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# A lignan extract from *Myristica fragrans* Houtt. prevents non-alcoholic fatty liver disease in mice by regulating the bile acid metabolism and microbes in the gut-liver axis

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## ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is intricately linked to dysregulated hepatic lipid metabolism and gut microbiota imbalances. This study explores the potential mechanisms by which AEN, a lignan-rich extract from *Myristica fragrans* Houtt., alleviates NAFLD by affecting gut bacteria, bile acid metabolism, and fecal chemical composition. We demonstrate that AEN prevents weight gain, lipid accumulation, and enhances liver function in mice subjected to a high-fat diet. Utilizing a multi-omics strategy that includes microbiome analysis and metabolomics, we observed significant shifts in gut microbial composition and fecal metabolites, notably an increase in lithocholic acid, taurocholic acid, and the ratio of non-12-OH to 12-OH conjugated bile acids. These metabolic changes were accompanied by a decrease in bile acid metabolism-related enzymes, such as bile salt hydrolase, in the AEN group compared to the high-fat diet group. Moreover, AEN downregulated the farnesoid X receptor-fibroblast growth factor 15 pathway in the intestine, promoting the alternative bile acid synthesis pathway. The elucidation of the correlation between changes in the microbiome and fecal metabolites suggests that AEN modulates specific gut microbes, thereby influencing bile acid metabolism. This modulation was evident in the increased abundance of beneficial bacteria related to bile acid production, such as *Parabacteroides* and *Bifidobacteria*, and the decreased abundance of harmful bacteria like *Helicobacter* and *Streptococcus*. Network pharmacology and GC × GC-TOF-MS suggested that licarin A and B could be key bioactive lignans in AEN. These findings underscore the potential of AEN in countering NAFLD by targeting the gut-liver axis, offering a novel therapeutic avenue for NAFLD management.

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## 1. Introduction

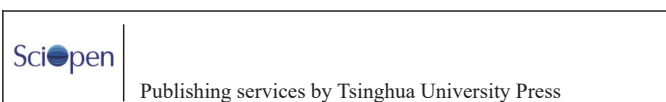
Non-alcoholic fatty liver disease (NAFLD), which manifests as simple fatty liver or non-alcoholic steatohepatitis (NASH), is the

most common chronic disease in the world<sup>[1]</sup>. According to ‘multiple parallel hits’ hypothesis, fatty liver disease is closely linked to oxidative stress, activation of the inflammatory cascade, and gut microbiota imbalance<sup>[2]</sup>. There is increasing evidence that changes in the gut microbiota are closely associated with hepatic glucose and lipid metabolism via affecting the gut-liver axis in patients with NAFLD<sup>[3]</sup>. It is generally believed that microbe-derived metabolites, such as short-chain fatty acids, bile acids, and amino acids, mediate

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communication *via* the gut-liver axis<sup>[4-5]</sup> can participate the pathogenesis of NAFLD.

In the field of traditional Chinese medicine (TCM), network pharmacology is used to discover drugs in herbal medicines and to identify their disease targets by applying pharmacology databases. These databases are used in combination with relevant disease and pathway databases<sup>[6-8]</sup>. Analysis of 16S rRNA genes can reveal the diversity and composition of the gut microbiome, and species analysis can identify the functions and correlations between microbes in the gut microbiome<sup>[9]</sup>. Metabolomics performs absolute or relative quantitative analysis of all metabolites in an organism, which is used to search for relationships between these metabolites and physiological and pathological changes<sup>[10]</sup>. Furthermore, a multi-omics analysis can be used to more comprehensively identify the direct relationships between related metabolites and targets<sup>[11-12]</sup>.

Nutmeg, the seed of the fruit of *Myristica fragrans* Houtt., is used as a spice to flavour beverages and baked goods. Studies on gastrointestinal diseases have also indicated a therapeutic effect of nutmeg, which is widely used in TCM<sup>[13-14]</sup>. The efficacies of many active components of nutmeg in the treatment of various diseases have been studied, and it has been demonstrated that these components have anti-inflammatory, antioxidant and antimicrobial effects<sup>[15-16]</sup>. In a previous study, we reported extracts of *M. fragrans* Houtt. alleviated obesity and inflammation in mice fed a high-fat diet (HFD)<sup>[17]</sup>. Other research has demonstrated that active components in nutmeg have therapeutic effects against hepatitis<sup>[18]</sup>. However, these studies have only demonstrated the effects of individual ingredients and the relevance of pathway targets in lipid metabolism and inflammation. Few studies have explored the effective material basis of these components and their corresponding direct mechanistic targets in metabolism.

This study innovatively demonstrates the potential of a lignan extract from *M. fragrans* Houtt. in preventing NAFLD in mice by modulating bile acid metabolism and gut microbiota. Through a multi-omics approach, the study highlights the extract's beneficial effects in preventing weight gain, improving liver function, and regulating inflammatory responses. The identification of active lignans and their mechanisms of action against NAFLD, along with the exploration of gut-liver axis interactions, offers a novel perspective in NAFLD research.

## 2. Materials and methods

### 2.1 Preparation and identification of active ingredients

Nutmeg and *M. fragrans* Houtt.'s seeds, generously provided by Tong Ren Tang (Beijing, China), were the basis for creating AEN, a lignan-rich extract from *M. fragrans* Houtt., detailed in our Supplementary Methods. For analysis, AEN samples underwent rigorous detection using an advanced extended two-dimensional gas chromatography time-of-flight mass spectrometer (GC × GC-TOF-MS; GGT 0620, Guangzhou Hexin Instrument Co., Ltd., Guangzhou, China). This process built upon previously established AEN sample preparation techniques and instrumentation<sup>[17]</sup>. To preserve their integrity for subsequent analysis, the AEN samples were stored at a stable −20 °C.

### 2.2 Animal experiment

Five-week-old male C57BL/6J mice with SPF grade were provided by Vital River (Beijing, China). Informing our

experimental design with existing references, we established three distinct concentration gradients of AEN<sup>[18]</sup>, specifically at doses of 62.5, 125, and 250 mg/kg body weight. Lovastatin (LOV), an  $\beta$ -hydroxy- $\beta$ -methylglutaryl-CoA reductase inhibitor, served as a positive control and reference (20 mg/kg body weight)<sup>[19-20]</sup>. A protocol for the animal experiments was described in the Supplementary Methods. All experiments were performed at the Jiangnan College Animal Experiment Center (JN. No.20160303-20160922[16]). Oral glucose tolerance tests (OGTT) and fasting blood glucose level determinations were conducted using a blood glucose meter (Roche, USA). Mice were sacrificed under deep anesthesia with isoflurane. The serum, feces, and other tissues were stored at −80 °C after the mice were euthanized.

This study's animal experiments adhere to the Animal Research: Reporting of *in vivo* Experiments guidelines and are conducted in accordance with the U.K. Animals (Scientific Procedures) Act, 1986, along with its related guidelines, the EU Directive 2010/63/EU on animal experiments, and the National Institutes of Health's guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

### 2.3 Blood biochemical index analysis in mice

Serum was extracted from mouse blood samples. A serum multiple automatic biochemical analyzer (BS-800M, Mindray, Shenzhen, China) was used to measure aspartate aminotransferase (AST), alanine aminotransferase (ALT), and high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), total cholesterol (TC) and serum triglycerides (TG).

### 2.4 Liver and white adipose tissue sections

Liver and epididymal adipose tissue were sectioned and stained with hematoxylin and eosin (H&E) and Oil Red O, separately (Wuhan Biotechnology Co., Ltd., Wuhan, China). The detailed method was shown in the Supplementary Methods. The stained sections were digitalized with a digital slide scanner (Pannoramic MIDI II, 3DHISTECH Ltd., Budapest, Hungary).

### 2.5 Gene expression analysis

The ileum and liver tissue were used for total RNA extraction with TRIzol (Invitrogen, Carlsbad, CA, USA). HiScript III-RT SuperMix (+ gDNA wiper; Vazyme, Nanjing, China) was used to perform reverse transcription. The expression of target genes was analyzed using quantitative real-time polymerase chain reaction (RT-qPCR) and agarose gel electrophoresis. The CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA) was used to perform the RT-qPCR. The  $2^{-\Delta\Delta C_t}$  cycle threshold method was used to analyse the results of qRT-PCR.

### 2.6 Gut microbiota analysis

During week 13, we collected mouse feces employing the FastDNA®Spin fit for feces (Santa Ana, USA). Amplification of the 16S rRNA V3–V4 region was conducted using specific universal primers. Post-amplification, the samples underwent purification and quantification *via* the TIANGel MiniPurification kit (TIANGEN, Beijing, China) and the Qubit dsDNA HS assay kit (Life Technologies Corporation, Carlsbad, CA, USA). Our sequencing approach was

adapted from a prior study in our lab<sup>[21]</sup>. Data analysis was performed using QIIME2 (<https://qiime2.org/>) and Microbiomeanalyst (<https://www.microbiomeanalyst.ca/>).

## 2.7 Non-targeted metabolomics analysis for fecal metabolome

Liquid chromatography (U3000; Thermo Scientific, Waltham, MA, USA) with tandem mass spectrometry (Q Elective, Thermo Scientific) was employed for analyzing treated fecal samples. The specific detection methodologies are elaborated in the Supplementary Methods section. We utilized Compound Discoverer 3.1 (CD 3.1; Thermo Scientific, USA) to meticulously identify, quantify, and discern differences in the relative abundance within the raw liquid chromatography-mass spectrometry data. For verifying metabolite mass spectra identities, we referred to several databases, including MassBank (<https://massbank.eu/>), ChemSpider ([www.chemspider.com](http://www.chemspider.com)), mzCloud (<https://www.mzcloud.org>), and PubChem (<http://ncbi.nlm.nih.gov/>). MetaboAnalyst 5.0 (<https://www.metaboanalyst.ca>) served as our tool for conducting principal component analysis (PCA) and Spearman correlation analysis. Furthermore, we employed the Omics Bean ([www.omicsbean.cn](http://www.omicsbean.cn)) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (<https://www.genome.jp/kegg>) databases for conducting comparative analyses between groups, enriching pathway data, and making predictive assessments.

## 2.8 Fecal bile acids analysis

Firstly, 50 mg fecal samples were dissolved in 1 mL methanol. The mixture was centrifuged to collect the supernatant, which was then filtered using a 0.22 µm syringe filter. A mixed standard solution was prepared by accurately weighing each bile acid standard, which was then diluted appropriately with methanol to obtain a working standard solution (see Table S1 for information on bile acids). Fecal bile acids were determined by liquid chromatography (U3000; Thermo Scientific, USA)-mass spectrometry (Q Elective, Thermo Scientific). The method for the determination of bile acids is described in the supplemental Methods. Xcalibur software was used to analyze the raw data.

## 2.9 Enzyme-linked immunosorbent assay (ELISA)

Mouse feces were subjected to ELISA kits following the standard protocol (7α-dehydroxylase (7α-DH) ELISA kit, 7α-hydroxysteroid dehydrogenase (7α-HSDH) kit, and bile salt hydrolase ELISA kit; SenBeiJia Biological Technology, Nanjing, China). The concentrations of analytes in ELISA were quantified using standard curves. Only ELISA plates that had  $R^2 \geq 0.99$  in their standard curve were included in the analysis.

## 2.10 Western blotting

Liver and ileum tissues were lysed with a RIPA buffer containing protease inhibitors from Beyotime (Shanghai, China), and samples were centrifuged at  $5000 \times g$  and 4 °C for 15 min. A 10% SDS-PAGE was used to separate the appropriate amounts of proteins, which were then measured by a BCA protein assay (Beyotime, Shanghai, China). After SDS-PAGE, proteins were transferred to a polyvinylidene

fluoride membrane. After blocking with 5% skim milk in Tris buffered saline-Tween 20 for 1 h at room temperature, the membrane was incubated with fatty acid synthase (FASN), GAPDH, farnesoid X receptor (FXR), fibroblast growth factor receptor 4 (FGFR4) (Cell Signaling Technology, MA, USA), sterol regulatory element binding protein 1c (SREBP-1c), and fibroblast growth factor 15 (FGF15) (Abcam, MA, USA) antibodies overnight at 4 °C. A blocking solution was then incubated with peroxidase-conjugated IgG antibody (Sigma, St Louis, MO, USA) and visualized with a chemiluminescent reagent (Amersham Bioscience, Piscataway, NJ, USA). The semi-quantitative protein expression was assessed using AlphaView software based on gray intensity analysis.

## 2.11 Pharmaceutical ingredients-disease targets-pathway network construction

The following approach was used to discover the active components in nutmeg that are linked to NAFLD. To examine active components in nutmeg (*M. fragrans* Houtt.), the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) database (<https://tcmsp-e.com/>) and the Encyclopedia of Traditional Chinese Medicine (ETCM) database (<http://www.tcmip.cn/ETCM>) were utilized. Combine reference searches by scanning PubMed (<http://pubmed.ncbi.nlm.nih.gov>) and comparing it to the National Center for Biotechnology Information (NCBI) database. Extract compounds from these databases that meet the drug similarity (DL) > 0.18 and blood barrier permeability (BBB)  $\geq -0.3$  requirements.

## 2.12 Statistical analysis

Post Shapiro-Wilk test, group contrasts were assessed using Student's *t*-test in GraphPad Prism 8. Multigroup data underwent one-way ANOVA. For broader comparisons, Dunnett's test was employed. A 95% confidence interval, with a 0.05 threshold, denoted statistical significance. Variables with  $P < 0.05$  in Spearman correlation analysis were considered correlated. Data is presented as means  $\pm$  SD.

# 3. Results

## 3.1 AEN prevent HFD-induced obesity and glucose intolerance in mice

Fig. 1A presents a streamlined flowchart of our experiments analyzing the impact of AEN lignans on HFD-fed mice. Post 12-week AEN administration *via* oral gavage, the AEN group showed notably less body weight and weight gain compared to the HFD group, as depicted in Figs. 1B-C. During initial 8 weeks food intake data revealed no significant appetite change post-AEN intervention (Fig. 1D). Weekly measurements indicated an increase in blood glucose levels in the HFD group (glucose > 6.1 mmol/L), with a marked deviation from the CON group at week 13. However, the AEN group maintained controlled blood glucose levels, mirroring the CON group's trend (Fig. 1E). The OGTT for the AEN group (Fig. 1F) and the area under the curve analysis (Fig. 1G) showed significant suppression compared to the HFD group, aligning closely with the CON and LOV treatment groups. Evaluating three AEN concentration gradients in HFD mice, we ascertained 125 mg/kg body

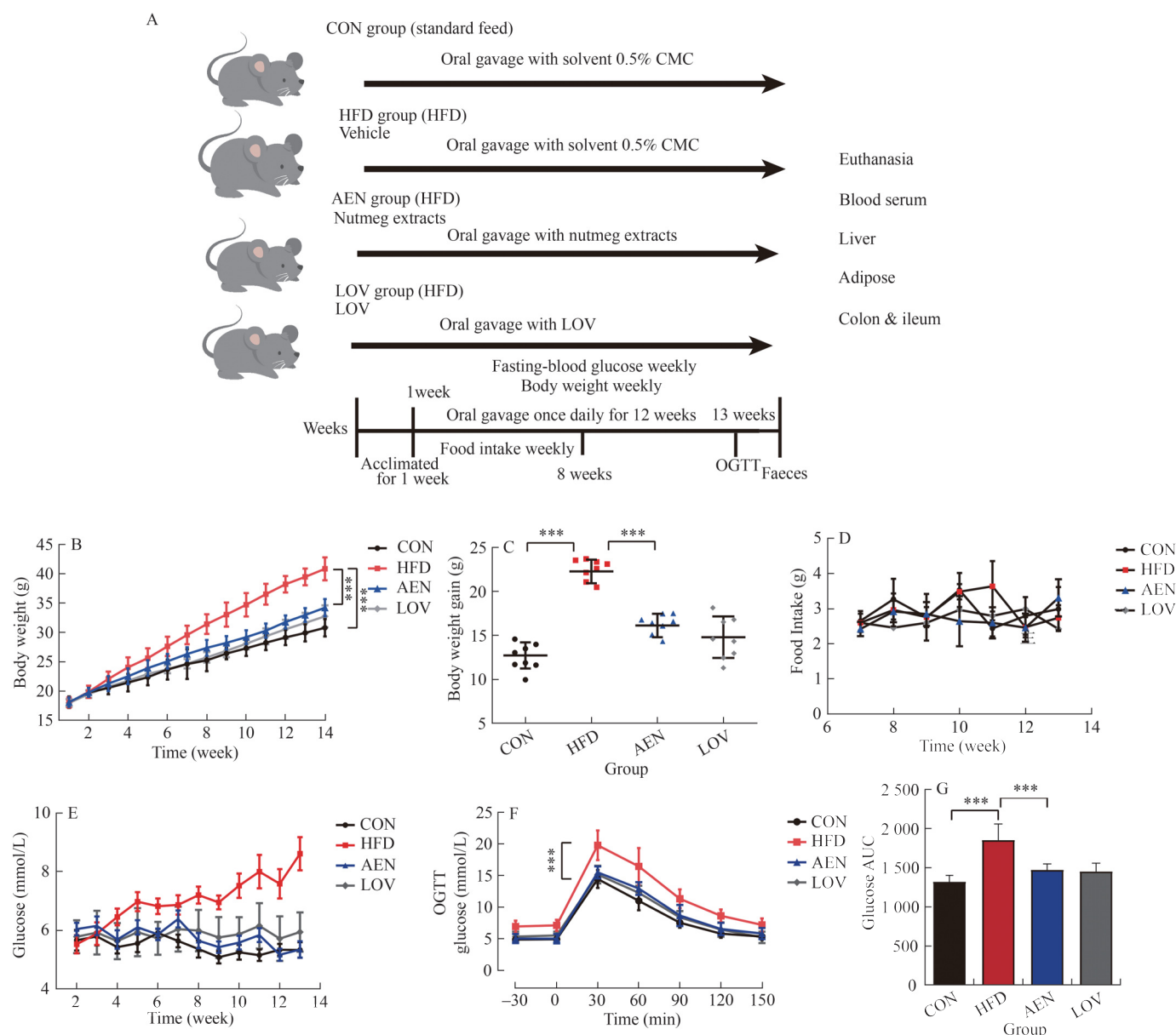


**Fig. 1** Impact of AEN on body weight and blood glucose in high-fat diet mice (C57BL/6J). Mice were divided into three groups: control (CON), HFD, and high-fat diet with lovastatin (LOV) or AEN treatment (AEN). (A) Experimental workflow for animals. (B) Trend in body weight changes over 14 weeks. (C) Body weight gain in mice. (D) Weekly food intake comparison from week. (E) Fasting blood glucose levels from weeks 2 to 14. (F) Oral glucose tolerance test results. (G) Oral glucose tolerance test (OGTT) area under the curve (AUC). Data shown as mean  $\pm$  SD,  $n = 8$  per group; \*\*\* $P < 0.001$ ; The same as below.

weight as the optimal AEN concentration (Tables S2-S3). In subsequent experiments, the efficacy of AEN will be evaluated using only this concentration.

### 3.2 AEN prevented hepatocyte steatosis and regulated mice's blood lipid concentrations and liver function

We investigated hepatic fat accumulation in mice, using liver and epididymal fat sections, to assess if AEN treatment could avert NAFLD. Liver samples from the HFD group differed markedly in staining, size (Fig. 2A), and weight (Fig. 2I), compared to the AEN and CON groups. Notably, in comparison to CON, the AEN group showed significantly reduced hepatocyte steatosis, lipid vacuolation, and inflammation in liver tissues. Similar reductions were evident in epididymal fat samples. These

observations suggest that AEN administration effectively curbed fat accumulation in both liver and epididymal fat areas.

These analyses in this study showed abnormally elevated serum TG, TC, and LDL-c concentrations in the HFD group; in contrast, mice in the AEN group tended to have normal blood lipid concentrations, indicating that disorders of lipid metabolism had been avoided (Figs. 2C-E). The HDL-c concentrations of the HFD and AEN groups, however, were the same (Fig. 2B). The levels of ALT and AST, two markers of liver function that are closely linked to hepatic adipocyte infiltration and hepatitis, were significantly lower in the AEN group (Figs. 2F-G). In the HFD group, the ratio of AST to ALT was significantly decreased, and in the AEN group of mice, hepatocellular damage brought by hepatocyte steatosis was not experienced (Fig. 2H). The AEN group also had substantially lower

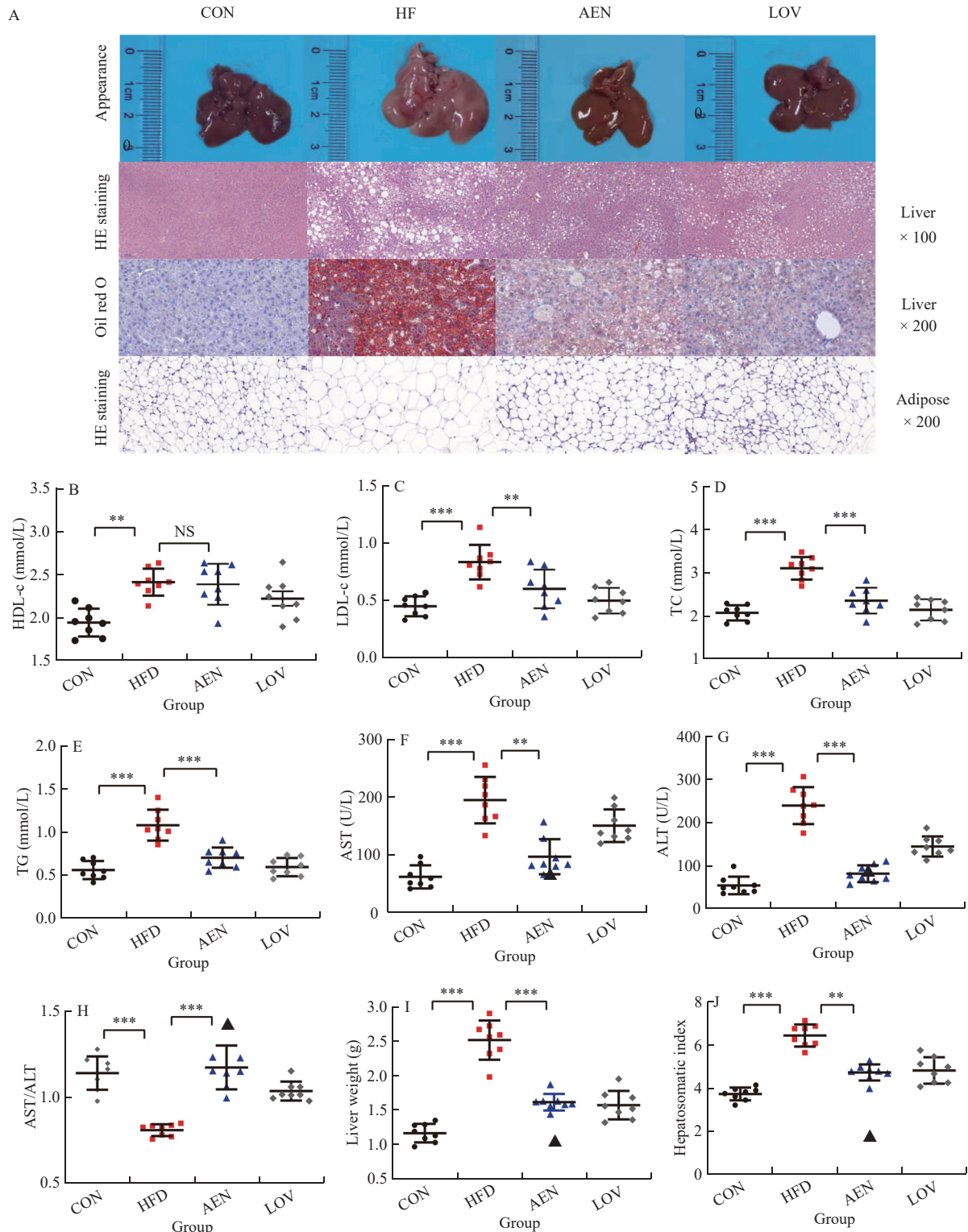


**Fig. 2** The effects on hepatic steatosis and liver function in mice by AEN. (A) Staining of epididymal fat sections with hematoxylin and eosin (H&E). Staining of liver sections with oil red O and H&E. The effect on blood lipid levels of mice with high-fat diet by AEN. (B-E) HDL-c, LDL-c, TC, and TG levels in mouse serum samples. (F-H) AST, ALT, and AST/ALT in mouse serum samples. (I) Mice liver weight in each group. (J) Hepatosomatic index of three groups of mice.

\*\* $P < 0.01$ , NS, non-significant;  $n = 8$  per group; The same as below.

hepatosomatic index (liver weight/body weight  $\times 100$ ) than the HFD group (Fig. 2J). In addition, AEN effectively reduced the levels of the hepatic cytokines TNF- $\alpha$ , IL-1 $\beta$  and IL-6 (Fig. S12). In conclusion, AEN treatment effectively prevented lipid accumulation and regulated blood lipid, liver function and inflammatory response in mice.

### 3.3 AEN modulated the gut microbiota in mice

Bacterial analyses in this study were based on 16S rRNA analysis. Figs. 3A and S4 show the gut microbial composition in each group. Bacteroidetes and Firmicutes were the dominant phyla

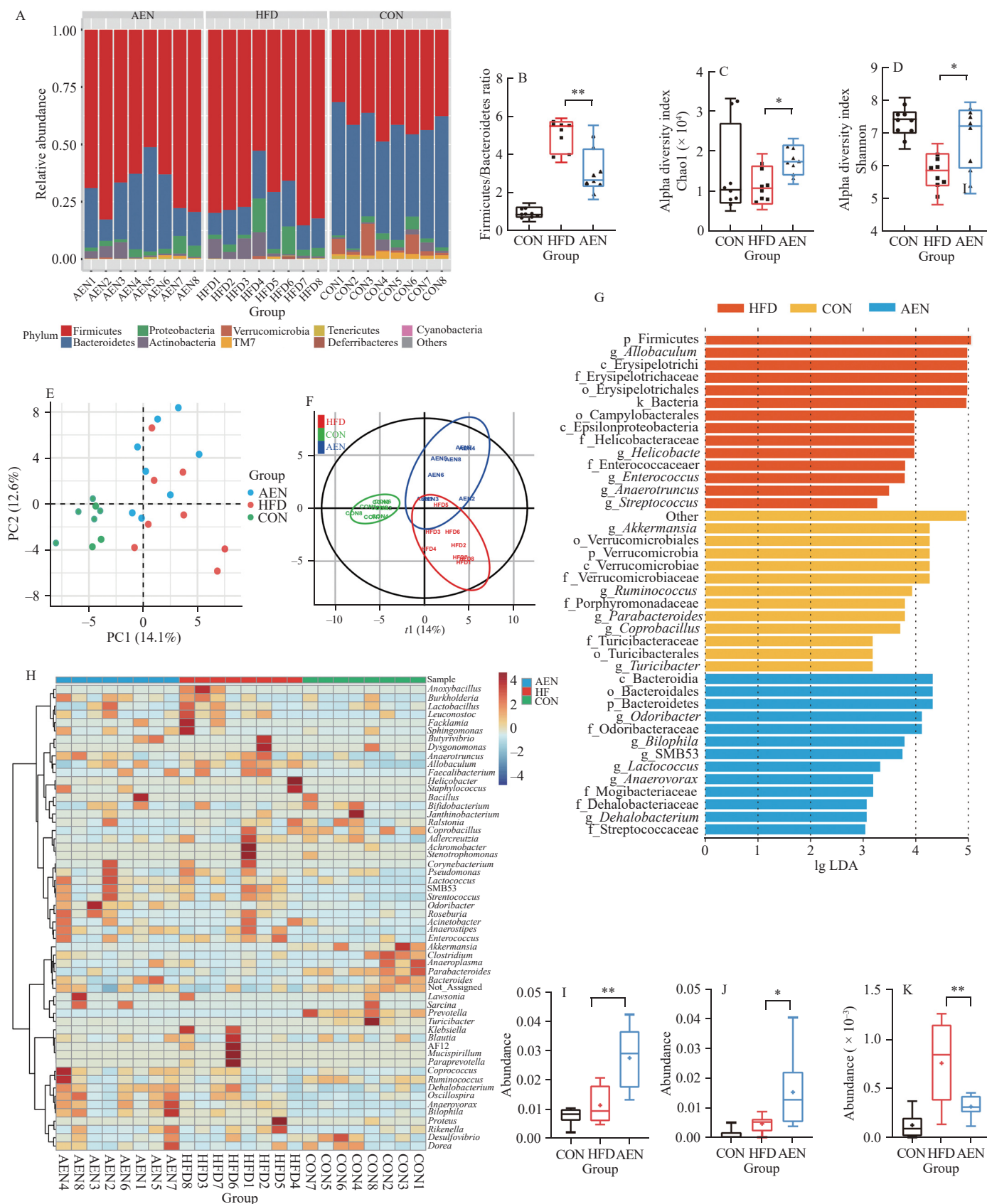


**Fig. 3** Modulation on gut microbiota in mice by AEN. (A) Bacterial community composition at phylum level (top 10). (B) Firmicutes/Bacteroidetes ratio in CON, HFD, and AEN groups. (C) Chao1 index species richness boxplots in three groups. (D) Shannon diversity index boxplots for each group. (E) PCoA of  $\beta$ -diversity across three groups. (F) PLS-DA of gut microbiota in three groups. (G) LefSe at OTUs level. (H) Heat map of genus-level relative abundances, with red indicating positive correlation and blue indicating negative correlation. (I-K) Increases in *Odoribacter* and *Bifidobacterium*, and decrease in *Streptococcus* in AEN-treated HFD mice. \* $P < 0.05$ ; The same as below.

detected in the gut microbiota. Firmicutes dominated in the HFD group, whereas the AEN group had significantly lower abundances of the Firmicutes and a lower Firmicutes/Bacteroidetes ratio (Fig. 3B). Compared with the HFD group, the AEN group exhibited a significant increase in the Chao1 index (Fig. 3C), and a similar increase in Shannon index (Fig. 3D). In addition,  $\beta$ -diversity was analysed, and the results are presented using a principal coordinate analysis (PCoA) plot (Fig. 3E). This analysis clearly distinguished the CON group from the HFD and AEN groups in ordination space along PC1 axis. Briefly, PC1 and PC2 account for 14.1% and 12.6% of the total variation, respectively. Partial least squares-discriminant analysis (PLS-DA) revealed analogous outcomes (Fig. 3F).

Further, we identified operational taxonomic units (OTUs) with different contents using linear discriminant analysis (LDA) effect sizes (LEfSe) (Fig. 3G). The dominant microbiota at the OTU level in each group was consistent with the results of cluster analysis (LDA score > 3). The dominant bacteria at OTU level in AEN group were c\_Bacteroidia, o\_Bacteroidales and p\_Bacteroidetes. See Fig. 3H for

a heat map of these results. At the genus level, the microbiota was enriched in *Enterococcus*, *Streptococcus*, *Allobaculum*, and *Anaerotruncus* in the HFD group, and *Odoribacter*, *Bilophila*, *Lactococcus*, and *Anaerovorax* in the AEN group. Significant differences in the microbes of each group at the order and genus levels are shown in Figs. 3I-K and S5A-D. Among the dominant taxa in the AEN group, the abundances of Bacteroidales, *Parabacteroides*, and *Lactococcus* had reverted towards the pattern in the CON group rather than that in the HFD group. The relative abundances of *Bilophila* and *Odoribacter* were significantly higher than those in the CON and HFD groups. In the AEN group, the abundances of *Streptococcus* and unclassified\_f\_Erysipelotrichaceae were significantly downregulated compared with the HFD group.

### 3.4 Intestinal metabolites analysis and pathway prediction

We next analysed and compared intestinal metabolites in the CON, HFD and AEN groups (Fig. 4). All ion chromatograms of the

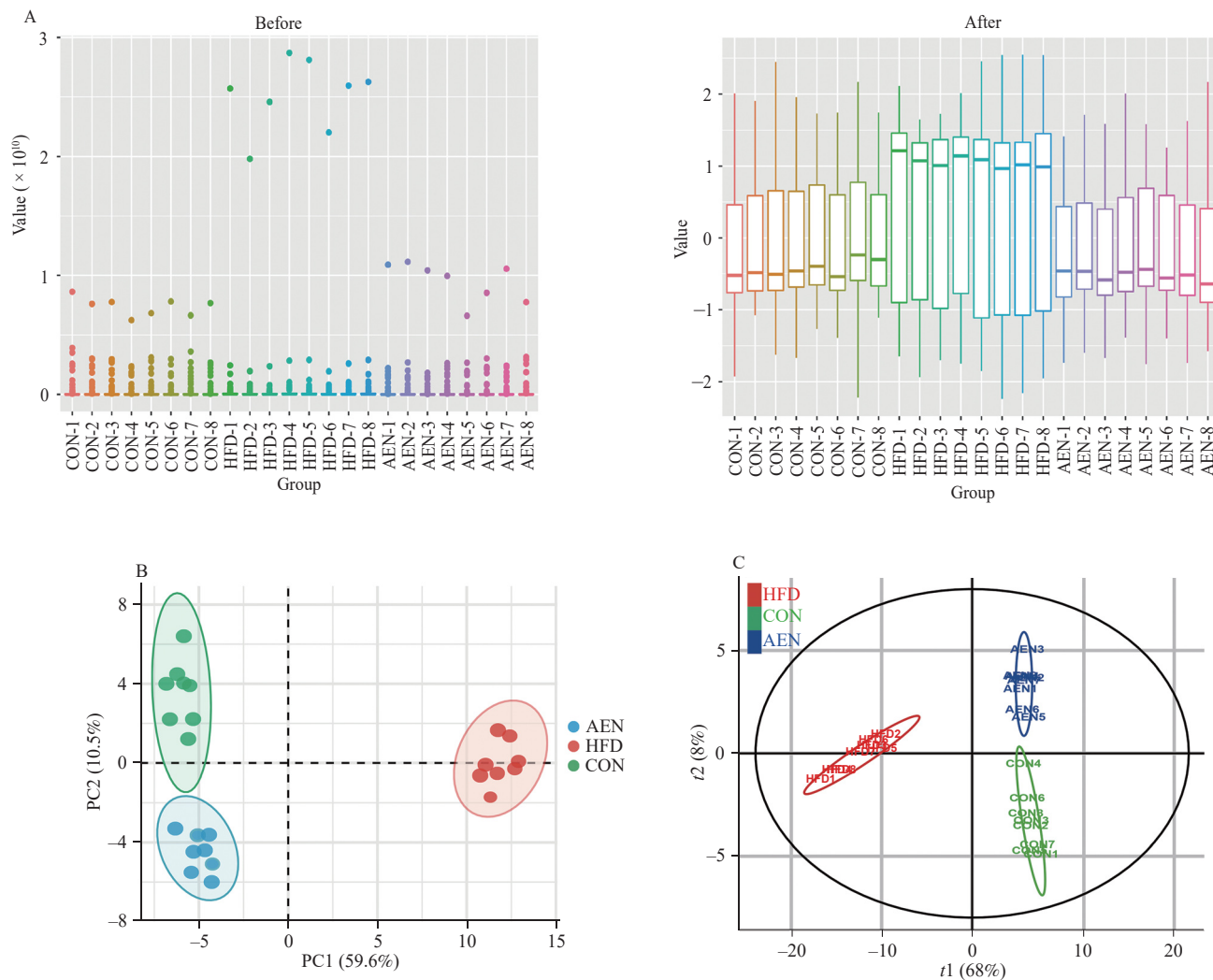


**Fig. 4** The effects on the intestinal flora metabolites in high-fat-fed mice by AEN. (A) Sample normalization by median standardization (before vs. after comparison). (B) PCA of metabolites in each group. (C) PLS-DA for group metabolites. (D) Correlation of metabolites in phylogenetic trees per group. (E) Pathway enrichment analysis between AEN and HFD groups.  $-\lg P$  value indicates enrichment level. (F) Network analysis of metabolite interactions, pathways, and targets (AEN-HFD, Student's  $t$ -test). Red indicates positive, and green indicates negative significant regulation in metabolites ( $|\text{Fold change}| > 1.5$ ). Rectangles are pathways, pentagons are metabolites.

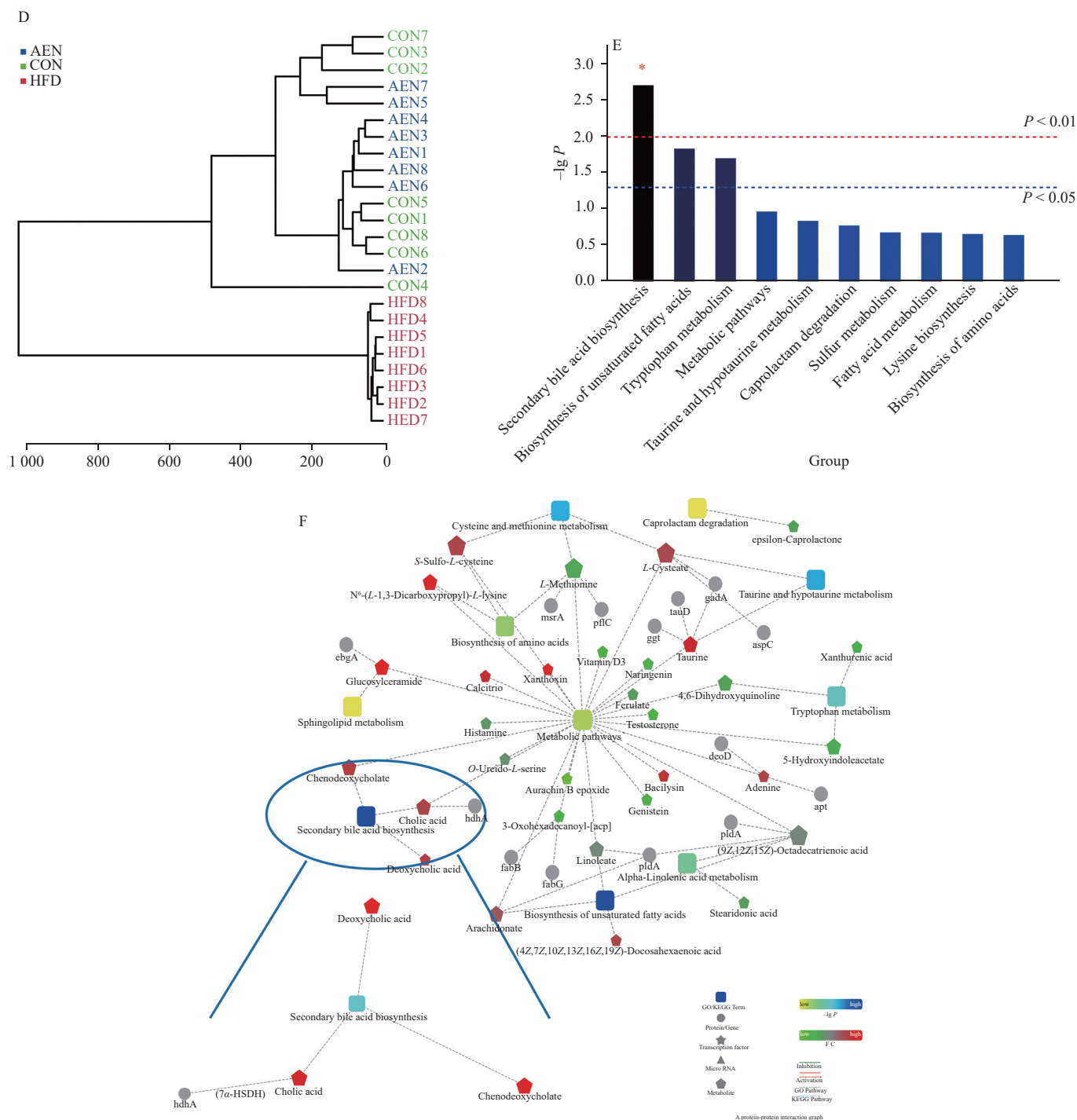


Fig. 4 (Continued)

quality control samples, obtained through ultra-high performance tandem mass spectrometry in both  $ESI^+$  and  $ESI^-$  modes, had well-defined peak shapes and consistent distributions (Fig. S1). The peak areas of the intestinal microbial metabolites were normalised to the mean for each fecal sample to reduce variation between mice (Fig. 4A). Fig. S6 showed the important features identified by ANOVA. Principal component analysis (PCA) revealed the underlying patterns that explained the variation between samples (Fig. 4B). PLS-DA showed a remarkable difference between the metabolites detected in AEN group samples versus the HFD group samples (Fig. 4C).

Cluster analysis dendrogram revealed that the HFD group samples clustered distinctly, separating from other groups (Fig. 4D). A heatmap displaying the top 50 metabolites (integrated positive and negative ion mode) is illustrated in Fig. S8. The overall disparity is captured in a volcano map (Fig. S7), facilitating a comparison between the AEN and HFD groups ( $t$ -test;  $P < 0.05$ , fold change  $> 2$ ). KEGG pathway enrichment analysis ( $P < 0.01$ ) pinpointed secondary bile acid biosynthesis as the most significantly enriched pathway in fecal metabolites (Fig. 4E). A metabolic pathway network interaction map demonstrates the interplay between metabolites, targets, and pathways (Fig. 4F).

### 3.5 Fecal bile acid composition and correlation with gut microbiota at the genus level

To further study the relationship between the composition of intestinal bile acids and the gut flora, 23 fecal bile acids were quantified by liquid chromatography MS/MS (Table S1). A total ion chromatogram of negative ion mode (standards and samples) is shown in Fig. S9. Among them, 15 sorts of bile acids were chosen to analyse significant differences between groups (Fig. 5A). Compared with the HFD group, the concentrations of  $\beta$ -muricholic acid ( $\beta$ -MCA), tauro- $\beta$ -MCA (T- $\beta$ -MCA), taurocholic acid (TCA), and tauroursodeoxycholic acid (TUDCA), hyodeoxycholic acid (HDCA) and lithocholic acid (LCA) were significantly increased in the AEN group. The concentrations of cholic acid (CA), ursodeoxycholic acid (UDCA) and deoxycholic acid (DCA) were downregulated in the

AEN group relative to the HFD group. However, the concentration of chenodeoxycholate (CDCA) did not differ significantly between the groups. To identify bile acids that were characteristic of the three groups, we performed a LefSe analysis ( $\lg \text{LDA} > 3$ ) (Fig. 5B). CA was found to be dominant in the HFD group ( $\lg \text{LDA} > 5$ ), and  $\beta$ -MCA, DCA and LCA were found to be dominant in the AEN group ( $\lg \text{LDA} > 4$ ).

We next focused on the ratios of the concentrations of non-12-OH bile acids (CDCA,  $\beta$ -MCA, UDCA, LCA, HCA, HDCA) produced by the bile acid replacement pathway to the concentrations of 12-OH bile acids (CA and DCA). The proportions of non-12OH bile acids were increased in the AEN group compared with those in the HFD group (Fig. 5C). In addition, different species have different types and proportions of BAs, for example, MCA is the main species in rodents. We observed that AEN treatment reduced the  $\beta$ -MCA/T- $\beta$ -MCA ratio

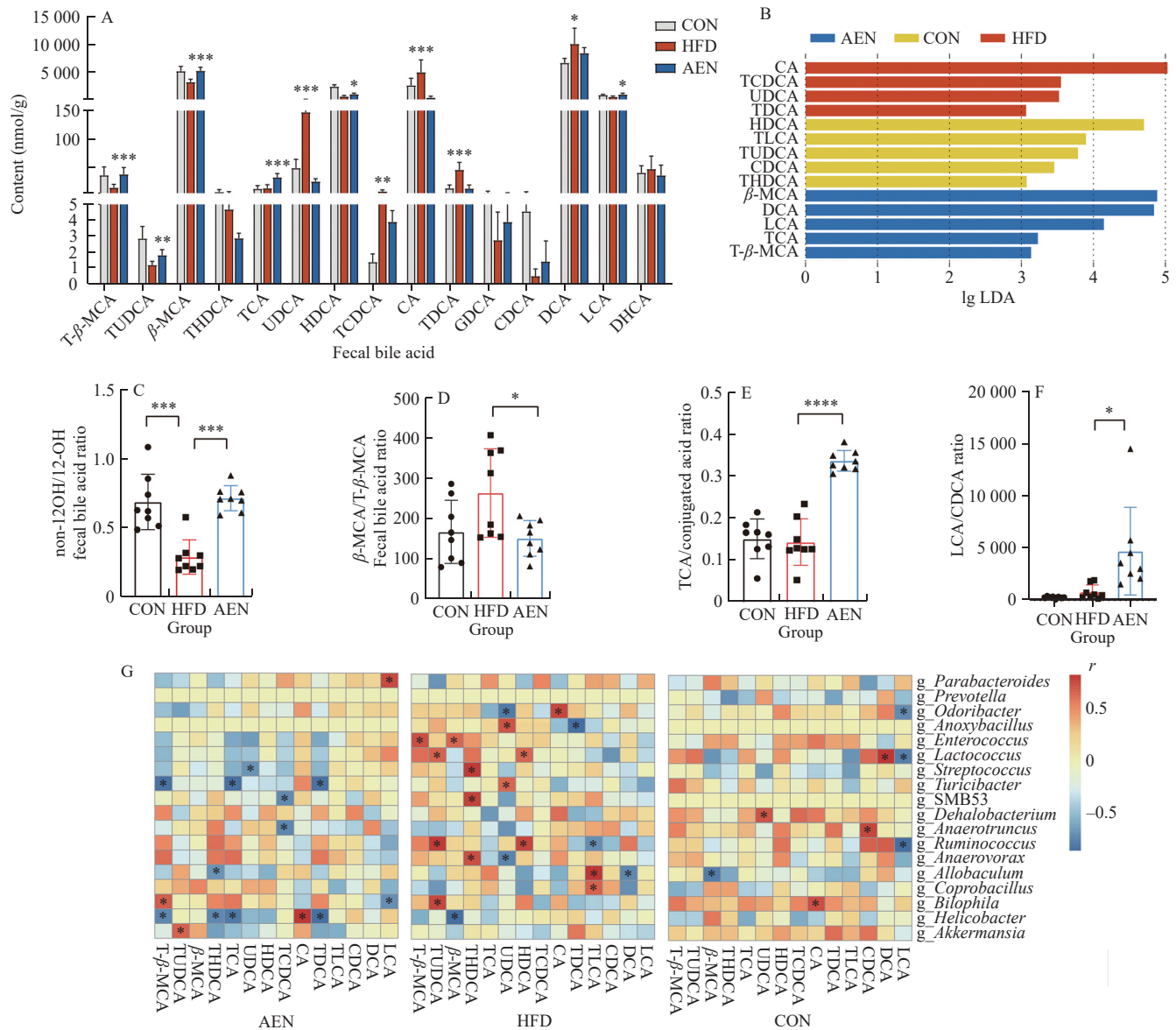


**Fig. 5** Regulation on intestinal metabolites-bile acids by AEN. (A) Fecal bile acid levels in each group. GDCA, glycodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; (B) LefSe analysis of bile acids in each group. (C) Ratio of non-12OH to 12-OH bile acids in feces. (D) Ratio of  $\beta$ -MCA to T- $\beta$ -MCA in feces. (E) Ratio of TCA to conjugated bile acids in feces. (F) Ratio of LCA to CDCA in feces. (G) Heat map of Spearman's correlation between bile acids and gut microbes at genus level. \*\*\*\* $P < 0.001$ .

(Fig. 5D), indicating an increase in the ratio of conjugated non-12-OH bile acids to unconjugated non-12-OH bile acids. The ratio of TCA to conjugated bile acids was also significantly upregulated in the AEN group (Fig. 5E). We also observed the noble higher ratio of LCA to CDCA in the AEN group than in other groups; the latter is transformed into the former in the gut (Fig. 5F).

Finally, we performed a Spearman's correlation analysis of the gut microbiota at the genus level and bile acids in the heatmap (Fig. 5G). In the AEN group, LCA was significantly positively correlated with the abundance of *Parabacteroides* and significantly negatively correlated with *Bilophila*, T- $\beta$ -MCA was positively correlated with *Bilophila* and notable negatively correlated with *Helicobacter* and *Turicibacter*; CA was notable positively correlated with *Helicobacter*; TCA was notable negatively correlated with *Helicobacter* and *Turicibacter*; Abundance of *Lactococcus* showed a significant positive correlation with TUDCA in the HFD group and inversely with TCA and T- $\beta$ -MCA in the AEN group. The abundance of *Streptococcus* has a significant positive correlation with taurohyodeoxycholic acid (THDCA) in the HFD group and a negative correlation with TCA and T- $\beta$ -MCA in the AEN group. In summary, AEN was shown to upregulate beneficial bacteria, such as *Parabacteroides* and *Bilophila* related to bile acid production and downregulate harmful bacteria, such as *Helicobacter* and *Streptococcus*.

### 3.6 AEN regulated bile acid metabolism in mice

We studied the effect of AEN intervention on the classical and alternative bile acid synthesis pathways (Fig. 6A). In the liver, the levels of cytochrome P450 family 7 subfamily B member 1 (*CYP7B1*) and *CYP27A1*, which are alternative pathway components, were increased by AEN treatment. The effects of AEN on gene expression of *CYP7A1* and *CYP8B1* of the classical pathway were not statistically significant in the liver. Significant increases in the expression of *FXR* and small heterodimer partner (*SHP*) were observed in the AEN group. AEN was found to reduce both the expression and function of the LXR/RXR (liver X receptor/retinoid X receptor) and SREBP-1c/FASN pathways (Fig. 6B). The expression of *FXR* and *FGFR4* in the ileum and the expression of *FGFR4* in the liver were decreased by AEN treatment and up-regulated the expression of the bile salt export pump (*BSEP*) gene in the liver simultaneously (Figs. 6B-C).

Microbial enzymes are pivotal in modulating bile acid homeostasis, transforming primary bile acids into secondary ones in the gut. We deployed ELISA analysis to quantify microbial enzymes involved in bile acid metabolism within fecal and colon samples (Figs. 6D-F). In comparison to the HFD group, the fecal and colon levels of bile salt hydrolase (BSH) and 7 $\alpha$ -HSDH were notably lower in the AEN group, while 7 $\alpha$ -DH levels rose significantly. We also confirmed the

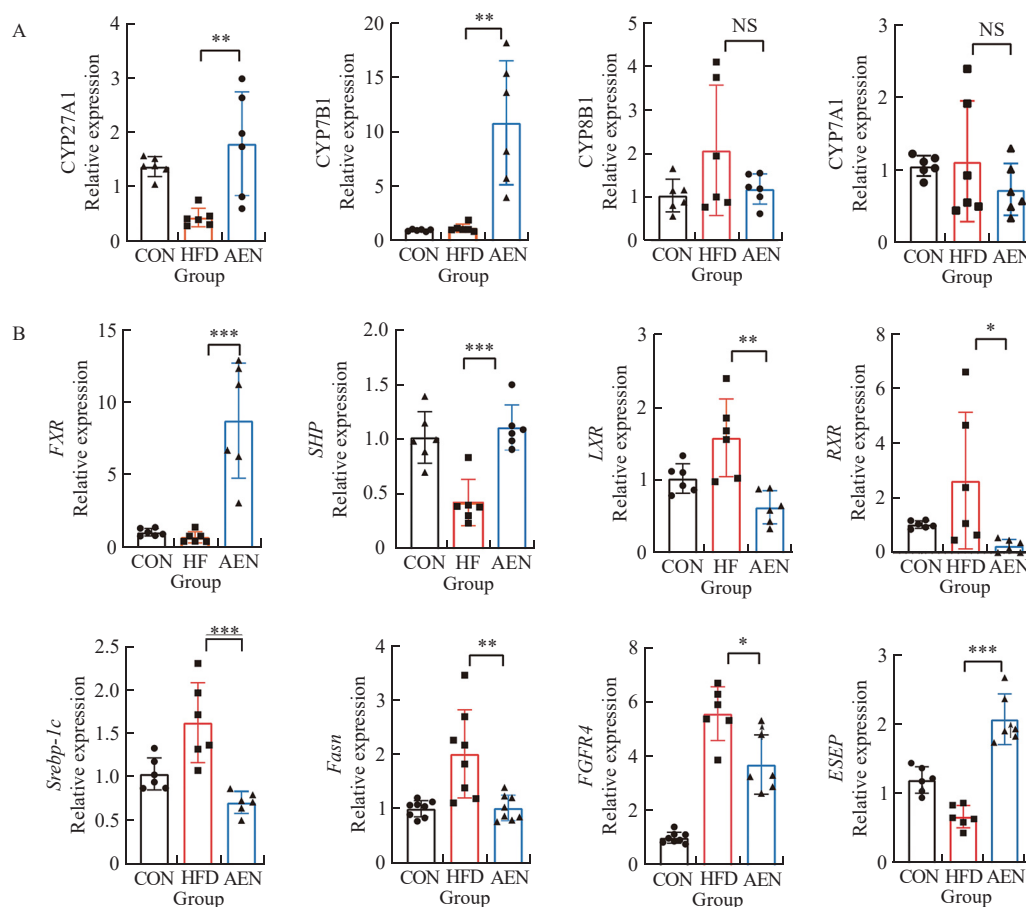


**Fig. 6** AEN's regulation of bile acid metabolic pathways and enzymes in liver and intestine. (A) AEN's impact on liver bile acid synthesis pathway. (B) AEN's effect on liver bile acid metabolism target genes. (C) Influence of AEN on ileum bile acid metabolism target genes. (D) AEN's effect on bile salt hydrolase (BSH) levels in colon and feces. (E) Impact of AEN on 7 $\alpha$ -HSDH in colon and feces. (F) AEN's effect on 7 $\alpha$ -DH levels in colon and feces. (G) Influence of AEN on bile acid metabolism target proteins in ileum. (H) Effect of AEN on liver bile acid metabolism target genes.

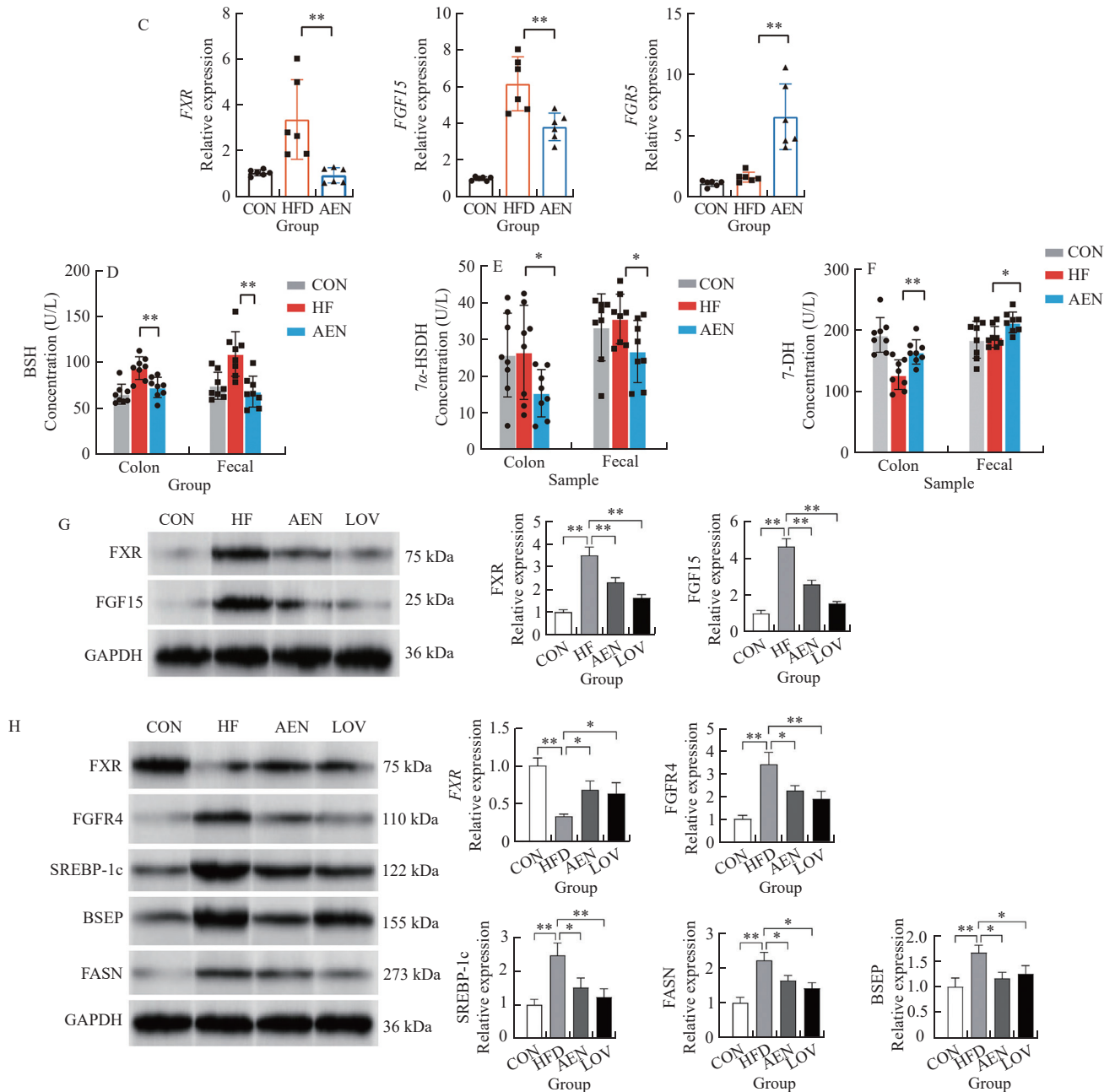


Fig. 6 (Continued)

protein expression of *FXR* and *FGF15* in the ileum (Fig. 6G), alongside *FXR*, *FGFR4*, *FASN*, *SREBP-1c*, and *BSEP* in the liver (Fig. 6H). These findings aligned with our gene expression observations.

### 3.7 Network pharmacology analysis of active *M. fragrans* components and NAFLD targets

To pinpoint nutmeg components effective against NAFLD, we employed network pharmacology analysis (Fig. 7A). We identified 158 potential herbal targets, focusing on the top 20 gene targets by degree (Fig. 7B). A disease database search revealed 1 040 related targets, and intersecting these, we found 52 overlapping gene targets (Fig. 7C). From these, we selected the 10 most active nutmeg components with drug-likeness (DL) > 0.18 for further analysis (Fig. 7D).

Chemical composition analysis of our AEN was performed using two-dimensional gas chromatography time-of-flight mass spectroscopy, as depicted in Fig. 7E. We identified seven key components in AEN, each with a peak intensity > 1% and match degree > 800 (Table S4). These components, when compared with database-mined compounds (Fig. 7F), highlighted three potentially active ones: licarin B with high oral bioavailability (OB  $\geq$  30%), and licarin A and myrislignan with lower OB (OB < 30%) but greater DL (> 0.18). Using Cytoscape v.3.9.0 software for network analysis of target-component pathways (Fig. 7G), we further identified potential hub targets and components (Tables S5, S6), enhancing our understanding of AEN's efficacy against NAFLD.

Utilizing databases, we conducted Gene Ontology (GO) and KEGG enrichment analyses. The GO analysis revealed that the biological process domain was the most prominent, accounting for

52%, followed by cellular component (22%) and molecular function (26%) domains (Fig. 8A). Within this domain, critical groups included response to nutrient levels (GO: 0031667), drug response (GO: 0042493), cell secretion regulation (GO: 1903530), and cellular response to nitrogen compounds (GO: 1901699), all significant (Fig. 8B). Potential targets showed enrichment primarily in NAFLD pathways, related to lipid metabolism like adipocytokine signaling, glucose metabolism pathways including insulin resistance, and inflammatory pathways, particularly hepatitis C (Fig. 8C).

We further conducted a protein-protein interaction network analysis to pinpoint significant hub genes affected by NAFLD-related pathways. Notably, MCODE1's hub proteins such as PPARA, RXRA, and RXRG, were actively expressed in the NAFLD pathway (Fig. 8D). Lastly, we examined the influence of AEN on targets within bile acid metabolism pathways in mice with HFD-induced NAFLD. This comprehensive assessment incorporated findings from our network pharmacology, microbiome, and metabolomics studies (Fig. 8E), providing a detailed view of AEN's regulatory impact.

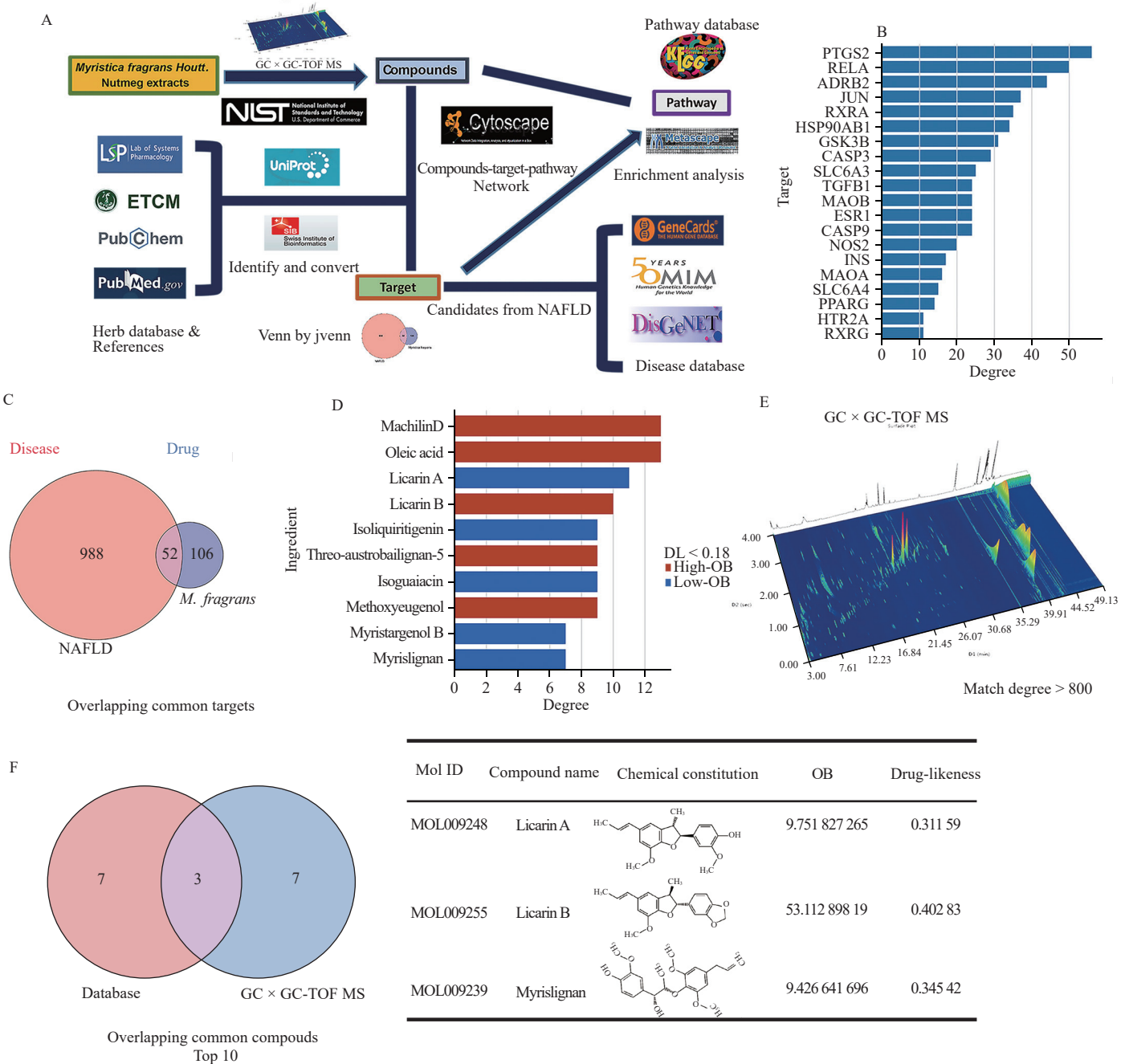


**Fig. 7** Prediction of the effect by nutmeg extract on NAFLD with network pharmacology and GC x GC-TOF MS associative analysis. (A) Network pharmacology analysis workflow. (B) Top 20 targets ranked by degree. (C) Venn diagram of common target genes from NAFLD and *M. fragrans* databases. (D) Top ten ingredients by degree. Red: Oral bioavailability (OB)  $\geq 30\%$ ; blue: OB  $< 30\%$ ; drug-likeness  $> 0.18$ . (E) AEN compounds identified by GC x GC-TOF MS. Match degree  $> 800$ ; data are peak intensities. (F) Venn diagram of top 10 common compounds from drug databases, GC x GC-TOF MS, and three-ingredient information table. (G) Interaction of *M. fragrans* active components, target genes, and pathways. Ingredients are represented, with squares for target genes and darts for pathways. Betweenness color gradient from yellow to purple indicates increasing significance.

G

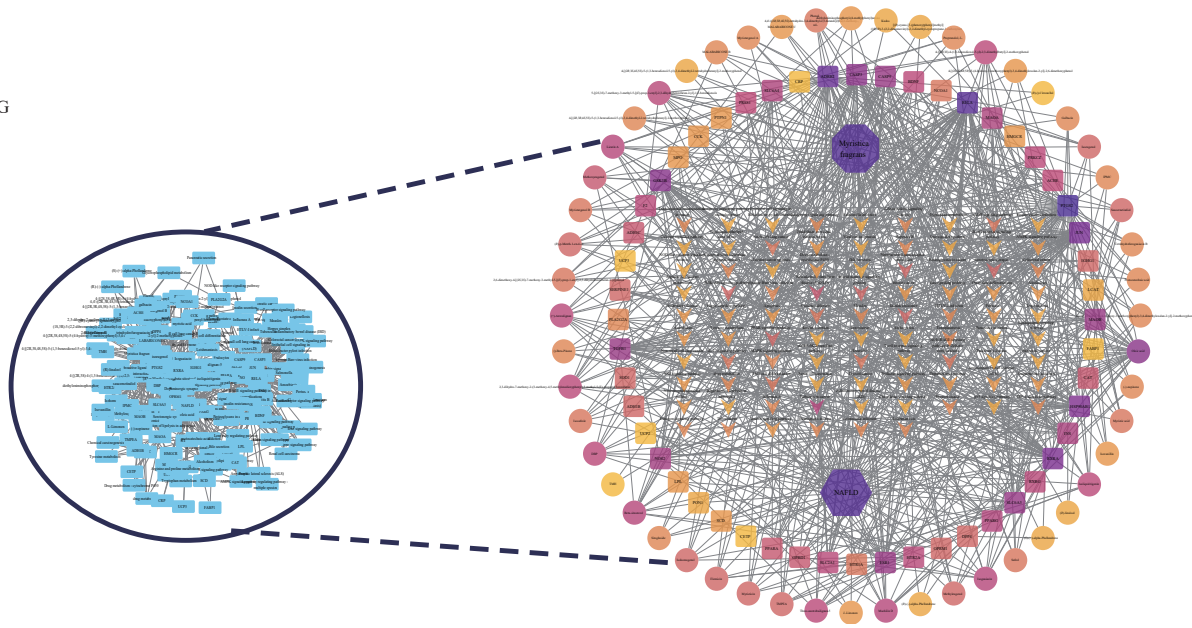
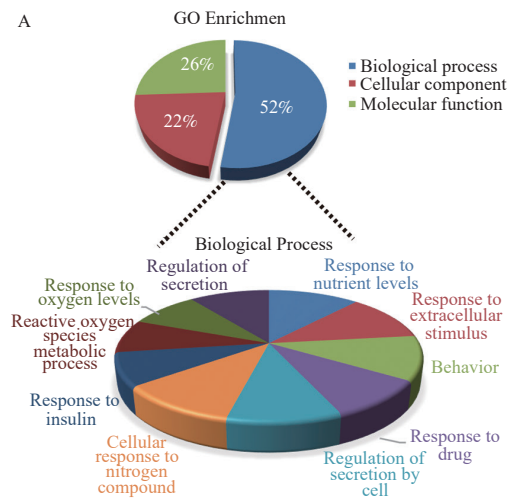
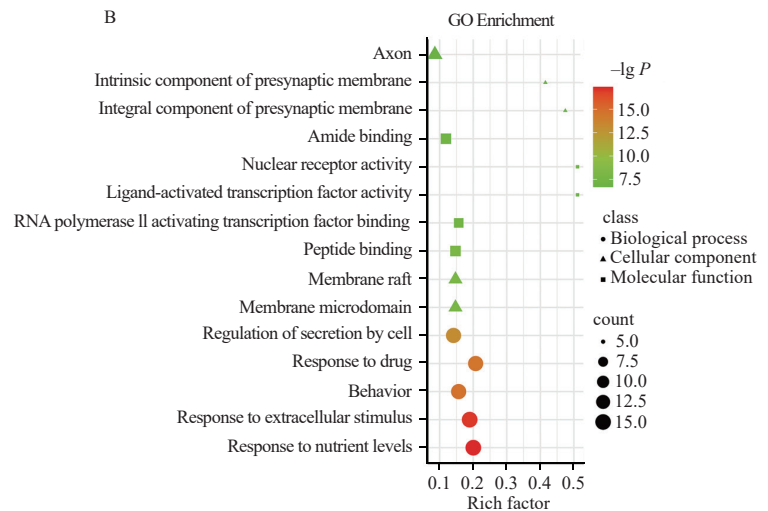


Fig. 7 (Continued)

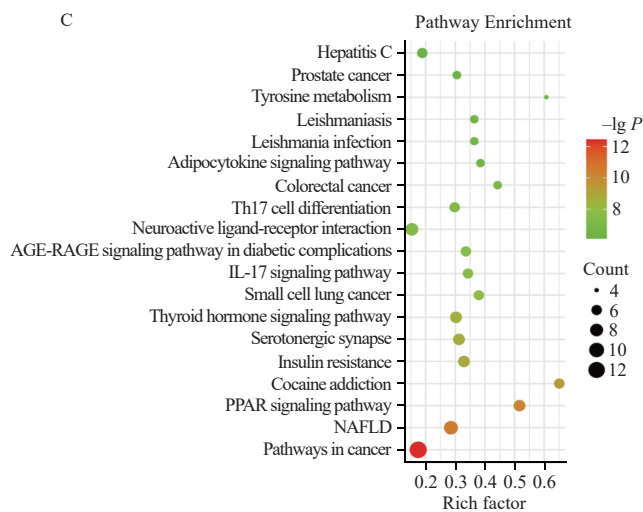
A



B



C



D

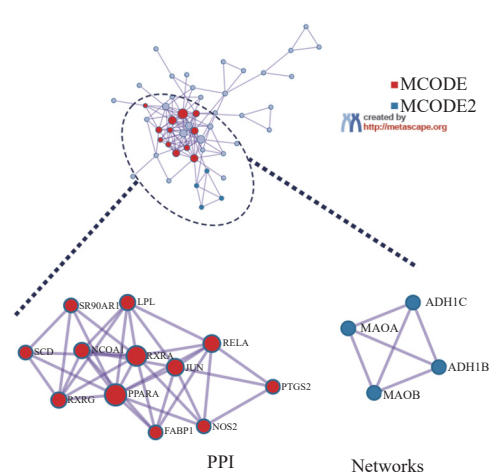


**Fig. 8** Database-based enrichment and prediction of GO and KEGG pathways. (A) Pie chart of GO enrichment analysis, the proportion of biological process, cellular component, and molecular function. (B) The GO enrichment analysis related to *M. fragrans* ( $P < 0.001$ , count  $> 5$ ) (C) The pathway enrichment analysis related to *M. fragrans* ( $P < 0.001$ , count  $> 4$ ) (D) Protein to protein interaction (PPI) network analysis related to *M. fragrans* from Metascape. (E) Analysis of the combination of metabolome and gut microbiome and network pharmacology in the KEGG pathway. Red represents up-regulation and blue represents down-regulation, BA, bile acid.

E

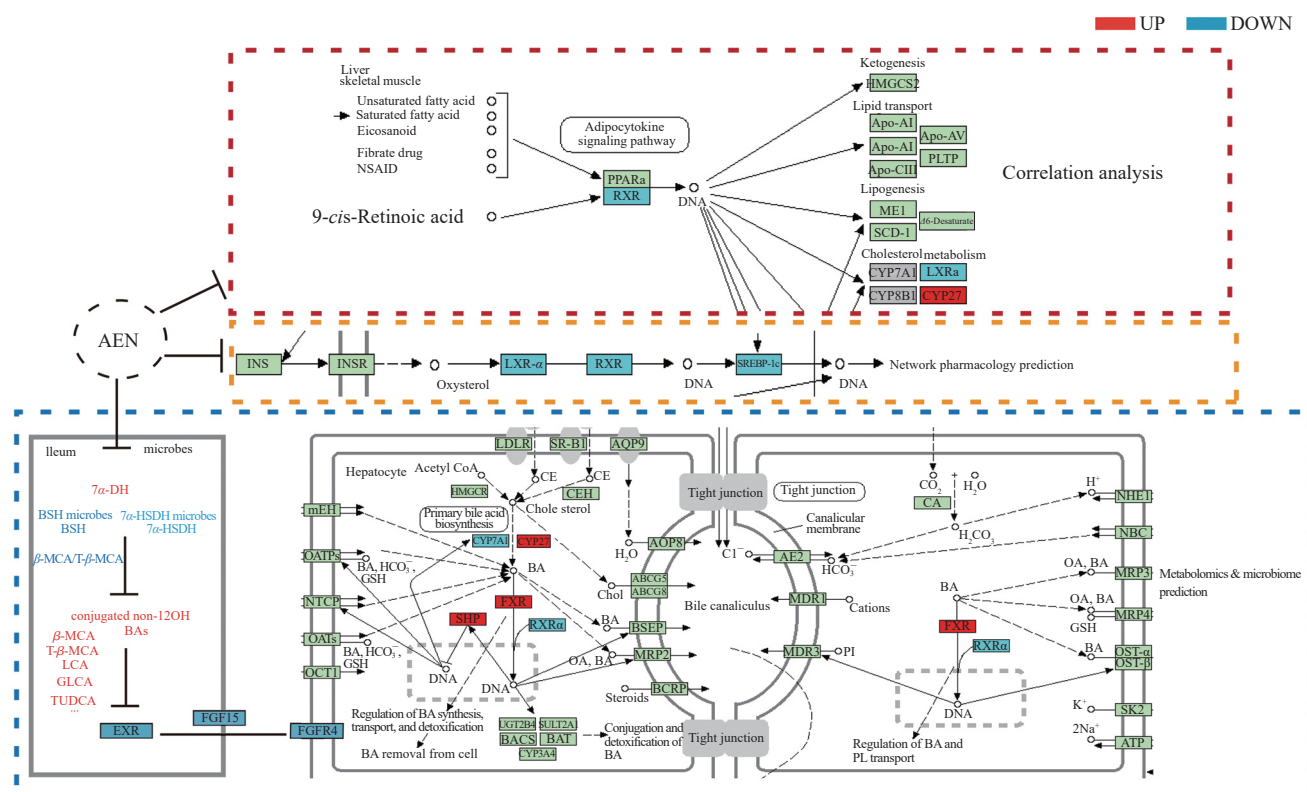


Fig. 8 (Continued)

#### 4. Discussion

Effective ingredients and compound prescriptions are increasingly used in TCM for the treatment or prevention of various metabolic diseases, including NAFLD. Nutmeg, the seed of *M. fragrans* Houtt., is a rich reservoir of bioactive molecules and has been used to treat diarrhoea, asthma, cough, malaria and other symptoms and diseases due to its antibacterial, anti-inflammatory and antioxidant effects<sup>[15-16]</sup>. Recently, studies have focused increasingly on the effects of nutmeg extracts against diabetes, obesity and other metabolic disorders<sup>[22-23]</sup>. However, few studies have clarified the effective material basis of these extracts and their direct targets in NAFLD.

Our study is the first to evaluate the efficacy of a nutmeg extract containing lignans, termed AEN, in the treatment of NAFLD from a gut-liver axis perspective using intestinal microbiome, metabolomics, and network pharmacology analyses. We considered AEN components with an OB < 30% in our analysis of the contributions of gut microbes and host metabolism to NAFLD, consistent with earlier studies that used berberine, which has low OB, to treat gastrointestinal disease<sup>[24-25]</sup>.

Our study led to the identification of two low OB components: licarin A and myrislignan. Myrislignan, despite its low plasma concentration post-oral administration, has shown notable hepatoprotective effects<sup>[18,26-27]</sup>. Licarin A, primarily known for its antibacterial and antiparasitic actions *in vitro*<sup>[28-29]</sup>, represents another compound of interest. Additionally, licarin B, another component identified, has demonstrated the potential to improve insulin sensitivity in adipocytes through the PPAR $\gamma$  pathway *in vitro*<sup>[30]</sup>. Our network pharmacology analysis not only pinpointed

these potential components but also highlighted NAFLD-related targets and pathways.

A HFD can lead to liver inflammation and lipid accumulation, which can also impair liver function. The ALT and AST levels are commonly considered as markers indicative of hepatic lipid accumulation and reflective of liver function<sup>[31]</sup>. The efficacy of AEN treatment on HFD mice is consistent with the previous results of oral gavage treatment<sup>[17,32]</sup>.

The important role of the gut microbiota in the gut-liver axis has been established clearly in both preclinical NAFLD/NASH models and in the analysis of clinical samples of patients with NASH<sup>[33-34]</sup>. Early studies have shown that individuals with higher adiposity levels and glucose concentrations have a lower Bacteroidetes/Firmicutes ratio than individuals with normal metabolic parameters<sup>[35-36]</sup>. In our study, the AEN treatment increased species diversity among and within groups. OTUs of Bacteroidetes were depleted in the HFD group, which is considered a biomarker closely related to obesity and NAFLD<sup>[37]</sup>.

In our study, we found intriguing shifts in gut microbiota between the HFD and AEN diet groups. Notably, the abundance of *Lactococcus*, typically thriving in the infant gut<sup>[38]</sup>, and *Parabacteroides*, inversely linked to obesity<sup>[39]</sup>, diminished in the HFD group but escalated in the AEN cohort. Concurrently, the AEN exhibited a pronounced increase in *Odoribacter*, renowned for butyrate production<sup>[40]</sup>. Moreover, the elevated presence of Erysipelotrichaceae, associated with increased inflammation and obesity risks<sup>[41]</sup>, indicates its potential as a target in obesity treatment strategies. Additionally, a marked reduction in the *Streptococcus* genus, housing pathogenic strains like *Streptococcus pyogenes*, was observed in the AEN group<sup>[42-43]</sup>, highlighting the nuanced effects of dietary interventions on gut microbial dynamics.

Intriguingly, AEN treatment boosted the abundance of the

*Bilophila*, which is often linked with increased lipid accumulation in the liver. Research indicates that elevated fecal bile acid concentrations, particularly specific conjugated forms like TCA, which can foster *Bilophila* growth<sup>[44-45]</sup>. Notably, the beneficial role of AEN extends to enhancing the intestinal luminal environment, thereby reducing liver lipid buildup through modulating obesity and fatty liver-associated bacterial genera within the intestinal microbiome.

Bile acids, crucial for enterohepatic circulation and closely linked with intestinal bacteria, stood out in our analysis, particularly secondary bile acid metabolism pathway-related metabolites. Thus, our focus shifted to exploring bile acid metabolism and its interaction with intestinal bacteria through the gut-liver axis. AEN treatment notably increased fecal concentrations of TCA and T- $\beta$ -MCA. TCA, known for activating G-protein-coupled bile acid receptor 1<sup>[46-47]</sup>, promotes heat generation in white adipose tissue, potentially reducing obesity in mice. Meanwhile, T- $\beta$ -MCA accumulation disrupts the FXR-FGF15/19 axis in the intestine, curbing bile acid reabsorption and modulating enterohepatic circulation through FGF-15 or-19. This axis plays a pivotal role within the gut-liver framework, where FGF15 regulates bile acid metabolism by indirectly suppressing *de novo* synthesis in the liver through inhibition of CYP7A1 and CYP27A1<sup>[48-49]</sup>.

After AEN treatment, an increase in the abundance of *Parabacteroides* was positively correlated with the increase in LCA in feces, consistent with the results of a previous study showing that *Parabacteroides distasonis* suppressed weight gain, hyperglycaemia and hepatic steatosis in mice fed an HFD<sup>[50]</sup>. We suspect that the lignans in AEN regulate certain bacterial genera or bile acids. Decreases in the abundances of some harmful bacterial genera, such as *Helicobacter*, were negatively correlated with the concentrations of T- $\beta$ -MCA, THDCA, TCA and TDCA. Interestingly, *Parabacteroides* and *Streptococcus* that were negatively correlated with conjugated bile acids such as T- $\beta$ -MCA and TCA in the AEN group but positively correlated in the HF group could produce BSH<sup>[51-52]</sup>.

In the liver, primary bile acids (CA, CDCA, MCA) combine with glycine and taurine, forming conjugated bile acids released into the intestine. Intestinal bacteria then mediate their hydrolysis, reverting them to primary forms. These primary acids are further transformed through dehydrogenation, dihydroxylation, and isomerisation into secondary bile acids: CA to DCA, CDCA to LCA, and MCA to HDCA<sup>[53-54]</sup>. Research suggests that increasing the ratio of conjugated to non-conjugated bile acids inhibits FXR in the intestine, thereby controlling liver bile acid synthesis. The balance between non-12-OH and 12-OH bile acids in the liver is regulated by bile acid synthesis enzymes. Certain endogenous conjugated bile acids (TUDCA, T- $\beta$ -MCA) and natural components act as FXR antagonists in the intestine<sup>[55-56]</sup>. In this study, AEN potentially influenced specific enzymes in intestinal bacteria, altering bile acid ratios.

BSH, an important component of bile acid degradation, is responsible for the dissociation of glycine and taurine from bile acids. However, there was no observed increase in the abundance of BSH-producing bacteria such as *Clostridium*, *Lactobacillus*, and *Bifidobacterium*. Furthermore, there was a downregulation in the abundance of BSH-producing genera like *Streptococcus*. We speculate that AEN inhibited part of the BSH-producing flora, thereby downregulating the level of BSH. 7 $\alpha$ -HSDH and 7 $\beta$ -HSDH mainly catalyse 2 processes in the intestine: the conversion of taurochenodeoxycholic acid to TUDCA, and the epimerisation of CDCA to UDCA<sup>[57-58]</sup>. The latter was significantly inhibited after

AEN treatment, and this is mainly attributable to the observed decrease in the concentration of 7 $\alpha$ -HSDH.

## 5. Conclusion

In summary, our research sheds light on how the lignan-enriched nutmeg extract influences the gut microbiome and bile acid metabolism in a HFD-induced NAFLD model. AEN acts to recalibrate gut flora, reducing levels of BSH and 7-HSDH and enhancing conjugated bile acids, including 12-OH variants. This reduction in intestinal FXR activity alters gene and protein expression in the FGF15-FGFR4 signaling pathway. The upshot is a boosted synthesis of bile acids in the liver and their increased release into the intestine.

The promising results of this study suggest that *M. fragrans* Hoult. holds significant potential as a natural therapeutic agent for preventing NAFLD in human health. Further investigations into the bioactive components of this plant and their mechanisms of action could lead to the development of effective treatments for NAFLD and related metabolic conditions. Harnessing the medicinal properties of *M. fragrans* Hoult. may offer new avenues for combating the global burden of NAFLD and improving public health outcomes.

## Conflict of interest

Gang Wang is an editorial board member for *Food Science and Human Wellness* and was not involved in the editorial review or the decision to publish this article. The authors report no conflict of interest.

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## Appendix A. Supplementary information

Supplementary data associated with this article can be found, in the online version, at <http://doi.org/10.26599/FSHW.2024.9250455>.

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