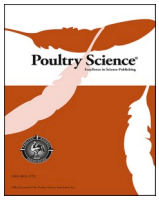
104 (2025) 104981

Contents lists available at ScienceDirect

Poultry Science

journal homepage: www.elsevier.com/locate/psj



Multi-omics reveal the effects and regulatory mechanism of dietary echinocystic acid supplementation on abdominal fat and liver steatosis in broiler chickens

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ARTICLE INFO

Keywords:
Broiler
Fat deposition
MAFLD
Gut microbiota
Echinocystic acid

ABSTRACT

The accumulation of abdominal fat and the metabolic dysfunction-associated fatty liver disease (MAFLD) are prevalent problems in the poultry industry, and seriously compromise broiler health and reduce economic benefits. Echinocystic acid (EA), a natural product with anti-inflammatory and antioxidant effects, has been demonstrated to reduce abdominal fat deposition and improve intestinal inflammation in mice. However, it has not been reported in poultry research.

In this study, we employed chicken hepatocytes (Leghorn male hepatoma cells, LMHs) to construct an oleic acid and palmitic acid (OA/PA)-induced MAFLD model in vitro and 60 male K90 chickens were induced MAFLD by a high-fat diet (HFD) to examine the impact of EA on liver-lipid metabolism and abdominal fat deposition. Moreover, metabolomic analysis, 16S rDNA gene sequencing, and transcriptomic profiling were performed to determine the mechanism of EA.

The results showed that EA (10 μM) significantly reduced triglyceride (TG) and total cholesterol (TC) levels in vitro. Moreover, EA reduced abdominal fat deposition without affecting growth performance. EA significantly decreased TC, TG, and low-density lipoprotein-cholesterol (LDL-C) levels, and increased high-density lipoprotein-cholesterol (HDL-C) levels in the blood. Additionally, EA supplementation altered the composition of the intestinal microbiota, particularly by decreasing the ratio of Firmicutes to Bacteroidetes. Furthermore, liver metabolomics analysis revealed that EA increased the abundance of metabolites related to arginine metabolism and mitochondrial oxidation pathways, and these metabolites were predicted to be positively correlated with the gut genera enriched by EA. EA also altered the expression patterns of genes related to liver lipid metabolism and inflammation, particularly *CYP7A1, CYP7B1, CYP3A5, and ACAT*, which are enriched in the PPAR signaling pathway and steroid hormone metabolism. Moreover, correlation analysis revealed that there was a close correlation between differential gut microbiota, metabolites, and gene expression profiles.

Collectively, the results indicated that EA may alleviate MAFLD by regulating steroid hormone metabolism and modulating the gut microbiota. EA may be a candidate feed additive to prevent abdominal fat deposition and MAFLD in the broiler industry.

Introduction

Poultry industry is playing a pivotal role in global food security by providing economical and high-quality protein to billions of humans worldwide (Abbas, 2020; Abbas et al., 2022). However, broiler production can be negatively impacted by high abdominal fat content, as excessive fat accumulation reduces meat quality and is less desirable for human consumption (Abbas et al., 2024). This excess fat is typically not utilized by humans and contributes to inefficiencies in poultry production, affecting both nutritional value and marketability.

Compared with other types of meat, chicken meat contains less fat and is a primary source of protein in the human diet (Do et al., 2022),

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https://doi.org/10.1016/j.psj.2025.104981
Received 2 December 2024; Accepted 2 March 2025
Available online 4 March 2025

making the development of broiler chickens a key focus of attention. In the poultry industry, a high-fat diet (HFD) has become a commonly used feeding strategy due to its efficient feed conversion rate (Chen et al., 2023). However, a long-term HFD can cause abdominal fat deposition in broiler chickens, further leading to MAFLD (Yang et al., 2023a). These problems have severely affected the development of the poultry industry. Excessive fat deposition in broilers adversely affects feed conversion, carcass yield, hatching rate, and fertility rate (Zhou et al., 2022).

The liver is the main organ for lipid metabolism in the body, and excessive fat accumulation in the liver can induce oxidative stress, leading to liver dysfunction (Yang et al., 2014; Pafili and Roden, 2021). A long-term HFD may trigger chronic inflammation, promote the secretion of inflammatory factors from the gut, which leads to intestinal epithelial barrier dysfunction (Deol et al., 2023). Concurrently, an HFD can also cause an imbalance in the gut microbiota, such as imbalances in the proportions of Firmicutes and Bacteroidetes, which can impair intestinal absorption functions (Wu et al., 2023). Numerous studies have shown that treating intestinal inflammation can effectively alleviate metabolic diseases caused by an HFD (Cheng et al., 2024). Recent studies have demonstrated that nutritional regulation can effectively alleviate abdominal fat deposition and improve the symptoms of MAFLD. A study by Yuan found that a low-protein diet in growing pullets affects liver lipid metabolism by altering gut microbiota and bile acid metabolism (Yuan et al., 2024). Additionally, folic acid reduced fat deposition in broilers by inhibiting abdominal adipocyte proliferation and differentiation (Liu et al., 2023). Natural products, as feed additives, play a significant role in preventing abdominal fat deposition and improving MAFLD in broiler chickens. Dietary supplementation with veratric acid alleviates liver steatosis and reduces abdominal fat deposition in broilers via the proliferator-activated receptor signaling pathway (Pan et al., 2024). Furthermore, hericium erinaceus polysaccharides has been shown to ameliorate the hepatic damage and metabolic disorders of NAFLD through regulating the gut-liver axis (Wu et al., 2024); however, current treatment options remain scarce, and strategies to address this problem are urgently needed.

Echinocystic acid (EA, shown in Fig. 1A), a natural plant extract, shows great anti-inflammatory and antioxidant activities in various diseases. Research has shown that EA can improve TNBS-induced colitis (Hyam et al., 2013). Our previous results revealed that EA can reduce abdominal fat deposition and liver inflammation in mice, possibly by modulating inflammatory and metabolic pathways (Xiao et al., 2023), but no studies have reported the application of EA in broiler fat deposition and oxidative stress. Therefore, we hypothesize that EA may play a role in alleviating fat deposition and improving MAFLD in broilers.

Hence, this study combines transcriptomics, metabolomics, and 16S rDNA sequencing to investigate the effects of EA supplementation on abdominal fat deposition and fatty liver disease in broiler chickens, aiming to uncover its underlying mechanisms.

Materials and methods

Animal ethics

All animal experiments were approved by the Guangxi University Animal Research Ethics Committee (Approval No. GXU-2024-159). The welfare and use of broiler chickens were conducted following the guidelines published by the Ministry of Science and Technology of the People's Republic of China (Approval number: 2006–398).

Reagents

Echinocystic acid (EA, purity $\geq$ 98 %) was purchased from Jing Cui Tian Cheng Pharmaceutical Technology Co., Ltd. (Chengdu, Sichuan, China). Triglyceride (TG) and total cholesterol (TC) test kits were purchased from Applygen Technologies Inc. (Beijing, China). Aspartate aminotransferase (AST), alanine aminotransferase (ALT), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), malondialdehyde (MDA), and total superoxide dismutase (T-SOD) test kits were purchased from the Nanjing Jiancheng Institute of Bioengineering (Nanjing, Jiangsu, China). Oleic acid and palmitic acid (OA/PA) were purchased from Sigma-Aldrich (Shanghai, China). A Cell Counting Kit-8 (CCK8) was purchased from Yeasen Biotechnology Co., Ltd. (Shanghai, China). A bicinchoninic acid (BCA) protein quantitative assay kit was purchased from Beyotime Biotechnology (Shanghai, China). LMHs (Leghorn male hepatoma cells) were obtained from Beijing Bena Chuanglian Biotechnology (Beijing, China). 1640 Dulbecco's modified Eagle's medium (DMEM) was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA).

Cell culture and treatment

LMH cells were maintained in DMEM supplemented with fetal bovine serum (FBS) (10 %, v/v) and penicillin-streptomycin (P/S) (1 %, v/v). The cells were cultured in a humidified incubator under an atmosphere of 5 % CO_2 at 37 °C. The cells were seeded into corresponding well plates and grown to 80 % confluence. After being cultured in DMEM without FBS for 6 h, oleic acid (200 μM, OA) and palmitic acid (100 μM, PA) were added to induce lipid deposition and an oxidative stress environment, and the cells were supplemented with EA for 24 h.

Cell viability assays

LMH cells were cultured in 96-well plates. When the cell concentration reached 60 %, different concentrations of EA (0, 5, 10, 25, 50, or 100 μM) were added for 24 h. Then, 100 μL of detection solution (DMEM: CCK8, 1:9, v/v) was added, and the mixture was incubated for 1 h. The fluorescence (450 nm) of the wells was measured according to the manufacturer's instructions. Each experiment was repeated at least three times.

Animals management and diet ingredient composition

60 healthy male K901 broiler chickens (1-day old) were purchased from a live poultry market (Nanning, Guangxi, China). In accordance with standard management practices, the chickens were housed in environmentally controlled cages with temperature and humidity starting at 35 °C and 65 % on day 1, decreasing by 2 °C every 3 days until reaching 26 °C after 21 days. During the first week, continuous 24-h lighting was provided, followed by a regimen of 23 h of light and 1 h of darkness for the remainder of the study. Every 5 broilers were raised in a single cage (size: 600 mm × 550 mm × 430 mm), and water was provided ad libitum.

Broiler chickens were randomly divided into four groups: a basal diet group (ND group), a high-fat diet group (HFD group), a high-fat diet group supplemented with 25 mg/kg EA (EAL group), and a high-fat diet group supplemented with 50 mg/kg EA (EA group), with 15 chickens per replicate. The feed and nutritional components of the diet are detailed in Table S1.

Growth performance measurement

During the experimental period, body weight (BW) and daily feed intake (DFI) of the broilers were recorded at 1, 7, 14, and 21 days of age. The body weight gain (BWG), average daily feed intake (ADFI), and feed-to-weight ratio (G/F) were subsequently calculated.

Slaughter performance detection and sample collection

After 21 days, the broiler chickens were sacrificed following a 12-h fasting period. Chickens are weighed before slaughter and after bloodletting. Blood samples were collected from the wing veins of broilers in

vacuum tubes without sodium heparin. After standing for 12 h at 4 °C, the serum was collected and stored at −20 °C until analysis. The chickens were then euthanized by cervical dislocation immediately. The weights of the abdominal fat, liver, breast muscle, and leg muscle were subsequently measured. After being eviscerated to remove feathers, feet, heads, abdominal fat, and viscera, the slaughter weight and eviscerated weight were measured, and then the slaughtering percentage and eviscerated weight were calculated following the Poultry Performance Standards and Measurement criteria (NY/T823-2020). Samples of the liver, small intestine, breast muscle, leg muscle, and cecal contents were collected and stored at –80 °C until analysis.

Detection of meat quality

The meat quality of the breast muscle was evaluated by measuring the color parameters of lightness (L*), redness (a*), and yellowness (b*) via a chromameter (CR400/410, Minolta, Osaka, Japan). Moreover, the pH values were measured via a pH meter (Testo 205, Testo, Germany). Drip loss was measured in approximately 2 g of each breast muscle sample via the plastic bag method described by Honikel (Xiao et al., 2022a).

Biochemical analysis in serum and liver

The total TG, TC, ALT, AST, MDA, HDL-C, and LDL-C levels in the serum and liver were measured using commercially available kits according to the manufacturer's instructions. Protein concentrations were measured via a bicinchoninic acid (BCA) protein quantitative assay kit. Each experiment was repeated at least three times, and the data were normalized to protein concentrations.

H&E and Oil Red O staining

Liver samples were fixed in 4 % paraformaldehyde, dehydrated, and embedded in paraffin. The paraffin sections were then subjected to hematoxylin and eosin (H&E) staining and Oil Red O staining. The slices were observed via microscopy and analyzed via a computerized image analysis system.

RNA Extraction and quantitative real-time PCR analysis

Total RNA was extracted from the liver using a TRIzol reagent (Genstar, Beijing, China), and cDNA was synthesized via reverse transcription. Real-time quantitative reverse transcription (qRT -PCR) was conducted using Real Star Green Fast Mixture (GenStar, Beijing, China) and a qTOWER (Analytik Jena, Germany). Gene expression levels were normalized to β-actin as an internal reference. The primer sequences used are listed in Table S2. Each experiment was repeated at least three times.

Fecal 16S rDNA sequencing and analysis

The intestinal contents from the ND, HFD and EA groups were chosen for gut microbiome analysis after 21 days of EA treatment. Total fecal microbial DNA was obtained with a Fecal Genome DNA Extraction Kit (Tiangen BioTech, Beijing, China). The DNA was quantified with a Qubit (Invitrogen, Carlsbad, CA). Total DNA was amplified via PCR using the universal primer 341F/805R (341F: CCT ACGGGNGGCWGCAG; 805R: GACTACHVGGGTATCTAATCC). The PCR products were purified via AMPure XT Beads (Beckman Coulter Genomics, Danvers, MA) and quantified via a Qubit (Invitrogen, Carlsbad, CA). The quality of the PCR products was evaluated via an Agilent 2100 Bioanalyzer (Invitrogen, Carlsbad, CA) and Illumina library quantitative kits (Kapa Biosciences, Wilmington, MA), which were further pooled together and sequenced on an Illumina NovaSeq 6000 from LC-Bio Technology Co., Ltd. (Hangzhou, Zhejiang, China).

The sequencing primers were removed from the raw de-multiplexed sequences using Cutadapt (v1.9). The paired-end reads were subsequently merged via FLASH (v1.2.8). The low-quality reads (quality scores <20), short reads (<100 bp), and reads containing more than 5 % "N" records were trimmed by using the sliding-window algorithm method in fqtrim (v 0.94). Quality fltering was performed to obtain high-quality clean tags according to fqtrim. Chimeric sequences were filtered via Vsearch software (v2.3.4). DADA2 was applied for denoising and generating amplicon sequence variants (ASVs). Species annotation and sequence alignment were performed using the QIIME2 plugin feature-classifier, with the alignment databases SILVA and NT-16S. Alpha and beta diversities were calculated using QIIME2, relative abundance was used in bacterial taxonomy. The Wilcoxon test was used to identify the differentially abundant genus, and significant differences were considered at $p < 0.05$. LDA effect size (LEfSe, LDA ≥ 3.0, $p < 0.05$) was performed using nsegata-lefse.

Metabolomics analyses

Metabolites were extracted with 80 % methanol buffer. Briefly, 50 mg of sample was extracted with 0.5 ml of precooled 80 % methanol. The extraction mixture was then stored for 30 min at −20 °C. After centrifugation at 20,00× g for 15 min, the supernatants were transferred to new tubes and vacuum dried. The samples were redissolved in 100 μL of 80 % methanol and stored at −80 °C prior to LC-MS analysis. In addition, pooled QC samples were also prepared by combining 10 μL of each extraction mixture. Statistical analysis was performed in R (version 4.0.0). The raw protein intensity was normalized via the method "medium", hierarchical clustering was performed using the pheatmap package. Principal component analysis (PCA) was performed using the metaX package. The PLSDA analysis is performed by using the R package ropls and the VIP values of each variable are calculated. Correlation analysis was performed by Pearson correlation coefficient of cor package. The three conditions of $p < 0.05$, difference multiple >1.2 obtained via the t-test and VIP calculated by PLSDA analysis simultaneously met the screening of the final metabolites with significant differences. Hypergeometric-based enrichment analysis with the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway was performed to annotate protein sequences. individually. GSEA (v4.1.0) and MSigDB software were used for gene set enrichment analysis to determine whether a set of genes in a specific KEGG pathway in different situations. When $|NES| > 1$, NOM $p < 0.05$, and FDR $q < 0.25$ were considered to indicate significant differences between the two groups. The network map is drawn according to the pathway where the metabolite is located.

Transcriptome analysis

Liver from the ND, HFD and EA groups were chosen for RNA-seq. Total RNA was extracted using Trizol reagent (Thermo Fisher, Waltham, MA) following the manufacturer's instrutions. The total RNA quantity and purity were analyzed with a Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, CA). cDNA library was constructed and the libraries were sequenced using the illumina Novaseq^{TM} 6000 (LC Bio Technology CO., Ltd. Hangzhou, China) platform following the vendor's recommended protocol, generating a total of millon 2 × 150 bp paired-end reads. Differentially expressed genes (DEGs) analysis was performed by DESeq2 software between two different groups. The genes with the parameter of false discovery rate (FDR) below 0.05 and absolute fold change ≥ 2 were considered DEGs. DEGs were then subjected to enrichment analysis of GO functions and KEGG pathways.

Statistical analysis

The data about the performance characteristics are expressed as mean ± standard error of the mean (SEM). Data were analyzed with unpaired two-tailed *t*-test for two groups or one-way analysis of variance

for multiple groups. Different letters represent differences that were statistically significant, $p < 0.05$. Figures were designed using GraphPad Prism version 8.0.

Results

EA reduced lipid accumulation in LMH cells

To evaluate the anti-steatotic effects of EA in vitro, we established a lipid accumulation model in LMH cells using OA/PA and subsequently analyzed EA-mediated alterations in hepatic lipid deposition. As shown in Fig. 1B, EA significantly inhibited cell viability at concentrations of 50 and 100 μM, while no significant effects were observed at concentrations below 10 μM. Additionally, the effects of different concentrations of EA on TG and TC levels in LMH cells were assessed. The results showed that EA decreased the intracellular TG content in a dose-dependent manner (Fig. 1C), with the most significant reduction at 10 μM (Fig. 1D); Notably, no effect was observed on extracellular TG content (Fig. 1E). Lipid levels were reduced as shown by Oil Red O staining (Fig. 1F). Concurrently, intracellular total cholesterol (TC) levels exhibited significant attenuation following EA treatment (Fig. 1G), but had no effect on the extracellular TC levels (Fig. 1H).

Effect of EA on growth performance in broilers

To investigate the effect of EA on growth performance in vivo, we used broilers as a model and added different doses of EA to the HFD. As shown in Table 1, HFD administration during days 1-21 significantly enhanced BW and ADG while reducing ADFI and G/F. EA supplementation significantly decreased BW, BWG, and ADG, whereas it

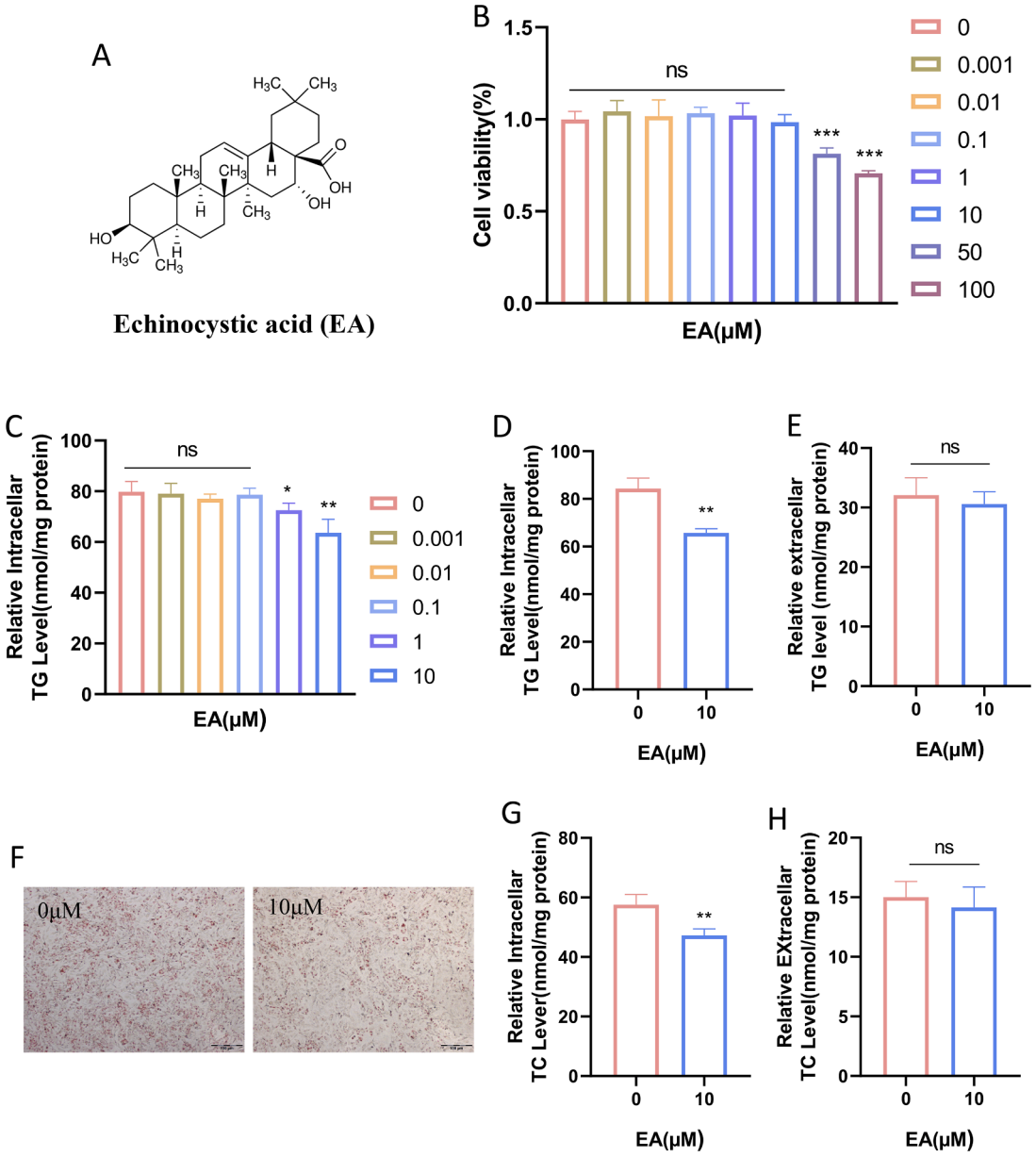

Fig. 1. Echinocystic acid (EA) inhibits lipid accumulation in chicken hepatocytes. (A) Molecular structure of EA. (B-H) LMH cells were treated with OA/PA or OA/PA supplementation EA for 24 h. (B) Cytotoxicity of EA in LMH cells (0, 5, 10, 25, 50, and 100 μM). (C) TG contents in LMH cells treated with different concentrations of EA (0, 5, 10, 25, and 50 μM). (D) Intracellular triglyceride (TG) level. (E) Extracellular triglyceride (TG) level. (F) Representative images of Oil Red O staining (scar bar = 100 μm). (G) Intracellular total cholesterol (TC) level. (H) Extracellular total cholesterol (TC) level. n = 3-6. Data are means ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = no significance.

Table 1
Effect of EA on growth performance of broilers.

Items	Treatment				*p*-value
	NC	HFHC	EAL	EA	
1 d BW, g	42.87 ± 0.16^{a}	42.70 ± 0.25^{a}	42.31 ± 0.27^{a}	42.32 ± 0.25^{a}	0.264
7 d BW, g	110.21 ± 0.81^{c}	114.26 ± 0.84^{a}	113.63 ± 0.95^{ab}	111.05 ± 1.11^{bc}	0.012
14 d BW, g	262.24 ± 5.04^{b}	284.93 ± 3.59^{a}	285.31 ± 2.19^{a}	256.26 ± 10.87^{b}	0.004
21 d BW, g	419.72 ± 4.35^{b}	446.63 ± 3.11^{a}	443.54 ± 3.77^{a}	426.70 ± 4.18^{b}	<0.001
1-21d BWG, g	376.85 ± .38^{b}	403.93 ± 3.16^{a}	401.23 ± 3.77^{a}	384.38 ± 4.13^{b}	<0.001
ADG, g/d	17.95 ± 0.21^{b}	19.23 ± 0.15^{a}	19.11 ± 0.18^{a}	18.30 ± 0.20^{b}	<0.001
ADFI, g/d	34.14 ± 0.32^{a}	32.73 ± 0.26^{b}	33.11 ± 0.36^{b}	32.98 ± 0.30^{b}	0.018
G/F, g/g	1.90 ± 0.02^{a}	1.70 ± 0.01^{c}	1.73 ± 0.03^{c}	1.80 ± 0.02^{b}	<0.001

Note: data are presented as mean ± SEM ($n = 10$). Different superscript letters are significantly different, p-value < 0.05.

significantly increased G/F, with no effect on ADFI.

Effects of dietary EA supplementation on fat accumulation in broilers

As shown in Fig. 2A, compared with the ND group, the HFD group presented increased abdominal fat deposition, subcutaneous fat thickness, intermuscular fat width, and abdominal fat to body weight ratio in broilers. However, EA supplementation significantly inhibited abdominal fat deposition and reduced subcutaneous fat thickness, intermuscular fat width, and the abdominal fat to body weight ratio (Fig. 2A-D). Moreover, EA reduced the liver weight ratio caused by the HFD (Fig. 2E). H&E staining results of abdominal fat further revealed that EA reduced the size of abdominal fat cells induced by the HFD (Fig. 2F). As shown in Table 2, EA supplementation significantly improved slaughter performance, but had no significant effect on the breast muscle and leg muscle ratios.

Effect of EA on the breast muscle quality of broilers

As depicted in Table S3, compared with the ND group, the pH value

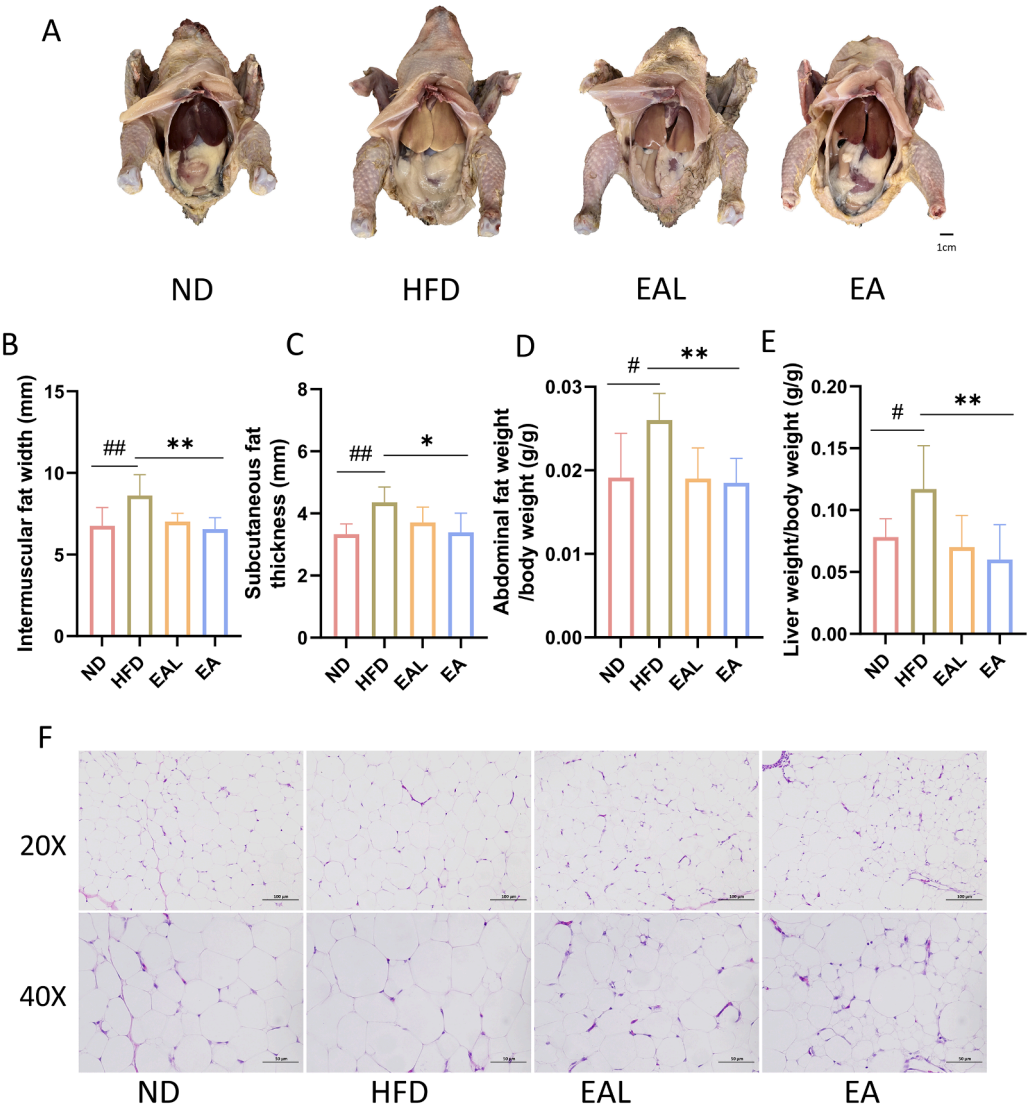

Fig. 2. Effects of dietary EA supplementation on fat accumulation in broiler chickens. (A) Representative photographs of liver and abdominal fat tissue of broilers. (B) Intramuscular fat width (mm). (C) Subcutaneous fat thickness (mm). (D) The ratio of abdominal fat weight to body weight (g/g). (E) The ratio of liver weight to body weight (g/g). (F) Representative hematoxylin and eosin (H&E) staining of abdominal fat. $n = 10$. Data are means ± SEM. A mark indicates a significant difference between the four groups ($^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = no significance).

Table 2
The effects of EA on the slaughter performance of broilers.

Items	Treatment				*p*-value
	ND	HFD	EAL	EA	
Slaughtering weight, g	385.76 ± 3.82^{c}	408.18 ± 2.23^{a}	406.80 ± 3.43^{a}	397.14 ± 3.50^{b}	<0.001
Slaughtering rate, %	91.91 ± 0.27^{a}	91.40 ± 0.33^{b}	0.93 ± 0.54^{a}	93.08 ± 0.37^{a}	0.027
Eviscerated weight, g	249.31 ± 2.91^{a}	249.65 ± 2.75^{a}	240.05 ± 2.04^{b}	241.70 ± 1.65^{b}	0.012
Eviscerated rate, %	59.40 ± 0.16^{a}	55.90 ± 0.48^{b}	54.13 ± 0.30^{c}	56.67 ± 0.51^{b}	<0.001
Chest muscle weight, g	65.89 ± 1.04^{a}	67.14 ± 0.84^{a}	64.85 ± 1.28^{a}	65.65 ± 0.70^{a}	0.443
Chest muscle rate, %	15.70 ± 0.24^{a}	15.04 ± 0.21^{ab}	14.62 ± 0.29^{bc}	15.39 ± 0.14^{ab}	0.014
Leg muscle weight, g	47.80 ± 1.04^{a}	49.51 ± 1.07^{a}	48.10 ± 1.59^{a}	50.02 ± 1.01^{a}	0.525
Leg muscle ratio, %	11.41 ± 0.23^{b}	11.10 ± 0.29^{ab}	10.85 ± 0.36^{ab}	11.72 ± 0.20^{a}	0.151

Note: data are presented as mean ± SEM ($n = 10$). Different superscript letters are significantly different, p-value < 0.05.

and drip loss (%) of chest muscle were decreased in the HFD group, but no significant effects on lightness (L*), redness (a*), or yellowness (b*) were observed. Notably, EA supplementation effectively attenuated HFD-induced drip loss, with no effects on L*, a*, or b* were detected.

Effects of EA supplementation on hepatic lipid metabolism and antioxidant stress in broilers

Chronic HFD exposure can lead to fat accumulation in the liver. Oil Red O staining showed that after 21 days of HFD, fat accumulated in hepatocytes, whereas EA inhibited HFD-induced hepatic lipid deposition in a dose-dependent manner (Fig. 3A). Fat accumulation can also lead to liver inflammation, and H&E staining of the liver revealed that EA significantly alleviated HFD-induced liver inflammation (Fig. 3B). Biochemical profiling indicated HFD significantly increased hepatic TG, TC, and MDA levels, whereas decreasing T-SOD levels, whereas EA intervention effectively normalized these parameters (Fig. 3C-F). Mechanistically, qPCR analysis demonstrated that EA significantly suppressing the expression of lipogenic genes, such as *FASN* and *ACC1*, as well as inflammation-related genes such as *NF-κB, IL-1*, and IL-6 (Fig. 3G).

Effects of EA on blood biochemical characteristics

A long-term HFD is a primary cause of liver fat accumulation and cholesterol synthesis, which further contribute to elevated blood lipids and chronic inflammation. We examined the effects of EA on blood lipids and inflammatory markers. As shown in Table S4, HFD significantly increased serum TG and TC levels, whereas EA intervention notably attenuated these elevations. Furthermore, EA treatment effectively mitigated HFD-induced dyslipidemia by reducing LDL-C and elevating HDL-C. AST and ALT are major markers of liver inflammation, and the HFD significantly increased ALT levels, but had no significant effect on AST levels. EA treatment reduced both ALT and AST levels. Additionally, antioxidant profiling further revealed EA-mediated reduction in serum MDA content, indicating systemic oxidative stress alleviation. These results suggest that EA can effectively reduce blood lipid levels and improve liver inflammation.

Impact of EA on the intestinal microbiota in broiler chickens induced by an HFD

To investigate the effects of EA on the gut microbiota of broiler chickens, we conducted 16S rDNA sequencing on the cecal contents of chickens in the ND, HFD, and EA groups. As shown in Fig. 4A, compared to the ND, the HFD significantly decreased the α-diversity and abundance of the gut microbiota, whereas EA supplementation increased both α-diversity and partially restored its abundance.

Further PCoA analysis indicated that the microbial clusters in the EA group were clearly distinct from those in the HFD group, suggesting a change in β-diversity (Fig. 4B). We also analyzed the changes in gut microbiota abundance after EA supplementation. Fig. 4C showed that Firmicutes, Proteobacteria, Actinobacteria, Bacteroidota, and Cyanobacteria were the top five microbes across the three treatments, with notable differences in their abundances. Compared to the ND group, EA supplementation reversed the HFD-induced decreases in Firmicutes and Bacteroidota and increased the ratio of Firmicutes to Bacteroidota (Fig. 4D-E). Moreover, compared to the HFD group, EA reduced the abundance of Escherichia-Shigella and Lactobacillus, whereas increasing the abundance of Faecalibacterium and Bacteroides at the genus level (Fig. 4F). LEfSe analysis identified six signature microbiota including Clostridia, Oscillospirales, Lactobacillales, Bacilli, Streptococcaceae, and Bacteroides as key discriminators (Fig. 4G). These results demonstrate that EA supplementation effectively recalibrated gut microbial ecology towards metabolic homeostasis.

Effects of EA supplementation on liver metabolites

PCA analysis was conducted on the metabolites of the ND, HFD, and EA groups, revealing significant separation between the groups (Fig. 5A), indicating that EA treatment caused significant changes in the liver metabolism of broiler chickens. The permutation test confirmed the reliability of the analytical model (Fig. 5B). Differential analysis identified 38 metabolites in EA vs HFD comparison, with 30 upregulated and 8 downregulated (Fig. 5C). KEGG enrichment analysis revealed that the DEMs were primarily enriched in 39 signaling pathways, with Glycerophospholipid metabolism, Vitamin B6 metabolism, and sucrose metabolism being the main metabolic pathways (Fig. 5D). Fig. 5E showed the DEMs between the HFD and EA groups. Among them, the upregulated metabolites were mainly 7-Hydroxy-4-cholesten-3-one, LysoPE, and 3a,7a-Dihydroxycoprostanic acid, whereas the downregulated metabolites were primarily 7-Hexadecynoic acid, Goshuyic acid, Opthalmic acid, and D-4-Phosphopantothenate. These pathways are closely related to lipid metabolism processes, and the metabolomics results are consistent with the transcriptomics analysis (Fig. 5F).

Impact of EA on liver gene expression patterns

To further validate the therapeutic mechanism of EA on NAFLD at the molecular level, we selected liver tissues from the ND, HFD, and EA groups for RNA-seq. PCA demonstrated clear segregation among ND, HFD, and EA groups, confirming diet-induced transcriptional remodeling and EA-mediated normalization (Fig. 6A). We subsequently analyzed the DEGs between the groups based on statistical criteria (fold change > 2.0; adjusted $p < 0.05$) (Fig. 6B). A total of 1102 DEGs were identified between ND and HFD groups, including 407 upregulated genes and 695 downregulated genes. Between ND and EA groups, 99 DEGs were identified, with 53 upregulated genes and 46 downregulated genes. Additionally, 761 DEGs were identified between the HFD and EA groups, including 500 upregulated genes and 261 downregulated genes. Hierarchical clustering analysis of DEGs between the HFD and EA groups revealed that the DEGs presented opposite expression patterns (Fig. 6C). GO and KEGG enrichment analyses were further performed on the DEGs. The GO analysis (Fig. 6D) indicated that the DEGs were primarily associated with biological process, cellular component, and molecular function, with enriched terms involving mainly cholesterol homeostasis and fatty acid metabolic processes. KEGG pathway enrichment analysis revealed that the DEGs were mainly associated with metabolic pathways, including the PPAR signaling pathway, fatty acid metabolism, ferroptosis, and steroid hormone biosynthesis (Fig. 6E).

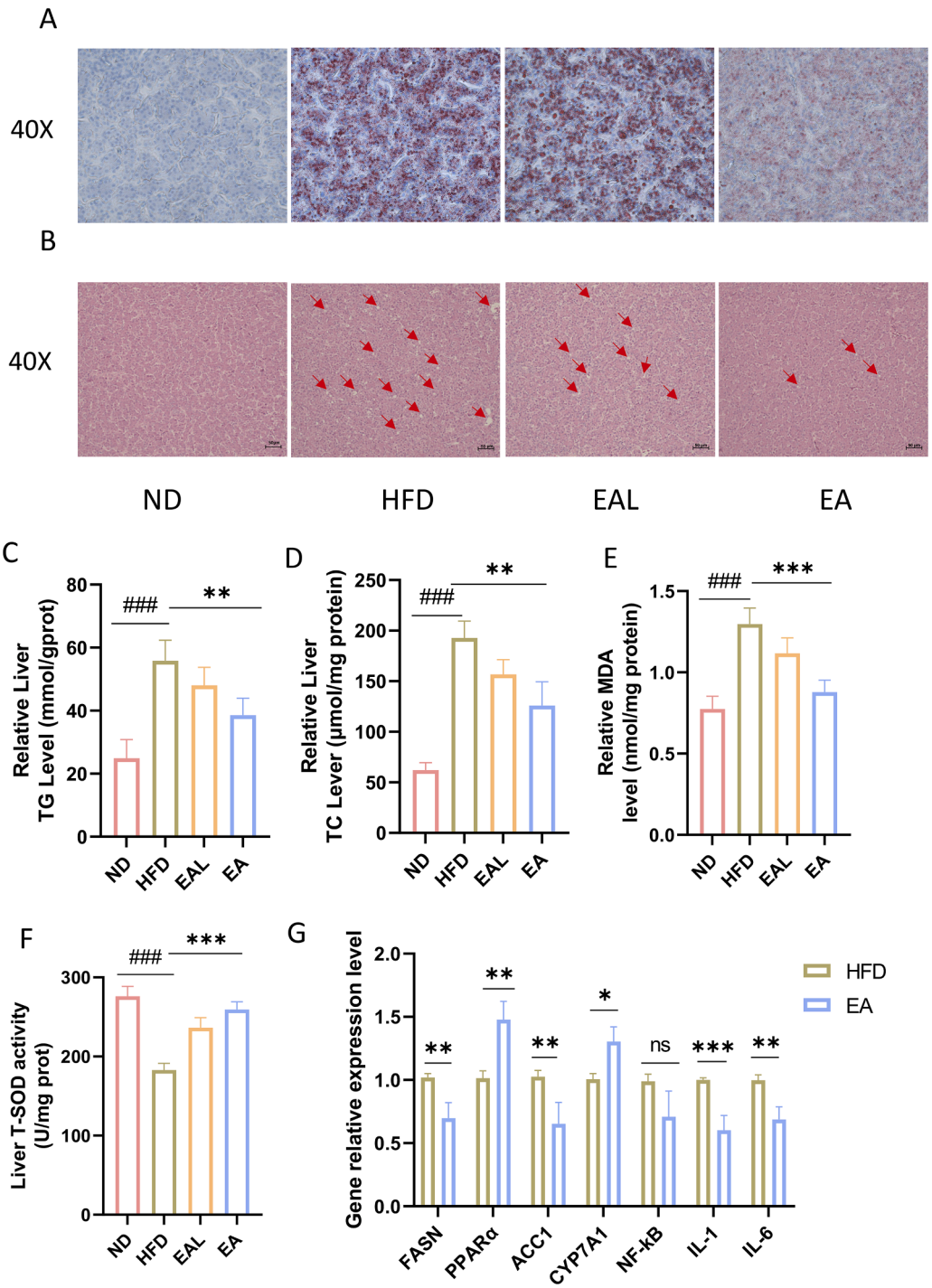

Fig. 3. Effects of EA supplementation on hepatic lipid metabolism and antioxidant stress in broilers. (A) hematoxylin and eosin (H&E) staining of liver tissue (scale bar = 100 μm). (B) Oil Red O staining of liver tissue. (C) Liver triglyceride (TG) level. (D) Liver total cholesterol (TC) level. (E)Liver Malondialdehyde (MDA) level. (F) Liver Total Superoxide Dismutase (T-SOD). (G) Quantitative real-time polymerase chain reaction (qPCR) assay. $n = 10$. Data are means ± SEM. A mark indicates a significant difference between the four groups ($^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = no significance).

Further analysis of genes related to the PPAR signaling pathway and steroid hormone biosynthesis (Fig. 6F) showed that FABP2, FABP4, and HSD17B1 were significantly downregulated in the EA group, whereas *CYP7A1, CYP3A5, CYP7B1*, and *CYP2D6* were significantly upregulated. These differences in gene expression were associated with bile acid metabolism.

Correlation analysis between the multi-omics data

To further elucidate the effects and underlying regulatory mechanisms of EA treatment on fat deposition and fatty liver disease in broiler chickens, Pearson correlation analysis was conducted on the cecal microbiota, DEMs, and DEGs. The results indicated that EA treatment was significantly correlated with both DEMs and liver DEGs. Specifically, the lipid transport-related genes *FABP2* and *FABP4* were negatively correlated with metabolites such as 6-phosphogluconic acid and

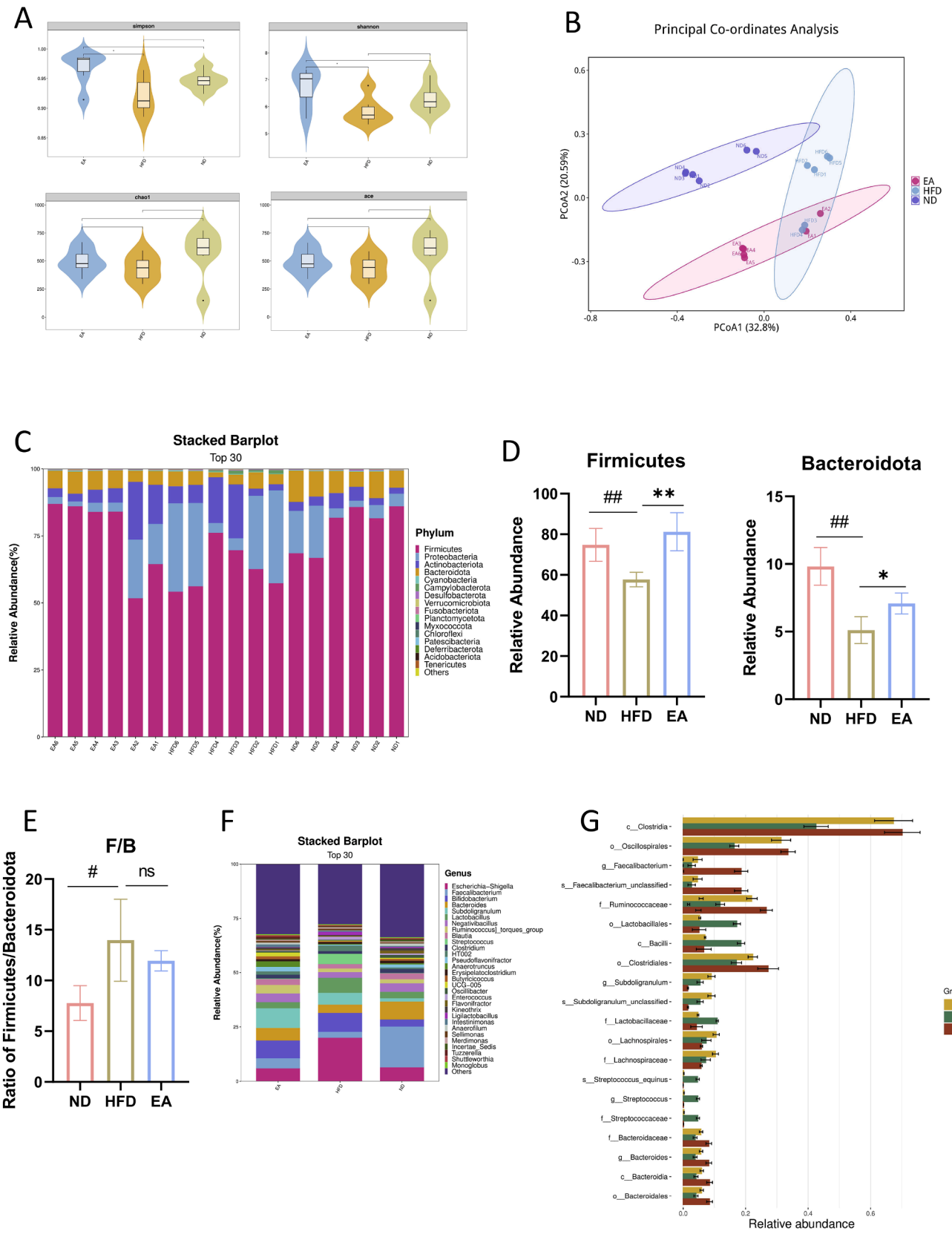

Fig. 4. Impact of EA on the intestinal microbiota in broiler chickens induced by an HFD. (A) Alpha diversity analysis of the microbiota (Simpson, Shannon, chao1, ace); (B) Beta diversity analysis of the microbiota (PCA analysis), Each point represents one sample; (C) Microbiota composition analysis at the phylum level; (D) Relative abundance of Firmicutes and Bacteroidetes; (E) The ratio of Firmicutes to Bacteroidetes. (F) Microbiota composition analysis at the genus level. (G) LEfSe analysis (Relative abundance). $n = 6$. Data are means ± SEM. An asterisk indicates a significant difference between the four groups ($^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = no significance).

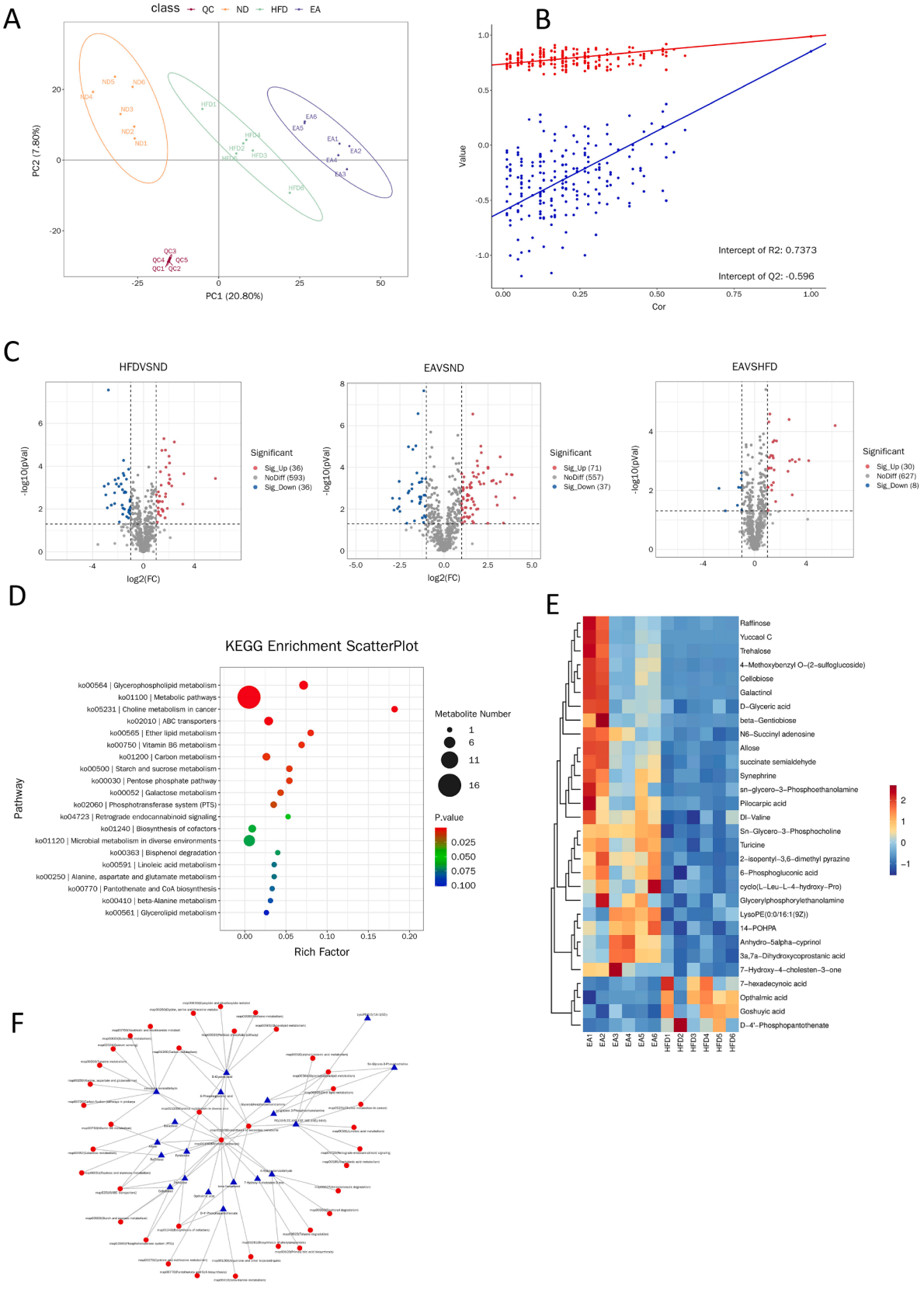

Fig. 5. Effects of EA supplementation on liver metabolites. (A) PCA analysis of the liver metabolites. (B) Permutation testing model between metabolites and experimental treatment. (C) Distribution of the metabolites in different groups. (D) KEGG analysis of the differential metabolites. (E) Cluster analysis of the differential metabolites in EA and HFD group. (F) The interaction between DEMs and signaling pathway. $n = 6$.

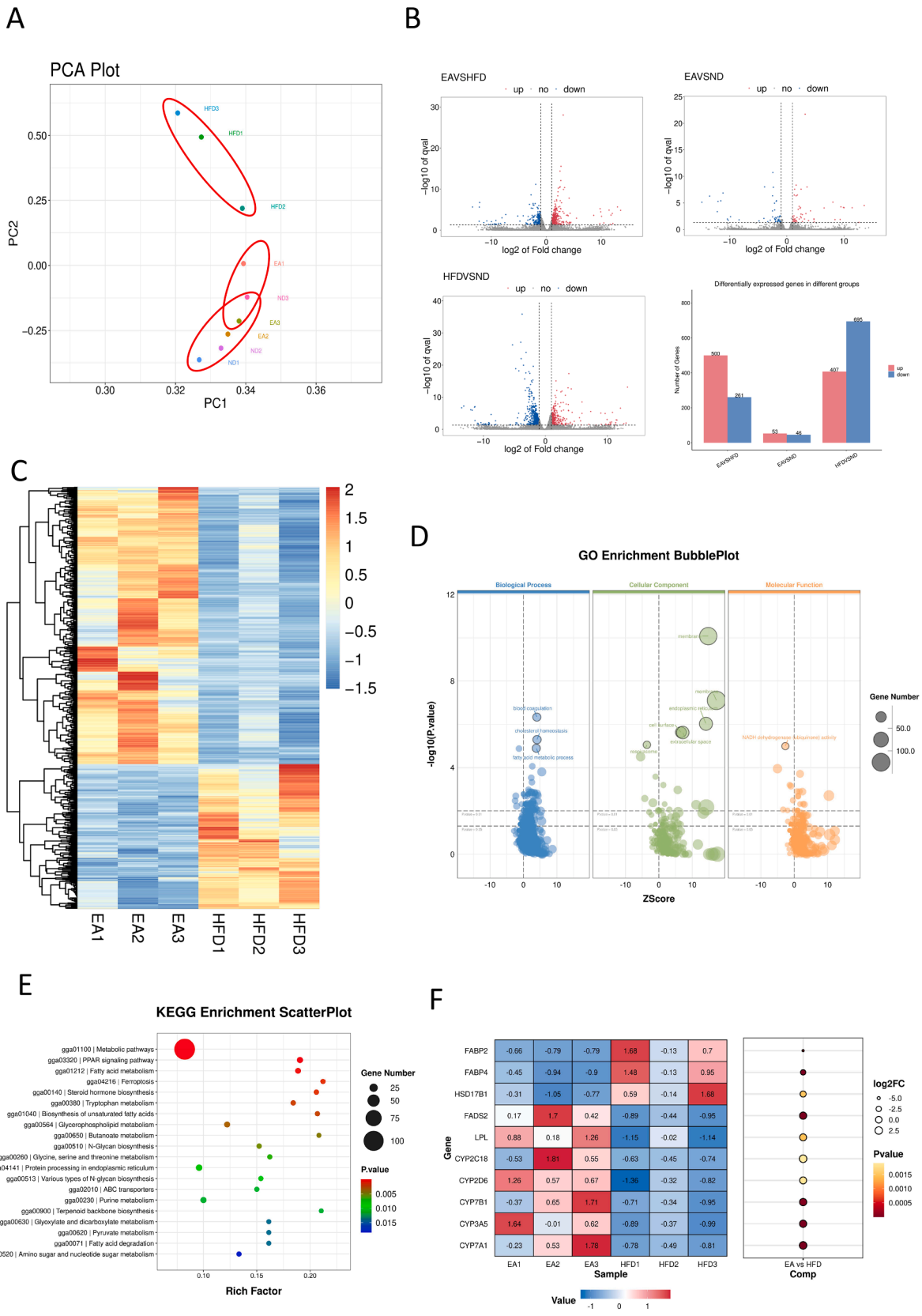

Fig. 6. Impact of EA on liver gene expression patterns. (A) PCA analysis of the samples in ND, HFD, and EA group. (B) Volcanic and bar charts distinguish and display the DEGs. (C) Cluster analysis of the DEGs in EA and HFD group. (D) GO analysis of the DEGs. (E) KEGG analysis of the DEGs. (F) heatmap on DEGs in PPAR and fatty acid metabolism. $n = 3$.

sn-glycero-3-phosphoethanolamine, and positively correlated with D-4-phosphopantothenate and opthalmic acid. Additionally, the cholesterol metabolism-related genes *CYP2C18* and *CYP2D6* were positively correlated with metabolites like succinate semialdehyde, Synephrine, and 6-Phosphogluconic acid and negatively correlated with D-4-Phosphopantothenate and Ophthalmic acid. We also found significant correlations between metabolites and the microbiota. Specifically, 6-Phosphogluconic acid and sn-glycero-3-Phosphoethanolamine were positively correlations with microorganisms such as g__Colidextribacter, and negatively correlations with g__Salmonella and g__Ligilactobacillus. D-4′-Phosphopantothenate and Opthalmic acid were positively correlated with g__Anaerotruncus and g__Christensenellaceae_R-7_group, and negatively correlations with g__Ligilactobacillus (Fig. 7). These analyses suggest that the gut microbiota contributes to liver lipid metabolism, with metabolites being regulated by specific genes.

Discussion

In this study, we found that EA significantly inhibited lipid and cholesterol accumulation in LMH cells in a dose-dependent manner. EA supplementation effectively reduces the abdominal fat deposition and alleviated fatty liver disease caused by an HFD, and decreases liver and serum TG, AST, and ALT levels. These are biomarkers related to NAFLD. 16S rDNA analysis revealed that EA altered gut microbiota abundance and diversity. Additionally, metabolomics analysis also revealed that the DEMs were mainly involved in lipid and steroid metabolism.

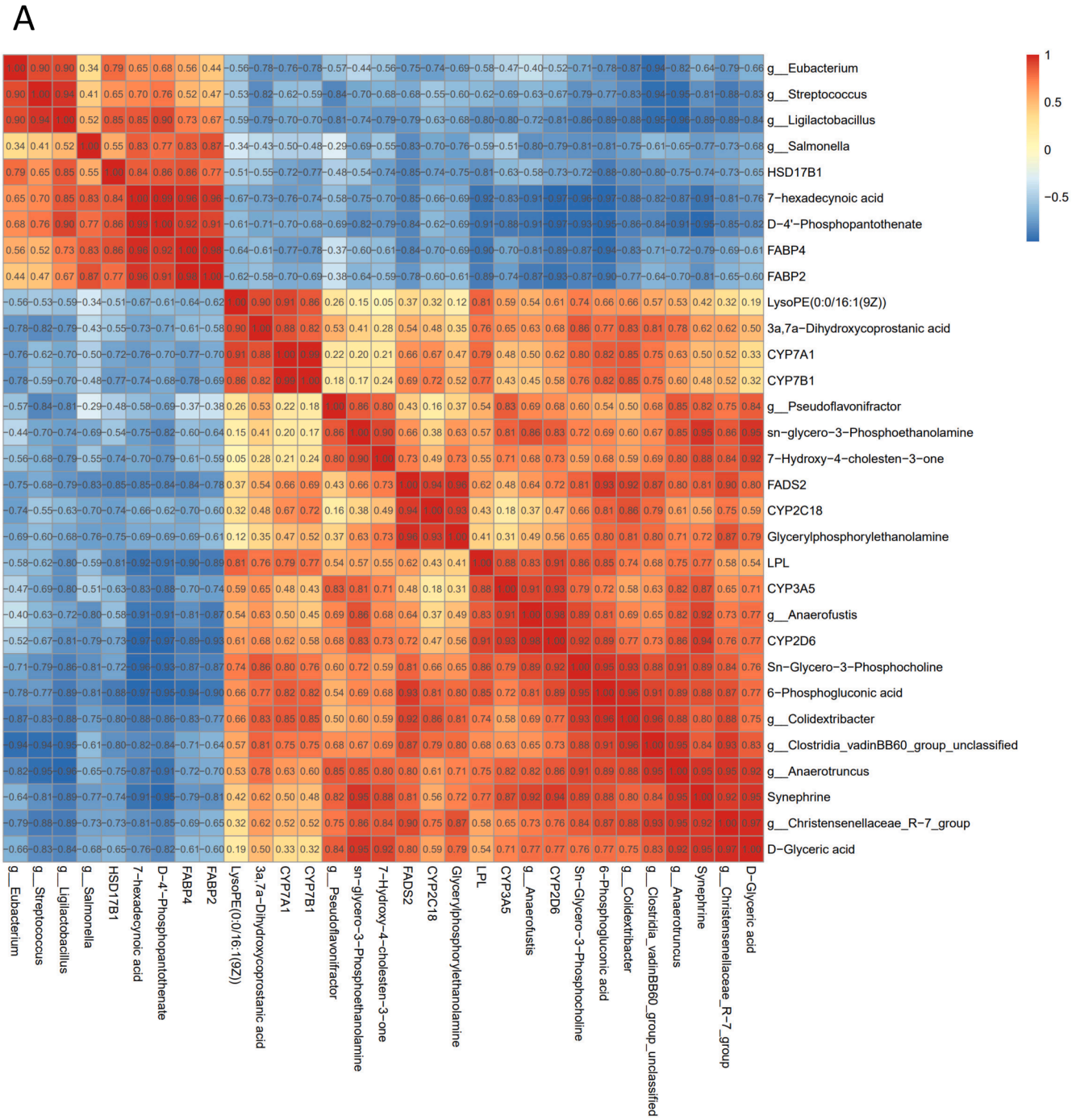

Fig. 7. Correlation analysis amongst microbiome metabolome, and transcriptome. (A) Correlation analysis amongst DEMs, metabolome and DEGs.

Transcriptomic analysis revealed that the DEGs are mainly enriched in signaling pathways related to PPAR, fatty acid metabolism, and steroid metabolism. Correlation analysis revealed that there was a correlation between DEMs, microbiota, and DEGs. These results suggest that EA may reduce abdominal fat deposition and improve fatty liver disease in broiler chickens by altering gut microbiota balance and regulating the steroid signaling pathway.

In poultry farming, an HFD increases the G/F ratio of chickens and further promotes fat deposition (Wang et al., 2024). Fat deposition in chickens is mainly manifested in abdominal fat deposition, which reduces the slaughter rate and affects the development of poultry industry (Zawacka et al., 2017). Our results revealed that, compared with the ND group, the HFD group presented increased abdominal fat deposition and the F/B ratios in chickens. EA reduced the BW and abdominal fat deposition of broiler chickens, improving the slaughter ratio, but had no effect on meat quality. H&E staining confirmed that EA reduces adipocyte size, inhibiting fat deposition. Our previous research revealed that EA supplementation effectively alleviates HFD-induced fat accumulation in mice (Xiao et al., 2023). These findings indicate that EA can inhibit fat deposition.

The liver is central to lipid metabolism, and prolonged fat accumulation can cause excessive TG synthesis and fatty liver disease (Shihana et al., 2023; Yang et al., 2023b). The accumulation of fat in liver cells can trigger lipid peroxidation, generating a large amount of ROS, leading to oxidative stress and liver damage (Li et al., 2021). Fatty liver is a common problem in broiler and egg chicken farming (Liu et al., 2022; Yuan et al., 2023). Our previous research revealed that supplementation with 100 mg/kg EA can effectively alleviates HFD-induced steatohepatitis in mice (Xiao et al., 2023). This study showed that EA effectively reduces HFD-induced liver fat deposition in broiler chickens. Elevated liver cholesterol levels are indicators of hepatitis and are characteristic of fatty hepatitis. Our results indicate that EA significantly inhibits both liver fat deposition and cholesterol levels. Oil Red O staining and H&E staining further confirmed that EA can reduce liver fat deposition and improve fatty hepatitis. The peroxisome proliferator activated receptor (PPAR) signaling pathway plays a critical role in various physiological processes, including lipid metabolism, energy balance, the inflammatory response, and cell differentiation (Yang et al., 2022; Li et al., 2023a). Specifically, PPARα activation promotes fatty acid oxidation in the liver and other tissues, thereby reducing fat accumulation (Li et al., 2020; Zhong et al., 2023). Transcriptomic analysis of liver tissue revealed that EA altered gene expression levels in vivo. These genes are mainly involved in metabolic pathways, as well as the PPAR signaling pathway, sterol homeostasis signaling pathway, fatty acid metabolism pathway, and fatty acid degradation pathway. These genes mainly include *FABP2, FABP4*, and steroid-related genes such as *CYP7A1, CYP3A5, CYP7B1, CYP2D6,* and *CYP2C18*. Research shows that *FABP2* is mainly expressed in the intestine and kidneys, transporting fatty acids from the extracellular space to the intracellular space and is often associated with intestinal inflammation (Gregor et al., 2021). Our study demonstrated that *FABP2*, a key transporter of fatty acids, was significantly downregulated after EA treatment. *FABP4* is expressed mainly in the liver. Studies have shown that *FABP4* expression is associated with obesity, insulin resistance, and metabolic syndrome, and affects the development of these diseases by regulating inflammatory responses and lipid metabolism (Lan et al., 2011; Chung et al., 2021). Additionally, the genes in the CYP family are involved mainly in bile acid metabolism, which is a product of cholesterol breakdown (Rizzolo et al., 2021; Kouno et al., 2022; Kuang et al., 2023). In our study, Biochemical indicators in liver tissue revealed that EA significantly reduced liver cholesterol content. These results suggest that EA may promote the breakdown of cholesterol into bile acids and transport out of the liver, alleviating fatty liver disease. Metabolomic analysis further revealed that the DEMs were identified where EA treatment was mainly related to lipid metabolism and sterol metabolism, with key metabolites including Sn-Gycero-3-Phosphatoline, 3a, 7a-Dihydroxycoptanic acid, synephrine, and glycerylphosphorylethanolamine.

Emerging evidence highlights the crucial role of the gut microbiota in fatty liver disease (Behary et al., 2021; Quesada-Vazquez et al., 2023). Fatty liver can lead to dysbiosis of the gut microbiota, typically characterized by decrease species diversity and a change in richness. Our results revealed that the proportion of Bacteroidetes in the gut microbiota of patients with fatty liver decreased, and the proportion of Firmicutes increased, promoting fat accumulation. Moreover, the gut microbiota has the capacity to regulate the production of SCFAs, increase SCFAs production, improve lipid metabolism and the inflammatory response, and prevent the occurrence of fatty liver (Xiao et al., 2022b; Song et al., 2023). Additionally, there is a correlation between the gut microbiota and intestinal inflammation (Zhang et al., 2021). In clinical practice, fecal microbiota transplantation has proven to be effective in improving the gut microbiota composition, reducing the production of endotoxin, and alleviating fatty liver disease (Zhang et al., 2021; Xue et al., 2022). Our results indicate that, compared to the ND group, the HFD reduced the abundance of Bacteroidetes and increased the abundance of Firmicutes, whereas EA supplementation reversed these changes, restoring the gut microbiota balance and reducing the F/B ratio. The Elife analysis revealed that supplementation with EA increased the abundance of microorganisms such as Faecalibacterium, Bacteroides, and Clostridium, which are key producers of SCFAs in the body. Moreover, EA also increased the abundance of Bifidobacterium. Studies have shown that Bifidobacterium helps decompose dietary fiber and produces SCFAs (Li et al., 2023b). Further joint multi-omics analysis indicated a correlation between these differential microbial communities and DEMs. These results found that EA might improve abdominal fat deposition and MAFLD via regulating steroid metabolism and modulating the gut microbiota.

Although this study combined multiple omics analyses to investigate the effects and mechanism of EA in alleviating HFD-induced abdominal fat deposition and fatty liver disease in broilers, there are several limitations. First, whereas the experimental period we selected was crucial for fat deposition in broiler chickens, a longer duration would be more appropriate for exploring the effects of small molecules on fat deposition. Second, our study focused on K90 broiler chickens and did not investigate the effect of EA on fat deposition in other broiler chickens. Additionally, the sample size in this study was small, and future studies should increase the number of repetitions for each group. Therefore, further studies involving multiple broiler breeds are necessary to better explore the effect of EA on fat accumulation and to provide a more solid foundation for its potential application in the poultry industry.

Conclusion

In this study, EA effectively inhibited TG and TC levels in LMH cells induced by OA/PA and reduced HFD-induced abdominal fat deposition and NAFLD in broiler chickens. Correlation analysis revealed that EA might promote the conversion of cholesterol into bile acids by regulating the expression of CYP family genes, thereby alleviating oxidative stress caused by fat accumulation. Moreover, EA may enhance the absorption and transformation of metabolites by improving the abundance of the gut microbiota, further inhibiting fat deposition in the body. This study provides new insights into improving fat accumulation and MAFLD in broiler chickens, and EA can be considered a natural, nontoxic food and therapeutic agent for preventing and alleviating fat accumulation and fatty liver disease.

Funding

This study was supported by grants from the Natural Science Foundation of Guangxi (Grant No. AD23026095) and the National Natural Science Foundation of China (Grant No. 32160171).

Availability for data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Lianggui Xiao: Data curation, Formal analysis, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Jiazhe Liu:** Methodology, Writing – original draft. **Liangshan Qin:** Methodology, Writing – original draft. **Shan Deng:** Methodology, Writing – original draft. **Guodong Mo:** Methodology, Writing – original draft. **Dandan Zhang:** Methodology, Writing – original draft. **Ben Huang:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors have declared that no competing interest exists.

Acknowledgements

Not applicable.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.104981.

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