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Different polarity polyphenol extracts from *P. cerasifera* fruits ameliorate non-alcoholic fatty liver disease via bile acid metabolism and gut microbiota

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ABSTRACT: Non-alcoholic fatty liver disease (NAFLD) is a global metabolic disease caused by abnormal accumulation of lipids in the liver, for which effective therapeutic targets and drugs are scarce. *P. cerasifera* polyphenol extract (PPE) has been proven to be able to alleviate NAFLD via the "gut microbiota - bile acid" signaling pathway, but its active ingredients that exert anti-NAFLD effects are still unclear. In this study, PPE was separated according to different polarities, and the optimal components for exerting anti-NAFLD effects based on the "gut microbiota - bile acid" signaling pathway were explored. Different polarity polyphenol extracts from *P. cerasifera,* especially aqueous PPE (APPE) enhanced hepatic bile acid synthesis and alleviated liver injury in NAFLD mice. Meanwhile, APPE significantly reduced the levels of BSH-producing bacteria, in particular Bacteroidetes, and reduced the presence of endogenous FXR ligands (CDCA, LCA, and DCA) in the intestines of NAFLD mice, thereby inhibiting the ileal FXR-FGF15 axis. In addition, activation of the hepatic FXR-SHP axis by APPE also supported CYP7A1- and CYP27A1-mediated bile acid synthesis. The data suggested that APPE demonstrated a notable anti-NAFLD effect through the "gut microbiota - bile acid" signaling pathway, indicating the potential for isolating individual compound from APPE for the treatment of NAFLD.

Keywords: *P. cerasifera*, polyphenol extracts, non-alcoholic fatty liver disease, bile acid metabolism, gut microbiota.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a systemic metabolic disease characterized by the abnormal accumulation of lipids in the liver. The global incidence of NAFLD is approximately 25%, with a significantly higher prevalence in developed regions compared to developing regions [1-3]. If left unmanaged, NAFLD can progress to a spectrum of liver conditions such as non-alcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [4]. Regrettably, there is currently a shortage of FDA-approved clinical drugs for treating NAFLD, highlighting the pressing requirement to identify compounds with potential therapeutic benefits for the prevention and management of NAFLD [5, 6].

Changes in gut microbiota and intestinal metabolites are closely related to the occurrence and development of NAFLD [7]. A recent meta-analysis of 8894 participants with/without NAFLD concluded that

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NAFLD patients are often accompanied by gut microbiota disorders [8]. At the phylum level, NAFLD mice and humans exhibit an increased abundance of Firmicutes and a decreased abundance of Bacteroidetes; at the genus level, NAFLD mice and humans exhibit an increased abundance of Bifidobacterium, Clostridium, Lactobacillus, Bacteroidetes, Eubacterium, Listeria, Streptococcus, and Escherichia [9-11]. These gut microbiota with bile salt hydrolase (BSH) activity and 7α-dehydroxylation activity are enriched in the intestines of NAFLD patients, leading to the metabolism of conjugated bile acids into secondary bile acids, such as deoxycholic acid, lithocholic acid, etc [12-14]. Farnesoid X receptor, a bile acid-activated transcription factor, after being activated by endogenous ligands (CDCA, DCA, LCA and CA), can inhibit the expression of CYP7A1 in the classic pathway through the liver FXR-SHP signaling pathway or the ileal FXR-FGF15/19 signaling pathway, thereby aggravating the occurrence and development of NAFLD [9, 15, 16]. Recent studies have shown that polyphenols such as caffeic acid phenethyl ester from propolis extract and saikosaponin D from Bupleurum can change the bile acid profile by reducing the abundance of BSH-producing bacteria, thereby improving NAFLD via inhibiting the ileal FXR-FGF15 signaling pathway [17, 18]. Therefore, searching for effective compounds against NAFLD from polyphenols based on the "gut microbiome-bile acid" axis has become the key to the current development of anti-NAFLD drugs.

P. cerasifera is a deciduous small tree or shrub belonging to the *Prunus* genus of the *Rosaceae* family. Its wild population in China is only distributed in Daxigou and Xiaoxigou in Huocheng County, Ili, Xinjiang [19, 20]. *P. cerasifera* fruit is a valuable plant resource with uses in both food and medicine. It contains a high concentration of plant-derived polyphenols, including phenolic acids, flavonoids, anthocyanins, and tannins, that exhibit antioxidant, anti-inflammatory, and antibacterial effects [21, 22]. Duan combined network pharmacology and in vitro experiments to confirm that the phenolic acids and flavonoids in *P. cerasifera* can inhibit the secretion of pro-inflammatory cytokines, NF-κB, MAPK pathways and NLRP3 inflammasome activation [23]. Another study pointed out that *P. cerasifera* anthocyanins can alleviate atherosclerosis in apoE-deficient (apoE-/-) mice by regulating glycerophospholipid metabolism [24]. Our previous research found that *P. cerasifera* polyphenol extracts (PPE) can alleviate NAFLD via the “gut microbiota - bile acid” axis [21]. However, the main active ingredients in PPE that alleviate NAFLD based on the “gut microbiota - bile acid” axis are currently unclear.

This study investigated the anti-NAFLD effect of different polar components of PPE derived from the n-butanol phase and aqueous phase. The focus is on the “gut microbiota - bile acid” axis to identify optimal polar fractions responsible for alleviating NAFLD. We used different polar fractions of PPE to intervene in HFD-induced NAFLD mice, and evaluated changes in fecal gut microbiota and bile acid profiles, as well as genes or proteins expression levels related to bile acid metabolism in the liver and ileum. This study demonstrated that APPE significantly decreased the abundance of gut microbiota with BSH activity, thereby reducing the levels of CDCA, DCA, and LCA, and finally improving NAFLD via suppressing ileal FXR-FGF15 signaling and promoting hepatic FXR-SHP signaling.

2. Materials and methods

2.1 Preparation of different polarity polyphenol extracts from P. cerasifera fruits

Wild *P. cerasifera* fruits were collected from Huocheng County, Ili, Xinjiang in mid-August 2023, and stored at -20℃. According to the previous research method, the prepared PPE was dissolved in ultrapure water [21]. The suspension was then partitioned with Petroleum ether and n-butanol to provide n-butanol PPE (BPPE) and aqueous PPE (APPE). Subsequently, BPPE and APPE powders were obtained after rotary evaporation, AB-8 macroporous resin purification and freeze-drying. Finally, ultra-high performance liquid chromatography was used to characterize the composition of APPE.

2.2 Reagents

Ordinary feed was purchased from the Animal Experiment Center of Xinjiang Medical University, while high-fat feed was produced according to the High Fat (60 FDC) Purified rodent diet formula provided by Daitz Biotechnology Co., Ltd. (Wuxi, China) [25, 26]. Triglyceride (TG) (A110-1-1), total cholesterol (TC) (A111-1-1) and total bile acid (T-BAs) (E003-1-1) were purchased from Nanjing Jiancheng Bioengineering Research Institute Co., Ltd. (Nanjing ,China). UnionScript First-strand cDNA Synthesis Mix for qPCR(with dsDNase) (SR511) was purchased from Beijing Genesand Biotechnology Co., Ltd. (Beijing, China). PerfectStart^{TM} Green qPCR SuperMix（TG-AQ601-04）was purchased from Beijing TransGen Biotechnology Co., Ltd. (Beijing, China). Super ECL Plus ultra-sensitive luminescent liquid (strong, P1050) was purchased from Beijing Applygen Technology Co., Ltd. (Beijing, China). The primary antibodies of CYP7A1 (AF6657), SHP (AF1093) and GAPDH (AF1186) were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). The primary antibodies of CYP27A1 (DF3571) was purchased from Affinity Biosciences Co., Ltd. (OH, USA). The primary antibody of FXR (A8320) was purchased from Abclonal (Wuhan, China). Horseradish peroxidase-labeled goat anti-rabbit IgG (H+L) secondary antibody was purchased from Hangzhou Beyotime Biotechnology Co., Ltd. (Hangzhou, China).

2.3 Animals experimental design

Four-week-old ICR male mice were purchased from the Experimental Animal Center of Xinjiang Medical University. All experiments and animal care in this study were conducted in accordance with the national and international directives (the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation and Guide for the Care and Use of Laboratory Animals, United States National Research Council, 2011) and approved by Animal Experimental Ethical Inspection of College of Life Sciences, Xinjiang Normal University (XJNU2023-12).

All mice were adaptively fed in a standard environment for 1 week and then randomly divided into a standard chow diet group (CK, n = 11) and a high-fat diet group (HFD, n = 35). After 24 weeks, 3 mice from each group were randomly selected for morphological analysis of liver tissue to evaluate whether the NAFLD modeling was successful. After successful modeling, the NAFLD mice were randomly divided into 4 groups, with 8 mice in each group. The CK group had free access to chow diet and water, while the other groups had free access to high-fat diet and water. All groups were subjected to intragastric administration every day till 5 weeks. The details were as follows: CK group (equal volume of saline), HFD group (equal

volume of saline), BPPE group (BPPE, 400mg/Kg/day), APPE group (APPE, 400mg/Kg/day) and S group (simvastatin 10mg/Kg/day). Water intake and body weight were recorded weekly (Fig. 1).

At the end of experiments, mice were anesthetized, and the samples of feces, serum, liver, fat, and ileum were collected. Part of the liver and fat samples from each group were collected and immersed in 4% paraformaldehyde for routine permanent section. The remaining portion was frozen in liquid nitrogen and temporarily stored at -80℃ to detect the relative expression of genes and proteins.

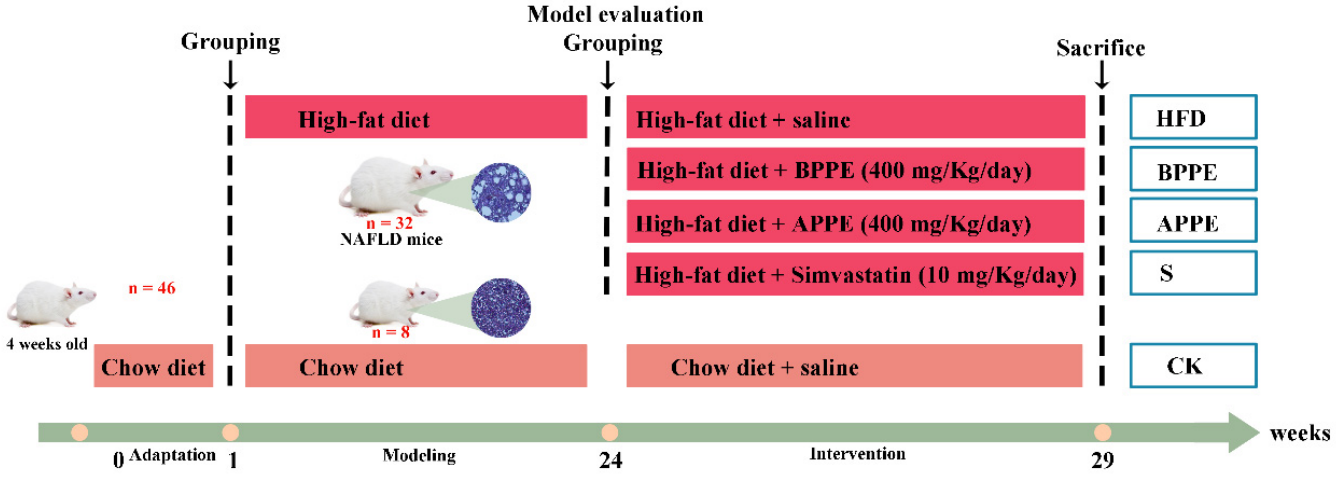

Fig. 1 The schematic diagram of the experimental design.

2.4 Determination of serum and liver biochemical indicators

The liver tissues were homogenized (-20℃, 50Hz, 120s) in saline, centrifuged (4℃, 3000 r/min, 10min), and the supernatant was collected. According to the recommended operation of the manufacturer, the TG, TC and T-BAs were quantitatively analyzed.

2.5 Histopathological observation of liver and fat

Paraffin-embedded or frozen liver and fat samples were sectioned and stained with hematoxylin and eosin (H&E) and Oil Red O (ORO) stains according to previous research method [21]. Subsequently, the pathological changes of the liver and fat tissue were observed and evaluated under a light microscope (Nikon, Japan).

2.6 Real-time PCR analysis

Total RNA from liver and ileum was isolated using Trizol and reverse-transcribed into cDNA using UnionScript First-strand cDNA Synthesis Mix for qPCR (with dsDNase). Quantitative polymerase chain reaction was performed using PerfectStart^{TM} Green qPCR SuperMix on a Real-time PCR machine (ABI StepOnePlus, USA). The relative expression of genes was normalized by β-actin and calculated by the $2^{-\Delta\Delta Ct}$ method. All mouse primer sequences are shown in Table 1.

Table 1 The primer sequences.

Gene name	Forward (5'→3')	Reverse (5'→3')
CYP7A1	TTC AAG ACC GCA CAT AAA GCC	GAG ATG CCC AGA GGA TCA CG
CYP27A1	AGG AAG TGA CCC AGT TTG TGT T	GGT GTT GGA CCC TGG AGT TT

FXR	GCT TGA TGT GCT ACA AAA GCT G	CGT GGT GAT GGT TGA ATG TCC
SHP	CAG GTC GTC CGA CTA TTC TGT	AGG CTA CTG TCT TGG CTA GGA
BSEP	CTG CCA AGG ATG CTA ATG CA	CGA TGG CTA CCC TTT GCT TCT
NTCP	CTT GCG CCA TAG GGA TCT TC	TGC CTG CCT TGA GGA CGT A
ASBT	GTC TGT CCC CCA AAT GCA ACT	CAC CCC ATA GAA AAC ATC ACC A
FGF15	GAC CAA AAC GAA CGA AAT TTG TT	ACG TCC TTG ATG GCA ATC G
β-actin	CCT AGA AGC ATT TGC GGT GCA CGA TG	TCA TGA AGT GTG ACG TTG ACA TCC GT

2.7 Western blot analysis

Total liver protein was prepared using RIPA lysis buffer containing protease inhibitors, and the protein concentration was detected using a BCA kit. The total protein was separated by 12% SDS-PAGE and transferred to PVDF membrane. Next, the target protein band can be obtained by blocking with milk, washing the membrane with TBST, incubating the primary antibody overnight at 4℃, washing the membrane, incubating the secondary antibody for 2 hours at room temperature, and visualizing the blot [21]. Finally, the target protein band was quantitatively analyzed using Image J2x 2.1.5.0 software.

2.8 Determination of fecal targeted bile acid metabolism profile and 16S rRNA sequencing

Aseptically collected mouse feces were snap-frozen in liquid nitrogen and sent to Shanghai Biotree Biomedical Technology Co., Ltd. in China for targeted bile acid metabolism profile and 16S rRNA sequencing. After data filtering, we performed comparative analysis on biologically meaningful data. Detailed information about the methods used can be found in the Supporting Information section.

2.9 Detection of fecal bile salt hydrolase activity

The stool samples were homogenized (-20℃, 50Hz, 60s) in saline, centrifuged (4℃, 3000 r/min, 10min), and the supernatant was collected. According to the recommended operation of the manufacturer, fecal bile salt hydrolase activity was quantitatively analyzed.

2.10 Statistical analysis

All data were presented as mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) and Two-tailed student's t-test in SPSS21.0 software, and $P < 0.05$ was considered statistically significant. GraphPad Prism software (version 8.0) and R Studio (version 4.0) were used for illustration creation.

3. Results

3.1 PPE of different polarities reduced the body weight and organ weight of NAFLD mice

Mice were randomly divided into HFD group (n=35) and CK group (n=11), and were fed high-fat diet and standard chow diet respectively. After 23 weeks, the body weight and serum TC, TG, LDL-C, AST and ALT in the HFD group were significantly increased compared with the CK group, while serum HDL-C was significantly decreased (Supplementary Fig. S1). At the same time, the liver of the HFD group showed massive steatosis, hepatocyte ballooning, and lobular inflammation (Supplementary Fig. S2).

Subsequently, mice showing NAFLD symptoms were randomly divided into HFD group, BPPE group, APPE group and S group for a 5-week intragastric intervention. Interestingly, the water intake of group S was

significantly higher than that of the other four groups, which was mainly attributed to water loss caused by osmotic diuresis due to excessive blood glucose concentrations induced by high-dose simvastatin intervention (Figure 2A). Compared with the HFD group, intragastric administration of BPPE, APPE and S could significantly reduce the body weight of NAFLD mice (Fig. 2B, 2D). Meanwhile, BPPE, APPE and S intervention effectively reversed the increase in fat weight and liver weight in NAFLD mice caused by long-term high-fat diet (Fig. 2C, 2E). In addition, observing the appearance of the mice, it was found that the coat color of the mice in the HFD group and the S group was greasy, dirty, and messy, and their livers were yellow (Fig. 2D). Therefore, different polarity PPE can effectively reduce weight gain and organ hypertrophy in NAFLD mice induced by long-term high-fat diet.

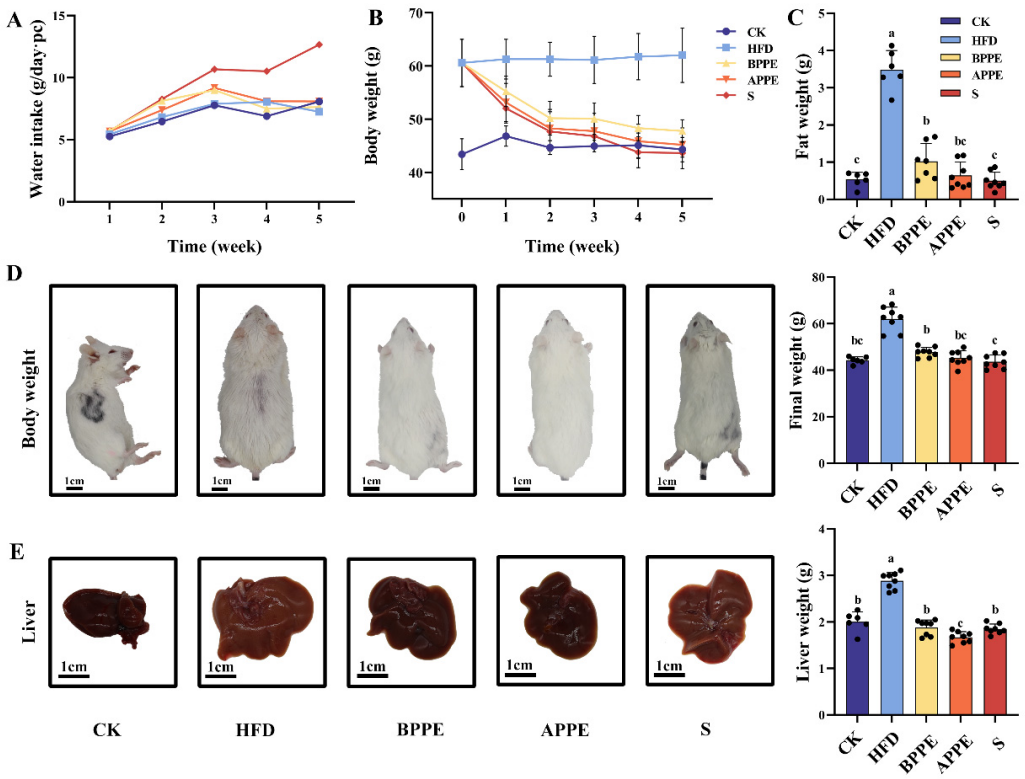

Fig. 2 Effects of different polarity PPE on body weight and organ weights in HFD-induced NAFLD mice. (A)Water intake. BPPE and APPE reduced body weight (B), fat weight (C), final body weight (D) and liver weight (E) of HFD-induced NAFLD mice. Data were expressed as mean ± SD (n = 6-8), and different letters indicated significant differences between groups ($P < 0.05$). CK, HFD: saline; BPPE: N-butanol PPE (400mg/Kg/day); APPE: Aqueous PPE (400mg/Kg/day); S: simvastatin (10mg/Kg/day).

3.2 PPE of different polarities alleviated TG, TC, T-BAs, liver damage and fat morphology in NAFLD mice

The effects of PPE of different polarities on liver lipid accumulation and liver damage in NAFLD mice were studied. Compared with the CK group, TG and TC in the liver of NAFLD mice increased to (2.51 ± 0.26)mg/g and (3.48 ± 0.25)mg/g respectively (Fig. 3A, 3B). It is worth noting that oral administration of both BPPE and APPE can effectively improve liver lipid accumulation in NAFLD mice, but the lipid-lowering effect of APPE is equivalent to that of S. Correspondingly, the levels of T-BAs in the livers of NAFLD mice were significantly lower than those in the CK group, indicating that long-term high-fat diet significantly inhibits the bioconversion of cholesterol into bile acids in the liver. After BPPE, APPE and S intervention,

T-BAs in the livers of NAFLD mice increased by 72.2%, 166.0% and 125.0% respectively (Fig. 3C). Analysis of the correlation between TC and T-BAs showed that heptic TC were negatively correlated with T-BAs (Fig. 3D, P=0.011). The results of H&E staining of liver and fat tissue sections and the results of Oil Red O staining of liver tissue sections were shown in Figure 3E-G. Compared with the CK group, the liver NAS score, hepatocyte red lipid droplets, and adipocyte area were significantly increased in the HFD group, indicating that long-term high-fat diet effectively simulated the pathological changes of NAFLD. After BPPE and APPE intervention, the liver NAS score, hepatocyte red lipid droplets, and adipocyte area were significantly reduced, especially in APPE. The above experimental results show that APPE can effectively improve liver lipid accumulation and liver damage caused by long-term high-fat diet.

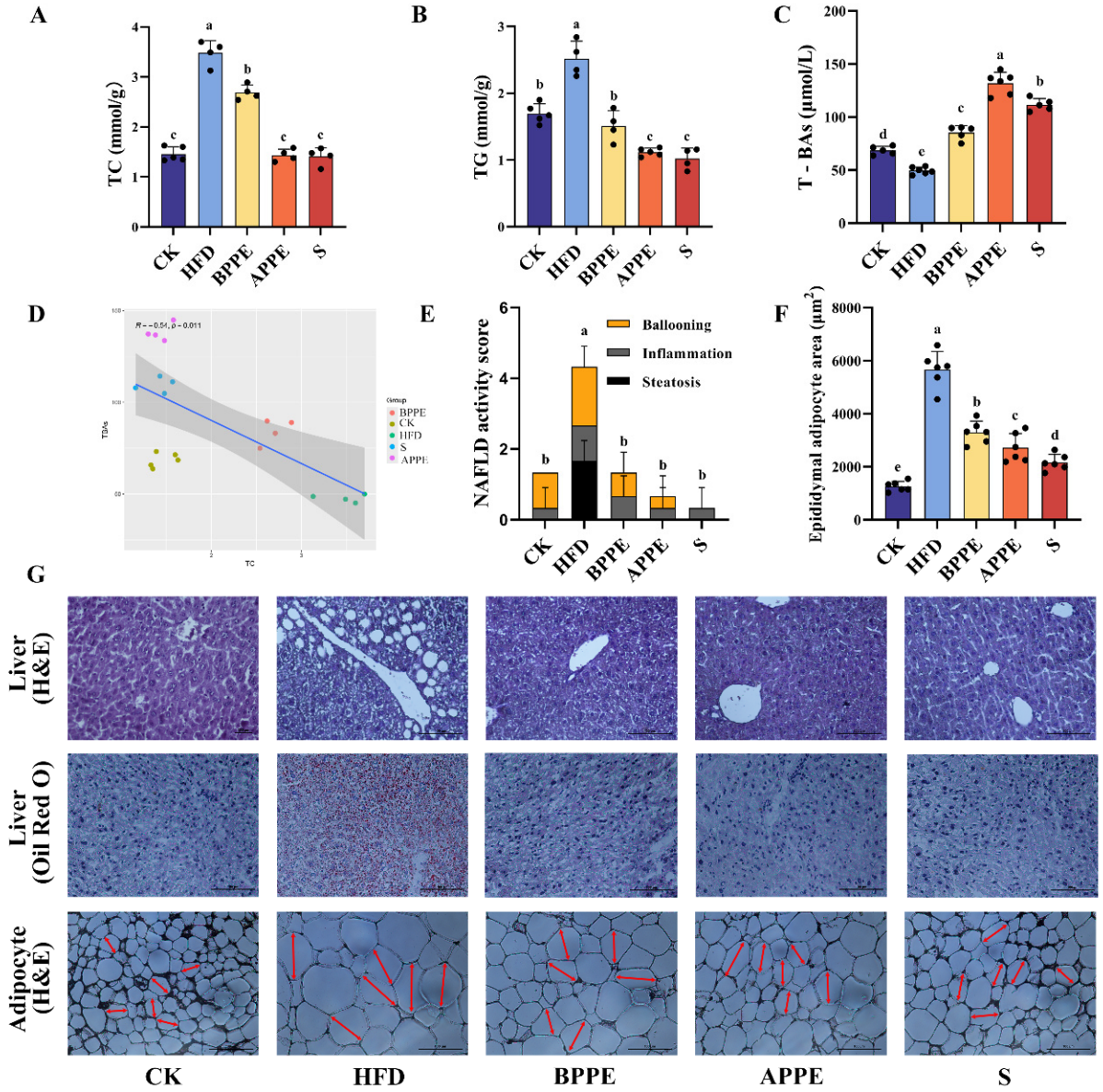

Fig. 3 Effects of different polarity PPE on TG, TC, T-BAs, liver damage and fat morphology in HFD-induced NAFLD mice. BPPE and APPE reduced the accumulation of liver TG (A) and TC (B) and enhanced the synthesis of liver T-BAs (C) in NAFLD mice. (D) Correlation analysis of liver TCs with T-BAs. Liver NAFLD activity score (E) and epididymal adipocyte area (F) in NAFLD mice were reduced by BPPE and APPE. Liver and epididymal adipocyte sections stained with H&E and Oil Red O (G). Scale bar, 100 μm. Data were expressed as mean ± SD (n = 5), and different letters indicated significant differences between groups (P < 0.05). CK, HFD: saline; BPPE: N-butanol PPE (400mg/Kg/day); APPE: Aqueous PPE (400mg/Kg/day); S: simvastatin (10mg/Kg/day).

3.3 PPE of different polarities reversed the abnormal fecal bile acid profile in NAFLD mice

Changes in the composition and content of bile acids in feces were observed to further verify the effects of PPE of different polarities on the fecal bile acid profile of NAFLD mice. The results showed that the T-BAs, proportions of other types of bile acids, DCA/(DCA+CA) and LCA/(LCA+CDCA) in the feces of NAFLD mice were significantly increased, and the unconjugated bile acids, taurine-conjugated bile acids, and secondary/primary bile acid ratio were significantly decreased, indicating NAFLD mice exhibit obvious bile acid profile disorder. Compared with the HFD group, both BPPE and APPE could significantly reduce fecal T-BAs, DCA/(DCA+CA) and LCA/(LCA+CDCA), and increase the proportions of unconjugated bile acids, taurine-conjugated bile acids and secondary/primary bile acid ratio. It is worth noting that the proportion of unconjugated bile acids in the APPE intervention was 2.48% lower than that in the BPPE intervention, and the taurine-conjugated bile acids were 1.42% higher than that in the BPPE intervention, indicating that APPE has a better reversal effect on fecal bile acid disorders in NAFLD mice (Fig. 4A-C).

Key differences in bile acids in the feces of mice in each group were further explored. Compared with the CK group, the DCA and LCA levels in the fecal bile acid profile of NAFLD mice were significantly increased, and the HDCA level was significantly decreased. After BPPE and APPE intervention, the DCA and LCA levels of NAFLD mice significantly decreased, and the HDCA level increased significantly, and the effect of APPE was better than that of BPPE. Compared with the CK group, CDCA, TCA, T-αMCA, αMCA, βMCA and UDCA were significantly increased and CDCA was significantly decreased in NAFLD mice, while TCDCA and T-βMCA had no significant difference. After intervention with BPPE and APPE, CDCA, TCA, T-αMCA, αMCA, βMCA and UDCA in the fecal bile acid profile of NAFLD mice significantly decreased, while CA content increased significantly, while TCDCA and T-βMCA had no significant difference (Fig. 4D).

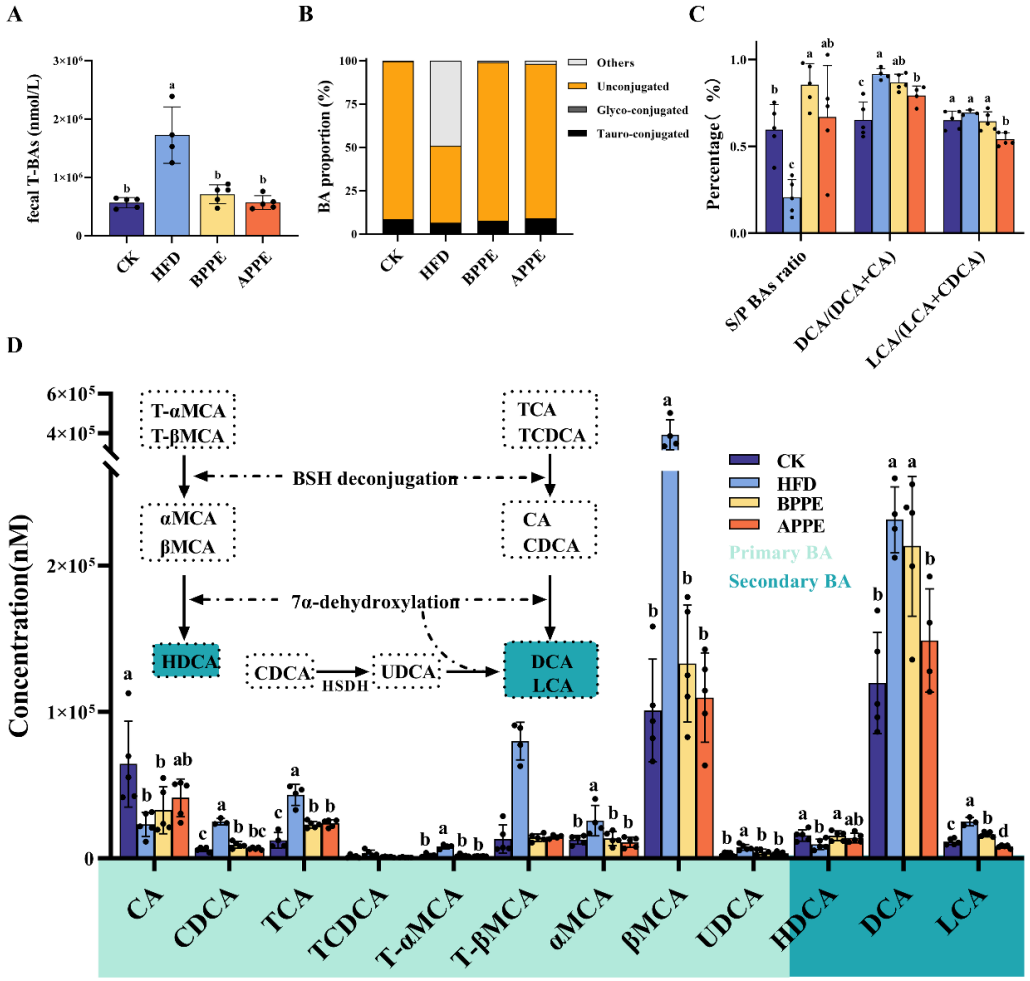

Fig. 4 Effects of different polarity PPE on fecal bile acid profile in HFD-induced NAFLD mice. BPPE and APPE reversed fecal T-BAs (A), BA proportion (B), secondary/primary BAs ratio, DCA/(DCA+CA) and LCA/(LCA+CDCA) (C) in HFD-induced NAFLD mice. (D) Fecal differential BAs. Data were expressed as mean ± SD (n = 5), and different letters indicated significant differences between groups ($P < 0.05$). CK, HFD: saline; BPPE: N-butanol PPE (400mg/Kg/day); APPE: Aqueous PPE (400mg/Kg/day); S: simvastatin (10mg/Kg/day).

3.4 PPE of different polarities showed significant improvement in bile acid metabolism in NAFLD mice

Accelerating cholesterol consumption is crucial to the treatment of NAFLD. Approximately 50% of cholesterol is consumed through the bile acid synthesis pathway mediated by CYP7A1 and CYP27A1. APPE significantly reduced the levels of FXR ligand (CDCA, DCA and LCA) in the feces of NAFLD mice. Whether this change can improve bile acid synthesis mediated by CYP7A1 and CYP27A1 remains to be further verified. At the gene and protein levels, hepatic FXR-SHP signaling and bile acid synthesis mediated by hepatic CYP7A1 and CYP27A1 were significantly upregulated by PPE intervention of different polarities, especially APPE. At the mRNA level, BSEP, which controls bile acid transport into bile, and NTCP, which controls bile acid reabsorption and transport into hepatocytes, were significantly downregulated in the livers of NAFLD mice, and this phenomenon was reversed by APPE, suggesting that APPE can effectively maintain the dynamic balance of liver bile acid profile in NAFLD mice. Conversely, APPE reduced high levels of FXR, FGF15, and ASBT mRNA expression in the ileum of NAFLD mice (Fig. 5A-D). These data indicate that APPE upregulates bile acid synthesis through inhibiting intestinal FXR-FGF15 signaling and activating FXR-SHP signaling.

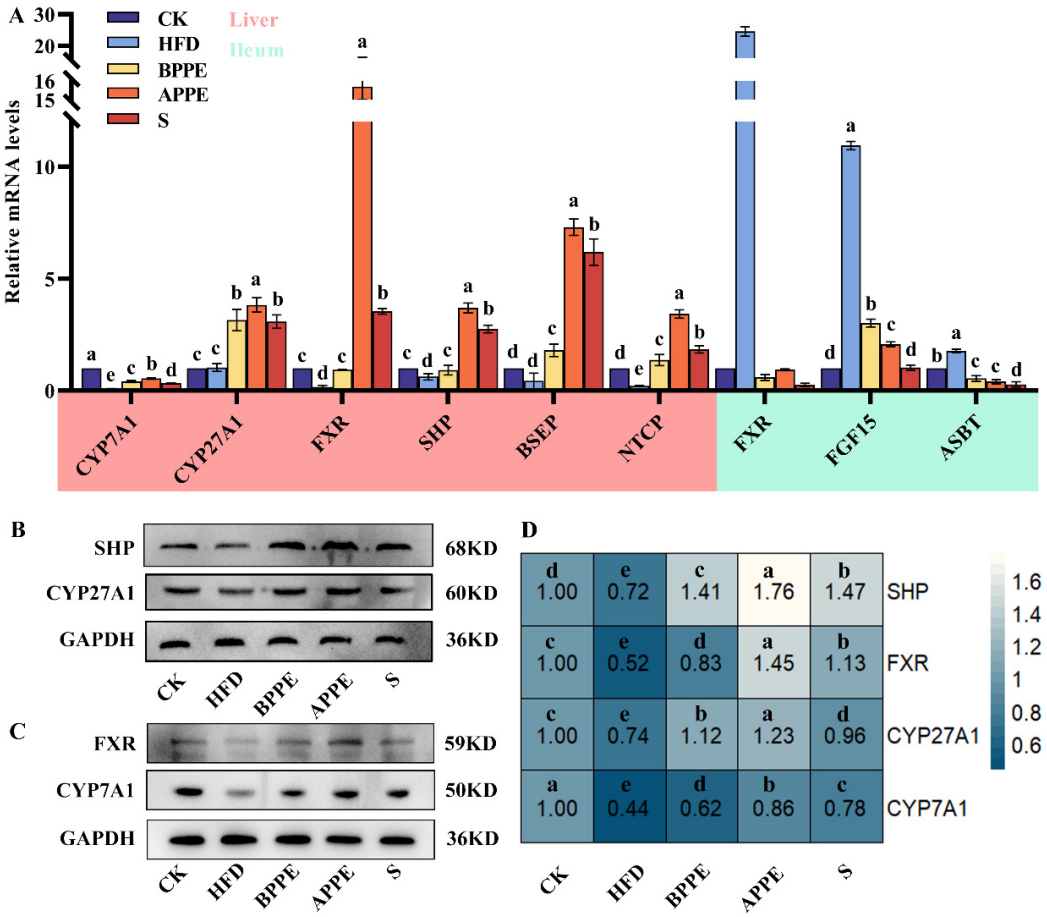

Fig. 5 Effects of different polarity PPE on bile acid metabolism in HFD-induced NAFLD mice. (A) Relative mRNA levels of bile acid metabolism-related genes in liver and ileum. (B-D)Western blot related to liver bile acid metabolism and its quantitative results. Data were expressed as mean ± SD (n = 3-5), and different letters indicated significant differences between groups ($P < 0.05$). CK, HFD: saline; BPPE: N-butanol PPE (400mg/Kg/day); APPE: Aqueous PPE (400mg/Kg/day); S: simvastatin (10mg/Kg/day).

3.5 Remodeling of gut microbiota in NAFLD mice by different polarity PPE

Targeted bile acid profiling results showed that APPE could reverse high levels of CDCA, DCA, and LCA in the feces of NAFLD mice, but whether this change was caused by the involvement of gut microbiota remains unclear. Therefore, we used 16S rRNA sequencing results to characterize the composition and structure of the gut microbiota in the feces of mice in each group. Analysis of the sequencing results revealed that the dilution curves of each group of samples tended to be flat, indicating that the sequencing data displayed in this study were reliable (Fig. 6A). The α-diversity results showed that the community richness and diversity of the gut microbiota of NAFLD mice were significantly lower than those of the CK group (CHAO1 index: P=0.032, Shannon index: P=0.014). BPPE (CHAO1 index: P = 0.168, Shannon index: P = 0.031) and APPE (CHAO1 index: P = 0.105, Shannon index: P = 0.055) altered the richness and diversity of the gut microbiota of NAFLD mouse (Fig. 6B, 6C). The β-diversity results indicated that the gut microbiota of the CK, BPPE and APPE group were significantly separated and spatially clustered from the HFD group (PCoA1=28.56%, PcoA2=16.12%), indicating that both BPPE and APPE can effectively reverse the intestinal flora disorder in NAFLD mice(Fig. 6D). At the phylum level, the abundance of Bacteroidetes increased and the abundance of Firmicutes decreased in the gut microbiota of NAFLD mice, while APPE intervention

reversed this change (Fig. 6E). In addition, the Firmicutes/Bacteroides ratio showed that APPE could also significantly alleviate intestinal flora disorder in NAFLD mice (Fig. 6F). At the genus level, we focused on intestinal microorganisms with bile salt hydrolase activity and 7α-dehydroxylation activity and found that both BPPE and APPE could significantly reduce high levels of Bacteroides in NAFLD mice (Fig. 6H, 6I). The measurement of mouse feces BSH activity confirmed that both BPPE and APPE reduced the high-level bile salt hydrolase activity in NAFLD mice, and the inhibitory effect of APPE was 32.9% lower than that of BPPE (Fig. 6J). The results suggested that Bacteroides might be the critical strain responded to the treatment on APPE in NAFLD.

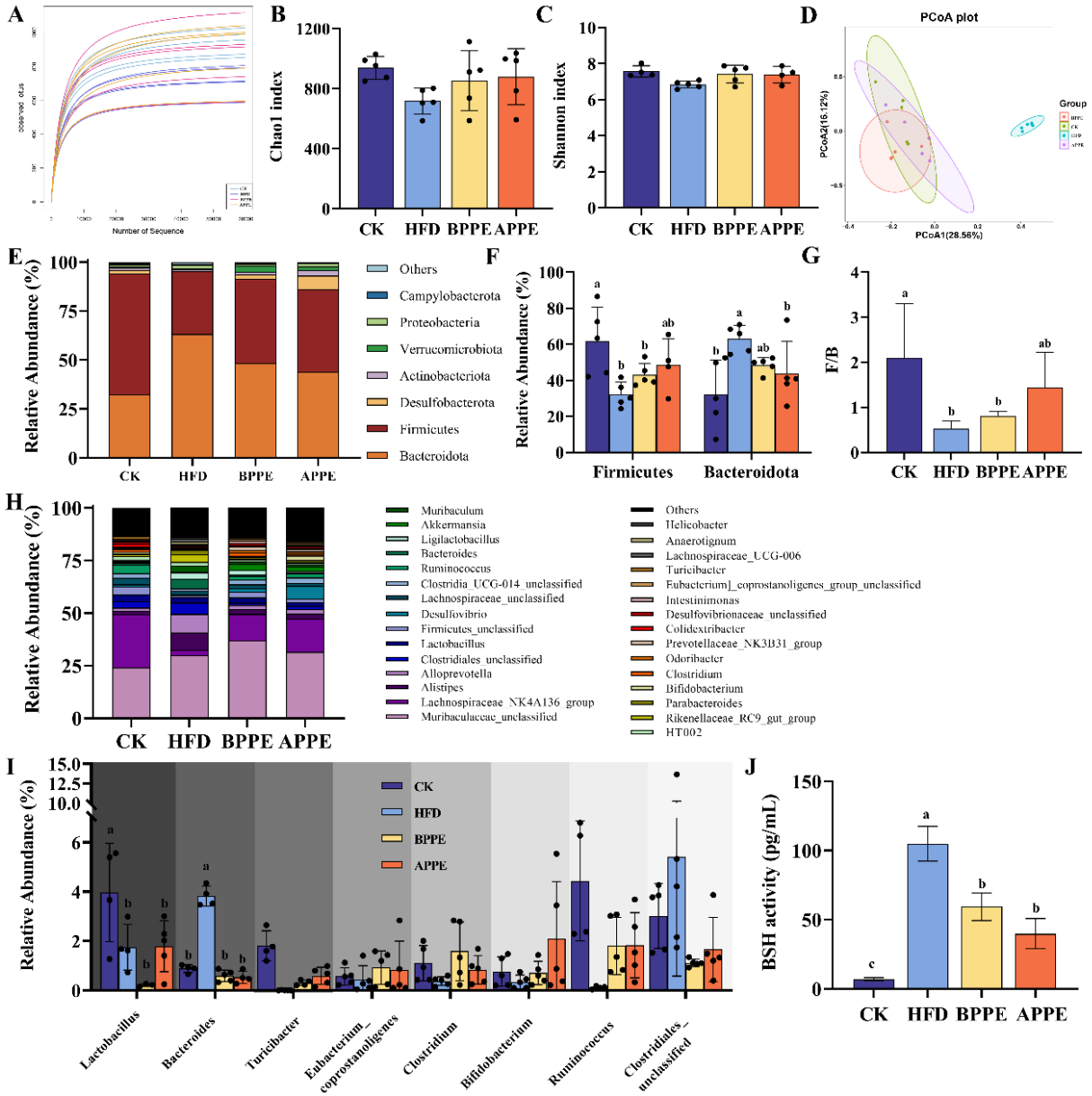

Fig. 6 Effects of different polarity PPE on gut microbiota in NAFLD mice. (A) Dilution curve of OTUs. Alpha diversity analysis based on Chao1 index (B) and Shannon index (C). (D) Principal component analysis. (E) Relative abundance of gut microbiota at the phylum level. (F) Relative abundance of Bacteroidota and Firmicutes at the phylum level. (G) Firmicutes/Bacteroidota ratio. (H) Relative abundance of gut microbiota at the genus level. (I) Relative abundance of gut microbiota with bile salt hydrolase activity and 7α-dehydroxylation activity at the genus level. (J) Fecal bile salt hydrolase activity. Data were expressed as mean ± SD (n = 5), and different letters indicated significant differences between groups ($P < 0.05$). CK, HFD: saline; BPPE: N-butanol PPE (400mg/Kg/day); APPE: Aqueous PPE (400mg/Kg/day); S: simvastatin (10mg/Kg/day).

3.6 Correlation analysis between gut microbiota and BAs metabolism

To further elucidate the relationship between fecal bile acid and gut microbiota under the intervention of APPE, we used Spearman correlation analysis to correlate fecal differential bile acids and the Top30 intestinal flora of NAFLD mice after APPE treatment. The results showed that Bacteroides reduced by APPE was significantly positively correlated with TCDCA, TCA, LCA, T-αMCA, UDCA, T-βMCA and CDCA in feces, and negatively correlated with CA. In addition, CDCA reduced by APPE was significantly positively correlated with Ligilactobacillus, Bacteroides, and Rikenellaceae_RC9_gut_group, and significantly negatively correlated with Desulfovibrio, Clostridia_UCG-014_unclassified, Odoribacter, Prevotellaceae_NK3B31_group, Turicibacter, and Desulfovibrionaceae_unclassified. LCA reduced by APPE intervention was significantly positively correlated with Anaerotignum, Lachnospiraceae_UCG-006, Bacteroides, Pikenellaceae_RC9_gut_group, Parabacteroides, Alloprevotella and Clostridiales_unclassified, and negatively correlated with Turicibacter and Akkermansia (Fig. 7). The above data are helpful to explain that APPE can regulate bile acid profile to alleviate NAFLD by reversing intestinal flora disorder.

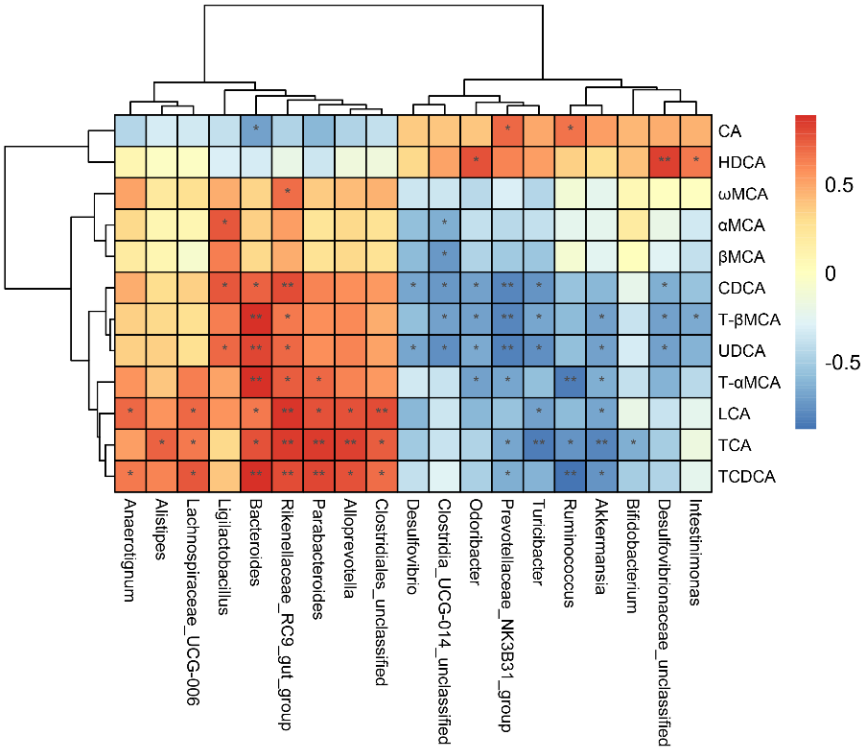

Fig. 7 Heat map of correlation between fecal bile acid and gut microbiota in NAFLD mice after APPE treatment. Compared with HFD, **P* < 0.05, ***P* < 0.05.

3.7 Chemical analysis of APPE and BPPE

The components of APPE and BPPE were identified by comparison with retention times and MS/MS fragmentation patterns of existing flavonoid commercial standards. By comparing the retention time, mass-to-charge ratio, and peak intensity with commercial flavonoid standards, 36 flavonoids were identified in APPE and BPPE, respectively (Fig. 8, Supplementary Table S2-3). The flavonoids with a content greater than 1 ng/mg in APPE mainly include 3,4-Dihydroxybenzoic acid (1.95ng/mg), Protocatechualdehyde (5.33ng/mg), Phthalic acid (289.77ng/mg), Rutin (1.26ng/mg), Salicylic acid (4.47ng/mg), Benzoic acid

(8.35ng/mg) and Quercetin (3.95ng/mg). Compared with BPPE, Luteoloside (0.0106 ng/mg) and Daidzein (0.0008 ng/mg) are unique in APPE. In addition, the contents of (+)-Dihydrokaempferol (0.0210 ng/mg > 0.0197 ng/mg) and Gossypol (0.0772 ng/mg > 0.0245 ng/mg) in APPE are higher than those in BPPE.

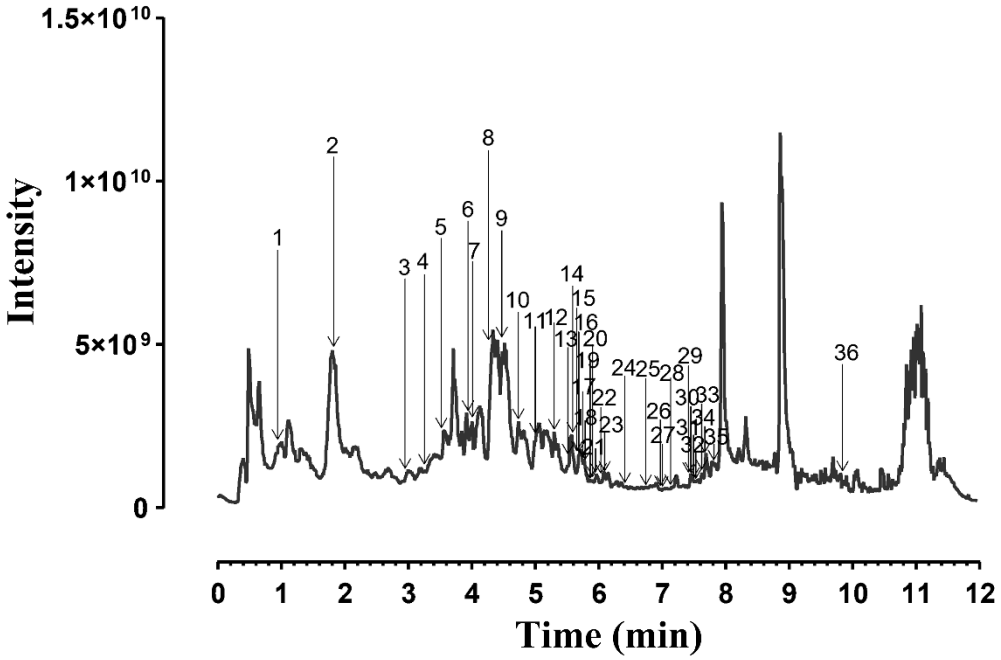

Fig. 8 Ultra-high performance liquid chromatography (UHPLC) analysis of APPE. 1. Gallic acid; 2. 3, 4-Dihydroxybenzoic acid; 3. Protocatechualdehyde; 4. 4-Hydroxybenzoic acid; 5. Phthalic acid; 6. Catechin; 7. Vanillic acid; 8. Caffeic acid; 9. Syringic acid; 10. Epicatechin; 11. Vanillin; 12. p-Hydroxycinnamic Acid; 13. Syringaldehyde; 14. Rutin; 15. Trans-Ferulic acid; 16. Salicylic acid; 17. Sinapic Acid; 18. Quercetin 3-β-D-glucoside; 19. Luteoloside; 20. (+)-Dihydroquercetin; 21. Genistin; 22. Benzoic acid; 23. Kaempferol-3-O-glucoside; 24. (+)-Dihydrokaempferol; 25. Daidzein; 26. Luteolin; 27. Quercetin; 28. Trans-Cinnamic acid; 29. Naringenin Chalcone; 30. Phloretin; 31. Naringenin; 32. Apigenin; 33. Kaempferol; 34. Isorhamnetin; 35. Isoliquiritigenin; 36. Gossypol.

4. Discussion

NAFLD is a systemic metabolic disease caused by abnormal accumulation of lipids in the liver. The complex pathogenesis and lack of clinical drugs have become two major factors limiting the management of NAFLD, and finding compounds with potential therapeutic effects is crucial for the treatment of NAFLD [1, 5]. *P. cerasifera*, a medicinal and edible plant, which multiple pharmacological properties determine its potential for the development of medicines and functional foods. Previous work in our laboratory have confirmed that PPE exerts excellent hepatoprotective properties by reducing abnormal lipid accumulation and chronic inflammation in the liver of obese mice [27]. Furthermore, the anti-NAFLD effect of PPE was found to be closely related to the regulation of cholesterol metabolism and bile acid metabolism and the reversal of gut microbiota [21]. Considering that PPE extraction faces the challenges of high cost and low output, we further clarified the components in PPE that exert anti-NAFLD effects based on the “gut microbiota - bile acid” axis, providing theoretical basis for the artificial synthesis of this substances for the treatment of NAFLD. This study found that APPE has a better anti-NAFLD effect and is mainly involved in the regulation of gut microbiota structure to change the bile acid profile composition, especially Bacteroides, thereby

inhibiting ileal FXR-FGF15 signaling pathway and activating hepatic FXR-SHP signaling pathway to enhance bile acid metabolism.

In recent years, gut microbiota and its metabolites have been frequently mentioned in studies on the pathogenesis of NAFLD. More and more studies have shown that the occurrence and development of NAFLD are often accompanied by changes in the composition and structure of gut microbiota, but the absorption of plant-derived polyphenols, such as Apple polyphenol, Berberine, Astragaloside IV, can significantly reverse this phenomenon [8, 12, 28, 29]. We found that APPE changed the alpha diversity (CHAO1 index, Shannon index) and beta diversity (PCoA) of the gut microbiota of NAFLD mice. At the same time, 16S RNA sequencing results displayed that the increase in the abundance of Bacteroidetes bacteria and the decrease in the abundance of Firmicutes bacteria at the phylum level in the feces of NAFLD mice induced by long-term HFD resulted in a decrease in the F/B ratio, which is consistent with previous research results [11]. Interestingly, an increase in the F/B ratio has also been observed in NAFLD mice induced by HFD, which may be caused by different detection methods [30, 31]. After APPE intervention, the bacterial abundance changes of Bacteroidetes and Firmicutes in the feces of NAFLD mice tended to normal, resulting in an increase in the F/B ratio. At the genus level, increased abundance of Bifidobacterium, Clostridium, Lactobacillus, and Bacteroidetes accelerated the hydrolysis of conjugated bile acids by BSH. This is a prerequisite for the production of secondary bile acids via 7α-dehydroxylation [9]. We found that the gut microbiota composition of NAFLD mice changed significantly at the genus level after APPE and BPPE intervention. The high abundance of Bacteroides in the intestines of NAFLD mice was significantly reduced by BPPE and APPE, and APPE had a better effect, indicating that PPE of different polarities have a regulatory effect on intestinal BSH activity. The fecal bile salt hydrolase activity was further measured, and it was found that both APPE and BPPE could reduce the bile salt hydrolase activity in the feces of NAFLD mice, and APPE was better than BPPE. In addition, 7α-dehydroxylation of Clostridium and Eubacterium can metabolize deconjugated bile acids into secondary bile acids to activate ileal FXR-FGF15 signaling pathway to inhibit bile acid synthesis [9-11]. However, there were no statistically significant differences between the groups with regard to the abundance of Clostridium and Eubacterium at the genus level. These results indicate that APPE can reduce intestinal BSH activity in NAFLD mice via reversing the structure of the gut microbiota, in particular Bacteroides.

Recent research has pointed out that changes in the structure of gut microbiota can affect bile acid metabolism by regulating bile acid profiles. We found that the high levels of T-BAs in the feces of NAFLD mice could be significantly reduced by changes in the gut microbiota structure induced by different polarity PPE. In addition, under the intervention of PPE of different polarities, the abundance of bacteria with BSH and 7α-dehydroxylation in the intestines of NAFLD mice decreased, resulting in an increase in the proportion of conjugated bile acids and the ratio of secondary/primary bile acids and a decrease in the ratios of DCA/(DCA+CA) and LCA/(LCA+CDCA). When FXR is activated by endogenous ligands in the ileum and liver, it can inhibit CYP7A1-mediated bile acid synthesis through the ileal FXR-FGF15/19 pathway and the

hepatic FXR-SHP pathway respectively [15, 16]. This change will promote the occurrence and development of NAFLD [9]. We found that after intervention with different polarity PPE, the reduction of CDCA, DCA, and LCA in the fecal bile acid profile of NAFLD mice was beneficial to activating hepatic CYP7A1-mediated bile acid synthesis by inhibiting the ileal FXR-FGF15 signaling pathway. It is worth noting that APPE has a stronger inhibitory effect on the ileal FXR-FGF15 signaling pathway and a stronger activating effect on the liver FXR-SHP signaling pathway in NAFLD mice than BPPE, and CYP7A1-mediated bile acid synthesis is significantly up-regulated, which is consistent with WAHLSTRöM's conclusion that ileal FXR inhibition leads to a stronger promotion of CYP7A1-mediated bile acid synthesis than hepatic FXR activation. [32]. In addition to the classic pathway of bile acid synthesis mediated by CYP7A1, the alternative pathway of bile acid synthesis mediated by CYP27A1 is also important for the conversion of cholesterol to bile acids. These results indicate that APPE promotes the bioconversion of cholesterol to bile acids better than BPPE, which mainly relies on the CYP7A1-mediated classical pathway and the CYP27A1-mediated alternative pathway. Futhermore, under the intervention of PPE of different polarities, the inhibition of the ileal FXR-FGF15 signaling pathway and the activation of the liver FXR-SHP signaling pathway in NAFLD mice decreased the expression of ASBT in the ileum and increased the expression of BSEP and NTCP in the liver, and the effect of APPE was more effective. The above data confirm that APPE-dependent enhancement of hepatic bile acid synthesis and secretion and reduction of ileal bile acid reabsorption have a positive effect on alleviating abnormal hepatic lipid accumulation in NAFLD mice.

5. Conclusion

In summary, this study demonstrated that APPE appeared to be the best anti-NAFLD ingredients among different polarity polyphenol extracts from *P. cerasifera* in HFD induced NAFLD mice. The potential anti-NAFLD mechanisms of APPE could at least partly be attributed to the fact that it reversed the structure of gut microbiota, especially Bacteroidetes, thereby affecting the composition of the fecal bile acid profile, and ultimately alleviated abnormal hepatic lipid accumulation by inhibiting ileal FXR-FGF15 signaling pathway and activating of hepatic FXR-SHP signaling pathway. This study further characterized the ingredients in PPE that exert anti-NAFLD effects and provided experimental basis for the artificial synthesis of anti-NAFLD drugs derived from PPE.

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Conflicts of interest

The authors report no conflict of interest.

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