

**Saikosaponin D Ameliorates Obesity and Metabolic Disorders *via* the Gut
Microbiota-SCFAs-thermogenic fat Axis**

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Abstract

Activating thermogenic programs in brown adipose tissue (BAT) represents promising approaches to combat obesity through dissipating energy. Saikosaponin D (Sa-D), a major triterpenoid saponin derived from *Radix bupleuri* (a traditional herbal product) could ameliorate obesity. Here, our results showed that Sa-D treatment for 12 weeks significantly suppressed weight gain and improved glycolipid abnormalities in high-fat-diet (HFD)-fed mice. Importantly, Sa-D treatment increased the expression of uncoupling protein 1 (UCP1) along with other thermogenic factors, as well as enhanced phosphorylation of PKA, AMPK, and p38 in BAT and inguinal white adipose tissue (iWAT). 16S rRNA sequence analysis revealed that Sa-D restored HFD-induced microbial dysbiosis and specific bacterial genera such as *unidentified_Lachnospiraceae*, *Tyzzarella*, *Romboutsia*, *Bilophila*, *Oscillibacter*, *Sphingomonas*, and *Companilactobacillus* were strongly associated with obesity-related traits and brown/beige-related markers. Moreover, Sa-D markedly increased the concentration of short-chain fatty acids (SCFAs), induced gene expression of SCFAs-producing enzymes (acetate kinase (Ack), methylmalonyl-CoA decarboxylase (Mcd), butyryl-CoA (BCoA) and propionaldehyde dehydrogenase (PduP)), and upregulated expression of SCFAs receptors (*Ffar3*, *Ffar2*, or *Hcar2*) in colon, iWAT, and BAT. Collectively, our findings revealed that Sa-D could ameliorate obesity by activating thermogenic fat, which may be associated with the alteration in microflora profiles and increased SCFAs levels.

Keywords: Saikosaponin D, Thermogenic fat, Microbiota, Short-chain fatty acids

1. Introduction

Excess energy intake over energy expenditure leads to an imbalance that promotes the accumulation of additional fat, thereby contributing to the onset of obesity. Therefore, strategies to ameliorate obesity are either through limiting dietary energy intake, enhancing energy expenditure, or a combination of both (Tseng, Cypess, & Kahn, 2010).

Adipose tissue serves as a key regulator of the body's energy metabolism, responsible for maintaining systemic energy balance (Zhang et al., 2014). Mammalian adipose tissue is generally categorized into two main types, white adipose tissue (WAT) and brown adipose tissue (BAT), based on their distinct functional and morphological characteristics. WAT is mainly distributed in the subcutaneous tissues and around the internal organs, with its primary role being the storage of surplus energy as triglycerides. BAT is mainly distributed in the scapular region and can be divided into two types: classical and recruitable (called beige adipose tissue or brown in white) which have similar functions. Brown adipocytes in BAT possess abundant mitochondria and express uncoupling protein 1 (UCP1), which promotes the oxidation of lipids, enabling energy to be transmitted in the form of heat, and enhancing whole-body metabolic function. Consequently, strategies that encourage the browning of fat could offer a novel approach to the management of obesity.

In addition to well-established stimuli such as cold exposure, exercise, and medications (e.g., β_3 -adrenergic agonist), several studies have reported that natural compounds (chlorogenic acid, luteolin, curcumin, quercetin, capsaicin, berberine) and dietary supplements (e.g., tea, cranberry, grape, apple) can potentially stimulate WAT browning

and BAT activity (Hu et al., 2020; Wang et al., 2023). In East Asia, Chaihu (*Radix Bupleuri*) is often processed into porridge, soup bases, or beverages and is widely used as a herbal remedy (Liu et al., 2023; Su et al., 2018, Zhu et al., 2017). Saikosaponin D (Sa-D) extracted from Chaihu is one of the major biologically active triterpenoid saponins and documented to exhibit diverse biological activities, including anti-aging (Kim, 2018), anti-leukemic (Sun et al., 2021), anti-obesity (Lim et al., 2021), anti-inflammatory (Lu et al., 2012), anti-cancer (Tang et al., 2023), anti-diabetic (Xiang et al., 2020), and anti-depressant-like effects (Su et al., 2020). In this study, we mainly investigate the underlying mechanism responsible for its efficacy in combating obesity. Most previous studies focus on its beneficial effect on glycolipid metabolism in the liver. For example, Sa-D treatment for 8 weeks inhibited body weight gain and promoted fatty acid catabolism in the liver of high-fat-diet (HFD)-fed mice (Gu et al., 2022). Integrated lipidomic and transcriptomic analyses demonstrated that Sa-D regulates hepatic glycerolipid metabolism by hampering fatty acid biosynthesis and enhancing fatty acid degradation (Li et al., 2021). However, little information on its effect on adipose tissue development and metabolism is available.

Given the significant role that thermogenic fat plays in facilitating weight loss, the effect of Sa-D on fat browning and its potential mechanism was investigated. Our research yielded promising results, demonstrating that Sa-D can reduce both blood lipid and glucose levels, which are key markers of metabolic health. Furthermore, we observed a notable increase in the activity of BAT, as well as the initiation of the browning process in inguinal WAT (iWAT), which was beneficial for increasing energy

expenditure. The mechanism behind this browning effect appears to be multifaceted, with a possible relationship to Sa-D's influence on the gut microbiota profiles and the subsequent production of short-chain fatty acids (SCFAs). Our findings provide insight into the role of Sa-D in obesity management, highlighting its potential as a therapeutic intervention for improving metabolic health by influencing multiple metabolic pathways.

2 Materials and Methods

2.1 Materials and reagents

Saikosaponin D was purchased from Chengdu Herbpurify Co., Ltd (Chengdu, China). Commercial assay kits of triglycerides (TG), total cholesterol (TC), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine (Cr) were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China).

2.2 Animals and Diets

Forty male C57BL/6J mice, aged five weeks, were obtained from Beijing Vitality River Laboratory Animal Technology Co. and accommodated at the Jiangnan University Animal Centre (permit number: JN.No20220615c1100925[233]). After one week of acclimatization, the mice were randomly assigned to five groups (n=10): normal diet (ND) group, HFD group, Sa-D group received HFD with oral administration of 20 mg/kg of Sa-D, and MI group (the positive group) received HFD with oral administration of 5 mg/kg of miglitol. The dose of Sa-D was chosen based on previous reports on mice (Gu et al., 2022; Li et al., 2021; Li et al., 2024). The formulation of the

feed is listed in Table S1.

2.3 Energy metabolism

Energy consumption was measured by the CLAMS-12 (Columbus Instruments, USA).

The metabolic cages were maintained at ambient temperature and were subjected to a

12h light/12h dark cycle. The individual mouse was placed in a metabolic cage and O₂

consumption, CO₂ production, and RER (Respiratory exchange ratio) (VCO₂/VO₂)

were continuously monitored for 24 h after the mice had acclimatized to the

environment (12 h), and the energy consumption was calculated.

2.4 Infrared thermography

Mice were placed on ice for 2 h of cold stimulation, then anaesthetized by

intraperitoneal injection of sodium pentobarbital, and immediately imaged with an

infrared thermography camera (Magnify Electronics, Shanghai, China).

2.5 Oral glucose tolerance test (OGTT)

After 12 hours of fasting, the blood glucose at different time points was measured post-

glucose injection at a dose of 1.5 g/kg.

2.6 Analysis of serum biochemical indicators

After 12 hours of fasting, mice were anesthetized with sodium pentobarbital, and

collected whole blood was centrifuged to obtain serum. Biochemical parameters were

determined following the kit manufacturer's instructions.

2.7 Histopathological analysis

The fixed tissues were rinsed under running water for 30 min, then dehydrated with

alcohol at graded concentrations (30%, 50%, 70%, 85%, and 95%) and placed in xylene.

The tissue became transparent and was placed in paraffin wax. After the paraffin cooled and solidified into a block, the paraffin was cut into thin slices (3-5 μm), adhered to slides, and dried at 45°C. The paraffin was stripped from the sections with xylene and washed with alcohol, and then the slides were stained with hematoxylin solution. After rinsing in running water, the slides were put into 70% and 90% alcohol for 10 min, and then stained with eosin for 2 min. After dehydration and transparency treatment, the sections were mounted with gum mountant and photographed for histopathological analysis.

2.8 Immunohistochemical (IHC) staining

The process of IHC staining for iWAT and BAT was performed as prescribed in the staining kit (#36312ES50, Yeasen, Shanghai, China). Briefly, tissue sections were heated, cleared with xylene, and dehydrated through a gradient of alcohol solutions. After antigen retrieval and endogenous enzyme inactivation with 3% (v/v) H_2O_2 , sections were blocked and incubated with primary antibodies (UCP1 and SIRT1 (1:100)) overnight at 4°C, followed by secondary antibody incubation. Staining was developed with DAB, counterstained with hematoxylin, dehydrated again, and mounted for microscopic observation.

2.9 Quantitative RT-PCR analysis

Total RNA extraction of BAT, iWAT, liver, and colon tissues, reverse transcription, and quantitative analysis was carried out as described in our previous study (Wang, Xue et al., 2022). The specific primer sequences are provided in Supplemental Table 2. Fecal genomic DNA was extracted utilizing the MolPure® Stool DNA Kit (Yeasten, China),

adhering to the protocol provided by the manufacturer. The gene expression was standardized against 16S rRNA. The sequences of the primers were referenced in the study by Guo et al. (2020) and Gopi et al. (2015).

2.10 Western blot analysis

The protein lysates derived from BAT and iWAT were analyzed *via* Western blot, consistent with the protocols detailed in our earlier investigation (Wang, Xue et al., 2022). Briefly, after protein electrophoresis and membrane transfer, the PVDF membranes were exposed to primary antibodies (1:1000, Proteintech), and incubated at 4°C for 12 h. Then, membranes were incubated with HRP-conjugated secondary antibody (1:4000, Proteintech) and visualized using an ECL detection reagent (Beyotime, China).

2.11 Gut Microbiota analysis

The bacterial composition within fecal samples from various groups was investigated through 16S rRNA gene sequencing, targeting the V3-V4 hypervariable regions. The amplicon sequencing was conducted utilizing the Illumina MiSeq PE250 platform by Metware Biotech Co., Ltd. (Wuhan, China). A full description of the sequencing process is provided in the supplementary documentation.

2.12 Quantification of SCFAs

Fecal samples were used to analyze SCFAs profiles by Gas Chromatography-Mass Spectrometry (GC-MS) following the method of Lin et al. (2022). Briefly, freeze-dried fecal samples were weighed, saturated NaCl and internal standard 2-Ethylbutyric acid were added and then the sample was shaken to remove any visible lumps. After

homogenization, SCFAs were sequentially extracted by adding 40 μ L of 10% sulphuric acid and 1 mL of ether, and the supernatant was separated by centrifugation after shaking for 30 s on a vortex shaker. Then, the supernatant was placed in a test tube containing 0.25 g of anhydrous sodium sulfate for 15 minutes and centrifuged to detect SCFAs by GC-MS.

2.13 Statistical Analysis

All data are expressed as the means $\pm$ standard deviation (SD). GraphPad Prism software was used to determine statistical significance by nonparametric one-way analysis of variance (ANOVA) with Duncan's test. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001. Values with different lowercase letters indicate a significant difference at P < 0.05 following Duncan's multiple range tests using DPS software.

3. Results

3.1 Effects of Sa-D on body weight, tissue weight, and blood biochemical parameters in HFD-fed mice

As shown in Fig. 1, Sa-D treatment reversed HFD-induced increase in body weight gain (Fig. 1A and 1B), liver (Fig.1D), eWAT (Fig.1G), pWAT (Fig.1H), iWAT (Fig.1I), and BAT weights (Fig.1J), which were similar to those in the MI group. Moreover, there were no notable differences in food intake (Fig.1C), or spleen (Fig.1E) and kidney (Fig.1F) weights of mice in the different treatment groups. Analysis of blood lipid profile showed that Sa-D supplementation dramatically attenuated TG (Fig. 1K), TC (Fig. 1L), and LDL-C (Fig. 1N) levels and increased HDL-C (Fig. 1M) levels. As HFD feeding tends to trigger insulin resistance, glucose tolerance was examined to investigate the effect of Sa-D on glucose homeostasis. It was observed that Sa-D

intervention significantly reduced fasting blood glucose (FBG) level (Fig. 1O) and enhanced OGTT (Fig. 1P and 1Q) in HFD-fed mice, indicating improved glucose homeostasis. These findings evidence the promising potential of Sa-D to inhibit weight gain and ameliorate glycolipid disorders.

3.2 Effects of Sa-D on energy consumption in HFD-fed mice

We further investigated whether the Sa-D-induced reduction in obesity was achieved by increasing energy consumption. As shown in Fig. 2, the VO_2 (Fig. 2A and 2B) and VCO_2 (Fig. 2C and 2D) in the Sa-D-treated mice were 1.51-fold and 1.26-fold of the HFD group, respectively. A similar effect was observed in energy expenditure (EE) (1.42-fold of the HFD group) (Fig. 2G and 2H). However, Sa-D did not exert a significant impact on the Respiratory Exchange Ratio (RER). The average values for RER in the ND, HFD, Sa-D, and MI groups were 0.98, 0.81, 0.84, and 0.85, respectively (Fig. 2E and 2F), indicating no substantial difference. The thermographic images upon cold stimulation showed increased skin temperature in the interscapular region of Sa-D-treated mice (34.41°C) compared to HFD-fed mice ($\text{HFD}_{\text{max}}: 30.86^{\circ}\text{C}$), approaching that of the MI group ($\text{MI}_{\text{max}}: 34.93^{\circ}\text{C}$) (Fig. 2I).

3.3 Histopathology analysis of HFD-fed mice

Administration of Sa-D significantly decreased the adipocyte size in both eWAT (Fig. 3A and 3B) and iWAT (Fig. 3C and 3D), and also diminished lipid accumulation in BAT (Fig. 3E and 3F) to comparable levels observed in the MI group. Hepatopathology revealed that Sa-D-treated mice had a decreased NAFLD activity score (NAS) as compared to the HFD group (Fig. 3G and 3H). Serum measurement of ALT and AST

demonstrated that Sa-D had not induced significant injury to the liver (Fig. 3I and 3J). Hepatic steatosis was further evaluated by mRNA analysis. Treatment of Sa-D suppressed the expression of lipogenesis-related genes, including *Srebp-1c* (Fig. 3K), *Fas* (Fig. 3L), *Pparγ* (Fig. 3M), and *Acc* (Fig. 3N), and increased lipolysis-related genes such as *Plin5* (Fig. 3O), *Plin2* (Fig. 3P), *Ppara* (Fig. 3Q) and *Cpt1α* (Fig. 3R). Furthermore, we examined the safety of Sa-D by analyzing tissue sections from the kidneys (Fig. 3S) and spleens (Fig. 3V). The data revealed that Sa-D provided a protective effect on both the kidney and the spleen, evidenced by decreased levels of BUN (Fig. 3T) and Cr (Fig. 3U), relative to the HFD group.

3.4 Effects of Sa-D on the activity of BAT in HFD-fed mice

As shown in Fig. 4A, Sa-D treatment upregulated mRNA expression for BAT-associated genes, including *Sirt1*, *Pparg1α*, *Cidea*, *Prdm16*, *Dio2*, *Nrf1*, *Tfam*, *Cox7a*, and *Ucp1* compared with the HFD group. Notably, expression of the lipid droplet-associated genes *Atgl*, *Hsl*, and *Abhd5* was induced by Sa-D treatment (Fig. 4A). In addition, increased immunostaining of SIRT1 (Fig. 4B) and UCP1 (Fig. 4B) was detected in Sa-D-treated mice. Similarly, Western blotting analysis showed Sa-D induced protein expression of brown-related markers such as SIRT1, PGC-1α, TFAM, and UCP1. Importantly, Sa-D significantly enhanced the ratio of p-PKA/PKA, p-p38/p38, p-AMPKα/AMPKα, and p-AMPKβ₁/AMPKβ₁.

3.5 Effects of Sa-D on the browning of iWAT in HFD-fed mice

Intracellular lipolysis is responsible for the physiological regulation of thermogenic processes in WAT browning. Investigation of lipolysis-related genes showed Sa-D

augmented mRNA expression of *Atgl*, *Hsl*, and *Abhd5* (Fig. 5A). Moreover, Sa-D induced BAT-rich genes, such as *Sirt1*, *Prdm16*, *Pparg1a*, *Cidea*, *Dio2*, *Tfam*, *Nrf1*, *Cox2*, and *Ucp1* in comparison to the HFD control (Fig. 5A). Moreover, beige adipocyte-specific genes, such as *Fgf21*, *Tbx1*, *Cd40*, *Tmem26*, *Cited1*, and *Cd137* were also upregulated in Sa-D-treated mice and the positive control (MI) group (Fig. 5A). Immunohistochemistry of SIRT1 and UCP1 showed higher positive immunostaining areas in iWAT for the Sa-D treatment group (Fig. 5B) and Western blotting analysis of SIRT1, TFAM and UCP1 also exhibited a similar trend (Fig. 5C). Importantly, Sa-D significantly enhanced the ratio of p-PKA/PKA, p-p38/p38, and p-AMPK α /AMPK α .

3.6 Sa-D remodels the gut microbiome in HFD-fed mice

To investigate the remodeling of the intestinal microflora by Sa-D treatment, fecal 16S rRNA gene sequencing analysis was performed. Observed OTUs showed a reduced trend in Sa-D-treated mice (Fig. 6A). Alpha diversity analysis showed no statistical difference in the Shannon and Simpson indexes (Fig. 6B and 6C), but reduced ACE and Chao1 indexes in Sa-D-treated mice compared the HFD group (Fig. 6D and 6E). The Venn diagram shows treatment with Sa-D decreased the number of OTUs, which was close to the ND group. A further 753 (24.6 %) of the total OTUs were common to all three groups; 1320 (37.2%) OTUs were shared between the ND and HFD groups; 1187 (33.4%) OTUs were shared between the HFD and Sa-D group (Fig. 6F). PCoA analysis showed that Sa-D-treated mice clustered separately from HFD mice, and were similar to ND mice (Fig. S1A). These results suggest that Sa-D could partially reverse changes in HFD-induced gut microbiota composition. Specifically, no significant differences in

phylum Firmicutes (F) and Bacteroidota (B) were found among the three groups (Fig. 6G(1-3)). However, the Sa-D treatment could reduce the F/B ratio, although this decrease was not statistically significant when compared to the HFD group (Fig. 6G(4)). The *Muribaculaceae* and *Akkermansiaceae* family encompassing many probiotic species were predominantly present in Sa-D-treated mice (Fig. 6H). The LEfSe (LDA>3) analysis identified a total of 93 OTUs, comprising 5 phyla and 36 genera, as pivotal phylotypes within the dataset (Fig. 6I and Fig. S1B). The *s_Faecalibaculum_rodentium*, *g_Faecalibaculum*, *c_Bacilli*, *f_Erysipelotrichaceae*, and *p_Erysipelotrichales* were predominantly located in the ND group (LDA>4); *s_Lachnospiraceae_bacterium_10_1*, *g_Roseburia*, *g_unidentified_lachnospiraceae*, *g_Lachnoclostridium*, and *p-Proteobacteria* were predominantly located in the HFD group (LDA>4); While after Sa-D treatment, the *o_unidentified_clostridia*, *c_Clostridia*, *f_Lachnospiraceae*, *g_Colidextribacter*, *s_Bacteroides_acidifaciens*, *f_oscillospiraceae*, *s_Akkermansia_muciniphila*, *s_Lachnospiraceae_bacterium_28_4*, *g_bacteroides*, *g_Akkermansia*, *g_Blautia*, *sParabacteroides_goldsteinii*, *g_Mucispirillum*, *g_unidentified_clostridia*, *g_Tyzzereella*, *s_Romboutsia_ileails*, and *g_bilophila* (LDA>4) were predominantly located in Sa-D group (LDA>4); For assignment on the genus-level, 34 genera with high abundance are shown in Fig. S2. The HFD group showed decreased abundance of *Faecalibaculum*, *Lactobacillus*, *Bifidobacterium*, and *Limosilactobacillus*, whereas enrichment of *Colidextribacter*, *unidentified_Lachnospiraceae*, *Lachnoclostridium*, *Roseburia*, *Tyzzereella*, *Negativibacillus*, *Acetatifactor*, and *Oscillibacter* was observed relative to the ND

group (Fig. 7A). Moreover, genera *Faecalibaculum*, *unidentified_Lachnospiraceae*, *Roseburia*, *Sphingomonas*, *Ralstonia*, *Bifidobacterium*, *unidentified_Ruminococcaceae*, *Achromobacter* were lower, but *Lactobacillus*, *Tyzzereella*, *Bilophila*, *Oscillibacter*, *Romboutsia*, *Companilactobacillus*, *Lactiplantibacillus*, and *Weissella* were higher in the Sa-D group versus HFD group (Fig. 7B). Spearman's correlation analysis indicated that the genera *Bifidobacterium*, *Faecalibaculum*, *Lactobacillus*, and *Limosilactobacillus* exhibited a negative association with brown/beige fat-associated markers and a positive link with serum lipid indicators and body weight gain ($p<0.05$), but *Alistipes*, *Mucispirillum*, *Erysipelatoclostridium*, *Companilactobacillus*, *Lactiplantibacillus*, *Weissella*, *Acetatifactor*, *Blautia*, *Lachnoclostridium*, *Roseburia*, *Tyzzereella*, *Colidextribacter*, *Oscillibacter*, and *Romboutsia* showed opposite trends (Fig. 7C).

3.7 Analysis of SCFAs and their correlation with key species in the fecal microbiota

To investigate whether Sa-D-induced browning of adipose tissue is related to intestinal flora, bacterial metabolites of SCFAs in the feces were examined by GC-MS. Sa-D augmented the levels of acetic, propionic and butyric acids as compared to the HFD group (Fig. 8A and Fig. S3). Consistently, Sa-D treatment significantly increased the expression of SCFA-producing enzymes in feces: acetate kinase (Ack), methylmalonyl-CoA decarboxylase (Mcd), propionaldehyde dehydrogenase (Pdup) and butyryl-CoA (BCoA) (Fig. 8B). The mRNA expression of SCFAs receptors (*Ffar3*, *Ffar2*, and *Hcar2*) in colon (Fig. 8C), *Ffar2* in WAT (Fig. 8D), and *Ffar2* in BAT (Fig. 8E), can be induced by Sa-D. However, Sa-D treatment did not significantly affect *Ffar3* expression in

iWAT and BAT (Fig. 8D and 8E). Spearman's correlation heatmap (Fig. 8F) demonstrated a notably positive correlation between *Lactobacillus* and the levels of SCFAs as well as the enzymes Ack, Mcd, and BCoA. *Lachnospiraceae*, *Roseburia*, *Negativibacillus*, *Alistipes*, and *Erysipelatoclostridium* were negatively correlated with acetic, propionic, and butyric acids, and SCFAs-producing enzymes (*Mcd*, *BcoA*, *PduP*). Meanwhile, *unidentified_Lachnospiraceae*, *Faecalibaculum*, *Sphingomonas*, *Ralstonia*, *Colidextribacter*, and *Achromobacter* were negatively correlated while *Romboutsia*, *unidentified_Ruminococcaceae*, *Companilactobacillus*, *Lactiplantibacillus*, *Weissella*, and *Limosilactobacillus* were positively correlated with certain SCFAs-producing enzymes. Thus, our results demonstrated a significant effect of Sa-D intervention on SCFAs levels and revealed a correlation between key microorganisms and SCFAs levels.

4 Discussion

BAT is a specialized fat depot that plays a key role in energy balance by promoting energy expenditure through thermogenesis. It burns calories to produce heat, thereby helping to regulate body temperature and metabolic rate. This unique ability of BAT makes it an important tissue for maintaining overall metabolic health and combating obesity. Previous findings have demonstrated that Sa-D could enhance fatty acid catabolism in BAT and white adipocytes (Gu et al., 2022), however, the mechanism remains poorly understood. Here, our findings revealed significant anti-hyperlipidemic, anti-hepatic steatosis, and anti-hyperglycemic effects of Sa-D in HFD-fed mice, in agreement with earlier findings (Gu et al., 2022). Furthermore, increased expression of

brown fat-associated factors, including SIRT1, PGC-1 α , PRDM16, and UCP1 support the role of Sa-D in activating BAT and iWAT browning. Notably, Sa-D supplementation enhanced O₂ consumption and CO₂ production without a significant effect on RER, suggesting that Sa-D-treated mice can utilize fat as an energy source along with increased energy expenditure. The PKA signaling pathway is a crucial modulator of energy expenditure. PKA activates AMPK and p38 MAPK, which in turn facilitates the transcription of PGC-1 α and UCP1, and is consequently essential in regulating the development and activation of BAT (Zhang et al., 2021). Here, Sa-D induced the phosphorylation of PKA, AMPK, and p38 in BAT and iWAT, supporting the activation of PKA-AMPK/p38 signaling in its mechanism of action.

The gut microbiota has been found to promote BAT thermogenesis, and in addition, alterations in the flora structure modulate the conversion of WAT into beige fat in patients with obesity (Wang et al., 2021). Earlier research reveals that Sa-D ameliorates gut microbiota disturbances in colitis mice (Li et al., 2020). In addition to identifying the microflora profiles in the improvement of Sa-D-treated mice exposed to HFD, the new findings of this study also uncover that microflora-derived SCFAs may be important factors for Sa-D-induced BAT thermogenesis. The microbiota of obese animals is mainly composed of Firmicutes (F) and Bacteroidetes (B), with F being predominant and B less abundant (Ley et al., 2006). However, it remains inconclusive whether the F/B ratio is an inevitable element in establishing obesity. Some studies have found no significant changes in this ratio, while others have reported a decreased F/B ratio among individuals with obesity (Magne et al., 2020). Evidence suggests that Sa-

354 D (15 mg/kg) for 12 weeks has no significant effect on the F/B ratio in HFD-fed mice
355 (Li et al., 2024). It may be more important to consider specific compositional changes
356 rather than solely focusing on the F/B ratio. Research suggests that *Faecalibaculum*
357 strains could be a notable genus implicated in obesity, with a demonstrated positive
358 relationship to body weight, WAT index, and serum glucose and inflammation levels
359 (Sun et al., 2020). Within *Firmicutes*, a high abundance of *Lachnospiraceae* contributes
360 to the disturbance of glucolipids (Vacca et al., 2020). Furthermore, a negative
361 correlation was observed between the genus *unidentified_Lachnospiraceae* and the
362 expression of leptin, HSL, and UCP1 in WAT as well as BAT function (Kang et al.,
363 2022). *Lactobacillus* is recognized as a probiotic and has shown promising anti-obesity
364 properties in both rodents and humans. The enhanced mitochondrial levels and function
365 by upregulating the thermogenic gene program (e.g., *Ucp1*, *PPAR γ* , and *PGC-1 α*) were
366 observed in iWAT and BAT of *Lactobacillus*-treated mice (Kang et al., 2022; Park et
367 al., 2019; Yoon et al., 2020). In the research of Zhang et al. (2022), the *Tyzzzeria* genus
368 positively correlated with *PPAR α* and *SIRT1* levels in iWAT, while *Roseburia* was
369 negatively associated with *PGC-1 α* , *PRDM16*, and *Cidea* levels in iWAT (Zhang et al.,
370 2022). *Bilophila* belongs to phylum *Proteobacteria* and some studies have shown that
371 this genus inversely correlates with HFD-induced multi-tissue metabolic disorders
372 (Gao et al., 2020), and was beneficial for the browning of iWAT (Zhu et al., 2020). The
373 genus *Oscillibacter* displayed a positive correlation with the levels of gene expression
374 related to thermogenesis, and concurrently, it was inversely associated with TC, TG,
375 and the mass of epididymal WAT (Li et al., 2022). Obesity-associated genera

376 *Romboutsia* showed a negative relationship with PGC-1 α , Cidea, and PRDM16 in
377 HFD-fed mice (Zhang et al., 2022). In our research, the altered microflora profiles such
378 as reduced *Faecalibaculum*, *unidentified_Lachnospiraceae*, and *Roseburia*, and
379 increased *Tyzzarella*, *Bilophila*, *Oscillibacter*, and *Romboutsia* may be responsible for
380 the anti-obesity and brown-promoting effects of Sa-D.

381 SCFAs are among the most thoroughly investigated metabolites that are generated by
382 the intestinal microbiota. They function as chemical messengers that bridge microbe-
383 host crosstalk, and thus may play beneficial metabolic actions in iWAT and BAT *via* the
384 microbiota-gut (brain)-adipose axis. SCFAs could act directly or indirectly on energy
385 expenditure by increasing BAT activity. Hu and collaborators demonstrated that
386 treatment with 10 mM acetate induced significant morphological alterations in
387 immortalized brown adipocytes, along with a boost in mitochondrial biogenesis, as well
388 as elevated expression levels of PGC-1 α , PPAR γ , aP2, and UCP1 (Hu et al., 2016). In
389 addition, research indicates that butyrate modulates the gut-brain neural circuits,
390 thereby boosting energy metabolism by stimulating BAT (Li et al., 2018). Several
391 studies have documented that *Lactobacillus* strains could ameliorate obesity-related
392 markers and provide a benefit by increasing the levels of acetate, butyrate, and
393 propionate (Ji et al., 2019; Larsen et al., 2013; Ma et al., 2022). Probiotic
394 *Lactiplantibacillus* and *Weissella* are key producers of SCFAs and strongly associated
395 with a range of SCFAs, namely acetic, propanoic, butyric, and valeric acids (He et al.,
396 2022; Sun et al., 2023). *Sphingomonas* being present in the obese strain showed a
397 negative relationship with acetic acid and butyric acid (Zhang et al., 2022).

Companilactobacillus is a potential gut microbiota-regulating probiotic and was found to alleviate colonic inflammation, which associates with increased SCFAs levels (Wang, Shi et al., 2022). Research has validated that certain herbal triterpenoid saponins, including ginsenoside Rk3 (Guo et al., 2023), ginsenoside Rh4 (Bai et al., 2021), and xanthoceraside (Zhou et al., 2022) could augment both the population of SCFAs-producing bacteria and the levels of SCFAs. Consistently, treatment with Sa-D markedly increased the abundance of *Lactobacillus*, *Lactiplantibacillus*, *Weissella*, *Sphingomonas*, and *Companilactobacillus* as well as the levels of acetate, butyrate and propionate in feces. Meanwhile, gut microbiota can secrete enzymes such as acetate kinase (*Ack*), methylmalonyl-CoA decarboxylase (*Mcd*), butyryl-CoA (*BCoA*), and propionaldehyde dehydrogenase (*PduP*) to metabolize carbohydrates into SCFAs. Studies have documented that Sa-D dramatically elevates the fecal expression of ACK, MMD, and BUT, thereby facilitating the production of SCFAs. Hence, Sa-D modified the microbial profile by reducing *Sphingomonas* and enhancing the abundance of *Lactobacillus*, *Lactiplantibacillus*, and *Weissella*, which consequently resulted in elevated levels of SCFAs.

As gut-derived signaling molecules, SCFAs are involved in energy metabolism by binding GPR43 (*Ffar2*), GPR41 (*Ffar3*), and GPR109a (*Hcar2*). Intestinal *Ffar3*, *Ffar2*, and *Hcar2* activation facilitates the secretion of GLP-1, GIP, and CCK, leading to fat browning and maintenance of energy balance (Wang et al., 2023). Furthermore, it has been documented that *Ffar2* and *Ffar3* are expressed in the WAT and BAT of HFD-fed mice (Ikuko Kimura et al., 2013; Lu et al., 2016). Previous research showed the body

temperature and Ucp1 expression in BAT were reduced in *Ffar3*^{-/-} mice and *Hcar2*^{-/-} mice compared to wild type mice (Kimura et al., 2011; Ye et al., 2019). Lu et al. (2016) found that treatment with 5% acetate, propionate, and butyrate could activate *Ffar3* and *Ffar2* receptors in iWAT and BAT and promoted the formation of beige adipocytes in HFD-fed mice. Here, Sa-D was found to greatly upregulate the mRNA expression of *Ffar3*, *Ffar2*, and *Hcar2* in colon, *Ffar2* in WAT, and *Ffar2* in BAT, potentially being beneficial for energy metabolism and BAT function. Importantly, a previous study has confirmed that SCFAs (acetate, propionate, and butyrate) promote a brown/beige adipocyte-like phenotype by PKA/AMPK signaling (Zhang et al., 2023). Moreover, the silencing of the GPR43 receptor through siRNA-mediated knockdown blocked the acetate and propionate-dependent activation of AMPK (Lu et al., 2021; Yoshida, Ishii, & Akagawa, 2019). Therefore, we propose that Sa-D enhances the GPR43/PKA-AMPK signaling pathways by elevated levels of SCFAs, which in turn enhance BAT activity and promote the browning of iWAT. Collectively, Sa-D-induced activation of BAT was potentially associated with gut microflora shifts and increased intestinal SCFAs levels. However, an in-depth understanding of the specific microflora and their SCFA metabolites that facilitate the browning process requires further studies using more direct methods. Approaches such as fecal transplantation or the use of sterile mice will be crucial in these investigations. These methods can help isolate the effects of the microbiota on browning, thereby providing more insight into their roles in metabolic regulation and potential therapeutic applications.

5 Conclusion

Sa-D exhibited multiple modulatory effects on obesity, including improvement of dyslipidemia and dysglycemia, enhancement of energy expenditure alongside mitochondrial biogenesis, and reduction of hepatic steatosis. The underlying mechanisms for these therapeutic effects involve the stimulation of adipose thermogenesis, a process that correlated with alterations in the gut microbiota and SCFAs profiles (Fig. 9). Our findings offer novel insights into utilizing Sa-D for obesity management and support the advancement of health products targeting weight loss.

Declarations of competing interest

The authors declare no conflicts of interest.

Acknowledgments

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List of Abbreviations

Abbreviation	Definition
Abhd5	Abhydrolase domain containing 5
ACC	Acetyl-CoA carboxylase
Ack	Acetate kinase
ALT	Alanine aminotransferase
AMPK	Adenosine 5'-monophosphate (AMP)-activated protein kinase
aP2	Adipocyte fatty acid binding protein
AST	Aspartate aminotransferase
Atgl	Adipose triglyceride lipase
BAT	Brown adipose tissue
BcoA	Butyryl-CoA
BG	Body weight gain
BUN	Blood urea nitrogen
CCK	Cholecystokinin
Cd40	Clusters of differentiation 40
Cd137	Tumor necrosis factor receptor superfamily member 9
Cidea	Cell death-inducing DFFA-like effector a
Cited1	Cbp/p300-interacting transactivator 1
Cox2	Cyclooxygenase-2
Cox7a	Cytochrome c oxidase subunit 7A

CPT1 α	Carnitine palmitoyltransferase 1 α
Cr	Creatinine
Dio2	Deiodinase type 2
eWAT	Epididymal white adipose tissue
Fas	Fatty acid synthetase
Fgf21	Fibroblast growth factor 21
GC-MS	Gas Chromatography-Mass Spectrometry
GLP-1	Glucagon-like peptide-1
GIP	Gastric inhibitory polypeptide
GPR43/41/109a	G-protein coupled receptor 43/41/109a
HFD	High-fat-diet
HDL-C	HDL-cholesterol
ND	Hormone-sensitive triglyceride lipase
IHC	Immunohistochemical
iWAT	inguinal white adipose tissue
LDL-C	LDL-cholesterol
Mcd	Methylmalonyl-CoA decarboxylase
MI	Miglitol
NAS	NAFLD activity score
ND	Normal diet
Nrf1	Nuclear factor erythroid 2-related factor 1
OGTT	Oral glucose tolerance test

PduP	Propionaldehyde dehydrogenase
PKA	protein kinase A
PPAR α/γ	Peroxisome proliferator-activated receptor α/γ
Prdm16	PR domain containing 16
Pparg1a	PPAR gama coactivator 1 alpha
Plin5	Perilipin-5
RER	Respiratory exchange ratio
Sa-D	Saikosaponin D
SCFAs	Short-chain fatty acids
SREBP-1c	Sterol regulatory element-binding protein-1c
Sirt1	Silent mating type information regula-tion 1
Tbx1	T-box transcription factor 1
TC	Total cholesterol
Tfam	Mitochondrial transcription factor A
TG	Triglycerides
Tmem26	Transmembrane protein 26
UCP1	Uncoupling protein 1
WAT	White adipose tissue

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Figure Legends

Fig. 1. Effects of Sa-D on body measurements and serum biochemical indicators. (A) Growth curve of mice in different groups. (B) Body weight gain (BG). (C) Food intake. (D) Liver weight. (E) Spleen weight. (F) Kidney weight. (G) eWAT weight. (H) pWAT weight. (I) iWAT weight. (J) BAT weight. (K) TG. (L) TC. (M) HDL-C. (N) LDL-C. (O) FBG Level. (P) OGTT. (Q) AUC of OGTT.

Fig. 2. Effects of Sa-D on the energy expenditure in HFD-fed mice. (A and B) O₂ consumption and AUC of O₂. (C and D) CO₂ production and AUC of CO₂. (E and F) RER and AUC of RER. (G and H) Energy expenditure (EE) and AUC of EE. (I) Thermal images of mice upon cold exposure.

Fig. 3. Effects of Sa-D on histopathology in HFD-fed mice. H&E staining and quantitative analysis of (A and B) eWAT, (C and D) iWAT, (E and F) BAT, and (G and H) liver. (I) serum ALT level. (J) serum AST level. (K) *Srebp-1c*, (I) *Fas*, (M) *Ppar γ* , (N) *Acc*, (O) *Plin5*, (P) *Plin2*, (Q) *Ppara* and (R) *Cpt1 α* mRNA expression in liver. H&E staining of kidney (S) and spleen (U) and levels of serum BUN (T) and Cr (V).

Fig. 4. Sa-D induces thermogenesis in BAT of HFD-fed mice. (A) mRNA expression levels of thermogenesis-related genes (*Sirt1*, *Pparg1 α* , *Cidea*, *Prdm16*, *Dio2*, *Nrf1*, *Tfam*, *Cox7 α* , *Ppara* and *Ucp1*) and lipid droplet-associated genes (*Atgl*, *Hsl* and *Plin5*). (B) Immunohistochemistry for SIRT1 and UCP1. (C and D) Protein expression levels of p-PKA, PKA, p-AMPK α , AMPK α , p-p38, p38, SIRT1, PGC-1 α , COX4, TFAM and UCP1 were measured by western blot.

Fig. 5. Sa-D induces WAT browning in HFD-fed mice. (A) mRNA expression levels of

lipid droplet-associated genes (*Atgl*, *Hsl* and *Plin5*), BAT-rich genes (*Sirt1*, *Prdm16*, *Pparg1a*, *Cidea*, *Dio2*, *Tfam*, *Nrf1*, *Cox2*, and *Ucp1*) and BeAT-specific genes (*Fgf21*, *Tbx1*, *Cd40*, *Tmem26*, *Cited1*, and *Cd137*). (B) Immunohistochemistry for SIRT1 and UCP1. (C and D) Protein expression levels of p-PKA, PKA, p-AMPK α , AMPK α , p-p38, p38, SIRT1, TFAM, and UCP1 were measured by Western blot.

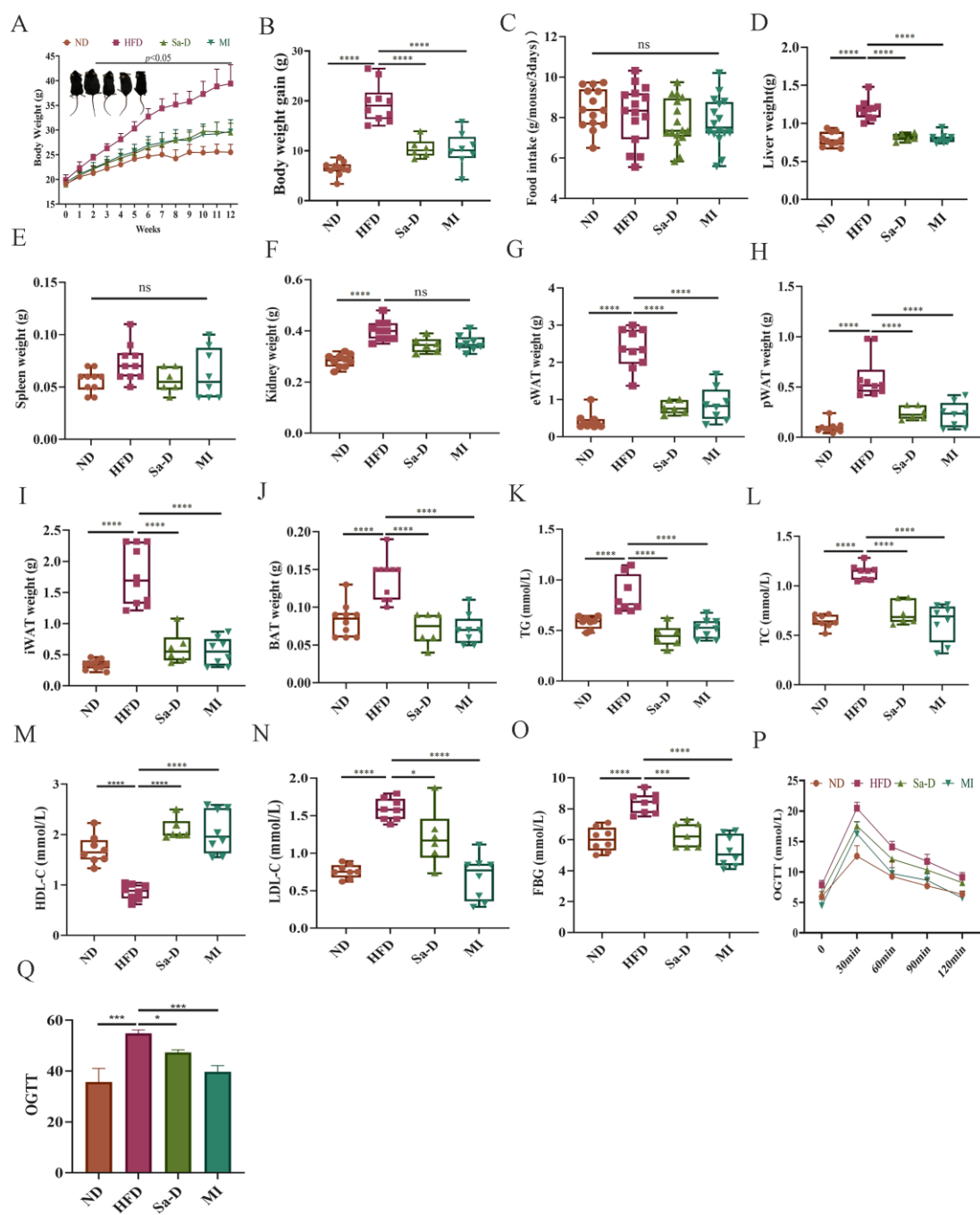
Fig. 6. Diversity and structural analysis of fecal flora. (A) Observed OTUs. (B) Shannon index. (C) Simpson index. (D) ACE index. (E) Chao1 index. (F) Venn diagram. (G) Bacterial taxonomic profiling at the phylum level. (H) Bacterial taxonomic profiling at the family level. (I) LDA score plot from LEfSE analysis.

Fig. 7. Effects of Sa-D on the composition of gut microbiota at the genus level. (A and B) Extended error bar plot identifying significant differences between ND versus HFD, HFD versus Sa-D. (C) Pearson's correlation analysis of the gut microbiota at genus level and obesity-related traits, beige fat-related factors, and brown fat-related factors.

Fig. 8. SCFAs production and associations with key species in the fecal microbiota (A) the levels of SCFAs (acetate, propionate, butyrate, valeric acid) in feces. (B) the gene expression levels of SCFA-producing enzymes in feces. (C-E) the mRNA expression of SCFAs receptors in the colon, WAT, and BAT. (F) Correlation of SCFAs production and key species in the fecal microbiota.

Fig. 9. Schematic representation of the effects of Sa-D on BAT activity and WAT browning. Administration of Sa-D increased the expression levels of several genes involved in adipose thermogenesis, and its effect may be related to the action of SCFAs produced by intestinal microflora through the GPR43/PKA-AMPK signaling pathways.

Fig. 1



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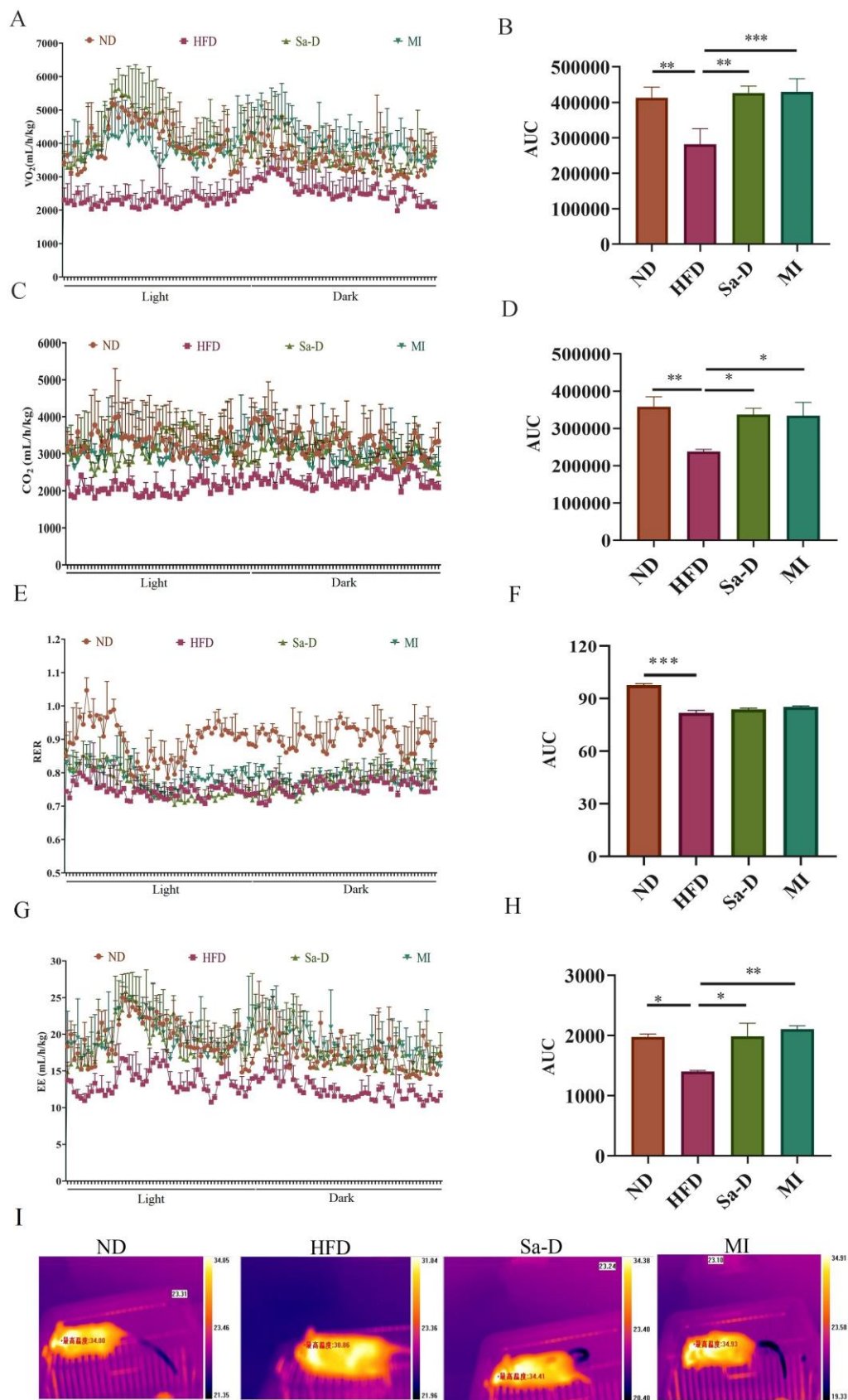
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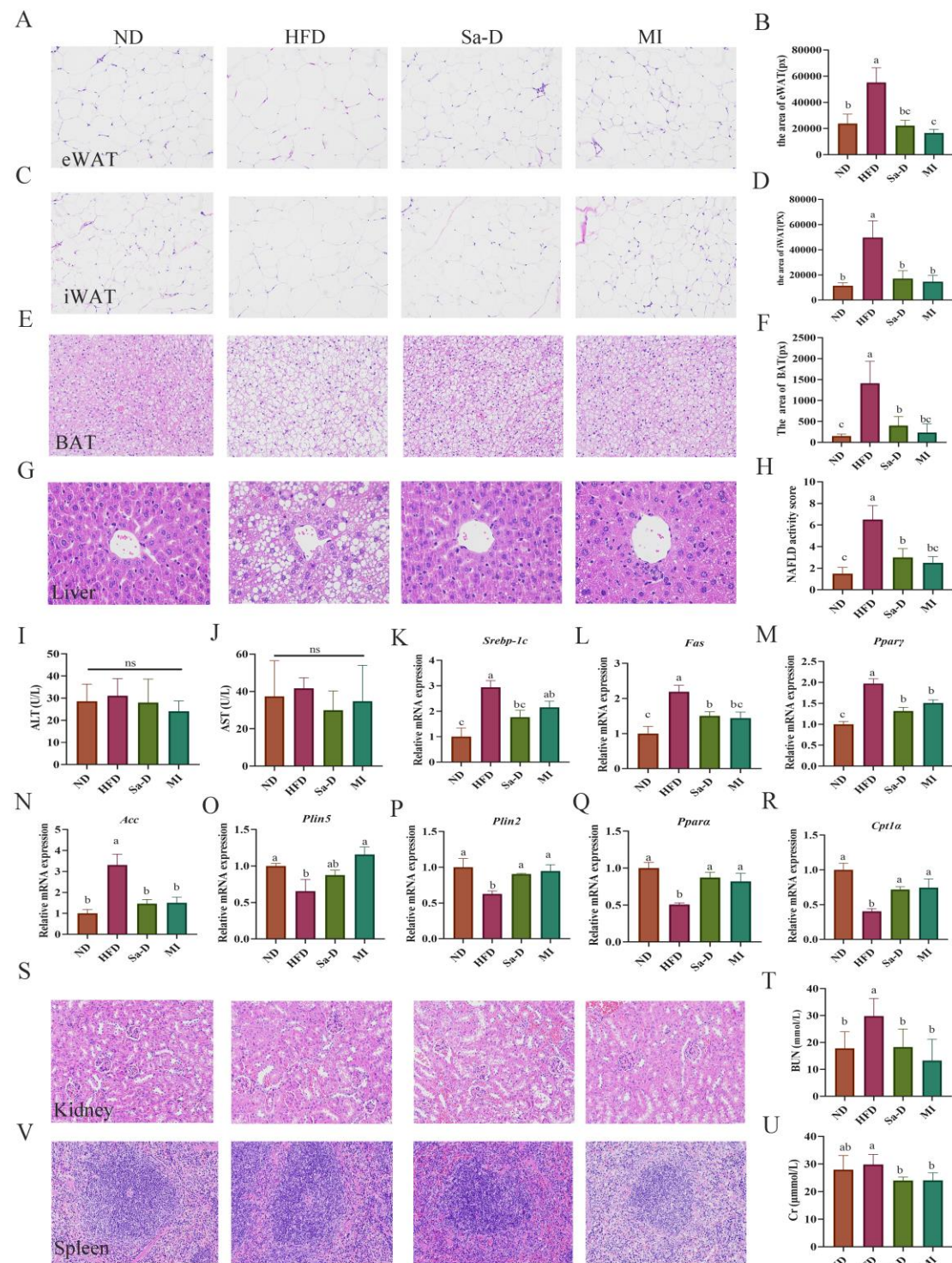


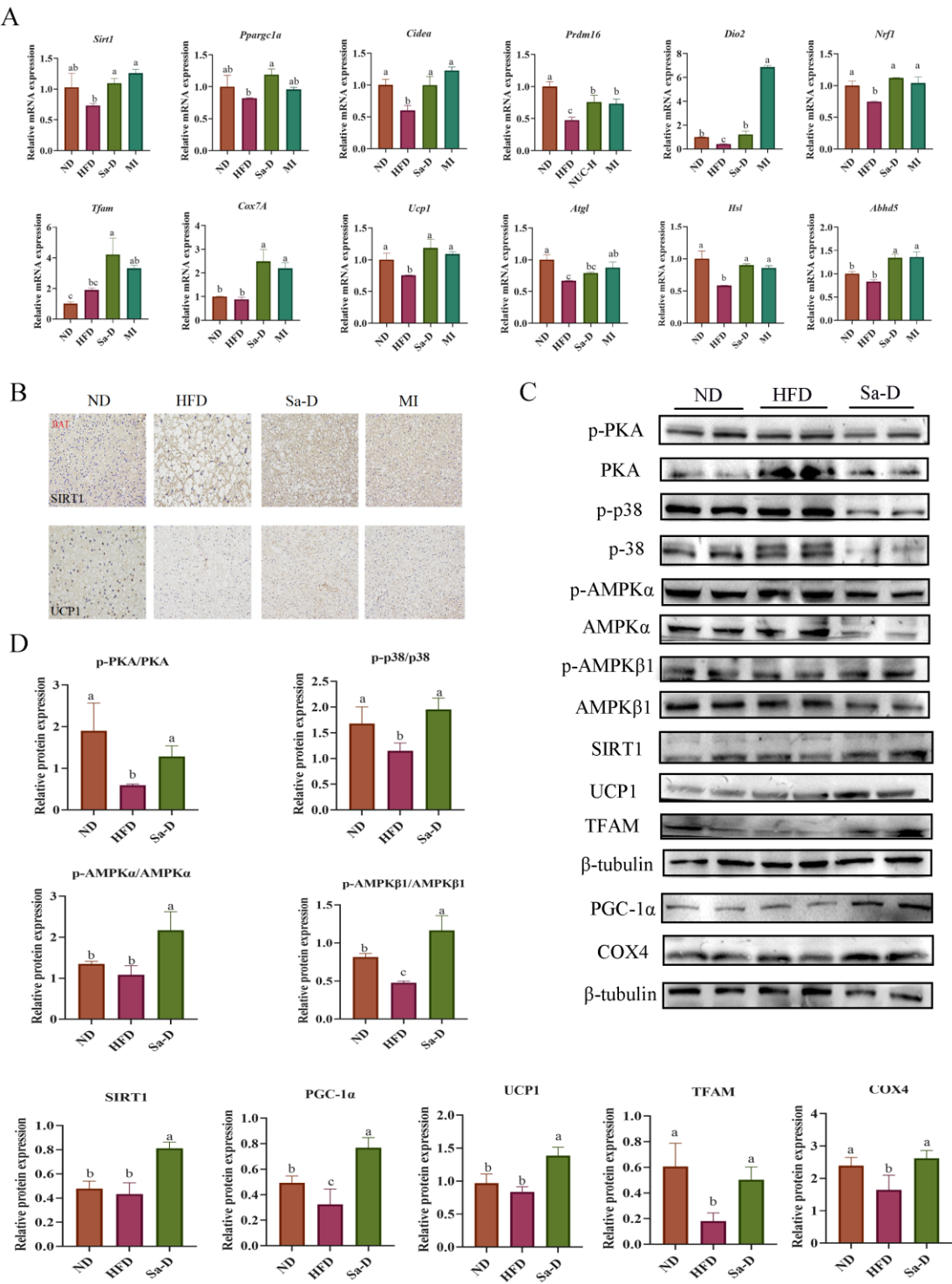
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Fig. 3





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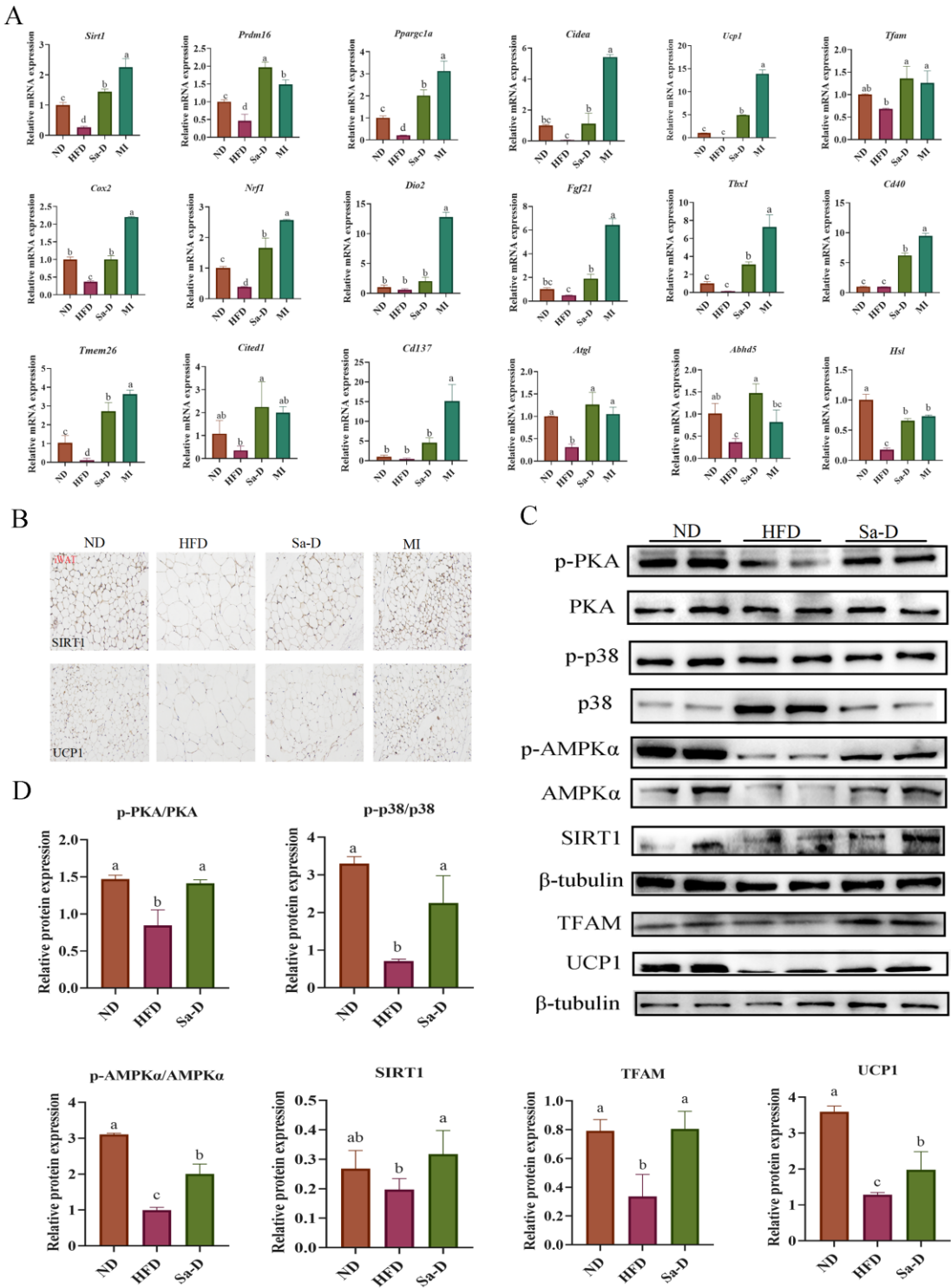
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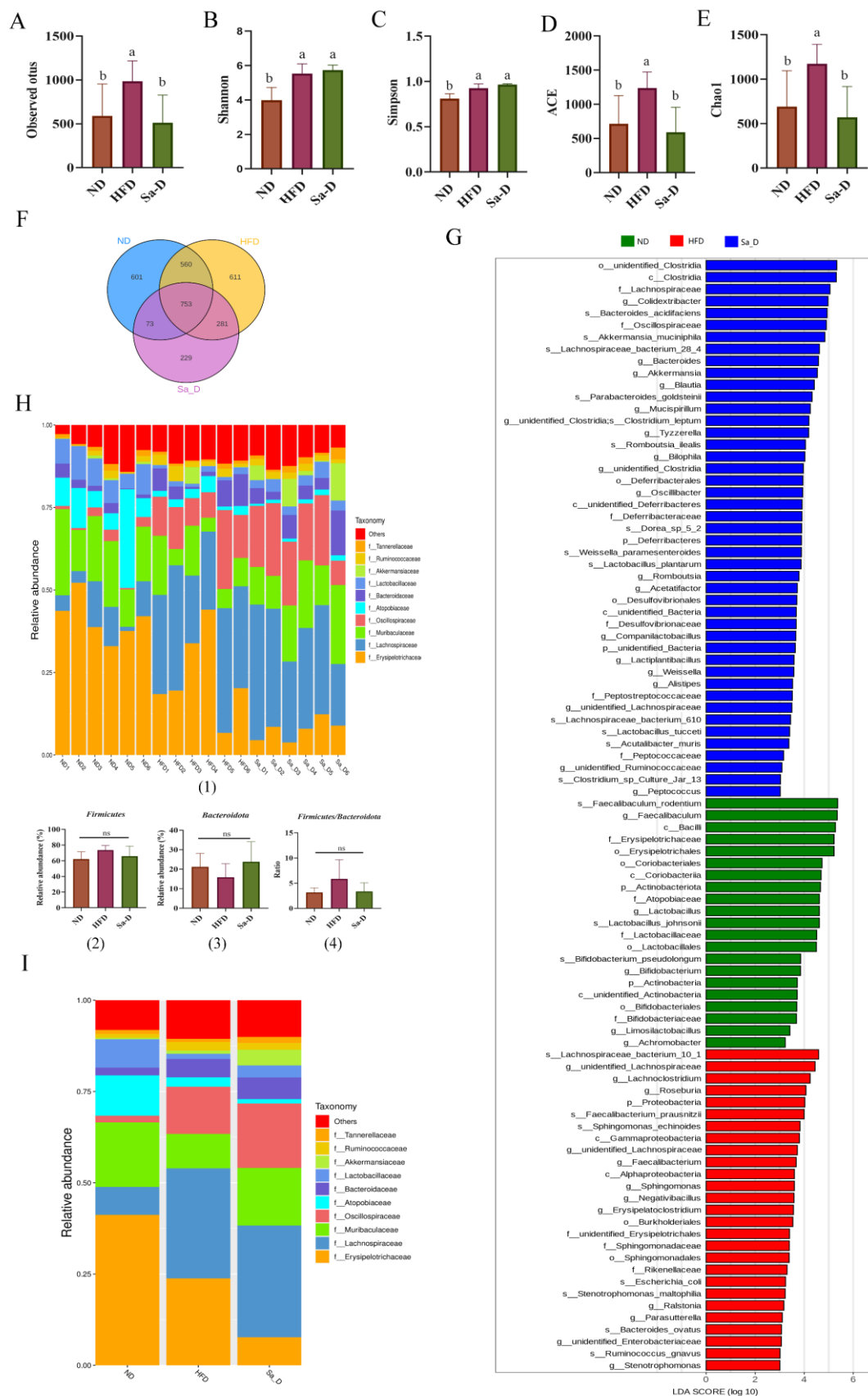
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Fig. 5



779 **Fig. 6**



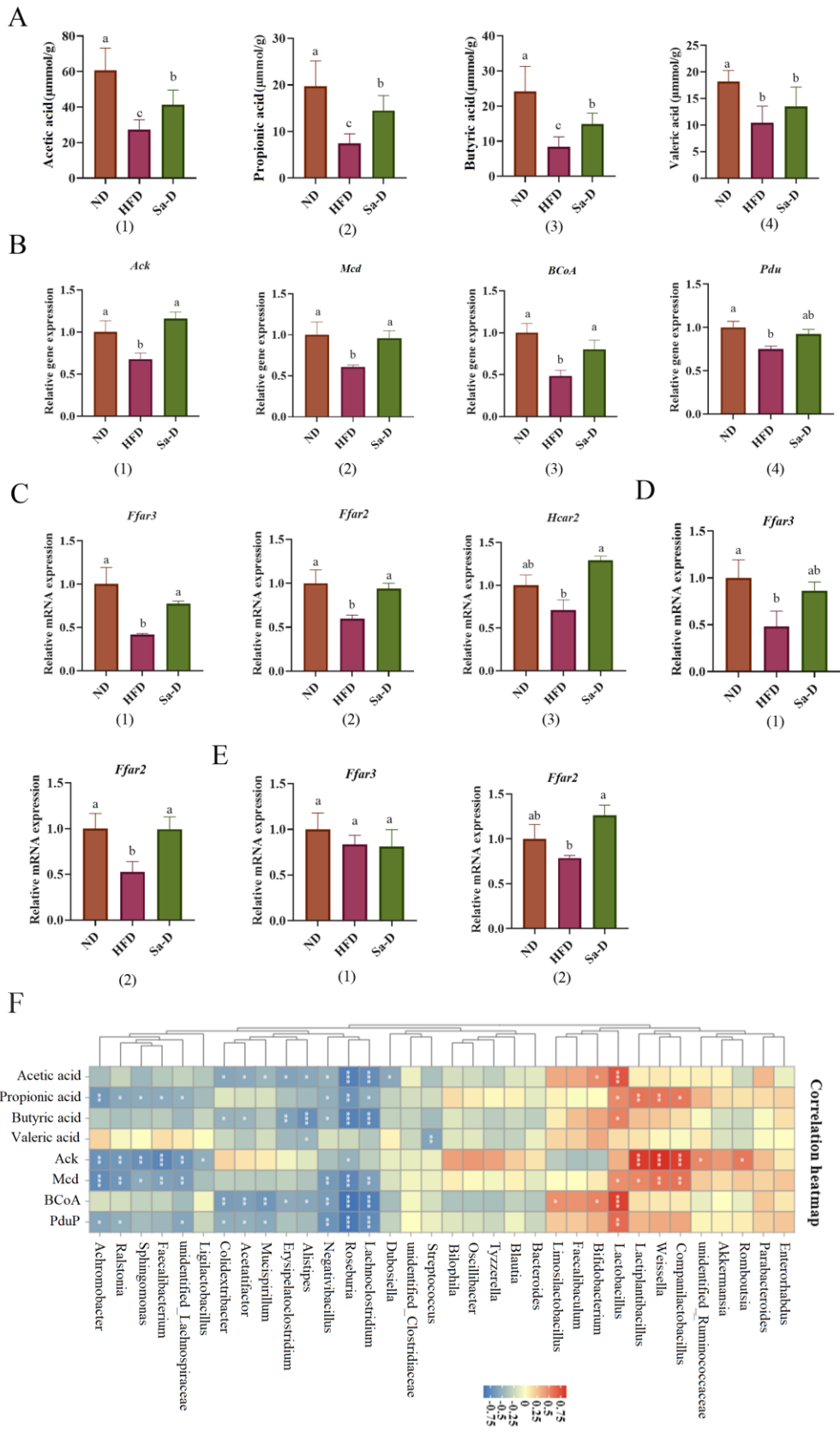
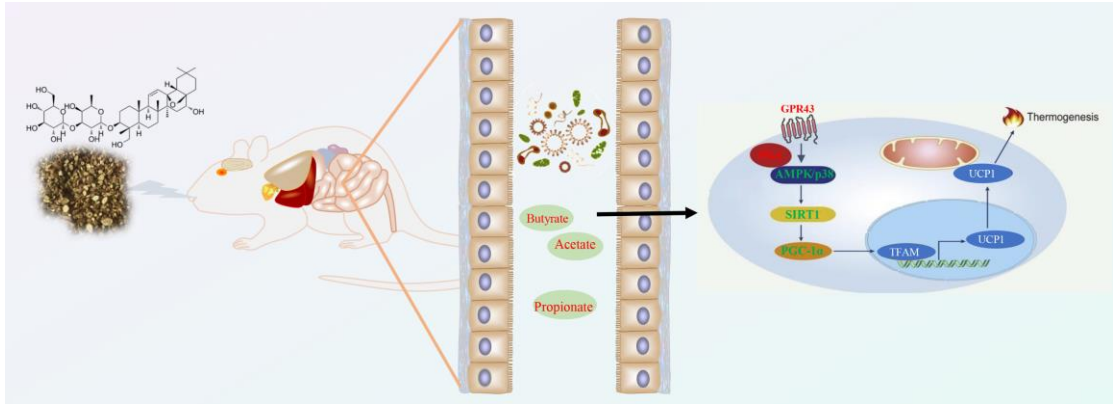


Fig. 9



Saikosaponin D Ameliorates Obesity and Metabolic Disorders *via* the Gut Microbiota-SCFAs-thermogenic fat Axis

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Abstract

Activating thermogenic programs in brown adipose tissue (BAT) represents promising approaches to combat obesity through dissipating energy. Saikosaponin D (Sa-D), a major triterpenoid saponin derived from *Radix bupleuri* (a traditional herbal product) could ameliorate obesity. Here, our results showed that Sa-D treatment for 12 weeks significantly suppressed weight gain and improved glycolipid abnormalities in high-fat-diet (HFD)-fed mice. Importantly, Sa-D treatment increased the expression of uncoupling protein 1 (UCP1) along with other thermogenic factors, as well as enhanced phosphorylation of PKA, AMPK, and p38 in BAT and inguinal white adipose tissue (iWAT). 16S rRNA sequence analysis revealed that Sa-D restored HFD-induced microbial dysbiosis and specific bacterial genera such as *unidentified_Lachnospiraceae*, *Tyzzarella*, *Romboutsia*, *Bilophila*, *Oscillibacter*, *Sphingomonas*, and *Companilactobacillus* were strongly associated with obesity-related traits and brown/beige-related markers. Moreover, Sa-D markedly increased the concentration of short-chain fatty acids (SCFAs), induced gene expression of SCFAs-producing enzymes (acetate kinase (Ack), methylmalonyl-CoA decarboxylase (Mcd), butyryl-CoA (BCoA) and propionaldehyde dehydrogenase (PduP)), and upregulated expression of SCFAs receptors (*Ffar3*, *Ffar2*, or *Hcar2*) in colon, iWAT, and BAT. Collectively, our findings revealed that Sa-D could ameliorate obesity by activating thermogenic fat, which may be associated with the alteration in microflora profiles and increased SCFAs levels.

Keywords: Saikosaponin D, Thermogenic fat, Microbiota, Short-chain fatty acids

1. Introduction

Excess energy intake over energy expenditure leads to an imbalance that promotes the accumulation of additional fat, thereby contributing to the onset of obesity. Therefore, strategies to ameliorate obesity are either through limiting dietary energy intake, enhancing energy expenditure, or a combination of both (Tseng, Cypess, & Kahn, 2010).

Adipose tissue serves as a key regulator of the body's energy metabolism, responsible for maintaining systemic energy balance (Zhang et al., 2014). Mammalian adipose tissue is generally categorized into two main types, white adipose tissue (WAT) and brown adipose tissue (BAT), based on their distinct functional and morphological characteristics. WAT is mainly distributed in the subcutaneous tissues and around the internal organs, with its primary role being the storage of surplus energy as triglycerides. BAT is mainly distributed in the scapular region and can be divided into two types: classical and recruitable (called beige adipose tissue or brown in white) which have similar functions. Brown adipocytes in BAT possess abundant mitochondria and express uncoupling protein 1 (UCP1), which promotes the oxidation of lipids, enabling energy to be transmitted in the form of heat, and enhancing whole-body metabolic function. Consequently, strategies that encourage the browning of fat could offer a novel approach to the management of obesity.

In addition to well-established stimuli such as cold exposure, exercise, and medications (e.g., β_3 -adrenergic agonist), several studies have reported that natural compounds (chlorogenic acid, luteolin, curcumin, quercetin, capsaicin, berberine) and dietary supplements (e.g., tea, cranberry, grape, apple) can potentially stimulate WAT browning

and BAT activity (Hu et al., 2020; Wang et al., 2023). In East Asia, Chaihu (*Radix Bupleuri*) is often processed into porridge, soup bases, or beverages and is widely used as a herbal remedy (Liu et al., 2023; Su et al., 2018, Zhu et al., 2017). Saikosaponin D (Sa-D) extracted from Chaihu is one of the major biologically active triterpenoid saponins and documented to exhibit diverse biological activities, including anti-aging (Kim, 2018), anti-leukemic (Sun et al., 2021), anti-obesity (Lim et al., 2021), anti-inflammatory (Lu et al., 2012), anti-cancer (Tang et al., 2023), anti-diabetic (Xiang et al., 2020), and anti-depressant-like effects (Su et al., 2020). In this study, we mainly investigate the underlying mechanism responsible for its efficacy in combating obesity. Most previous studies focus on its beneficial effect on glycolipid metabolism in the liver. For example, Sa-D treatment for 8 weeks inhibited body weight gain and promoted fatty acid catabolism in the liver of high-fat-diet (HFD)-fed mice (Gu et al., 2022). Integrated lipidomic and transcriptomic analyses demonstrated that Sa-D regulates hepatic glycerolipid metabolism by hampering fatty acid biosynthesis and enhancing fatty acid degradation (Li et al., 2021). However, little information on its effect on adipose tissue development and metabolism is available.

Given the significant role that thermogenic fat plays in facilitating weight loss, the effect of Sa-D on fat browning and its potential mechanism was investigated. Our research yielded promising results, demonstrating that Sa-D can reduce both blood lipid and glucose levels, which are key markers of metabolic health. Furthermore, we observed a notable increase in the activity of BAT, as well as the initiation of the browning process in inguinal WAT (iWAT), which was beneficial for increasing energy

expenditure. The mechanism behind this browning effect appears to be multifaceted, with a possible relationship to Sa-D's influence on the gut microbiota profiles and the subsequent production of short-chain fatty acids (SCFAs). Our findings provide insight into the role of Sa-D in obesity management, highlighting its potential as a therapeutic intervention for improving metabolic health by influencing multiple metabolic pathways.

2 Materials and Methods

2.1 Materials and reagents

Saikosaponin D was purchased from Chengdu Herbpurify Co., Ltd (Chengdu, China). Commercial assay kits of triglycerides (TG), total cholesterol (TC), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine (Cr) were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China).

2.2 Animals and Diets

Forty male C57BL/6J mice, aged five weeks, were obtained from Beijing Vitality River Laboratory Animal Technology Co. and accommodated at the Jiangnan University Animal Centre (permit number: JN.No20220615c1100925[233]). After one week of acclimatization, the mice were randomly assigned to five groups (n=10): normal diet (ND) group, HFD group, Sa-D group received HFD with oral administration of 20 mg/kg of Sa-D, and MI group (the positive group) received HFD with oral administration of 5 mg/kg of miglitol. The dose of Sa-D was chosen based on previous reports on mice (Gu et al., 2022; Li et al., 2021; Li et al., 2024). The formulation of the

feed is listed in Table S1.

2.3 Energy metabolism

Energy consumption was measured by the CLAMS-12 (Columbus Instruments, USA).

The metabolic cages were maintained at ambient temperature and were subjected to a

12h light/12h dark cycle. The individual mouse was placed in a metabolic cage and O₂

consumption, CO₂ production, and RER (Respiratory exchange ratio) (VCO₂/VO₂)

were continuously monitored for 24 h after the mice had acclimatized to the

environment (12 h), and the energy consumption was calculated.

2.4 Infrared thermography

Mice were placed on ice for 2 h of cold stimulation, then anaesthetized by

intraperitoneal injection of sodium pentobarbital, and immediately imaged with an

infrared thermography camera (Magnify Electronics, Shanghai, China).

2.5 Oral glucose tolerance test (OGTT)

After 12 hours of fasting, the blood glucose at different time points was measured post-

glucose injection at a dose of 1.5 g/kg.

2.6 Analysis of serum biochemical indicators

After 12 hours of fasting, mice were anesthetized with sodium pentobarbital, and

collected whole blood was centrifuged to obtain serum. Biochemical parameters were

determined following the kit manufacturer's instructions.

2.7 Histopathological analysis

The fixed tissues were rinsed under running water for 30 min, then dehydrated with

alcohol at graded concentrations (30%, 50%, 70%, 85%, and 95%) and placed in xylene.

The tissue became transparent and was placed in paraffin wax. After the paraffin cooled and solidified into a block, the paraffin was cut into thin slices (3-5 μm), adhered to slides, and dried at 45°C. The paraffin was stripped from the sections with xylene and washed with alcohol, and then the slides were stained with hematoxylin solution. After rinsing in running water, the slides were put into 70% and 90% alcohol for 10 min, and then stained with eosin for 2 min. After dehydration and transparency treatment, the sections were mounted with gum mountant and photographed for histopathological analysis.

2.8 Immunohistochemical (IHC) staining

The process of IHC staining for iWAT and BAT was performed as prescribed in the staining kit (#36312ES50, Yeasen, Shanghai, China). Briefly, tissue sections were heated, cleared with xylene, and dehydrated through a gradient of alcohol solutions. After antigen retrieval and endogenous enzyme inactivation with 3% (v/v) H_2O_2 , sections were blocked and incubated with primary antibodies (UCP1 and SIRT1 (1:100)) overnight at 4°C, followed by secondary antibody incubation. Staining was developed with DAB, counterstained with hematoxylin, dehydrated again, and mounted for microscopic observation.

2.9 Quantitative RT-PCR analysis

Total RNA extraction of BAT, iWAT, liver, and colon tissues, reverse transcription, and quantitative analysis was carried out as described in our previous study (Wang, Xue et al., 2022). The specific primer sequences are provided in Supplemental Table 2. Fecal genomic DNA was extracted utilizing the MolPure® Stool DNA Kit (Yeast, China),

adhering to the protocol provided by the manufacturer. The gene expression was standardized against 16S rRNA. The sequences of the primers were referenced in the study by Guo et al. (2020) and Gopi et al. (2015).

2.10 Western blot analysis

The protein lysates derived from BAT and iWAT were analyzed *via* Western blot, consistent with the protocols detailed in our earlier investigation (Wang, Xue et al., 2022). Briefly, after protein electrophoresis and membrane transfer, the PVDF membranes were exposed to primary antibodies (1:1000, Proteintech), and incubated at 4°C for 12 h. Then, membranes were incubated with HRP-conjugated secondary antibody (1:4000, Proteintech) and visualized using an ECL detection reagent (Beyotime, China).

2.11 Gut Microbiota analysis

The bacterial composition within fecal samples from various groups was investigated through 16S rRNA gene sequencing, targeting the V3-V4 hypervariable regions. The amplicon sequencing was conducted utilizing the Illumina MiSeq PE250 platform by Metware Biotech Co., Ltd. (Wuhan, China). A full description of the sequencing process is provided in the supplementary documentation.

2.12 Quantification of SCFAs

Fecal samples were used to analyze SCFAs profiles by Gas Chromatography-Mass Spectrometry (GC-MS) following the method of Lin et al. (2022). Briefly, freeze-dried fecal samples were weighed, saturated NaCl and internal standard 2-Ethylbutyric acid were added and then the sample was shaken to remove any visible lumps. After

homogenization, SCFAs were sequentially extracted by adding 40 μ L of 10% sulphuric acid and 1 mL of ether, and the supernatant was separated by centrifugation after shaking for 30 s on a vortex shaker. Then, the supernatant was placed in a test tube containing 0.25 g of anhydrous sodium sulfate for 15 minutes and centrifuged to detect SCFAs by GC-MS.

2.13 Statistical Analysis

All data are expressed as the means $\pm$ standard deviation (SD). GraphPad Prism software was used to determine statistical significance by nonparametric one-way analysis of variance (ANOVA) with Duncan's test. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001. Values with different lowercase letters indicate a significant difference at P < 0.05 following Duncan's multiple range tests using DPS software.

3. Results

3.1 Effects of Sa-D on body weight, tissue weight, and blood biochemical parameters in HFD-fed mice

As shown in Fig. 1, Sa-D treatment reversed HFD-induced increase in body weight gain (Fig. 1A and 1B), liver (Fig.1D), eWAT (Fig.1G), pWAT (Fig.1H), iWAT (Fig.1I), and BAT weights (Fig.1J), which were similar to those in the MI group. Moreover, there were no notable differences in food intake (Fig.1C), or spleen (Fig.1E) and kidney (Fig.1F) weights of mice in the different treatment groups. Analysis of blood lipid profile showed that Sa-D supplementation dramatically attenuated TG (Fig. 1K), TC (Fig. 1L), and LDL-C (Fig. 1N) levels and increased HDL-C (Fig. 1M) levels. As HFD feeding tends to trigger insulin resistance, glucose tolerance was examined to investigate the effect of Sa-D on glucose homeostasis. It was observed that Sa-D

intervention significantly reduced fasting blood glucose (FBG) level (Fig. 1O) and enhanced OGTT (Fig. 1P and 1Q) in HFD-fed mice, indicating improved glucose homeostasis. These findings evidence the promising potential of Sa-D to inhibit weight gain and ameliorate glycolipid disorders.

3.2 Effects of Sa-D on energy consumption in HFD-fed mice

We further investigated whether the Sa-D-induced reduction in obesity was achieved by increasing energy consumption. As shown in Fig. 2, the VO_2 (Fig. 2A and 2B) and VCO_2 (Fig. 2C and 2D) in the Sa-D-treated mice were 1.51-fold and 1.26-fold of the HFD group, respectively. A similar effect was observed in energy expenditure (EE) (1.42-fold of the HFD group) (Fig. 2G and 2H). However, Sa-D did not exert a significant impact on the Respiratory Exchange Ratio (RER). The average values for RER in the ND, HFD, Sa-D, and MI groups were 0.98, 0.81, 0.84, and 0.85, respectively (Fig. 2E and 2F), indicating no substantial difference. The thermographic images upon cold stimulation showed increased skin temperature in the interscapular region of Sa-D-treated mice (34.41°C) compared to HFD-fed mice ($\text{HFD}_{\text{max}}: 30.86^{\circ}\text{C}$), approaching that of the MI group ($\text{MI}_{\text{max}}: 34.93^{\circ}\text{C}$) (Fig. 2I).

3.3 Histopathology analysis of HFD-fed mice

Administration of Sa-D significantly decreased the adipocyte size in both eWAT (Fig. 3A and 3B) and iWAT (Fig. 3C and 3D), and also diminished lipid accumulation in BAT (Fig. 3E and 3F) to comparable levels observed in the MI group. Hepatopathology revealed that Sa-D-treated mice had a decreased NAFLD activity score (NAS) as compared to the HFD group (Fig. 3G and 3H). Serum measurement of ALT and AST

demonstrated that Sa-D had not induced significant injury to the liver (Fig. 3I and 3J). Hepatic steatosis was further evaluated by mRNA analysis. Treatment of Sa-D suppressed the expression of lipogenesis-related genes, including *Srebp-1c* (Fig. 3K), *Fas* (Fig. 3L), *Pparγ* (Fig. 3M), and *Acc* (Fig. 3N), and increased lipolysis-related genes such as *Plin5* (Fig. 3O), *Plin2* (Fig. 3P), *Ppara* (Fig. 3Q) and *Cpt1α* (Fig. 3R). Furthermore, we examined the safety of Sa-D by analyzing tissue sections from the kidneys (Fig. 3S) and spleens (Fig. 3V). The data revealed that Sa-D provided a protective effect on both the kidney and the spleen, evidenced by decreased levels of BUN (Fig. 3T) and Cr (Fig. 3U), relative to the HFD group.

3.4 Effects of Sa-D on the activity of BAT in HFD-fed mice

As shown in Fig. 4A, Sa-D treatment upregulated mRNA expression for BAT-associated genes, including *Sirt1*, *Pparg1α*, *Cidea*, *Prdm16*, *Dio2*, *Nrf1*, *Tfam*, *Cox7a*, and *Ucp1* compared with the HFD group. Notably, expression of the lipid droplet-associated genes *Atgl*, *Hsl*, and *Abhd5* was induced by Sa-D treatment (Fig. 4A). In addition, increased immunostaining of SIRT1 (Fig. 4B) and UCP1 (Fig. 4B) was detected in Sa-D-treated mice. Similarly, Western blotting analysis showed Sa-D induced protein expression of brown-related markers such as SIRT1, PGC-1α, TFAM, and UCP1. Importantly, Sa-D significantly enhanced the ratio of p-PKA/PKA, p-p38/p38, p-AMPKα/AMPKα, and p-AMPKβ₁/AMPKβ₁.

3.5 Effects of Sa-D on the browning of iWAT in HFD-fed mice

Intracellular lipolysis is responsible for the physiological regulation of thermogenic processes in WAT browning. Investigation of lipolysis-related genes showed Sa-D

augmented mRNA expression of *Atgl*, *Hsl*, and *Abhd5* (Fig. 5A). Moreover, Sa-D induced BAT-rich genes, such as *Sirt1*, *Prdm16*, *Pparg1a*, *Cidea*, *Dio2*, *Tfam*, *Nrf1*, *Cox2*, and *Ucp1* in comparison to the HFD control (Fig. 5A). Moreover, beige adipocyte-specific genes, such as *Fgf21*, *Tbx1*, *Cd40*, *Tmem26*, *Cited1*, and *Cd137* were also upregulated in Sa-D-treated mice and the positive control (MI) group (Fig. 5A). Immunohistochemistry of SIRT1 and UCP1 showed higher positive immunostaining areas in iWAT for the Sa-D treatment group (Fig. 5B) and Western blotting analysis of SIRT1, TFAM and UCP1 also exhibited a similar trend (Fig. 5C). Importantly, Sa-D significantly enhanced the ratio of p-PKA/PKA, p-p38/p38, and p-AMPK α /AMPK α .

3.6 Sa-D remodels the gut microbiome in HFD-fed mice

To investigate the remodeling of the intestinal microflora by Sa-D treatment, fecal 16S rRNA gene sequencing analysis was performed. Observed OTUs showed a reduced trend in Sa-D-treated mice (Fig. 6A). Alpha diversity analysis showed no statistical difference in the Shannon and Simpson indexes (Fig. 6B and 6C), but reduced ACE and Chao1 indexes in Sa-D-treated mice compared the HFD group (Fig. 6D and 6E). The Venn diagram shows treatment with Sa-D decreased the number of OTUs, which was close to the ND group. A further 753 (24.6 %) of the total OTUs were common to all three groups; 1320 (37.2%) OTUs were shared between the ND and HFD groups; 1187 (33.4%) OTUs were shared between the HFD and Sa-D group (Fig. 6F). PCoA analysis showed that Sa-D-treated mice clustered separately from HFD mice, and were similar to ND mice (Fig. S1A). These results suggest that Sa-D could partially reverse changes in HFD-induced gut microbiota composition. Specifically, no significant differences in

phylum Firmicutes (F) and Bacteroidota (B) were found among the three groups (Fig. 6G(1-3)). However, the Sa-D treatment could reduce the F/B ratio, although this decrease was not statistically significant when compared to the HFD group (Fig. 6G(4)). The *Muribaculaceae* and *Akkermansiaceae* family encompassing many probiotic species were predominantly present in Sa-D-treated mice (Fig. 6H). The LEfSe (LDA>3) analysis identified a total of 93 OTUs, comprising 5 phyla and 36 genera, as pivotal phylotypes within the dataset (Fig. 6I and Fig. S1B). The *s_Faecalibaculum_rodentium*, *g_Faecalibaculum*, *c_Bacilli*, *f_Erysipelotrichaceae*, and *p_Erysipelotrichales* were predominantly located in the ND group (LDA>4); *s_Lachnospiraceae_bacterium_10_1*, *g_Roseburia*, *g_unidentified_lachnospiraceae*, *g_Lachnoclostridium*, and *p-Proteobacteria* were predominantly located in the HFD group (LDA>4); While after Sa-D treatment, the *o_unidentified_clostridia*, *c_Clostridia*, *f_Lachnospiraceae*, *g_Colidextribacter*, *s_Bacteroides_acidifaciens*, *f_oscillospiraceae*, *s_Akkermansia_muciniphila*, *s_Lachnospiraceae_bacterium_28_4*, *g_bacteroides*, *g_Akkermansia*, *g_Blautia*, *sParabacteroides_goldsteinii*, *g_Mucispirillum*, *g_unidentified_clostridia*, *g_Tyzzereella*, *s_Romboutsia_ileails*, and *g_bilophila* (LDA>4) were predominantly located in Sa-D group (LDA>4); For assignment on the genus-level, 34 genera with high abundance are shown in Fig. S2. The HFD group showed decreased abundance of *Faecalibaculum*, *Lactobacillus*, *Bifidobacterium*, and *Limosilactobacillus*, whereas enrichment of *Colidextribacter*, *unidentified_Lachnospiraceae*, *Lachnoclostridium*, *Roseburia*, *Tyzzereella*, *Negativibacillus*, *Acetatifactor*, and *Oscillibacter* was observed relative to the ND

group (Fig. 7A). Moreover, genera *Faecalibaculum*, *unidentified_Lachnospiraceae*, *Roseburia*, *Sphingomonas*, *Ralstonia*, *Bifidobacterium*, *unidentified_Ruminococcaceae*, *Achromobacter* were lower, but *Lactobacillus*, *Tyzzereella*, *Bilophila*, *Oscillibacter*, *Romboutsia*, *Companilactobacillus*, *Lactiplantibacillus*, and *weissella* were higher in the Sa-D group versus HFD group (Fig. 7B). Spearman's correlation analysis indicated that the genera *Bifidobacterium*, *Faecalibaculum*, *Lactobacillus*, and *Limosilactobacillus* exhibited a negative association with brown/beige fat-associated markers and a positive link with serum lipid indicators and body weight gain ($p<0.05$), but *Alistipes*, *Mucispirillum*, *Erysipelatoclostridium*, *Companilactobacillus*, *Lactiplantibacillus*, *Weissella*, *Acetatifactors*, *Blautia*, *Lachnoclostridium*, *Roseburia*, *Tyzzereella*, *Colidextribacter*, *Oscillibacter*, and *Romboutsia* showed opposite trends (Fig. 7C).

3.7 Analysis of SCFAs and their correlation with key species in the fecal microbiota

To investigate whether Sa-D-induced browning of adipose tissue is related to intestinal flora, bacterial metabolites of SCFAs in the feces were examined by GC-MS. Sa-D augmented the levels of acetic, propionic and butyric acids as compared to the HFD group (Fig. 8A and Fig. S3). Consistently, Sa-D treatment significantly increased the expression of SCFA-producing enzymes in feces: acetate kinase (Ack), methylmalonyl-CoA decarboxylase (Mcd), propionaldehyde dehydrogenase (Pdup) and butyryl-CoA (BCoA) (Fig. 8B). The mRNA expression of SCFAs receptors (*Ffar3*, *Ffar2*, and *Hcar2*) in colon (Fig. 8C), *Ffar2* in WAT (Fig. 8D), and *Ffar2* in BAT (Fig. 8E), can be induced by Sa-D. However, Sa-D treatment did not significantly affect *Ffar3* expression in

iWAT and BAT (Fig. 8D and 8E). Spearman's correlation heatmap (Fig. 8F) demonstrated a notably positive correlation between *Lactobacillus* and the levels of SCFAs as well as the enzymes Ack, Mcd, and BCoA. *Lachnospiraceae*, *Roseburia*, *Negativibacillus*, *Alistipes*, and *Erysipelatoclostridium* were negatively correlated with acetic, propionic, and butyric acids, and SCFAs-producing enzymes (*Mcd*, *BcoA*, *PduP*). Meanwhile, *unidentified_Lachnospiraceae*, *Faecalibaculum*, *Sphingomonas*, *Ralstonia*, *Colidextribacter*, and *Achromobacter* were negatively correlated while *Romboutsia*, *unidentified_Ruminococcaceae*, *Companilactobacillus*, *Lactiplantibacillus*, *Weissella*, and *Limosilactobacillus* were positively correlated with certain SCFAs-producing enzymes. Thus, our results demonstrated a significant effect of Sa-D intervention on SCFAs levels and revealed a correlation between key microorganisms and SCFAs levels.

4 Discussion

BAT is a specialized fat depot that plays a key role in energy balance by promoting energy expenditure through thermogenesis. It burns calories to produce heat, thereby helping to regulate body temperature and metabolic rate. This unique ability of BAT makes it an important tissue for maintaining overall metabolic health and combating obesity. Previous findings have demonstrated that Sa-D could enhance fatty acid catabolism in BAT and white adipocytes (Gu et al., 2022), however, the mechanism remains poorly understood. Here, our findings revealed significant anti-hyperlipidemic, anti-hepatic steatosis, and anti-hyperglycemic effects of Sa-D in HFD-fed mice, in agreement with earlier findings (Gu et al., 2022). Furthermore, increased expression of

brown fat-associated factors, including SIRT1, PGC-1 α , PRDM16, and UCP1 support the role of Sa-D in activating BAT and iWAT browning. Notably, Sa-D supplementation enhanced O₂ consumption and CO₂ production without a significant effect on RER, suggesting that Sa-D-treated mice can utilize fat as an energy source along with increased energy expenditure. The PKA signaling pathway is a crucial modulator of energy expenditure. PKA activates AMPK and p38 MAPK, which in turn facilitates the transcription of PGC-1 α and UCP1, and is consequently essential in regulating the development and activation of BAT (Zhang et al., 2021). Here, Sa-D induced the phosphorylation of PKA, AMPK, and p38 in BAT and iWAT, supporting the activation of PKA-AMPK/p38 signaling in its mechanism of action.

The gut microbiota has been found to promote BAT thermogenesis, and in addition, alterations in the flora structure modulate the conversion of WAT into beige fat in patients with obesity (Wang et al., 2021). Earlier research reveals that Sa-D ameliorates gut microbiota disturbances in colitis mice (Li et al., 2020). In addition to identifying the microflora profiles in the improvement of Sa-D-treated mice exposed to HFD, the new findings of this study also uncover that microflora-derived SCFAs may be important factors for Sa-D-induced BAT thermogenesis. The microbiota of obese animals is mainly composed of Firmicutes (F) and Bacteroidetes (B), with F being predominant and B less abundant (Ley et al., 2006). However, it remains inconclusive whether the F/B ratio is an inevitable element in establishing obesity. Some studies have found no significant changes in this ratio, while others have reported a decreased F/B ratio among individuals with obesity (Magne et al., 2020). Evidence suggests that Sa-

354 D (15 mg/kg) for 12 weeks has no significant effect on the F/B ratio in HFD-fed mice
355 (Li et al., 2024). It may be more important to consider specific compositional changes
356 rather than solely focusing on the F/B ratio. Research suggests that *Faecalibaculum*
357 strains could be a notable genus implicated in obesity, with a demonstrated positive
358 relationship to body weight, WAT index, and serum glucose and inflammation levels
359 (Sun et al., 2020). Within *Firmicutes*, a high abundance of *Lachnospiraceae* contributes
360 to the disturbance of glucolipids (Vacca et al., 2020). Furthermore, a negative
361 correlation was observed between the genus *unidentified_Lachnospiraceae* and the
362 expression of leptin, HSL, and UCP1 in WAT as well as BAT function (Kang et al.,
363 2022). *Lactobacillus* is recognized as a probiotic and has shown promising anti-obesity
364 properties in both rodents and humans. The enhanced mitochondrial levels and function
365 by upregulating the thermogenic gene program (e.g., *Ucp1*, *PPAR γ* , and *PGC-1 α*) were
366 observed in iWAT and BAT of *Lactobacillus*-treated mice (Kang et al., 2022; Park et
367 al., 2019; Yoon et al., 2020). In the research of Zhang et al. (2022), the *Tyzzzerella* genus
368 positively correlated with *PPAR α* and *SIRT1* levels in iWAT, while *Roseburia* was
369 negatively associated with *PGC-1 α* , *PRDM16*, and *Cidea* levels in iWAT (Zhang et al.,
370 2022). *Bilophila* belongs to phylum *Proteobacteria* and some studies have shown that
371 this genus inversely correlates with HFD-induced multi-tissue metabolic disorders
372 (Gao et al., 2020), and was beneficial for the browning of iWAT (Zhu et al., 2020). The
373 genus *Oscillibacter* displayed a positive correlation with the levels of gene expression
374 related to thermogenesis, and concurrently, it was inversely associated with TC, TG,
375 and the mass of epididymal WAT (Li et al., 2022). Obesity-associated genera

376 *Romboutsia* showed a negative relationship with PGC-1 α , Cidea, and PRDM16 in
377 HFD-fed mice (Zhang et al., 2022). In our research, the altered microflora profiles such
378 as reduced *Faecalibaculum*, *unidentified_Lachnospiraceae*, and *Roseburia*, and
379 increased *Tyzzereella*, *Bilophila*, *Oscillibacter*, and *Romboutsia* may be responsible for
380 the anti-obesity and brown-promoting effects of Sa-D.

381 SCFAs are among the most thoroughly investigated metabolites that are generated by
382 the intestinal microbiota. They function as chemical messengers that bridge microbe-
383 host crosstalk, and thus may play beneficial metabolic actions in iWAT and BAT *via* the
384 microbiota-gut (brain)-adipose axis. SCFAs could act directly or indirectly on energy
385 expenditure by increasing BAT activity. Hu and collaborators demonstrated that
386 treatment with 10 mM acetate induced significant morphological alterations in
387 immortalized brown adipocytes, along with a boost in mitochondrial biogenesis, as well
388 as elevated expression levels of PGC-1 α , PPAR γ , aP2, and UCP1 (Hu et al., 2016). In
389 addition, research indicates that butyrate modulates the gut-brain neural circuits,
390 thereby boosting energy metabolism by stimulating BAT (Li et al., 2018). Several
391 studies have documented that *Lactobacillus* strains could ameliorate obesity-related
392 markers and provide a benefit by increasing the levels of acetate, butyrate, and
393 propionate (Ji et al., 2019; Larsen et al., 2013; Ma et al., 2022). Probiotic
394 *Lactiplantibacillus* and *Weissella* are key producers of SCFAs and strongly associated
395 with a range of SCFAs, namely acetic, propanoic, butyric, and valeric acids (He et al.,
396 2022; Sun et al., 2023). *Sphingomonas* being present in the obese strain showed a
397 negative relationship with acetic acid and butyric acid (Zhang et al., 2022).

Companilactobacillus is a potential gut microbiota-regulating probiotic and was found to alleviate colonic inflammation, which associates with increased SCFAs levels (Wang, Shi et al., 2022). Research has validated that certain herbal triterpenoid saponins, including ginsenoside Rk3 (Guo et al., 2023), ginsenoside Rh4 (Bai et al., 2021), and xanthoceraside (Zhou et al., 2022) could augment both the population of SCFAs-producing bacteria and the levels of SCFAs. Consistently, treatment with Sa-D markedly increased the abundance of *Lactobacillus*, *Lactiplantibacillus*, *Weissella*, *Sphingomonas*, and *Companilactobacillus* as well as the levels of acetate, butyrate and propionate in feces. Meanwhile, gut microbiota can secrete enzymes such as acetate kinase (*Ack*), methylmalonyl-CoA decarboxylase (*Mcd*), butyryl-CoA (*BCoA*), and propionaldehyde dehydrogenase (*PduP*) to metabolize carbohydrates into SCFAs. Studies have documented that Sa-D dramatically elevates the fecal expression of ACK, MMD, and BUT, thereby facilitating the production of SCFAs. Hence, Sa-D modified the microbial profile by reducing *Sphingomonas* and enhancing the abundance of *Lactobacillus*, *Lactiplantibacillus*, and *Weissella*, which consequently resulted in elevated levels of SCFAs.

As gut-derived signaling molecules, SCFAs are involved in energy metabolism by binding GPR43 (*Ffar2*), GPR41 (*Ffar3*), and GPR109a (*Hcar2*). Intestinal *Ffar3*, *Ffar2*, and *Hcar2* activation facilitates the secretion of GLP-1, GIP, and CCK, leading to fat browning and maintenance of energy balance (Wang et al., 2023). Furthermore, it has been documented that *Ffar2* and *Ffar3* are expressed in the WAT and BAT of HFD-fed mice (Ikuo Kimura et al., 2013; Lu et al., 2016). Previous research showed the body

temperature and *Ucp1* expression in BAT were reduced in *Ffar3*^{-/-} mice and *Hcar2*^{-/-} mice compared to wild type mice (Kimura et al., 2011; Ye et al., 2019). Lu et al. (2016) found that treatment with 5% acetate, propionate, and butyrate could activate *Ffar3* and *Ffar2* receptors in iWAT and BAT and promoted the formation of beige adipocytes in HFD-fed mice. Here, Sa-D was found to greatly upregulate the mRNA expression of *Ffar3*, *Ffar2*, and *Hcar2* in colon, *Ffar2* in WAT, and *Ffar2* in BAT, potentially being beneficial for energy metabolism and BAT function. Importantly, a previous study has confirmed that SCFAs (acetate, propionate, and butyrate) promote a brown/beige adipocyte-like phenotype by PKA/AMPK signaling (Zhang et al., 2023). Moreover, the silencing of the GPR43 receptor through siRNA-mediated knockdown blocked the acetate and propionate-dependent activation of AMPK (Lu et al., 2021; Yoshida, Ishii, & Akagawa, 2019). Therefore, we propose that Sa-D enhances the GPR43/PKA-AMPK signaling pathways by elevated levels of SCFAs, which in turn enhance BAT activity and promote the browning of iWAT. Collectively, Sa-D-induced activation of BAT was potentially associated with gut microflora shifts and increased intestinal SCFAs levels. However, an in-depth understanding of the specific microflora and their SCFA metabolites that facilitate the browning process requires further studies using more direct methods. Approaches such as fecal transplantation or the use of sterile mice will be crucial in these investigations. These methods can help isolate the effects of the microbiota on browning, thereby providing more insight into their roles in metabolic regulation and potential therapeutic applications.

5 Conclusion

Sa-D exhibited multiple modulatory effects on obesity, including improvement of dyslipidemia and dysglycemia, enhancement of energy expenditure alongside mitochondrial biogenesis, and reduction of hepatic steatosis. The underlying mechanisms for these therapeutic effects involve the stimulation of adipose thermogenesis, a process that correlated with alterations in the gut microbiota and SCFAs profiles (Fig. 9). Our findings offer novel insights into utilizing Sa-D for obesity management and support the advancement of health products targeting weight loss.

Declarations of competing interest

The authors declare no conflicts of interest.

Acknowledgments

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List of Abbreviations

Abbreviation	Definition
Abhd5	Abhydrolase domain containing 5
ACC	Acetyl-CoA carboxylase
Ack	Acetate kinase
ALT	Alanine aminotransferase
AMPK	Adenosine 5'-monophosphate (AMP)-activated protein kinase
aP2	Adipocyte fatty acid binding protein
AST	Aspartate aminotransferase
Atgl	Adipose triglyceride lipase
BAT	Brown adipose tissue
BcoA	Butyryl-CoA
BG	Body weight gain
BUN	Blood urea nitrogen
CCK	Cholecystokinin
Cd40	Clusters of differentiation 40
Cd137	Tumor necrosis factor receptor superfamily member 9
Cidea	Cell death-inducing DFFA-like effector a
Cited1	Cbp/p300-interacting transactivator 1
Cox2	Cyclooxygenase-2
Cox7a	Cytochrome c oxidase subunit 7A

CPT1 α	Carnitine palmitoyltransferase 1 α
Cr	Creatinine
Dio2	Deiodinase type 2
eWAT	Epididymal white adipose tissue
Fas	Fatty acid synthetase
Fgf21	Fibroblast growth factor 21
GC-MS	Gas Chromatography-Mass Spectrometry
GLP-1	Glucagon-like peptide-1
GIP	Gastric inhibitory polypeptide
GPR43/41/109a	G-protein coupled receptor 43/41/109a
HFD	High-fat-diet
HDL-C	HDL-cholesterol
ND	Hormone-sensitive triglyceride lipase
IHC	Immunohistochemical
iWAT	inguinal white adipose tissue
LDL-C	LDL-cholesterol
Mcd	Methylmalonyl-CoA decarboxylase
MI	Miglitol
NAS	NAFLD activity score
ND	Normal diet
Nrf1	Nuclear factor erythroid 2-related factor 1
OGTT	Oral glucose tolerance test

PduP	Propionaldehyde dehydrogenase
PKA	protein kinase A
PPAR α/γ	Peroxisome proliferator-activated receptor α/γ
Prdm16	PR domain containing 16
Pparg1a	PPAR gama coactivator 1 alpha
Plin5	Perilipin-5
RER	Respiratory exchange ratio
Sa-D	Saikosaponin D
SCFAs	Short-chain fatty acids
SREBP-1c	Sterol regulatory element-binding protein-1c
Sirt1	Silent mating type information regula-tion 1
Tbx1	T-box transcription factor 1
TC	Total cholesterol
Tfam	Mitochondrial transcription factor A
TG	Triglycerides
Tmem26	Transmembrane protein 26
UCP1	Uncoupling protein 1
WAT	White adipose tissue

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Figure Legends

Fig. 1. Effects of Sa-D on body measurements and serum biochemical indicators. (A) Growth curve of mice in different groups. (B) Body weight gain (BG). (C) Food intake. (D) Liver weight. (E) Spleen weight. (F) Kidney weight. (G) eWAT weight. (H) pWAT weight. (I) iWAT weight. (J) BAT weight. (K) TG. (L) TC. (M) HDL-C. (N) LDL-C. (O) FBG Level. (P) OGTT. (Q) AUC of OGTT.

Fig. 2. Effects of Sa-D on the energy expenditure in HFD-fed mice. (A and B) O₂ consumption and AUC of O₂. (C and D) CO₂ production and AUC of CO₂. (E and F) RER and AUC of RER. (G and H) Energy expenditure (EE) and AUC of EE. (I) Thermal images of mice upon cold exposure.

Fig. 3. Effects of Sa-D on histopathology in HFD-fed mice. H&E staining and quantitative analysis of (A and B) eWAT, (C and D) iWAT, (E and F) BAT, and (G and H) liver. (I) serum ALT level. (J) serum AST level. (K) *Srebp-1c*, (I) *Fas*, (M) *Ppar γ* , (N) *Acc*, (O) *Plin5*, (P) *Plin2*, (Q) *Ppara* and (R) *Cpt1 α* mRNA expression in liver. H&E staining of kidney (S) and spleen (U) and levels of serum BUN (T) and Cr (V).

Fig. 4. Sa-D induces thermogenesis in BAT of HFD-fed mice. (A) mRNA expression levels of thermogenesis-related genes (*Sirt1*, *Pparg1 α* , *Cidea*, *Prdm16*, *Dio2*, *Nrf1*, *Tfam*, *Cox7 α* , *Ppara* and *Ucp1*) and lipid droplet-associated genes (*Atgl*, *Hsl* and *Plin5*). (B) Immunohistochemistry for SIRT1 and UCP1. (C and D) Protein expression levels of p-PKA, PKA, p-AMPK α , AMPK α , p-p38, p38, SIRT1, PGC-1 α , COX4, TFAM and UCP1 were measured by western blot.

Fig. 5. Sa-D induces WAT browning in HFD-fed mice. (A) mRNA expression levels of

lipid droplet-associated genes (*Atgl*, *Hsl* and *Plin5*), BAT-rich genes (*Sirt1*, *Prdm16*, *Pparg1a*, *Cidea*, *Dio2*, *Tfam*, *Nrf1*, *Cox2*, and *Ucp1*) and BeAT-specific genes (*Fgf21*, *Tbx1*, *Cd40*, *Tmem26*, *Cited1*, and *Cd137*). (B) Immunohistochemistry for SIRT1 and UCP1. (C and D) Protein expression levels of p-PKA, PKA, p-AMPK α , AMPK α , p-p38, p38, SIRT1, TFAM, and UCP1 were measured by Western blot.

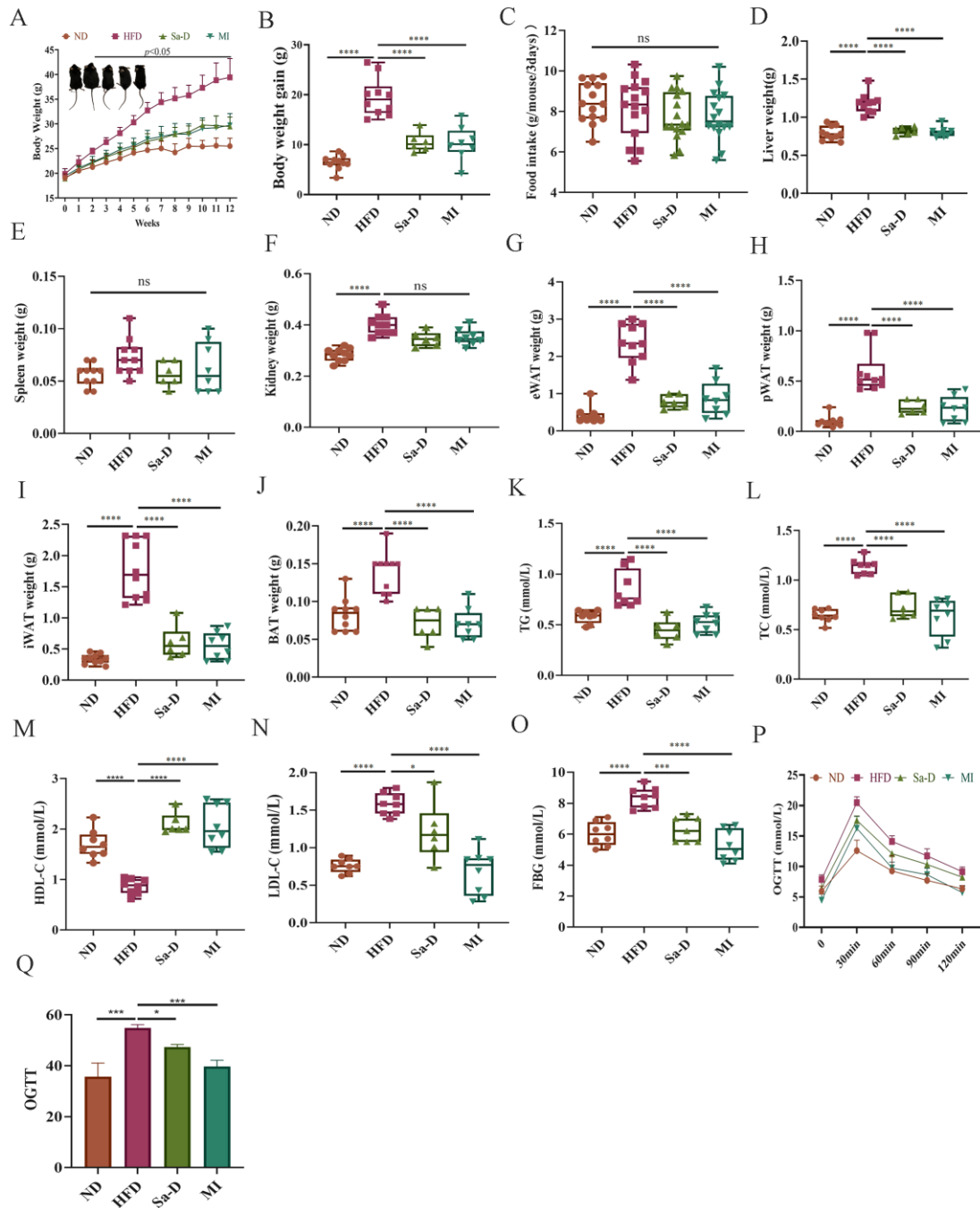
Fig. 6. Diversity and structural analysis of fecal flora. (A) Observed OTUs. (B) Shannon index. (C) Simpson index. (D) ACE index. (E) Chao1 index. (F) Venn diagram. (G) Bacterial taxonomic profiling at the phylum level. (H) Bacterial taxonomic profiling at the family level. (I) LDA score plot from LEfSE analysis.

Fig. 7. Effects of Sa-D on the composition of gut microbiota at the genus level. (A and B) Extended error bar plot identifying significant differences between ND versus HFD, HFD versus Sa-D. (C) Pearson's correlation analysis of the gut microbiota at genus level and obesity-related traits, beige fat-related factors, and brown fat-related factors.

Fig. 8. SCFAs production and associations with key species in the fecal microbiota (A) the levels of SCFAs (acetate, propionate, butyrate, valeric acid) in feces. (B) the gene expression levels of SCFA-producing enzymes in feces. (C-E) the mRNA expression of SCFAs receptors in the colon, WAT, and BAT. (F) Correlation of SCFAs production and key species in the fecal microbiota.

Fig. 9. Schematic representation of the effects of Sa-D on BAT activity and WAT browning. Administration of Sa-D increased the expression levels of several genes involved in adipose thermogenesis, and its effect may be related to the action of SCFAs produced by intestinal microflora through the GPR43/PKA-AMPK signaling pathways.

Fig. 1



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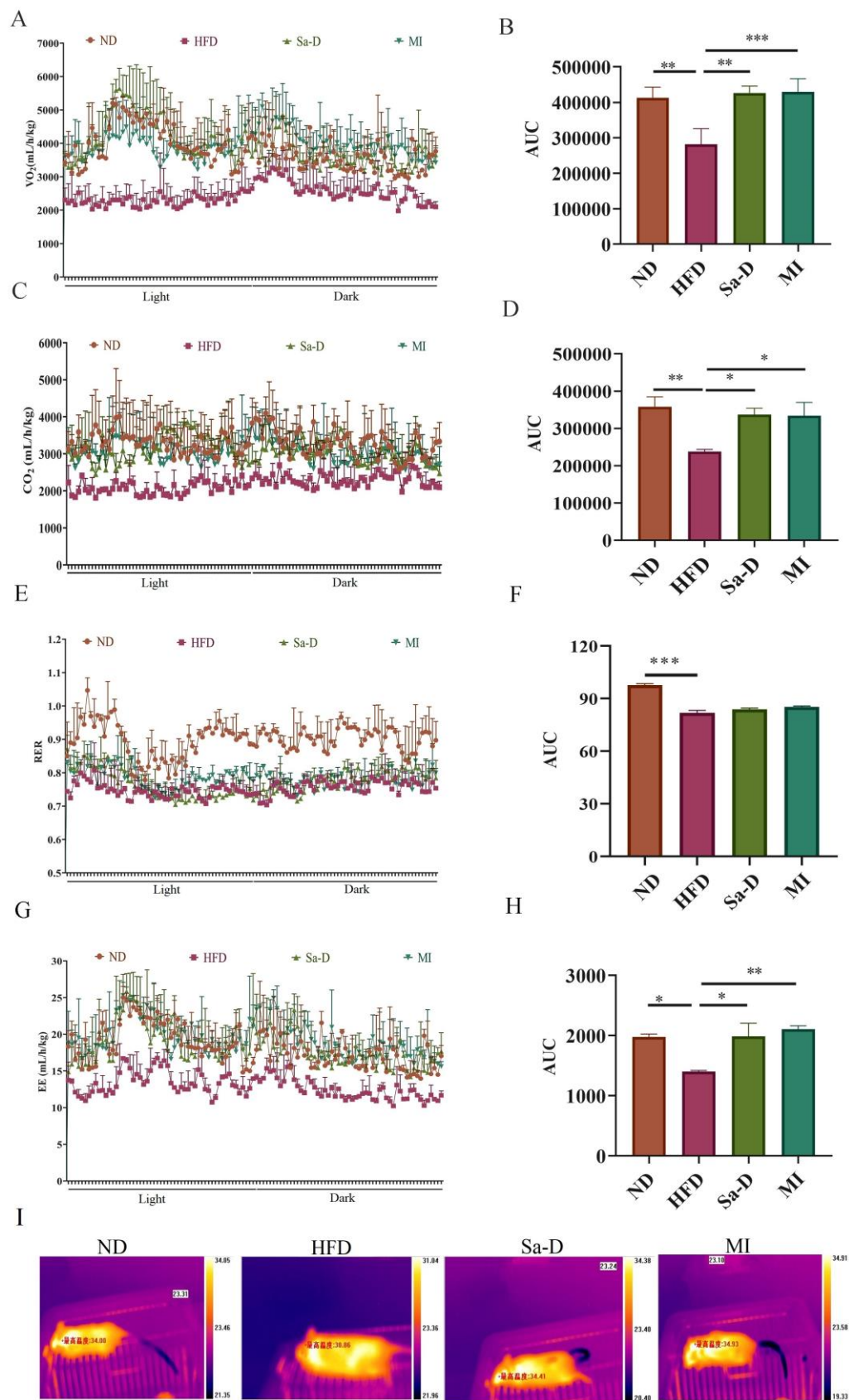
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748 **Fig. 2**

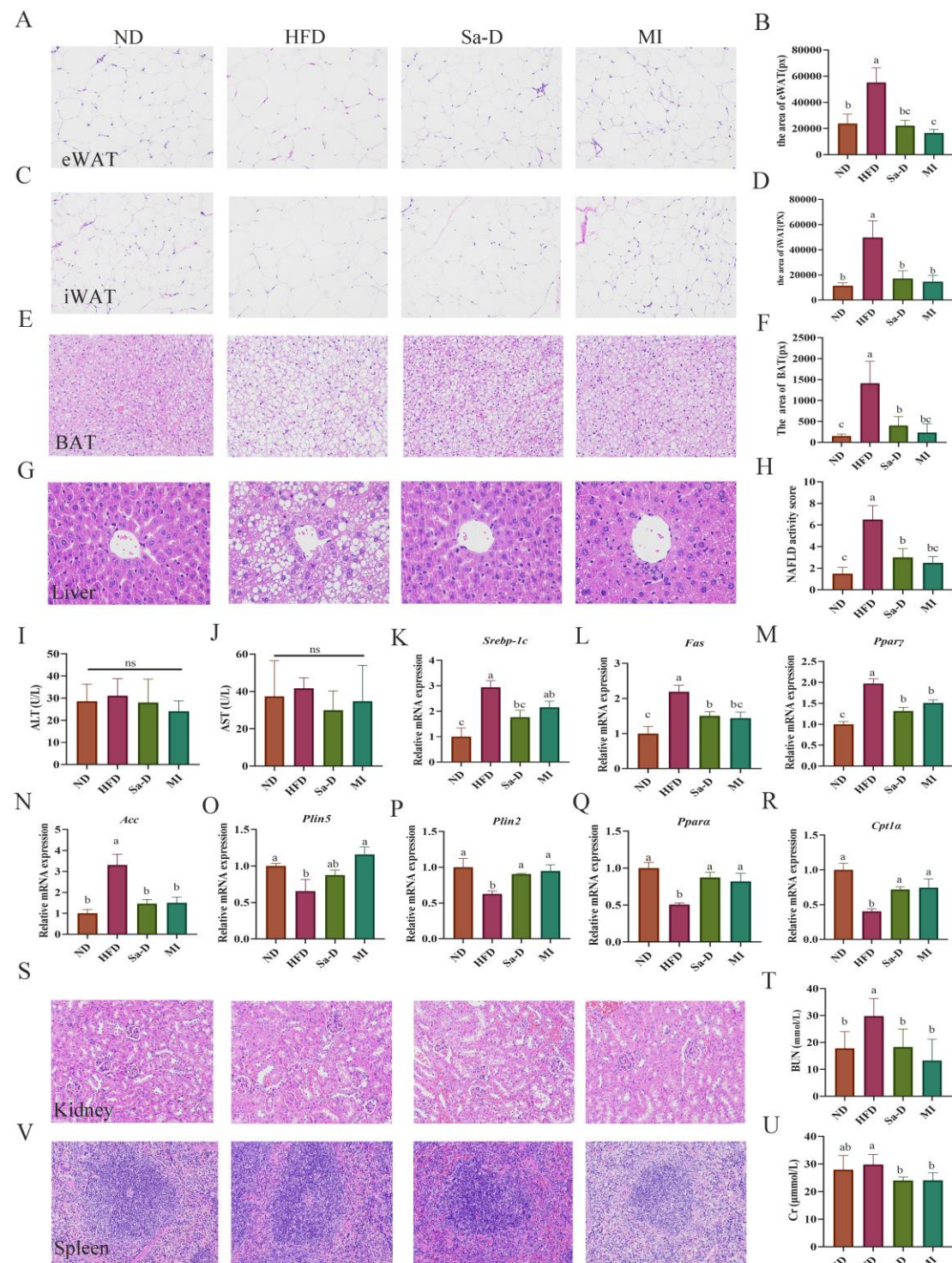


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Fig. 3



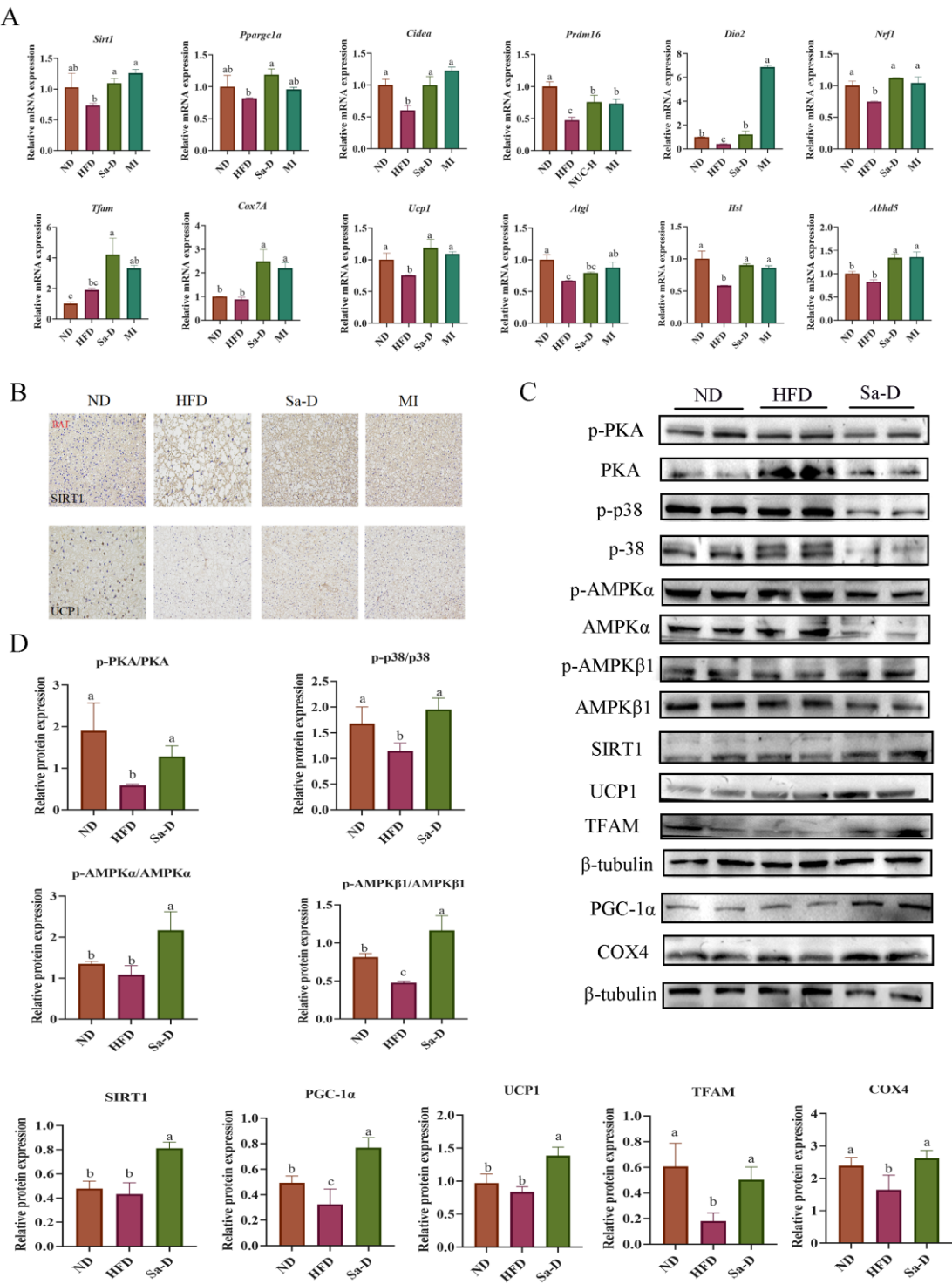
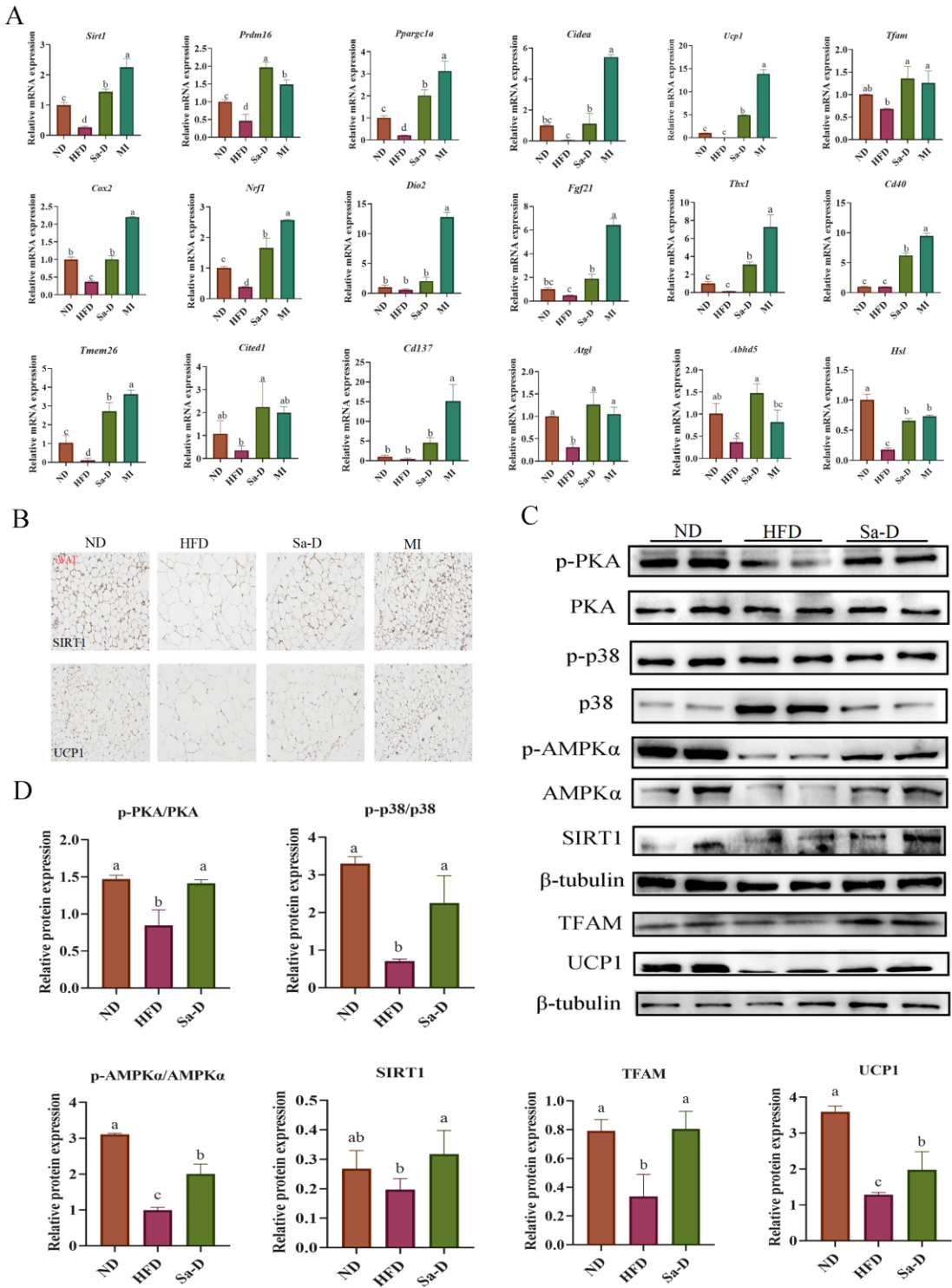


Fig. 5



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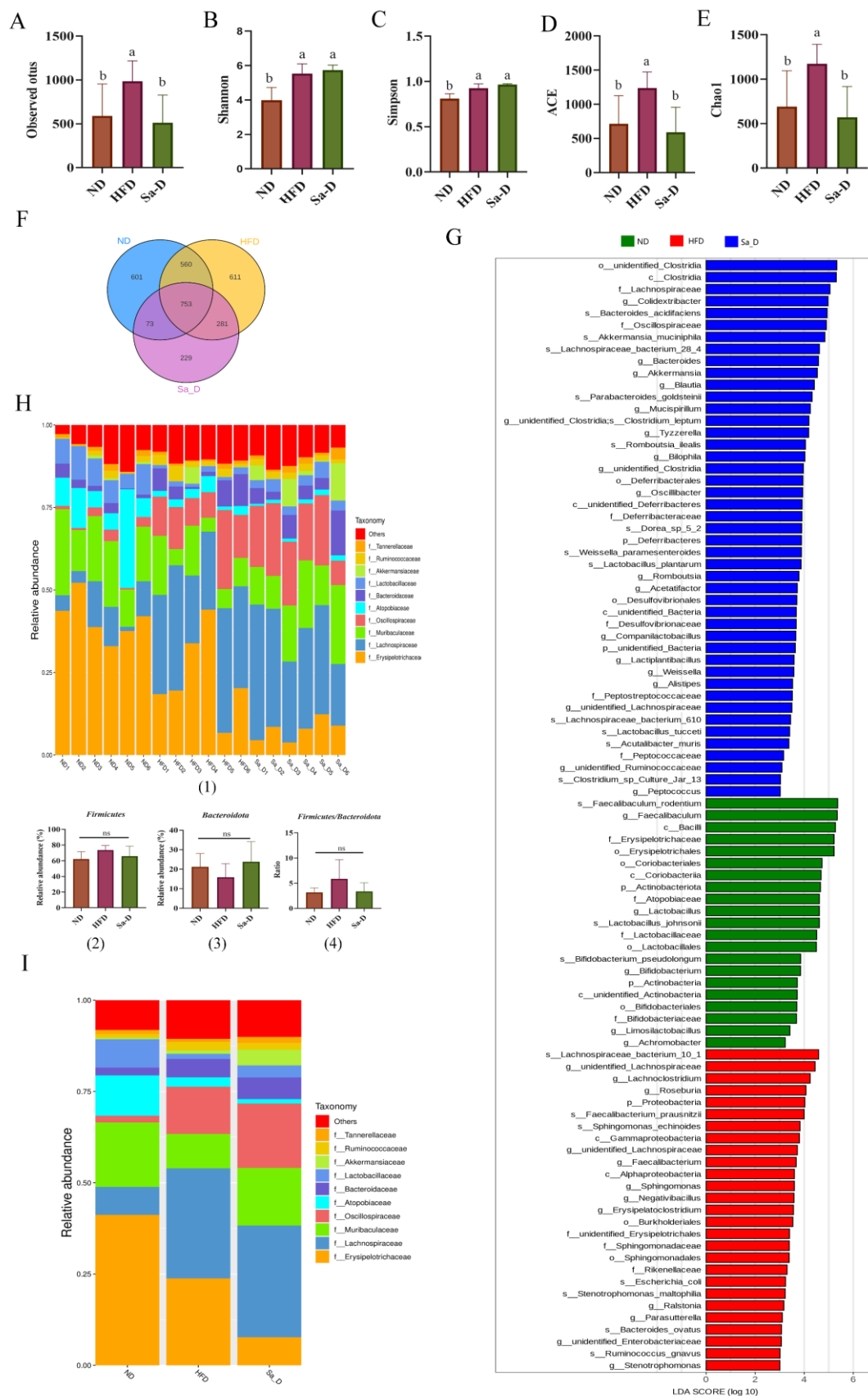


Fig. 9

