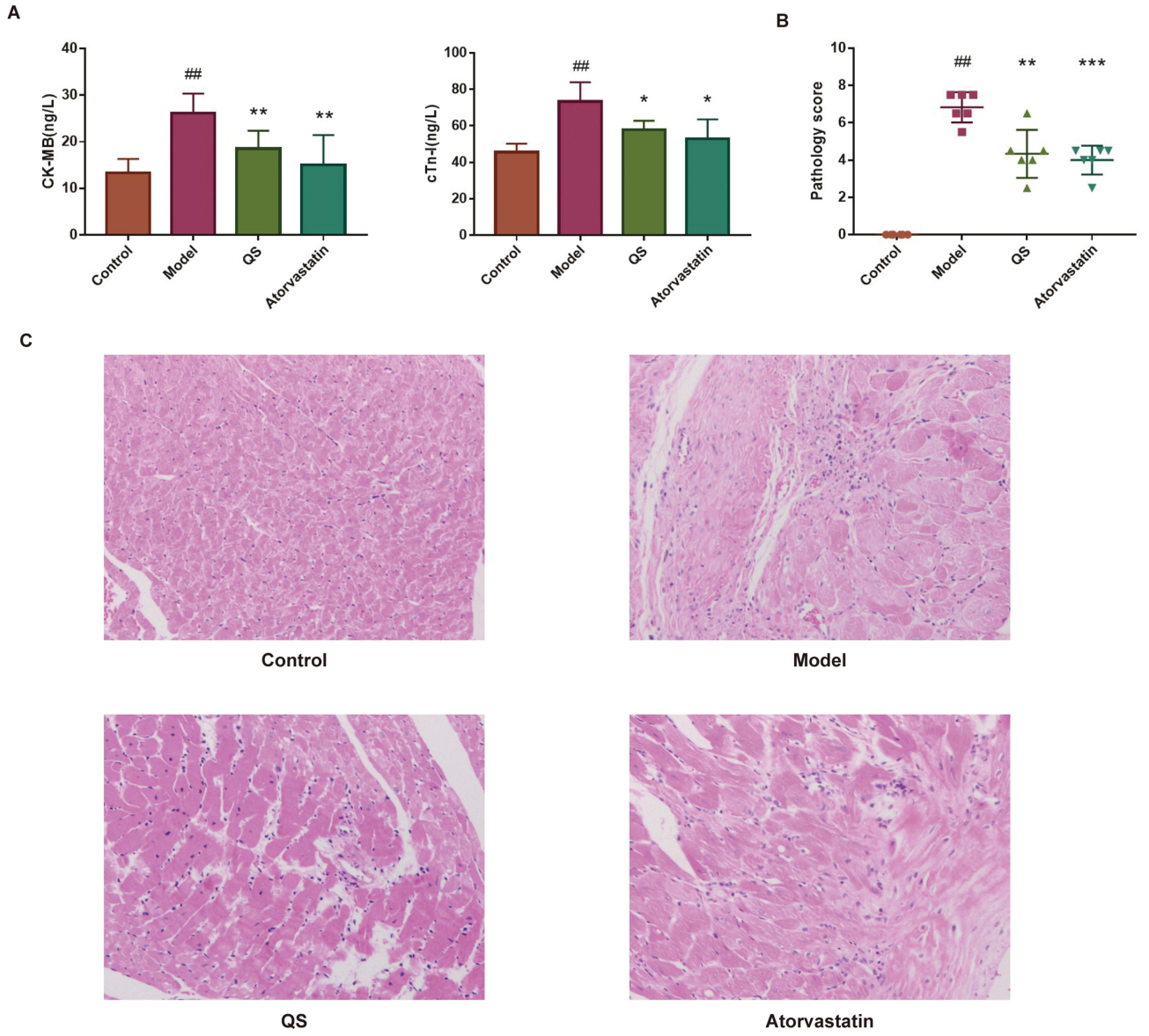


**Figure 3** Effect of Qianshan Huoxue Gao (QS) on the heart of acute coronary syndrome (ACS) model rats. (**A**) Detection of cardiac enzymes in different groups. (**B**) Pathological score of the heart tissue. (**C**) Hematoxylin and eosin (HE) staining of the heart tissue. $^{\#\#}P<0.01$ compared with the control group; $^{*}P< 0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with the model group.

changes in the diversity of rat intestinal flora, the alpha diversity of intestinal flora was estimated by calculating the Ace index, Chao index, Shannon index, and Simpson index. From the Rank–Abundance plot (Figure 5A), the distribution of rat intestinal flora species was evenly distributed among the groups, with differences in flora abundance. The coverage index of each group indicates the coverage of sequencing (Figure 5B), and all of them were above 99%, indicating that the sequencing has a high coverage of sequences in the samples and the possibility of sequences in the samples not being sequenced being low. Ace and Chao indexes are OTU numbers representing the species richness of the flora. Shannon and Simpson indexes are quantitative indicators of the diversity of the flora. The larger the Ace, Chao, and Shannon indices, and the smaller the Simpson index, the richer the species in the sample. As shown in Figure 5C–F, the intestinal microbial diversity was reduced in the model group compared with the control group ($P<0.05$), and QS played a role in restoring the intestinal flora diversity.

Using principal co-ordinates analysis (PCoA) and non-metric multidimensional scaling (NMDS) analysis, the groups of samples were found to be largely separated; the QS group deviated from the model group. QS could regulate the return of normal intestinal flora (Figure 5G and H, P=0.001 in the "anosim" test for PCoA analysis and NMDS analysis).

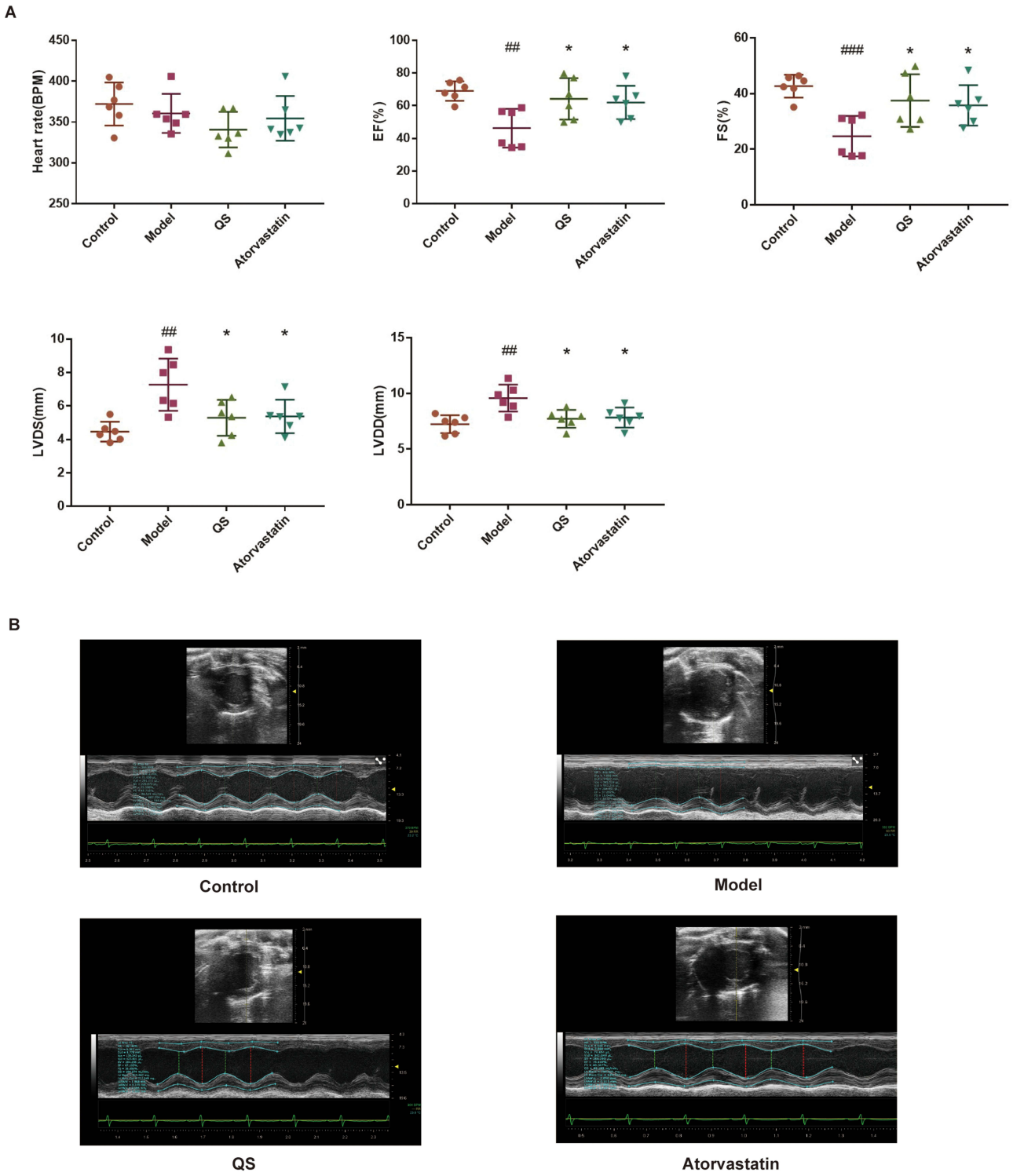


**Figure 4** Effect of Qianshan Huoxue Gao (QS) on cardiac function in acute coronary syndrome (ACS) model rats. (**A**) Echocardiogram data. (**B**) Photos of the echocardiogram. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared with the control group; $^{*}P< 0.05$ compared with the model group.

At the phylum level and the genus level, QS and atorvastatin had regulatory effects on the intestinal flora of the ACS model rats (Figure 6A and C), especially the ratio at the phylum level (F/B) (Figure 6B). As well as at the genus level, QS inversely regulated the abundance of *Bacteroides*, significantly elevating Lactobacillus ($P<0.01$) and decreasing *Rikenellaceae_RC9_gut_group* ($P<0.01$) (Figure 6D–F).

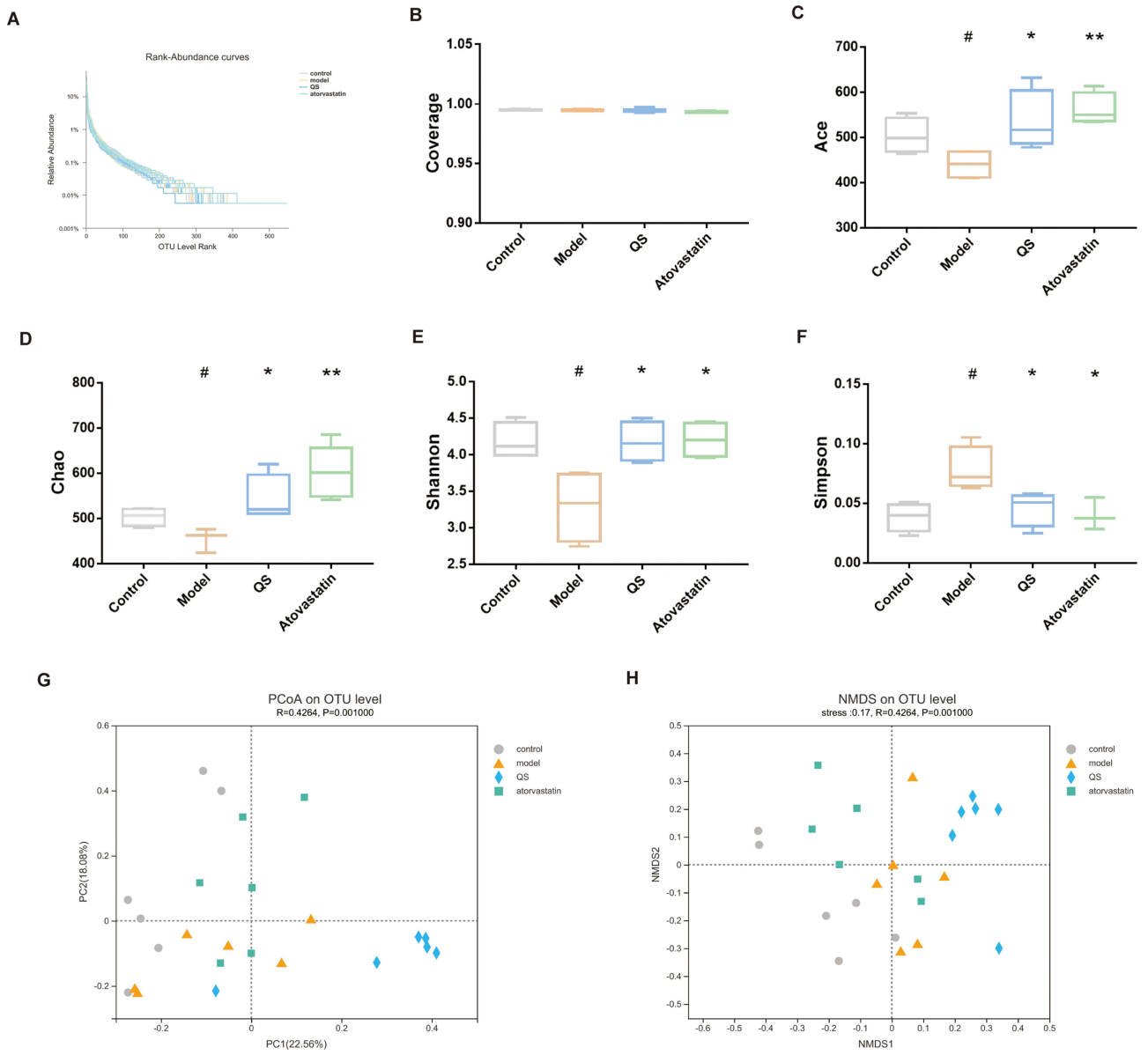


**Figure 5** Microbial community structure of the different groups. (**A**) Rank-abundance. (**B**) Coverage. Alpha diversity in groups: (**C**) Ace, (**D**) Chao, (**E**) Shannon, (**F**) Simpson, (**G**) principal co-ordinates analysis (PCoA), and (**H**) non-metric multidimensional scaling (NMDS) analysis. #*P*<0.05 compared with the control group; **P*< 0.05, ***P*<0.01 compared with the model group.

## Effect of QS on the Metabolites of ACS Models

The effects of QS on the regulation of intestinal flora in a rat model of ACS led us to hypothesize that certain active metabolites may play a vital role in the therapeutic effects of QS in ACS. To address this question, we performed a metabolomic analysis of rat feces using GC-TOF/MS. The results showed that a total of 219 different classes of metabolites were identified and differed in content (eg, Figure 7A and B). Similar to the intestinal flora analysis, the metabolomics also showed significant inter-group differences (Figure 7C and D).

## EA May Be a Critical Factor in the Intestinal Microflora of ACS

Correlation analysis between individual metabolites and blood parameters were generated using Spearman rank correlation coefficients, and the results showed that 36 metabolites were significantly positively or negatively correlated with inflammation or cardiac function serum parameters (Figure 8A). Further analysis of the differences in the levels of these

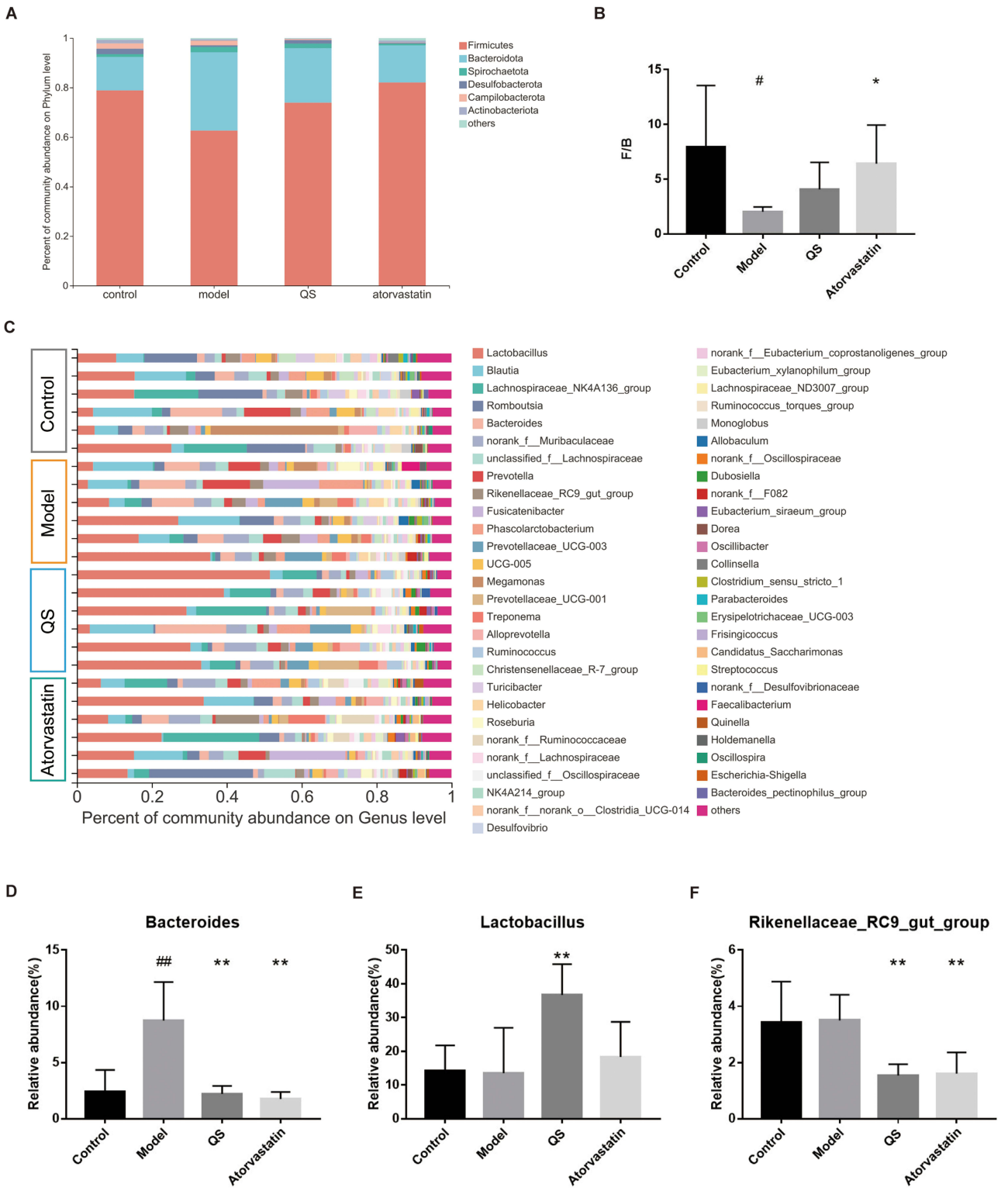


**Figure 6** Variation of bacterial groups within different groups at the phylum level and genus level. (**A**) Comparison of community composition at the phylum level. (**B**) Rate of Firmicutes/Bacteroidota at the phylum level. (**C**) Comparison of community composition at the genus level. (**D–F**) Significantly differently bacteria at the genus level in different groups. $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with the control group; $^{*}P< 0.05$, $^{**}P<0.01$ compared with the model group.

36 metabolites in each group yielded that ethanolamine (EA), glutaric acid, phenylethylamine, and lathosterol were the metabolites most strongly associated with the treatment of ACS with QS (Figure 8B). Among them, EA was significantly increased in the model group compared with the control group. In contrast, the EA content of the QS group was

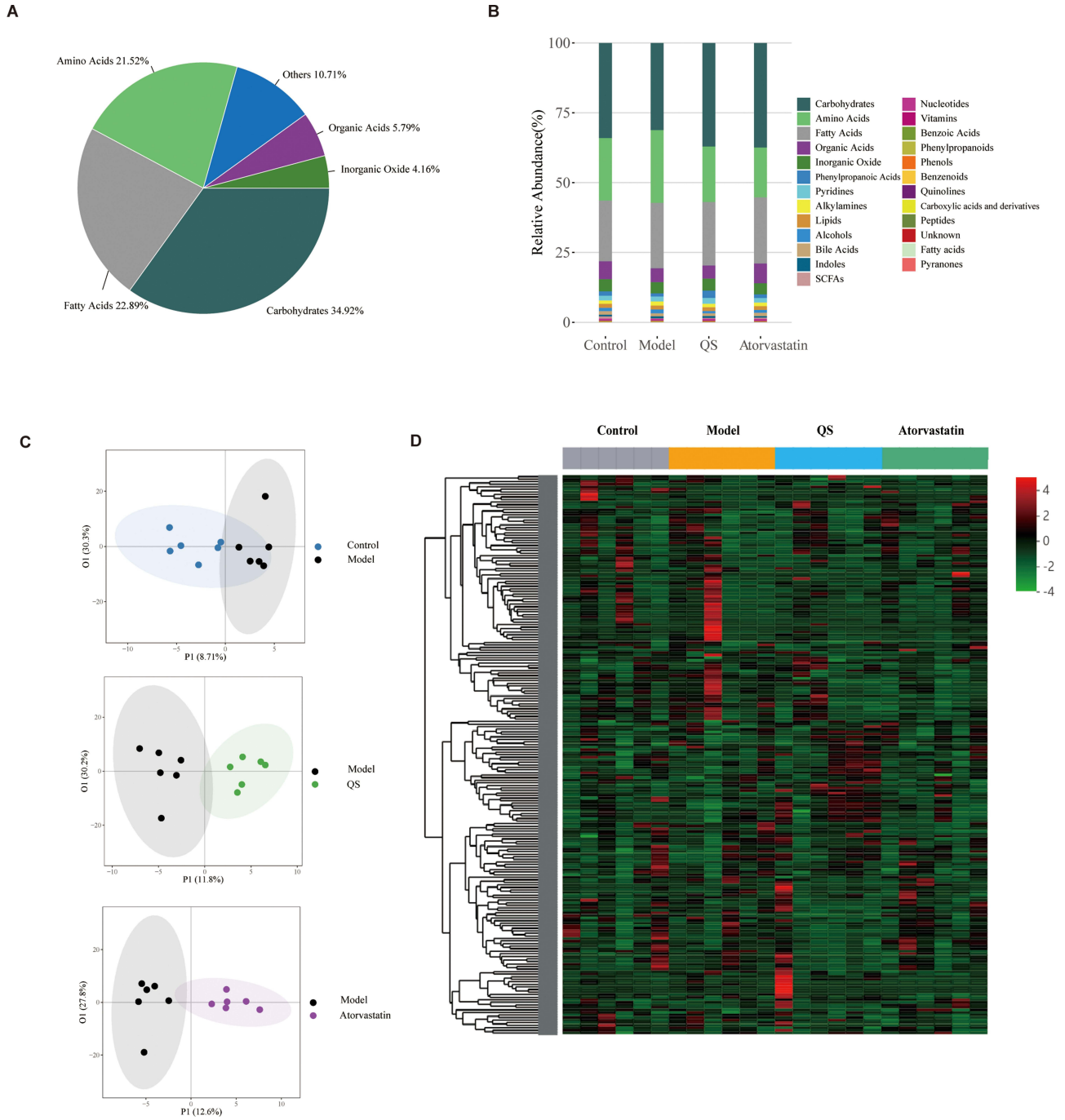


**Figure 7** Effect of Qianshan Huoxue Gao (QS) on metabolites in acute coronary syndrome (ACS) model rats. (**A** and **B**) Distribution of metabolites in different groups. (**C**) Orthogonal projections to latent structures discriminant analysis (OPLS-DA) of metabolomic results in different groups. (**D**) Heatmap of the fecal metabolite profile.

significantly decreased, indicating that EA may be a critical factor in the formation or alteration of the intestinal microflora in ACS. We further correlated EA content with the abundance of each genus of intestinal flora to identify the significant EA-producing intestinal flora. The *Eubacterium xylanophilum group, Ruminococcus, unclassified f__Oscillospiraceae, Intestinimonas, Eubacterium siraeum group, Lachnospiraceae NK4A136 group*, and *norank f__Desulfovibrionaceae* were the only genera that were significantly positively correlated for EA content (Figure 8C), suggesting that they may be the critical EA-producing intestinal bacteria.

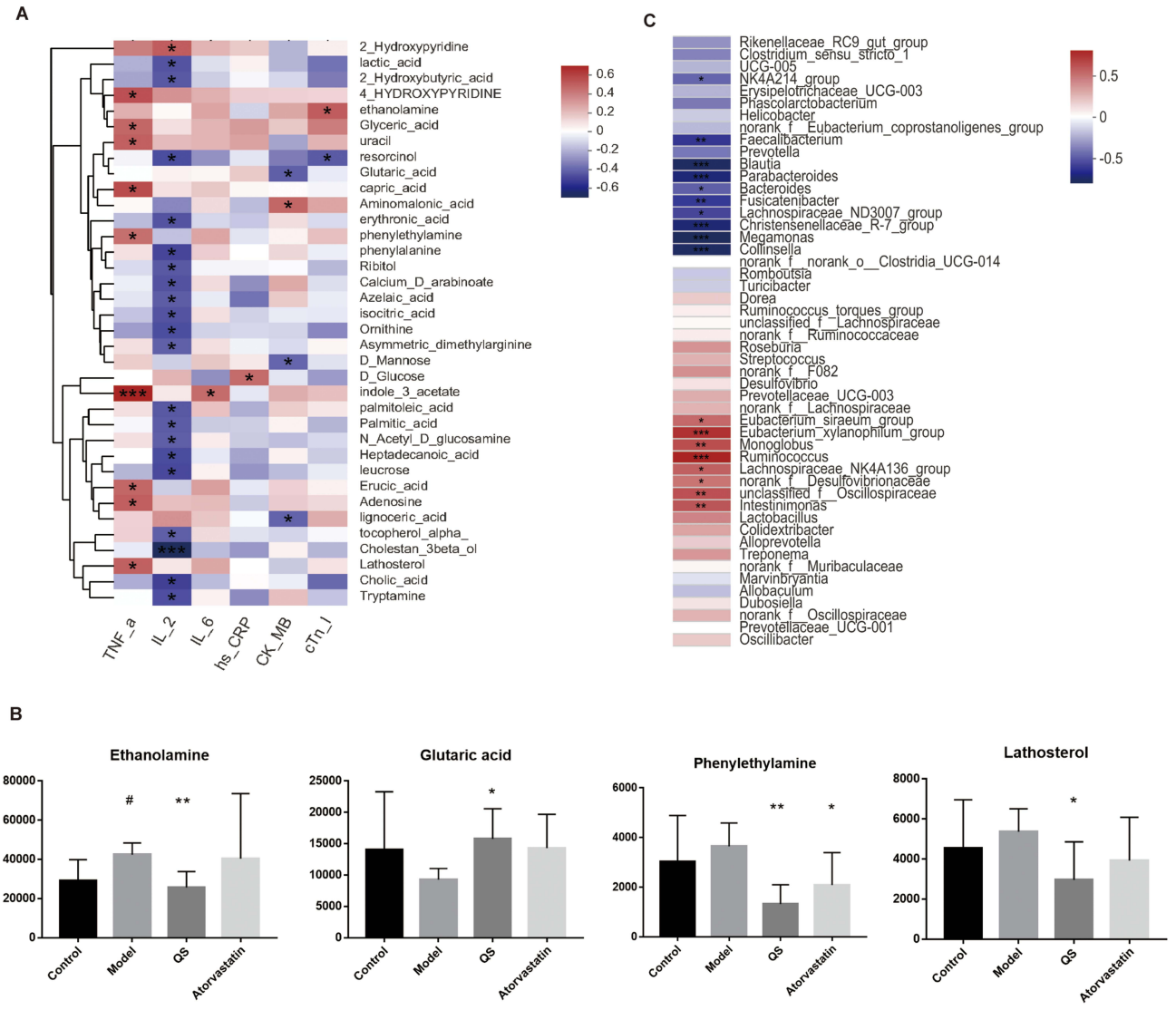


**Figure 8** Microbial community structure of different groups. (**A**) Heatmap for the correlation analysis of metabolites and clinical factors. (**B**) Main differential metabolites between different groups. (**C**) Heatmap analysis of the correlation between EA and intestinal flora. $^{\#}P<0.05$ compared with the control group; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with the model group.

Some studies have reported that EA is a valuable source of carbon and/or nitrogen for bacteria capable of catabolism, is prevalent in the gastrointestinal tract, and is associated with inflammation.[27] The increased EA content in the intestine of ACS model rats and the decreased EA content after using QS or atorvastatin suggests that EA is the most likely metabolite involved in the beneficial effects of QS on ACS and is associated with some intestinal flora.

Based on the above findings, we demonstrate that QS could ameliorate the inflammatory response and myocardial injury in the ACS model. Furthermore, EA may be an effective metabolite for treating ACS, as it is related with intestinal flora such as the *Eubacterium xylanophilum group, Ruminococcus, unclassified f__Oscillospiraceae, Intestinimonas, Eubacterium siraeum group, Lachnospiraceae NK4A136 group*, and *norank f__Desulfovibrionaceae*.

## Discussion

In recent years, with the in-depth research on the modernization of traditional Chinese medicine, its use in the treatment of cardiovascular diseases has gradually gained recognition. Among traditional Chinese medicines, topical ointment for the treatment of cardiovascular diseases has the advantages of being safe, a simple application method, fast onset of action, and improved compliance with medication.[28] For example, angina cream can improve the symptoms of patients

with angina, improve ST-T abnormalities on the electrocardiogram, and reduce C-reactive protein levels.[29] Furthermore, the pretreatment of angina pectoris with acupressure with a topical paste can reduce the degree of myocardial injury and necrosis in a rat model of acute myocardial infarction and may reduce inflammatory cell infiltration by inhibiting the expression of TNF-α and IL-6, thus playing a role in improving myocardial ischemia. The combination of coronary artery paste and conventional Western medicine can improve the clinical symptoms of patients with coronary angina, reduce the levels of TG, TC, and LDL-C, and improve the clinical efficacy.[30] Therefore, the topical paste has reasonable prospects for development for the treatment of cardiovascular diseases.

ACS is a typical clinical cardiovascular emergency; the pathology is based on the sudden rupture of a vulnerable plaque under special circumstances, which causes thrombosis through a series of chain reactions and eventually leads to partial or even complete blockage of coronary arteries, resulting in a clinical syndrome. The occurrence of ACS is closely related to AS. Our previous study found that QS can regulate serum ADPN levels in terms of lipid metabolism and anti-inflammation, slowing down the occurrence of AS. Therefore, we hypothesized that QS might have a preventive or mitigating effect on ACS. The present study verified the hypothesis and explored its possible mechanism of action using a combination of gut microbiology and metabolomics analyses.

Topical creams are a form of transdermal drug delivery. A special requirement for experimental animals in studies related to transdermal drug delivery is that the skin permeability of that animal should be similar to that of human skin. Among the commonly used experimental animal species, including mice, rats, guinea pigs, rabbits, pigs, and monkeys, rats were selected as a better model for the treatment of ACS with topical creams, considering the commonly used experimental animals for the disease, whether the skin permeability is close to that of humans, and the price. In this study, we used coronary artery left anterior descending branch ligation to trigger ST-segment elevation to establish the SD rat model of ACS, a commonly used animal model of ACS.

Cardiac ultrasound, cardiac enzymes, and inflammation-related indices were used to evaluate the efficacy of QS for the treatment of ACS, and atorvastatin was used as a control drug. QS significantly reduced the levels of cardiac enzymes and inflammatory indexes and improved cardiac function. HE staining to observe changes in cardiac tissue yielded results similar to those of the cardiac ultrasound and serum indexes. In the study on the safety of drug administration, no significant abnormalities were found on the skin surface when QS was used for 28 consecutive days at a dose of 6 g/kg (equivalent to 60 times ACD). Furthermore, no significant abnormalities were observed regarding the pathological findings of the skin, liver, and kidney by HE staining. Therefore, topical treatment of ACS with QS has good efficacy and safety, and its effect may be comparable to atorvastatin.

ACS is closely related to the intestinal flora. We also conducted a study of changes in the intestinal flora during QS treatment of ACS to explore the possible mechanism of action. The results revealed that *Bacteroides, Lactobacillus*, and *Rikenellaceae_RC9_gut_group* were the groups with significant differential changes in the QS treatment of ACS. Chen[31] reported that *Bacteroides* were found in data from all 16 cohort studies (2210 patients) in the systematic evaluation assessing the association between commensal microflora and coronary artery disease. A clinical study published in Nature in 2020 found that[32] a high abundance of *Bacteroides* and a low abundance of *Faecalibacterium* were associated with inflammation and obesity, and that the administration of atorvastatin was negatively associated with dysbiosis of this flora. This result was validated in the accompanying MetaCardis cardiovascular disease data (n = 282) and in the independent Flemish Gut Flora Project population cohort (n = 2345). Gil-Cruz[33] found that symbiotic *Bacteroides* triggered a cross-immune response against bacterial proteins and cardiac epitopes, leading to cardiomyopathy. In our study, QS significantly reduced the abundance of *Bacteroides*, suggesting a modulatory effect of QS on the dysbiosis of *Bacteroides* flora associated with inflammation, obesity, and cardiovascular disease.

*Lactobacillus* is a non-gas-producing lactic acid bacterium that is generally considered safe and has multiple beneficial effects on intestinal health, metabolic disorders, and brain health. It is essential in the "gut-cardio-brain axis" theory.[34] The cardiovascular-related effects of Lactobacillus include lowering inflammation levels, cholesterol levels, and hypertension.[35] One study found that Lactobacillus significantly reduced the effect of a high salt diet on hypertension in salt-sensitive rats,[36] highlighting a potential therapeutic role in salt-induced hypertension. In the treatment of spontaneous hypertension, the mechanisms of *Lactobacillus* include an improvement of the oxidative, inflammatory state of blood vessels and reduction of lipid levels, which may be due to the production of ACE (angiotensin-converting enzyme) inhibitors and modulation of the

downstream sympathetic nervous system.[37–39] A meta-analysis study found that *Lactobacillus* supplementation significantly reduced diastolic blood pressure in a total of 653 participants in seven randomized controlled trials.[40] In our study, the abundance of *Lactobacillus* was elevated in the intestinal flora of rats in the QS group ($P<0.01$), suggesting that *Lactobacillus* may be a relevant probiotic for the treatment of ACS by QS.

A high-fat diet (HFD) is one of the risk factors for cardiovascular disease. Wang[41] found that a HFD increased intestinal microbial diversity and increased the abundance of flora such as the *Rikenellaceae_RC9_gut_group*. Gao[42] found that *Rikenellaceae_RC9_gut_group* was positively correlated with "harmful indicators" and negatively associated with "beneficial indicator" caused by HFD. In our study, QS significantly reduced ($P<0.01$) *RRikenellaceae_RC9_gut_group* abundance, similar to atorvastatin. In summary, regulation of inflammation, obesity, and cardiovascular disease-related dysbiosis may be one of the mechanisms by which QS exerts its therapeutic effects on ACS, involving mainly *Bacteroides, Lactobacillus*, and *Rikenellaceae_RC9_gut_group*.

In further studies of metabolites, 36 identified metabolites were found to be significantly positively or negatively correlated with at least one serum parameter indicating an association with inflammation or cardiac function. EA was the metabolite most strongly associated with Chiyama Revitalizing Blood Cream for the treatment of ACS. EA is essential for life and is present in every cell of the body as the head group of polyethylene (and other lipids) and in body fluids in the form of free EA in varying concentrations.[43] EA has common biological effects, including promoting rapid growth of mammalian cells,[44] a cardioprotective effect in ischemia/reperfusion injury through activation of the transcription factor STAT-3,[45] reversal of low serum-induced apoptosis in a mouse neuroblastoma cell line,[46] etc. The dual role of EA as a carbon/nitrogen source and signaling molecule is beginning to emerge after decades of research. *Clostridium, Listeria, Enterococcus, Escherichia*, and *Salmonella*[47] contain genes that enable the catabolism of EA.[48] EA catabolism occurs in a multiprotein compartment called the carbon vesicle.[49] Ethanolamine deaminase (EutBC) converts EA to acetaldehyde and ammonia.[50] Acetaldehyde can be converted to ethanol or, more likely, to acetyl coenzyme a, which can be used in many cellular processes (Krebs cycle, glyoxylate bypass, lipid biosynthesis, or other methods).[48] In our study, we found that EA content was increased in the intestine of rats in the ACS model group compared with the control group ($P<0.05$), suggesting that the decreased utilization of EA by the flora in the ACS state may be related to the change in flora abundance. The EA of the QS group was significantly higher compared with the model group ($P<0.01$), indicating that QS had a regulatory effect on the flora of the ACS model rats and restored the utilization of EA. We found that EA was significantly and positively correlated with the *Eubacterium xylanophilum group, Ruminococcus, unclassified f__Oscillospiraceae, Intestinimonas, Eubacterium siraeum group, Lachnospiraceae NK4A136 group*, and *norank f__Desulfovibrionaceae*; however, their mechanisms of action require further investigation.

## Conclusion

In conclusion, QS can effectively improve cardiac function and the inflammatory response in a rat model of ACS, with similar efficacy to atorvastatin. Meanwhile, QS could regulate the structure and abundance of the intestinal flora in ACS model rats, in addition to affecting the metabolites. EA was found to be a potential metabolite that mediates the effect of QS in treating ACS effectively and was positively correlated with some intestinal flora.

## Author Contributions

All authors made a significant contribution to the work reported. D.B. conceived and designed the project. N.Z., Y.Z., X. L. and Y.W. carried out the experiments, analyzed the data, and drafted the manuscript. N.Z., Y.M. and D.H., participated in the design and prepared the paper. All authors revised the manuscript and have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## Funding

This research was funded by State Drug Administration "Science and technology help the economy 2020" key special projects and China Academy of Chinese Medical Independent Selection (YZ-1782) and also supported by the Chinese Government Scholarship (No. 202005350010).

## Disclosure

The authors report no conflicts of interest in this work.

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