DOI: 10.26599/FSHW.2026.9251186

Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: https://www.sciopen.com/journal/2097-0765

China Food Publishing Co.

Sexual Dimorphism in the Regulation of AMPK/PGC-1α/ERRα and gut microbiota by blueberry leaf polyphenols in the high-fat diet induced obesity mice

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ABSTRACT: Blueberry leaf polyphenols (BLP) have shown therapeutic potential against obesity-related metabolic syndromes. However, the sexually dimorphic mechanisms underlying these effects remain underexplored. Our study demonstrates significant sex-dependent effects of BLP in ameliorating HFD-induced obesity in mice. Specifically, BLP administration resulted in a more pronounced reduction in body weight and hepatic steatosis in male mice, while it induced a more substantial decrease in subcutaneous white adipose tissue mass in females. Integrated network pharmacology and molecular validation further demonstrated that the anti-obesity effect of BLP was predominantly mediated through activation of the AMPK/PGC-1α/ERRα pathway to enhance fatty acid oxidation in male mice. Conversely, in female mice, the effect was primarily achieved through suppression of the PPAR-γ/SREBP-1/FAS pathway, thereby reducing lipid synthesis. Integrated multi-omics analysis revealed that in obese male mice, BLP intervention significantly enriched beneficial gut microbes such as *Akkermansiaceae* and *Oscillibacter*, which subsequently elevated levels of metabolites including palmitic acid, linoelaidyl carnitine, and LysoPC (18:0). These changes collectively promoted fatty acid oxidation, accelerated hepatic lipid consumption, and ameliorated lipid accumulation-induced liver injury and hepatomegaly. In contrast, that in obese female mice, BLP intervention significantly increased abundances of Lactobacillus, Bifidobacterium, and Enterorhabdus, along with elevated expression of arachidonic acid, FAD, and LysoPC (16:0). This microbial and metabolic profile ultimately suppressed lipogenesis, significantly reduced adipocyte size, and effectively attenuated HFD-induced subcutaneous white adipose tissue accumulation. These multi-level interactions collectively drive hepatic lipid clearance in males and adipose remodeling in females, establishing a "microbiome-metabolite-signaling-host" axis that underpins BLP's sex-specific therapeutic efficacy against obesity.

Keywords: Blueberry Leaf Polyphenols, Network Pharmacology, Gut Microbiota, Metabolomics, AMPK/PGC-1α/ERRα pathway, Gender Differences.

1. Introduction

Blueberry (*Vaccinium.spp.*) has gained global popularity for its flavorful fruits rich in anthocyanins[1]. The chemical composition and health effects of blueberry fruits have been extensively studied[2]. However,

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Received 27 August 2025
Received in revised from 21 October 2025
Accepted 11 February 2026

blueberry leaves are often neglected as agricultural and sideline products. In reality, blueberry leaves contain significantly higher polyphenol content compared to the fruits. These leaf extracts are enriched with bioactive compounds such as Luteolin, Kaempferol, Quercetin and its glycoside derivatives, which exhibit potent anti-oxidant, anti-diabetic, and anti-obesity properties[3]. Furthermore, blueberry leaves have a history of consumption as herbal tea in regions such as Japan and Europe. These attributes position them as promising candidates for dietary supplements aimed at prevention and treatment of metabolic syndrome-related disorders[4].

Obesity, a hallmark of metabolic syndrome characterized by excessive lipid accumulation, is a primary risk factor for nonalcoholic fatty liver disease (NAFLD) and type 2 diabetes[5]. Currently, regulating obesity-related signaling molecules represents a primary strategy in anti-obesity research. AMPK (AMP-activated protein kinase) serves as a central regulator of cellular energy homeostasis[6]. On the one hand, AMPK phosphorylation inhibits the expression of PPAR-γ and SREBP-1, thereby inhibiting fatty acid synthase (FAS) at the transcriptional level, suppressing lipid synthesis, and reducing fat storage. On the other hand, AMPK phosphorylation activates the PGC-1α pathway to enhance energy metabolism and improve fatty acid consumption efficiency, thereby contributing to its anti-obesity effects[7,8]. PGC-1α, a key transcriptional coactivator, plays a pivotal role in regulating mitochondrial biogenesis and energy metabolism. It interacts with multiple transcription factors to promote mitochondrial generation and bolster cellular antioxidant capacity, thereby comprehensively elevating energy metabolic levels[9]. Notably, PGC-1α acts as a critical coactivator for ERRα, and the complex they form synergistically enhances the expression of genes involved in fatty acid oxidation, thus facilitating energy production and increasing the body's efficiency in fat utilization[9]. For instance, Daniela D.'s research demonstrated that anthocyanins in blueberry extracts can enhance the expression levels of enzymes involved in glycogen decomposition and lipolysis by activating the AMPK signaling pathway, thereby effectively alleviating hyperlipidemia[10].

Furthermore, gut microbiota has emerged as a crucial focus in obesity research. Previous studies indicate that dietary polyphenols can enrich beneficial gut microbial populations, thereby conferring health benefits to the host such as weight loss, improved glycemic control, and chemopreventive effects[11]. Specifically, certain beneficial bacteria produce short-chain fatty acids (SCFAs), which activate AMPK and inhibit FAS expression, consequently reducing fat accumulation. For example, blueberry polyphenols can remodel the gut microbiota by enriching genera such as Lachnoclostridium and Roseburia, increasing SCFA production, promoting AMPK expression to enhance energy expenditure, while simultaneously suppressing FAS expression to reduce lipid accumulation, ultimately ameliorating obesity in high-fat diet-fed mice[12].

As research in the field of antiobesity becomes more intensive, some interesting studies showed that the effects of plant polyphenols differ significantly between genders. For example, *Honokiol* were more enriched with *Akkermansia* in males but more enriched with *Allobacuium* in females when treating obese mice by regulating the gut microbiota, thereby leading to gender differences in the anti-obesity effect[13]. In the study of Wankhade, there were also gender differences in the gut microbiota regulation effect of

blueberry polyphenols on obese mice, the results showed that differences in the enrichment of gut microbiota between the sexes may be a key factor contributing to the superiority of male mice over females in functions such as energy metabolism[14]. Although these plant extracts have been shown to be effective in anti-obesity and triggering a significant gender difference effect, the potential sex-specific anti-obesity effects of blueberry leaf polyphenols (BLP) have not been investigated, and existing studies have only demonstrated that this gender difference is closely related to the alteration of gut microbiota. In fact, the reduction in obesity is often the result of a combination of factors. The role of factors other than the gut microbiota in gender differences has not been fully explored, such as responses at the molecular level or alterations in metabolites. These uncharted territories deserve further investigation.

In our study, the blueberry leaves as the source of polyphenols are selected, and the anti-obesity effect and mechanism of BLP are fully investigated. Our results demonstrate that BLP effectively ameliorates HFD-induced obesity in mice and exerts sex-specific anti-obesity effects. Specifically, BLP predominantly attenuated hepatic steatosis in male mice, whereas it primarily reduced white adipose tissue mass in female mice. In order to explore the potential mechanisms, we firstly identified BLP compound components by mass spectrometry and analyzed the relevant pathways by network pharmacology. Subsequently, differences in expression of the relevant AMPK/PGC-1α/ERRα pathways in male and female mice were verified by qPCR and WB. Meanwhile, the differences in the regulation of gut microbiota and metabolic pathway by BLP in male and female mice were also determined by 16S rRNA sequencing and metabolomics. Ultimately, the relationships among molecular mechanisms, gut microbiota and metabolites were determined by Pearson correlation analysis. The results were clearly elucidated the specific mechanisms by which BLP exerts different anti-obesity effects in mice of different sexes, that focus on promoting the energy metabolism in male mice, but inhibiting adipogenesis in female mice. This provides a theoretical basis for constructing refined and personalized interventions for obese groups of different genders, and offers a reference point for natural polyphenol drugs in the design of future clinical practice (Fig. 1).

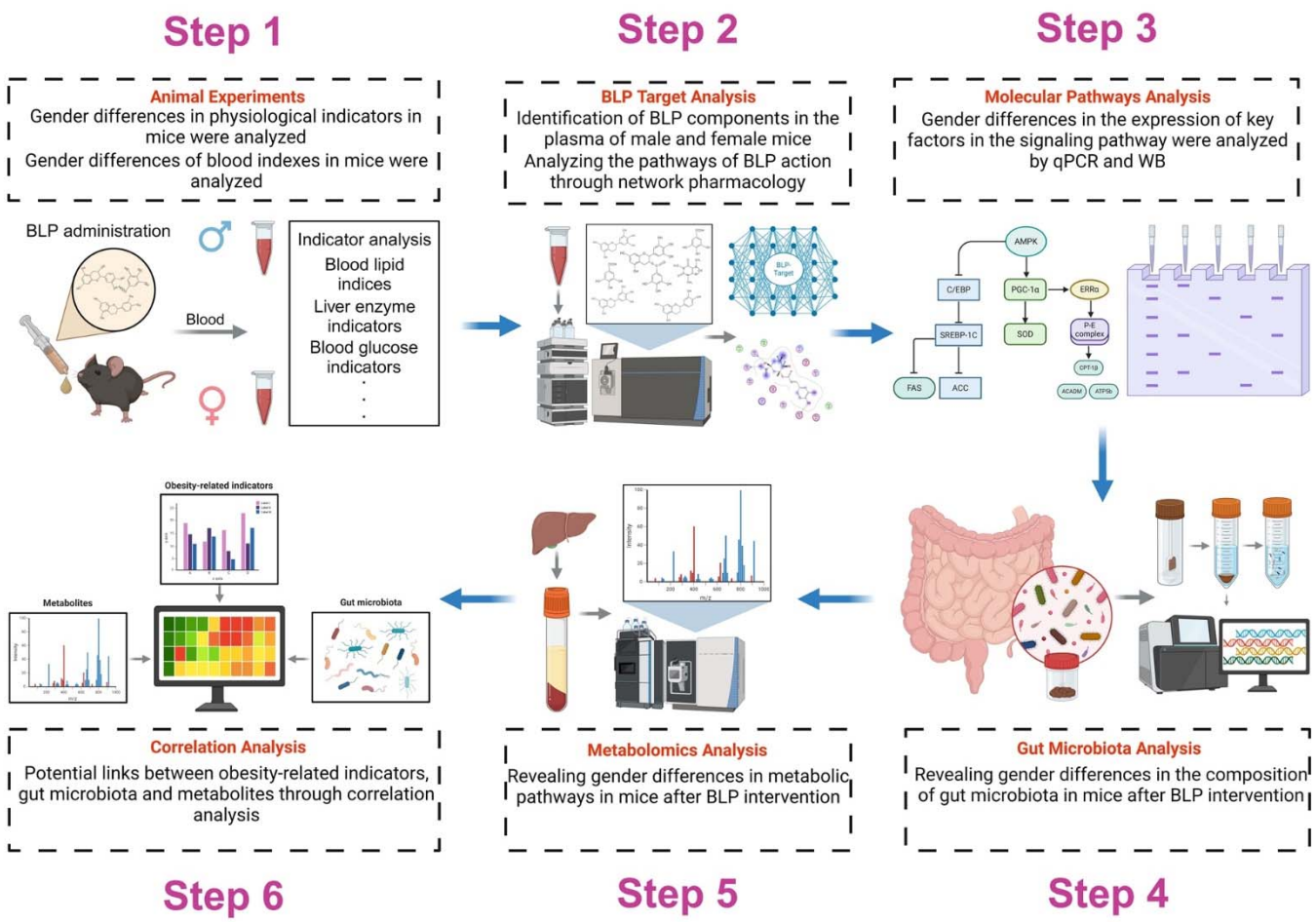

Figure 1. Summary of research lines.

2. Materials and methods

2.1 In vitro experiments of antioxidant capacity and anti-adipose differentiation ability of BLP

In the in vitro study on BLP antioxidant capacity, HepG2 cells stimulated by H_2O_2 had their ROS levels detected via DHE staining per the kit supplier's instructions. The blank control group was untreated cells (negative control), and cells treated with 1 mM H_2O_2 served as the positive control. Experimental group cells were incubated with 1 mM H_2O_2 and BLP (6.25, 12.5, 25 μg/mL) for 2 hours. Fluorescence intensity, indicative of intracellular ROS levels, was quantified using ImageJ V1.8.0.112 at an excitation wavelength of 535 nm.

For the Oil Red O staining, HepG2 cells were incubated with 300 μM PA and BLP (6.25, 12.5, 25 μg/mL). The blank control was untreated cells (negative control), and cells with 300 μM PA alone were the positive control. After 48 hours, cells were rinsed twice with PBS, stained with Oil Red O (50 mg/mL) for 15 minutes, and observed and photographed under an optical microscope.

2.2 In vivo experiment of BLP improving obesity in mice

2.2.1 Experimental animal design

All mice were fed in an animal experimental center (temperature 20-24°C, humidity 40% -45%, light-dark cycle 12 h). 80 mice were randomly divided into eight groups after one week of adaptive feeding

(Male:40,Female:40). Control group, Male (M-BC, n=10) and female (F-BC, n=10) C57BL / 6 mice (6 weeks old) were fed with standard maintenance diet (BC). Male (M-HFD, n=10) and female (F-HFD, n=10) C57BL / 6 mice (6 weeks old) were fed a 60 % fat diet (HFD). Drug group Male (M-BP, n=10) and female (F-BP, n=10) C57BL / 6 mice (6 weeks old) were fed a standard maintenance diet and supplemented with BLP (200mg/kg/day) by gavage. Intervention group Male (M-PIN, n=10) and female (F-PIN, n=10) C57BL / 6 mice (6 weeks old) were fed a 60 % fat diet and supplemented with BLP (200mg/kg/day) by gavage (Fig. 2). The formulation tables for the standard maintenance diet and the 60% high-fat diet are presented in Supplementary Table 1. The dosage of BLP administration was determined according to Jiao et al.'s report, with detailed animal experimental protocols provided in the Supplementary File[15]. All animals and diets were sourced from Zhuhai Baishitong Biotechnology Co., Ltd.

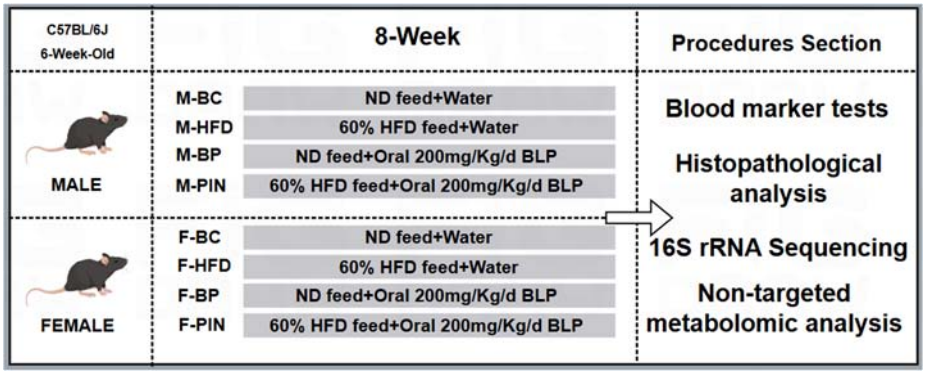

Figure 2. Schematic diagram of animal grouping.

Mice were weighed weekly during the 8-week experimental period. After a 12-hour fast, animals were euthanized under Zoletil 50 anesthesia (0.01 mg/kg). Blood, white adipose tissues (epididymal, subcutaneous, mesenteric, and perirenal), liver, and colon contents, weighed, and stored at -80°C. Portions of liver and adipose tissue were preserved in 4% paraformaldehyde for histological examination and biochemical analysis.

2.2.2 Biochemical Analysis

The blood was centrifuged (3000 rpm, 15 min) to obtain serum, which was used to measure the levels of fasting FBG, TG, TC, LDL-C, HDL-C, MDA, AST and ALT using commercial kits. Meanwhile, the levels of TG, TC, LDL-C, and HDL-C in the liver were also determined.

2.2.3 Histological Analysis

Adipose tissue and liver were subjected to hematoxylin-eosin (HE) and Oil Red O staining. After imaging under an optical microscope, the average size of adipocytes was calculated using ImageJ (image processing) V1.8.0.112 (total area of adipocytes/total number of adipocytes).

2.3 Network pharmacology and molecular docking studies

2.3.1 Screening of active ingredients and targets for BLP

In our previous study, we identified the composition of BLP in mice serum based on the method of Tan et al.(Supplementary Table 2)[16], with potential human targets identified through TCMSP, SwissTargetPrediction, PharmMapper, and PubMed databases (after redundancy removal). Obesity-related

targets were similarly compiled from DisGenet, GeneCards, OMIM, and TTD. Common targets were analyzed via STRING 11.5 (confidence score ≥0.7; Homo sapiens) and visualized using Cytoscape 3.7.2. Pathway enrichment was performed with DAVID 6.7, applying thresholds of $p \leq 0.05$ and Benjamin≤0.05 for KEGG analysis, while GO analysis covered cellular components, molecular functions, and biological processes. Results were visualized as bubble charts and histograms using a bioinformatics platform.

2.3.2 Molecular docking

AMPK structure (PDB:4CFH) was retrieved from Protein Data Bank (https://www.rcsb.org/). Using MOE 2019, BLP compounds were prepared (water removal/hydrogen addition) and docked against 5'-Adenylic acid (positive control). Compounds with lower binding energy than the control were selected for analysis[17].

2.4 Verification of molecular mechanism

2.4.1 qPCR Analyses

Total RNA was extracted from HepG2 cells and liver tissue using Trizol reagent, and reverse transcribed into complementary DNA using Superscript III reverse transcriptase and random primers according to the manufacturer 's instructions. Quantitative analysis of gene expression using Power Up TM SYBR TM Green Master Mix on the ViiATM 7 Real-Time PCR system according to the manufacturer 's recommended standard operating procedures. The data were normalized with GAPDH gene. The primers and reagents used in the study were purchased from Sangon Biotech Co., Ltd (Shanghai China). The primer sequences are shown in Supplementary Table 4.

2.4.2 Protein expression analysis

Western blot was performed to observe the expression of AMPK, pAMPK, PGC-1α and ERRα in the liver. Total protein of liver tissue and colon was extracted with lysis buffer and quantified with the protein concentration assay kit. After denaturation, 20 μg of protein was electrophorized and transferred to nitrocellulose filter membrane. After being sealed with 5% skim milk and incubated with the primary and secondary antibodies, protein bands were detected using a fully automated bioluminescence instrument and the enhanced chemiluminescence detection kit. Image J 1.8.0 software was used to analyze the results.

2.5 Gut Microbiota Analysis

Total genomic DNA of the gut microbiota was extracted from mouse Intestinal contents for 16S rRNA sequencing. Illumina Novaseq 6000 platform was used for sequencing. Trimmomatic 0.33, Cutadapt 1.9.1, USEARCH 10.0 and UCHIME 4.2 were used to splice and filter the original data and obtain high-quality sequences for subsequent analysis. Then, Silva (http://www.arb-silva.de) was used as a reference database to annotate the feature sequences with a Naive Bayes classifier to obtain the species classification information. QIIME 2 2020.6 was used for alpha and beta diversity analysis. Alpha and beta diversity analyses were performed using QIIME2 2020.6. All data result visualizations were plotted in the Genedenovo OmicShare Bioinformatics Cloud Platform (http://www.omicshare.com/tools).

2.6 Metabolomics Analysis

0.2 g of mouse liver tissue was homogenized in saline (1:3, w/v). The homogenate was centrifuged at 14,800 rpm for 10 min (4°C), and 200 μL of supernatant was collected. Protein precipitation was performed by adding 600 μL methanol, followed by vortexing for 2 min and centrifugation under the same conditions. Subsequently, 200 μL of the supernatant was dried under nitrogen at 37°C. The residue was reconstituted in 1 mL methanol, vortexed for 10 min, and centrifuged (14,800 rpm, 10 min, 4°C). The final supernatant was filtered through a 0.22 μm membrane and subjected to metabolomic analysis.

Chromatographic conditions: ACQUITY UPLC BEH C18 column (2.1×50 mm, 1.7 μm) at 40°C with 5°C sample temperature. Mobile phase: 0.1% formic acid (A) and acetonitrile (B) at 0.3 mL/min (2 μL injection). Gradient: 10% B (0-2 min), 10→90% B (2-16 min), 90% B (16-17 min), 90→10% B (17-18 min), 10% B (18-20 min). MS conditions: ESI-MSE in positive/negative modes (m/z 100-1500). Parameters: capillary 3.0 kV, cone 35 V, source 120°C, desolvation 350°C; gas flows: cone 50 L/h, desolvation 600 L/h; collision energy 10-40 eV. Mass spectrometry data processing methods and detailed parameters are provided in the Supplementary Materials.

2.7 Correlation Analysis

Pearson correlation analysis (-1 to 1 scale) assessed data relationships, with $P < 0.05$ as significance threshold. Visualization on OmicShare platform used color gradients (light to dark) to represent coefficient magnitude.

2.8 Statistical Analysis

All statistical analyses were performed using GraphPad Prism 9.0 and R 4.2.1. Significant difference was determined by one-way analysis of variance (ANOVA). A value of $P < 0.05$, or $P < 0.01$, was regarded as significant difference or extremely significant.

3. Result

3. 1 BLP ameliorates oxidative stress and adipose differentiation in HepG2 cells

Fig 3.A.B demonstrates the results of the assessment of the ability of different concentrations of BLP to scavenge ROS and inhibit adipose differentiation in HepG2 cells. As shown in Fig 3.A, C that BLP treatment significantly decreased H_2O_2-induced ROS levels, and the antioxidant effect was dependent on BLP concentration in a dose-dependent manner. Fig 3.B, D showed that BLP was able to effectively reduce the lipid accumulation induced by PA, and this inhibition of lipid differentiation also had a concentration-dependent manner. The above results suggest that BLP has the ability to ameliorate oxidative stress and reduce lipid accumulation in HepG2 cells.

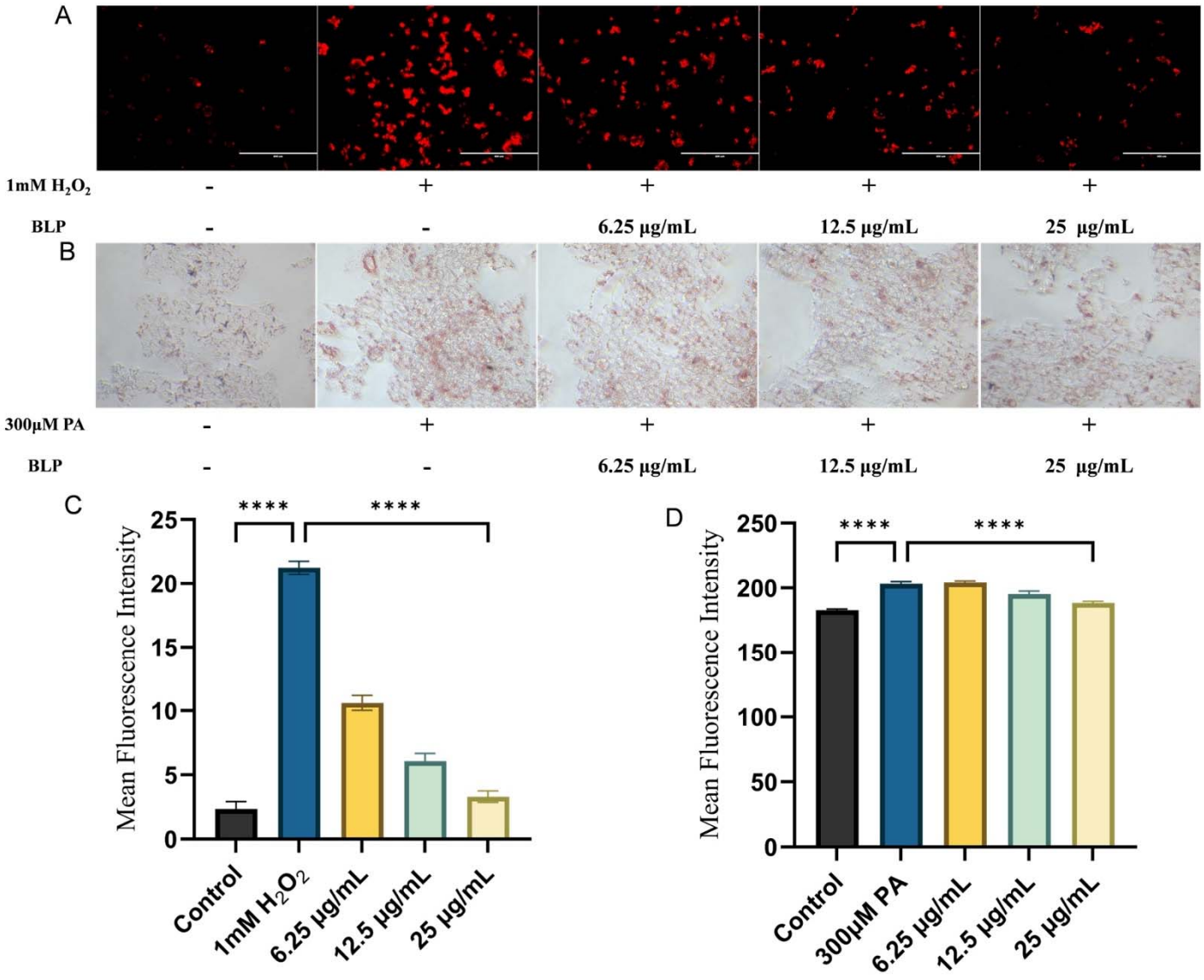

Figure.3. BLP ameliorates cellular oxidative stress status (A) and fat accumulation (B). fluorescence quantification of ROS (C) and quantification of oil red O (D).N=3, $P < 0.05$, "**" $P < 0.01$, "***" $P < 0.001$, "****" $P < 0.0001$.

3.2 BLP ameliorates obesity syndrome in HFD mice and shows different intervention effects in different sexes.

Fig 4. A, B show the food intake curves of mice, revealing no significant differences in dietary intake between groups fed a standard diet and those fed a high-fat diet. Fig 4. C, D demonstrates the weight reduction effect of BLP in HFD-induced male and female mice. The results showed that BLP decreased body weight in both HFD-induced male and female mice, but the weight reduction effect was more significant in male mice. Specifically, the weight growth rate of female mice was reduced by 34.00% (Fig 4.F), while reduced by 43.57% in male mice after BLP intervention (Fig 4.E).

Fig 4.G reveals the effect of BLP on liver fat accumulation in male and female mice. Firstly, BLP significantly reduced HFD-induced liver hypertrophy in male mice. In contrast, in female mice, although HFD did not lead to significant liver hypertrophy, it did trigger a certain degree of fatty liver symptoms, and this symptom that was also ameliorated after BLP intervention. In addition, Fig 4.H-I shows that liver mass was significantly reduced in male mice by BLP intervention ($P < 0.0001$), whereas the mentioned alteration was

non-significant in female mice ($P < 0.05$). HE and Oil Red O staining images showed that BLP effectively reversed liver steatosis induced by HFD in both males and females.

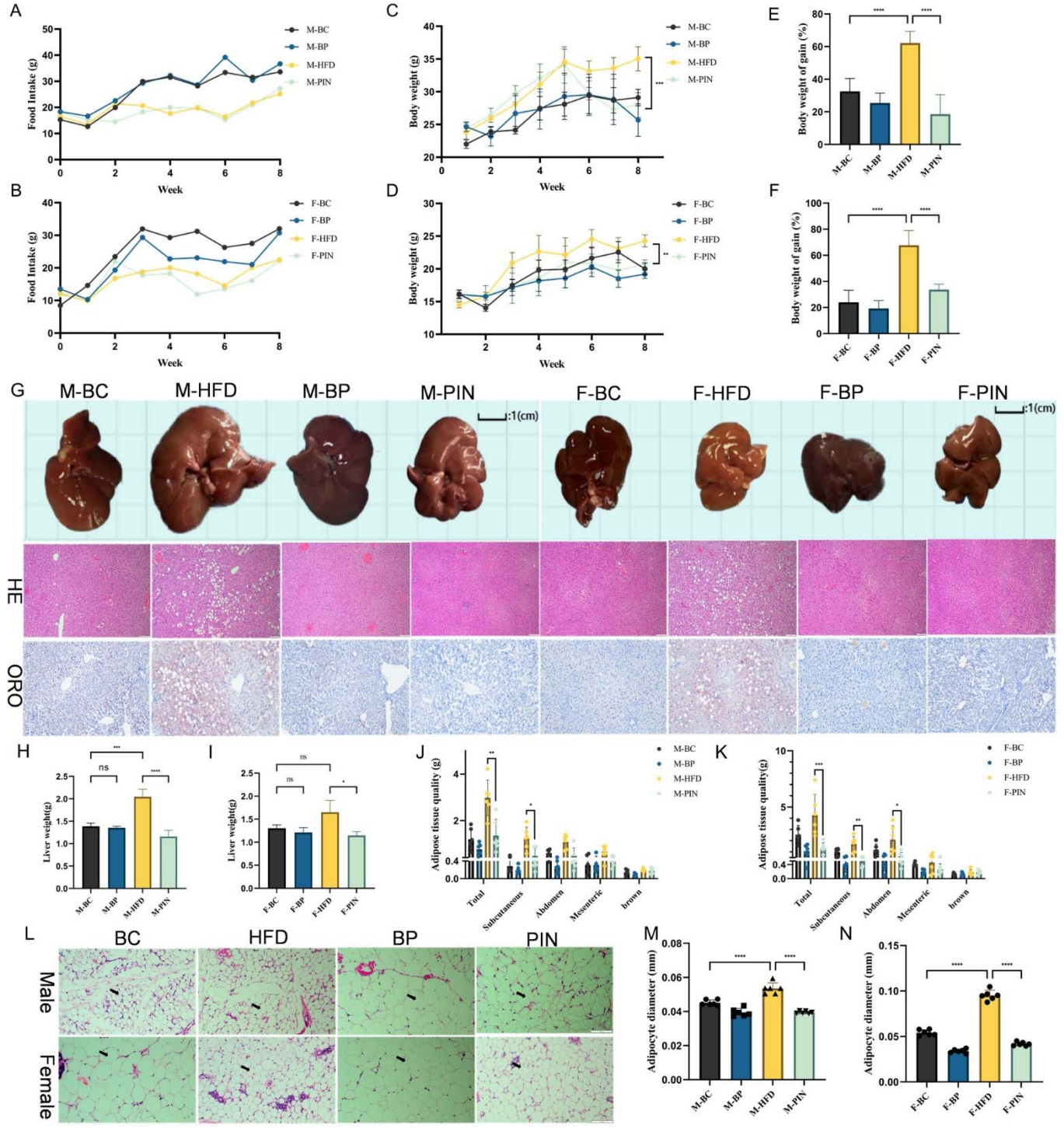

Figure.4. Food intake changes under different conditions in male mice (A) and female mice (B), body weight changes under different conditions in male mice (C) and female mice (D), body weight growth rates under different conditions in male mice (E) and female mice (F), HE staining analysis and oil red O staining analysis of liver in male mice and female mice (G), changes in liver mass under different conditions in male mice (H) and female mice (I), changes in white fat mass in male mice (J) and female mice (K) of different conditions, HE analysis of white fat in male and female mice (L), changes in white fat cell size in male (M) and female mice (N)

Fig 4. J-K demonstrate the effects of BLP on white fat volume and mass in male and female mice. As shown in Fig 4.J-K that BLP intervention reduced total white fat ($P < 0.01$) and subcutaneous white fat mass

($P < 0.05$) in male mice. Whereas, the reduction of total white fat ($P < 0.001$), subcutaneous white fat ($P < 0.01$) and peri-ovarian white fat ($P < 0.05$) mass was more significant in female mice after BLP intervention compared to male mice. In addition, the results of Fig 4.L-N showed that the subcutaneous adipocyte size was reduced by 25.97% in male mice (Fig 4.N) but 55.99% reduction in female mice (Fig 4.M) after BLP intervention.

The above results indicate that there is a significant gender difference in the effect of BLP on liver and white fat mass in male and female mice. The reduction in liver mass was more pronounced in male mice after BLP intervention, while female mice were more prominent in white fat mass reduction.

Next, the blood biochemical indices, such as FBG, MDA, TG, TC, HDL, LDL, ALT, AST, in mice of different sexes after BLP intervention were measured. The results in Fig 5 show that, except for LDL, other HFD-induced blood-related indices in both male and female mice were reduced to some extent after BLP intervention. Interestingly, the results in Fig 5.E-H show that BLP more significantly reduced TC ($P < 0.0001$) and TG ($P < 0.0001$) serum concentrations in male mice compared to TG ($P < 0.001$) and TC ($P < 0.01$) in female mice. Fig 5.M-P also confirmed that BLP was more effective in reducing AST ($P < 0.0001$) and ALT ($P < 0.0001$) serum concentrations in male mice, but had no significant change in female mice. These results suggest that there are also gender differences in blood biochemistry after BLP intervention.

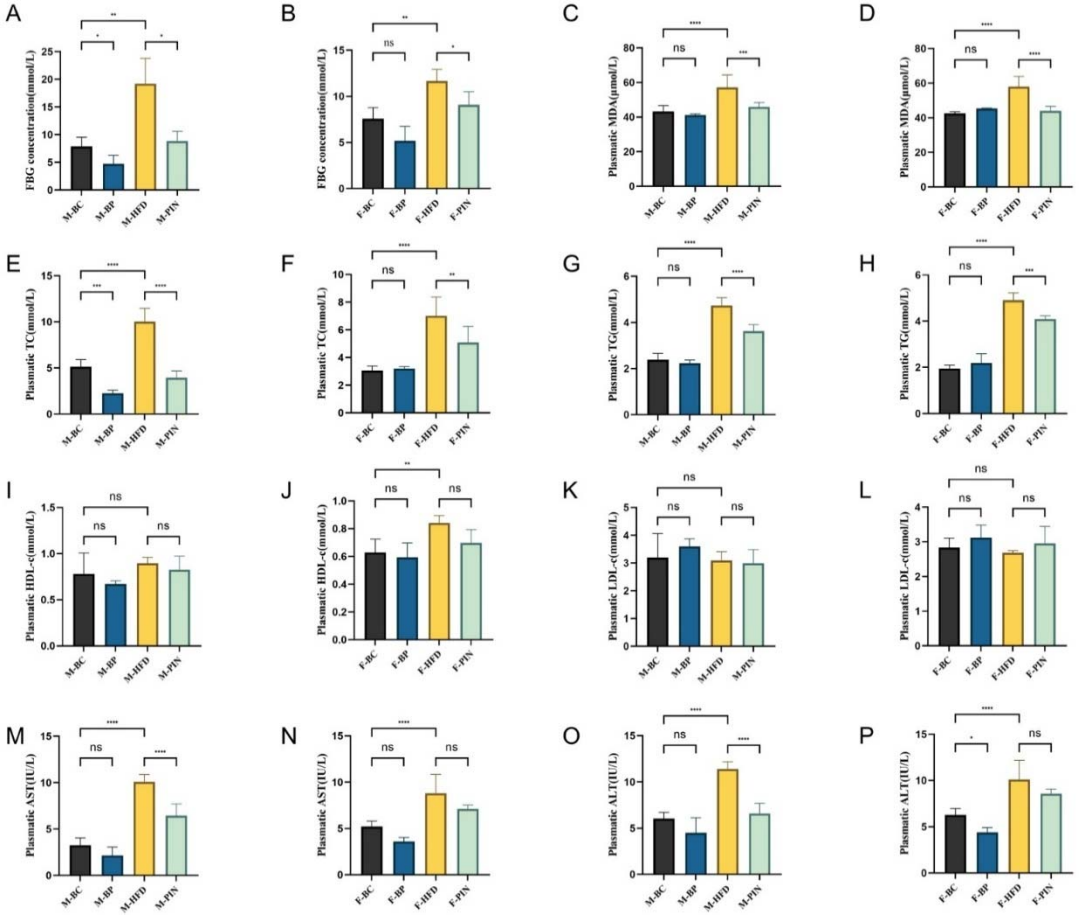

Figure. 5. Comparison of FBG concentration in male (A) and female (B) mice, comparison of MDA concentration in male (C) and female (D) mice, changes in TC concentration in male (E) and female (F) mice, changes in TG concentration in male (G) and female (H) mice, changes in HDL-c concentration in male (I) and female (J) mice, changes in LDL-c concentration in male (K) and female (L) mice. concentration, AST concentration in male (M) and female (N) mice, ALT concentration in male (O) and female (P) mice. N=6 "*"$P < 0.05$, "**"$P < 0.01$, "***"$P < 0.001$, "****" $P < 0.0001$.

As shown in Supplementary Figure 2, analysis of hepatic biochemical indicators (Figures S2 A-D) demonstrated that BLP intervention significantly reduced hepatic total cholesterol (TC, $P < 0.001$) and triglycerides (TG, $P < 0.0001$) in male mice. Although female mice also showed downward trends in liver TG ($P < 0.05$) and TC, the reduction in TC did not reach statistical significance. Figures S2 E-H further showed that BLP treatment significantly increased high-density lipoprotein cholesterol (HDL-c, $P < 0.0001$) while decreasing low-density lipoprotein cholesterol (LDL-c, $P < 0.0001$) in male mice, but induced no significant alterations in females. These results are consistent with our previous blood biochemical findings.

3.3 Identification and network pharmacology analysis of BLP blood components

The results of UPLC/Q-TOF-MS/MS showed that 45 compounds were identified in BLP solution (Supplementary Fig 1.A). There were 24 BLP components in the plasma of male mice, of which 13 were flavonoids and 9 were phenolic acids (Supplementary Fig 1.B). In female mice, there were 29 BLP components in the plasma, of which, 13 were flavonoids and 12 were phenolic acids (Supplementary Fig 1.C). The total number of BLP components in the plasma of male and female mice was 21, and the retention times and mass spectrometry data of the above substances are shown in Supplementary Table 2.

We further enriched potential targets in the plasma of different sexes mice based on the BLP compounds by a network pharmacology approach. The results showed that there were 208 targets in the plasma of male and female mice where BLP compounds acted together on obesity and mitochondrial dysfunction (Fig 6.A). GO enrichment analysis revealed that BLP may combat obesity and related metabolic diseases in mice by activating energy metabolism, lipid metabolism, protein kinase phosphorylation and estrogen receptor signaling (Fig 6.B). To further analyze the target proteins interacting with BLP, we performed protein interaction network analysis (PPI) and network graph analysis (Supplementary Fig. 3) for 208 common targets. As shown in Fig 6.C, the protein targets that BLP may act on are PRKAA1, PRKAA2, PPARGC1A, ESRRA, and ESR1. Among them, PRKAA1 and PRKAA2 are the 2 subunits of AMPK protein, and PPARGC1A and ESRRA represent PGC-1α and ERRα. The PPI results showed that BLP could regulate ERRα by acting on AMPK and exert anti-obesity effects by affecting ERRα-related functions such as energy metabolism and lipid metabolism.

Molecular docking analysis results demonstrate that the binding strength (expressed as S-score) of the main bioactive compounds in BLP to AMPK was lower than that between AMPK and its endogenous ligand 5'-Adenylic Acid (S=-5.372). Specifically, representative compounds such as quercetin (S=-6.233), luteolin-4'-O-glucoside (S=-6.983), granatin A (S=-8.495), and kamphiol-3-O-glucoside amide (S=-7.437) all exhibited stronger binding affinity (Supplementary Table 3). These compounds primarily enhance binding stability by forming hydrogen bond networks with key amino acid residues in the AMPK active pocket(Fig. 6.D-G).

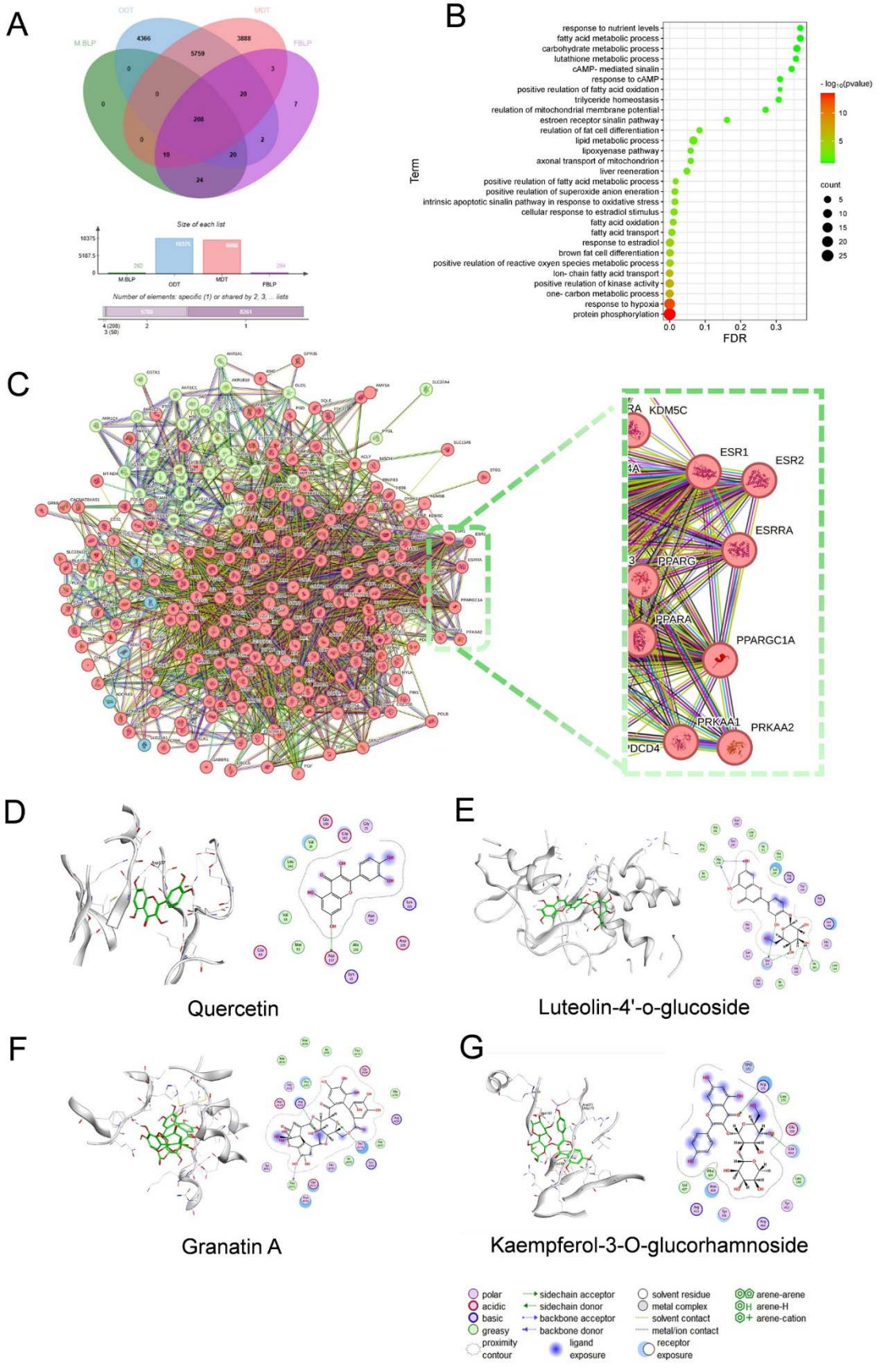

Figure 6. Common target screening of BLP, obesity metabolic syndrome target (ODT) and mitochondrial dysfunction target (MDT) (A); GO pathway enrichment analysis (B and protein interaction network analysis of common targets (C). Note: MBLP is the target set of blood BLP components in male mice and FBLP is the target set of blood BLP components in female mice. Local and two-dimensional images of the active pocket of BLP entry components (Quercetin (D), Luteolin-4'-o-glucoside (E), Granatin A (F), Kaempferol-3-O-glucorhamnoside (G)) upon docking with *AMPK* proteins.

3.5 BLP ameliorates obesity and contributes to gender differences in mice through the AMPK/PGC-1α/ERRα and PPAR-γ/SREBP-1/FAS pathways.

We next explored the expression of AMPK-related genes and proteins through in vitro and in vivo experiments. Firstly, the relevant genes were analyzed in HepG2 cells and the results in Supplementary Fig 4.A-B shows that BLP intervention increased the mRNA levels of AMPK, PGC-1α, ERRα, ACADM, SIRT1, SIRT3, and SOD2. Moreover, BLP also decreased the mRNA levels of FAS in a concentration-dependent manner, suggesting that BLP can promote fatty acid beta oxidation and ROS scavenging, and inhibit fat synthesis through the AMPK/PGC-1α/ERRα pathway. These data preliminarily confirm the above results we obtained through network pharmacology.

To further investigate whether BLP in mice leads to gender differences through the AMPK/PGC-1α/ERRα pathway. The expression of AMPK/PGC-1α/ERRα pathway and downstream related genes and proteins were examined using qPCR and WB. As shown in Fig 7.A-B, after BLP intervention, mRNA levels of SIRT1, SIRT3, and SOD2($P < 0.05$) increased in male and female mice. In addition, inflammation-related genes are also significantly altered in both different sex mice. Specifically, BLP intervention down-regulated the mRNA levels of pro-inflammatory-related genes such as TNF-α, IL-1β, and IL-6, and up-regulated the mRNA levels of anti-inflammatory-related gene IL-10. However, it is worth to note that the mRNA levels of fatty acid β-oxidation, oxidative phosphorylation, and adipose synthesis factors were altered and showed a clear gender difference. Specifically, the mRNA levels of factors related to fatty acid β-oxidation and oxidative phosphorylation, such as AMPK, PGC-1α, ERRα ($P < 0.05$), ACADM, PDK-4, CPT-1β ($P < 0.05$), COX-1, Cycs, Cytp, and ATP5b ($P < 0.05$) were increased in male mice, while there was no significant change in female mice. In addition, BLP intervention in female mice significantly inhibited the mRNA levels of FAS ($P < 0.05$), but showed no change in male mice.

WB analysis further validated gender differences on the expression of the AMPK/PGC-1α/ERRα related protein after BLP intervention (Fig 7.C). In male mice, BLP significantly increased the level of AMPK phosphorylation (Fig 7.D), along with the protein expression of PGC-1α (Fig 7.G) and ERRα (Fig 7.H). In contrast, in female mice, there was no significant change in AMPK phosphorylation level, AMPK, PGC-1α and ERRα protein expression.

In summary, BLP intervention significantly enhanced energy metabolism-related functions in male mice through activation of the AMPK/PGC-1α/ERRα pathway, whereas in female mice, BLP reduced fat accumulation mainly through inhibition of the AMPK/FAS-related fat synthesis pathway. These results suggest that the BLP-mediated AMPK-related pathway is one of the key factors contributing to gender differences in the anti-obesity effects of BLP.

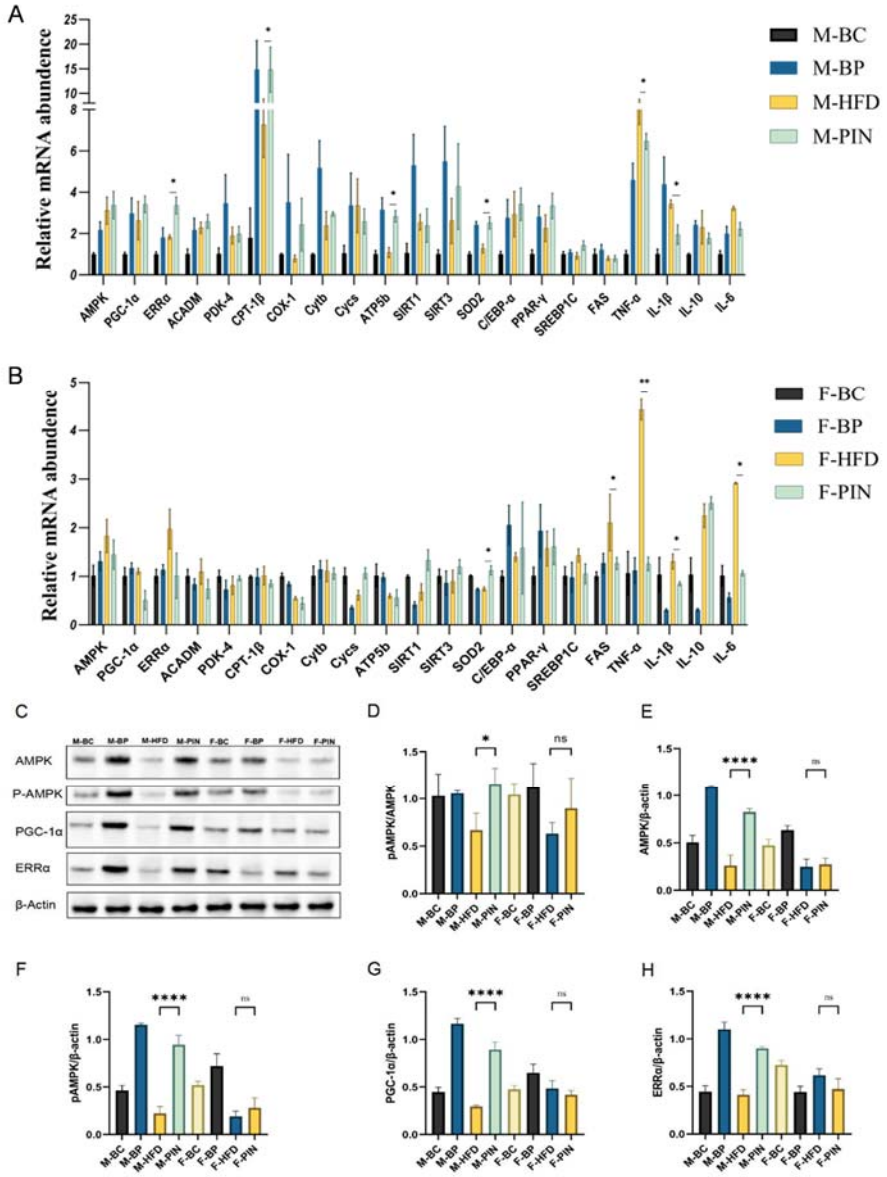

Figure 7: Effects of BLP intervention on expression of related factors in the liver of obese mice, qPCR analyses of gene expression in males (A) and females (B), and WB assessment of AMPK/PGC-1 α/ERRα signaling pathway expression differences in different sexes (C), AMPK phosphorylation flux (D), AMPK (E), pAMPK (F), PGC-1α (G), ERRα (H). N=3 "*" $P < 0.05$, "**" $P < 0.01$, "***" $P < 0.001$, "****" $P < 0.0001$.

3.6 Effect of BLP on the composition of intestinal flora in mice of different sexes.

16S rRNA sequencing was utilized to investigate the composition of the gut microbiota of differently sexed mice after BLP intervention. The results of PCoA and NMDS analyses showed that the samples of each group were well differentiated (Fig 8.A and B). The results of the Chao1 index, the ACE index, the Simpson index, and the Shannon index indicated that BLP altered the species diversity of the gut microbiota, but did not reach a significant difference (Supplementary Fig 5).

The results in Fig 8.C and D show that the abundance of *Bacteroides* was significantly increased in both male and female mice after HFD induction, whereas the number of *Firmicutes* was suppressed, but this phenomenon was reversed after BLP intervention. Interestingly, Fig 8.C showed that BLP intervention significantly increased *Verrucomicrobiota* abundance in male mice, whereas in female mice, the change in *Verrucomicrobiota* abundance was not significant (Fig 8.D).

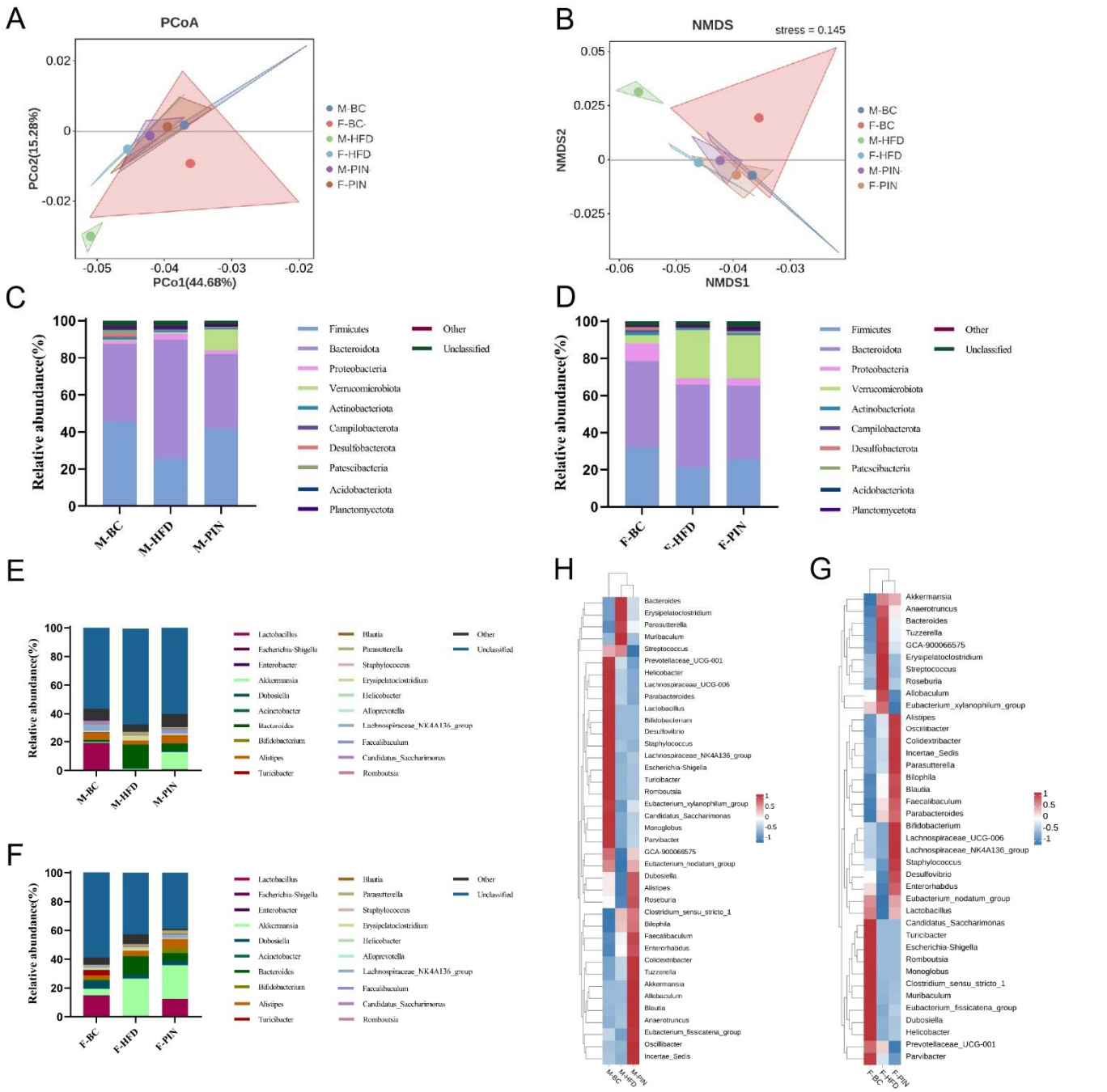

Figure. 8. PCoA analysis (A) and NMDS clustering analysis(B), Phylum-level stacked plots of intestinal flora composition in male (C) and female (D) mice. Genus-level stacked plots of intestinal flora composition in male (E) and female (F) mice. And heatmap of abundance for the top 40 abundance-ranked species at the genus level in male (G) and female (H) mice.

Fig 8.E and F show that the abundance of the top 40 genus-level species fluctuates among male and female mice across groups. Specifically, in male mice, the BLP intervention increased the abundance of 13 beneficial species of *Akkermansiaceae, Faecallbaculum* and *Oscillibacter* (Supplementary Fig 6), while decreasing the abundance of *Bacteroidaceae, Muribaculaceae* and *Erysipelatoclostridium* and other bacterial species in abundance (Fig 8.G). In contrast, in female mice, BLP intervention increased the abundance of 16 strains of *Lactobacillus, Bifidobacterium* and *Enterorhabdus,* (Supplementary Fig 6), while decreasing the abundance of 9 strains of *Bacteroidaceae, Roseburia* and *Dubosiella* (Fig 8.H).

PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) analyzes the possible effects of gut microbiota on metabolic pathways in mice. Fig 9.A-B shows that BLP promotes energy metabolism and carbohydrate metabolism in male mice. In female mice, BLP intervention promoted amino acid metabolism, nucleic acid metabolism, and energy metabolism. We further compared the results of PICRUSt analysis in male and female mice subjected to BLP intervention, which showed that male mice showed more significant expression in bile acid synthesis, short-chain fatty acid metabolism, pantothenic acid and CoA synthesis, citric acid cycle, Fatty acid degradation and oxidative phosphorylation, whereas females showed more significant expression in linoleic acid metabolism and biosynthesis of steroid hormones (Fig. 9.C).

In addition, we determined the indicator species responsible for the differences in the gut microbiota of mice of different sexes by LEfSe (Linear Discriminant Analysis Effect Size) analysis, and we screened for species with LDA values greater than 2 to identify species with significant differences after BLP intervention. Fig 9.D demonstrates that BLP predominantly altered the gut microbiota at the level of 24 different species in male mice, including *Roseburia, Oscillibacter* and *Faecallbaculum*, in female mice, BLP predominantly affected the gut microbiota at the level of 39 different species, including *Lactobacillus, Bifidobacterium* and *Staphylococcus.*

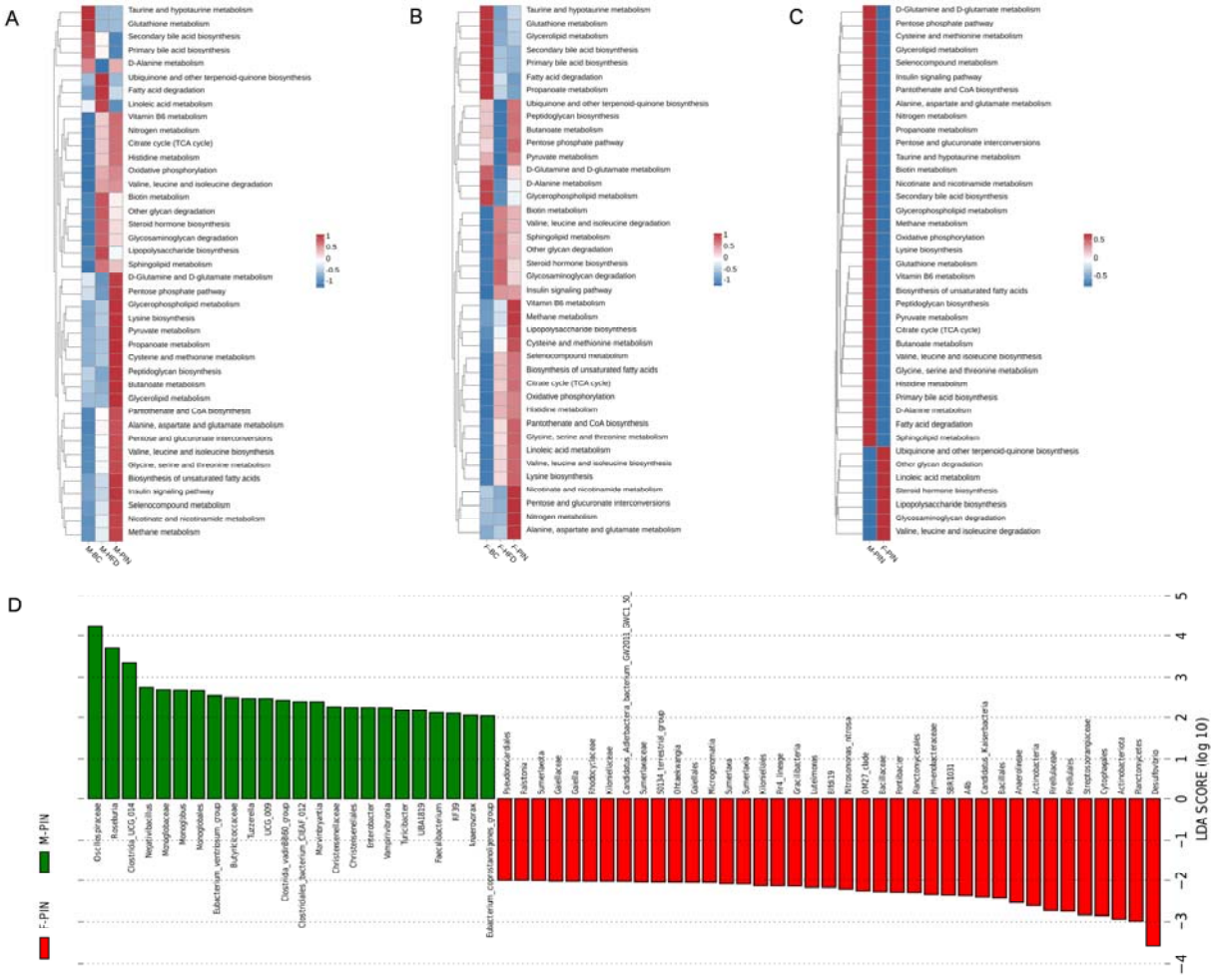

Figure. 9. PICRUSt analysis predicts the function of the gut microbiota in male (A) and female (B) mice after BLP intervention, gender differences between male and female mice in the analysis of PICRUSt function (C), and LEfSe analysis reveals the main species responsible for gender differences in PICRUSt function (D), LDA score≥2，$P<0.05$.

3.7 Effect of BLP on the expression of endogenous metabolites in liver tissues of mice of different sexes and analysis of metabolic pathways.

EZinfo V 3.0 software was used to perform PCA analysis, which resulted in a good clustering of samples in each group (Fig 10.A), and OPSL-DA analysis of each comparison group showed the same results (Supplementary Fig. 7). BLP intervention significantly altered the expression levels of 198 endogenous metabolites in male mice and 226 endogenous metabolites in female mice. The top 40 metabolites with significant abundance differences ($P < 0.05$,VIP > 1.0, fold change > 2) in all cohorts are considered as common differential metabolites. As shown in Fig 10.B, 27 metabolites including Palmitic acid, Linoelaidyl carnitine, and LysoPC (18:0) were significantly increased in expression levels, while Taurocholic acid 3-sulfate, Oxidized glutathione and PE, 13 metabolites were effectively reduced after BLP intervention in male mice. In female mice, 23 metabolites, including LysoPC (16:0), Arachidonic acid and FAD, were significantly increased in expression levels. In contrast, seven metabolites, including Taurocholic acid-3-sulfate, Oxidized glutathione and 1-(13Z,16Z-docosadienoyl)-glycero-3-phosphate, were effectively reduced after BLP intervention (Fig 10.C).

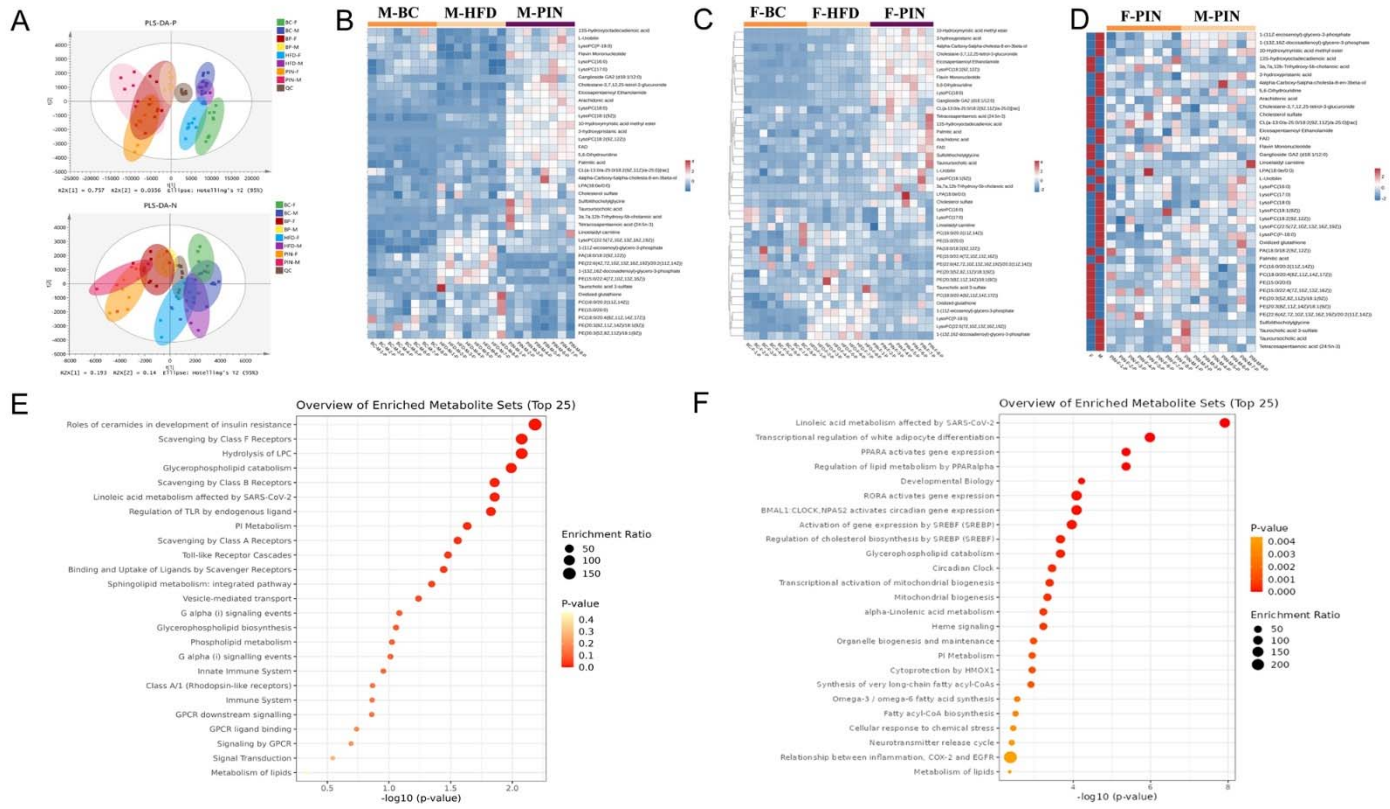

Figure 10. Score plots of PLS-DA supervision of liver metabolites in positive and negative mode (A). Effects of BLP on common differential metabolites in male mice (B) and female mice (C), differential metabolite expression between male and female mice after BLP intervention (D), pathway enrichment bubble plots for male differential metabolites (E) and female differential metabolites (F).

We examined the differences in the expression of these metabolites between male and female mice. The results in Fig 10.D showed that 22 metabolites such as 5,6-Dihydrouridine, Linoelaidyl carnitine and Palmitic acid were significantly expressed in male mice. While the expression of 18 metabolites such as Arachidonic acid, L-Urobilin and Cholesterol was significantly enhanced in female mice after BLP intervention.

Fig 10.E and F show the metabolic pathways for differential metabolite enrichment in mice of different sexes, with enrichment pathways selected on the basis of enrichment ratio >1.5 and $P < 0.05$. The results showed that the differential metabolites in male mice were associated with metabolic pathways such as clearance of F receptor, hydrolysis of LPC and catabolism of glycerophospholipids (Fig 10.E). The metabolite profiles of female mice were associated with metabolic pathways such as linoleic acid anabolism, white fat differentiation and PPAR activator expression (Fig 10.F).

3.8 Correlation analysis of BLP intervention on intestinal flora-metabolites-health indicators in mice of different sexes

Our first step was to use Pearson correlation analysis to examine the possibility of a correlation between gut microbiota and obesity-related indicators. The results showed that in male mice, 8 male-associated beneficial species such as *Faecalibaculum, Akkermansiaceae* and *Oscillibacter* were significant positive correlation with AMPK, PGC-1α, ERRα, ACADM, CPT-1β, COX-1, Cytb, Cycs, ATP5b and SOD2 (Fig 11.A). Fig 11.B shows that in female mice, 4 female beneficial species such as *Lactobacillus, Bifidobacterium and Enterorhabdus* showed significant negative correlation with obesity-related indicators such as ALT, AST, TC, TG, HDL-c, IL-1β, TNF-α, liver mass, fat mass and FAS,while they were positively correlated with antioxidant indices such as SOD2, SIRT1.

Fig 11.C, D shows the results of the correlation analysis of differential metabolites and obesity-related indicators. The results showed that 24 beneficial androgenic differential metabolites including 5,6-Dihydrouridine, Linoelaidyl carnitine and Palmitic acid were significant positively correlated with AMPK, PGC-1α, ERRα, ACADM, CPT-1β, COX-1, Cytb, Cycs, ATP5b and SOD2, whereas they were significant negatively correlated with ALT, AST, TC, TG, HDL-c, IL-1β, TNF-α, liver mass and fat mass (Fig 11.C). Fig 10.D shows that 5 beneficial female differential metabolites such as arachidonic acid, FAD and LysoPC (16:0) were significant negatively correlated with ALT, AST, TC, TG, HDL-c, IL-1β, TNF-α, liver mass and fat mass.

Finally, the relationship between gut microbiota and different metabolites was shown by Pearson analysis. The results showed that 7 differential species such as *Faecalibaculum, Akkermansiaceae* and *Oscillibacter* were significantly and positively correlated with Palmitic acid, Linoelaidyl carnitine,and LysoPC (18:0) in male mice (Fig 11.E). *Lactobacillus, Bifidobacterium and Enterorhabdus* were the 7 differentiating species significantly and positively correlated with Arachidonic acid, LysoPC (16:0), and FAD in female mice (Fig 11.F).

Figure 11. Correlation analysis of gut microbiota with obesity-related indicators in male (A) and female mice (B). Correlation analysis of differential metabolites with obesity-related indicators in male mice (C) and female mice (D). Correlation analysis of differential metabolites with gut microbiota in male mice (E) and female mice (F). "*" $P < 0.05$, "**" $P < 0.01$, "***" $P < 0.001$, "****" $P < 0.0001$.

4. Discussion

Energy metabolism and lipid synthesis are important considerations in studying the anti-obesity effects of plant polyphony[18]. Many studies suggested that the development of obesity leads to excessive accumulation of lipid in cells, increasing the production of reactive oxygen species (ROS) and leading to oxidative damage in mitochondria. Previous studies have proved that activation of AMPK promotes energy metabolism, inhibits lipid synthesis, reduces oxidative stress, and improves mitochondrial function, thereby ameliorating the adverse effects caused by obesity[19]. Specifically, AMPK phosphorylation can inhibit the expression of acetyl-CoA carboxylase (ACC), SREBP1C, and FAS, which reduces lipid synthesis[20]. In addition, AMPK phosphorylation can also increase the expression level of PGC-1α, which is an important mediator in the regulation of mitochondrial biosynthesis and energy metabolism by AMPK, and it binds to a variety of transcription factors and promotes the expression of mitochondrial production and antioxidant enzyme systems, which in turn improves the efficiency of cellular energy metabolism[21]. Importantly, PGC-1α can also bind to ERRα to form a complex that promotes the expression of genes related to fatty acid oxidation and oxidative phosphorylation, which promotes energy production and glucose-lipid metabolism, thereby reducing cellular fat accumulation[22]. Wu et al found that extract from Sechium edule promoted the expression of fatty acid β-oxidation-related factors as CPT-1 by activating the AMPK pathway. Kim et al found that Red Ginseng extract could regulate the expression of AMPK/PGC-1α/ERRα signaling pathway, which in turn promotes the expression of oxidative phosphorylation genes and antioxidant-related genes, and consequently promotes the efficiency of cellular energy metabolism and restores the normal function of mitochondria[23].

In our study, we firstly found that BLP reduced ROS production and fat accumulation in HepG2 cells. Furthermore, in vivo animal experiments revealed that after BLP administration, male mice exhibited a more pronounced reduction in body weight compared to female mice. Specifically, the variation in male mice were mainly characterized by a reduction in liver weight and volume, whereas the changes in female mice were mainly characterized by a significant reduction in white adipose tissue. This demonstrates that BLP not only has antioxidant and anti-obesity effects, but also display gender differences in weight loss effects between male and female mice. Mass spectrometry analysis revealed that mouse plasma contains several BLP compound components, such as Quercetin, Catechin, Luteolin and other active substances that contributed to the antioxidant and anti-obesity activities[24]. In addition, network pharmacology predicts that BLP can exert its anti-obesity effect through the AMPK/PGC-1α/ERRα pathway. In order to investigate the mechanisms responsible for the gender differences of BLP in obesity management, the expression of genes related to anti-inflammatory, antioxidant, energy metabolism and inhibition of fat synthesis between male and female mice were examined. Molecular studies revealed that BLP induced differential expression of the AMPK/PGC-1α/ERRα and PPAR-γ/SREBP-1/FAS pathways between male and female mice, further resulted in gender differences in lipid lowering and weight loss in male and female mice. Specifically, in male mice, BLP promotes the expression of the AMPK/PGC-1α/ERRα pathway in liver tissues, which promotes beta

oxidation of fatty acids and oxidative phosphorylation, depletes fatty acid accumulation within the body, and thus attenuates liver steatosis and oxidative damage. Previous study has proved that ERRα promotes TG hydrolysis in adipocytes by up-regulating the expression of lipolytic enzymes such as Hormone-Sensitive Lipase (HSL) and Adipose Triglyceride Lipase (ATGL), which promotes fat mobilization and increases fatty acid release[25]. And fat mobilization and beta oxidation of fatty acids can effectively reduce TC and TG in blood[26]. In our study, the blood levels of TG and TC were significantly decreased in male obese mice after BLP treatment, and this result further demonstrated that BLP intervention in male mice could promote fat mobilization through up-regulation of the AMPK/PGC-1α/ERRα pathway, which led to a decrease in the blood levels of TG and TC. In addition, the promotion of beta oxidation of fatty acids could also attenuate liver steatosis and restore liver injury in mice, which explains the more pronounced decrease of liver injury indexes in male mice. In contrast, in female mice, BLP did not significantly activate the AMPK/PGC-1α/ERRα pathway, but rather significantly inhibited the expression of FAS, one of the key enzymes in fat synthesis, and the inhibition of its expression directly limited the fat synthesis process. Thus, female mice were significantly reduced the accumulation of white fat in the body by inhibiting the PPAR-γ/SREBP-1/FAS pathways.

After explaining gender differences through molecular mechanisms, multi-omics analysis of gut microbiota and metabolites were also utilized in our studies to reveal the gender differences. Based on 16S rRNA gene sequencing, we analyzed the sex-specific modulation of gut microbiota by BLP in mice. The results demonstrated that BLP significantly altered the gut microbial composition in both male and female mice, particularly leading to a marked reduction in *Bacteroidetes* abundance and an increase in *Firmicutes*, thereby elevating the *Firmicutes*-to-*Bacteroidetes* (F/B) ratio. Traditionally, an increase in F/B has been thought to be one of the hallmarks of obesity. However, as research has progressed, it has been found that taxonomic attributes at the gate level may mask functionally distinct subpopulations. More in-depth species- or genus-level analyses can truly reflect the modulatory effects of pharmacological interventions on the gut microbiota[27]. Previous study proved that *Faecalibaculum* from *Firmicutes* could enhance energy metabolism and reduce obesity[28]. Ni et al. found that *Bacteroides* was significantly increased in patients with NAFLD and positively correlated with hepatic triglyceride and serum aminotransferase (ALT/AST) levels[29]. In our study, BLP intervention not only reduced Bacteroides abundance, but also promoted the proliferation of beneficial Firmicutes genera (Faecallbaculum and Bifidobacterium) in both sexes, ultimately increasing the F/B ratio while mitigating obesity. It is worth noting that the differences in gut microbiota after BLP intervention are specifically manifested in the regulation of *Firmicutes* genera. Specifically, in male mice, BLP increased the abundance of species mainly *Akkermansiaceae, Faecallbaculum* and *Oscillibacter.* In female mice, BLP increased the abundance of *Lactobacillus, Bifidobacterium* and *Enterorhabdus* suggesting that gut microbiota also play an essential role in the gender difference anti-obesity effects of BLP. Functional analysis of PICRUSt showed that male mice expressed better bile acid synthesis, butyric acid and propionic acid metabolism, pantothenic acid and CoA biosynthesis, fatty acid degradation, TCA cycle and oxidative phosphorylation than female mice. Among them, bile acids can activate FXR

receptors, which in turn promotes enhanced hepatic fatty acid β-oxidation, promotes the catabolism of fat in the liver for energy supply, and also inhibits hepatic fat synthesis, in addition to promoting the catabolism and utilization of blood triglycerides, and ultimately promotes the catabolism and oxidation of fat in vivo[30,31]. In addition, butyric acid and propionic acid can increase the rate of fatty acid β-oxidation by activating PPARγ and CPT-1A, respectively[32,33]. The main role of pantothenic acid (vitamin B5) in the human body is to act as a precursor of CoA, which by binding to fatty acids and forming lipoyl coenzyme A, thus enabling fatty acids to enter the mitochondria for β-oxidation, and ultimately generating acetyl-coenzyme A, NADH, and $FADH_2$, promotes the tricarboxylic acid cycle and oxidative phosphorylation[34,35]. Pearson correlation analysis further showed that beneficial species expressed in male mice, such as *Akkermansiaceae, Faecallbaculum*, and *Oscillibacter*, were significantly and positively correlated with the expression of AMPK/PGC-1α/ERRα downstream factors, such as fatty acid β-oxidation genes, oxidative phosphorylation genes, and antioxidant genes, which was in agreement with the PICRUSt functional analysis results. It should be noted that species such as *Akkermansiaceae, Faecallbaculum* and *Oscillibacter* have been shown to alleviate symptoms associated with obesity. Of these, *Akkermansiaceae* is considered one of the candidate probiotic therapies, which could promote the metabolism of the intestinal mucosa, effectively repair the intestinal barrier, and reduce the colonization of harmful bacteria related to obesity[36]. Furthermore, related studies have indicated that microbes such as *Akkermansiaceae, Faecalibaculum,* and *Oscillibacter* are capable of producing short-chain fatty acids (SCFAs) like butyrate and propionate. These SCFAs have been found to activate the AMPK/PGC-1α/ERRα pathway through multiple mechanisms[37,38].

As for female mice, functional analysis of PICRUSt showed that female mice expressed better steroid biosynthesis and linoleic acid metabolism than male mice. In particular, steroid biosynthesis can be inhibited by activation of estrogen receptor α to inhibit SREBP-1c and FAS, thereby reducing fat synthesis[39,40]. In addition, metabolites of linoleic acid, such as conjugated linoleic acid, can inhibit the expression of FAS through activation of PPARα, which reduces the expression of lipid synthesis[41]. Pearson correlation analysis further showed that the beneficial species of *Lactobacillus, Bifidobacterium* and *Enterorhabdus* were significantly negatively correlated with the expression of FAS. This suggests that the alteration of the gut microbiota by BLP in female mice ameliorates obesity mainly through the inhibition of fat synthesis. Noteworthy, metabolic products of Lactobacillus, such as SCFA and linoleic acid, can stimulate and regulate appetite and satiety through the gut-brain axis, thereby achieving weight loss. Simultaneously, these metabolites can modulate expression of FAS, thereby inhibiting lipid synthesis[42]. In addition, studies have shown that *Bifidobacterium* can inhibit fat synthesis and reduce fat accumulation by suppressing the expression of genes such as ACC. This mechanism directly interferes with fat storage and metabolism, helping to alleviate obesity and its related metabolic diseases[43].

Non-targeted metabolomics is an approach that reacts to changes in metabolic pathways by altering the levels of metabolites. Our metabolomics analysis also discovered significant gender differences in metabolite composition between male and female mice after BLP intervention. Specifically, BLP intervention increased

the expression levels of metabolites such as Palmitic acid, Linoelaidyl carnitine, and LysoPC (18:0) in male mice, and further pathway enrichment analysis showed that these metabolites were involved in the regulation of lipid metabolism, transcriptional activation of mitochondrial biosynthesis, and fatty acyl-coenzyme A biosynthesis by PPARα. These results suggest that the differential metabolites in male mice are associated with energy metabolic pathways. In female mice, the expression of LysoPC (16:0), Arachidonic acid and FAD was increased, and these differential metabolites were involved in the catabolism of glycerophospholipids, the regulation of cholesterol biosynthesis by SREBP, and the metabolism and synthesis of white fat, demonstrating the differential metabolites in female mice were related to the catabolic pathway of lipids.

Pearson correlation analysis further showed that the metabolites Palmitic acid, Linoelaidyl carnitine and LysoPC (18:0), which were highly expressed in male mice, showed significant positive correlation associations with the AMPK/PGC-1α/ERRα pathway, fatty acid β-oxidation and oxidative phosphorylation. Moreover, Palmitic acid and LysoPC (18:0) showed significant positive synergistic relationships with energy metabolism-promoting species such as *Akkermansiaceae, Faecallbaculum* and *Oscillibacter*. Indeed, Palmitic acid is one of the products of fat mobilization that stimulates the intracellular transfer of histone deacetylase (SIRT6) from the nucleus to the cytoplasm. In the cytoplasm, SIRT6 triggers the activity of deacetylated long-chain lipoyl coenzyme A synthetase 5 (ACSL5), which allows more long-chain fatty acids to enter the mitochondria for β-oxidation, generating acetyl-coenzyme A, that provides raw materials for the tricarboxylic acid cycle and improves the efficiency of cellular metabolism of fatty acids[44]. LysoPC (18:0) is a lipid molecule that is widely found in living organisms and plays an important role in a variety of physiological and pathological processes. During regeneration after liver injury, LysoPC (18:0) regulates hepatocyte proliferation as well as depletion of liver fat accumulation through activation of hepatocyte AMPK, PGC-1α, and mitochondrial uncoupling protein (UCP1)[45]. Linoelaidyl carnitine is an acylcarnitine that functions primarily to promote fatty acid β-oxidation in cells, supplying cellular energy requirements and ultimately reducing lipid accumulation[46]. Kaiyan Gong et al. further revealed that enrichment of microbial genera such as *Akkermansiaceae* and *Faecalicatena* promotes hepatic synthesis of linoelaidyl carnitine by consuming δ-valerobetaine[47]. This mechanism provides further insight into why BLP treated male mice are more inclined to activate the AMPK/PGC-1α/ERRα pathway to exert anti-obesity effects.

Whereas, Pearson correlation analysis in female mice showed that Arachidonic acid, LysoPC (16:0) and FAD were significantly positively correlated with SITR1 and SOD2, while significantly negatively correlated with SREBP1C and FAS. In addition, Arachidonic acid, LysoPC (16:0) and FAD showed significant positive coordination with *Lactobacillus, Bifidobacterium* and *Staphylococcus*. Relevant studies have shown that Arachidonic acid can promote lipolysis by increasing the activity of adipocyte lipid-binding protein 2 (AP2) in adipose tissue, thereby reducing the size of adipose tissue cells[48-50]. LysoPC (16:0) is a bioactive lipid molecule, which plays an important signaling role in organisms. During lipid synthesis, LysoPC (16:0) can play an inhibitory role through various mechanisms. Previous study showed that LysoPC (16:0) can inhibit the expression of FAS by activating specific receptors or affecting the intracellular second messenger system, and

LysoPC (16:0) can also promote the catabolism or translocation of lipids out of the adipocyte, which reduces the precursors required for the synthesis of new fat[51]. FAD (flavin adenine dinucleotide) serves not only as an essential coenzyme for acyl-CoA dehydrogenases in catalyzing key steps of lipid catabolism, but also acts as a cofactor for linoleic acid isomerase in *Lactobacillus* and *Bifidobacterium,* promoting the conversion of linoleic acid to conjugated linoleic acid (CLA)[52-55]. Studies have shown that CLA significantly suppresses the expression of SREBP1C and FAS, thereby inhibiting adipocyte differentiation in 3T3-L1 cells[56]. Together, these findings demonstrate that in female mice, BLP promotes the proliferation of beneficial bacteria such as Lactobacillus, which modulates metabolites including arachidonic acid, LysoPC(16:0), and FAD, collectively forming a synergistic network that suppresses lipogenesis. This provides a mechanistic explanation for why BLP treated female mice preferentially inhibit the PPAR-γ/SREBP-1/FAS pathway to exert anti-obesity effects.

In summary, multi-omics evidence clearly delineates the mechanism by which BLP exerts sex-specific anti-obesity effects through regulation of the "gut microbiota-metabolite-signaling pathway" axis. However, many other factors may also lead to the sex-specific anti-obesity effects. For instance, previous studies showed that dynamic changes in estrogen and androgen levels, and the estrogen receptors activation are closely associated with the regulation of obesity[57,58]. In addition, some compounds like quercetin, gallic acid, and catechin have been shown to activate estrogen receptors,thereby exerting an anti-obesity effect, while are core components of BLP[59]. Therefore, we hypothesize that BLP may induce the activation of sex hormone receptors and affect the estrogen and androgen levels, and subsequently lead to the sexually dimorphic anti-obesity effects. Future studies will focus on elucidating the precise mechanisms through which BLP intervention alters sex hormone levels and determining the contribution of these changes to its sex-dependent anti-obesity efficacy, providing a theoretical foundation for personalized BLP applications.

5. Conclusion

In summary, BLP showed significant gender differences in ameliorating HFD-induced obesity in mice. Specifically, in male mice, BLP enhanced the expression of genes related to fatty acid β-oxidation and oxidative phosphorylation through activation of the AMPK/PGC-1α/ERRα pathway. In addition, BLP increased the abundance of *Akkermansiaceae, Faecallbaculum,* and *Oscillibacter* in the mouse intestine. These factors promote the expression of Palmitic acid, Linoelaidyl carnitine and LysoPC (18:0), which increase the level of energy metabolism and thus improve the metabolic dysfunction associated with obesity. In female mice, BLP reduces lipid synthesis by inhibiting the expression of *PPAR-γ/SREBP-1/FAS* pathways, and increases the abundance of *Lactobacillus, Bifidobacterium and Enterorhabdus*. These factors promote the expression of Arachidonic acid and LysoPC (16:0), thereby inhibiting adipogenesis and inducing lipid translocation, and finally effectively reduced lipid accumulation in adipocytes (Fig 12). In our studies, the phenomenon of gender difference in mice due to BLP was explained for the first time in terms of molecular mechanism, gut microbiota and metabolomics. These findings provide a theoretical basis for developing refined, individualized interventions for sex-specific obesity subgroups.

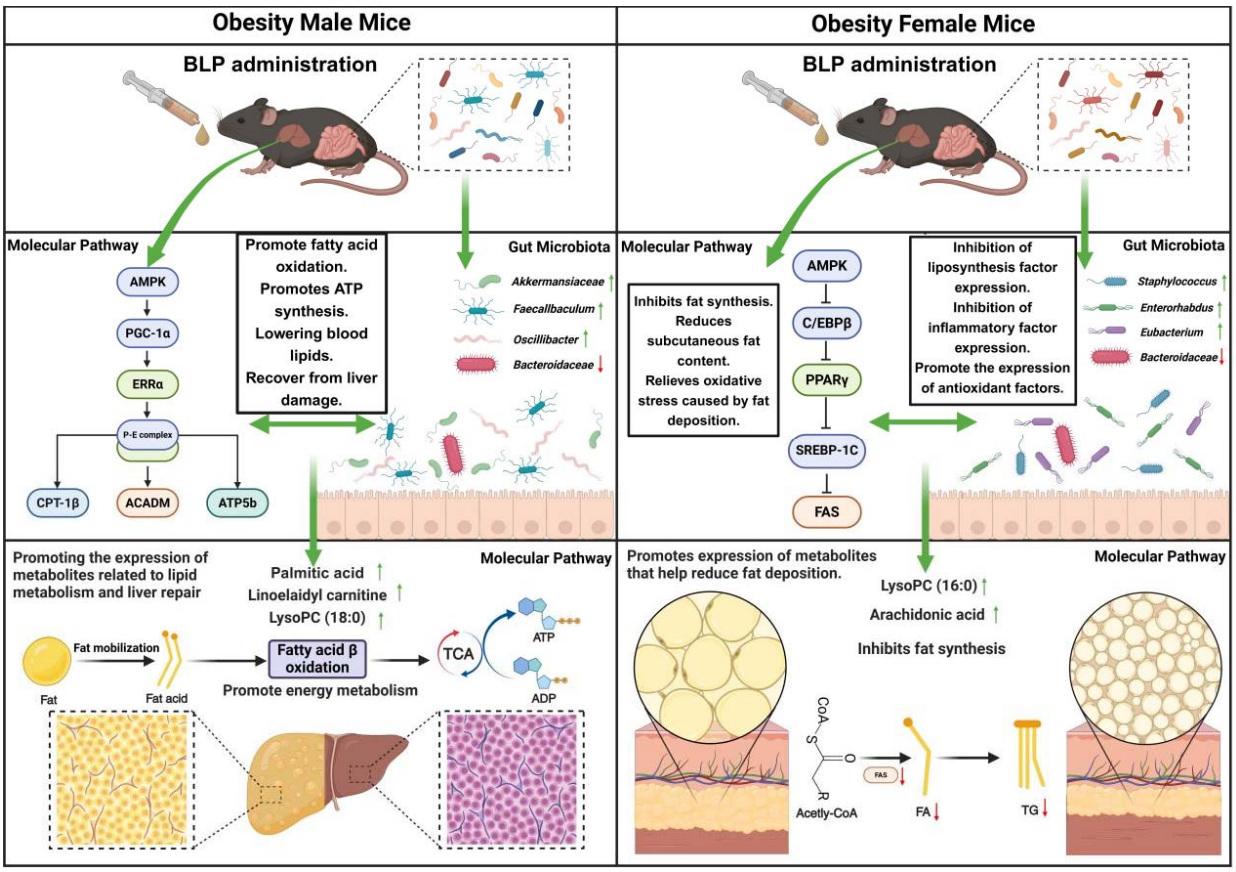

Figure 12. Summary of the anti-obesity effects of BLP in obese mice and sex-differentiated pathways.

Availability of data and materials

All data supporting the findings of this study are available within the paper and its Supplementary Information. Microsatellite primer sequences are provided in Supplementary Table 4. The raw sequencing data has been deposited into China National Center for Bioinformatics (CNCB) with the accession numbers CRA021156 (https://bigd.big.ac.cn/gsa/browse/CRA021156).

Ethics approval and consent to participate

All animal assays were carried out abided by the standard of the National Regulation of China for the Care and Use of Laboratory Animals complied with the National Research Council Guide for the Care and Use of Laboratory Animals. And all animal experiments in this study were approved by the Medical Ethics Committee of Zunyi Medical University, with the ethics number [ZHSC-2-[2024]047].

Funding

This study was financially supported by the future ‘Science and technology elite’ project of Zunyi Medical University [ZYSE-2021-02], key construction discipline of immunology and pathogen biology, Zunyi medical university zhuhai campus [ZHGF2024-1], Zunyi Medical University 2018 Academic New Seedling Cultivation and Innovative Exploration Special Project [QKH[2018]5772- 028].

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