Moderate red wine alleviates high-fat diet-induced atherosclerosis in ApoE−/− mice via modulations of liver metabolism through gut microbiota remodeling

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ABSTRACT: Red wine has a good potential for alleviating atherosclerosis, but the mechanisms related to hepatointestinal circulation remain to be elucidated. This study showed that administration of a high-polyphenol red wine (16 mL/kg/day) for 16 weeks significantly reduced the atherosclerotic lesion in high-fat diet-fed ApoE−/− mice. The total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels of plasma were lowered by 11.54% and 18.98%. The pro-inflammatory cytokines including interleukin-6 (IL-6) and tumor necrosis factor (TNF-α) levels were decreased by 27.59% and 31.92%. Red wine also reduced triglyceride (TG) level and lipid deposition in the liver, and increased the concentration of total bile acids (TBA). Untargeted metabolomics analysis indicated that red wine modulated the disorder of liver metabolism by regulating sphingolipid signaling pathway, sphingolipid metabolism, glycerophospholipid metabolism, choline metabolism and bile secretion. 16S rRNA sequencing revealed that red wine increased the abundance of Akkermansia and Bifidobacterium and reduced the abundance of Mucispirillum, Romboutsia, Lactobacillus, Bilophila and Blautia, along with the increased concentrations of short-chain fatty acids (SCFAs) in feces. These findings indicated that red wine could exert anti-atherosclerotic effect by regulating gut microbiota, restoring SCFAs, alleviating liver metabolic disorders.

Keywords: Wine; Atherosclerosis; Gut microbiota; Untargeted metabolomics; Short chain fatty acids

1. Introduction

Atherosclerosis (AS) is a chronic vascular disease driven by disorders of lipid metabolism, and characterized by accumulation of lipids in the arterial wall with inflammatory response and endothelial dysfunction [1, 2]. Liver plays a key role in lipid homeostasis, including intake, synthesis and transport cholesterol, synthesis triglycerides, intake and secretion lipoproteins [3, 4]. Hepatic dysfunction can accelerate atherosclerosis via pro-atherogenic lipid changes [5], increasing pro-inflammatory factors and dyslipidemia [6]. Hence, regulating liver metabolism is one of the effective measures to reduce the incidence of atherosclerosis.
Moderate red wine consumption can decrease the incidence of cardiovascular diseases, including AS [7]. It is widely recognized that the red wine polyphenols confer cardioprotective effects [8, 9]. Previous reports mainly focused on the antioxidant [10], anti-inflammatory [11], inhibition of platelet aggregation [12] and reduction of endothelin synthesis [13, 14] to explain red wine’s cardioprotective effect. So far, there is a lack of reports on red wine inhibiting the development of AS by regulating liver metabolism.

Besides, gut microbiota plays important roles on AS by changing the microbial structure and their metabolites [15]. Accumulating evidences have showed that some gut microbiota participated in modulating cardiovascular health through producing intermediate metabolites [16-18], and some gut microbiota exerted anti-atherosclerotic effects by decreasing inflammatory factors and improving the gut barrier [19]. In addition, most phenolic components in red wine were not absorbed in small intestine and passed into the large intestine, in which it modulated the composition of the gut microbiota [20]. Meanwhile, phenolic components could be decomposed by gut microbiota and affect the host metabolism via enterohepatic circulation [21]. In the present study, we hypothesis that red wine can reduce the development of AS by improving the disorder of liver through hepatointestinal circulation.

Several specific bioactive compounds in red wine have been shown to attenuate AS by modulating the gut microbiota in mice. For example, the oral administration of quercetin changed the structure of the gut microbiota and reduced the contents of atherogenic lipid metabolites [22]. Resveratrol attenuated AS by regulating bile acid metabolism via gut microbiota remodeling [23]. The main substances in red wine are alcohol and kinds of polyphenols and there may be synergistic effects among different components, which could not be fully explored using specific bioactive compounds. While there are few reports on how direct intervention of red wine alleviate AS in ApoE−/− mice.

ApoE−/− mice are ideal experimental models for studying the pathogenesis of AS since they are unable to carry out reverse cholesterol transport. Our previous research found that red wines from high-altitude Shangri-La region of Yunnan province, China, have higher polyphenol contents, and may possess a good potential to inhibit the development of AS. This study was conducted to evaluate the alleviation effect of high-fat diet-induced AS in ApoE−/− mice by a high-polyphenol red wine intervention and to explore the potential mechanisms based on remodeling of gut microbiota and liver metabolites. A group of ApoE−/− mice gavaged with the same alcohol content in the red wine was set as a control. Gut microbiota profiles were analyzed by 16S rRNA sequencing and liver metabolites were investigated using untargeted metabolomics. Correlation analysis was carried out to reveal the possible relationships among gut microbiota, liver metabolites and anti-atherosclerotic function of red wine.

2. Materials and methods

2.1 Wine samples

A high-quality Cabernet Sauvignon red wine from Shangri-La wine region (Yunnan, China) was used for the animal experiments. The basic chemical composition of wine was alcohol content 14.6 (vol%), total acid
6.6 g/L expressed as tartaric acid, total sugar 6.7 g/L, pH 3.45, volatile acid 0.66 g/L and total phenolic content 3553.4 mg/L expressed as mg of gallic acid equivalents.

2.2 Animals and experimental design

Animal experiments were strictly conducted under the guidelines of Ethical Inspection of Animal Experimental at Laboratory Animal Centre of Huazhong Agriculture University (Approval NO.: 202111090010). Fifty 8-week-old male ApoE⁻/⁻ mice were bought from Beijing Vital River Laboratory Animal Technology Co. Ltd (SCXK2021-0006) (Beijing, China). After a week of adaption, 50 mice were randomly divided into five groups (10 mice/group), and were fed and orally administered as follows: normal diet group (ND); high-fat diet group (HFD); saline-treated group (HFD_S), HFD gavaged with 16 mL/kg/day body weight of physiologic saline solution; red wine-treated group (HFD_W), HFD gavaged with 16 mL/kg/day body weight of red wine; alcohol-treated group (HFD_A), HFD gavaged with 16 mL/kg/day body weight of alcohol solution. The ND group was fed with normal diet (TP26312, Nantong Trophic Co., Ltd. China). The other four groups were fed with high-fat diet (TP26300, Nantong Trophic Co., Ltd. China). The specific diet information was shown in Table S1.

Mice were placed in a 12-hour light/dark cycle room at 25°C and were free to eat and drink water. Foods were changed and recorded per 2 days, and body weight of each mice was weekly recorded. Collect fecal samples on the last three days of the experiment and store at −80 ℃ for microbiota analysis. After 16 weeks, mice were sacrificed at the last day after fasting for 12 hours. Blood were collected and centrifuge at 4 ℃, 3000 g, for 20 minutes to separate plasma. Organs were carefully taken (liver, kidney, spleen, small intestine and colon) and washed with normal saline. Parts of liver tissues were put in 4% formalin solution; other samples were snap-frozen and then stored at −80 ℃ for further use.

2.3 Dosage information

Mice in HFD_W and HFD_A groups were gavaged red wine and alcohol solution (edible distilled ethanol 14.6 vol%) at a dose of 16 mL/kg/day body weight, corresponding to 310-420 mg of ethanol per mouse per week. The weight range of three groups of mice treated by gavage from week 0 to week 16 was 24.35-35.27 g. Range of gavage dose was 0.39-0.56 mL. Based on previous report [24], the administration dosage was consistent with a human equivalent dose of 100 g ethanol per week for a 70 kg adult human, and was calculated by the conversion factor between mice and humans.

2.4 Evaluation of atherosclerosis

Atherosclerotic lesions were measured in the entire aorta and aortic root as previously described [25]. The aorta was fixed in 4% paraformaldehyde for 6 hours, opened longitudinally, and stained with Oil Red O to determine the size of the lesion. The degree of atherosclerosis was determined by calculating the percentage of the aorta area covered by plaque. To assess the atherosclerotic lesions at the aortic sinus, the upper portions of the hearts were preserved in an optimal cutting temperature compound, and 10-µm-thick frozen sections were stained with Oil Red O. Image Pro Plus 6.0 was used to calculate the area of stained section.
2.5 Histological analysis of liver tissues

Hematoxylin, eosin staining and Oil Red O staining of the liver tissues were conducted as previous method [26].

2.6 Biochemical analysis of plasma and liver tissues

Total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and total bile acid (TBA) of plasma were measured using an automatic biochemistry analyzer (Biobase BK-280, China). Plasma cytokines, including tumor necrosis factor (TNF-α) and interleukin-6 (IL-6) were analyzed enzymatically by immunosorbent assay (ELISA) following the manufacturers' protocols. Liver TC, TG and TBA levels were assessed enzymatically by commercial kits (Nanjing Jiancheng Bioengineering Institute, Shanghai, China).

2.7 Metabolomics analysis

Liver samples were processed referring to previous study with a modification [27]. Briefly, take 50 mg of liver sample, add 400 µl of the extract solution (methanol: water 4:1) with 0.02 mg/mL of 2-Chloro-L-phenylalanine and with an addition of isotopically labeled standard). The samples were mixed by vortex for 30 s and homogenized at 50 Hz for 6 min, and then ultrasonic extracted for 30 min. The extracted samples were then centrifuged for 15 min (13,000 × g, 4 ℃). Take the supernatant for liquid chromatography-tandem mass spectrometry (LC-MS) analysis.

The chromatographic separation was performed using an ultrahigh-performance liquid chromatography system (Vanquish; Thermo Fisher Scientific) equipped with an Acquity UPLC HSS T3 column (100 mm × 2.1 mm i.d., 1.8 µm; Waters). The mobile phase consisted a mixture of 0.1% formic acid in acetonitrile: water (95:5) (A) and in acetonitrile: isopropanol (1:1) (B) at a flow rate of 0.4 mL·min⁻¹. The volume of sample injected was 2 µL. The gradient profile for mobile phase B was programmed a 0% at 0 min, 24.5% at 3.5 min, 65% at 5 min, 100 % at 5.5 min, 100% at 7.4min, 51.5% at 7.6 min, 0% at 7.8 min, and 10% at 10 min. A Q-Exactive HF-X mass spectrometer (Orbitrap MS; Thermo) was connected to the UPLC system and operated in both negative and positive ionization modes using an electrospray source. The mass conditions were set as follows: heater temperature, 425 ℃; capillary temperature, 325 ℃; sheath gas flow rate, 50 arb; aux gas flow rate, 13 arb; ion-spray voltage floating, −3,500 V and 3,500 V in negative and positive mode, respectively; S-Lens RF level, 50; normalized collision energy, 204,060 eV. The scan range was of 70 to 1050 m/z. The metabolomics data were analyzed using Progenesis QI software for baseline filtering, peak identification, integration and normalization. Potential liver biomarkers were tentatively identified using the HMDB database.

2.8 SCFAs analysis

To analysis the fecal SCFAs, a gas chromatography tandem mass-spectrometry (GC-MS) (7000D, Agilent, USA) was used according to the previously described method [28]. In brief, the feces samples were homogenized, and the supernatant was derivatized by adding propanol/pyridine mixture solvent and propyl
chloroformate followed by ultrasonication. After derivatization, the derivatives were extracted by hexane and analyzed by GC-MS.

2.9 16S rRNA sequencing of gut microbiota

The total bacterial DNA of fecal samples was extracted using a DNA extraction kit (BioTeke Corporation, Beijing, China) and the quality of extracted DNA was confirmed by agarose gel electrophoresis. Bacterial primers 341F (5'-CCTAYGGGRBGCASCAG-3’) and 806R (5'-GGACTACNNGGGTATCTAAT-3’) were used to amplify the region with a hypervariable region targeting V3-V4, followed by high-throughput sequencing on an Illumina Sequencer Miseq platform by Majorbio Bio-Pharm Technology Co. Ltd (Shanghai, China). Illumina paired end reads were merged and filtered with the QIIME program (v 1.9.1). Then, the Uparse software (version 7.0.1090) was used to cluster all quality filtered sequencing reads into OTUs (operational taxonomic units) at 97% pairwise identity, and RDP classifier with Bayesian algorithm was conducted to annotate the taxonomic information.

2.10 Statistical analysis

Experimental data were expressed as mean ± standard error (SEM). Statistical significances among different groups were analyzed by one-way ANOVA with Duncan's multiple range tests. Statistical significance was considered at $P < 0.05$.

3. Results

3.1 Red wine alleviated AS in high-fat diet-fed ApoE⁻/⁻ mice

There was no significant difference in weekly dietary intake in five groups (Fig. 1 A). However, the bodyweight of the HFD_W group was significantly lower than that of the HFD_S group from week 10 to week 14 ($P < 0.05$) (Fig. 1 B). Both the alcohol and red wine treatment significantly reduced the weight gain of the high-fat diet-fed mice ($P < 0.05$) (Fig. 1 C).

The development of AS was measured by the percentage of atherosclerotic lesion areas in the whole aorta and total vessel. Compared with the ND group, high-fat diet increased the plaque formation, while alcohol and red wine administration reduced the atherosclerotic plaques to different degrees in the aorta (Fig. 1 D, F). The quantitative results revealed that the atherosclerotic plaque rates of total aorta in HFD_A and HFD_W groups were decreased by 20% and 34.4%, respectively, compared with the HFD_S group (Fig. 1 E). Similarly, alleviation of atherosclerosis was observed in the aortic sinus. The atherosclerotic plaque area rates of aortic sinus in HFD_A and HFD_W groups were reduced by 12.58% and 46.32%, respectively, compared with the HFD_S group (Fig. 1 G).
Figure 1. The growth data and atherosclerotic lesion status in ApoE−/− mice. (A) Food intake for each week; (B) Body weight for each week; (C) Weight gain of 16 weeks; (D) Representative images of the full-length aorta stained with oil red O; (E) Quantification of lesion area percentage of the total aorta; (F) Representative images of the aortic sinus stained with oil red O; (G) Quantification of lesion area percentage of the total vessel. The line and *means $P < 0.05$ for HFD_W vs. HFD_S from week 10-14. Different letters (a–d) represent significant differences ($P < 0.05$) among groups by one-way ANOVA with Duncan’s multiple range test. ND, control; HFD, high fat diet; HFD_S, HFD administered with saline; HFD_A, HFD administered with alcohol; HFD_W, HFD administered with wine.

3.2 Red wine improved the lipids and inflammatory status in plasma of high-fat diet-fed ApoE−/− mice

Abnormal lipid metabolism, including elevated plasma TC, TG, LDL-C, TBA and reduced plasma HDL-C, is closely related to the metabolic diseases [29]. The plasma lipid profiles of different groups were shown in Fig. 2 (A-E). Compared with the HFD_S group, the TC and LDL-C levels of the HFD_W group were significantly reduced by 11.54% and 18.98%, and the TG, HDL-C and TBA levels were not significantly changed. Besides, alcohol treatment did not reduce the TC, TG, LDL-C, HDL-C and TBA levels, compared with the HFD_S group.

IL-6 and TNF-α are important pro-inflammatory factors in atherosclerosis. As shown in Fig. 2 (F, G), the HFD and HFD_S groups exhibited significantly higher plasma IL-6 and TNF-α level than the ND group. The red wine treatment significantly decreased the overexpression of IL-6 and TNF-α by 27.59% and 31.92%, respectively, compared with HFD_S group. Besides, alcohol administration also reduced the IL-6 and TNF-α levels by 8.03% and 10.91%, respectively, compared with HFD_S group.
Figure 2. Lipids (TC, TG, LDL-C, HDL-C, TBA) and inflammatory (IL-6 and TNF-α) levels of the plasma in ApoE−/− mice. (A) Total cholesterol; (B) Triglyceride; (C) Low density lipoprotein cholesterol; (D) High density lipoprotein cholesterol; (E) Total bile acids; (F) IL-6; (G) TNF-α. Values are expressed as mean ± SD, and different letters (a–d) represent significant differences ($P < 0.05$) among groups by one-way ANOVA with Duncan’s multiple range test. ND, normal diet; HFD, high fat diet; HFD_S, high-fat diet administered with saline; HFD_A, high-fat diet administered with alcohol; HFD_W, high-fat diet administered with red wine.

3.3 Red wine attenuated hepatic steatosis in high-fat diet-fed ApoE−/− mice

The plasma lipid and inflammatory levels associated with atherosclerosis were closely related to that in the liver tissues. H&E staining showed obvious fat vacuolization and inflammatory cell infiltration in the HFD and HFD_S groups, while they were reduced in both the HFD_A and HFD_W groups. Besides, the Oil Red O-staining results showed significantly reduced lipid deposition in the liver of the HFD_W group compared to HFD and HFD_S groups (Fig. 3 A, B). In addition, the concentration of liver TC showed a tendency to decrease, and that of TG was significantly decreased by red wine treatment ($P < 0.05$) (Fig. 3 C, D). The concentration of liver TBA was significantly increased by red wine treatment ($P < 0.05$) (Fig. 3 E). Therefore, the red wine intervention may modulate hepatic lipid and bile acid metabolism.

Figure 3. Histological analysis and lipids levels of the liver in ApoE−/− mice. (A) H&E stains and oil red stains; (B) Quantification of steatosis area of Oil Red O; (C) Total cholesterol; (D) Triglyceride; (E) Total bile acids. Values are expressed as mean ± SD, and different letters (a–d) represent significant differences ($P < 0.05$) among groups by one-way ANOVA with Duncan’s multiple range test. ND, normal diet; HFD, high fat diet; HFD_S, high-fat diet administered with saline; HFD_A, high-fat diet administered with alcohol; HFD_W, high-fat diet administered with red wine.
3.4 Red wine altered liver metabolites in high-fat diet-fed ApoE−/− mice

Since the above results showed that there was no significant difference in plaque rates, lipids and inflammatory levels between HFD_S and HFD groups, the liver tissue and fecal samples of the ND, HFD_S, HFD_A and HFD_W groups were prepared for liver metabolome and gut microbiome analysis.

3.4.1 Overall changes in liver metabolites

Untargeted metabolomics analysis was carried out to determine the differential metabolites in the liver tissues. A total of 752 metabolites (463 for the positive and 289 for the negative ion modes) were identified in the liver samples. Organic acids and derivatives (20.7%) and organoheterocyclic compounds (10.6%) were predominant at the superclass level (Fig. S1 A), amino acids, peptides and analogues (16.7%), fatty acids and conjugates (7.5%) were major components at the subclass level (Fig. S1 B). The PLS-DA score plots displayed significant differences among the 4 groups with good intragroup reproducibility (Fig. 4A). Subsequently, both HFD_W group and HFD_A group were clearly separated from the HFD_S group in the OPLS-DA models (Fig. S2 A, B). Permutation test of 200 response sorting indicated that the statistical model was stable and reliable (Fig. S2 C, D). To screen significantly differential metabolites, the following criteria were used: VIP value > 1 in OPLS-DA model and $P$ value < 0.05 in Student’s t-test. The Volcano plot showed that compared with the HFD_S group, 46 metabolites were up-regulated and 146 metabolites were down-regulated in the HFD_W group (Fig. 4 B), and 32 metabolites were up-regulated and 50 metabolites were down-regulated in the HFD_A group (Fig. 4 C). Heatmap of Fig. S3 (A, B) showed the top 50 metabolites significantly altered in HFD_W and HFD_A groups compared to HFD-S group, respectively.

3.4.2 KEGG pathways and key metabolites

Based on the differential metabolites, several metabolic pathways were enriched using Metabolanalyst. The KEGG classification results showed that the lipid metabolism was the main altered metabolic pathway between the HFD_W and HFD_S groups (Fig. S4 A), and the amino acid metabolism was mainly enriched between the HFD_A and HFD_S groups (Fig. S4 B). Besides, the KEGG pathway enrichment analysis indicated the top 15 most significant metabolic pathways affected. Compared to the HFD_S group, the main cardiovascular-related metabolic pathways altered by red wine administration were sphingolipid signaling pathway, sphingolipid metabolism, glycerophospholipid metabolism, choline metabolism in cancer, and bile secretion (Fig. 4 D). The main cardiovascular-related metabolic pathways altered by the alcohol treatment were vascular smooth muscle contraction, VEGF signaling pathway, platelet activation, phenylalanine metabolism, glycerophospholipid metabolism and choline metabolism in cancer (Fig. 4 E). Moreover, the top 30 differential metabolites in different groups were shown in Fig. 4 F, of which were mainly lipids and lipid-like molecules and organic oxygen compounds. The core metabolites involved in the 5 main cardiovascular-related metabolic pathways altered by red wine were lipids. Specifically, red wine significantly down-regulated TXB2, spermidine, cortisol and L-carnitine involved in bile secretion pathway; up-regulated PC(18:1/18:1) and down-regulated PE(15:0/18:3), PE(15:0/16:1), PE(18:3/16:0), PE(15:0/20:4) and dimethylethanolamine related to glycerophospholipid metabolism pathway; down-regulated sphingosine
and sphinganine involved in sphingolipid metabolism pathway. For metabolites related to pathways of sphingolipid signaling pathway and choline metabolism in cancer, DG(20:1/18:3), sphingosine and sphinganine were down-regulated and DG(18:0/18:2), DG(18:0/18:3), DG(18:0/16:1) and PC(18:1/18:1) were upregulated by red wine (Fig. 4 G). In addition, red wine significantly increased bile acids including glycolcolic acid, 3-oxo-4,6-choladienoic acid, 3,7-dihydroxy-12-oxocholanolic acid, tauroursodeoxycholic acid and taurodeoxycholic acid (Fig. 4 H).

Figure 4. Effect of red wine on the liver metabolites in ApoE−/− mice. (A) PLS-DA score of liver samples from different groups; (B, C) Volcanic plot of differential metabolites between groups. Red dots represent up-regulated metabolites and blue dots represent down-regulated metabolites; (D, E) KEGG enrichment analysis. The size of bubbles in the figure represents the number of compounds enriched in the metabolic pathway, and the color of each bubble was based on the P-value; (F) The top 30 differential metabolites in different groups, the X axis represents the average relative abundance of metabolites; (G) Relative levels of metabolites involved in the main cardiovascular-related metabolic pathways altered by red wine administration; (H) Relative levels of bile acids altered by red wine administration. * 0.01 < P ≤ 0.05, ** 0.001 < P ≤ 0.01, *** P ≤ 0.001; ND, normal diet; HFD_S, high-fat diet administered with saline; HFD_A, high-fat diet administered with alcohol; HFD_W, high-fat diet administered with red wine.
3.5 Red wine increased SCFAs concentrations in high-fat diet-fed ApoE−/− mice

Red wine significantly increased the concentrations of acetic acid, propionic acid and butyric acid in fecal samples, which were decreased by high-fat diet (Fig. 5 A-C). Correlation analysis showed that the SCFAs were significantly negatively related with lesion area, plasma TC, LDL-C, IL-6 and TNF-α levels, liver TC and TG levels, and positively related with liver TBA levels (Fig. 5 D).

![Figure 5](image)

**Figure 5.** Effect of red wine on SCFAs concentrations in ApoE−/− mice. (A) Acetic acid concentrations; (B) Propionic acid concentrations; (C) Butyric acid concentrations; (D) Correlation analysis of SCFAs with other metabolic phenotype indexes. Lesion Area-1, lesion area percentage of the total aorta; Lesion Area-2, lesion area percentage of the total vessel. Values are expressed as mean ± SD, and different letters (a – d) represent significant differences (P < 0.05) among groups by one-way ANOVA with Duncan’s multiple range test. * P < 0.05: significance of Spearman correlation. ND, normal diet; HFD, high-fat diet; HFD_S, high-fat diet administered with saline; HFD_A, high-fat diet administered with alcohol; HFD_W, high-fat diet administered with red wine.

3.6 Red wine regulated gut microbiota in high-fat diet-fed ApoE−/− mice

A total of 2,810,568 high-quality sequences of the 16S rRNA were obtained from fecal samples and were clustered into 431 OTUs, and 101 genera or the next higher taxonomic rank were identified. The α-diversity based on Chao and Shannon index indicated that red wine reduced intestinal flora diversity in mice fed a high-fat diet (Fig. 6 A&B). β-diversity based on the PLS-DA scores showed that red wine significantly modulated the community structure of gut microbiota (Fig. 6 C). The HFD_S group was clustered separately from ND group along COMP1, which explained 15% of the total variation. The red wine treatment (HFD_W) apparently migrated the gut microbiota structure along the negative direction of COMP1, closer to the ND group, while the HFD_A group clustered away from the ND group (Fig. 6 C). At the phylum level, Firmicutes and Actinobacteriota were the most abundant phyla in all samples, approximately 90% of the total bacterial sequences. Compared with the HFD_S group, both the HFD_W and HFD_A groups increased the abundance of Actinobacteriota and decreased the Firmicutes (Fig. 6 D).
Figure 6. Effects of red wine on the gut microbiota in ApoE−/− mice. (A, B) The Chao and Shannon index; (C) Partial least square discriminant analysis (PLS-DA) score plot; (D) Relative abundance of dominant bacteria at the phylum level (mean relative abundance > 0.1%) (E) LEfSe at different taxonomy levels: Evolutionary branch diagram; (F) Relative abundance of key bacteria genera; *P < 0.05 vs HFD_S. ND, normal diet; HFD_S, high-fat diet administered with saline; HFD_A, high-fat diet administered with alcohol; HFD_W, high-fat diet administered with red wine.

To identify the specific bacterial taxa that present among the administration groups, the discriminative features of microbiota compositions were compared by the LEfSe analysis (linear discriminant analysis score > 2.0). In total, the cladogram revealed 26 discriminative features from phylum to genus clades. The most specific bacterial taxa in response to red wine treatment was the g_unclassified_o_Bacteroidales, belonging to the phylum Bacteroidota. And the most specific bacterial detected in the alcohol administration group were the g_unclassified_f_Atopobiaceae and g_Coriobacteriaceae_UCG-002, belonging to the phylum Actinobacteriota (Fig.6 E). In addition, the relative abundance of bacteria associated with atherosclerosis at the genus level were showed in Fig.6 F. Compared to the ND group, high-fat diet increased the abundance of Lactobacillus, Blautia, Bilophila, Mucispirillum and Romboutsia, while red wine treatment attenuated these changes. Furthermore, the abundance of Coriobacteriaceae_UCG-002, Bifidobacterium, Dubosiella, unclassified_f__Atopobiaceae, unclassified_f__Erysipelotrichaceae and Akkermansia were lower in the
HFD_S group than that in the ND group. However, red wine treatment (HFD_W) restored the abundance of *Coriobacteriaceae_UCG-002*, *Bifidobacterium*, unclassified_f__Atopobiaceae and *Akkermansia* compared with the HFD_S group. In addition, alcohol treatment (HFD_A) mainly decreased the abundance of *Mucispirillum*, and restored the abundance of *Coriobacteriaceae_UCG-002*, *Dubosiella*, unclassified_f__Atopobiaceae and unclassified_f__Erysipelotrichaceae*, compared with the HFD_S group (Fig. 6 F).

3.7 Correlation analysis linking the liver metabolites and gut microbiota with atherosclerotic parameters

Red wine treatment significantly altered the liver metabolites and the composition of gut microbiota, and reduced the atherosclerotic parameters in high-fat diet-fed ApoE<sup>−/−</sup> mice. To determine whether potential correlations existed among the liver metabolites, gut microbiota and atherosclerosis indicators (lesion areas, plasma TC, TG, HDL-C, LDL-C, IL-6, TNF-α, liver TC, TG, TBA, and fecal SCFAs), the correlation analysis was investigated using Spearman’s correlation coefficient (Fig. 7). Noticeably, the liver metabolites of taurine, betaine aldehyde (R)-1-O-[b-D-apiofuranosyl-(1->2)-b-D-glucopyranoside]-1,3-octanediol, N-methyl-a-aminoisobutyric acid, PC(16:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), and L-alloisoleucine were significantly positively correlated with liver TC, plasma TC, TG, LDL-C, IL-6 and lesion areas (r > 0.5), and negatively correlated with liver TBA and fecal SCFAs (r < −0.5). In addition, the correlation of 3-deoxyarabinohexonic acid, uridine, PC(18:2/0:0) and atherosclerosis indicators were opposite with the above metabolites (Fig. 7 A).

![Figure 7](image-url)

**Figure 7.** Heatmap of correlation analysis. (A) Correlation coefficients of liver metabolites with metabolic phenotype indexes; (B) Correlation coefficients of gut microbiota with metabolic phenotype indexes. Lesion Area-1, lesion area percentage of the total aorta; Lesion Area-2, lesion area percentage of the total vessel. Red color indicates positive correlation whereas blue denotes negative correlation. *P < 0.05, **P < 0.01, ***P < 0.001 : significance of Spearman correlation.

Besides, some bacteria showed strong correlations with atherosclerosis indicators (|r| > 0.5). The abundance of *unclassified_p__Firmicutes*, *Eubacterium_brachy_group*, *Romboutsia*, *Unclassified_f__Peptostreptococcaceae*, *Clostridium_sensu_stricto_1* and *Blautia* were positively correlated
with liver TC, TG, plasma TC, TG, LDL-C, IL-6, TNF-α, and lesion areas, and negatively correlated with liver TBA and fecal SCFAs. In contrast, *Mucispirillum, Akkermansia, Erysipelatoclostridium, Lactobacillus, Alistipes, Bifidobacterium, norank_f__Muribaculaceae* and *norank_f__norank_o__Clostridia_UCG-014* were negatively correlated with liver TC, TG, plasma TC, TG, LDL-C, IL-6, TNF-α, and lesion areas (−0.5 < r < 0.5) (Fig. 7 B).

### 4. Discussion

The present study demonstrated that red wine alleviated AS in high-fat diet-fed ApoE−/− mice by regulating the gut microbiota community, increasing SCFAs, attenuating liver metabolic disorder, reducing plasma lipids and inflammatory levels.

In this study, red wine treatment for 16 weeks reduced the full-length aortic plaque rates by 34.4% in comparison with the placebo group. It was also reported that consumption of red wine for 6 weeks significantly reduced the atherosclerotic lesion in ApoE−/− mice [30]. While Chassot et al. found that red wine had no significant effect on the prevention of AS in LDLr−/− mice, though the wine consumption altered biomarkers of oxidative stress and lipidemia [31]. These inconsistencies can be explained in part by the different total phenol content of red wines and feeding methods. The red wine used in our experiment was from the high-altitude Shangri-la region with a high total phenolic content of 3553.4 mg/L (expressed as mg of gallic acid equivalents). In addition, red wine was daily gavaged in our experiment, but were added into water for free drinking by mice in Chassot’s work. Another important finding in our study was that the alcohol treatment also alleviated AS. It was reported that moderate consumption of alcohol (100 grams/week) may associate with reducing risk of CVD in humans [32]. These findings suggest that both the moderate red wine and alcohol were beneficial for alleviating the development of AS.

High blood levels of cholesterol, particularly LDL-C, favor the transformation of macrophages into foam cells, contributing to the development of AS [33]. In this study, red wine administration significantly decreased the TC and LDL-C levels of plasma in ApoE−/− mice. In addition, alcohol treatment did not change lipids levels significantly. The results indicated that the lipids-lowering effect of red wine was not due to the alcohol, but maybe the phenolics in it. This supported the previous work that the polyphenols in wine, rather than alcohol, played important roles in explaining red wine’s lipid-lowering properties [34]. Furthermore, dyslipidemia and liver lipid accumulation also accelerate the development of AS. In this work, red wine administration significantly reduced the high TC and TG levels in liver tissues induced by high-fat diet, further supported the important role of red wine's lipid-lowering effect in anti-atherosclerotic effect.

Proinflammatory cytokines of IL-6 and TNF-α are involved in atherosclerotic plaque formation [35]. Intriguingly, both red wine and alcohol significantly inhibited the overexpression of IL-6 and TNF-α in plasma, and reduced the inflammatory infiltration of liver cell structure in high-fat diet-fed mice. Previous studies also confirmed the anti-inflammatory effect of red wines. Especially, a research demonstrated that de-alcoholised red wine decreased inflammatory markers in atheroma plaques in ApoE−/− mice [11]. In our experiment, the anti-inflammatory effect of red wine was significantly higher than that of alcohol treatment.
Further study should concentrate on the anti-inflammation of red wine, alcohol and polyphenols, to investigate whether there is a synergistic effect between alcohol and polyphenols.

The differences of liver metabolites could partly explain the changes in physiological activities. In our results, the ND, HFD_S, HFD_W and HFD_A groups showed different metabolic profiles. The differences of some core metabolites may explain the mechanism of red wine’s effect on alleviating AS, especially lipids and bile acids. The regulations of lipid substances by red wine led to significant changes of glycerophospholipid metabolism, sphingolipid metabolism, sphingolipid signaling pathway and choline metabolism in cancer. Previous studies have reported that glycerophospholipid and sphingolipid metabolism were the most significantly changed pathways in atherosclerotic development [36]. The lipids involved in sphingolipid and glycerophospholipid accelerate atherosclerosis-related cellular processes, such as angiogenesis and inflammation [37]. Sphingosine and sphinganine are precursors of sphingolipids and have been implicated in impaired insulin signaling and hepatic lipoprotein overproduction [38]. Changes in the phosphoethanolamines (PE) content of various tissues are implicated in atherosclerosis [39]. Inhibition of sphingolipid synthesis could reduce plasma cholesterol levels and prevent atherogenesis in LDLR−/− mice [40], ApoE−/− mice and rabbits [41]. Red wine also reduced liver TC, TG and plasma TC, TG, LDL-C levels, which may be the result of the regulation of liver metabolites.

Besides, the metabolic results showed that red wine increased the concentrations of 5 kinds of bile acids, consistent with the results of liver TBA levels. Bile acid is involved in the absorption, transport and secretion of fat, and closely associated with the catabolism of cholesterol [42]. In addition, red wine significantly decreased the TC level and increased the TBA level of the liver, indicating that red wine modulated hepatic lipid and bile acid metabolism by increasing the converting from cholesterol into bile acid. Previous study reported that the gut microbiota could regulate secondary bile acid metabolism in the liver [43]. Therefore, red wine might induce the synthesis of hepatic BA via regulation of gut microbiota.

Red wine increased the content of SCFAs including acetate, propionate and butyrate, which were decreased by high-fat diet. SCFAs are mainly produced by gut microbes from undigested carbohydrates and act as a substrate or regulator in lipid metabolism [44], cross the intestinal epithelium and influence the mucosal immune responses, beneficial for blood vessels (Verhaar, Prodan et al. 2020), and play anti-inflammatory roles through different signaling pathways [45]. Combine with our correlation analysis, red wine may reduce the inflammatory levels and improve the disorder of lipid metabolism in the plasma and liver of high-fat diet ApoE−/− mice by increasing the content of SCFAs.

Recently, evidence has highlighted the cross talk between the gut microbiota and AS [46, 47]. Our results showed that red wine significantly regulated the gut microbiota compositions. Specifically, red wine increased the abundance of Akkermansia and Bifidobacterium associated with SCFAs production. Besides, red wine treatment reduced the abundance of bacteria associated with inflammation, including genus of Mucispirillum, Romboutsia, Lactobacillus and Bilophila. Mucispirillum has been associated with increased mucosal pro-inflammatory responses and promoted plasma levels of inflammatory cytokines [48, 49]. Romboutsia was
correlated with circulating inflammatory (IL-1β) [50], and played a pro-inflammatory role in colitis [51]. In our study, the abundance of *Romboutsia* was significantly positively correlated with the plasma level of IL-6, indicating that *Romboutsia* may play a pro-inflammatory role in preventing atherosclerosis. Though *Lactobacillus* species are generally recognized as safe, some investigations showed that specific *Lactobacillus* species stimulated dendritic cells to produce inflammatory cytokines [52, 53]. *Bilophila* was reported to be bound up with a pro-inflammatory T helper type 1 immune response [54] and acted synergistically with high fat-diet to promote higher inflammation [55], promoted intestinal barrier dysfunction and bile acid dysmetabolism in human and mice [55, 56]. The alleviation effect of peanut skin and berberine on high-fat diet induced AS were related to a decrease in the abundance of *Bilophila* [57, 58]. The reduction in the abundance of *Mucispirillum, Romboutsia, Lactobacillus* and *Bilophila* may also be one of the ways by which red wine exerts its anti-AS effect.

In addition, our results revealed that the alcohol treatment significantly restored the abundance of *Coriobacteriaceae_UCG-002*, which was decreased in high-fat diet-fed ApoE<sup>−/−</sup> mice and was negatively related with TNF-α level and atherosclerotic plaque lesion areas. *Coriobacteriaceae_UCG-002* has been reported to be beneficial for insulin sensitivity [59] and involved in promoting intestinal cholesterol absorption [60-62]. The restoring of *Coriobacteriaceae_UCG-002* may contribute to the anti-atherosclerosis effect of alcohol, and specific functioning mechanism remains to be further clarified.

5. Conclusion

In summary, a high-polyphenol red wine exhibited significantly alleviation effect on high-fat diet-induced atherosclerosis in ApoE<sup>−/−</sup> mice, as proved by reduction of aortic plaque, along with decreased lipid and inflammatory levels in plasma and liver. These effects were associated with the regulations of liver metabolic disorders mediated by modulating of gut microbiota and increasing of SCFAs. Metabolomic analysis indicated that the core metabolites regulated by red wine were lipids and bile acids. Further microbiome analysis revealed that red wine modulated the stucture of gut microbiota including increased the abundance of *Akkermansia* and *Bifidobacterium* and reduced the abundance of *Mucispirillum, Romboutsia, Lactobacillus* and *Bilophila*, which inducing an increase in SCFAs. Our study indicated that high-polyphenol red wine effectively reduced atherosclerosis and explained this effect from the perspective of hepatointestinal circulation.

Conflict of Interest

The authors declare that they have no competing interests.

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