

**Flavonoid-rich extracts of *Nelumbo nucifera* leaves alleviate obesity in HFD-fed mice via microbiota-dependent modulation of brown fat thermogenesis**

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## **Abstract**

### **Ethnopharmacological relevance**

*Nelumbo nucifera* Gaertn (lotus) leaf is a commonly used traditional Chinese herbal medicine with a wide range of pharmacological properties, especially lipid-lowering and weight-loss effects. Accumulating evidence highlights activation of the thermogenic program of brown adipose tissue (BAT) as a promising anti-obesity strategy. However, it remains unclear whether such beneficial metabolic effects induced by the lotus leaf are related to its regulatory role in BAT function.

### **Aim of the study**

This work aims to investigate whether the lotus leaf reduces obesity by activating BAT and to elucidate whether the mechanism behind it is related to the regulation of gut microbiota.

### **Material and methods**

A mouse model of obesity was established using a high-fat diet (HFD), and the anti-obesity effect of flavonoid-rich lotus leaf extract (LLE) was determined in vivo. An animal energy metabolism monitoring system confirmed that LLE promoted energy expenditure. Then, RT-qPCR, immunohistochemistry, and Western blotting were conducted to detect the expression of genes and proteins involved in BAT thermogenesis. Subsequently, the underlying mechanisms were demonstrated by 16S rRNA gene sequencing and non-targeted metabolism analysis. Finally, fecal microbiota transplantation (FMT) was performed to investigate the LLE-dependent alleviation of obesity *via* the gut microbiota-BAT axis.

## Results

Our study demonstrated that LLE effectively reduced weight gain, ameliorated glucolipid disorders, and enhanced energy expenditure in HFD-fed mice. Notably, LLE augmented BAT activity by increasing thermogenic markers (e.g., SIRT1, PGC-1 $\alpha$ , UCP1) and repressing inflammatory responses, potentially through activation of  $\beta_3$ -AR/AMPK/p38 signaling pathways. Importantly, LLE could mitigate HFD-induced microbial dysbiosis (decrease in *Proteobacteria*, *Verrucomicrobiota*, *Acidobacteriota*, *Bacteroides*, *Dubosiella*, and increase in *Bilophila*, *Tyzzereella*, *Oscillibacter*, *Akkermansia*, and *Alistipes*) and significantly altered 5 metabolite pathways, especially primary bile acid biosynthesis and linoleic acid metabolism. The FMT experiment confirmed that the microbial changes induced by LLE were associated with reduced body weight, enhanced energy expenditure, increased BAT activity, and thermogenesis.

## Conclusions

Collectively, our findings reveal that lotus leaf promotes brown fat thermogenesis by modulating gut microbiota, identifying it as a promising new treatment target for obesity.

**Keywords:** Lotus leaves extract, Obesity, Brown adipose tissues, Gut microbiota, Metabolomics, Fecal microbiota transplantation

## Abbreviations

AMPK: adenosine 5'-monophosphate-activated protein kinase; AUC: area under the curve;  $\beta_3$ -AR:  $\beta_3$ -adrenoceptor; BAs: Bile acids; BAT: Brown adipose tissue; BG: Blood glucose; CD11c: Cluster of differentiation 11c; Cd40: Tumor necrosis factor receptor superfamily member 5; Cd137: 4-1BB; COX2: Cyclooxygenase-2; COX7a: Cytochrome c oxidase subunit 7A; Cidea: Cell death-inducing DNA fragmentation factor alpha subunit-like effector A; Dio2: Iodothyronine deiodinase 2; ECL: Enhanced chemiluminescent; eWAT: Epididymal WAT; EE: Energy expenditure; Fgf21: Fibroblast Growth Factor 21; FMT: Fecal microbiota transplantation; GPR120: G-protein coupled receptor 120; H&E: Hematoxylin-eosin; HDL-C: High-density lipoprotein cholesterol; HFD: High-fat diet; HRP: Horseradish peroxidase; IL-1 $\beta$ : Interleukin-1 beta; IL-6: Interleukin-6; iNOS: Inducible nitric oxide synthase; iWAT: Inguinal WAT; LDL-C: Low-density lipoprotein cholesterol; LLE: Flavonoid-rich lotus leaf extract; MCP1: Monocyte chemotactic protein-1; NOX2: NADPH oxidase 2; NRF1: Nuclear respiratory factor 1; OGTT: Oral glucose tolerance test; pWAT: Perirenal WAT; PGC-1 $\alpha$ : Peroxisome proliferator activated receptor  $\gamma$  coactivator 1 $\alpha$ ; PKA: Protein Kinase A; PPAR $\alpha$ : Peroxisome proliferator-activated receptor  $\alpha$ ; PRDM16: PR domain containing 16; PUFAs: Polyunsaturated fatty acids; PVDF: Polyvinylidene fluoride; RER: Respiratory exchange ratio; RT-qPCR: Quantitative real-time PCR; SCFAs: Short-chain fatty acids; SIRT1: Silent information regulator 1; TAFM: Mitochondrial transcription factor A; Tbx1: T-box transcription factor 1; TC: Total cholesterol; TG: Triglyceride; Tmem26: Transmembrane protein 26; TNF- $\alpha$ : Tumor necrosis factor-alpha; TRPV1: Transient receptor potential vanilloid 1; UCP1: Uncoupling protein 1; WAT: White adipose tissue

## 1. Introduction

The approach commonly used to combat obesity is to restrict calorie intake, but another alternative is to boost the energy-burning capacity of significant metabolic organs, such as brown adipose tissue (BAT) (Zhang et al., 2014). Brown adipocytes in BAT are distinguished by numerous small lipid droplets, a high density of mitochondria, and a high expression of uncoupling protein 1 (UCP1), which plays a key role in their thermogenic function. In adults, the average mass of BAT is 50 to 70 grams (Virtanen et al., 2009), and upon activation increases energy expenditure by about 20% in a person who consumes 2500 kcal/day, which is sufficient to prevent weight rebound after dieting (Feng et al., 2013). Consequently, the pursuit of diverse strategies aimed at enhancing not only the quantity but also the metabolic activity of BAT is gaining recognition as a promising therapeutic approach for effectively managing and combating the global obesity epidemic, offering new hope for improved health outcomes in affected individuals.

The lotus leaf, derived from the plant *Nelumbo nucifera*, is a medicine and food homology resource that has long been utilized as both a culinary ingredient and a medicinal herb in Asian, including China, Japan, Korea, India, and Thailand (Huang et al., 2021; Je and Lee, 2015). In China, the yearly yield of lotus leaves is in the range of 7000 to 10000 tons, with just 1% being applied in the tea and catering industry (Wang et al., 2021). The recommended daily consumption of lotus leaf, as stated in the Pharmacopoeia of the People's Republic of China, is between 3 to 10 grams for an adult (Huang et al., 2013). This dosage is equivalent to a daily intake of

0.56 to 1.85 grams of lotus leaf extract. The lotus leaf contains a wealth of bioactive substances, such as phenolic acids, flavonoids, and alkaloids, and is often included in dietary supplements aimed at facilitating weight loss. Research has identified a range of mechanisms by which the bioactive constituents in lotus leaves can confer positive effects on metabolic health. These mechanisms include: 1) reduction of pancreatic lipase activity; 2) suppression of adipocyte differentiation; 3) promotion of WAT lipolysis; 4) inhibition of inflammation; 5) enhancement of hepatic lipase activity (Gong et al., 2020). In addition to the above mechanism, our preliminary study found that lotus leaf extract (LLE), rich in quercetin 3-O-glucuronide, enhanced the thermogenic and metabolic functions of brown adipocytes by upregulating key genes such as SIRT1, CIDEA, TFAM, and UCP1, as demonstrated in differentiated C<sub>3</sub>H<sub>10</sub>T<sub>1/2</sub> cells (Wang et al., 2023a). Despite these promising results, significant questions still exist about whether this beneficial effect is also observed *in vivo*, and the precise mechanisms are yet to be fully understood. This uncertainty underscores the necessity for additional research to explore the *in vivo* efficacy and identify the specific biological pathways at play.

Research findings emerging from extensive clinical trials as well as detailed experimental studies have consistently highlighted the pivotal role that the gut microbiota plays in the regulation of energy expenditure and the proper functioning of BAT (Liu et al., 2025; Richard et al., 2022). Mestdagh et al. (2012) first proposed the critical role of microbiota in the regulation of brown fat functionality. They discovered that the BAT in germ-free mice seemed to exhibit greater activity and a

faster rate of calorie combustion than that in normal mice (Mestdagh et al., 2012). Consistent with this study, Li and colleagues documented that microbiota depletion (by antibiotic treatment) led to improved glucose tolerance and an increased capacity for glucose uptake in BAT (Li, M. et al., 2021). Notably, metabolites produced by the microbiota function as chemical messengers mediating interactions between microbes and the host and can play beneficial roles in the browning process. Metagenomic and metabolomic studies have uncovered associations between metabolites [i.e., bile acids (BAs), short-chain fatty acids (SCFAs), polyunsaturated fatty acids (PUFAs)], and BAT thermogenesis (Xiao and Kang, 2020). Here, integrated analysis of microbiome and metabolomics with fecal microbiota transplantation (FMT) was employed to decipher the precise mechanism through which LLE alleviates obesity. Our research not only identifies LLE as a potentially beneficial compound in the improvement of glycolipid metabolism but also sheds new light on its ability to boost the functional activity of BAT. This finding is particularly noteworthy because it underscores the pivotal importance of the "gut microbiota-metabolite-BAT" axis in conferring the anti-obesity effects of LLE. In summary, our expanded analysis indicates that LLE's anti-obesity effects likely result from a complex interplay between the gut microbiota and BAT, and that manipulating this axis could represent a novel and effective strategy to combat obesity and related metabolic disorders.

## **2. Materials and Methods**

### **2.1. Preparation of LLE**

*Nelumbo nucifera* (lotus) leaves were collected from Bozhou, Anhui Province, China,

in July of 2022. A voucher specimen has been deposited in the State Key Laboratory of Food Science and Resources, Jiangnan University (accession number SKLFSR1608). The flavonoid-rich lotus leaf extract (LLE) was obtained and identified as reported in our prior publication (Wang et al., 2023a). Briefly, the procedure involved soaking dried leaves in 80% ethanol, followed by filtering the obtained extract through a 0.45 µm filter. The filtrate was then concentrated and freeze-dried to yield the LLE, which was subsequently stored at -20°C. Colourimetric results showed that LLE contained 383.7 mg/g of total phenolics and 178.3 mg/g of total flavonoids. By UPLC-MS/MS analysis, seven flavonoids were identified in LLE, including procyanidin B2, quercetin 3-O-arabinopyranosyl-(1→2)-galactopyranoside, rutin, quercetin 3-O-glucuronide, kaempferol 3-O-glucoside, isorhamnetin 3-O-rutinoside, and isorhamnetin 3-O-glucoside, which accounted for 12.8% of the dry weight.

## **2.2. Animals and experimental design**

Experiment 1: A total of 40 male C57BL/6J mice, aged 5 weeks, were accommodated in the Animal Center at Jiangnan University (approval number: JN.No20230415c1300730[142]) and were allowed to adapt for a week. Then, the mice were assigned to five groups (n=8): ND group fed a normal diet, HFD group fed a high-fat diet, LLE-L group receiving HFD supplemented with 100 mg/kg of LLE dissolved in 0.5% carboxymethyl cellulose sodium (CMC-Na) by oral gavage, LLE-H group receiving HFD supplemented with 200 mg/kg of LLE dissolved in 0.5% CMC-Na by oral gavage, and MI group given HFD plus 5 mg/kg of miglitol dissolved

in 0.5% CMC-Na by oral gavage as a positive control. Notably, HFD feeding and LLE or MI treatment were initiated simultaneously. The food and water were available ad libitum during the 12-week administration. The components of the ND and HFD can be found in Supplementary Table 1.

Experiment 2: In preparation for fecal microbiota transplantation (FMT), extra 24 mice (recipient mice) were orally administrated with a 0.2 mL cocktail of broad-spectrum antibiotics, including ampicillin (1 g/L), metronidazole (1 g/L), vancomycin (0.5 g/L), and neomycin (0.5 g/L) for 7 days (Caesar et al., 2015). All experiments were conducted in accordance with the relevant regulations of the Animal Center at Jiangnan University (approval number: JN.No20230415c1300730[142]). Briefly, fecal samples were procured from donor mice (Experiment 1) in the ND group, the HFD group, and the LLE group (mice on an HFD plus 200 mg/kg of LLE). These samples were further processed by homogenization by dilution in 0.9% sterile saline (200 mg/2 mL), and centrifugation at 600 g for 5 minutes. The resulting bacterial suspensions were then administered intragastrically to recipient mice (200  $\mu$ L) daily as follows: ND→HFD group (ND-FMT, n=8), HFD→HFD group (HFD-FMT, n=8), and LLE→HFD group (LLE-FMT, n=8). After 12 weeks of fecal bacteria gavage, serum was isolated for biochemical analysis; tissues were isolated for weighing, histological analysis, and browning potential testing.

### **2.3. Analysis of respiratory metabolism**

The energy consumption was measured by the CLAMS-12 (Columbus Instruments,

USA). Mice were individually housed in metabolic cages, and following a 24-hour acclimation period, their O<sub>2</sub> consumption and CO<sub>2</sub> production rates were continuously tracked for an additional 24 hours, allowing for the calculation of their energy expenditure.

#### **2.4. Oral glucose tolerance test (OGTT)**

The mice were fasted for 12 hours and then orally dosed with a glucose solution at 1.5 g/kg body weight. After dosing, the levels of blood glucose (BG) were measured at different time points, with a BG meter (Roche) used for the monitoring process. A OGTT curve was constructed, mapping time in minutes along the x-axis and BG value in millimoles per liter (mM) along the y-axis, with the area under the curve (AUC) subsequently computed.

#### **2.5. Serum biochemical parameters**

Anesthetized mice were used to collect blood samples from their eyeballs. The serum was subsequently isolated by centrifuging the blood at 3500g for 10 minutes. Once separated, the serum was stored at a freezing temperature of -80°C. Analysis was later performed on this serum to assess the four lipid-based biomarkers: TG, TC, LDL-C, and HDL-C, following the protocols of the kit method.

#### **2.6. Histopathological analysis**

Interscapular BAT and colon tissues fixed in 10% formalin were washed with running water and ethanol. The tissues were dehydrated through a graded series of alcohols (70%, 85%, 90%, 95%, and 100%) and then embedded in paraffin wax. Five-micrometer sections were prepared and stained with hematoxylin and eosin

(H&E). The sections were observed under an optical microscope (Leica DM2500, Wetzlar, Germany).

## **2.7. Quantitative RT-PCR analysis**

The experimental procedures for total RNA extraction, cDNA synthesis, and quantitative PCR analysis were performed according to the established protocols from our prior research (Wang et al., 2023a). The primer sequences are listed in Supplementary Table 2.

## **2.8. Immunohistochemistry**

Immunohistochemical staining was performed in accordance with the manufacturer's protocol (Servicebio, Wuhan, China) using paraffin-embedded BAT sections. The procedure involved deparaffinization in xylene followed by rehydration through a graded ethanol series (100%, 95%, 85%, 75%). After antigen retrieval, endogenous peroxidases were quenched with 3% H<sub>2</sub>O<sub>2</sub> in PBS, followed by blocking with 5% bovine serum albumin in Tris-buffered saline to reduce non-specific binding. The samples were incubated overnight at 4°C with rabbit-derived primary antibodies against SIRT1 and UCP1 (1:1000), followed by a 1-hour room temperature incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies. DAB substrate was added to generate a brown precipitate at the antigen-antibody complex, followed by hematoxylin counterstaining to visualize cell nuclei. Sections were mounted with neutral resin and examined under a light microscope to assess protein localization, intensity, and distribution.

## **2.9. Western blotting**

Tissue lysates were processed for Western blot analysis using our previously established protocol (Wang et al., 2023a). PVDF membranes underwent sequential probing with primary antibodies targeting  $\beta_3$ -AR, PKA, p-PKA, AMPK, p-AMPK, p38, p-p38, SIRT1, TFAM, and UCP1 (Proteintech, 1:1000), with overnight incubation at 4°C. Following this, HRP-conjugated secondary antibodies (Proteintech, 1:400) were used to bind to the primary antibodies. The immobilized antibody-antigen complexes were then visualized using a chemiluminescent detection system, which involved the application of an enhanced chemiluminescent (ECL) detection reagent supplied by Beyotime (China).

#### **2.10. Gut microbiota analysis**

Fecal microbial DNA was extracted using the Omega DNA Kit as described in the manufacturer's instructions. The 16S rRNA sequencing was performed as previously described (Wang et al., 2023b) and is detailed in the supplementary material. Raw data were analyzed by Metware Biotechnology Co., Ltd. (Wuhan, China).

#### **2.11. Untargeted metabolomics analysis**

The fecal specimens were rapidly frozen using liquid nitrogen and subsequently dispatched to Metware Biotechnology Co., Ltd. (Wuhan, China) for untargeted metabolomics analysis. Once the analysis was completed, the experimental data were transferred to the Metware cloud platform for further analysis. The specific methods for sample preparation, the detection procedures, and the data processing are detailed in the Supplementary Materials section.

#### **2.12. Statistical analysis**

All data are expressed as the means  $\pm$  standard deviation (SD). One-way analysis of variance (ANOVA) with Dunnett's comparisons test using GraphPad Prism 8.0 was used for multiple comparisons. \*, \*\*, and \*\*\* indicate  $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.001$ , respectively. Furthermore, ANOVA with Duncan's comparisons test using DPS software 7.05 was used for multiple comparisons. Different lowercase letters indicate significance ( $p < 0.05$ ).

### 3. Results

#### 3.1. Effect of LLE on obesity-related indicators in HFD-fed mice

LLE treatment inhibited HFD-induced increase in body weight gain (Fig. 1A and 1B), eWAT (Fig. 1C), iWAT (Fig. 1D), pWAT (Fig. 1E), and BAT weights (Fig. 1F) and reached a similar effect to that of the MI group. Blood biochemical analysis revealed that LLE supplementation could significantly decrease serum TG (Fig. 1G) and TC (Fig. 1H) levels without dramatically affecting LDL-C (Fig. 1I) and HDL-C (Fig. 1J) levels. Meanwhile, we observed that the mice treated with LLE showed a notable reduction in BG level during OGTT (Fig. 1K and 1L), supporting the potential of LLE supplementation in weight loss and improvement of dyslipidemia.

#### 3.2. Effect of LLE on energy metabolism in HFD-fed mice

The results show that LLE can significantly enhance  $O_2$  consumption (Fig. 1M and Fig. 1N),  $CO_2$  production (Fig. 1O and 1P), and energy expenditure (EE) (Fig. 1S and 1T) levels without affecting the respiratory exchange ratio (RER,  $RER = VO_2/VCO_2$ ) (Fig. 1Q and 1R). Moreover, LLE treatment did not affect the physical activity (Fig. 1U), suggesting that the LLE-induced increase in energy expenditure was independent

of the mice's activity level.

### **3.3. Effects of LLE on BAT activity in HFD-fed mice**

Histological analysis *via* H&E staining revealed that LLE administration significantly reduced lipid droplet accumulation in BAT, as indicated by diminished white vacuolar spaces, with effects comparable to those observed in the MI control group (Fig. 2A). Furthermore, BAT from LLE-treated mice exhibited enhanced immunostaining positivity for SIRT1 (Fig. 2B) and UCP1 (Fig. 2C), suggesting activation of thermogenesis. At the transcriptional level, LLE upregulated mRNA expression of key thermogenic regulators, including *Sirt1*, *Pgc-1 $\alpha$* , *Cidea*, *Prdm16*, *Dio2*, *Tfam*, *Nrf1*, *Cox2*, *Cox7a*, and *Ucp1* (Fig. 3A). Concurrently, it suppressed inflammatory gene expression (*Nox2*, *IL-6*, *TNF $\alpha$* , *Cd11c*, *Mcp1*, *IL-1 $\beta$* , and *iNOS*) (Fig. 3A). Western blot analysis revealed LLE enhanced key signaling pathways in BAT, demonstrating elevated phosphorylation ratios of PKA (p-PKA/PKA), AMPK $\alpha$  (p-AMPK $\alpha$ /AMPK $\alpha$ ), and p38 (p-p38/p38) and confirmed at the protein level increased expression of  $\beta_3$ -AR, SIRT1, TFAM, and UCP1 (Fig. 3B).

### **3.4. Effects of LLE on fecal microbiota profile in HFD-fed mice**

mRNA analysis of inflammatory markers in colon tissue showed that high-dose LLE significantly reduced the expression of *Mcp1*, *iNOS*, *TNF $\alpha$* , *IL-6*, and *IL-1 $\beta$* , while significantly increasing the expression of *Sod3*, *Sod2*, and *Nox2* (Fig. 4A), suggesting its beneficial effects on gut health. 16S rRNA gene analysis showed that LLE treatment reduced ACE without affecting the Shannon index, which was similar to the ND group, indicating that LLE treatment normalizes the microbiota profile (Fig. 4B

and 4C). The Venn diagram shows a total of 2111, 2498, 2203, and 2116 OTUs were identified in the ND, HFD, LLE-L, and LLE-H groups, respectively (Fig. 4D). In addition, PCA scatter plot analysis demonstrated that microbiota structures in the LLE and HFD groups were separated from the ND group (Fig. 4E). Specifically, administration of LLE counteracted HFD-induced increase in the *Firmicutes* phylum (Fig. 4F and 4G) and accompanying reduction in the *Bacteroidota* phylum (Fig. 4F and 4G), resulting in a lower ratio of *Firmicutes/Bacteroidota* (Fig. 4G). Furthermore, *Proteobacteria*, *Verrucomicrobiota*, and *Acidobacteriota* were of higher abundance in HFD-fed mice, but LLE treatment could restore them to normal levels (Fig. 4F and 4G). The LefSe analysis (LDA score>3) identified 80 statistically significant differential OTUs (comprising 7 families and 31 genera) as key phlotypes distinguishing the four groups (Fig. 5A and 5B). Fig. 5C highlights 59 high-abundance genera, with LLE treatment significantly decreasing *Achromobacter*, *Faecalibaculum*, *Bacteroides*, *Dubosiella*, *Colidextribacter*, and *Sphingomonas*, while markedly increasing *Oscillibacter*, *Alistipes*, *Akkermansia*, *Bilophila*, *Lachnoclostridium*, and *Tyzzzerella* compared to the HFD group (Fig. 5D).

### 3.5. Effects of LLE on fecal metabolites in HFD-fed mice

Figure 6A-D illustrates OPLS-DA score plots comparing ND vs. HFD and HFD vs. LLE (200 mg/kg) groups, with permutation testing confirming model validity. The ND vs. HFD groups (Fig. 6A) showed distinct separation ( $R^2Y=0.998$ ,  $Q^2=0.944$ ), exceeding thresholds for predictive reliability ( $Q^2>0.90$ ). Similarly, HFD vs. LLE groups exhibited robust differentiation ( $R^2Y=0.992$ ,  $Q^2=0.762$ ) (Fig. 6C), validating

model stability ( $Q^2 > 0.5$ ). Hierarchical clustering revealed distinct metabolic patterns between LLE and HFD groups (Fig. 6E). Volcano plot analysis identified statistically significant biomarkers distinguishing experimental groups, with 2751 significant alteration of metabolites (802 upregulated, 1749 downregulated) between HFD and ND groups ( $VIP > 1$ ,  $p < 0.05$ ), and 1195 significant alteration of metabolites (430 upregulated, 765 downregulated) between HFD and LLE groups (Fig. 6F and 6G). KEGG pathway analysis of differential metabolites ( $VIP > 1$ ,  $p < 0.05$ ) (Supplementary Table 3), 7 specific pathways ( $p < 0.05$ ) including 70 metabolites were enriched in HFD vs ND groups (Fig. 6H). After high-dose of LLE intervention, 30 metabolites were observed in the LLE group compared to the HFD group involved in 5 key metabolic pathways ( $p < 0.05$ ), including primary bile acid biosynthesis, biosynthesis of unsaturated fatty acids, linoleic acid metabolism, pyrimidine metabolism, and sphingolipid metabolism (Fig. 6I). The above-mentioned 5 key metabolic pathway-related metabolites are shown in Fig. 6J and 6K.

### 3.6. Correlation analysis

A close correlation was observed between the gut microbiota and BAT function (thermogenesis and inflammation) (Fig. 7A). Among those genera, *Faecalibaculum*, *Bacteroides*, *Achromobacter*, *Sphingomonas*, and *Colidextribacter* exhibited inverse correlations with markers of thermogenesis and, conversely, positive associations with obesity-related traits and BAT inflammation; *Oscillibacter*, *Lachnoclostridium*, *Tyzzereella*, and *Bilophila* were positively correlated with thermogenesis-related markers and negatively correlated with BAT inflammation. To assess potential

linkages between metabolite profiles and intestinal microbial communities/BAT thermogenic activity, a statistical association analysis was performed (Fig. 7B). Strong correlations were identified between lipid-modulating metabolites-particularly those participating in biosynthesis of unsaturated fatty acids and linoleic acid metabolism-and specific bacterial taxa including *Alistipes*, *Dubosiella*, *Bilophila*, *Oscillibacter*, *Faecalibaculum*, *Lachnoclostridium*, *Tyzzerella*, and *Colidextribacter*; BAs biosynthesis-related metabolites including cholic acid and taurine showed negative correlation with *Achromobacter* and *Sphingomonas*. Furthermore, correlation analysis showed metabolites, including cholic acid, taurine, vernolic acid, FFA(16:0), FFA(18:0), linoleic acid, 5S,8R-DiHODE, and gamma-linolenic acid positively associated with BAT function and negatively associated with obesity-related traits. Additionally, an examination of the mRNA expression levels for the fatty acid receptor GPR120 and TRPV ion channels within the colon demonstrated that treatment with LLE led to a notable upregulation of these receptors, as illustrated in Fig. 7C.

### **3.7. Identification of microflora regulating LLE-induced BAT activation by FMT**

To confirm that microbiota was indeed involved in LLE-related obesity and browning, an FMT experiment was carried out (Fig. 8A). The body weight gain and food consumption of recipients were monitored over this period. The results showed that the mice receiving fecal transplantation from the LLE group (LLE-FMT) significantly attenuated the increase in body weight (Fig. 8B and 8C), liver weight (Fig. 8E), kidney weight (Fig. 8G), iWAT weight (Fig. 8H), pWAT weight (Fig. 8I), eWAT

weight (Fig. 8J), and BAT weight (Fig. 8K) without affecting food intake (Fig. 8D) and spleen weight (Fig. 8F) compared with the HFD-FMT mice. Blood biochemical analysis revealed that LLE recipient mice exhibited improved glucolipid metabolism, evidenced by significantly decreased BG level (Fig. 8P and 8Q), serum TG (Fig. 8L), TC (Fig. 8M), and LDL-C levels (Fig. 8N) without affecting HDL-C (Fig. 8O) level. Analysis of H&E-stained histopathology images demonstrated that after the LLE-modulated microbiota was transplanted into HFD-fed mice, there was a reduction in lipid accumulation within BAT, as well as a decrease in the size of both iWAT and eWAT, along with mitigation of liver steatosis (Fig. 8R). Moreover, transplantation of feces from the LLE mice could beneficially improve energy metabolism, as evidenced by the increased O<sub>2</sub> consumption (Fig. 8S), CO<sub>2</sub> production (Fig. 8T), and EE (Fig. 8U). qPCR analysis of thermogenesis- and inflammation-related genes in BAT revealed that LLE-FMT mice displayed the upregulated expression of *Sirt1*, *Cidea*, *Prdm16*, *Dio2*, *Cox2*, and *Ucp1* but reduced levels of *iNOS*, *IL-6*, *Nox2*, and *TNFα* without changing *Pgc-1α*, *IL-6* and *IL-1β* expression (Fig. 9A). Meanwhile, the brown fat-related markers such as *Sirt1*, *Cidea*, *Pgc-1α*, *Prdm16*, *Cox2*, and *Ucp1*, and beige fat-specific markers such as *Fgf21*, *Tmem26*, *Cd40*, *Tbx1*, and *Cited1* in iWAT were also significantly induced in LLE recipient mice compared with the HFD-FMT mice (Fig. 9E). Moreover, increased positive immunostaining of UCP1 was observed in BAT (Fig. 9B) and iWAT (Fig. 9F) of LLE-FMT mice. Further Western blotting assays confirmed a substantial enhancement of SIRT1 and UCP1 protein expression within the BAT (Fig. 9C and 9D)

and iWAT (Fig. 9G and 9H) of LLE recipient mice. Collectively, these data support that LLE-modulated microbiota facilitate the induction of fat browning and thermogenesis.

#### **4. Discussion**

BAT has recently gained considerable attention due to its significant role in regulating energy metabolism. It has been found that BAT activity is inversely related to the accumulation of excess fat and the development of metabolic disorders, making it a promising target for therapeutic interventions. Our findings reveal that LLE not only ameliorated glucolipid disturbances associated with HFD consumption but also has a broader effect on metabolic health. Importantly, LLE treatment enhanced energy expenditure, suggesting an increased caloric burn. The observed upregulation of thermogenic markers such as *Prdm16*, *Sirt1*, *Pgc-1 $\alpha$* , and *Ucp1* in BAT following LLE treatment suggests a direct modulation of the metabolic machinery responsible for energy expenditure. Notably, *PRDM16* serves as a master transcriptional coregulator in brown adipocytes and exerts a bifunctional role in facilitating the expression of brown fat-selective genes (e.g., *UCP1*) while repressing white fat-selective genes (Hu et al., 2020). *Sirt1*, a class III histone deacetylase, regulates energy metabolism by deacetylating key transcriptional coactivators such as *Pgc-1 $\alpha$* , thereby promoting the expression of thermogenic and mitochondrial biosynthesis-related genes (e.g., *NRF1*, *TFAM*, *UCP1*) (Hu et al., 2020). Specifically, the  $\beta_3$ -AR is a key sensor for catecholamine signaling, and its activation leads to the phosphorylation of AMPK and p38, which in turn promotes thermogenesis (Kim and Plutzky, 2016). The high

expression of  $\beta_3$ -AR, p-AMPK/AMPK, and p-p38/p38, suggesting the induction of these genes is likely mediated through the activation of the  $\beta_3$ -AR/AMPK/p38 signaling pathway. Furthermore, AMPK signaling also mediates the inhibition of inflammatory pathways in adipose tissue (Bijland et al., 2013). Here, the reduction in inflammation-related markers in BAT further supports the concept that LLE enhances BAT function. Importantly, these findings corroborate our previous observations, which demonstrated that LLE induced a brown fat-like phenotype in C<sub>3</sub>H<sub>10</sub>T<sub>1/2</sub> mesenchymal stem cells *via* the  $\beta_3$ -AR/AMPK/p38 signaling (Wang et al., 2023a). The consistency between *in vitro* and *in vivo* results strengthens the mechanistic validity of this pathway in mediating LLE's effects on BAT function and its potential as a therapeutic target for obesity and metabolic disorders.

The intestinal microbiota plays a crucial role in thermogenesis throughout the body, with BAT serving as the main regulator. In the intestine of animals and humans with obesity, the predominant phyla are Firmicutes (F) and Bacteroidetes (B), with an overabundance of F and a deficiency of B (Ley et al., 2006). However, the relationship between the F/B ratio and obesity remains a topic of debate. While some research suggests a higher ratio in individuals with obesity, several studies have not detected any significant changes in this parameter. In fact, there are reports of a decreased F/B ratio in both animals and humans with obesity, complicating the understanding of the role this bacterial balance plays in the context of obesity (Magne et al., 2020). Consequently, it appears that the variation in other elements within the microbiome at sub-levels such as genera or species might be more critical than the

F/B ratio itself (Montandon and Jornayvaz, 2017; Yang et al., 2021). Investigations by Chai and colleagues have shown that an enriched population of *Faecalibaculum* species is linked to elevated serum lipid concentrations, which include TC and TG, as well as increased levels of inflammatory markers such as interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-6 (Chai et al., 2023). Here, the abundance of *Faecalibaculum* spp. and its species *F. rodentium* were greatly reduced in the LLE group, and correlation analysis showed *Faecalibaculum* was negatively associated with brown-related markers (e.g., *Nrfl*, *Cox2*, *Cox7a*) and HDL-C level, but positively associated with LDL-C level and BAT inflammation (e.g., *Mcp1*, *iNOS*). *Bilophila* (Proteobacteria phylum) is considered a crucial taxon involved in the enhancement of metabolic homeostasis in HFD-fed mice mediated by metabolite  $\alpha$ -linolenic acid. The negative correlation found between the *Bilophila* spp. and metabolic disruption, such as serum lipid levels, intestinal inflammation, and intestinal barrier dysfunction, implies a role for *Bilophila* spp. in these metabolic processes (Gao et al., 2020; Li et al., 2022). Consistent with these reports, our result showed that *Bilophila* abundance was induced by LLE and positively associated with BAT function, which may be due to the byproducts of *Bilophila*, BAs. The research conducted by Haro et al. (2016), which analyzed the microbial communities of 239 individuals (138 with MetS and 101 without), found that the abundance of *Bacteroides* was notably higher in the group of patients with MetS compared to those without the condition (Louis et al., 2016). In addition, the baseline microbiota of participants who succeeded in sustaining a loss of their weight over two years was enriched in *Alistipes* spp. (phylum *Bacteroidetes*) compared to

466 patients who were unsuccessful in weight loss (Louis et al., 2016). A significant  
467 increase in the number of *Alistipes* was found to strongly and positively correlate with  
468 the level of thermogenic activity of BAT (Du et al., 2021). The finding indicates that  
469 *Alistipes* might be involved in regulating or enhancing BAT's ability to produce heat,  
470 which is a critical aspect of energy balance and temperature maintenance in the body.  
471 Furthermore, research conducted on animal models has demonstrated that the oral  
472 intake of *Akkermansia* can effectively counteract HFD-induced obesity in mice, as  
473 well as improve several metabolic disorders, including hyperglycemia, insulin  
474 resistance, and hyperlipidemia (Deng et al., 2020). Hence, modifications in the  
475 microbiota, including changes in *Faecalibaculum*, *Bilophila*, *Bacteroides*, *Alistipes*,  
476 and *Akkermansia* induced by LLE, could account for its therapeutic potential against  
477 obesity and its ability to promote brown fat activity.

478 Metabolites originating from the microbiota act as chemical signals that facilitate  
479 communication between microbes and the host, exerting positive metabolic effects on  
480 adipose tissue through the gut (brain)-adipose tissue axis. Evidence suggests BAs  
481 perform hormone-like functions (e.g., GLP-1) by activating nuclear and membrane  
482 receptors (e.g., TGR5, FXR) in intestinal L cells, thereby regulating energy  
483 metabolism (Li et al., 2021). FXR activation in the intestine robustly induces enteric  
484 fibroblast growth factor 15 (FGF15), leading to activation of BAT function by  
485 reducing ectopic lipid deposition and systemic inflammation (Fang et al., 2015).  
486 Moreover, BAs-TGR5 signaling stimulates UCP1 expression and mitochondrial  
487 respiration, directly enhancing thermogenesis (Velazquez-Villega et al., 2018). It was

found that fecal BAs levels, especially cholic acid, were significantly increased after BAT activation upon ginsenoside Rg1 treatment (Liu et al., 2023). Animal studies have confirmed that cholic acid elevates the rate of whole-body energy usage and stimulates adaptive thermogenesis in BAT of mice (Watanabe et al., 2006). Taurine is essential for the metabolism of BAs, and its supplementation has been demonstrated to improve insulin sensitivity, boost energy expenditure, and promote fat burning in HFD-fed mice, primarily through the AMPK-PGC-1 $\alpha$  signaling pathway (Guo et al., 2019). Moreover, research has indicated that certain metabolites within the linoleic acid metabolic pathway, such as linoleic acid,  $\gamma$ -linolenic acid, vernolic acid (12,13-EpOME), and 5S,8R-DiHODE, have a crucial function in regulating both glucolipid homeostasis and the body's inflammatory responses (Silveira et al., 2007). For example, consuming supplements rich in PUFAs, particularly linoleic acid and  $\gamma$ -linolenic acid, results in a lower accumulation of body fat and is associated with the induction of thermogenesis in BAT (Snoke et al., 2023; Takahashi et al., 2000). GPR120 serves as a receptor for long-chain fatty acids, and its stimulation within the intestinal tract can augment the secretion of GLP-1 (Paulsen et al., 2014), thereby boosting brown BAT thermogenesis through the gut-brain axis (González-García et al., 2019). Additionally, linoleic acid metabolism also impacts membrane lipid composition, altering ion channel activity (e.g., TRPV1) in the intestine to improve energy metabolism and promote thermogenesis in BAT (Matta et al., 2014; Kim et al., 2017). The potential mechanism is attributed to the activation of SNS (sympathetic nervous system)/ $\beta_3$ -AR/PKA/AMPK signaling (Zapata et al., 2022). In parallel with

these findings, the treatment with LLE was observed to significantly enhance the mRNA expression of the receptors GPR120 and TRPV1 in colonic tissue. This increase in gene expression implies that LLE may have a regulatory effect on the biological functions mediated by these receptors in the colon. The collective evidence underscores the major therapeutic implications of gut microbiota alterations mediated by LLE, specifically the metabolites that influence the metabolism of BAs and linoleic acids. These alterations are shown to improve the functional capacity of BAT and positively modify multiple traits related to obesity, representing a new route for treating metabolic disorders therapeutically.

FMT is a method of reconstructing the recipient's intestinal microbial environment by transplanting fecal microbiota from a donor into the recipient (Borody et al., 2012). This method has proven to be efficacious for investigating the role of the gut microbiota in the therapeutic approach to obesity. Transferring the gut microbiota from HFD mice to germ-free mice can induce a rise in both body weight and fat accumulation in recipient mice (Park et al., 2022). In a parallel experiment, the researchers noted the onset of obesity in the recipient germ-free mice following the transplantation of gut microbiota from ob/ob mice (Turnbaugh et al., 2006). Liao et al. (2018) found that the gut microbiota altered by resveratrol administration was capable of markedly reducing body weight, enhancing glycolipid metabolism, and promoting WAT browning in HFD-fed mice through FMT (Liao et al., 2018). Moreover, the transplantation of microbiota from nobiletin-treated mice into HFD-fed mice resulted in a substantial enhancement of energy expenditure, along with a marked increase in

thermogenesis-associated factors such as UCP1, PRDM16, Cidea, TFAM, and NRF1 in both the BAT and iWAT of the recipient mice (Kou et al., 2021). To additionally substantiate the gut microbiota as a potential target for LLE-induced browning, an experiment utilizing FMT was carried out. Administering LLE-modified microbiota triggered a browning effect of iWAT and BAT thermogenesis, accompanied by weight loss, elevated energy metabolism, and improved glucolipid metabolism, suggesting that LLE-mediated microbial is responsible for obesity alleviation and promoting the browning process. Take together, our findings establish LLE as a dual-targeted therapeutic agent that synchronizes gut microbiome modulation with adipose signaling activation, offering a novel mechanism to combat obesity through BAT-mediated thermogenic programming. However, our current study has certain limitations. First, the polypharmaceutical nature of LLE makes it challenging to attribute observed effects to specific bioactive compounds. Future research should focus on fractionating LLE to isolate and identify individual active constituents, such as quercetin 3-O glucuronide, and systematically investigating their direct interactions with BAT and the gut microbiota. Second, while FMT supports the involvement of gut microbiota in mediating LLE's benefits, the precise bacterial strains and metabolites responsible for BAT activation remain unclear. To address this, future studies could employ single-strain gavage experiments or utilize germ-free mouse models to dissect the causal relationships between specific microbes and metabolic outcomes. Finally, the long-term safety and optimal dosing of LLE require further investigation. Chronic toxicity studies and dose-response analyses are essential to establish the therapeutic

window and ensure the clinical translatability of our findings.

## **5. Conclusion**

This study demonstrated that LLE treatment reduced adiposity, improved glucolipid homeostasis, and elevated energy expenditure by upregulating BAT thermogenic markers. The underlying mechanism for these effects were potentially related to changes in the gut microbiota and associated metabolites, particularly BAs and linoleic acid derivatives. FMT directly linked these microbial shifts to LLE's metabolic benefits, confirming microbiota-driven enhancements in BAT activity and thermogenesis.

## **CRedit authorship contribution statement**

Zhenyu Wang: Writing-review & editing, Methodology, Funding acquisition, Conceptualization. Xiangwen Ren: Writing-original draft, Data curation, Formal analysis. Zhihanxin Peng: Writing-original draft, Software, Data curation. Maomao Zeng: Investigation, Supervision. Zhaojun Wang: Supervision. Qiuming Chen: Supervision. Jie Chen: Supervision. Xianjun Dai: Validation. Mark Christian: Writing-review & editing. Xuejiao Qie: Writing-review & editing, Validation, Supervision. Zhiyong He: Writing-review & editing, Funding acquisition, Project administration, Supervision.

## **Declarations of competing interest**

The authors declare no conflicts of interest.

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## Figure Legends

**Fig. 1.** Effects of LLE on body measurements, serum biochemical indicators, and energy expenditure. (A) Body weight change curve. (B) Body weight gain. (C) eWAT weight. (D) iWAT weight. (E) pWAT weight. (F) BAT weight. (G) TG level. (H) TC level. (I) LDL-C level. (J) HDL-C level. (K) BG. (L) AUC of BG. (M and N) O<sub>2</sub> consumption and AUC of O<sub>2</sub>. (O and P) CO<sub>2</sub> production and AUC of CO<sub>2</sub>. (Q and R) RER (VO<sub>2</sub>/VCO<sub>2</sub>) in light and dark. (S and T) EE and AUC of EE. (U) Physical activity. Statistical significance was assessed using nonparametric one-way ANOVA with GraphPad Prism. p-values were indicated as follows: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001.

**Fig. 2.** Effects of LLE on BAT pathology and function in HFD-fed mice. (A) H&E staining of BAT in different groups. (B and C) Immunohistochemistry for SIRT1 and UCP1.

**Fig. 3.** LLE induces  $\beta_3$ -AR/AMPK/p38 signaling in BAT of HFD-fed mice. (A) mRNA expression levels of thermogenesis-related genes (e.g., *Sirt1*, *Pgc-1a*, *Prdm16*, *Cidea*, *Ucp1*, *Dio2*, *Cox2/7A*, *Nrf1*, *Tfam*) and inflammation-associated genes (e.g., *Nox2*, *IL-6*, *TNF- $\alpha$* , *Cd11c*, *Mcp1*, *IL-1 $\beta$* , and *iNOS*) in BAT. (B) Protein expression levels of  $\beta_3$ -AR, p-PKA, PKA, p-AMPK $\alpha$ , AMPK $\alpha$ , p-p38, p38, SIRT1, TFAM, and UCP1 were measured by western blot. Statistical significance was assessed using nonparametric one-way ANOVA with GraphPad Prism. p-values were indicated as follows: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001.

**Fig. 4.** Effects of LLE on gut inflammation and microbiota structural analysis in

HFD-fed mice. (A) mRNA expression levels of inflammation-related genes (e.g., *Mcp1*, *iNOS*, *TNF $\alpha$* , *IL-6*, *Nox2*, *IL-1 $\beta$* , *Sod2* and *Sod3*). (B and C) Shannon and ACE index. (D) Venn diagram. (E) PCA scatter plot analysis. (F) Bacterial taxonomic profiling at the phylum level. (G) The relative abundances of Firmicutes, Bacteroidetes, Acidobacteriota, Actinobacteriota, Proteobacteria, and Firmicutes/Bacteroidetes ratio. Statistical significance was assessed using nonparametric one-way ANOVA with GraphPad Prism. p-values were indicated as follows: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

**Fig. 5.** Effects of LLE on key microbiota genera in HFD-fed mice. (A) LDA value distribution histogram. (B) Evolutionary branch diagram. (C) Heatmap of gut microbiota at the genus level with higher abundance. (D) The relative abundance of genera with statistical differences upon LLE treatment. Values with different lowercase letters indicate a significant difference at  $p < 0.05$  according to Duncan's multiple range tests using DPS software.

**Fig. 6.** Effects of LLE on fecal metabolites in HFD-fed mice. (A-B) OPLS-DA score scatter plot and permutation plot in ND vs. HFD groups. (C-D) OPLS-DA score scatter plot and permutation plot in HFD vs. LLE groups. (E) Hierarchical clustering dendrogram. (F and G) Volcano plot in ND vs. HFD groups and HFD vs. LLE groups. (H and I) The metabolic pathway impact prediction in ND vs. HFD groups and HFD vs. LLE groups based on the KEGG database. (J and K) Heatmap of 5 key metabolic pathway-related metabolites and the peak intensity of representative metabolites in HFD vs. LLE groups. Statistical significance was assessed using Statistical

significance was determined with a t-test with GraphPad Prism. p-values were indicated as follows: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

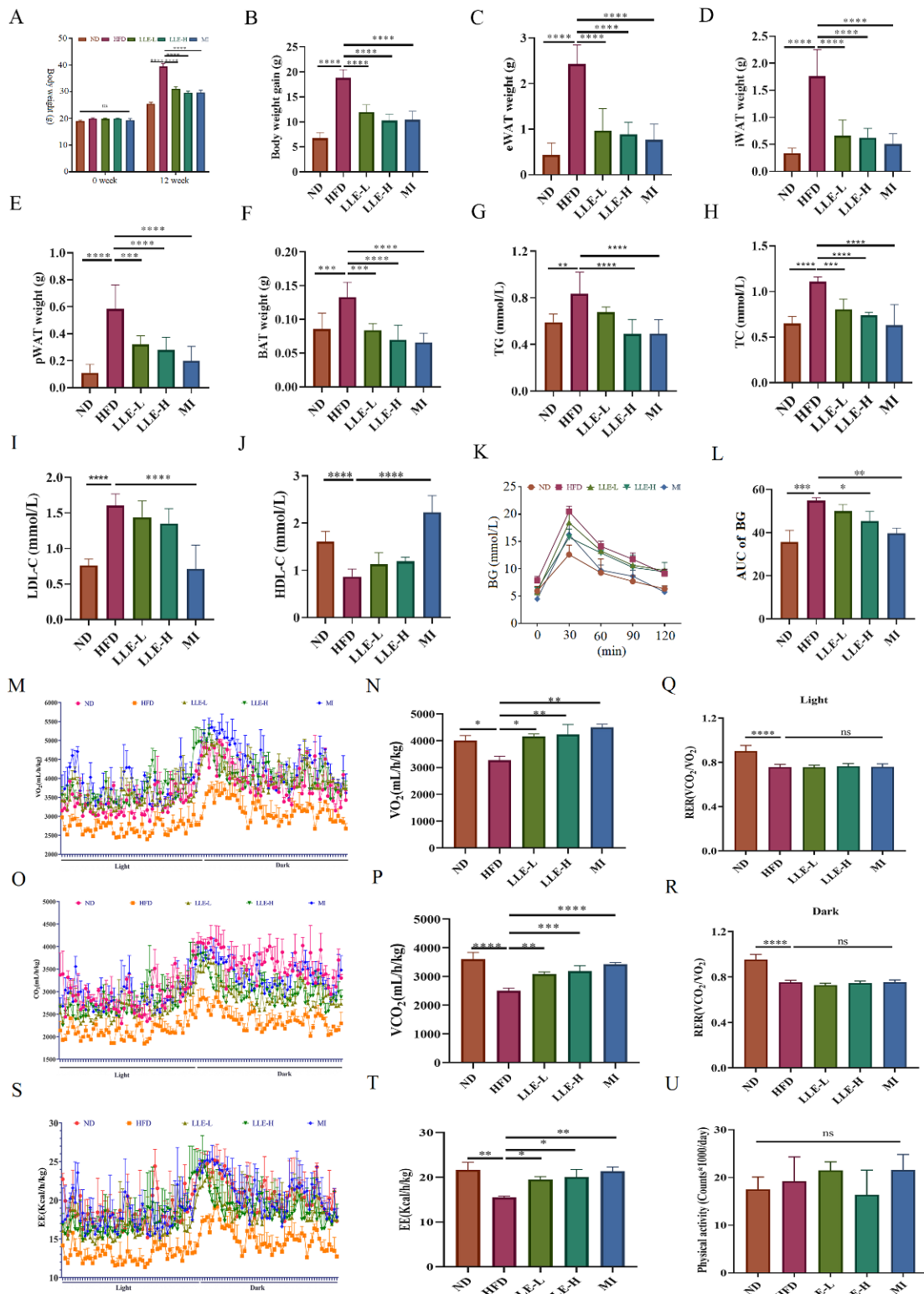
**Fig. 7.** The interrelationship between gut microbiota, key metabolic pathway-related metabolites, and the obesity- and brown fat-related indicators. (A) Correlation analysis of genera and obesity-related, brown fat-related, and inflammation-related indicators. (B) Correlation analysis of metabolites and genera or obesity- and brown fat-related indicators. (C) qPCR analysis of GPR120 and TRPV1 expression in the colon. WG: Body weight gain. Statistical significance was assessed using nonparametric one-way ANOVA with GraphPad Prism. p-values were indicated as follows: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

**Fig. 8.** Transplantation of feces from mice fed LLE attenuated HFD-induced obesity and promoted energy metabolism. (A) Schematic diagram of FMT in different groups. (B) Changes in body weight. (C) The body weight gain. (D) Food intake. (E) Liver weight. (F) Spleen weight. (G) Kidney weight. (H) iWAT weight. (I) pWAT weight. (J) eWAT weight. (K) BAT weight. (L) TG level. (M) TC level. (N) LDL-C level. (O) HDL-C level. (P) BG. (Q) AUC of BG. (R) H&E staining of BAT, iWAT, eWAT, and liver, respectively (200x). (S)  $O_2$  consumption and AUC of  $O_2$ . (T)  $CO_2$  production and AUC of  $CO_2$ . (U) EE and AUC of EE. Statistical significance was assessed using nonparametric one-way ANOVA with GraphPad Prism. p-values were indicated as follows: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

**Fig. 9.** Transplantation of feces from LLE donor mice enhanced BAT activity and induced WAT browning. (A) mRNA expression levels of brown fat and

inflammation-related genes in BAT. (B) Immunohistochemistry for UCP1 in BAT. (C and D) protein expression levels of SIRT1 and UCP1 in BAT. (E) mRNA expression levels of brown fat-related genes in iWAT. (F) Immunohistochemistry for UCP1 in iWAT. (G and H) protein expression levels of SIRT1 and UCP1 in iWAT. Statistical significance was assessed using nonparametric one-way ANOVA with GraphPad Prism. p-values were indicated as follows: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001.

867 **Fig. 1**



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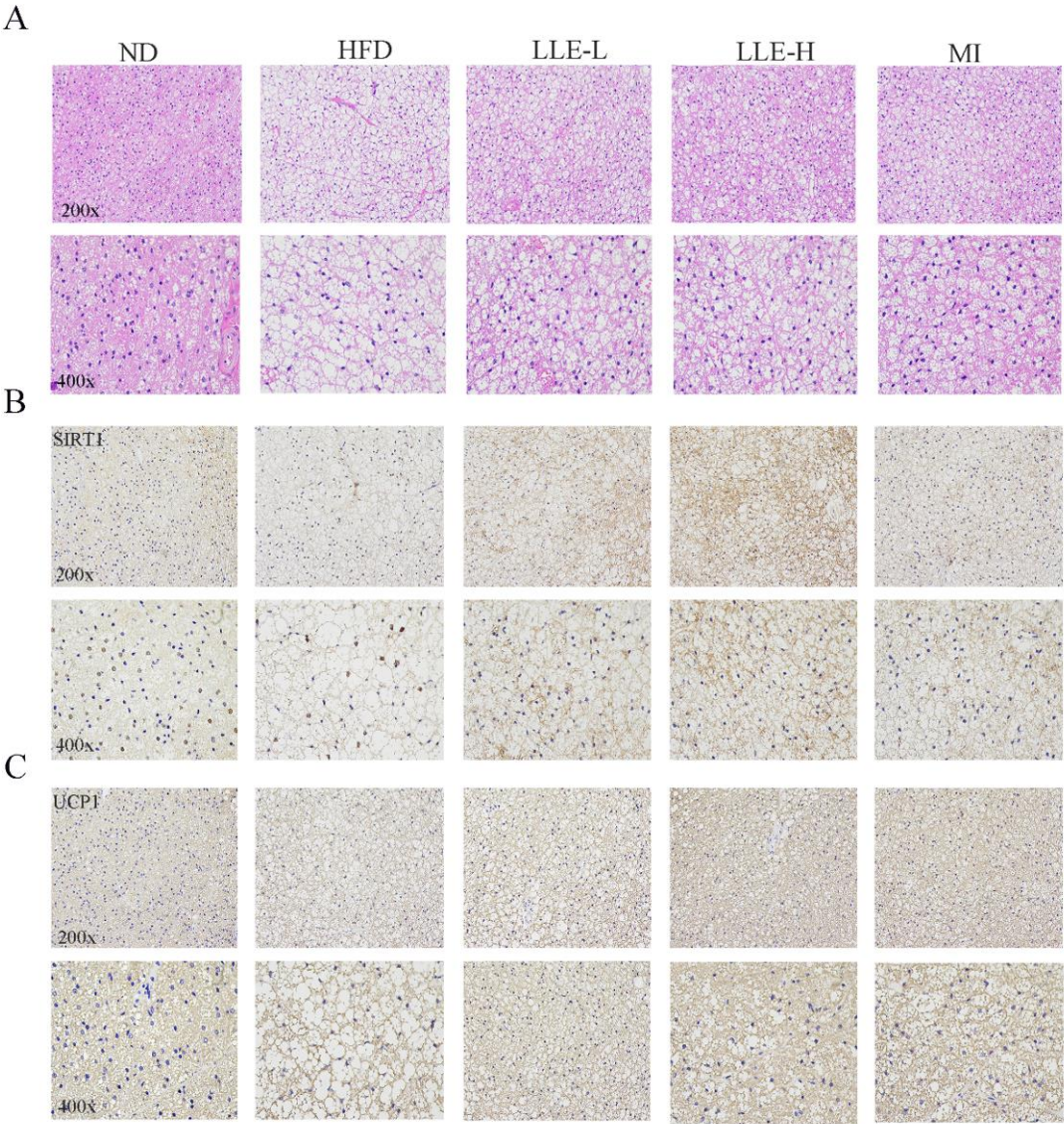
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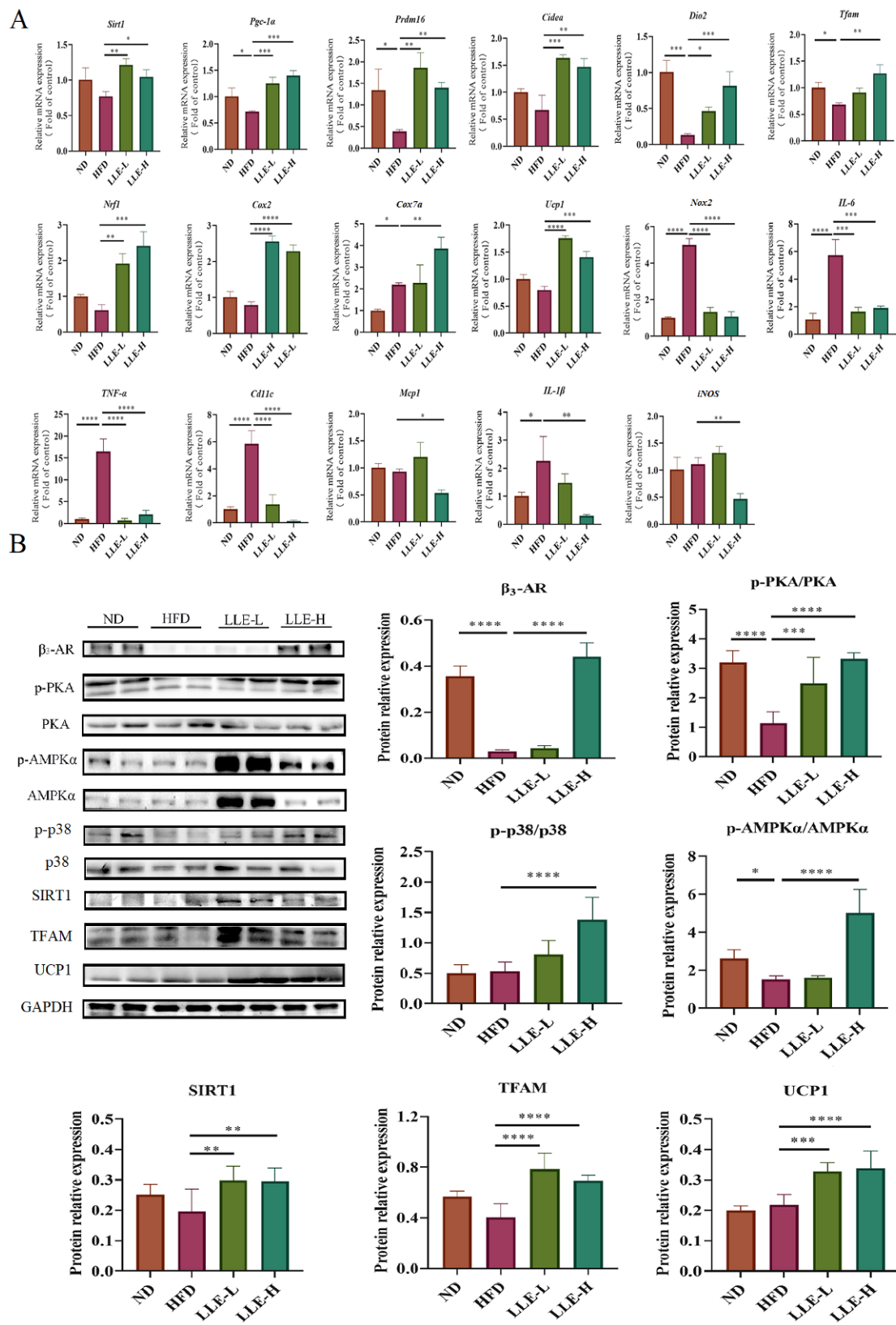
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**Fig. 2**





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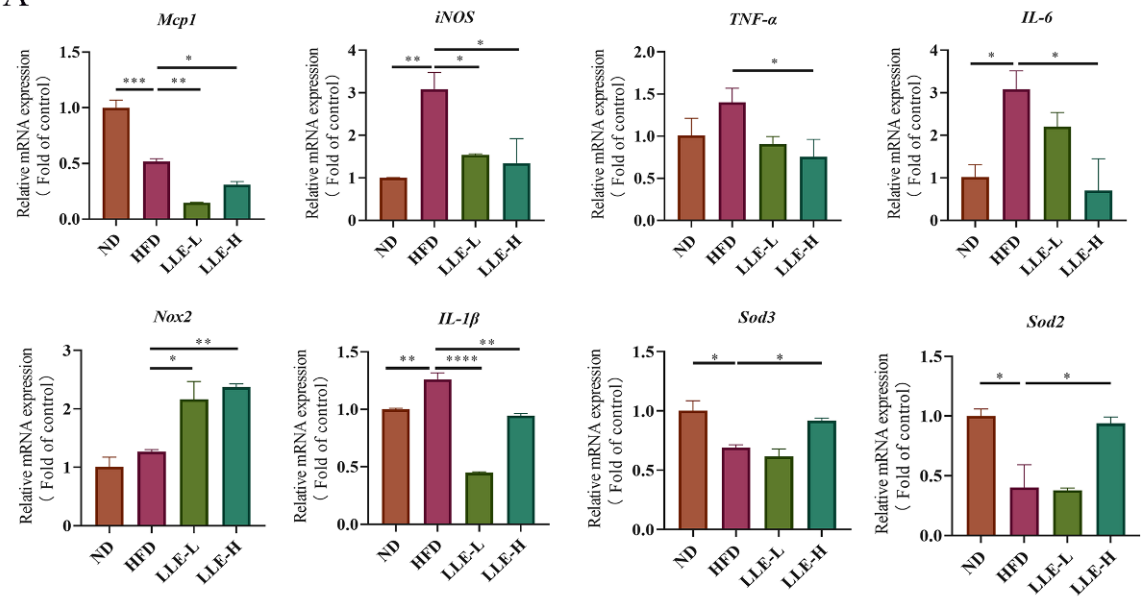
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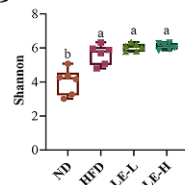
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895 **Fig. 4**

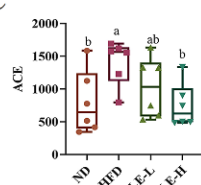
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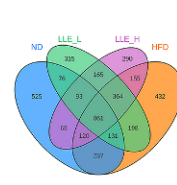
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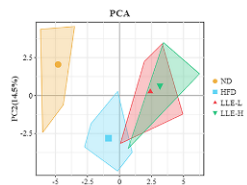
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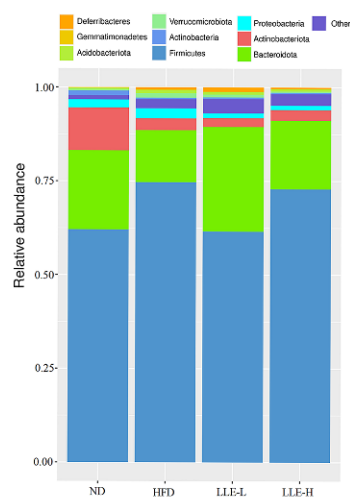
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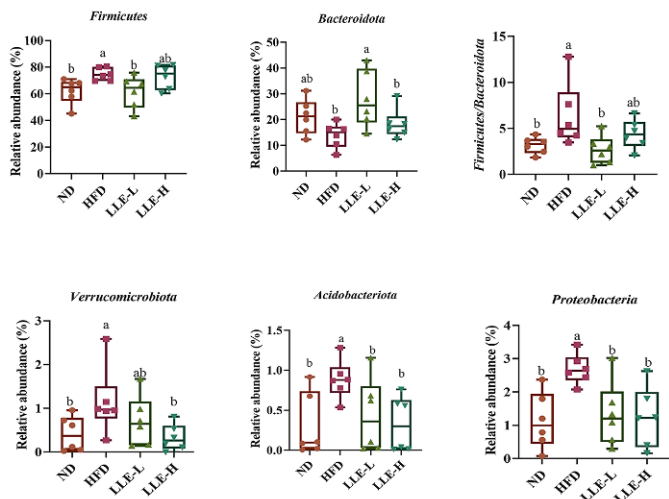
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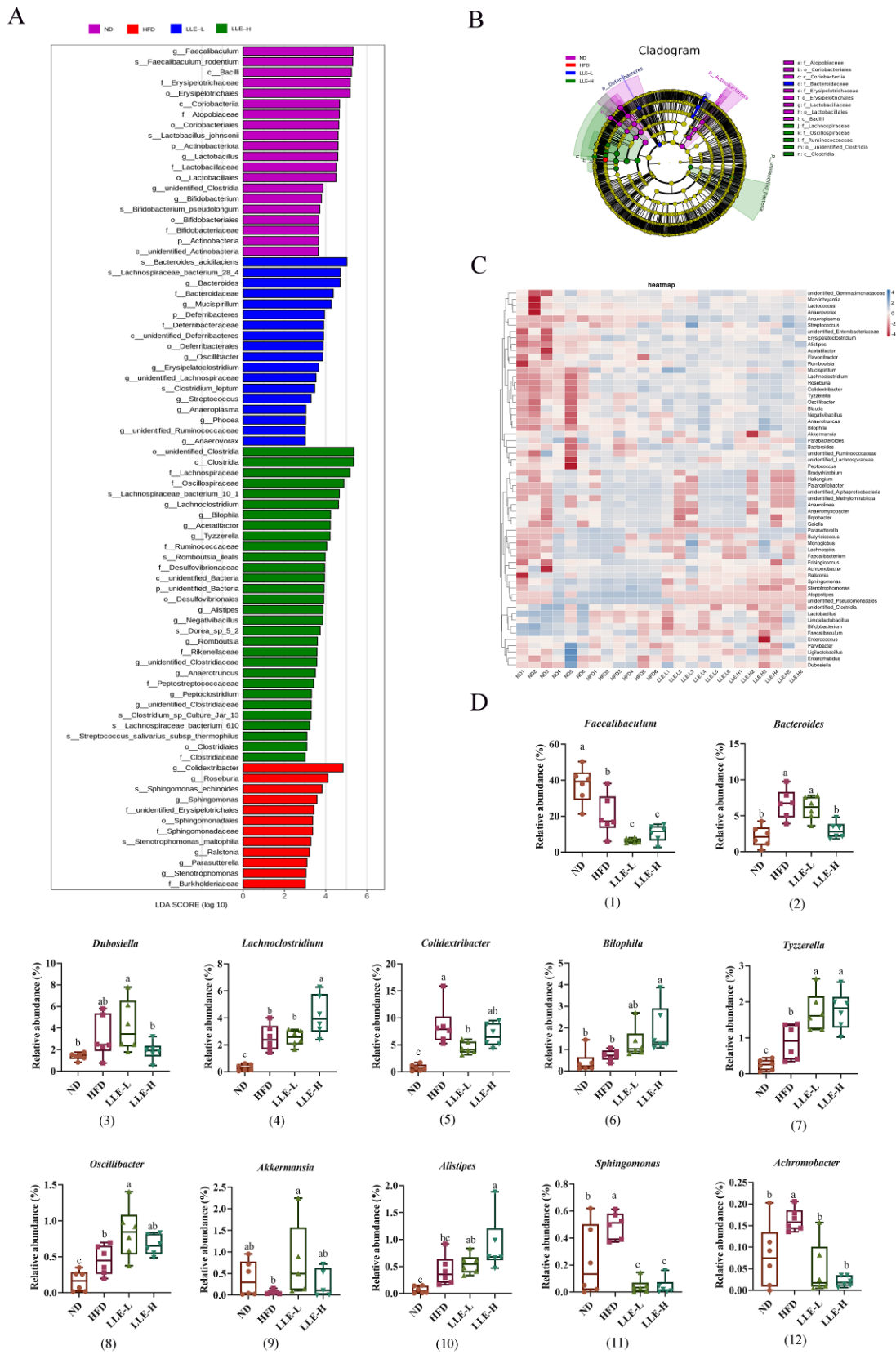
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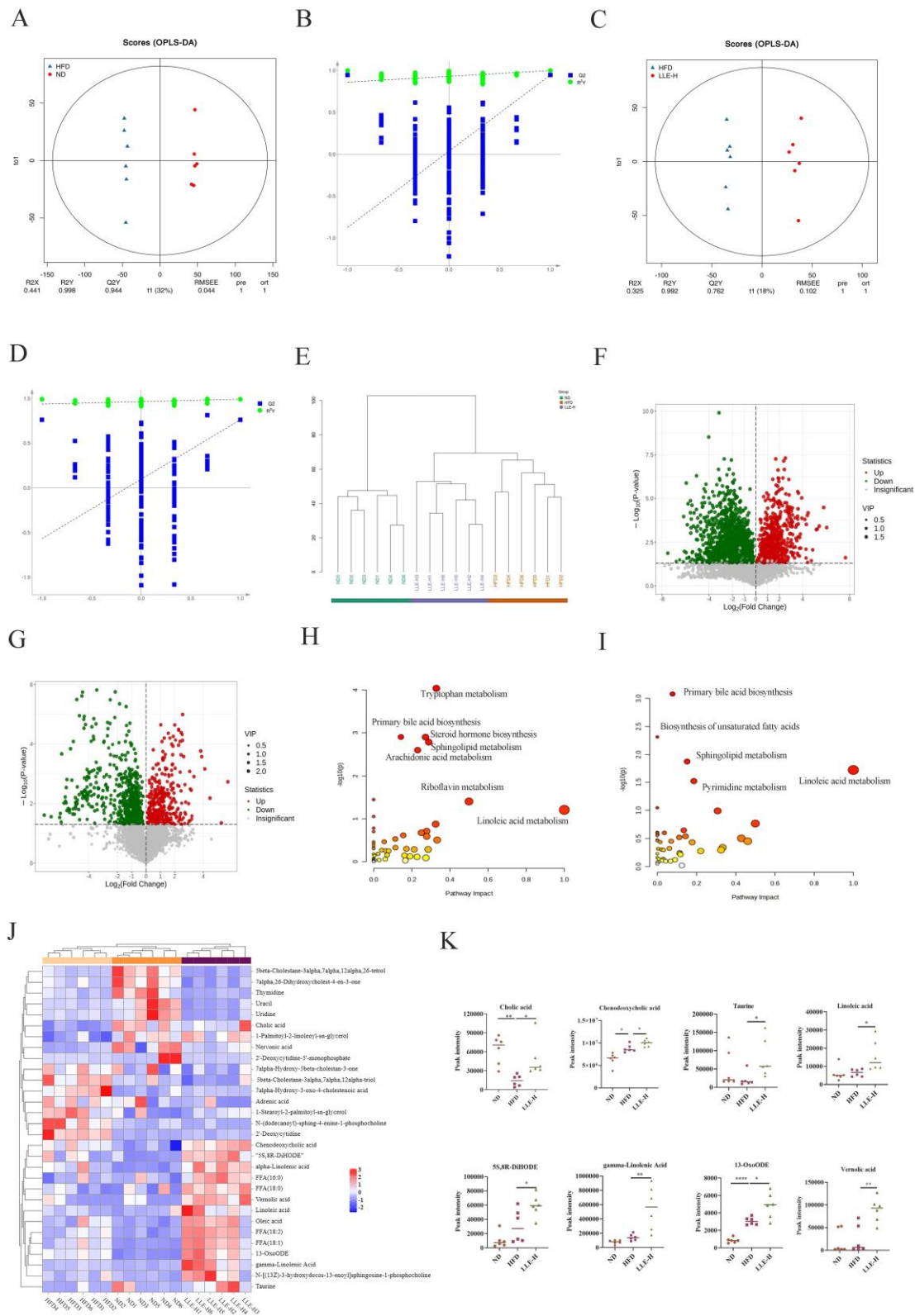
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908 **Fig. 6**



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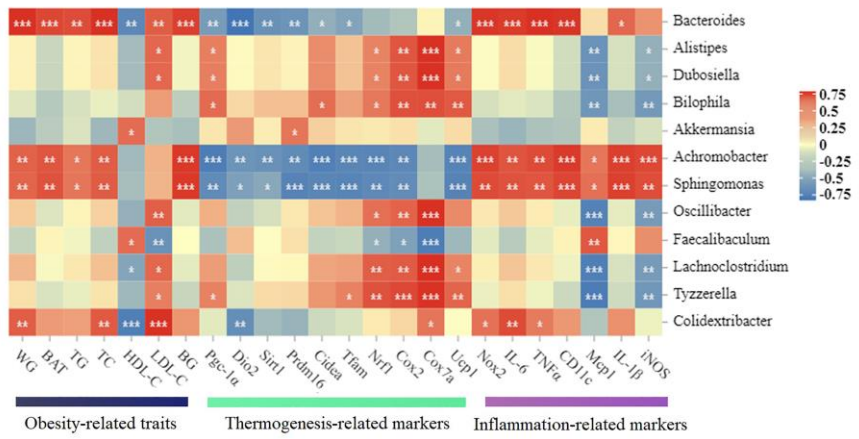
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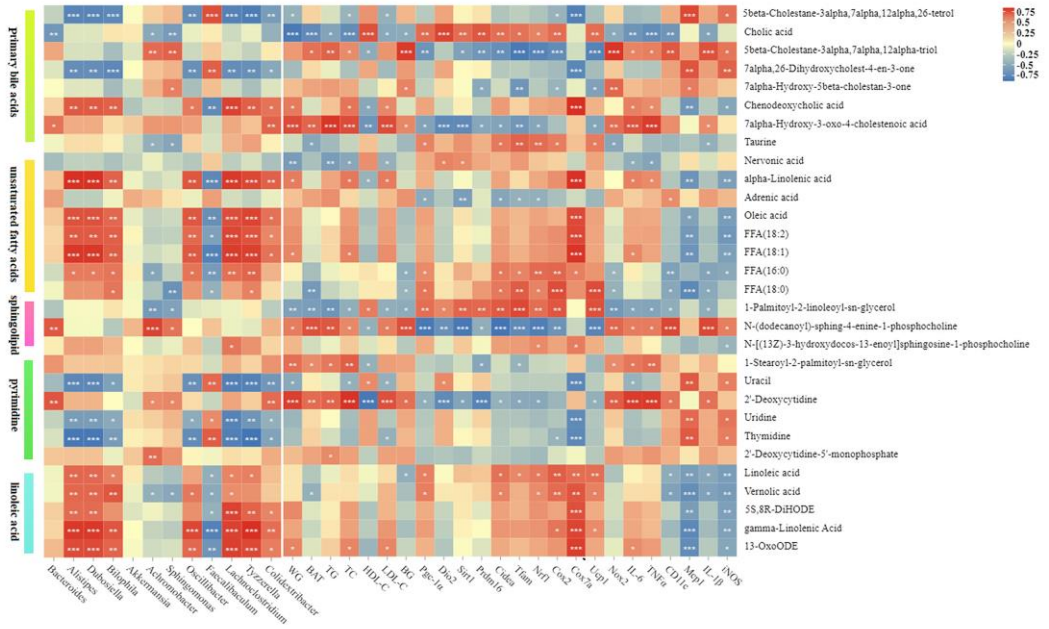
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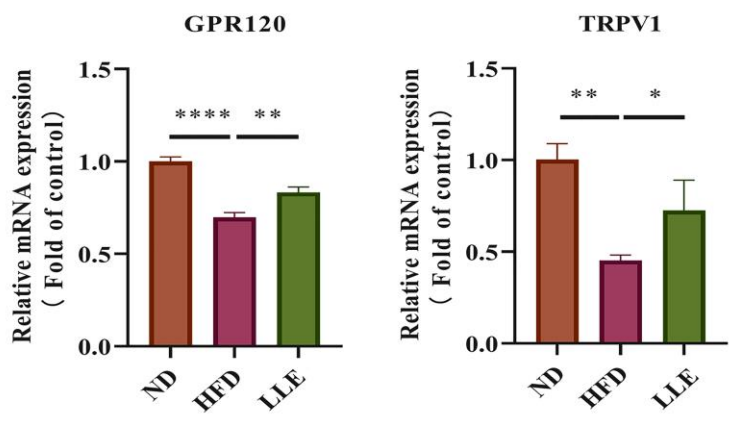


Fig. 8

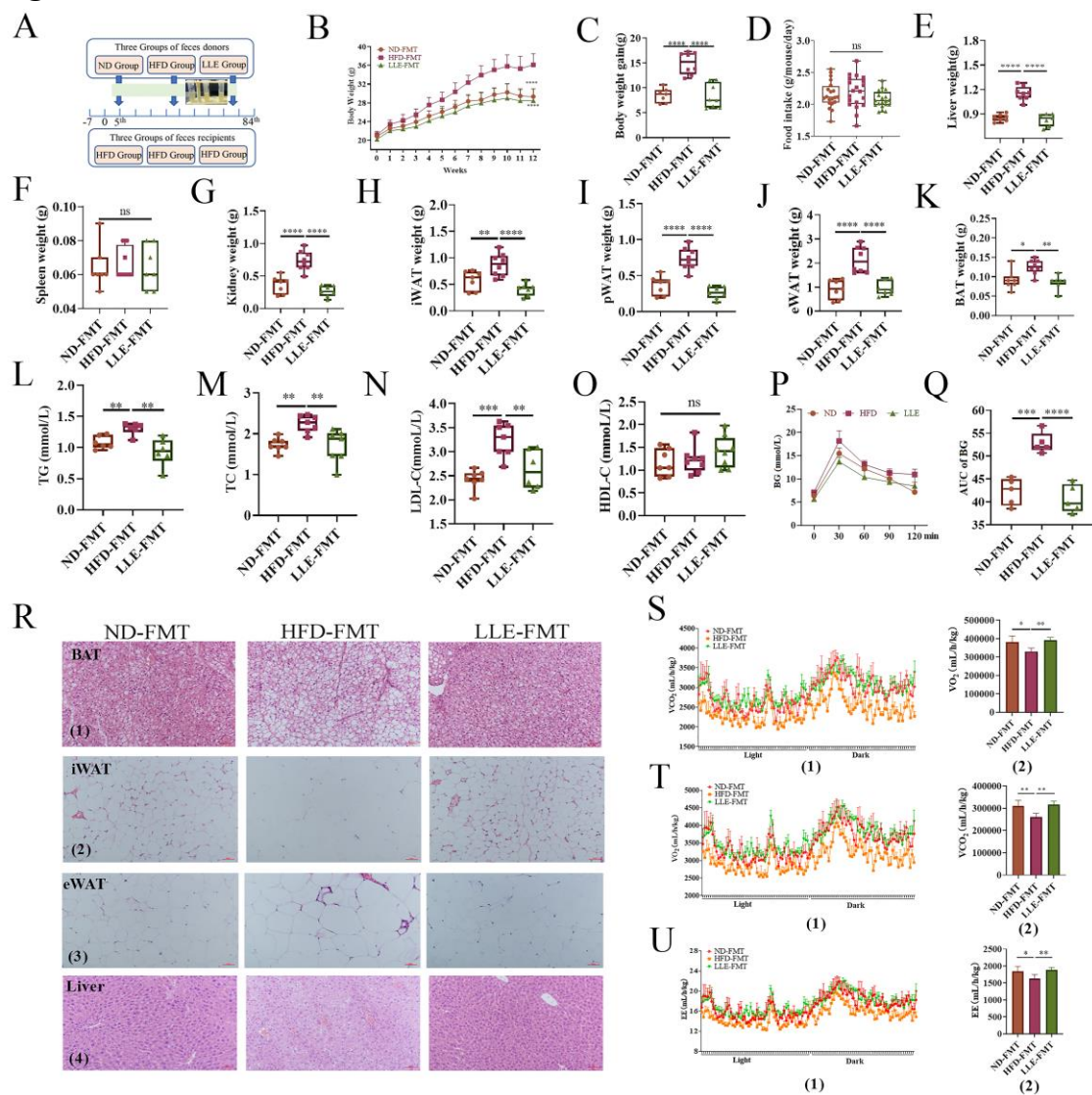
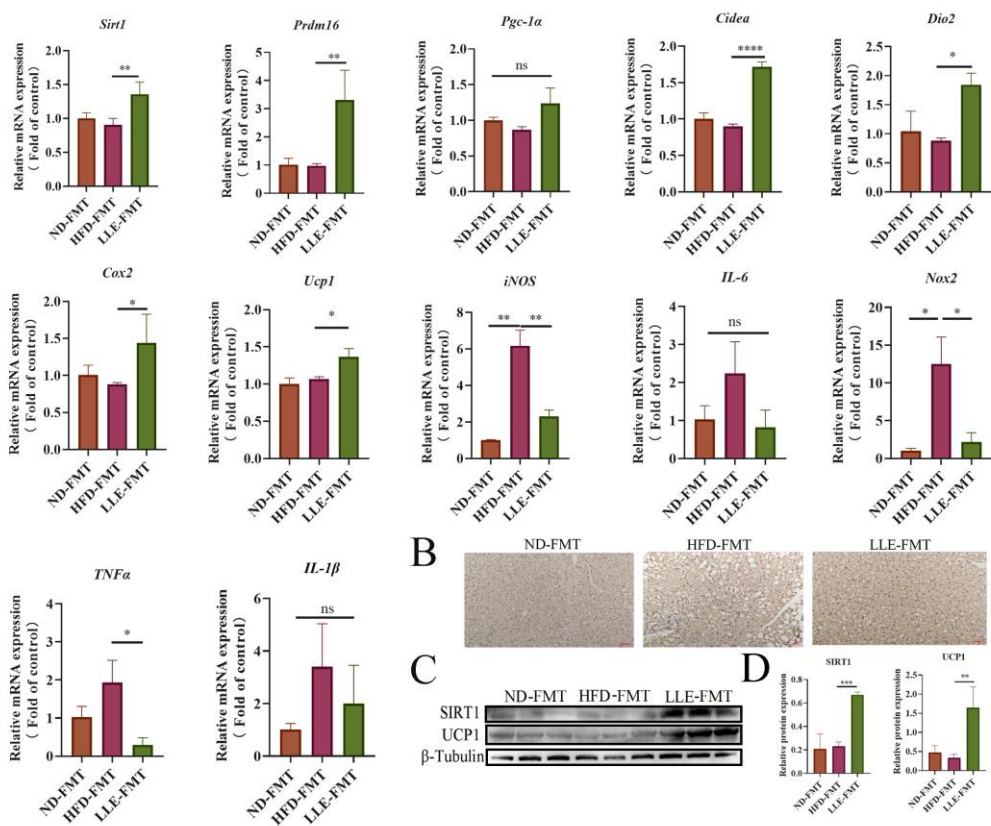
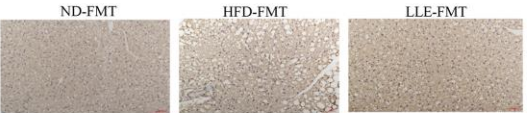


Fig. 9

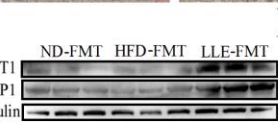
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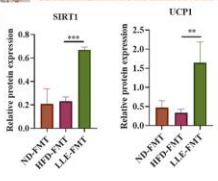
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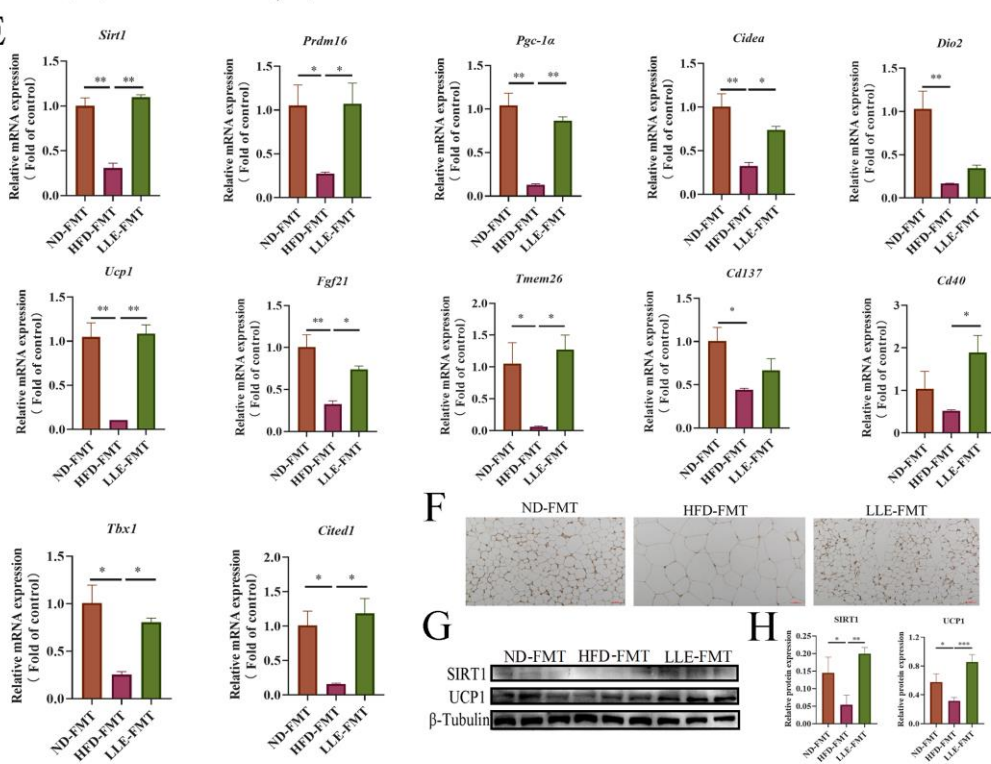
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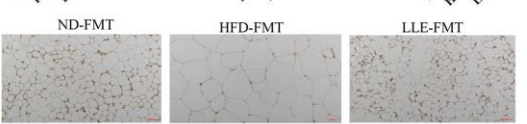
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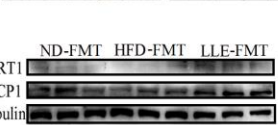
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