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## A new mechanism action of nuciferine to ameliorate high-fat diet-induced obesity: microbiota-dependent modulation of adipose browning and thermogenesis

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**ABSTRACT:** Nuciferine (NUC) is a major porphine alkaloid derived from *Folium Nelumbinis* with anti-obesity activity. Our previous *in vitro* findings demonstrated that the beneficial effects of NUC were associated with its induction of thermogenic fat, although the specific mechanisms of action remain to be fully elucidated. Here, our results showed that NUC (12.8 and 25.6 mg/kg/day) treatment for 12 weeks could effectively attenuate weight gain, activate brown adipose tissues (BAT), and promote browning of inguinal white adipose tissues (iWAT) in high-fat diet (HFD)-fed mice. Subsequently, the 16S rRNA sequencing showed that NUC was effective in ameliorating the HFD-induced microbiota imbalance, and the specific genera (e.g., *Lachnoclostridium*, *Roseburia*, *Limosilactobacillus*, *Tyzzelerella*, *Bilophila*, and *Weissella*) correlated strongly with brown-related features in BAT and iWAT. Meanwhile, metabolomics analysis revealed that NUC significantly increased the levels of microbial metabolites such as bile acids (e.g., taurocholic acid, chenodeoxycholic acid), myristic acid, and  $\gamma$ -linolenic acid, which have been previously described to turn fat “brown”. Importantly, *in vitro* experiments provided the first evidence of N-Acetylneuraminic acid (NANA)'s potential to activate brown adipocytes. Finally, a fecal microbiota transplantation (FMT) experiment identified that NUC-induced microbial alterations could increase energy expenditure (EE), BAT activity, and iWAT browning. Collectively, our findings demonstrated that NUC effectively promoted adipose browning and enhanced thermogenesis, linked to alterations in the gut microbiota and its metabolic byproducts, offering a novel therapeutic target for addressing obesity and associated metabolic disorders.

**Keywords:** Nuciferine, adipose browning, brown fat, gut microbiota, metabolomics, fecal microbiota transplantation

### 1. Introduction

Obesity develops as a consequence of sustained energy imbalance, where caloric input chronically exceeds physiological energy output. Guided by thermodynamic fundamentals, effective weight management strategies necessitate suppressing caloric intake and/or elevating metabolic energy dissipation<sup>[1]</sup>. While calorie restriction remains the primary approach to combat obesity, modulating metabolic efficiency, such as enhancing energy expenditure (EE) in adipose tissue, is a significant alternative strategy<sup>[2]</sup>. Research has

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identified two distinct types of adipose tissue in mammals: white adipose tissue (WAT) and brown adipose tissue (BAT)<sup>[3]</sup>. In terms of function, WAT primarily serves as the main site for triglyceride storage, whereas BAT specializes in EE and adaptive thermogenesis due to its high levels of uncoupling protein 1 (UCP1)<sup>[4]</sup>. Under specific stimuli, WAT can undergo a phenotypic transformation into thermogenically active beige adipocytes, a process termed "browning", which mimics the metabolic characteristics of BAT. As a result, promoting thermogenesis through the activation of BAT and browning of WAT is now viewed as a potential therapeutic approach to combat obesity and associated metabolic conditions. Recent findings imply that a variety of transcriptional regulators, along with their coregulators (e.g., PRDM16, PGC-1 $\alpha$ ), are pivotal in both the development and physiological functions of brown adipocytes and the metabolic conversion of white adipocytes to beige adipocytes<sup>[5]</sup>.

Nuciferine (NUC), a predominant aporphine-class alkaloid isolated from *Nelumbo nucifera* leaves, demonstrates significant therapeutic potential in mitigating adiposity and its associated metabolic complications, including insulin resistance and hepatic steatosis<sup>[6, 7]</sup>. Its effects have garnered substantial interest from the fields of medicine, dietetics, and pharmaceuticals. The suggested daily intake of raw lotus leaves for adults is in the range of 3 to 10 grams, as per the guidelines of the National Pharmacopoeia of the People's Republic of China, while the traditional decoction He-Ye Jiang-Zhi or the formula Shen-He Zhi-Gan, which incorporates lotus leaves, advises a dosage of 30 grams per day.<sup>8</sup> The raw lotus leaves contain about 0.1%-1.1% of nuciferine, so the dosage of NUC for adult humans is about 19-190 mg/day, which equates to about 2.9-28.5 mg/kg/day for mice<sup>[8]</sup>. Yan et al. (2023) discovered that NUC increased mitochondrial and thermogenesis-related genes in primary brown adipocytes through direct binding to RXRA<sup>[9]</sup>. Our previous studies demonstrated that NUC may also activate thermogenic fat through the 5-HT<sub>2A</sub>/AMPK signaling pathway<sup>[10]</sup>. Although the beneficial roles of NUC at the molecular level have been demonstrated, the contribution of other mechanisms to its action remains to be explored.

Emerging translational studies across preclinical models and human cohorts have established a causal link between gut microbial remodeling and metabolic reprogramming<sup>[11]</sup>, with microbiota-driven modulation of thermogenesis and adipose tissue browning now recognized as a key regulator of energy homeostasis. The study by Mestdagh et al.<sup>[12]</sup> is the first to propose a potential role for the gut microbiota in BAT activity. They found that germ-free mice whose BAT seemed to be more active burned calories faster than normal mice. In line with this investigation, Suárez-Zamorano et al. (2015) documented that microbiota depletion through antibiotic administration induced WAT browning in mice, characterized by elevated expression of thermogenic markers *Ucp1*, *Cidea*, *Pgc-1 $\alpha$* , and *Ppar $\alpha$* , along with increased mitochondrial density and multilocular lipid droplet formation in subcutaneous fat depots<sup>[13]</sup>. Integrated omics investigations now confirm that bacterial metabolites such as bile acids (BAs), short-chain fatty acids (SCFAs), and trimethylamine N-oxide (TMAO) serve as molecular intermediaries connecting microbial ecology to adipose tissue browning via epigenetic remodeling of thermogenesis-related gene clusters<sup>[14]</sup>. In this study, high-fat diet (HFD)-induced mice were used to investigate the preventive effect of NUC on obesity. Importantly,

microbiomics was coupled with non-targeted metabolomics and integrated with fecal microbiota transplantation (FMT) to elucidate whether the gut microbiota and its metabolites are important pathways by which NUC promotes WAT browning and BAT thermogenesis. Our findings reveal a novel therapeutic target for NUC by demonstrating its role in promoting adipose browning that holds significant promise for combating obesity and its associated metabolic disorders through enhanced EE and metabolic regulation.

## 2. Materials and Methods

### 2.1. Animals and Diets

**Experiment 1:** Five-week-old male C57BL/6J mice (n=40, weighing 15–18 g) were obtained from Beijing Witonglihua Laboratory Animal Technology Co., Ltd., China, and all procedures were conducted with the approval of the Animal Center of Jiangnan University (Approval No. JN.No20230415c1300730[142]). After one week of acclimation, the mice were randomly allocated into five groups for 12-week feeding: the normal diet (ND) group, the high-fat diet (HFD) group, the NUC-L group [HFD with oral gavage of 12.8 mg/kg NUC (purity: 98%, isolated from *Folium Nelumbinis* by Chengdu Herpurify Co., Ltd)], the NUC-H group (HFD with oral gavage of 25.6 mg/kg nuciferine), and the MI group (the positive control group, HFD with oral gavage of 5 mg/kg miglitol). The components of the ND and HFD can be found in Supplemental Table 1.

**Experiment 2:** Fecal microbiota transplantation (FMT) was performed based on an established protocol<sup>[15]</sup>. The additional mice (n=24) designated as recipients were administered a 200  $\mu$ L 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) through oral gavage for 7 days. Fecal samples were procured daily from donor mice (Experiment 1): the ND group, the HFD group, and the NUC group (mice on an HFD plus 25.6 mg/kg of NUC). These samples were 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): ND→HFD group (ND-FMT, n=8), HFD→HFD group (HFD-FMT, n=8), and NUC→HFD group (NUC-FMT, n=8).

### 2.2. Thermography and energy metabolism

At week 11, the rectal temperature of mice was determined by an electronic thermometer after oral administration bacterial solution for 30 min. The thermal imaging of mice was performed with an infrared thermal imaging camera (MAG32, Juge Electronic Technology Co., Ltd., Shanghai, China) under ambient temperature or cold exposure (4°C) conditions. To determine the EE, the CLAMS-12 system (Columbus Instruments, USA) was utilized. Each mouse was singly housed in a metabolic cage, and after 12 hours of acclimatization, their O<sub>2</sub> consumption and CO<sub>2</sub> production were continuously monitored for 24 hours. The data collected were then used to calculate the energy consumption of the mice.

### 2.3. Oral glucose tolerance test (OGTT)

At week 11, mice were fasted for 12 hours before collecting blood samples from the tail vein to assess fasting blood glucose (FBG) levels using a Roche glucometer. Afterward, an oral glucose solution was administered at a dose of 1.5 g/kg body weight. Blood glucose concentrations were measured at intervals of 0, 30, 60, 90, and 120 minutes following administration. The oral glucose tolerance test (OGTT) curve was constructed with time (minutes) on the x-axis and blood glucose concentration (mmol/L) on the y-axis, and the area under the curve (AUC) was subsequently calculated.

#### 2.4. Serum biochemical parameters

After a 12-hour fast, mice were anesthetized with sodium pentobarbital, and whole blood was obtained from the eyes and centrifuged at 3500 r/min for 10 minutes to obtain serum. Triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), blood urea nitrogen (BUN), creatinine (Cr), aspartate transaminase (AST), and alanine transaminase (ALT) were performed according to kit instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

#### 2.5. Histopathological analysis

The standard H&E staining protocol involved dewaxing paraffin-embedded tissue sections, including epididymal WAT (eWAT), inguinal WAT (iWAT), ~~BAT~~, liver, spleen, kidney, and colon in xylene, rehydration through graded ethanol series (100%, 95%, 80%, 70%), nuclear staining with Harris hematoxylin (10 min), differentiation in 1% acid alcohol (3 sec), bluing in Scott's tap water substitute (1 min), cytoplasmic counterstaining with 1% eosin Y (1 min), dehydration through ascending alcohols (70%, 80%, 95%, 100%), clearing in xylene, and mounting with neutral resin under coverslips for microscopic examination.

#### 2.6. Quantitative RT-PCR analysis

The total RNA was extracted using FastPure® Cell/Tissue Total RNA Isolation Kit V2 (Vazyme Biotech Co., Ltd., Nanjing, China). The cDNA synthesis and qPCR were conducted following our previous protocols<sup>[16]</sup>. The primer sequences are listed in Supplemental Table 2.

#### 2.7. Immunohistochemistry

Immunohistochemical staining of SIRT1 and UCP1 was carried out following the Immunohistochemistry Kit instruction (Servicebio, Wuhan, China) in agreement with our previous study<sup>[16]</sup>.

#### 2.8. Western blotting

Tissue lysates were prepared and analyzed via Western blotting following established protocols from our prior study<sup>[16]</sup>. Briefly, protein samples were separated by 10% SDS-PAGE, transferred to PVDF membranes (Millipore), and incubated with a primary antibody against UCP1 (Proteintech; 1:1000) at 4°C for 24 hours. The membranes were then washed with TBST and incubated with a secondary antibody (Proteintech; 1:4000) at 25°C for 1 hour. Finally, the immunoreactive bands were developed using BeyoECL Plus chemiluminescent substrate (Beyotime).

#### 2.9. Gut Microbiota analysis

Fecal microbial genomic DNA was isolated from mice stool specimens using the QIAamp Fast DNA Stool Mini Kit (Qiagen), followed by amplification of the 16S rRNA gene V3-V4 hypervariable regions and paired-end sequencing ( $2 \times 250$  bp) on the Illumina NovaSeq 6000 platform conducted by Metware Biotech Co., Ltd. (Wuhan, China). Bioinformatics processing involved quality filtering with Fastp, OTU clustering via USEARCH at 97% similarity, and taxonomic classification against the SILVA 138.1 database. The detailed procedure is shown in the Supplemental document.

### 2.10. Untargeted metabolomics analysis

Untargeted metabolomic profiling was conducted on fecal and serum samples ( $n=6$ ) using HPLC-QTOF-MS (Waters ACQUITY UPLC I-Class/Xevo G2-XS). Fecal metabolites were extracted via bead-beating homogenization in methanol-water (80:20 v/v) followed by centrifugation ( $15000 \times g$ , 10 min,  $4^\circ\text{C}$ ), while serum proteins were precipitated using cold acetonitrile-methanol (1:1 v/v). Raw data processing included peak alignment, annotation via CAMERA against HMDB and LipidMaps databases, and multivariate analysis (MetaboAnalyst 5.0).

### 2.11. Statistical Analysis

All data are expressed as the means  $\pm$  standard deviation (SD). One-way analysis of variance (ANOVA) with Duncan's test was used to determine statistical significance. Graph bars marked with different letters on top represent a significant difference.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  for comparisons of the ND, NUC-L, and NUC-H with the HFD group.

## 3. Results

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

HFD-fed mice exhibited pronounced increases in body weight gain (Fig. 1A and 1B), and increased weights of liver (Fig. 1C), eWAT (Fig. 1F), pWAT (Fig. 1G), iWAT (Fig. 1H), and BAT compared to ND control. However, NUC supplementation demonstrated dose-dependent attenuation of HFD-induced weight gain and metabolic dysregulation, achieving therapeutic outcomes equivalent to the MI group. Notably, neither food intake (Fig. 1B) nor spleen (Fig. 1D) and kidney weights (Fig. 1E) exhibited statistically significant alteration. Serum lipid analysis revealed that NUC effectively counteracted HFD-driven dyslipidemia by reducing TG (Fig. 1J), TC (Fig. 1K), and LDL-C (Fig. 1L) and OGTT (Fig. 1S and 1T) levels while elevating HDL-C (Fig. 1M) level, mirroring MI's therapeutic effects. Safety assessments indicated that no renal and splenic toxicity was observed in NUC-treated mice by measuring indicators, including ALT, AST, BUN, and Cr (Fig. 1N-Q).

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

To investigate whether the weight loss of NUC was attributed to the increase in energy metabolism,  $\text{O}_2$  consumption (Fig. 1U),  $\text{CO}_2$  production (Fig. 1V), and EE (Fig. 1W) were monitored, and the results showed that NUC significantly enhanced their levels. To evaluate the thermogenic capacity in mice, they were subjected to a cold environment at  $4^\circ\text{C}$  for 2 hours, during which their surface temperatures were monitored.

Thermographic imaging revealed that the skin temperature in the interscapular area of mice treated with NUC increased (NUC-L<sub>max</sub>: 35.51°C, NUC-H<sub>max</sub>: 35.59°C) following cold stimulation, which was closer to the temperatures observed in the ND group (ND<sub>max</sub>:34.12°C) and MI group (MI<sub>max</sub>: 34.70°C), and was notably higher than that of the HFD-fed mice (HFD<sub>max</sub>:32.28°C) (Fig. 1X).

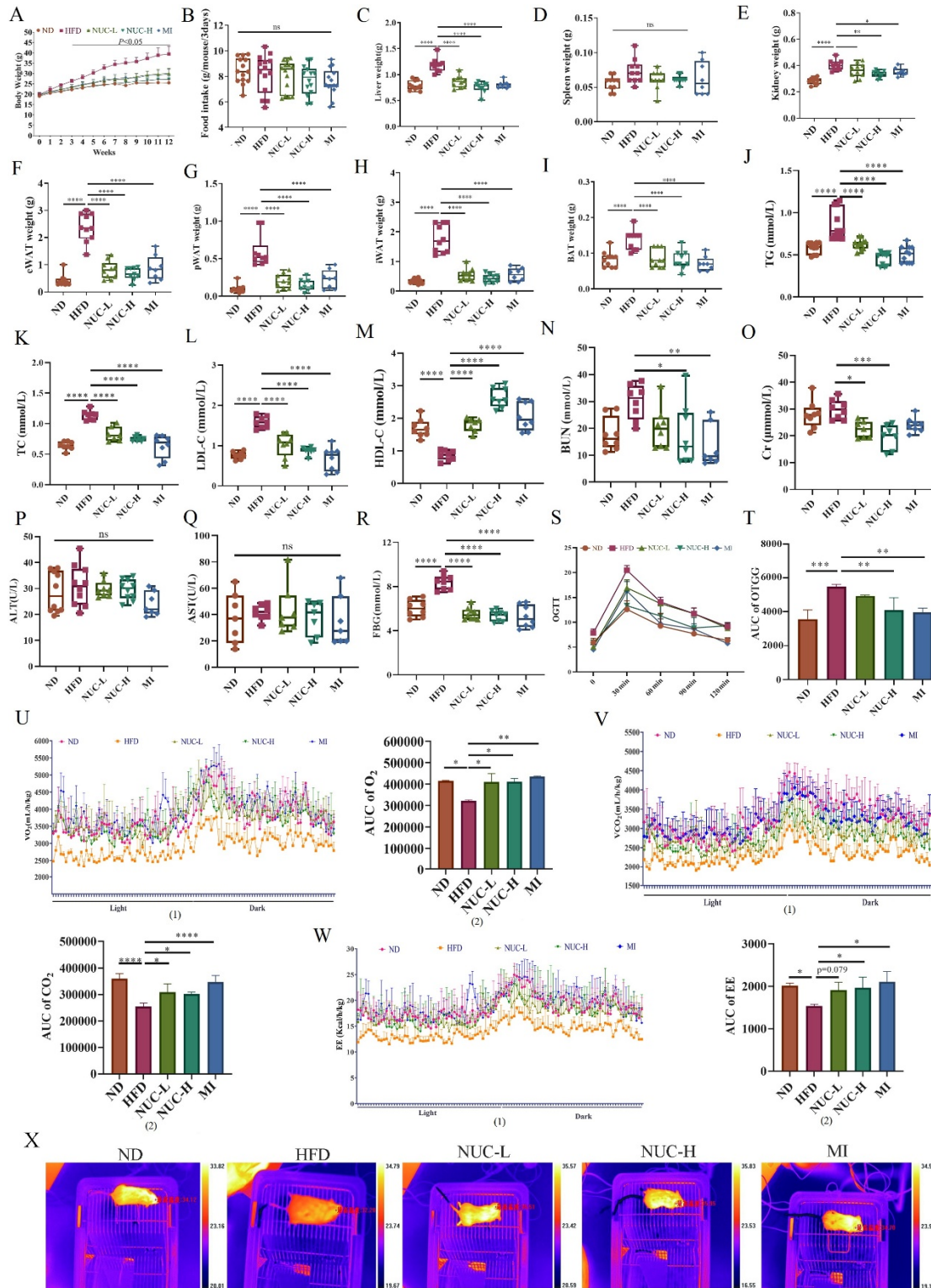
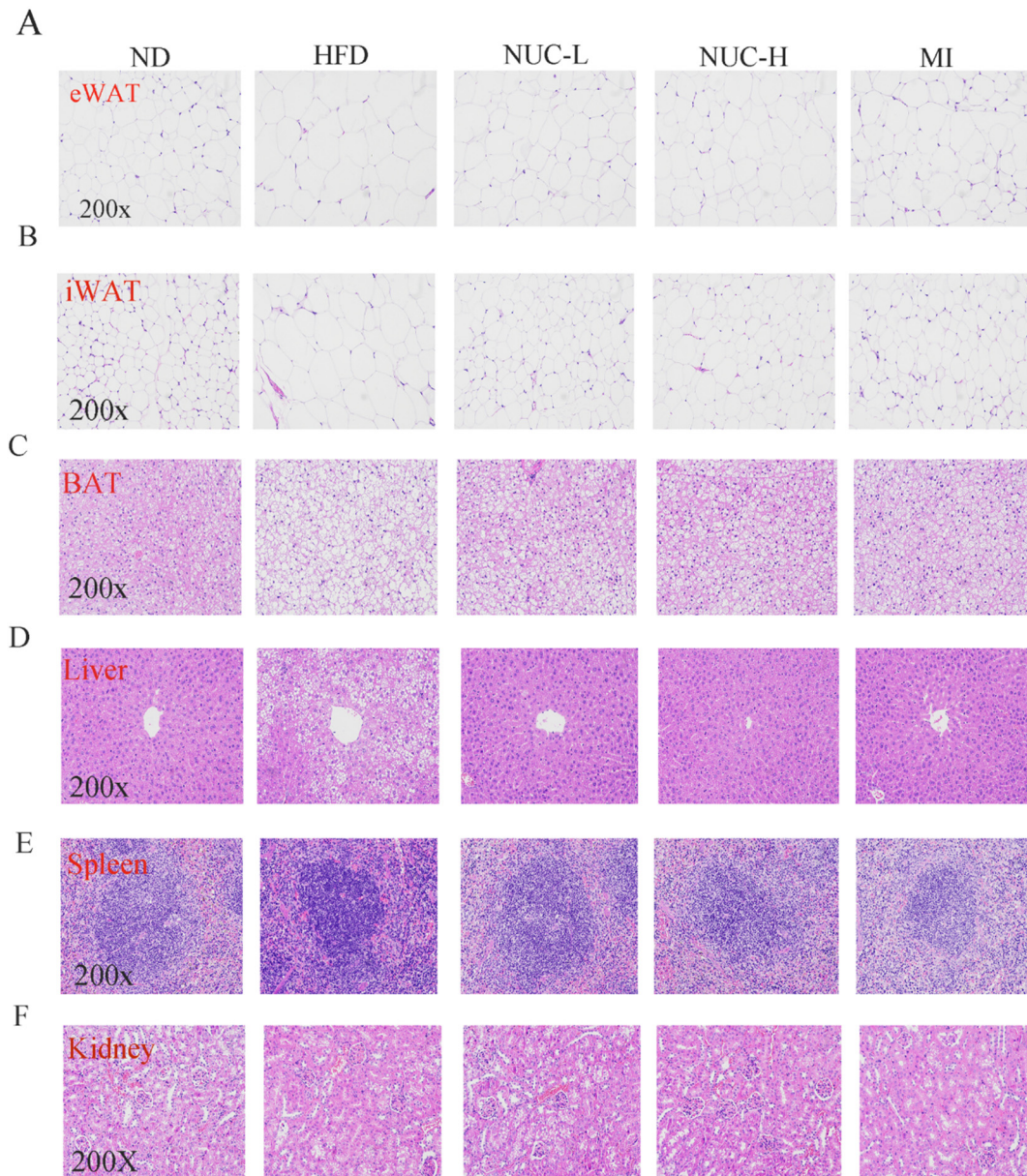


**Fig. 1** Effects of NUC on obesity-related indicators and energy metabolism. (A) Body weight change curve. (B) Food intake. (C) Liver weight. (D) Spleen weight. (E) Kidney weight. (F) eWAT weight. (G) pWAT weight. (H) iWAT weight. (I) BAT weight. (J) Serum TG level. (K) Serum TC level. (L) Serum LDL-C level. (M) Serum HDL-C level. (N) Serum BUN level. (O) Serum Cr level. (P) Serum ALT level. (Q) Serum AST level. (R) FBG level. (S) OGTT level. (T) AUC of glucose. (U) O<sub>2</sub> consumption curves and AUC of O<sub>2</sub> consumption. (V) CO<sub>2</sub> production curve and AUC of CO<sub>2</sub> production. (W) EE and AUC of EE. (X) Thermal images of mice in different groups (4 °C).

### 3.3. Effects of NUC on histopathology of HFD-fed mice

Thermogenic activation of adipose tissue enhances lipid catabolism, as evidenced by NUC treatment significantly reducing adipocyte hypertrophy in eWAT (Fig. 2A and 2G) and iWAT (Fig. 2B and 2H), while the area occupied by unilocular brown adipocytes in BAT (Fig. 2C and 2I) is reduced to levels comparable to the MI group. Microscopic analysis of liver sections (Fig. 2D and 2J) indicated that HFD-fed mice developed severe fatty liver disease with extensive lipid droplet deposition relative to the ND control group. However, administration of NUC significantly attenuated these hepatic alterations, resulting in a lower NAFLD activity score. Moreover, renal cortical sections revealed preserved glomerular architecture with attenuated tubular vacuolization in NUC-treated mice (Fig. 2F), consistent with significantly reduced serum biomarkers (BUN and Cr) of renal dysfunction (Fig. 1N and 1O). Splenic histology showed mitigated red pulp hyperplasia and retained follicular organization (Fig. 2E), consistent with stable splenic weight (Fig. 1D). These findings collectively confirm NUC's safety without observable treatment-related histotoxicological alterations.



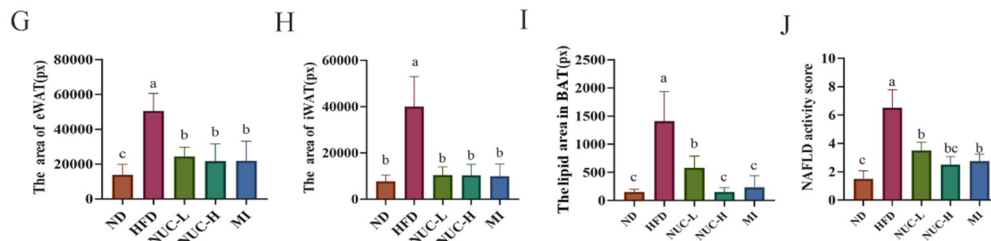


**Fig. 2** Effects of NUC on tissue pathology in HFD-fed mice. (A-F) H&E staining of eWAT, iWAT, BAT, liver, spleen, and kidney in different groups, respectively. (G and H) Adipocyte size of eWAT and iWAT. (I) Lipid accumulation in BAT. (J) NAFLD activity score.

### 3.4. Effects of NUC on the activity of BAT in HFD-fed mice

To identify NUC's impact on BAT activation, RT-qPCR analysis revealed dose-dependent upregulation of BAT-enriched thermogenic regulators, including *Pgc-1α*, *Cidea*, *Dio2*, *Tfam*, *Nrf1*, *Prdm16*, *Cox7a*, *Ppara*, and *Ucp1*, whereas *Nrf2* remained unaffected (Fig. 3A). Concurrently, lipid droplet remodeling genes *Atgl* and *Hsl* exhibited graded induction, suggesting enhanced lipolytic priming for fatty acid substrate supply. Immunohistochemical staining confirmed elevated SIRT1 and UCP1-positive area in the BAT of NUC-treated mice (Fig. 3B).

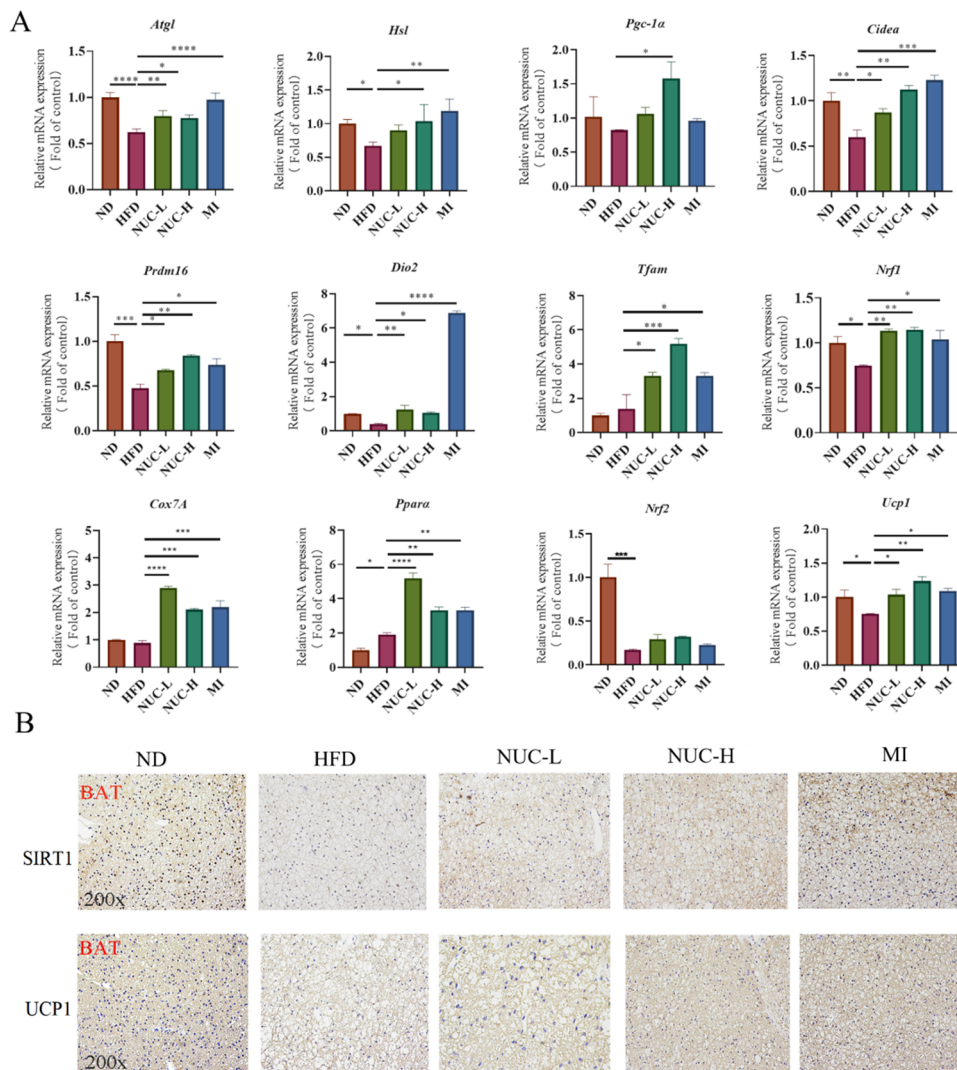
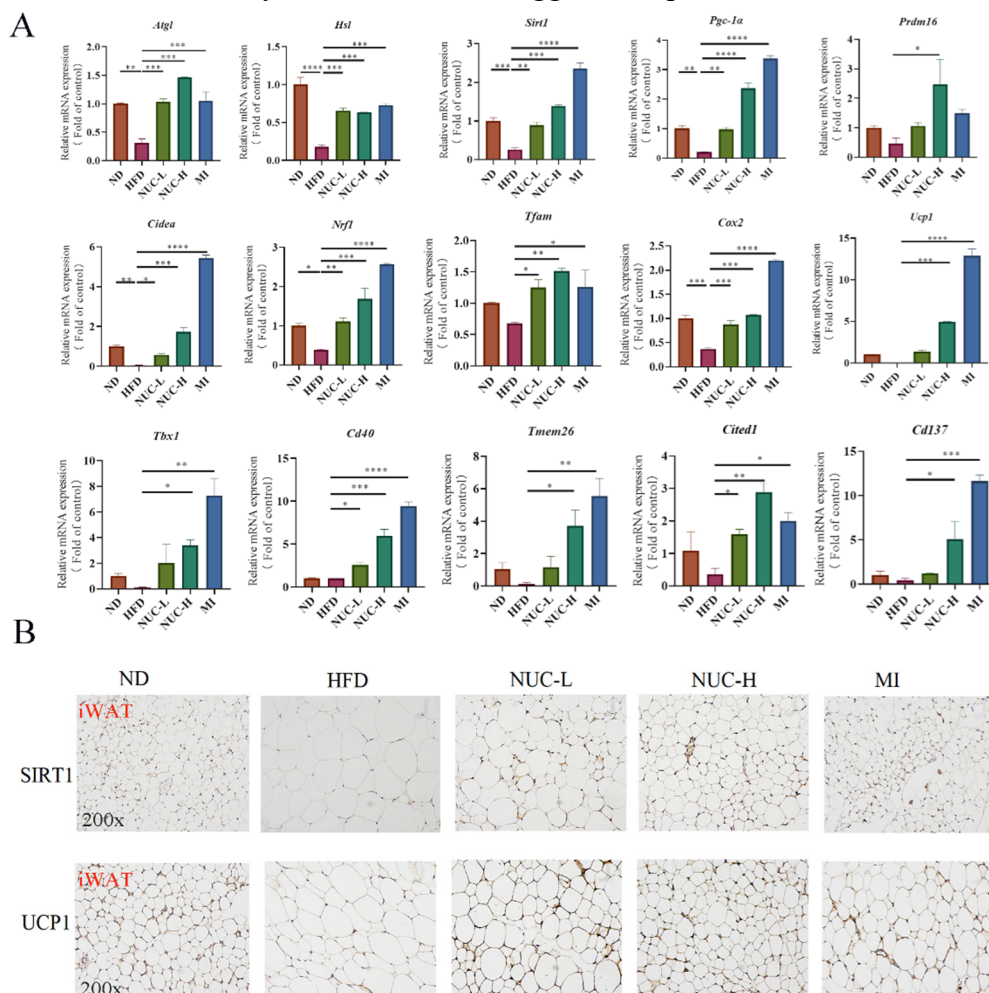


**Fig. 3** NUC enhances BAT activity in HFD-fed mice. (A) mRNA expression levels of brown and lipolysis-related genes (*Pgc-1α*, *Sirt1*, *Prdm16*, *Dio2*, *Cidea*, *Ucp1*, *Ppara*, *Cox2*, *Nrf1*, *Tfam*, *Atgl*, *Hsl*) in BAT. (B) Immunohistochemistry for SIRT1 and UCP1.

### 3.5. Effects of NUC on the beiging of iWAT in HFD-fed mice

WAT browning is intricately regulated by intracellular lipolysis, which mobilizes stored triglycerides to supply fatty acids for thermogenesis. Our investigations revealed that NUC treatment robustly enhanced the expression of key lipolytic genes (*Atgl* and *Hsl*) in a dose-dependent manner (Fig. 4A). Concurrently, NUC induced a robust thermogenic transcriptome signature in iWAT by upregulating BAT-rich genes, such as *Sirt1*, *Pgc-1α*, *Cidea*, *Prdm16*, *Tfam*, *Nrf1*, *Cox2*, and *Ucp1* in comparison to the untreated HFD control (Fig. 4A). Moreover, specific beige marker genes, such as *Tbx1*, *Cd40*, *Tmem26*, *Cited1*, and *Cd137* were also dose-dependently enhanced in NUC-treated mice, which were close to the positive group (Fig. 4A). Immunostaining of SIRT1 and UCP1 showed a higher positive area was observed in NUC-treated iWAT (Fig. 4B). To further elucidate the role of NUC in promoting fat browning, the functionality of NUC on mitochondrial respiration in 3T3-L1 adipocytes was assessed using the Agilent Seahorse XFe96 Analyser (experimental methods described in the Supplemental Material). Mitochondrial stress analysis revealed NUC increased maximum respiration and non-mitochondrial oxygen consumption in 3T3-L1 cells. ATP rate assay showed NUC treatment significantly elevated oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) relative to the control (Fig. 4C). The proportional glycoATP production rate increased by 2.30-fold, while the proportional mitoATP production rate was 1.23-fold higher compared to the control group, indicating that ATP production induced by NUC was mainly dependent on glycolysis. Together, the improvement of mitochondrial biosynthesis and OCR suggests the potential of NUC in WAT browning.



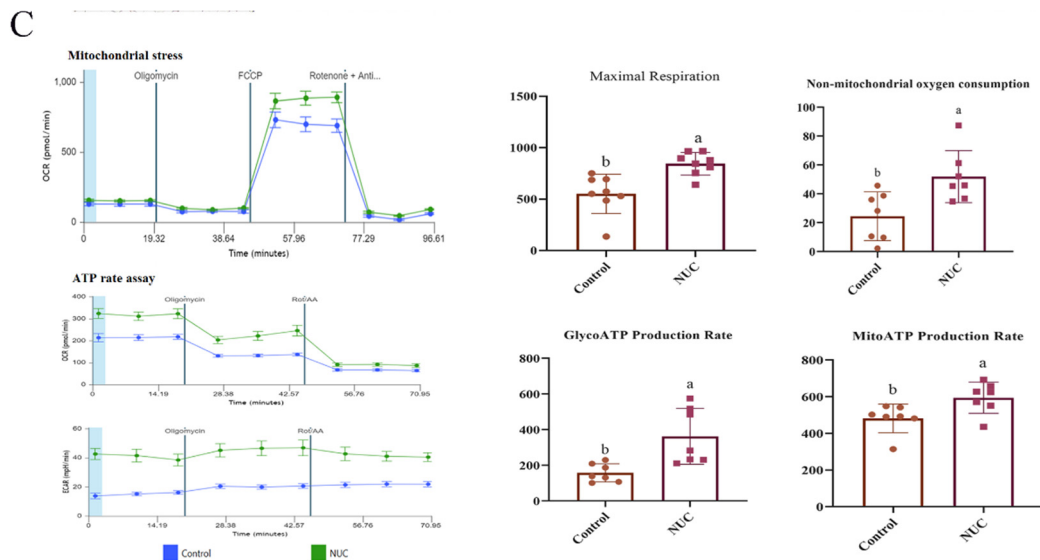


**Fig. 4** NUC stimulates WAT beigeing in HFD-fed mice. (A) mRNA expression levels of beige and lipolysis-related genes (*Pgc-1 $\alpha$* , *Sirt1*, *Prdm16*, *Cidea*, *Ucp1*, *Cox2*, *Nrf1*, *Tfam*, *Cd40*, *Cd147*, *Cited1*, *Tmem26*, *Tbx1*, *Atgl*, *Hsl*) in iWAT were determined by qPCR. (B) immunohistochemistry for SIRT1 and UCP1. (C) mitochondrial stress test (up) and ATP rate assay (down).

### 3.6. Effects of NUC on intestinal microecology in HFD-fed mice

The low bioavailability of NUC results in incomplete absorption, allowing it to reach the colon. Once there, it could be metabolized by the gut microbiota, which in turn could have an indirect impact on the host's metabolic processes. Assessment of gut inflammation *via* colon histology demonstrated that NUC treatment effectively prevented inflammatory cell infiltration, as evidenced by H&E staining results showing clear differences from the HFD group (Fig. 5A). Subsequently, mRNA analysis of inflammation markers in colon revealed the high dose of NUC markedly reduced *Mcp1*, *iNOS*, *TNF $\alpha$* , *IL-6*, *Nox2*, *Nox1*, and *IL-1 $\beta$*  without affecting *Sod3* expression (Fig. 5B), suggesting its beneficial effects on gut health. Analysis of fecal 16S rRNA sequencing in HFD-fed mice revealed comparable Shannon indices across HFD, NUC-L, and NUC-H groups (Fig. 6A), suggesting NUC treatment maintained microbial community diversity. Moreover, the reduced ACE was found in the NUC-treated mice compared with the HFD group, which was similar to that of the ND group (Fig. 6B), indicating that NUC treatment potentially decreased the community richness of microbiota. PCA scatter plot analysis demonstrated that microbiota structures in the NUC and HFD groups were separated from the ND group (Fig. 6C). In addition, the Venn diagram illustrates that the administration of NUC led to a reduction in the number of operational taxonomic units (OTUs), with the count resembling that of the ND group. It was observed that 873 OTUs (24.6% of the total) were common across all four groups. Additionally, there were 1320 (37.2%) OTUs shared exclusively between the ND and HFD groups, 1187 (33.4%) OTUs shared between the HFD and NUC-L groups, and 972 (27.4%) OTUs shared between the HFD and NUC-H groups. (Fig. 6D). UPGMA trees of gut microbiomes of the four groups based on unweighted UniFrac distance showed that the NUC-treated mice formed a distinct cluster that was separate from the HFD mice and more closely aligned with the ND mice. Specifically, HFD increased the *Firmicutes* (F) phylum relative to the ND group, but high dose of NUC treatment slightly reduced its richness (Fig. 6E and 6F (1)); HFD potentially decreased the *Bacteroidota* (B) phylum, but high dose of NUC treatment could reverse the

trend (Fig. 6E and 6F (2)). Furthermore, the F/B ratio in the NUC-H-treated mice exhibited a downward trend, although the change was not statistically significant when compared to the F/B ratio observed in the HFD group (Fig. 6E and 6F (3)). *Proteobacteria*, an important phylum of gram-negative bacteria, presented higher abundance in HFD-fed mice, but NUC treatment restored it to a normal level (Fig. 6E and 6F (4)). LEfSe analysis identified 83 key OTUs across dietary groups, with 6 phyla and 28 genera showing significant differences (Fig. 6G). The NUC-L group was enriched in *g\_Colidextribacter*, *g\_unidentified\_Lachnospiraceae*, *g\_Lachnospiraceae\_bacterium\_610*, while the NUC-H group had higher prevalence of *g\_Mucispirillum*, *g\_Oscillibacter*, *g\_Bilophila*, and *g\_Tyzzereella*. In addition, 39 genera with high abundance are shown in Supplemental Fig. 1. Relative to the ND group, the abundance of *Colidextribacter*, *Lachnospiraceae*, *Lachnociostridium*, *Roseburia*, *Mucispirillum*, *Tyzzereella*, *unidentified Enterobacteriaceae*, *Acetatifactor*, *Oscillibacter*, *Alistipes*, and *Stenotrophomonas* were enriched in the HFD group (Fig. 6H). Compared to the HFD group, NUC-L significantly reversed the changes of *Roseburia* and *unidentified Enterobacteriaceae* (Fig. 6I); NUC-H markedly reversed the changes of *unidentified Lachnospiraceae*, *Stenotrophomonas*, *Lachnoclostridium*, *Roseburia*, and *unidentified Enterobacteriaceae* (Fig. 6J). Notably, some bacteria, including *Bilophila*, *Limosilactobacillus*, *unidentified\_Clostridia*, and *Weissella*, were dominantly present in the NUC-H group (Fig. 6J). Spearman's correlation analysis showed *unidentified\_Lachnospiraceae*, *Lachnoclostridium*, *Roseburia*, *Ligilactobacillus*, *Stenotrophomonas*, and *unidentified\_Enterobacteriaceae* were negatively correlated with brown/beige fat-related markers in BAT/iWAT and positively correlated with obesity-related indicators (e.g., TG, TC, HDL-C, LDL-C, FBG, BG) ( $P < 0.05$ ,  $R > 0.6$ ) (Fig. 6K and 6L). In addition, *Bilophila*, *Limosilactobacillus*, *Lactiplantibacillus*, *Peptococcus*, *Tyzzereella*, *Anaeroplasma*, *unidentified\_clostridia*, and *Weissella* highly correlated positively with brown/beige fat-related markers in BAT/iWAT and correlated negatively with obesity-related indicators ( $P < 0.05$ ,  $R > 0.6$ ) (Fig. 6K and 6L).

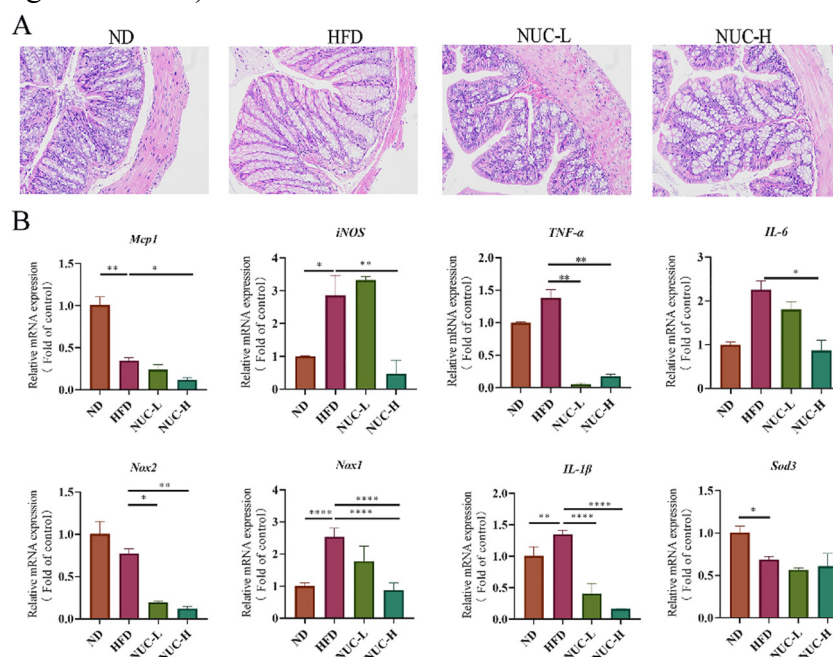


**Fig. 5** NUC reduces gut inflammation in HFD-fed mice. (A) H&E staining of colon. (2) mRNA expression levels of inflammation-related genes (e.g., *Mcp1*, *iNOS*, *TNFα*, *IL-6*, *Nox2*, *Nox1*, *IL-1β*, and *Sod3*) were determined by qPCR.

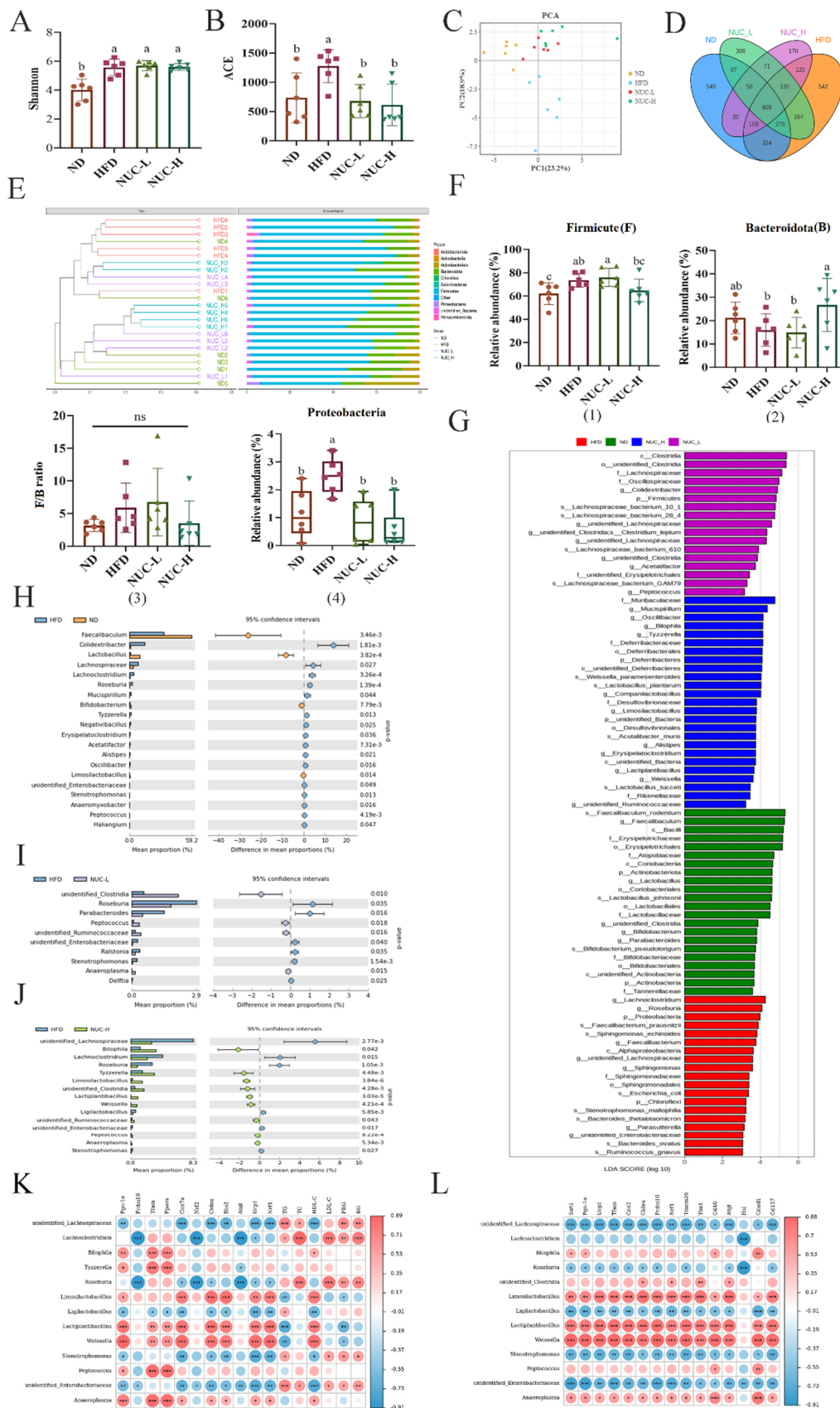
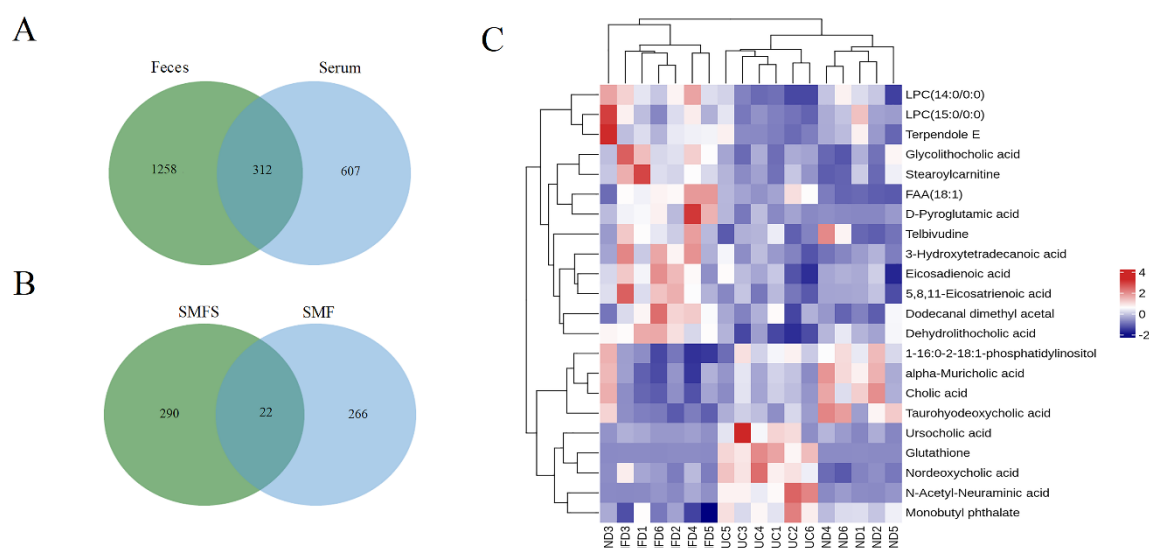


**Fig. 6** NUC reshapes the microbiota composition in HFD-fed mice. (A) Shannon index. (B) Ace index. (C) PCA scatter plot analysis of the fecal microbiota among the three groups. (D) Venn diagram. (E) UPGMA cluster analysis and dendrogram. (F) Differences in the abundances of *Firmicutes*, *Bacteroidetes*, the ratio of *Firmicutes/Bacteroidetes*, and *Proteobacteria*. (G) LefSe analysis (LDA value distribution histogram) identifies the gut microbiota phylotypes in different groups (LDA>3). (H-J) Extended error bar plot identifying significant differences between ND versus HFD, HFD versus NUC-L, and HFD versus NUC-H. (K and L) Spearman's correlation analysis of the 13 genera and obesity-related indicators and brown fat-enriched genes in BAT and iWAT, respectively. BG: body weight gain.

### 3.7. Effects of NUC on fecal and serum metabolites in HFD-fed mice

VIP >1,  $P < 0.05$ , and  $|FC| > 1.5$  were used as thresholds to screen for significant metabolites in feces. Among 1568 metabolites, 164 metabolites were significantly increased and 344 metabolites were significantly decreased in the HFD vs. ND group (Supplemental Fig. 2A). In the HFD vs. NUC (high dose at 25.6 mg/kg) group, 81 metabolites were significantly increased and 201 metabolites were significantly decreased (Supplemental Fig. 2B). With the same thresholds for differential metabolites screening in serum, among 919 metabolites, 108 metabolites were significantly increased and 45 metabolites were significantly decreased in the HFD vs. ND group (Supplemental Fig. 2C). In the HFD vs. NUC group, 39 metabolites were significantly increased and 71 metabolites were significantly decreased (Supplemental Fig. 2D). When the fecal and serum metabolites of the HFD vs. NUC group were analyzed with a Venn diagram for intersection, a total of 312 shared metabolites were identified (Fig. 7A), with 22 significantly changed metabolites in the feces (Fig. 7B), primarily consisting of bile acids (BAs) as indicated by the heatmap (Fig. 7C and 7D). Notably, a significant increase in the intensity of the N-Acetylneuraminic acid (NANA) peak was also found in the HFD vs. NUC group (Fig. 7D). Metabolites with significant changes in serum were 19 (Fig. 7E), as shown in the heatmap (Fig. 7F). It is noteworthy that the peak intensity of taurocholic acid, chenodeoxycholic acid (CDCA), myristic acid,  $\gamma$ -linolenic acid, and NANA were significantly increased in the serum of the NUC group (Fig. 7G). Serum-targeted metabolic analysis showed that NUC increased NANA levels (1.5-fold) (Supplemental Fig. 3). Correlation analysis showed that NANA showed positive correlation with *Oscillibacter Tyzzerella*, *Bilophila*, and *Limosilactobacillus* while negative correlation with *Faecalibaculum*, *Sphingomonas*, and *unidentified\_Lachnospiraceae* (Supplemental Fig. 4). Importantly, NANA positively correlates with UCP1 expression in BAT and iWAT (Supplemental Fig. 5). NANA (1 and 10  $\mu\text{g/mL}$ ) stimulation for 24 h induced mRNA levels of thermogenic genes (*Sirt1*, *Ppargc1a*, *Prdm16*, *Cidea*, *Dio2*, *Cox2*, *Nrf2*, and *Ucp1*) and lipolytic genes (*Atgl* and *Hsl*) in C3H10T1/2 brown adipocytes (Supplemental Fig. 6). Therefore, NANA has a role in activating brown fat.



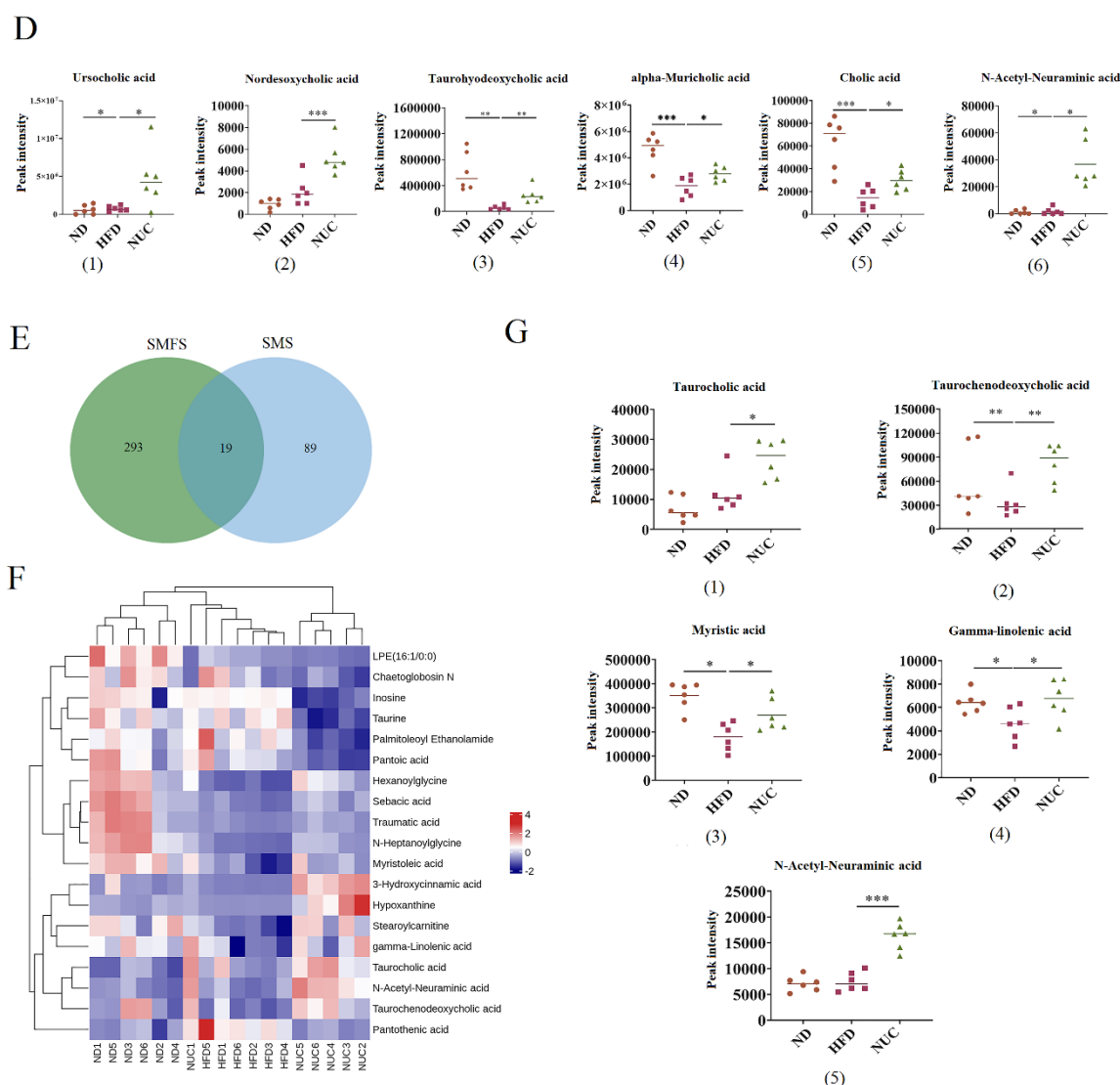
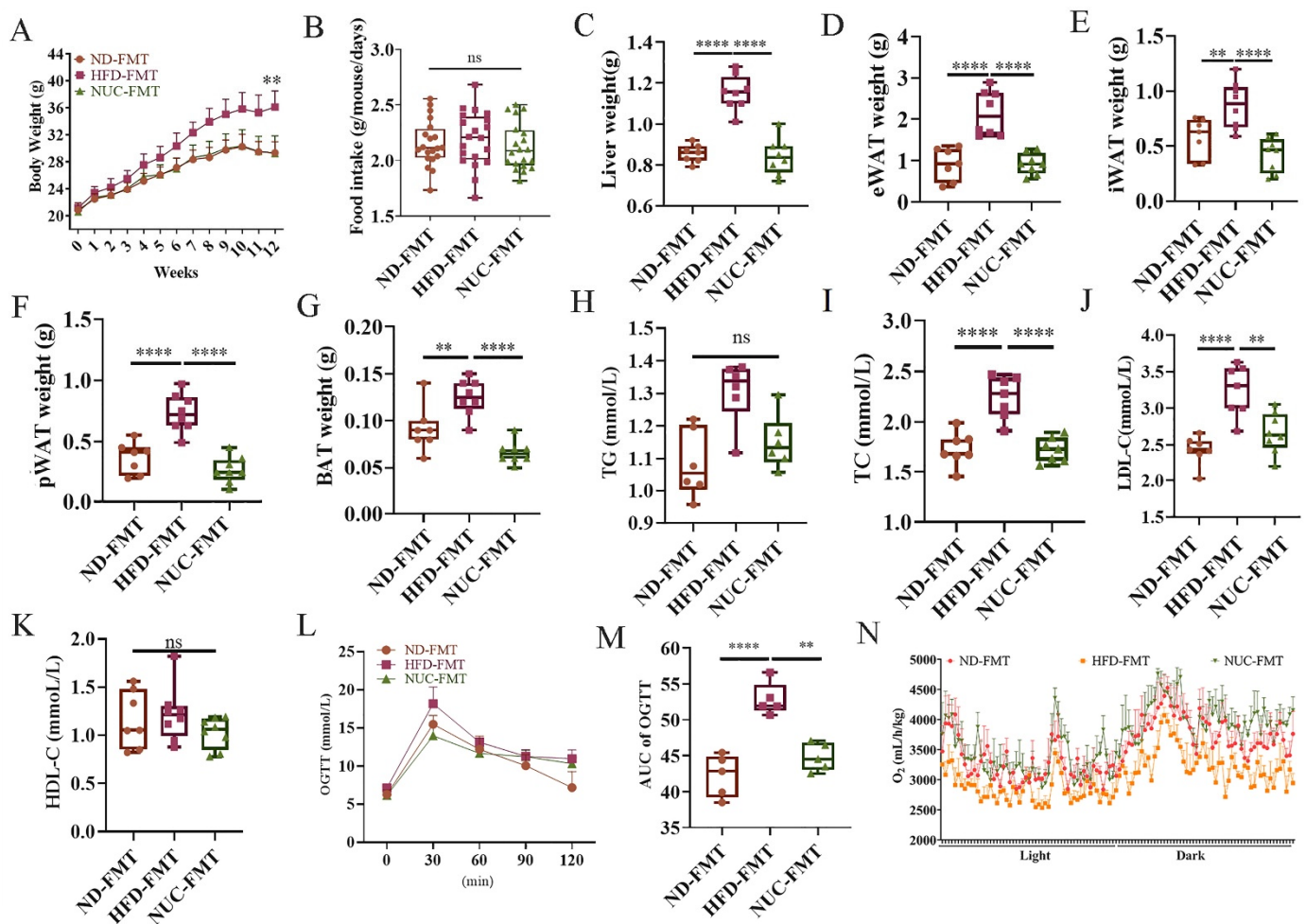


**Fig. 7** Effects of NUC on the fecal and serum metabolic profile in HFD-fed mice. (A) Venn diagram of metabolites in feces and serum. (B) Venn diagram of SMF and SMFS. (C) Heatmap of 22 metabolites in feces. (D) Peak intensities of six bile acids (BAs) in feces. (E) Venn diagram of significant variable metabolites in feces (SMF) and shared metabolites in feces and serum (SMFS). (F) Heatmap of 19 metabolites in serum. (G) Peak intensity of taurocholic acid, taurochenodeoxycholic acid (TUDCA), myristic acid,  $\gamma$ -linolenic acid, and N-acetylneuraminic acid (NANA) in serum.

### 3.8. Identification of NUC-promoted fat browning dependent on gut microbiota

To confirm that the intestinal flora was indeed involved in NUC-related obesity and browning, an FMT experiment was carried out. Fecal transplants from three different groups of donor mice were performed on newly acclimatized recipients and then placed on an HFD for 12 weeks. The results showed that the mice receiving fecal transplantation from the NUC-H group (NUC-FMT) significantly attenuated the increase in body weight (Fig. 8A), liver weight (Fig. 8C), eWAT weight (Fig. 8D), iWAT weight (Fig. 8E), pWAT weight (Fig. 8F), and BAT weight (Fig. 8G) without affecting food intake (Fig. 8B) compared with the HFD-FMT mice. Blood biochemical analysis revealed that NUC recipient mice exhibited improved glucolipid metabolism, evidenced by significantly decreased serum TG (Fig. 8H), TC (Fig. 8I), LDL-C levels (Fig. 8J), and OGTT (Fig. 8L and 8M), without affecting HDL-C (Fig. 8K) level. Moreover, transplantation of feces from the NUC-treated mice could beneficially improve energy metabolism, as evidenced by the increased  $O_2$  consumption (Fig. 8N and 8O),  $CO_2$  production (Fig. 8P and 8Q), and EE (Fig. 8R and 8S). It is noteworthy

that NUC increased the rectal temperature (Fig. 8T) and skin temperature in the interscapular region of NUC-FMT mice compared with the HFD-FMT mice under ambient temperature (34.38°C vs. 35.18°C) (Fig. 8U) and cold exposure (33.86°C vs. 35.05°C) (Fig. 8U) conditions. Analysis of H&E-stained histopathology images demonstrated that after the NUC-modulated microbiota was transplanted into HFD-fed mice, there was a decrease in lipid accumulation within BAT (Fig. 9A) as well as a reduction in the size of iWAT (Fig. 9B). qPCR analysis of thermogenesis- and inflammation-related genes in BAT revealed that NUC-FMT mice displayed upregulated expression of *Pgc-1 $\alpha$* , *Sirt1*, *Cidea*, *Prdm16*, *Dio2*, *Cox2*, and *Ucp1* but reduced levels of *iNOS*, *IL-6*, *Nox2*, and *TNF $\alpha$*  without changing *Tfam* expression (Fig. 9C). Western blotting assays confirmed a substantial enhancement of UCP1 protein expression in the BAT of NUC recipient mice (Fig. 9D). Meanwhile, the brown fat-related markers such as *Sirt1*, *Cidea*, *Pgc-1 $\alpha$* , *Prdm16*, *Cox2*, and *Ucp1*, and beige fat-specific markers such as *Fgf21*, *Tmem26*, *Cd40*, *Tbx1*, and *Cited1* were also significantly upregulated in the iWAT of NUC-FMT mice compared to those in HFD-FMT mice (Fig. 9E). Furthermore, fecal bacteria from NUC-treated group significantly upregulated the expression of UCP1 protein compared to the HFD-FMT group (Fig. 9F). Collectively, NUC-modulated microbiota is a driving factor in the induction of fat browning and thermogenesis.



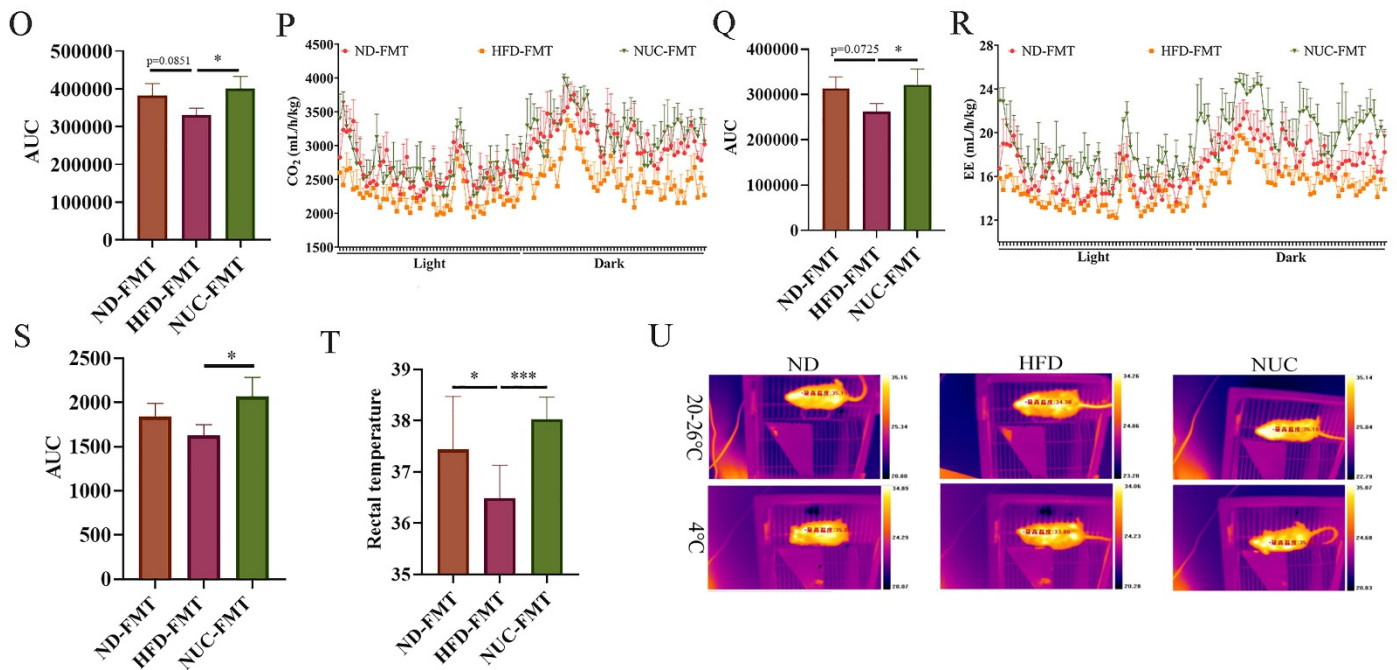
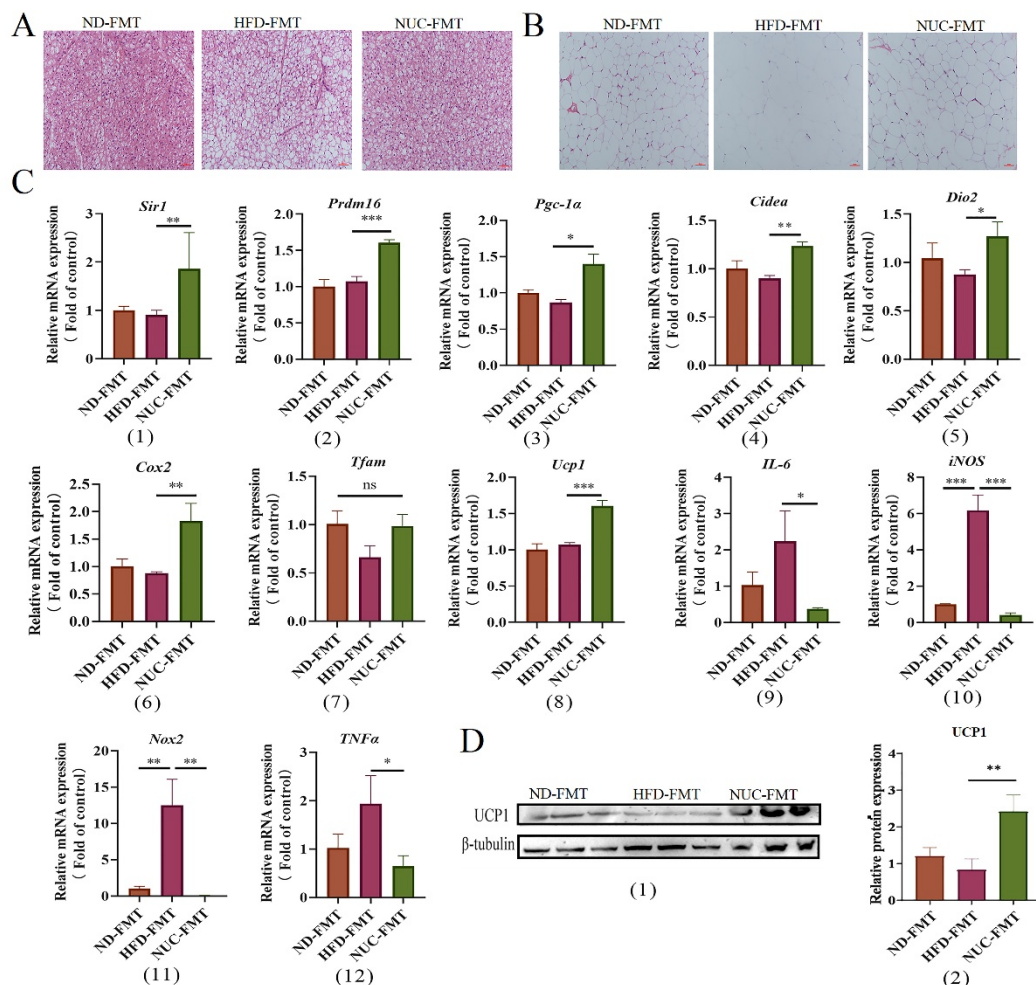


**Fig. 8** Transplantation of feces from NUC-treated mice attenuated obesity in HFD-fed mice. (A) Body weight change curve. (B) Food intake. (C) Liver weight. (D) eWAT weight. (E) iWAT weight. (F) pWAT weight. (G) BAT weight. (H) TG level. (I) TC level. (J) LDL-C level. (K) HDL-C level. (L) OGTT. (M) AUC of OGTT. (N) O<sub>2</sub> consumption and (O) AUC of O<sub>2</sub>. (P) CO<sub>2</sub> production and (Q) AUC of CO<sub>2</sub>. (R) EE and (S) AUC of EE. (T) The rectal temperature was determined after FMT for 30 min. (U) Skin temperature in the interscapular region of FMT mice was determined under ambient temperature (23 ± 3)°C and cold exposure (4°C) conditions, respectively. Statistical significance was assessed using nonparametric one-way ANOVA with GraphPad Prism. p-values were indicated as follows:  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ,  $****P < 0.0001$ .



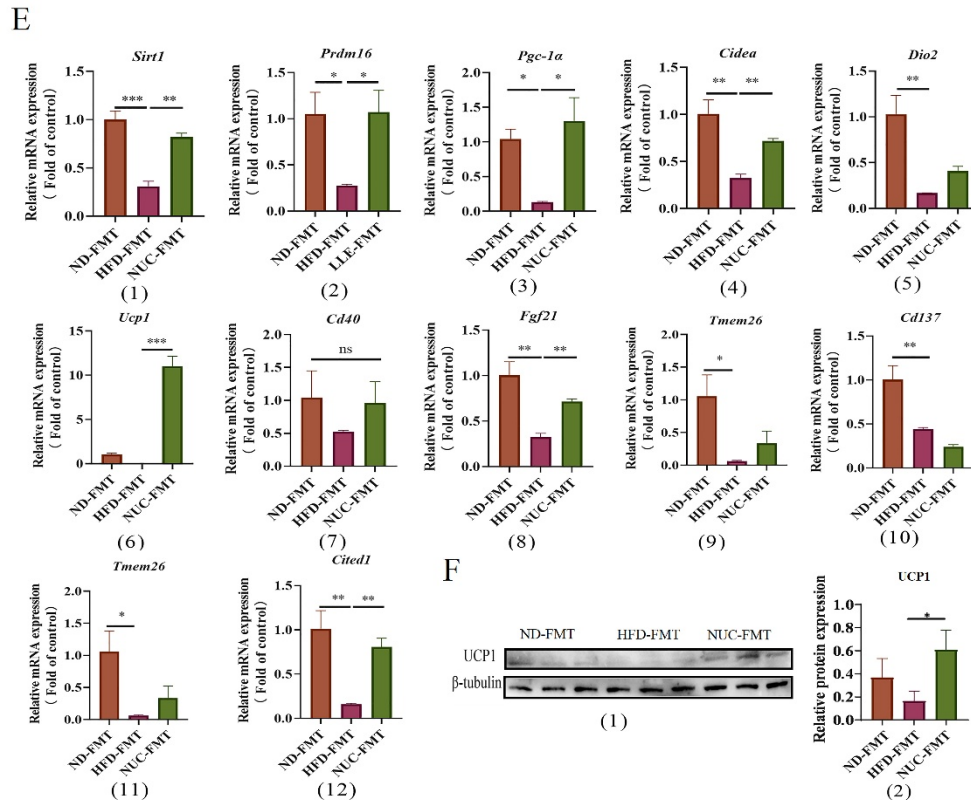


**Fig. 9** Transplantation of feces from NUC-treated mice induces adipose browning in HFD-fed mice. (A and B) H&E staining of BAT and iWAT. (C) mRNA expression levels of brown fat-enriched and inflammation-related genes in BAT. (D) protein expression of UCP1 in BAT. (E) mRNA expression levels of brown fat-related genes in iWAT. (F) protein expression of UCP1 in iWAT. Statistical significance was assessed using nonparametric one-way ANOVA with GraphPad Prism. p-values were indicated as follows: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .

#### 4. Discussion

BAT has become a focal point of research due to its thermogenic capacity and potential to counteract obesity by burning excess fat and stabilizing metabolic processes. The possibility of harnessing this tissue's thermogenic capabilities through dietary modifications that incorporate high levels of phytochemicals is an exciting development in the field of nutrition. Among the substances being studied for their anti-obesity potential, NUC has consistently shown promising results. Our research aimed to delve deeper into the mechanisms behind NUC's benefits. We not only substantiated previous findings that NUC can prevent weight gain in HFD-fed mice and enhance glycolipid metabolism but also discovered new and intriguing effects of NUC on metabolic health. A notable observation was the increase in skin temperatures following NUC treatment, an indirect indication of enhanced thermogenesis, suggesting that the body is actively burning calories to produce heat. Our molecular analysis revealed that NUC significantly increased the expression of brown adipocyte-specific genes, such as *Sirt1*, *Pgc-1α*, *Prdm16*, and *Ucp1*, in BAT. This upregulation indicates that NUC may be stimulating BAT to increase its metabolic activity, thereby contributing to the body's EE. Moreover, we detected key molecular changes in iWAT indicative of transition to beige fat. This was evidenced by increased mitochondrial bioenergetics and the expression of beige-related markers, including *Ucp1*, *Cidea*, *Prdm16*, *Tmem26*, *Cd40*, *Cd147*, *Cited1*, *Tbx1*, and *Fgf21*. The recruitment of brown adipocytes within WAT by NUC represents a significant finding, as it suggests

that NUC can promote the conversion of less metabolically active white fat into a more energy-dissipating brown fat. This multifaceted action of NUC on adipose tissue could lead to a reduction in fat accumulation and an improvement in metabolic homeostasis, offering a promising strategy for the prevention and management of obesity and its associated metabolic disorders.

The gut microbiome affects thermogenesis through systemic pathways, whereas BAT serves as the primary agent responsible for heat production. Furthermore, the configuration of the gut flora is instrumental in altering the structure of WAT, guiding its transformation towards a beige adipose phenotype in individuals with obesity<sup>[17]</sup>. Previous studies revealed the significant impact of NUC on alleviating HFD-induced dysbiosis of gut microbiota<sup>[18–21]</sup>. However, differences in microflora structures are reported, which may be due to variations in animal models (rats or mice), intake patterns (NUC added into feed, NUC added into water, or oral gavage), dose, and duration of intervention. In addition to identifying the improvement mediated by NUC in obese mice, this research also disclosed that alterations to the gut microbiota resulting from NUC exposure could be significant contributors to the heightened thermogenesis in BAT and the process of WAT browning. The primary phyla observed in obese animals and individuals were Firmicutes (F) and Bacteroidetes (B), characterized by a high prevalence of F and a lower prevalence of B<sup>[22]</sup>. However, there is controversy regarding the alteration of the F/B ratio in obesity, with some studies not observing any change in this parameter and even reporting a decrease in the F/B ratio in obese animals and humans<sup>[23]</sup>. Therefore, variations in other components at the phylum, family, genus, or species level may hold greater relevance than the F/B ratio. An exclusive focus on the F/B ratio risks neglecting important biological variations that emerge across different taxonomic ranks. *Proteobacteria*, a phylum of gram-negative bacteria, were found to be negatively associated with BAT/iWAT glucose uptake in HFD-fed offspring mice, suggesting their detrimental effect on BAT/iWAT thermogenesis<sup>[24]</sup>. Evidence suggests that HFD feeding results in an increase in the abundance of *Lachnospiraceae* bacteria<sup>[25]</sup>. A positive correlation has been found between *unidentified\_Lachnospiraceae* (phylum *Firmicutes*) and obesity-related indices (WG, iWAT, TG, TC, HDL, and BG) and colon inflammation. Conversely, a negative correlation was identified between *unidentified\_Lachnospiraceae* and levels of leptin, HSL, and UCP1 expression in WAT, along with BAT function<sup>[26]</sup>. The *Bilophila* genus belongs to the phylum *Proteobacteria*, and its increase has been considered to positively associate with obesity<sup>[27]</sup>. However, previous studies have also shown conflicting results. Some studies have shown that a reduction in *Bilophila* spp. is associated with disease onset. For example, in the research of Gao and colleagues, *Bilophila* inversely correlated with HFD-induced multi-tissue metabolic disorders and positively associated with most beneficial indicators<sup>[28]</sup>. Spearman's correlation of *Bilophila* and obesity-related indicators showed *Bilophila* was negatively correlated with body weight, blood glucose, and BAT weight<sup>[29, 30]</sup>. This discrepancy may also depend on the species level and the location. Studies have identified that *Bilophila wadsworthia* (a species from the genus *Bilophila*) exacerbates HFD-induced metabolic dysfunction in mice<sup>[31]</sup>. However, the abundance of *Bilophila wadsworthia* was significantly reduced by NUC treatment (Supplemental Fig. 7). In addition, *Bilophila* was

found to positively correlate with intestinal total, secondary, and unconjugated bile acids, including cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), taurodeoxycholic acid (TDCA)<sup>[27, 32]</sup>, and these metabolites may contribute to browning of adipose. Moreover, evidence supports that thermogenesis is enhanced with the enrichment of *Limosilactobacillus*<sup>[33]</sup>. A study performed on the feces of 82 young adults found that the *Lachnospiraceae* genus was negatively correlated with BAT volume and activity<sup>[34]</sup>. In HFD-fed mice/rats, the *Lachnoclostridium* genus was significantly enriched and positively correlated with OGTT, fasting insulin, serum TC, and TG content<sup>[35, 36]</sup>. *Weissella* exhibits antioxidant and anti-inflammatory properties, helps maintain intestinal homeostasis<sup>[37]</sup>, and contributes to inhibiting fat accumulation in association with high expression of UCP1 and PGC1 $\alpha$  in HFD-fed mice<sup>[38]</sup>. *Lactiplantibacillus* has been shown to elevate UCP1 mRNA level in WAT<sup>[39]</sup>. Furthermore, multi-strain probiotics containing *Limosilactobacillus*, *Lactiplantibacillus*, and *Lactobacillus* induced the expression of thermogenesis-related genes (UCP1 and PGC-1 $\alpha$ ) and increased the core temperature in high-carbohydrate diet-fed mice<sup>[40]</sup>. In the research of Zhang et al. (2022)<sup>[41]</sup>, *Tyzzereella* showed a positive correlation with PPAR $\alpha$  and SIRT1 levels in iWAT; *Roseburia* showed a negative correlation with PGC-1 $\alpha$ , PRDM16, and Cidea levels in iWAT. Relationships linking PET-CT imaging data and microbiota showed *unclassified\_Enterococcaceae* negatively associated with BAT/iWAT glucose uptake, suggesting their beneficial effects on BAT/iWAT metabolic function<sup>[24]</sup>. Thus, NUC may modulate fat browning *via* gut microflora.

The physiological functions of gut microbiota are primarily mediated through their metabolites *via* two main mechanisms: First, metabolites directly act on local colonic receptors (e.g., G protein-coupled receptors), stimulating the secretion of gut hormones (such as GLP-1 and PYY). These hormones transmit signals to the central nervous system *via* the gut-brain axis, thereby regulating appetite and glucose-lipid metabolic processes<sup>[42]</sup>. Second, some metabolites are absorbed by the colonic epithelium into the systemic circulation and transported *via* blood to peripheral target organs (e.g., adipose tissue)<sup>[43]</sup>. Here, fecal metabolite analysis revealed that BAs, including arbutinosocholic acid, nordesoxycholic acid, taurohyodeoxycholic acid, CDCA,  $\alpha$ -Muricholic acid, and cholic acid, were significantly increased in the NUC group, which may be related to intestinal FXR receptors and thus contribute to the browning of fat. Research revealed that BAs selectively activate intestinal FXRs to enhance thermogenesis and WAT browning, ultimately reducing obesity and insulin resistance<sup>[44]</sup>. *Bilophila* was found to be positively correlated with conjugated BAs, secondary BAs, total BAs, and the FXR signalling pathway<sup>[27]</sup>, and previous analysis of the colon bacteria showed that NUC treatment enriched *Bilophila* abundance. Importantly, the notable BAs metabolites in serum are taurocholic acid and taurochenodeoxycholic acid (TUDCA). TGR5 is highly expressed in BAT, and BAs can increase BAT thermogenesis and white fat browning by acting on TGR5 in adipose tissue<sup>[45, 46]</sup>. Taurocholic acid (4 and 12 mmol/L) stimulation for 48 h or 72 h up-regulated TGR5, D2, and cAMP expression in brown adipocytes, suggesting that it may increase BAT activity and energy metabolism through the TGR5-cAMP-mediated pathway<sup>[47]</sup>. Here, these

two BAs were significantly increased in the serum of NUC-treated mice and may be regulated by *Bilophila* (Supplemental Fig. 4), which is responsible for adipose browning. Polyunsaturated fatty acids (PUFA) are thought to activate UCP1 expression, enabling 3T3-L1 adipocytes to acquire a brown adipocyte phenotype and to promote mitochondrial biogenesis and glucose consumption<sup>[48]</sup>. Animal studies have shown that a diet rich in  $\gamma$ -linolenic acid reduces the accumulation of body fat while increasing the levels of UCP1 mRNA in BAT<sup>[49]</sup>. Lactic acid bacteria are thought to produce conjugated fatty acids<sup>[50]</sup>; *Limosilactobacillus*, a rod-shaped lactic acid bacterium, was significantly increased in the NUC-intervention group and showed a positive correlation with serum levels of  $\gamma$ -linolenic acid (Supplemental Fig. 4). In addition, NUC also facilitated an increase in serum levels of the BAT-activating factor myristic acid, which may be associated with *Limosilactobacillus*, *Bacteroides*, *Lachnoclostridium*, and unidentified *Lachnospiraceae* (Supplemental Fig. 4). Notably, a dramatically increased level of NANA was found in the serum and feces of the NUC group, with NANA being able to activate brown adipocytes. Evidence supports that NANA could be a promising biomarker for mitigating the HFD-induced metabolic disturbances, as determined by serum metabolomics profiling<sup>[51]</sup>. Han et al.<sup>[52]</sup> identified an NANA-producing bacterium, *Escherichia. coli*, which was isolated from a human microbiome. However, colonic microbiota analysis showed that the NUC intervention was detrimental to the growth of *Escherichia coli*, possibly because of other organisms, such as *Oscillibacter* (thermophilic bacteria), *Tyzzereella*, *Bilophila*, and *Limosilactobacillus* (thermophilic bacteria) promote an increase in NANA (Supplemental Fig. 4), as it has been suggested that some thermophilic bacteria express neuraminic acid synthase, which is responsible for NANA production<sup>[53]</sup>.

To confirm that gut bacteria modulate fat browning, the FMT experiment was carried out. In previous studies, researchers noted the onset of obesity in the recipient germ-free mice following the transplantation of gut microbiota from ob/ob mice<sup>[54]</sup>. Kou et al.<sup>[55]</sup> found that the transplantation of fecal bacteria from nobiletin-treated mice into HFD-fed mice resulted in a substantial enhancement of EE, 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. Although fecal transplantation in this study demonstrated that gut microbes facilitate NUC to promote BAT thermogenesis, the limitation of 16S rRNA sequencing failed to screen for particularly critical strains. The untargeted metabolome screening identified a number of significant metabolites contributing to thermogenic adipose activity, but these require validation of their effect on whole-body metabolism. Moreover, the need for additional dose-escalation experiments and the continuous exploration of nuciferine's pharmacological attributes are essential to fully understand its effects.

## 5. Conclusions

NUC treatment inhibited weight gain, improved glucolipid metabolism, activated BAT, and promoted the recruitment of brown-like fat in WAT. Additionally, preliminary microbiome and metabolome analyses indicate a strong correlation between NUC-induced fat browning and gut microbes and their metabolites (e.g., BAs, myristic acid,  $\gamma$ -linolenic acid, and NANA). To further investigate this mechanism, FMT

experiment was conducted to reveal that the microbial changes induced by NUC were associated with increased EE, enhanced BAT activity, and elevated thermogenesis. Collectively, NUC mitigated obesity potentially through microbiota-dependent modulation of adipose browning and thermogenesis. Importantly, our findings reveal a previously unrecognized mechanism underlying NUC's anti-obesity effects-its capacity to potentiate the “gut microflora-metabolites-thermogenic fats” crosstalk, thereby advancing our understanding of lotus leaf intervention for metabolic syndrome.

### Declarations of competing interest

The authors declare no conflicts of interest.

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