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Polysaccharides from Millettia speciosa Champ. alleviate obesity-associated metabolic dysfunction through the 'gut microbiota-leptin' axis

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ABSTRACT: The roots of *Millettia speciosa* Champ., a medicinal-edible plant, contain bioactive polysaccharides (MSCPs) that demonstrate potential prebiotic effects. Studies in diet-induced obese (DIO) mice have shown that MSCPs can modulate gut microbiota dysbiosis, suppress appetite, and ultimately alleviate obesity. However, the mechanism by which MSCPs regulate appetite and ameliorate obesity-associated metabolic dysfunction through modulating the gut microbiota still requires further investigation. In this study, we observed that MSCPs improved diet-induced obesity to the same extent as the pair-fed group, suggesting that MSCPs can ameliorate obesity in DIO mice by reducing appetite and energy intake. Through RT-qPCR, Western blot, and immunohistochemical experiments, it was found that MSCPs regulate the expression of appetite-related peptides and activate the JAK2/STAT3 signaling pathway to improve obesity in DIO mice; however, MSCPs did not ameliorate obesity in leptin receptor-deficient (db/db) mice, indicating that the leptin pathway is a critical target for treating DIO mice. Furthermore, MSCPs beneficially modulate gut microbiota; fecal microbiota transplantation experiment (FMT) restored the gut microbiota structure in mice, and the FMT group still alleviated obesity through the same signaling pathway. Additionally, 16S rRNA gene sequencing revealed that both MSCPs and FMT upregulated beneficial microbiota such as Lachnospiraceae_UCG_006 and *Faecalibaculum*, and promoted the production of short-chain fatty acids (SCFAs). Notably, in db/db mice, although MSCPs significantly elevated SCFAs levels, they failed to ameliorate obesity or metabolic disorders, further confirming that the leptin pathway serves as a critical requirement for MSCPs-mediated obesity improvement. In summary, MSCPs may alleviate obesity in DIO mice by enriching Lachnospiraceae_UCG_006 and *Faecalibaculum* to promote SCFAs production, thereby modulating the leptin pathway.

Key words: obesity-associated metabolic dysfunction; leptin; gut microbiota; MSCPs

1. Introduction

Obesity is not only an excess of body weight but also a high-risk trigger for various metabolic-related diseases such as type 2 diabetes, cardiovascular diseases, non-alcoholic fatty liver disease [1]. It is a

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pathological condition resulting from prolonged energy imbalance. The hypothalamus within the central nervous system played a crucial role in maintaining energy homeostasis. It received signals from peripheral hormones and regulated food intake and energy expenditure by managing appetite through neural pathways [2, 3].

Leptin is one of the appetite-regulating hormones produced by adipose tissue. It promotes the regulation of food intake and energy expenditure by binding to leptin receptors in the hypothalamus, thereby inhibiting fat accumulation and facilitating weight reduction [4]. Obese individuals often exhibit hyperleptinemia, characterized by elevated leptin levels that fail to exert their normal physiological effects. This condition, known as 'leptin resistance', contributes to obesity and metabolic disorders [5].

Several signaling pathways are involved in leptin resistance, with the JAK2/STAT3 pathway playing a critical role in energy homeostasis and neuroendocrine function [6]. Upon binding to LepRb, leptin activates JAK2 phosphorylation, which subsequently phosphorylates and dimerizes STAT3. The phosphorylated STAT3 translocates into the nucleus, where it activates the transcription of anorexigenic POMC and inhibits the transcription of orexigenic AgRP and NPY. Additionally, the JAK2/STAT3 pathway is positively regulated by SH2B1 and negatively regulated by SOCS3 and PTP1B, thereby modulating leptin resistance [7].

The gut microbiota can influence the formation and progression of obesity by regulating the production and release of related hormones. A study by Xu et al. demonstrated that alterations in the gut microbiota could induce leptin pathway activation, influence beige adipocyte remodeling, and participate in the regulation of the leptin-AMPK/STAT3 signaling pathway [8]. Research by Everard et al. showed that *Bifidobacterium* spp. and *Akkermansia muciniphila* could reduce the host's resistance to leptin and decrease fat accumulation [9]. Furthermore, changes in short-chain fatty acids (SCFAs) driven by gut microbiota were found to interfere with leptin expression [10]. Bacterial-derived SCFAs, such as propionate, can increase leptin expression via specific G protein-coupled receptor (GPR43， GPR41) [11, 12].

Chinese medicine Niudali (commonly known as Mountain Lotus Root, Nine-dragon Pearl Chain, or Dali Tuber) is the dried root of *Millettia speciosa* Champ., a plant of the *Millettia* genus in the Leguminosae family. Growing in hillside grasslands, it is mainly distributed in China's Hainan, Guangdong, Guangxi and other provinces, as well as some Central and South Asian countries [13, 14]. In recent years, due to the potential applications of *Millettia speciosa* Champ. in the food, pharmaceutical and agricultural fields, its commercial planting scope has been continuously expanding, making contributions to the local agricultural economy through domestic sales and export potential [15, 16]. The roots of this plant are rich in protein and starch, and possess both medicinal and edible value. In folk medicine, the roots have been used commonly as a soup ingredient in the South of China for hundreds of years, which can strengthen the functions of the immune system and promote anti-inflammatory and anti-tumor effects [17]. The polysaccharides from the roots of *M. speciosa* (MSCPs) have garnered significant attention due to their health-promoting properties, including anti-inflammatory, immunomodulatory, and anti-obesity effects [18]. Our previous studies

demonstrated that MSCPs could reduce energy intake and beneficially modulate the gut microbiota in diet-induced obese (DIO) mice [19]. Building on these findings, we hypothesize that MCSPs regulating gut microbiota to influence leptin levels and thereby control appetite. To test this hypothesis, we employed a DIO mouse model, db/db mice, and FMT experiments, utilizing modern immunological and molecular biological techniques to investigate the specific underlying mechanisms.

2. Materials and methods

2.1 Preparation of MSCPs

MSCPs were prepared using a hydrothermal ethanol precipitation method. First, *M. speciosa* powder was pulverized and passed through a 40-mesh sieve. The resulting powder was soaked in ethanol (1:5, W/V) for 24 hours to remove lipids. Subsequently, the residue was extracted twice with distilled water (1:6, W/V) at 100℃, each extraction lasting 2 hours. The combined extracts were concentrated and mixed with four volumes of ethanol, then left to precipitate at 4℃ for 24 hours. The precipitate was washed three times with anhydrous ethanol. After redissolving the precipitate, Sevage reagent was applied to remove proteins, and dialysis bags were used to eliminate small molecular impurities from the polysaccharides. The dialyzed polysaccharide solution was then freeze-dried to yield MSCPs.

The methods used to determine the monosaccharide composition and protein content of MSCPs were included in the **Supplymentary information** and results were shown in **Fig. S1**.

2.2 Animal experiment

All SPF-grade male C57BL/6J mice (10 weeks old) were purchased from Zhuhai Bestone Biotechnology Co., Ltd. (Zhuhai, China). and all SPF-grade male db/db mice (8 weeks old) were purchased from Guangdong Yaokang Biotechnology Co., Ltd. All mice were acclimatized for one week under conditions of (25 ± 2)℃ with a 12-hour light/dark cycle before starting the experiments.

Experiment 1: C57BL/6J mice (10 weeks old) were randomly divided into four groups: (1) Chow – normal diet group (10% fat, 3.5 kcal/g); (2) HFD – high-fat diet group (45% fat, 4.7 kcal/g); (3) HFD-MSCPs –MSCPs treatment group (150 mg/kg MSCPs, 45% fat, 4.7 kcal/g); (4) Pair -feed group (given the same amount of feed as the HFD-MSCPs group, 45% fat, 4.7 kcal/g). Each group consisted of 10 mice, and the feeding continued for 12 weeks.

Experiment 2: db/db mice were randomly divided into two groups: db/db group and db/db-MSCPs group (150 mg/kg MSCPs gavage intervention). Both groups were fed standard chow, with 5-6 mice per group, and feeding continued for 12 weeks.

Experiment 3: For the FMT experiment, C57BL/6J mice (10 weeks old) were randomly divided into two groups, each group consisted of 10 mice, and the feeding continued for 9 weeks: FMT– fecal microbiota transplantation group (45% fat, 4.7 kcal/g). SFF– fecal microbiota transplantation control group (45% fat, 4.7 kcal/g). The detailed procedures are provided in the **Supplementary information**.

Body weight and food intake of the mice were recorded weekly on Monday. Before the end of the experiment, fresh fecal samples were collected under sterile conditions and stored in cryovials. At the end of the experiment, plasma, adipose tissue, and other organs were harvested. The collected samples were either fixed in paraformaldehyde or rapidly frozen in liquid nitrogen for subsequent analysis.

2.3 Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT)

At week 11 of the experiment, OGTT were performed after a 12-hour fast. Blood glucose levels at 0 minutes were measured using tail-tip blood samples. Glucose solution (0.1 mL/10 g BW) was administered via oral gavage, and timing was initiated immediately. Blood glucose levels were measured at 15, 30, 60, 90, and 120 minutes using a Roche glucometer. For ITT, conducted four days after the OGTT, mice were fasted for 6 hours and then administered an intraperitoneal injection of insulin solution (0.3 U/kg BW). Blood glucose levels were similarly measured at 0, 15, 30, 60, 90, and 120 minutes using the same glucometer (Roche, Switzerland).

2.4 The biochemical analysis of plasma and liver

Hepatic triglyceride (TG) content were measured using kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) and the plasma leptin levels using the mouse leptin ELISA kit (Multi Sciences, China) according to the manufacturer's protocol.

2.5 Hematoxylin-eosin (H&E) staining

The fixed liver, subcutaneous fat, epididymal fat, and distal ileum tissue were embedded in paraffin. Then, sections were cut and dewaxed using xylene and anhydrous ethanol, followed by rehydration through xylene and a gradient of ethanol. Subsequently, the sections were stained with H&E, and tissue pathological changes were observed under a standard optical microscope (OLYMPUS, Tokyo).

2.6 Quantitative RT-PCR analysis

The mRNA expression levels of leptin, JAK2, SH2B1, PTP1B, and SOCS3 were measured in the iWAT with the qRT-PCR assay. Primer information is provided in **Supplementary Table S1**. Total RNA was extracted using the Fatty Tissue RNA Extraction Kit (GENENODE, China), the concentration and purity were determined using a Nanodrop micro-ultraviolet spectrophotometer (Thermo Scientific, USA) at 260 and 280 nm. cDNA was synthesized using a reverse transcriptase (TOYOBO, China) according to the manufacturer's instructions. The expression of individual genes was normalized to the expression of β-tubulin, as measured using a LightCycler96 Real-Time PCR System (Roche, USA).

2.7 Immunohistochemical (IHC) experimentation

The immunohistochemistry experiment was conducted as follows. Hypothalamic tissue sections were baked at 60℃ for 2 hours, then deparaffinized, hydrated, and antigen retrieved. Sections were washed in PBS, blocked with 10% goat serum at room temperature for 30 minutes, incubated overnight with POMC or NPY antibody at 4℃, then at 37℃ for 45 minutes. After washing, diluted secondary antibody was applied and

incubated at room temperature for 60 minutes. Sections were washed in PBS, mounted on glass slides, and imaged using an automated chemiluminescence imaging system (OLYMPUS, Tokyo).

2.8 Western blot analysis

A total of 100 mg of adipose tissue was weighed lysed in a tube containing RIPA buffer (Beyotime, China), protease inhibitors (Servicebio, China), and phosphatase inhibitors (Servicebio, China). The supernatant was collected, and its protein concentration was determined using a BCA protein assay kit (Thermo, USA). Proteins were separated using SDS-PAGE, followed by membrane transfer and blocking. The membrane was then subjected to immunoblotting with appropriate primary and secondary antibodies. Finally, the membrane was scanned using an imaging system (Sapphire RGBNIR). Details of the antibodies used are provided in **Supplementary Table S2.**

2.9 16S rRNA gene sequence

The experimental methods were conducted according to previously published protocols [20, 21]. Briefly, fresh fecal samples were collected from the mice, and total genomic DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). The 16S rRNA gene was amplified with specific primers containing barcodes and subsequently validated and sequenced on the PacBio platform (Biomarker Technologies Co., Ltd., Beijing, China). The samples were classified into operational taxonomic units (OTUs), and the taxonomic composition of the gut microbiota was analyzed at the phylum, family, and genus levels.

2.10 Determination of SCFAs

The concentrations of acetate, propionate, butyrate, and valerate in fecal samples were determined following previously reported methods [22]. Briefly, the sample solution was derivatized with 3-nitrophenylhydrazine hydrochloride and 3-dimethylaminopropyl reagents containing 6% pyridine. Quantification of SCFAs was performed using ultra-performance liquid chromatography coupled with triple quadrupole tandem mass spectrometry (UPLC-QqQ-MS/MS), as detailed in the **Supplementary information.**

2.11 Statistical analysis

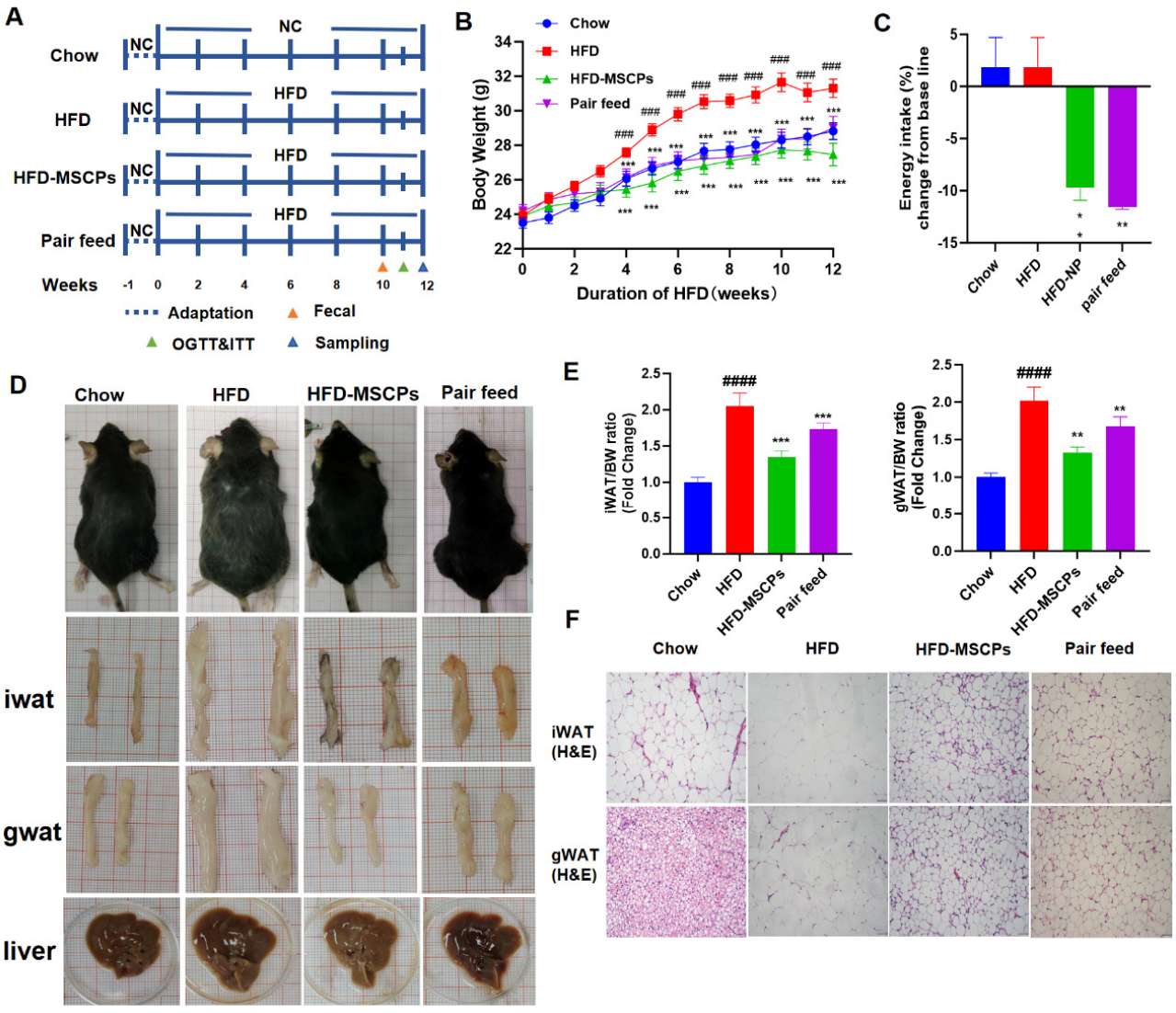
Statistical analysis was performed using GraphPad Prism 10.0 software (USA). Data were presented as the mean ± SEM of biological replicates. Significance between two groups was determined using an unpaired Student's t-test, while differences among more than two groups were analyzed using one-way ANOVA followed by multiple comparisons.

3. Result

3.1 *MSCPs decreased energy intake and reduced appetit in DIO mice*

Based on previous findings by our research group revealed that MSCPs reduced energy intake in DIO mice; therefore, this study aimed to establish a DIO mouse model, with additional paired feeding experiments

(**Fig. 1A**). Compared with normal diet-fed mice, the HFD-fed group showed a significant increase in body weight ($P < 0.001$), but the group treated with MSCPs or paired feeding group exhibited a significant reduction in body weight in the fourth week (**Fig. 1B**) ($P < 0.001$). MSCPs also lowered energy intake in DIO mice (**Fig. 1C**). Adipose tissue weights (iWAT and gWAT) significantly increased in DIO mice but were reduced by MSCPs and paired feeding (**Fig. 1D**). However, intervention with MSCPs and paired feeding effectively reduced these fat indices. Histological analysis revealed reduced fat area and alleviated adipocyte hypertrophy in both iWAT and gWAT following interventions (**Fig. 1E, F**). Furthermore, MSCPs and pair feed groups exhibited decreased liver weight and TG levels (**Fig. 1G**). H&E and Oil Red O staining of liver tissues indicated that these interventions reduced hepatic lipid droplet accumulation, ameliorating hepatic steatosis in DIO mice (**Fig. 1H**). Additionally, HFD mice treated with MSCPs and pair feed interventions exhibited improved insulin sensitivity and enhanced glucose tolerance (**Fig. 1I, J**). Intestinal damage observed in HFD-fed mice, including villus atrophy, fragmentation, and submucosal inflammation, was alleviated by MSCPs and dietary interventions, indicating restored intestinal barrier integrity (**Fig. S2**). Collectively, these results suggest that the anti-obesity effects of MSCPs are likely to be primarily mediated through appetite suppression and subsequent reduction in caloric intake.



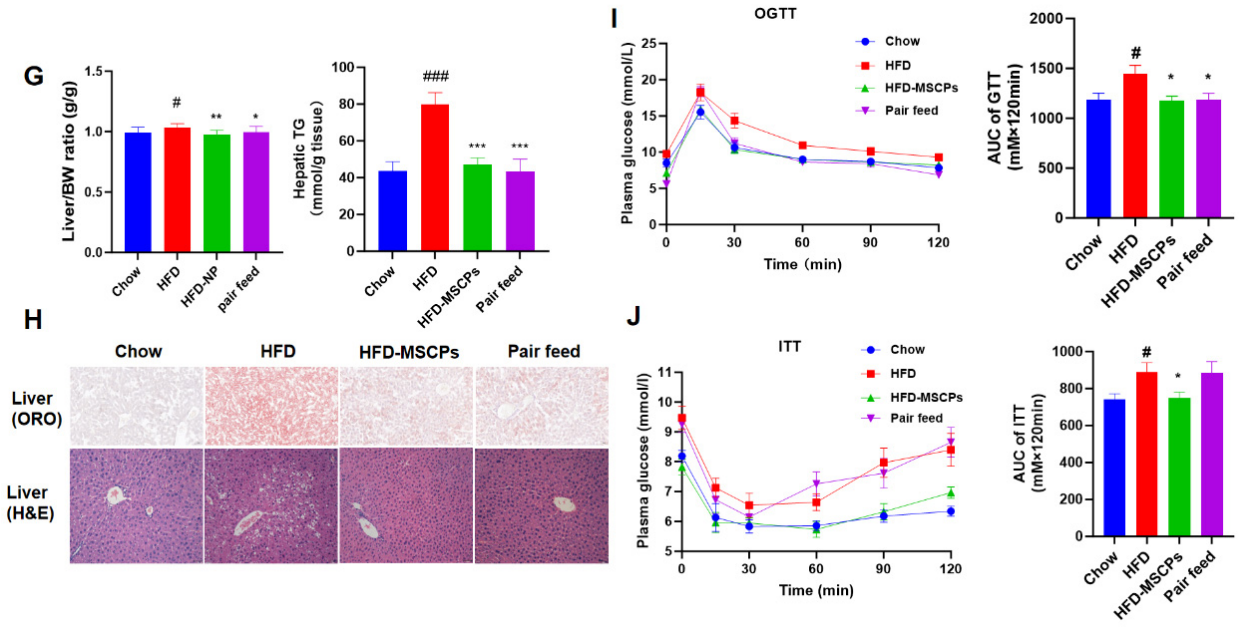


Fig. 1. MSCPs were shown to reduce energy intake, promote weight loss, and improve lipid deposition, adipocyte hypertrophy, and glucose homeostasis in DIO mice. (A) Experimental design for DIO mouse model. (B) The body weight trends over a twelve-week period. (C) Energy intake of mice. (D) The phenotypes of mice in each group, including body size, iWAT, gWAT and liver. (E) iWAT/BW ratio and gWAT/BW ratio. (F) H&E staining of iWAT and gWAT (magnification bar, 1 mm). (G) Liver/BW ratio and liver triglyceride content. (H) Oil red staining (magnification bar, 50 μm) or H&E staining (magnification bar, 1 mm) of liver sections. (I, J) GTT, ITT at time point of 0 to 120 min and AUC of GTT and ITT. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ $^{\#\#\#}P < 0.001$ vs. Chow group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. HFD group.

3.2 MSCPs reduced serum leptin levels and modulated the leptin signaling pathway

Leptin, a protein hormone secreted by adipose tissue, plays a critical role in regulating appetite [23]. We measured plasma leptin levels in mice and found that MSCPs and pair-feeding significantly reduced leptin levels ($P < 0.01$, $P < 0.05$) (**Fig. 2A**). Central leptin resistance in the hypothalamus increases food intake, while peripheral resistance in tissues like adipose, liver, and muscle disrupts glucose and lipid metabolism, contributing to obesity [24]. Since the pair-feeding group has already confirmed that this drug promotes weight loss by suppressing appetite, we will focus on comparing the potential mechanisms of the model group and the drug administration group, and will no longer explore the mechanisms of the pair-feeding group. To investigate the effects of MSCPs on leptin signaling pathways, the expression of related proteins and genes in the hypothalamus and adipose tissues of mice was analyzed.

POMC and NPY are neurotransmitters in the hypothalamic arcuate nucleus, respectively playing anorexigenic and orexigenic roles [25, 26]. Therefore, IHC was performed to assess the expression levels of POMC and NPY in the hypothalamus. The results showed that after 12 weeks of MSCPs intervention, the hypothalamic POMC staining in DIO mice became significantly darker, indicating an 82.1% increase in its expression; but NPY staining became noticeably lighter, suggesting that MSCPs promoted the expression of POMC while inhibiting the expression of NPY (**Fig. 2B**). In peripheral tissues, it was observed that MSCPs reduced the mRNA expression of leptin and PTP1B, while increased the mRNA expression of JAK2 and SH2B1 (**Fig. 2C**). Additionally, MSCPs elevated STAT3 phosphorylation (**Fig. 2D**).

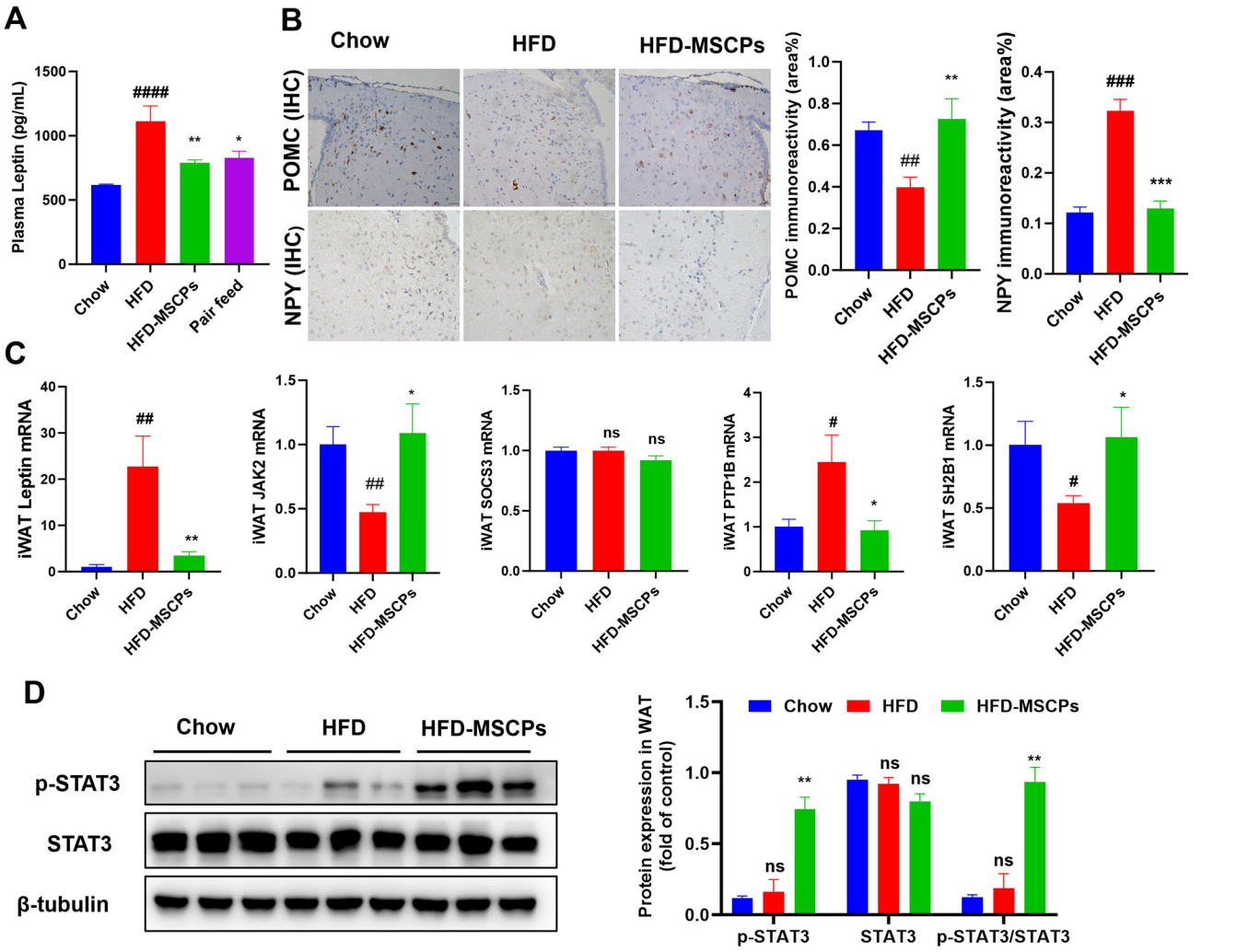


Fig. 2. MSCPs improved leptin resistance by promoting hypothalamic POMC expression and inhibiting NPY expression in DIO mice, while also modulating the leptin signaling pathway in adipose tissue. (A) Leptin levels in serum of mice in each group. (B) IHC staining and expression levels of POMC and NPY in the hypothalamus (magnification bar, 1 mm). (C) The q-PCR analysis of Leptin, JAK2, SH2B1, SOCS3 and PTP1B gene expression in iWAT (n=6-9). (D) Western Blot analysis of p-STAT3, STAT3 in iWAT (n=3). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ $^{\#\#\#}P < 0.001$ vs. Chow group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. HFD group.

3.3 MSCPs exerted no significant effect on obesity in db/db mice

Leptin receptor-deficient mice were used to investigate whether the leptin signaling pathway serves as a primary target of MSCPs in alleviating obesity-associated metabolic dysfunction in diet-induced obesity. A 12-week MSCPs gavage was administered (**Fig. 3A**). The results indicated that MSCPs failed to significantly mitigate metabolic dysfunctions, including excessive weight gain, increased energy intake, fat deposition, adipocyte hypertrophy, hepatic steatosis, and leptin resistance (**Fig. 3B-G**). In addition, the results demonstrated that a 12-week MSCPs gavage neither enhanced hypothalamic POMC expression (**Fig. 3H**). In peripheral tissues, polysaccharide supplementation did not alter the mRNA expression of leptin or its pathway regulators (JAK2, SH2B1, SOCS3, PTP1B) in subcutaneous adipose tissue and had no effect on the leptin signaling pathway in db/db mice (**Fig. 3I**). These results suggest that the leptin signaling pathway may represent a central target of MSCPs in mitigating obesity-associated metabolic dysfunction in DIO mice.

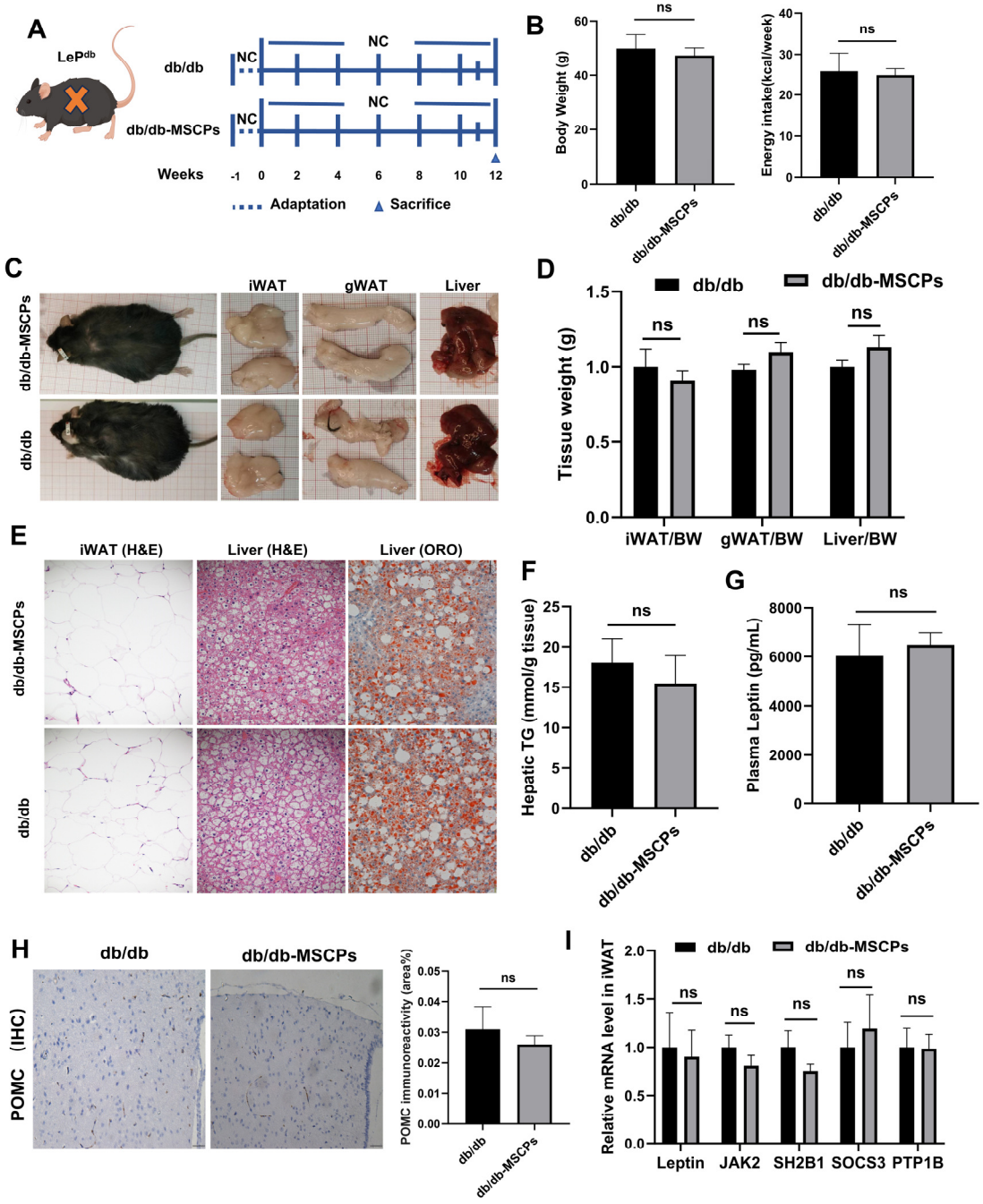
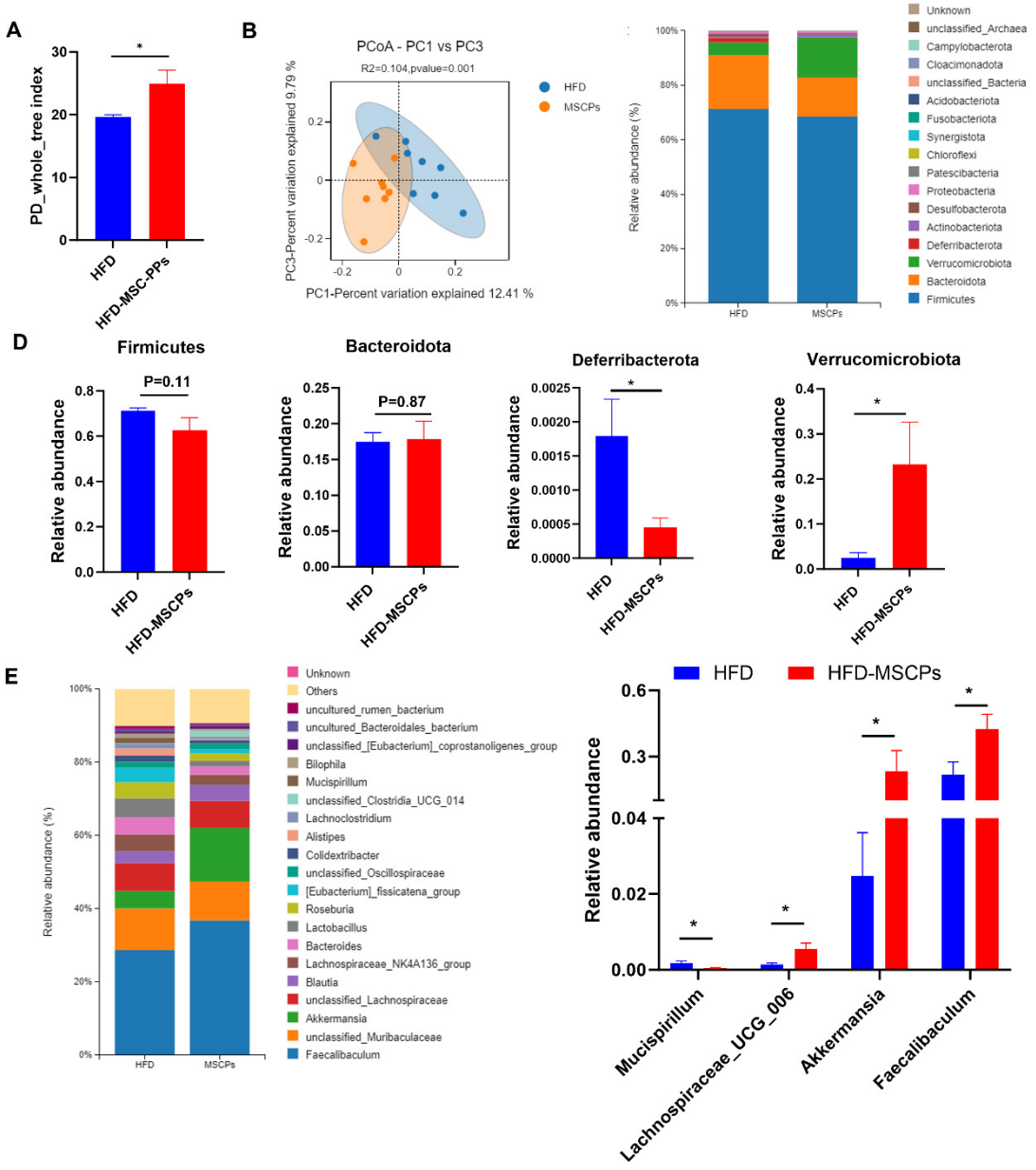


Fig. 3. MSCPs cannot slow down obesity syndrome in db/db mice. (A) Experimental design for LeP^{db} mouse model. (B) The body weight trends over a eight-week period and energy intake of mice. (C) The phenotypes of mice in each group, including body size, iWAT, gWAT and liver. (D) The ratio of iWAT/BW, gWAT/BW and liver/BW. (E) Oil red staining of liver sections (magnification bar, 50 μm) and H&E staining of iWAT and liver (magnification bar, 1 mm). (F) The hepatic TG content. (G) The levels of leptin in mouse serum. (H) IHC of hypothalamic POMC (magnification bar, 1 mm). (I) The q-PCR analysis of Leptin, JAK2, SH2B1, SOCS3 and PTP1B gene expression in iWAT (n=4-6). *P < 0.05, **P < 0.01, ***P < 0.001 vs. db/db group.

3.4 MSCPs beneficially modulated gut microbiota in DIO mice

Modulating the gut microbiota has been shown to influence host energy balance and metabolic health, thereby exerting beneficial effects on obesity [27]. In this study, the fecal microbiota of mice in the HFD group and HFD-MSCPs group was analyzed based on 16S rRNA gene sequencing. The results that supplementation with MSCPs significantly increased the PD_whole_tree index in DIO mice (P < 0.05) (**Fig. 4A**). These findings suggest that MSCPs supplementation enriched the microbial species diversity in DIO mice. Principal

coordinate analysis (PCoA) further demonstrated distinct microbial community structures between the HFD and HFD-MSCPs groups, indicating that MSCPs supplementation significantly altered gut microbiota composition (**Fig. 4B**). Taxonomic comparisons of the gut microbiota revealed the dominant phyla to be Firmicutes, Bacteroidota, Verrucomicrobiota, and Deferribacterota. Compared to the HFD group, MSCPs supplementation significantly increased the abundance of Verrucomicrobiota while decreasing the relative abundance of Deferribacterota ($P < 0.05$) (**Fig. 4C, D**). At the genus level, MSCPs supplementation significantly upregulated Lachnospiraceae_UCG_006, *Faecalibaculum*, and *Akkermansia*, while notably downregulating *Mucispirillum* ($P < 0.05$) (**Fig. 4E, F**). A phylogenetic tree analysis was performed to explore the evolutionary and phylogenetic relationships among microbial taxa. The results revealed that the microbial taxa altered by MSCPs exhibited close evolutionary relationships with other species or genera (**Fig. 4G**). These findings suggest that MSCPs supplementation modulated the gut bacterial ecosystem, thereby altering the diversity and richness of the gut microbiota in DIO mice. This modulation of the microbial community may contribute to the observed metabolic benefits.



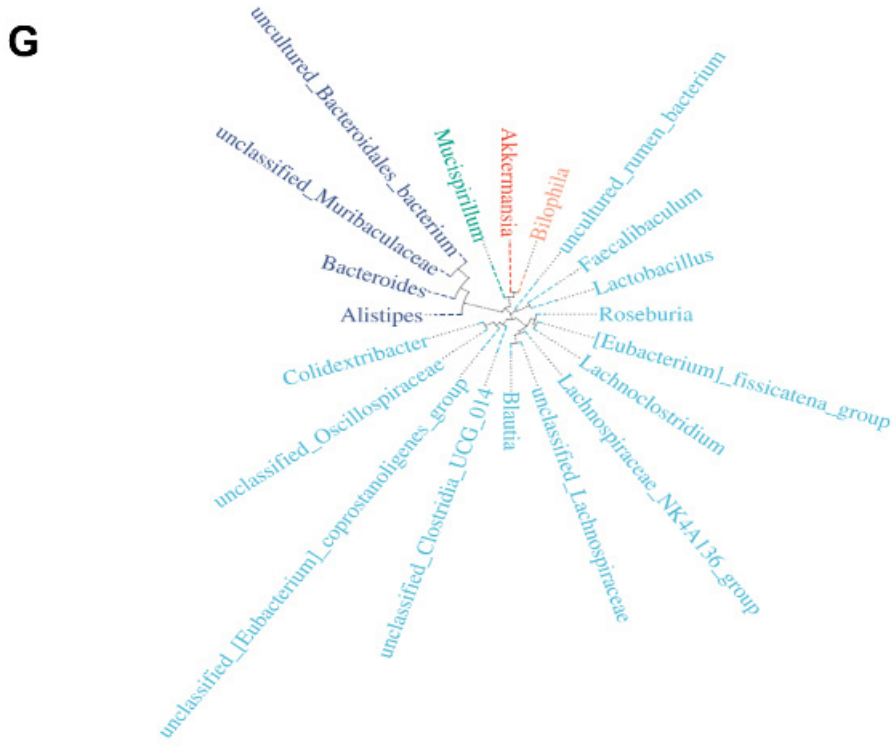


Fig. 4. MSCPs regulate gut microbiota diversity. (A) PD_whole_tree index of microbiota. (B) PCoA score plot analysis based on the relative abundance of OTUs. (C, D) Bar chart of taxonomic distribution and relative abundance of Firmicutes, Bacteroidota, Verrucomicrobiota, and Deferribacterota at the phylum level. (E, F) Bar chart of taxonomic distribution and relative abundance of *Mucispirillum*, Lachnospiraceae_UCG_006, *Akkermansia* and *Faecalibaculum* at the genus level. (G) A phylogenetic tree among various biological species based upon similarities and differences in their physical or genetic characteristics. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. HFD group.

3.5 Gut microbiota mediated the regulation of the leptin pathway by MSCPs to improve obesity in DIO mice

A new gut microbiota structure was reconstructed following the protocol described in **Fig. S3A**, to further investigate the effects of MSCPs on the gut microbiota of mice. As shown (**Fig. 5A-D**), the FMT group significantly reduced body weight ($P < 0.001$) , decreased energy intake ($P < 0.01$), and lowered the gWAT and liver index. Histological analysis of H&E-stained sections revealed that, compared to the SFF group, the FMT group exhibited a significant reduction in adipose tissue area and improved the intestinal barrier environment in DIO mice. Furthermore, TG content and staining results indicated that the FMT intervention effectively mitigated liver steatosis (**Fig. S3B** and **Fig. 5E**). Additionally, the FMT group improved impaired glucose tolerance and insulin sensitivity (**Fig. 5F, G**). Notably, leptin resistance was ameliorated in the FMT group. This was achieved by regulating appetite through modulating the expression of appetite-related peptides (**Fig. 5H, I**), and improved peripheral leptin resistance by regulating leptin levels in adipose tissue, leptin signaling factors, and STAT3 phosphorylation. (**Fig. 5J, K**). These findings suggest that reconstructing gut microbiota can similarly regulate the leptin pathway, appetite peptide expression, and lipid metabolism in DIO mice.

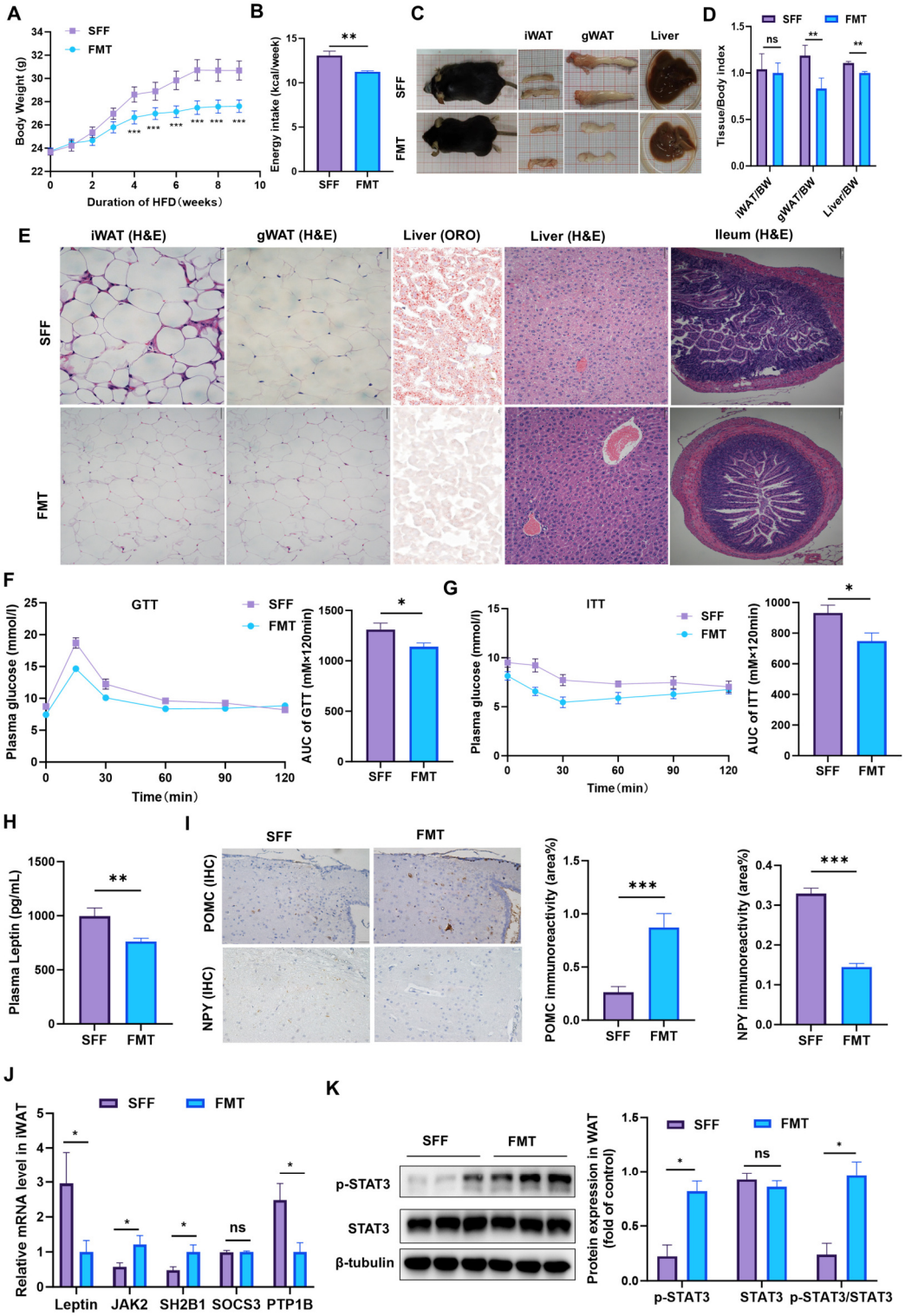


Fig. 5. FMT were shown to reduce energy intake, promote weight loss, and improve lipid deposition, adipocyte hypertrophy, and glucose homeostasis in DIO mice. (A) The body weight trends over a nine-week period. (B) Energy intake of mice. (C) The mouse phenotype diagram including body size, iWAT, gWAT and liver. (D)The ratio of iWAT/BW, gWAT/BW and liver/BW. (E) Oil red staining of liver, H&E staining of liver, iWAT, gWAT and ileum. (F, G) GTT, ITT at time point of 0 to 120 min and AUC of GTT, ITT. (H) The levels of leptin in mouse serum. (I) The expression levels of POMC and NPY. (J) The q-PCR analysis of Leptin, JAK2, SH2B1, SOCS3 and PTP1B gene expression in iWAT (n=6-9). (K) Western Blot analysis of p-STAT3 and STAT3 in iWAT (n=3). *P < 0.05, **P < 0.01, ***P < 0.001 vs. SFF group.

In this study, we also performed 16S rRNA gene sequencing on the feces of both groups of mice. Alpha-diversity analysis showed that the FMT group shared 520 OTUs with the SFF group, while 114 OTUs were unique to the FMT group, representing 41 more than the SFF group (**Fig. 6A**). This suggests that

diversity increased in the mice treated with FMT. PCoA and NMDS analysis revealed distinct differences in the microbial community structure between the SFF and FMT groups, indicating that FMT significantly altered the community composition (**Fig. 6B, C**). We also conducted analysis at the phylum and genus levels. The results indicated that at the phylum level, the enriched microbiota primarily consisted of Firmicutes, Bacteroidota, Verrucomicrobiota, and Deferribacterota (**Fig. 6D, E**). At the genus level, the FMT group exhibited an increase in Lachnospiraceae_UCG_006, *Faecalibaculum* (**Fig. 6F, G**). These findings suggest that MSCPs may improve obesity-associated metabolic dysfunction by altering the gut microbiota structure and composition, particularly by increasing beneficial bacteria such as Lachnospiraceae_UCG_006 and *Faecalibaculum*, which in turn influence the leptin signaling pathway.

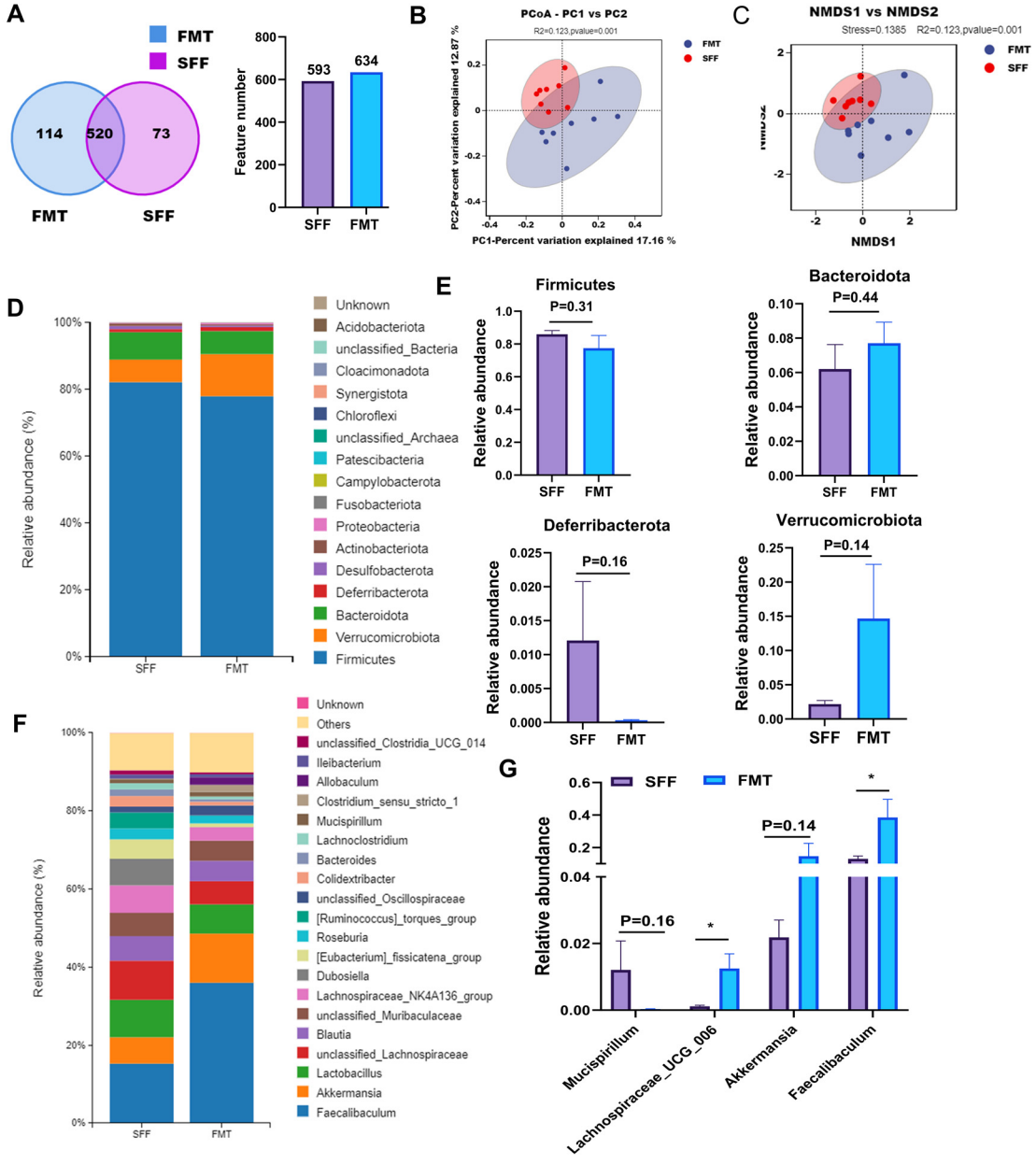


Fig. 6. FMT regulate gut microbiota diversity. (A) Venn diagram and feature number of all the microorganisms detected. (B, C) PCA and NMDs score plot analysis based on the relative abundance of OTUs. (D, E) Bar chart of taxonomic distribution and relative abundance of Firmicutes, Bacteroidota, Verrucomicrobiota, and Deferribacterota at the phylum level. (F, G) Bar chart of taxonomic distribution and relative abundance of *Mucispirillum*, Lachnospiraceae_UCG_006, *Akkermansia* and *Faecalibaculum* at the genus level (n=6-8). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. SFF group.

3.6 MSCPs regulated SCFAs in feces

Evidence has shown that SCFAs are microbial metabolites of polysaccharides, and that SCFAs influence leptin secretion [28]. The SCFAs levels in the feces of DIO mice were further assessed. It was found that, compared to the HFD group, oral administration of MSCPs significantly enhanced SCFAs production, particularly acetate and propionate **(Fig. 7A-C).** Similarly, FMT group significantly increased acetate production when compared to the SFF group **(Fig. 7D-F)**. Notably, consistent with this regulatory effect, MSCPs supplementation also elevated fecal SCFAs content in leptin receptor-deficient db/db mice **(Fig. 7G-I)**.

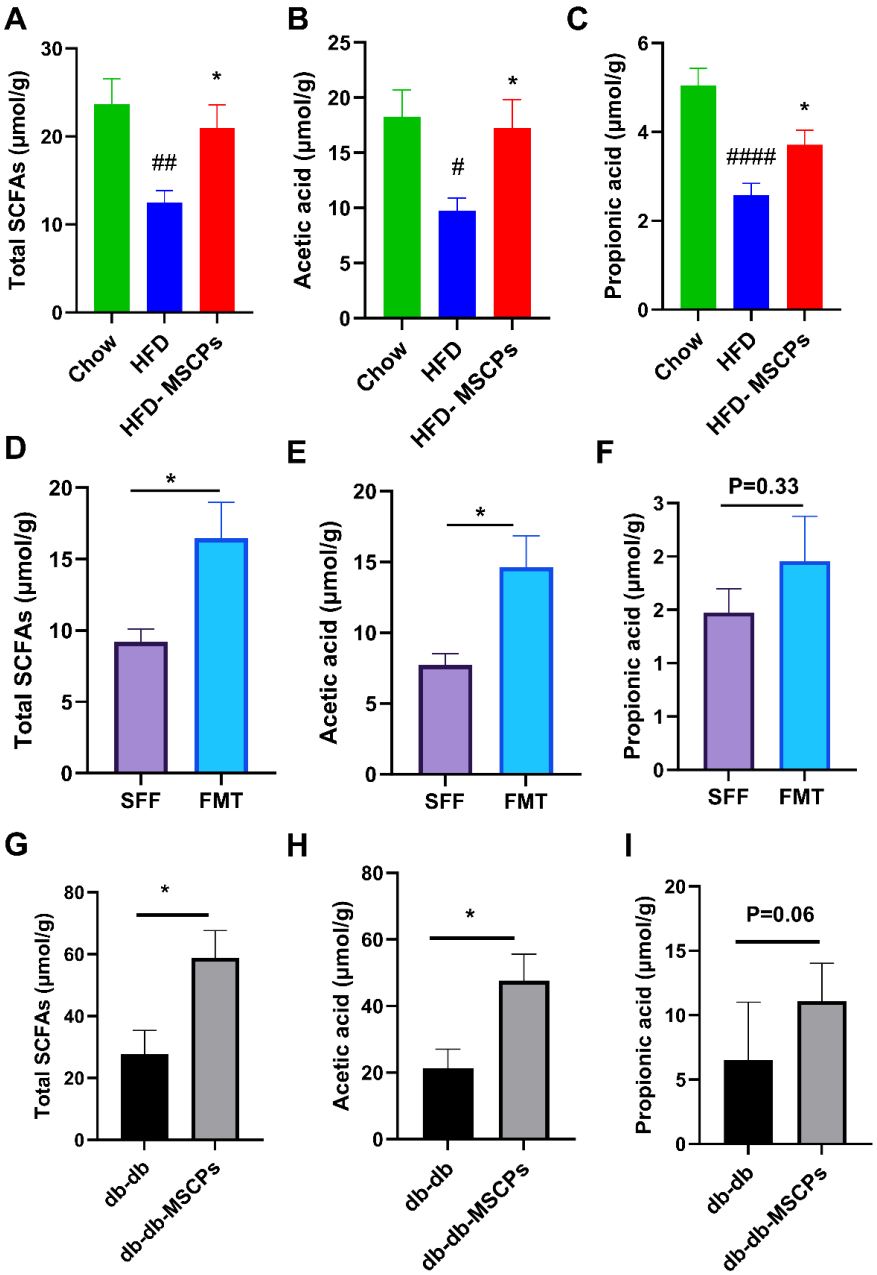


Fig. 7. MSCPs regulated SCFAs production in the feces of mice. (A) The total SCFAs levels in the HFD and HFD-MSCPs groups. (B, C) The acetic acid and propionic acid levels in the HFD and HFD-MSCPs groups. (D) The total SCFAs levels in the SFF and FMT groups. (E, F) The acetic acid and propionic acid levels in the SFF and FMT groups. (G) The total SCFAs levels in the db/db and db/db-MSCPs groups. (H, I) The acetic acid and propionic acid levels in the db/db and db/db-MSCPs groups. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ $^{\#\#\#}P < 0.001$ vs. Chow group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. HFD，SFF or db/db group.

4. Discussion

Although previous studies by our group demonstrated that MSCPs exhibit anti-obesity effects by reducing appetite and alleviating gut microbiota dysbiosis, establishing their role as a potential prebiotic, the mechanisms through which MSCPs mediate gut microbiota to suppress appetite and improve obesity-associated metabolic dysfunction remain unclear.

In this study, a high-fat diet induced obese mouse model was established, with an additional pair-fed group included. After 12 weeks of MSCPs administration and pair-feeding intervention, both treatments significantly improved the obesity phenotype in DIO mice. These improvements included reductions in body weight, fat deposition, and disruptions in glucose and lipid metabolism. These findings suggest that the anti-obesity effects of MSCPs on metabolic syndrome are likely mediated by appetite suppression and reduced energy intake.

Leptin played a critical role in regulating appetite, energy expenditure, and body weight, establishing itself as a key factor in maintaining metabolic balance [29]. Leptin binding to its receptor activates multiple signaling pathways, including the JAK2/STAT3 pathway, which plays a critical role in regulating energy homeostasis. Dysfunction in this pathway has been associated with the development of metabolic diseases, with obesity as a central feature [30, 31]. In HFD-induced obesity mouse model, we found that MSCPs upregulated POMC protein expression and downregulated NPY protein expression in the hypothalamus, thereby reducing appetite and energy intake. In peripheral tissues, MSCPs reduced leptin levels in adipose tissue and enhanced leptin receptor downstream signaling pathways, thereby alleviating peripheral leptin resistance and regulating lipid metabolism. Notably, MSCPs elevated SCFAs, but failed to improve obesity-associated metabolic dysfunction in db/db mice. Furthermore, MSCPs did not influence the hypothalamic expression of the anorexigenic peptide POMC or modulate leptin signaling pathways in db/db mice. These findings suggest that the leptin pathway is likely a central target through which MSCPs exerts its anti-obesity effects in DIO mice.

An increasing number of studies demonstrated that the relative abundance of gut microbiota influenced leptin levels and regulated leptin signaling pathways, thereby improving obesity-related disorders [32]. In this study, MSCPs were found to modulate the gut microbiota structure and composition in DIO mice, Following FMT group to reconstruct the intestinal microbiota, the FMT group reproduced the anti-obesity effect of MSCPs, and the changes in microbiota structure were highly consistent with those in the HFD-MSCPs group. Specifically, both MSCPs supplementation and FMT significantly increased the abundance of beneficial bacteria, including Lachnospiraceae_UCG_006, *Faecalibaculum*, and *Akkermansia* [33, 34], while decreasing the abundance of harmful bacteria, such as Deferribacterota and *Mucispirillum* [35]. Notably, changes in Lachnospiraceae_UCG_006 and *Faecalibaculum* were particularly significant. Previous studies have demonstrated that fiber polysaccharides enrich Lachnospiraceae and *Faecalibaculum*, leading to increased SCFAs production [36, 37]. In this study, both MSCPs intervention and FMT significantly elevated acetate and propionate levels in the feces of DIO mice. It is known that SCFAs can

promote leptin secretion from adipocytes through the activation of specific G protein-coupled receptors (such as GPR43) [38]. The dysbiosis of gut microbiota and their related metabolites, including bile acids, trimethylamine, and SCFAs, have been confirmed to modulate the gut microbiota and contribute to participate in the relieving obesity [39]. Additionally, SCFAs have been reported to enhance intestinal epithelial barrier function, reduce intestinal permeability, and decrease the entry of endotoxins into the bloodstream, thereby improving obesity [40, 41]. Although this study did not directly assess intestinal barrier integrity or systemic inflammatory markers, elevated SCFAs are crucial for maintaining intestinal barrier integrity [42]. An intact intestinal barrier effectively prevents pro-inflammatory microbial components (such as lipopolysaccharide LPS) from entering the systemic circulation. Based on our previous research, MSCPs have been shown to inhibit the secretion of pro-inflammatory cytokines, promote the secretion of anti-inflammatory cytokines, and repair intestinal epithelial barrier dysfunction [43]. Therefore, we speculate that the elevated SCFAs levels observed after MSCPs intervention in FMT in this study not only directly regulate leptin signaling but may also contribute to anti-obesity effects through indirect pathways by improving intestinal barrier function and reducing inflammatory responses critically. However, our data demonstrate that functional leptin signaling is the indispensable gateway for MSCPs' efficacy—a conclusion unequivocally supported by the key observation that despite significantly increasing SCFAs levels in leptin receptor-deficient (db/db) mice, MSCPs failed to alleviate obesity or activate hypothalamic anorexigenic pathways (e.g., POMC upregulation). This compelling evidence positions leptin receptor activation (rather than SCFAs elevation per se) as the non-redundant checkpoint for translating MSCPs' effects into metabolic improvements. Thus, while SCFAs may confer ancillary benefits for gut health, their anti-obesity efficacy is intrinsically dependent on intact leptin signaling to drive appetite suppression and weight loss. In the future, it is hoped that MSCPs can be incorporated into human dietary supplements. It is particularly important to emphasize that translating these findings into human applications requires specialized clinical dose exploration studies. Future research should systematically establish the minimum effective dose and safety characteristics of MSCPs in humans through clinical trials, and utilize bioavailability-enhancing formulations (such as nano-encapsulation technology) to potentially bridge efficacy gaps. The mechanistic insights revealed in this study provide a foundational basis for such translational research and development.

5. Conclusion

The paired feeding experiments provided indirect evidence that MSCPs reduced energy intake, regulated the expression of appetite-related peptides, and activated the JAK2/STAT3 pathway, thereby alleviating obesity in DIO mice. However, further studies demonstrated that MSCPs failed to improve obesity in db/db mice, highlighting the leptin signaling pathway as a critical mediator of MSCPs' anti-obesity effects. Additionally, MSCPs were shown to enrich beneficial gut bacteria, modulate the gut microbiota, and increase SCFAs production. Results from FMT experiments further indicated that the anti-obesity effects of MSCPs in DIO mice were mediated by the gut microbiota. Therefore, we propose that MSCPs may alleviate obesity in

DIO mice by modulating the gut microbiota and increasing SCFAs levels to regulate the leptin signaling pathway.

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Ethics statement

The animal experiment scheme of this study was approved by the Ethics Committee for Use of Experimental Animals at International Healthcare Innovation Institute (Jiangmen) (Guangdong, China) (approval number: N2023016).

Conflicts of interest

The authors declare no conflict of interest.

Data availability

Date will be made available on request.

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