doi.org/10.36721/PJPS.2025.38.3.REG.13406.1

Study on the mechanism of Yajieyixin formula in improving atherosclerosis

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Abstract: Yajieyixin Formula (YJYXF) is an effective prescription commonly used by Dai medicine practitioners to treat cardiovascular diseases. This study explored how YJYXF helps ApoE mice with atherosclerosis (AS). An atherosclerosis model was established by feeding ApoE mice with a high-fat diet, and treatment with 36.075 g/kg YJYXF and 12.025 g/kg YJYXF for 12 weeks were the optimal treatment conditions for the ankylosis spondylitis mouse model. The mechanism of action of YJYXF against atherosclerosis was comprehensively analyzed by observing the AS-related indexes (blood biochemical indexes, inflammation indexes, TMAO), changes in atherosclerotic plaques observed by HE staining and oil red O staining, liver metabolisms, microbiome, changes in bile acid content, and the expression of key genes and proteins of cholesterol metabolism. The present study showed that YJYXF could lower blood lipid levels, reduce inflammation and aortic plaque accumulation, regulate hepatic lipid metabolism, and regulate bile acid metabolism by modulating the diversity, composition and abundance of intestinal flora, and by decreasing the expression levels of intestinal FXR, FGF-15 mRNA and protein, and by increasing the expression levels of hepatic CYP7A1 mRNA and protein. The study findings that YJYXF can improve AS, and the mechanism is associated with intestinal microbiota regulation by trimethylamine N-oxide (TMAO).

Keywords: Yajieyixin formula, atherosclerosis, intestinal microbiota, trimethylamine N-oxide, lipid metabolism

Submitted on 13-09-2024 – Revised on 19-12-2024 – Accepted on 01-02-2025

INTRODUCTION

Atherosclerosis (AS) is a steadily worsening chronic inflammatory and metabolic condition characterized by plaque formation, thickening of the artery wall, and lipid deposition caused by abnormalities in lipid metabolism (Song and Chen, 2021, Sheng *et al*., 2023). WHO data show that cardiovascular disease kills up to 18 million people each year, and AS is the leading cause of cardiovascular disease worldwide (Fan *et al*., 2024).AS has a detrimental impact on health and quality of life (Li *et al*., 2024d), AS can induce heart attack, angina, etc., and in severe cases, death. Evidence suggests intestinal microbiota can affect AS (Ai *et al*., 2024, Mao *et al*., 2024, Jiang *et al*., 2024b). Regulating gut flora in the fight against AS is very important (2024). For example, Bacteroidetes, Lactobacillus, Firmicutes, Prevotella, Blautia and Desulfovibrio have been associated with slowing AS (Wan *et al*., 2024). Therefore, exploring the effect of YJZL on intestinal flora can provide ideas for YJZL against AS. Currently, the drugs used to treat AS mainly inhibit lipid deposition in vascular plaques, such as statins, but these drugs cause tendon ruptures and tendinopathies(Gillard *et al*., 2024), which severely impair liver function (Sobukawa *et al*., 2024). The discovery of a secure and efficient clinical formula for the treatment of AS has become crucial for medical professionals and researchers.

Dai medicine believes that atherosclerosis is the imbalance of the “four towers” and “five elements” caused by people's excessive diet, and the poisonous evils are embedded in the internal organs and injure the internal organs, which is referred to as “food poison”. YJYXF is a national effective prescription collected and organized from the clinical literature of Dai medicine, which has the effect of relieving toxins, regulating the physiological functions of human body and maintaining the balance of the functions of “four towers” and “five elements” of human body. Experimental research has demonstrated that YJYXF, a prescription drug frequently used in Dai medicine, has strong antioxidant and anti-atherosclerotic properties (Luo *et al*., 2023a, Luo *et al*., 2023b). However, its exact mode of action against AS is unclear.

Therefore, in the present study, an AS model was established using ApoE mice fed a high-fat diet with YJYXF intervention. We examined the mechanism of action of YJYXF against AS in detail, taking into account changes in atherosclerotic plaques, hepatic metabolomics, microbiome, bile acid content, and the expression of important genes and proteins for cholesterol metabolism, as well as AS-related indices (blood biochemical indices, inflammation indices, TMAO). The objectives of the study was to investigate the mechanism of action of YJYXF against AS.

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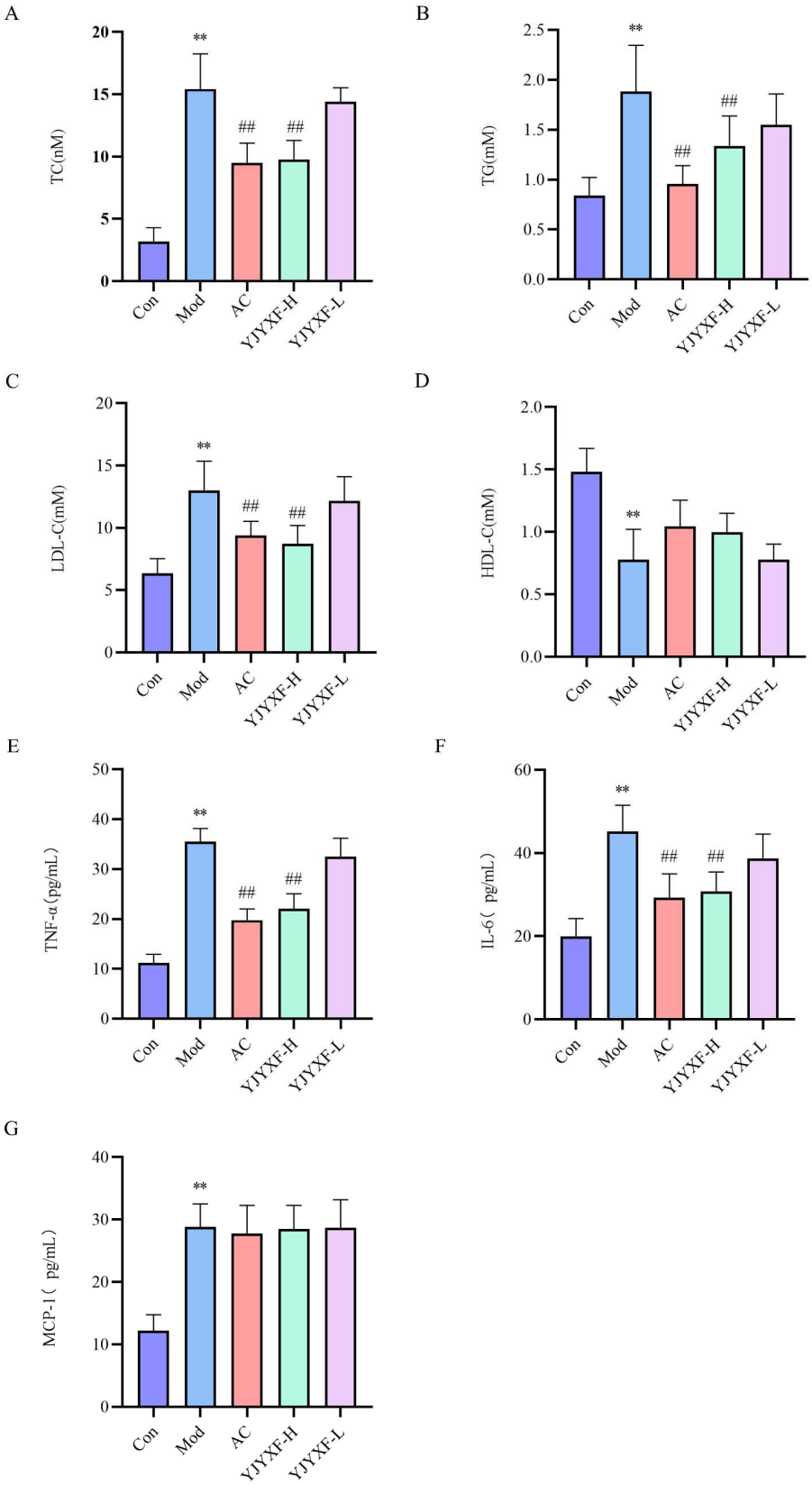

Fig. 1: Effects of YJYXF on lipid and inflammatory factors in mice with AS (A) TC, (B) TG, (C) LDL-C, (D) HDL-C, (E) TNF-α, (F) IL-6, and (G) MCP-1, n=6. In contrast to the Con group, $^{*}p<0.05$, $^{**}p<0.01$; In contrast to the Mod group, $^{\#}p<0.05$, $^{\#\#}p<0.01$.

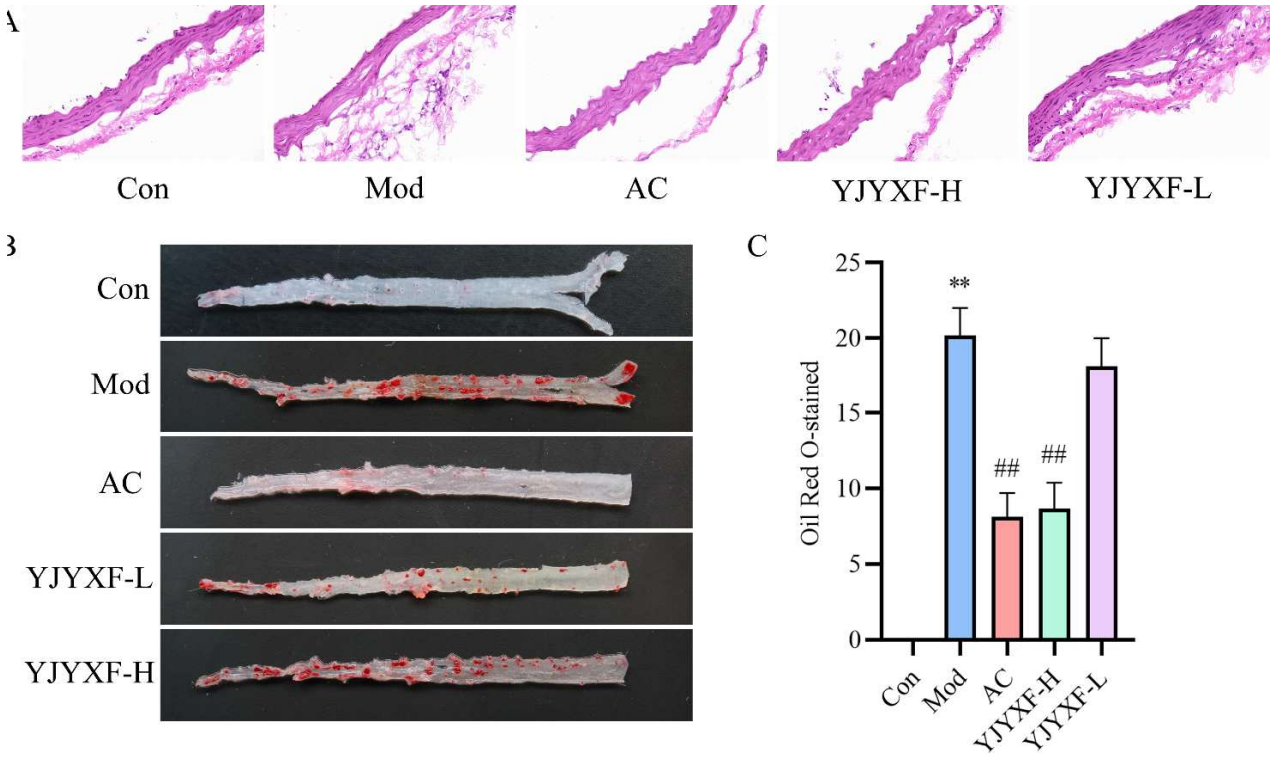

Fig. 2: YJYXF reduced atherosclerosis. (A) Hematoxylin and eosin (HE) staining; (B) Oil red O staining of the whole aorta; (C) area of red staining by Oil red O staining. In contrast to the Con group, $^{*}p<0.05$, $^{**}p<0.01$; In contrast to the Mod group, $^{\#}p<0.05$, $^{\#\#}p<0.01$.

MATERIALS AND METHODS

Preparation of YJYXF

YJYXF is composed of *Daibaijie* (*Marsdenia tenacissima (Roxb.) Wight et Arn*) 20 g, *Baiyangjie* (*Arundia graminifolia (D.Don) Hochr*) 20 g, *Dahuangteng* (*Fibraurea recisa Pierre*) 15 g, *Jianghuang* (*Curcuma Longa L.*) 15 g, *Zhanglang* (*Blatta orientalis (L.)* 15 g,*Miduoling* (*Blaps japonensis yunnanensis Mars*) 15 g, *Dingxinteng* (*Mappianthus iodoides Hand. - Mazz.*) 15 g, *Baihuachoumudan* (*Clerodendrum chinense (Osfeck) Mabferlley*) 15 g, *Sanqi* (*Panax notoginseng (Burk.) F. H. Chen*) 5 g, *Naiziteng* (*Anodendron formicinum (Tsiang et P.T.Li) D.J.Middl.*) 10 g, *Gegen* (*Pueraria lobata (Willd.) Ohwi*) 20 g, *Sanyemanjing* (*Vitex trifolia Linn.*) 10 g, *Yegancao* (*Scoparia dulcis L.*) 10 g. In this study, YJYXF dosages of 12.025 g/kg and 36.075 g/kg were chosen based on the conversion of doses by humans and animals, respectively. A dosage of 36.075 g/kg was selected for the high-dose YJYXF group (YJYXF-H), and a dosage of 12.025 g/kg was selected for the low-dose YJYXF group (YJYXF-L). The preparation method was as follows: decoction three times with eight times, six times, and six times of water. It was then filtered three times, which was collected, heated, and concentrated until it contained raw herbs 3.6g·mL-1) and stored at 20 °C in a refrigerator.

Animals

32 male ApoE-/- and eight male C57BL/6J mice, both 8 weeks old and 20–25 g in weight, were bought from SPF (Beijing) Biotechnology Company (no. SCXK (Beijing) 2019-0010). The facility where mice were kept had temperatures that ranged broadly from 23°C to 27°C, and humidity was controlled between 45% and 55%, with a fixed 12-hour day and night cycle. High-fat diets (40% fat and 1.25% cholesterol) were bought from Chengdu BioPike Technology Company. Authorization was granted for the experiment by the animal ethics committee from Yunnan University of Traditional Chinese Medicine (R-062022053).

Construction of an AS model in mice and treatment

After one week of acclimatization, the mice were split into five groups: control group (Con), model group (Mod), positive group (Atorvastatin, AC; 2.5 mg/kg/d), YJYXF-H group (36.075 g/kg/d), and YJYXF-L group (12.025 g/kg/d), each group including eight mice. The C57BL/6J mice in the Con group were given a regular diet. The high-fat diet was given to the remaining four groups of ApoE-/- animals; the YJYXF-H group and the YJYXF-L groups were orally administered with YJYXF at 10:00 every day, oral atorvastatin was given to the AC group, and an equivalent amount of saline was given to the Mod group, and all of them were administered for 12 weeks.

Fig. 3: Effects of YJYXF on hepatic metabolism in AS mice. (A) PCA, (B) differential metabolite volcano plot, (C) differential metabolite histogram, (D) clustered heatmap, and (E) KEGG pathway enrichment analysis. M, model group; T, treatment group (YJYXF-H group).

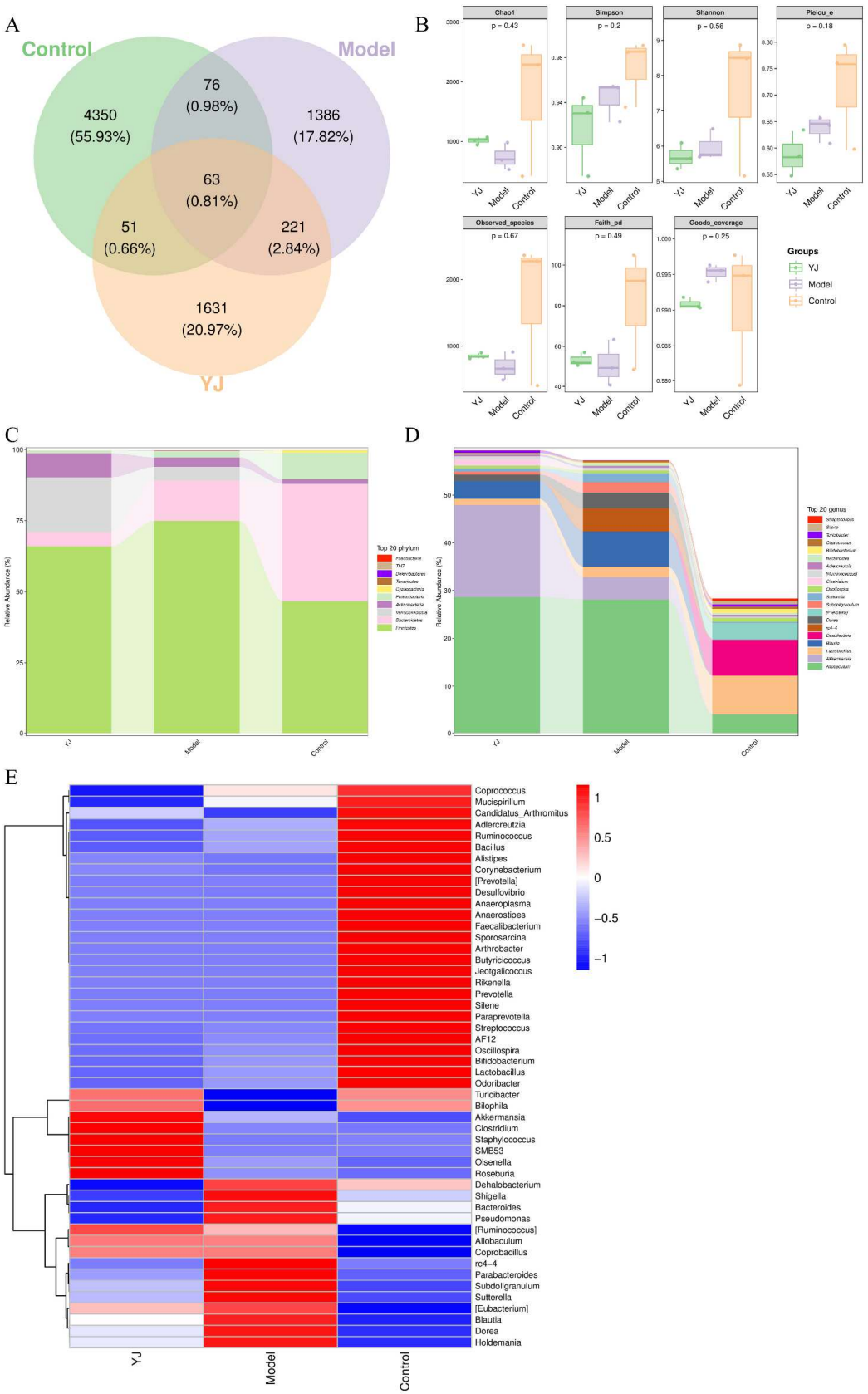

Fig. 4: Impact of YJYXF on AS mice's intestinal microbiota. (A) OUT Wayne plots; (B) alpha diversity analysis; (C) phylum level; (D) genus level; (E) clustered heatmap. YJ, YJYXF; Model, model group; Control, control group.

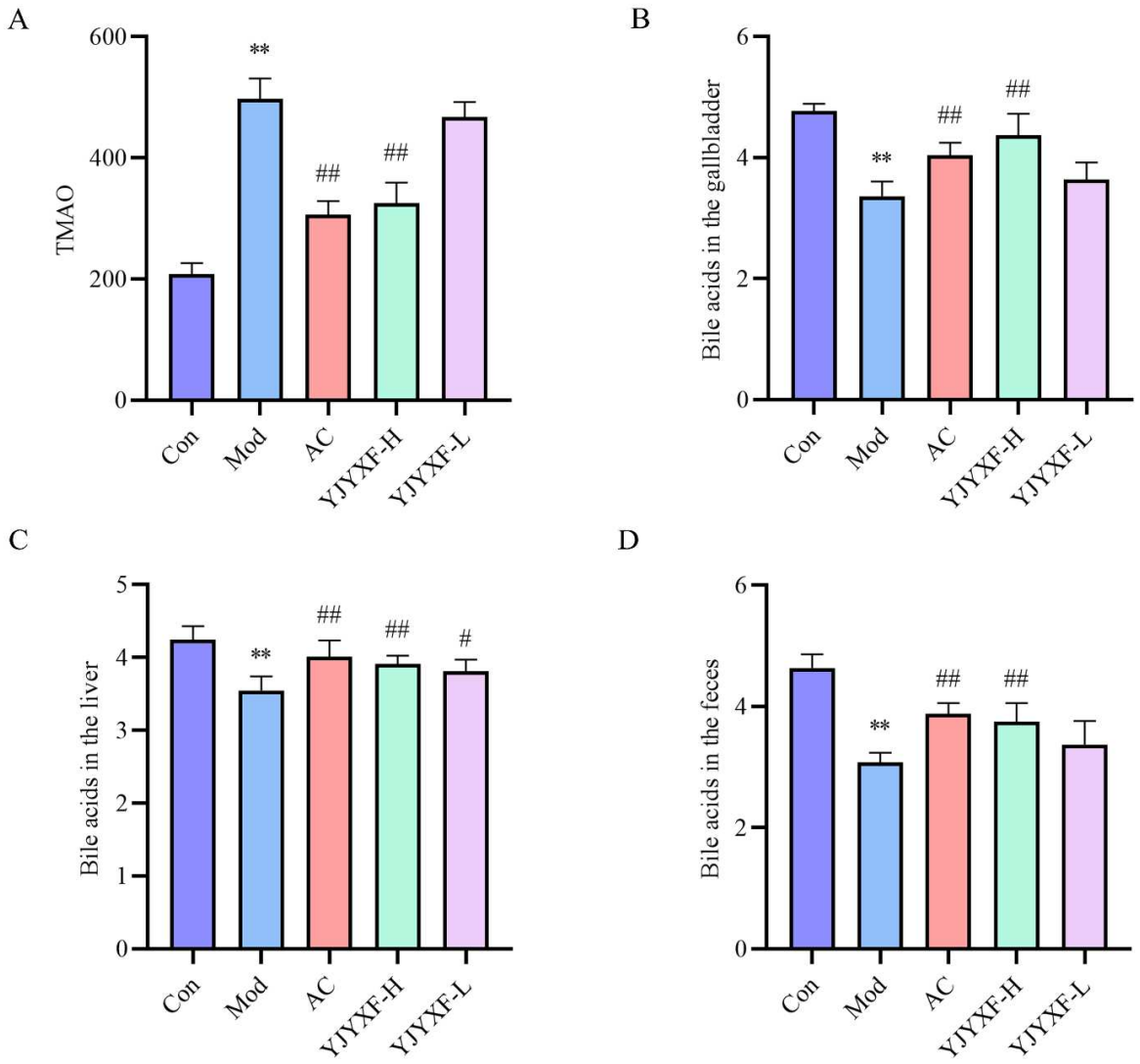

Fig. 5: YJYXF decreased TMAO and increased bile acids in AS mice. (A) TMAO, (B) bile acids in the gallbladder, (C) bile acids in the liver, and (D) bile acids in feces. n=6. In contrast to the Con group, $^{*}p<0.05$, $^{**}p<0.01$; in contrast to the Mod group, $^{\#}p<0.05$, $^{\#\#}p<0.01$.

Blood lipid level determination

Pentobarbital sodium was injected intraperitoneally to anesthetize mice. Blood was centrifuged at 4000 revolutions/min for 15 minutes at a temperature of 4°C. The serum and total cholesterol following the instructions provided by the kits was measured. (TC; no. A111-1-1), triglyceride (TG; no. A110-1-1), low-density lipoprotein cholesterol (LDL-C; no. A113-1-1), high-density lipoprotein cholesterol (HDL-C; no. A112-1-1). Every kit was bought from the Nanjing Jiancheng Bioengineering Institute.

Serum inflammatory factor level determination

Serum was measured using a TNF-α ELISA Kit (No. H052-1-2), IL-6ELISA Kit (No. H007-1-2), and MCP-1ELISA kits (no. H115-1-2). The Nanjing Jiancheng Bioengineering Institute was the source of all kits.

Atherosclerotic Lesion Analysis

After the mice were decapitated and sacrificed, they were placed in the supine position, and their abdomens were sterilized using iodophor. Select the skin of the abdomen with forceps, hold the scissors, cut open the abdomen along the midline to the neck, and cut open the septum and ribs to expose the cardiac components. Using forceps to gently hold the heart, insert the infusion needle into the left ventricle along the tip of the heart, cut open the right auricle of the heart, and perfuse the saline solution until the color of the liver slowly changes to light pink, which indicates that the cardiac perfusion had been successful. At the end of the perfusion, the infusion needle was deleted. The lungs, liver, spleen, gastrointestinal tissues, and organs only only was removed to expose the aorta from the heart to a position below the iliac bifurcation.

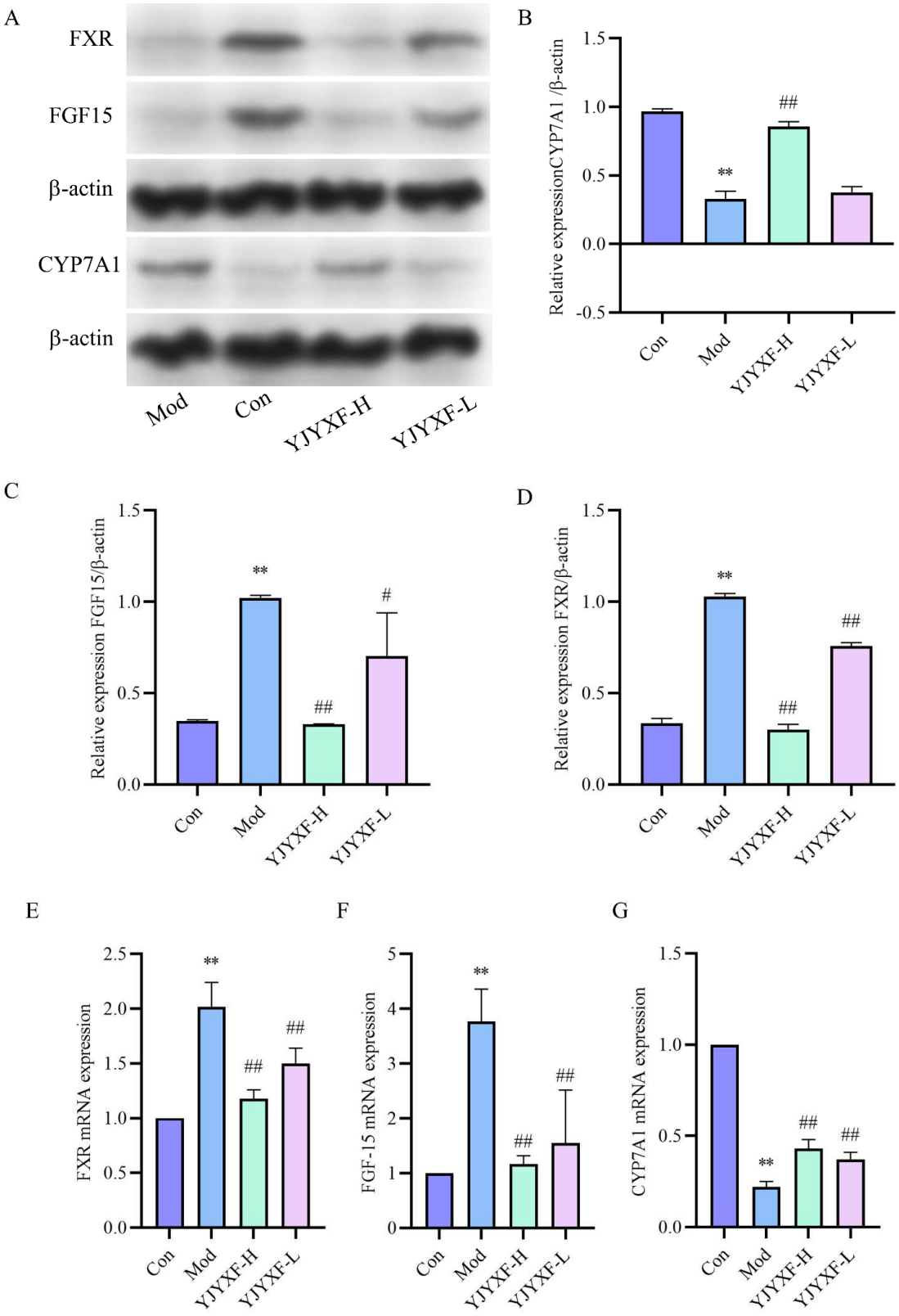

Fig. 6: YJYXF affects the expression of key genes and proteins for cholesterol metabolism in AS mice. (A) Cholesterol metabolism key protein expression;(B) quantified protein levels of CYP7A1;(C) quantified protein levels of FGF15; (D) quantified protein levels of FXR; (E) FXR mRNA expression; (F) FGF15 mRNA expression; (G) CYP7A1 mRNA expression. In contrast to the Con group, $^{*}p<0.05$, $^{**}p<0.01$; in contrast to the Mod group, $^{\#}p<0.05$, $^{\#\#}p<0.01$.

The entire aorta was harvested by detaching the aorta along the spine of the mice, as far as the arterial arches and as far as 2-3 mm below the iliac bifurcation. The aorta was divorced, transferred to a black vinyl dish, and placed under a stereomicroscope. The Adipose tissue around the entire aorta was carefully peeled off using precision scissors and tweezers, and fixed in a general-purpose tissue fixative (No. G1101 was bought from Wuhan Servicebio Technology Company).

Oil red O staining

After removing the tissue from the fixative and washing twice with PBS, the vessels were gently cut longitudinally along the vessel walls using dissecting scissors. The cut arteries were then briefly cleaned with tap water for five seconds. After being submerged for three seconds in 60% isopropyl alcohol (no.80109218, bought from Sinopharm Chemical Reagent Company), the blood vessels were submerged at 37 °C in Oil Red O staining solution (no. G1015, bought from Wuhan Servicebio Technology Company), protected from light for 60 min, and then removed. The vessels were removed using forceps and submerged in 60% isopropyl alcohol for differentiation. One minute was allotted for differentiation or until the fatty plaques in the lumen were bright red or orange, while the surrounding tissue was almost completely colorless. Differentiation was terminated by washing the cells with distilled water. Finally, the vessels were removed and used to absorb excess water with filter paper, and a slide was placed on a scale-equipped black background plate. The blood vessels were spread out on a slide, a well-lit area was chosen, and the camera (D70, purchased from Canon) was adjusted for focus and exposure to capture pictures.

Aorta hematoxylin-eosin (H&E) staining

After removing the tissues and using filter paper to absorb some of the surface water, the tissues were placed cut-side up on the embedding table for cryo-embedded sectioning at 8 μm thickness. The tissue was attached to the slide by placing a clean slide flat on top of the cut tissue piece. Following a 15-minute fixing period using a tissue fixative, the tissue was washed under running water. The slices were submerged in an HD constant staining pretreatment solution. After immersing the slices in hematoxylin staining solution (no. G1004) for three to five minutes, rinsed under running water, and differentiated using differentiation solution (no. G1039), and then rinsed again with tap water. Finally, they were dyed back to blue using Blue Dako Bluing Buffer (No. G1040). After being dehydrated for one minute in 95% alcohol, sections were stained for 15 s with an eosin staining solution (No. G1076). The slices were sealed with neutral gum after being sequentially placed in Ethyl Alcohol (No.100092683) I, Ethyl Alcohol II, and Ethyl Alcohol III for two minutes each, butyl alcohol (No.100052190) I and II for two minutes each, and xylene (No.10023418) I and II for two minutes each. A light microscope (NIKON ECLIPSE E100, Nikon) (magnification, ×20) was used for the last observation. Wuhan Servicebio Technology Company supplied the differentiation solution, hematoxylin staining solution, Dako Bluing Buffer, and Hematoxylin and Eosin Staining Kit; Sinopharm Chemical Reagent Company supplied the xylene, butyl alcohol, and ethyl alcohol.

H650 targeted metabolomics assay

Shanghai Applied Protein Technology Company (Shanghai, China) conducted a targeted metabolomics analysis. Ultrahigh-performance Liquid Chromatography Triple Quadrupole Mass Spectrometry (UHPLC-QTRAP MS) was used to identify the metabolites in the samples monitored in MRM mode. The peaks from the MRM raw data were extracted using MultiQuant or Analyst software. The content was computed based on the standard curve and the ratio of each material's peak area to the internal standard's peak area. Data were analyzed using KEGG pathway analysis, differential metabolite screening, multidimensional statistical analysis, and univariate statistical analysis.

16S rRNA high-throughput sequencing for the detection of the intestinal microbiota in mice.

The Fecal Genomic DNA Extraction Kit was used to gather the cecum contents and extract the DNA. Following the concentration detection, 1% agarose electrophoresis was used to measure the size of the DNA fragments, and the DNA was kept for later analysis at -20°C. The V4-V5 region and whole 16S rRNA gene were amplified, and the amplified products were subjected to high-throughput sequencing. After each sample's PCR amplification product was extracted in 30 ng for equal mixing. For separation, 1.2% agarose gel electrophoresis was employed. The gel strips of DNA were collected and purified using a kit, and the purified mixed DNA samples were analyzed by sequencing.

Mouse serum TMAO Assay

Serum samples were added to 75 μL of 80% acetonitrile to precipitate the proteins, and D9-TMAO was added to the mixture as an appropriate internal standard. The samples were mixed and centrifuged for 30 minutes at 3000 r/min after being incubated for 30 minutes at 4°C. To quantify the samples, TMAO standard solutions were prepared at concentrations of 1, 8, 40, 200, 500, 1000, 2500, and 5000 ng/mL. Y=490.287164*X+13000.065469, with a correlation coefficient of R2=0.9995, is the standard curve equation that LC/MS was able to determine.

Determination of bile acids in mouse gallbladder, liver, and intestinal contents

The specimen of the gall bladder, liver, small intestine and cecum contents were collected. Using the bile acid ELISA kit (no. ml037200, Shanghai Enzyme-linked Biotechnology Co., Ltd.), the amount of bile acid in each sample was ascertained.

Western blot was used to measure intestinal FXR and FGF15 protein expression, and liver CYP7A1 protein expression.

Intestinal and liver specimens were repeatedly ground to a powder in liquid nitrogen, and tissue proteins were collected by lysis centrifugation on ice. The protein content, which was assessed using the BCA method, gave results that allowed further processing. For each sample, 20 μL per well was loaded for the assay to proceed. After quantification, the protein was boiled for 5 min, and then subjected to SDS polyacrylamide gel electrophoresis. After membrane transfer, the membrane was closed with 5% skimmed milk for 2 h, rinsed with TBST for 3 times and then added with primary antibody at 4° overnight, and then rinsed with TBST for 3 times and then added with secondary antibody and incubated at 37° for 1 h, and then visualized by ECL. Gray scale values of target protein bands were collected using Quantity One software, and the target protein to β-actin grayscale ratio was computed.

RT-PCR analysis of mRNA levels for intestinal FXR, FGF15, and hepatic Cyp7a1 in mouse

Liver and intestinal tissue samples were collected to isolate total RNA. This RNA underwent reverse transcription to produce complementary DNA (cDNA). The cDNA obtained served as the template material for further amplification procedures using primers that were specific and targeted to genes like GAPDH, the farnesoid X receptor (FXR), fibroblast growth factor 15 (FGF15), and cholesterol 7 alpha-hydroxylase (CYP7A1). The procedure is described in the instruction manual. Each sample was analyzed three times for real-time data collection and quantitative analysis. The primer sequences used were FXR (forward: 5′-AATGAGGACGACAGCGAAGG-3,' reverse: 5′-TCTGTTGGTCTGCCGTGAGT-3′), FGF15 (forward: 5′-GAGGACCAAAACGAACGAAATT-3,' reverse: 5′-ACGTCCTTGATGGCAATCG-3′), CYP7A1 (forward: 5′-GGGGATTGCTGTGGTAGTGA-3,' reverse: 5′-CAGCCCAGGTATGGAATCAAC-3′), GAPDH (forward: 5′-GGTTGTCTCCTGCGACTTCA-3,' reverse: 5′-TGGTCCAGGGTTTCTTACTCC-3′), GAPDH as a reference gene. Quantified using the $2^{-\Delta\Delta CT}$ comparative threshold cycle method.

STATISTICAL ANALYSIS

Using GraphPad Prism 8, one-way analysis of variance (ANOVA) was employed to assess all of the data. Each result is presented as $\bar{x} \pm s$, with a p-value of less than 0.05 being considered statistically significant.

RESULTS

Effects of YJYXF on blood lipids and inflammatory factors in AS mice

The outcomes of lipid level measurements in mice (fig. 1) demonstrated that, compared to the Con group, the Mod group showed much elevated TC, TG, and LDL-C levels ($p<0.01$), and at the same time, their HDL-C levels were noticeably reduced ($p<0.01$). In contrast to the Mod group, the mice in the AC group and the YJXYF-H group had considerably lower serum levels of TC, TG, and LDL-C ($p<0.01$). The HDL levels were not significantly elevated (See fig. 1). No notable changes were observed in the YJXYF-L group ($p>0.05$). The results of inflammatory factors showed (fig. 1) that mice in the Mod group had remarkably higher TNF-α, IL-6, and MCP-1 levels in serum than mice in the Con group ($p<0.01$), and that mice in the AC and YJYXF-H groups had remarkably lower TNF-α and IL-6 levels in serum ($p<0.01$) than mice in the Mod group, but MCP-1 levels did not change significantly ($p>0.05$). No notable changes were observed in the YJXYF-L group ($p>0.05$).

Effects of YJYXF on the aorta of AS mice

The HE staining results (fig. 2A) showed that the aortic vessel wall was structurally intact, and no atheromatous plaques were observed in the Con group. In contrast to the Con group, the Mod group had a greater degree of atheromatous plaques, inflammatory cell infiltration within the plaques, and markedly thicker, structurally disordered, and mesangial hyperplasia in the intima of the arteries at the plaque attachment. In contrast to the Mod group, the inner walls of the vessels in the YJYXF-L, YJYXF-H, and AC groups were less smooth, a small amount of atheromatous plaque was observed, and the degree of inflammatory cell infiltration and the thickness of the vessel wall showed different degrees of improvement. Oil Red O staining (fig. 2B) showed no atherosclerotic plaques in the whole vessels of the Con group, and the aorta of the Mod group had significant atherosclerotic plaques with a block-like distribution. Aortic atheromatous plaque in the model group was remarkably higher than in the control group and remarkably reduced after treatment with YJYXF-H and AC ($p<0.01$). However, the YJYXF-L group's total aortic atheromatous plaque did not significantly change.

Effects of YJYXF on the hepatic metabolomics in AS mice

Fig. 3A shows that there was a high degree of aggregation within the groups and a clear separation between the groups, indicating a reliable model and a significant difference between the groups. The differential metabolite analysis results (fig. 3B) showed 74 metabolites that differed between the Mod group and YJYXF group, of which 17 were increased and 57 were reduced. These metabolites were categorized broadly into types like fatty acids, carboxylic acids and their derivatives, along with steroids and related derivatives. The metabolite change (FC) values after log2 treatment are shown in Figure 3C. This indicated that YJYXF effectively regulated lipid metabolism. Differential metabolite clustering analysis showed (fig. 3D) that the metabolites within the same

cluster had comparable expression patterns or functional similarities. Differential metabolites were performed by KEGG pathway enrichment analysis. It was observed (fig. 3E) that the differential metabolites, mostly seen in areas like protein digestion and absorption, were also found in pathways like central carbon metabolism in cancer, ABC transporters, biosynthesis of amino acids, aminoacyl-tRNA biosynthesis, biosynthesis of unsaturated fatty acids, and mineral absorption.

Effects of YJYXF on intestinal microbiota in AS mice

OTU clustering analysis is shown in Figure 4A; there are 4350 specific asv in the Con group, 1386 specific asv in the Mod group, 1631 specific asv in the YJYXF-H group, and 63 asv common to all three groups, which shows that YJYXF can affect the intestinal microbiota. The results of alpha diversity analysis (Fig. 4B) that the Chao1 and Observed-species indices decreased in the Mod group compared to the Con group, and these indices increased in mice treated with YJYXF. The Goods-coverage index of fecal microbial communities exceeded 0.9 in all three groups, indicating high microbial coverage. The Pielou index of microbial communities was less than 0.7 in all three groups. Microbial Simpson, Shannon, and Faith indices were greater in the Con group than in the Mod group and decreased after YJYXF treatment. Diversity analysis showed that after receiving YJYXF therapy, the model group's intestinal flora changed in diversity.

At the phylum level (fig. 4C), the dominant microorganisms in each group of mice were Firmicutes, Bacteroidetes, Verrucomicrobia, Actinobacteria, Proteobacteria, and Cyanobacteria. After receiving YJYXF treatment, the abundances of Firmicutes, Bacteroidetes, and Proteobacteria declined in comparison to the Mod group, whereas the abundances of Verrucomicrobia and Actinobacteria increased. Cyanobacterial levels were not significantly altered. At the genus level (fig. 4D), when contrasting the Mod group with the YJYXF group, there were no appreciable variations in the abundances of Allobaculum, Prevotella, Silene, Streptococcus, Dorea, Oscillospira, Ruminococcus, Adlercreutzia, and Desulfovibrio. The abundance of Akkermansia, Turiciabacter, and Clostridium increased, and the abundance of Lactobacillus, Blautia, rc4-4, Dorea, Subdoligranulum, Sutterella, Bacteroides, Bifidobacterium, Coprococcus, and other microbial communities decreased after treatment. Heat maps were created using the abundance data at the genus level for the top 50 average abundances. The clustered heat map (fig. 4E), the abundances of Turicibacter, Bilophila, Akkermansia, Clostridium, Staphylococcus, SMB53, Olsenella, and Roseburia were elevated after YJYXF treatment, while the abundances of Dehalobacterium, Shigella, Bacteroides, Pseudomonas, rc4-4, Parabacteroides, Subdoligranulum, Sutterella, Blautia, Dorea, and Holdemania, and other colonies decreased after treatment, which is consistent with the genus level species composition histogram analysis.

Effects of YJYXF on TMAO in AS mice

The levels of TMAO in mice that were in the high-fat diet-induced AS model were clearly higher ($p<0.01$) than in those that were in the control group. When looking at the YJYXF-H and AC groups compared to the Mod group, a significant reduction was noted ($p<0.01$). However, the TMAO levels in the YJYXF-L group didn't remarkably change ($p>0.05$)(fig. 5A).

Effects of YJYXF on bile acid content in AS mice

Bile acid content is lower in model group according to the figure 5. However, looking at the bile acid contents in the AC and YJYXF-H groups, it increased clearly ($p<0.01$) compared to the Mod group. As for the YJYXF-L group, the bile acid levels in the gallbladder, liver, and intestines showed no statistically significant changes. (fig. 5).

Effects of YJYXF on the expression of key genes and proteins of cholesterol metabolism in AS mice

Compared to what was seen in the Con group, there was a noticeable increase in FXR and FGF-15 mRNA and protein expression levels in the intestine ($p<0.01$), while a clear decrease was found in the levels of hepatic CYP7A1 mRNA and protein expression ($p<0.01$) in the Mod group. In contrast, the YJYXF-H group showed a significant rise in CYP7A1 mRNA and protein levels in the liver ($p<0.01$), along with a marked reduction ($p<0.01$) in the intestinal FXR and FGF-15 mRNA and protein expression compared to the Mod group (fig. 6).

DISCUSSION

Most mouse models of atherosclerosis are manipulated by genes that play a key role in lipid metabolism, such as apolipoproteins; therefore, in the experiment, ApoE-/- mice were selected to be fed a high-fat diet to establish an atherosclerosis model (Wang *et al*., 2022, Li *et al*., 2024a), and C57BL/6J mice were selected as the normal group (Ilyas *et al*., 2022). Dai medicine believes that atherosclerosis is the result of people eating too much, leading to the imbalance of the "four towers," "five elements," poisonous evil within, and internal injury to internal organs, referred to as "food poisoning" (Luo *et al*., 2023b). YJYXF can regulate the functions of the body's "four towers and five elements," remove toxins from the human body, protect the functions of internal organs, and has been proven to improve AS (Luo *et al*., 2023a), but the mechanism of its action is still unclear. Therefore, this study designed Con, Mod, AC, YJYXF-H, and YJYXF-L groups to investigate the effect of YJYXF in AS mice. The experimental results showed that YJYXF could improve AS.

Lipid metabolism problems are known to be risk factors for AS, which is the build-up of vascular plaques in the arterial wall (Wan *et al*., 2024). Inflammation is the primary cause

of plaque development, rupture, and arterial thrombosis. Lipids are deposited in the vascular intima as a result of arterial thrombosis, which accelerates plaque production(Liang *et al*., 2024b). Lipid levels are an important indicator of lipid metabolism, and inflammatory factors can be used to identify high-risk plaques (Zheng *et al*., 2024). The experiment's findings demonstrated that YJYXF may considerably raise HDL-C levels while considerably lowering TNF-α, IL-6, TC, TG, and LDL-C levels. The results of aortic oil red O and HE staining showed that atorvastatin and YJYXF could slow down the proliferation of aortic plaques, indicating that YJYXF could regulate lipid metabolism and inflammation.

AS is linked to lipid build-up in the liver (Li *et al*., 2024b). The liver is the primary site of lipid synthesis in the body and plays a significant role in lipid metabolism (Xu *et al*., 2022, Du *et al*., 2023, Li *et al*., 2015). The results of hepatic metabolomics showed that the differential metabolites in the liver are associated with lipid metabolism, indicating that YJYXF can improve lipid metabolism in the liver.

Growing evidence shows intestinal microbiota can modulate AS (Ai *et al*., 2024, Mao *et al*., 2024, Jiang *et al*., 2024b). The study shows that Verrucomicrobia, Turicibacter, Desulfovibrio, Akkermansia, and Firmicutes were significantly associated with AS.(Wang *et al*., 2024, Wan *et al*., 2024, Depommier *et al*., 2019, Centner *et al*., 2023, He *et al*., 2024, Liang *et al*., 2024b, Huang *et al*., 2023). Beneficial bacteria, such as Akkermansia and Lactobacillus, can reduce the risk of AS (Jiang *et al*., 2023). Our research is proving just that YJYXF modulated the diversity and abundance of gut microbiota, especially decreasing the abundances of Firmicutes, Bacteroidetes, and Proteobacteria and increasing the abundance of Verrucomicrobia at the phylum level, increasing the abundances of Akkermansia and Turiciabacter, and decreasing the abundances of Blautia, Bacteroides, *et al* at genus level. Therefore, YJYXF can regulate atherosclerosis through the intestinal microbiota.

Clinically, plaque scores in AS patients are positively correlated with TMAO, and a large amount of literature suggests that the production of TMAO aggravates the progression of AS (Zheng *et al*., 2024, Zhu *et al*., 2023). TMAO is an intestinal bacteria-derived metabolite (Bao *et al*., 2024) that contains choline, L-carnitine, and phosphatidylcholine from red meat (Li *et al*., 2024c, Jiang *et al*., 2024a)through intestinal microbiota to produce TMA, which is transported through the bloodstream to the liver, where it is further metabolized to TMAO by flavin monooxygenase 3 (FMO3) (Ma *et al*., 2022). However, TMAO can promote AS development through lipid metabolism, oxidative stress, inflammation, platelet activation, and aggregation (Zheng *et al*., 2024, Bao *et al*., 2024, Zhuo *et al*., 2023, Luo *et al*., 2024). TMA can be produced by intestinal microorganisms, including Clostridium, Firmicutes and Proteobacteria (Zhuo *et al*., 2023, Fei *et al*., 2024). It has been demonstrated that remodeling the abundances of intestinal microbiota such as Prevotella, Bacteroides, Akkermansia, and Lactobacillus can inhibit the production of TMA, which in turn affects TMAO levels and alleviates AS (Chen *et al*., 2016). The experimental results show that YJYXF can reduce the synthesis of TMAO by regulating the intestinal microbiota to alleviate AS.

Hypercholesterolemia plays a crucial role in accelerating the progression of AS, as it is an independent risk factor for the condition (Wang *et al*., 2023). The main pathway of cholesterol metabolism is bile acid metabolism, regulated by FXR and cyp7a1 (Sheng *et al*., 2024). Bile acids may alleviate AS progression by modulating the inflammatory response, endothelial function, and lipid metabolism. Bile acid metabolism mediated by intestinal microbiota plays a crucial role in maintaining bile acid homeostasis (Chi *et al*., 2024, Schoeler and Caesar, 2019). Bile acids are synthesized in the liver (Chen *et al*., 2016)and are transformed by the intestinal microbiota upon excretion into the gut, thus affecting host metabolism (Huang *et al*., 2024). TMAO can also affect cholesterol metabolism by modulating the bile acid synthesis pathway (Li *et al*., 2024c, Chen *et al*., 2016). The results of this study showed that YJYXF could elevate the bile acid content in the gallbladder, liver, and intestinal of mice, suggesting that YJYXF could regulate the synthesis of bile acids to regulate cholesterol metabolism in mice.

FXR plays a crucial role in the synthesis of bile acids, which in turn can influence AS by promoting the breakdown of cholesterol into bile acids, regulating intestinal FXR, inducing FGF15/19 expression, and changing CYP7A1 expression through the liver-gut axis (Liang *et al*., 2024a, Kliewer and Mangelsdorf, 2015). Alteration of the abundances of Lactobacillus, Bacteroides, Blautia, Roseburia, and Clostridium can regulate bile acid production through the expression of CYP7A1 and FXR/FGF15 (Wang *et al*., 2024, Wang *et al*., 2020). Research has revealed that bile acid levels can be regulated by down-regulating FXR and FGF15 levels and up-regulating CYP7A1 levels (Yu *et al*., 2024). It has been shown to promote bile acid metabolism by remodeling the microbiota and inhibiting intestinal FXR-FGF15 (Wang *et al*., 2024). Naringin has been reported to inhibit CYP7A1 expression to regulate bile acid synthesis by down-regulating FXR-FGF15 (Wang *et al*., 2020). Studies have demonstrated that AS can be prevented by activating FXR and bile acid excretion (Sheng *et al*., 2024). Additionally, YJYXF dramatically boosted the levels of CYP7A1 mRNA and protein expression in the liver while considerably reducing the levels of FXR, FGF-15 mRNA, and protein expression, according to our studies. Therefore, YJYXF can regulate bile acid metabolism through intestinal microbiota to prevent AS.

CONCLUSION

In summary, YJYXF alleviates the development of AS. Our study demonstrated that YJYXF regulates lipid metabolism and inflammation by modulating intestinal microbiota and TMAO to prevent and treat AS. This research offers YJYXF a fresh foundation for treating and preventing AS.

ACKNOWLEDGMENTS

Thanks for the support of Yunnan Yunnan Provincial Department of Science and Technology Basic Research Special Program - Top Level Project (202101AT070250), Yunnan Provincial Department of Science and Technology Joint Special Project on Traditional Chinese Medicine - Top Level Project (202101AZ070001-231), High-level Chinese Medicine Talents (Reserve Talents) in Yunnan Province, Training Program for Young Clinical Talents in Traditional Chinese Medicine under the Eagle Program of the Chinese Society of Traditional Chinese Medicine (Phase I), The First Affiliated Hospital of Yunnan University of Traditional Chinese Medicine "Excellence in Young Talent Program (Composite)" Project (No.: Yunzhong attached to the Party word [2022] No. 3), Yunnan Provincial Science and Technology Department of Chinese Medicine Joint Program-Youth Program (202001AZ070001-094), Open Project of Yunnan Provincial Key Laboratory of Dai Medicine and Yi Medicine (2021K002) and Open Subject Project of Yunnan Provincial Key Laboratory of Dai Medicine and Yi Medicine (202210ZD2211).

Conflict of interest

The authors declare that they have no competing interests.

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