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Polysaccharides from *Platycodon grandiflorus* attenuates high-fat diet induced obesity in mice through targeting gut microbiota

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**ABSTRACT**

The root of *Platycodon grandiflorus* (PG), abundant in soluble polysaccharides, has a long history in traditional Asian diets and herbal medicine due to its anti-inflammatory activity and anti-obesity effects. Our previous study was the first to establish a link between the beneficial effects of PG and changes in the gut microbiota, and suggested potential roles that the polysaccharide components play. However, more evidence was needed to understand the anti-obesity functions of polysaccharides from PG (PS) and their relationship with the regulation of the gut microbiota. In this study, we first performed an experiment to explore the anti-obesity activities of PS: Male C57BL/6 mice (six-weeks-old) were fed either a standard control diet (CON), or a high-fat diet (HFD) to induce obesity, or a HFD supplemented with PS (HFPS) for 8 weeks. Body weight and food intake were monitored throughout. Lipid metabolism were determined and related gene expression changes in adipose tissues were analyzed by RNA-seq. Amplicon sequencing of the bacterial 16S rRNA gene was used to explore gut microbiota structure in fecal samples. Then, we performed the second experiment to explore whether the anti-obesity activities of PS were dependent on the regulation of the gut microbiota: Male C57BL/6 mice (six-weeks-old), treated with an antibiotic cocktail to reduce the gut microbial load, were fed either a standard control diet (CON), or a high-fat diet (HFD) supplemented with PS; TG, Serum total triacylglycerol; TC, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; OTUs, operational taxonomic units; PCA, principal component analysis; LEfSe, linear discriminant analysis effect size; LDA, linear discriminant analysis; PPAR-\(\gamma\), peroxisome proliferators-activator receptor-\(\gamma\); SCFAs, short chain fatty acids.

1. **Introduction**

Obesity is a chronic metabolic disease, characterized by lipid metabolism disorder and excessive fat accumulation [1]. As a result, complications such as nonalcoholic fatty liver disease, type 2 diabetes and cardiovascular disease are common in obese populations [2].

**Abbreviations:** PG, *Platycodon grandiflorus*; PS, Polysaccharides from *Platycodon grandiflorus*; HFD, standard high-fat diet; CON, standard control diet; HFPP, HFD supplemented with PS; TG, Serum total triacylglycerol; TC, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; WAT, white adipose tissue; OTUs, operational taxonomic units; PCA, principal component analysis; LEfSe, linear discriminant analysis effect size; LDA, linear discriminant analysis; PPAR-\(\gamma\), peroxisome proliferators-activator receptor-\(\gamma\); SCFAs, short chain fatty acids.

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High-fat diet (HFD), a common modern dietary habit, is a major risk factor for obesity globally and has a profound impact on morbidity, mortality and cost of health care [3]. Despite the existence of various medical treatments for obesity, dietary intervention is the most promising due to its low cost and minimal risk of complications compared with surgical or other treatments [4].

Diet has a major influence on the structural and functional capacity of the gut microbiota which plays a key role in the development of obesity [5]. The gut microbiota is the complex ecosystem of bacteria harbored in the intestinal tract. The commensal gut microbiota prevent colonization of enteropathogenic organisms [6], modulate the immune system [7] and help with nutrient extraction from undigested dietary components and/or conversion to nutrients that are beneficial to the host [8–10]. For example, polysaccharides from plants can’t be broken-down by mammalian enzymes, but can be utilized by gut microbiota to produce short chain fatty acids (SCFAs) that nourish the epithelial cells or influence other target tissues [11–13]. It is believed that a symbiotic host-microbe relationship benefits human health, while dysbiosis aggravates certain host diseases, including obesity. Previous studies have demonstrated that the interplay between diet and gut microbiota influenced the development of obesity and diabetic phenotypes [5]. One notable characteristic of obesity is the diminished diversity of gut microbiota [14]. Hence, further study of gut microbiota is necessary to enable targeted dietary intervention for prevention and/or treatment of obesity.

The root of the balloon flower Platycodon grandiflorus (Jacquin) DeCandolle (Asterales:Campanulaceae) (PG), known as jiegeng in China, has a long history in Asian diets. It is also used as herbal medicine because of its anti-inflammatory [15–17] and anti-obesity functions [18]. PG is abundant in various soluble polysaccharides including a selenium polysaccharide [19], a pectin-type polysaccharide [20] and an inulin-like polysaccharide [21]. A number of in vitro studies have
reported beneficial effects of polysaccharides from PG (PS), including immunity-enhancing [17,22,23] and antioxidant activities [19,20]. However, these effects haven’t been verified in vivo. As polysaccharides are utilized by the gut microbiota, a link between the gut microbiota and the beneficial effects of PS is likely but has not been investigated to date. Our previous in vivo study has shown that PS is likely a major driver of the health promoting properties of PG; including reducing HFD-induced obesity, suppressing systematic inflammation and intestinal barrier dysfunction, and, mitigating changes in the gut microbiota caused by a HFD [24,25]. However, the effects of PS on obesity prevention and the mechanisms that underpin this need deeper exploration to facilitate informed dietary interventions for obesity.

Therefore, in this study, we designed in vivo and in vitro experiments to further investigate and understand the mechanism by which PS acts on HFD-induced obesity. We developed an antibiotic-induced gut microbiota depleted mouse model to explore the link between PS-induced changes in obesity-related phenotypes and the gut microbiota.

2. Materials and methods

2.1. Preparation of PS

Naturally dried PG root was purchased from Chifeng, Inner Mongolia Autonomous Region, China. A total of 1 kg PG were cut into pieces and further dried for 48 h with a freeze-dryer (LJG-10 C, Beijing, China). Then, the dried samples were milled using a commercial hand-carry milling machine to produce PG powder, which was kept in a moisture-controlled cabinet at 4 °C for PS extraction. Twenty-gram of freeze-dried PG powder was dissolved to 200 ml of n-hexane with continuous magnetic stirring for 30 min. The mixture was centrifuged at 2000 rpm for 3 min at room temperature, and the supernatant was discarded. The process was repeated once. The solution was refluxed twice using 80% ethanol at 80 °C for 1 h per reflux, after which the residue was collected by suction filtration. The polysaccharide was then extracted from the residue by adding 25 times its volume of distilled water and subjected to magnetic stirring for two hours at 80 °C. The mixture was centrifuged at 4000 rpm for 10 min to separate the supernatant and residue. The residue was extracted again, and the two extracts were combined. The extract was then concentrated by means of a rotary evaporator under vacuum at 50 °C. After concentration, 4 times its volume of anhydrous ethanol was added to the concentrate. The mixture was then allowed to stand overnight, during which time the polysaccharides precipitated from the extract. The polysaccharide precipitate was then centrifugated for 10 min at 8000 rpm, and the supernatant was discarded. The resulting polysaccharide precipitate was lyophilized to obtain crude PS.

2.2. Animal experimental design

Six-week-old C57BL/6 mice (n = 45) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed three per cage with free access to food and water in a standard specific pathogen-free facility, with 12 h day and night cycles at 22 °C. After one-week acclimatization, the mice were randomly allocated to different treatments groups shown in Fig. 1A and Fig. 8A and stayed in their cages with three mice per cage for the whole experiment.

For the experiment 1, the mice were fed either a standard control diet (CON, n = 9), a high fat diet (HFD, n = 9) or an HFD supplemented with PS (HFPS, n = 9) for 8 weeks. The ingredients of the experimental diets are shown in Supplementary Table 1.

For experiment 2, the mice were fed either a HFD (A-HFD) or a HFPSS (A-HFPS) with free access to sterile drinking water with an added antibiotic cocktail for 8 weeks. The drinking water was changed twice a week. The antibiotic cocktail was composed of 100 μg/ml neomycin, 50 μg/ml streptomycin, 100 U/ml penicillin, 50 μg/ml vancomycin, 100 μg/ml metronidazole, 100 μg/ml cefazolin, 125 μg/ml ciprofloxine hydrochloride, 1 mg/ml bacitracin [26].

The HFPS and A-HFPS group mice had PS administered by oral gavage at a dose of 500 mg/kg body weight, once a day by trained personnel. The dosage was based on recommendations of PG in the Chinese Pharmacopoeia and the relative content of PS in PG. Mice of the CON, HFD and A-HFD groups received an equivalent dose of sterile physiological saline by oral gavage as a control.

The average food intake of mice per cage was weighed once a week. Each mouse was weighed once per week throughout the experiments. At the end of each experiment, the mice were starved for 12 h before euthanasia. Mice were anesthetized by 3% isoflurane before blood samples were collected by retro-orbital bleeding and immediately centrifuged at 1050 × g at 4 °C for 20 min to obtain the serum samples. Then the mice were immediately euthanized by cervical dislocation. After euthanasia, adipose tissues (epididymal, inguinal, and perirenal adipose tissues) and livers were dissected out, weighed and either immersed into formalin (4% paraformaldehyde) for later histological analysis or immersed in liquid nitrogen and stored at −80 °C for further analysis. Cecal contents were separated from ceca and fresh fecal samples were stored in sterilized tubes at −80 °C. Both animal experiments were approved by the Animal Care Committee of China Agricultural University (approval No. AW21603202–4–1) and conformed to the Guide for the Care and Use of Laboratory Animals [National Institutes of Health (NIH), Bethesda, MD, USA].

2.3. Biochemical analysis

Serum total triacylglycerol (TG), total cholesterol (TC), total protein (TP), glucose (GLU), alanine transaminase (ALT), aspartate transaminase (AST), low-density lipoprotein cholesterol (LDL) and high-density lipoprotein cholesterol (HDL) were measured using commercial kits (Applygen Technologies Inc., Beijing, China) and an OLYMPUS Automatic Biochemical Analyzer (AU480, Japan Olympus Corporation, Tokyo, Japan).

2.4. Histological analysis

The formalin fixed epididymal white adipose tissue (eWAT) and livers were paraffin-embedded, cut into 5–7-μm sections, and stained with hematoxylin and eosin (H&E, Sigma-Aldrich, St. Louis, MO, USA) or Oil-Red O. Stained slides were viewed under a Zeiss Observer (Carl Zeiss, Oberkochen, Germany) at 200 × magnification, and images were taken by an Olympus digital camera (Nikon DS-L1, Tokyo, Japan). Finally, the images were analyzed with the Adiposoft software (CIMA, University of Navarra, Spain) to measure the size of adipocytes.

2.5. In vitro anaerobic fecal bacterial fermentation

Fresh fecal samples (4 g) from mice fed on the HFD diet were suspended in 40 ml sterile PBS and the bacterial suspension was divided into 24 samples with each being 1.5 ml. Twelve samples were diluted by 2.5 ml BHI-medium (Oxoid, Basingstoke, UK) (named as Bac group) and the rest of the samples were co-cultured with PS (named as PS-Bac group) at a final concentration of 40 mg/ml BHI-medium under anaerobic conditions (10% H2, 10% CO2, 80% N2, 37 °C). After incubation for 0, 6, 12, and 24 h (three replicates per time point), cultures were centrifuged at 4 °C, 1000 × g for 3 min to separate the supernatant and precipitate, and respectively stored at −80 °C for later metabolites detection and bacterial composition analysis. The bacteria were quantified by RT-qPCR.

2.6. Short-Chain Fatty Acids (SCFAs) analyses

To analyze the SCFAs content in cecal samples and bacterial culture, both 0.8 ml of the supernatant of bacterial culture and 30 mg of lyophilized cecal samples diluted by distilled water to 0.8 ml were mixed with 0.4 ml 50% aqueous H2SO4 solution. The extraction of
SCFAs was performed with 1.5 ml of diethyl ether followed by 3 min of shaking and centrifugation at 1050 \( \times \) g for 5 min at room temperature. Anhydrous CaCl\(_2\) was added into the collected supernatant to remove residual water, and then 2 \( \mu \)l supernatant was analyzed by injection into the GC system. Acetic acid, propionic acid, butyric acid and valeric acid were used as the standards. The analysis was performed using an Agilent 5975 GC equipped with a 30 m \( \times \) 0.32 mm \( \times \) 0.25 \( \mu \)m polar GC column (ZB-FFAP, Phenomenex, Torrance, CA, USA). The solvent was delayed for 3.5 min. The initial oven temperature was set to 90 \( ^\circ \)C for 2 min, followed by a ramp at 15 \( ^\circ \)C/min to 220 \( ^\circ \)C, which was held for 5 min. The flame ionization detector (FID) and inlet temperatures were set to 175 \( ^\circ \)C. The carrier gas was He (1.0 ml/min). The selected ion monitoring (SIM) mode and Agilent Mass Hunter WorkStation software (Agilent Technologies) were used for data acquisition. The sample peaks

Fig. 2. PS reduced adipocytes hyperplasia and fat accumulation in the adipose tissue and liver of HFD mice. (A) Epididymis WAT morphology as assessed by H&E staining (scale: 50 \( \mu \)m) (\( n = 9 \)). (B) Total white adipose tissue mass (epididymal, inguinal, and perirenal white adipose tissues) (\( n = 9 \)). (C) Mean epididymal adipocyte size (\( n = 9 \)). Liver histology as determined by (C) H&E and (E) Oil Red O staining (scale: 100 \( \mu \)m) (\( n = 9 \)). Labeled means without a common letter differ, \( P < 0.05 \). CON, standard control diet; HFD, standard high-fat diet; HFPS, HFD supplemented with PS; PS, polysaccharides from Platycodon grandiflorus; H&E, hematoxylin and eosin.
Table 1

| Parameter | CON | HFD | HFPSS
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>ALT (mg/dL)</td>
<td>30.67 ± 1.96a</td>
<td>32.22 ± 1.96a</td>
<td>0.28 ± 1.96a</td>
</tr>
<tr>
<td>AST (mg/dL)</td>
<td>127.22 ± 5.51b</td>
<td>130.78 ± 5.51b</td>
<td>125.33 ± 5.51a</td>
</tr>
<tr>
<td>TP (mmol/L)</td>
<td>6.29 ± 0.12a</td>
<td>6.23 ± 0.12a</td>
<td>6.27 ± 0.12a</td>
</tr>
<tr>
<td>GLU (mmol/L)</td>
<td>3.98 ± 0.24</td>
<td>4.68 ± 0.06a</td>
<td>5.48 ± 0.28a</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>0.22 ± 0.02a</td>
<td>0.38 ± 0.02a</td>
<td>0.27 ± 0.02a</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>2.60 ± 0.14a</td>
<td>5.01 ± 0.14a</td>
<td>3.28 ± 0.14a</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>2.35 ± 0.13b</td>
<td>3.64 ± 0.13b</td>
<td>2.85 ± 0.13b</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>4.22 ± 0.03b</td>
<td>1.13 ± 0.03c</td>
<td>0.51 ± 0.03c</td>
</tr>
<tr>
<td>ALT/ALT</td>
<td>4.18 ± 0.20c</td>
<td>4.16 ± 0.20c</td>
<td>4.19 ± 0.20c</td>
</tr>
<tr>
<td>LDL/HDL</td>
<td>0.18 ± 0.01b</td>
<td>0.32 ± 0.01a</td>
<td>0.18 ± 0.01b</td>
</tr>
</tbody>
</table>

Data shown are mean ± SEM of 9 mice per group. One-way ANOVA, P < 0.05. Labeled means without a common letter differ. CON, standard control diet; HFD, high-fat diet; HFPSS, HFD supplemented with PS; PS, Polyaccharides from Platycodon grandiflorus.

were identified by comparing their retention times with internal standards acetate, butyrate, propionate, and valerate (Sigma-Aldrich).

2.7. Quantitative PCRs

Quantitative reverse transcription PCR (RT-qPCR) was used to assess the expression levels of target genes in eWAT and quantitative real-time PCR (qPCR) was used to quantify the specific bacteria in fecal and fecal culture samples determined by 16 s rRNA gene copies.

Total RNA of eWAT was isolated using a Trizol reagent (Invitrogen, Waltham, MA, USA), purified by using a RNeasy Mini Kit (Qiagen, Venlo, Limburg, Netherlands) and reverse as template cDNA by using FastQuant RT Kit (TianGen, Beijing, China) following the manufacturer’s instructions. The template cDNA (1.5 μl) were mixed with 0.5 μl of the target primers, 5 μl of SYBR Green 1 Master Mix (Roche Diagnostics, Basel, Switzerland) and 3 μl of water. PCRs were performed in triplicates on a LightCycler 480 Real-Time PCR system as follows: initial preincubation at 95 °C for 5 min, 40 PCR cycles of 95 °C for 10 s, 60 °C for 30 s, 72 °C for 15 s, followed by a melting curve. Relative quantification was performed using the comparative 2-ΔΔCT method with three biological replicates and was normalized against GAPDH gene expression. The mean expression levels of the CON mice were set at a value of 1. The primers used are listed in Supplementary Table 2.

Total genomic DNA of fecal and cultured fecal samples was extracted using a QIAamp-DNA Stool Mini Kit (Qiagen, Hilden, Germany). The integrity of the extracted DNA was examined by electrophoresis in 1% (wt/vol) agarose gels. The template DNA (0.5 μl) were mixed with 1 μl of the target primers, 5 μl of SYBR Green 1 Master Mix (Roche Diagnostics, Basel, Switzerland) and 3.5 μl of water. PCRs were performed in triplicates on a LightCycler 480 Real-Time PCR system using the following PCR program: initial preincubation at 95 °C for 30 s, 40 PCR cycles of 95 °C for 5 s, 72 °C for 30 s, followed by a melting curve. For bacteria quantification, standard curves were prepared using continuous dilution of purified and quantified PCR products generated from the bacterial genomic DNA. The primers used are listed in the Supplemental Methods.

2.8. Transcriptome analysis (RNA-seq)

The total RNA of eWAT was extracted (n = 3 per group) using a Trizol reagent (Invitrogen, USA) and quantified by electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, San Diego, CA, USA). RNA-seq libraries were constructed using the TruSeq™ RNA Sample Prep kit (Illumina, San Diego, CA, USA) when 5 μg samples had RNA integrity numbers (RINs) > 9.4 and 260/280 nm absorbance ratios within 1.9–2.1. Sequencing and processing of the libraries were performed on an Illumina HiSeq2000 platform by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The data were analyzed on the free online platform of Majorbio Cloud Platform (www.majorbio. com). The raw reads were trimmed with Trimmmomatic (v 0.35) to remove the adaptors and low-quality bases. The quality of raw data was evaluated by FastQC and reads passing quality control parameters were aligned to the mouse reference genome (GRCm39) with the STAR v 2.7.1a aligner [27]. Aligned reads were then quantified at the gene level using HTSeq (v 0.7.2).

2.9. 16S rRNA amplicon sequencing

Total DNA was extracted from feces (n = 6 per group) using a QIAamp-DNA Stool Mini Kit (Qiagen, Hilden, Germany). Based on the quantity and the quality of the DNA extracted, samples were selected to perform the consequent sequencing. The V3–V4 hypervariable regions of the 16 S ribosomal RNA (rRNA) gene were amplified using the primers 338 F (5’-GTGCCACGCMGCCGCGG-3’) and 806 R (5’-CCGTCAATTCMTTTRAGTTT-3’) (21). PCR amplification was performed on an ABI GeneAmp®9700 PCR instrument (AppliedBiosystems) and the products were quantified with a QuantiFluor™-ST Handheld Fluorometer with UV/Blue Channels (Promega Corporation). Sequencing and processing of the PCR amplicons were performed on the Illumina Miseq platform by Majorbio Bio-Pharm Technology Co., Ltd. The Supplemental Methods describe the sequencing and data analysis in detail.

3. Statistical analysis

For data measured in experiment 1, one-way ANOVA models were initially used to analyze differences in the dependent variables (weight gain, white adipose tissue mass, mean adipocyte size, serum biochemical analysis, SCFAs and gene expression levels) between groups (CON, HFD and HFPSS). The presence of heteroskedasticity was tested using the Breusch-Pagan / Cook-Weisberg test. If the homogeneity assumption was violated, the corresponding ANOVA models were refitted using the robust standard errors. The normality assumption was visually assessed using qqplots. If normality was violated, then the dependent variable was either transformed (i.e. 1/mean adipocyte size in the analysis) or a non-parametric alternative – Kruskal–Wallis method was used (i.e. SCFAs). Once the models were decided, pairwise multiple comparison tests were conducted using either Sidak correction (if normality held) or Dunn’s test (followed by Kruskal-Wallis method). The difference in energy intake between groups was analyzed using a two-way ANOVA model including both time and study group with the robust standard errors, followed by pairwise multiple comparisons using Sidak correction. Finally, the effect of the treatment on body weight was analyzed using an order one autoregressive linear model with the study group, time and their interaction included as fixed effects.

For RNA-Seq data, principal component analysis (PCA) was used to verify the results of the grouping based on the gene expression levels. Normalization of read counts and differential expression analysis of genes (DEG) between sample groups were performed using DESeq2 (28). Statistical significance of the differentially expressed genes was defined with the adjusted p-value < 0.05 correcting for multiple testing. All DEGs were used to perform functional enrichment cluster based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. A heatmap of DEGs from PPAR signaling pathway were plotted.

Since experiment 2 and the in vitro experiment involved only two groups, two sample t test was used for studying the differences in dependent variables between groups. The data analysis was performed using Stata/SE 15.1, except the Kruskal–Wallis method and the Dunn’s test which were implemented in R 4.2.2. Throughout, a p-value < 0.05 was considered as statistically significant.
Fig. 3. PS regulated the expression of lipid-metabolic genes in eWAT. (A) Between-sample diversity and clustering shown by PCA of the eWAT transcriptomes \((n = 3)\). Differentially expressed genes \((\log_{2}FC \geq |1|\) and adjust p-value < 0.05\) between (B) CON and HFD groups, (C) HFD and HFPS groups, shown by Volcano Plot \((n = 3)\). (D) KEGG pathway enrichment analysis of the most significantly changed pathways \((n = 3)\). The size of the dot indicates the number of differently expressed genes in the signaling pathway. CON, standard control diet; HFD, standard high-fat diet; HFPS, HFD supplemented with PS; PS, polysaccharides from Platy- 
codon grandiflorus; eWAT, epididymal white adipose tissue; PCA, principal component analysis; KEGG, Kyoto Ency-
clopedia of Genes and Genomes.
Fig. 4. PS affected the PPAR signaling pathway in eWAT. (A) Heatmap of differentially expressed genes in PPAR signaling pathway among three dietary treatments. Genes with fold changes > 1 and adjust p-value < 0.05 were considered to be differentially expressed (n = 3). (B) Real-time qPCR assay for the differentially expressed genes in PPAR signaling pathway (n = 9). Labeled means without a common letter differ, P < 0.05. CON, standard control diet; HFD, standard high-fat diet; HFPS, HFD supplemented with PS; PS, polysaccharides from Platycodon grandiflorus.
4. Results

4.1. PS reduced body weight, fat accumulation and ameliorated dyslipidemia in HFD-fed mice

At the end of the 8-week study the body weight of the HFD mice was significantly higher than the CON mice, with this difference observed from week 3 onwards (Fig. 1B, C). In contrast, there was no statistically significant difference in body weight between HFPS and CON group mice during the entire experiment. Notably, the HFPS group mice had a slower weight gain compared to the HFD mice (the slope of the line was smaller), yet no difference in energy intake between the two groups was observed (Fig. 1B, C). Such observation indicated that the effects of PS on body weight is not related to any adverse effects on eating habits. After 8 weeks the mice in the three groups showed a significant difference in total weight gain (Fig. 1D). Additionally, there were differences in fat accumulation in white adipose tissues (WAT), with the HFPS group mice having smaller WAT mass and adipocyte size than the HFD mice (Fig. 2B). Histological analyses of epididymal adipocytes and liver tissues showed less hypertrophy in adipose tissues and steatosis in the livers of HFPS mice compared to HFD mice (Fig. 2A-E). In addition, the HFPS mice had decreased plasma TC, TG, HDL, LDL levels and LDL to HDL ratios, compared to HFD mice (Table 1), indicating a reduction in the risk of obesity.

4.2. PS regulated the expression of lipid-metabolic genes in eWAT

RNA-sequencing on eWAT was performed to test differential expression of genes among the dietary treatments, which may lead to the reduced fat accumulation in HFPS mice. In total, we sequenced 79,147,248,564 base pairs, which were mapped to 11,773 genes after quality-filtering. There were differences in fat accumulation in white adipose tissues (WAT), with the HFPS group mice having smaller WAT mass and adipocyte size than the HFD mice (Fig. 2B). Histological analyses of epididymal adipocytes and liver tissues showed less hypertrophy in adipose tissues and steatosis in the livers of HFPS mice compared to HFD mice (Fig. 2A-E). In addition, the HFPS mice had decreased plasma TC, TG, HDL, LDL levels and LDL to HDL ratios, compared to HFD mice (Table 1), indicating a reduction in the risk of obesity.
mice in the different groups with principal component analysis (PCA) of the transcripts identifying clustering based on treatment (CON, HFD, HFPS) (Fig. 3A), indicating that all mice exhibited dietary-specific transcriptional responses. The comparison of CON versus HFD group exhibited the higher amount of DEGs (log$_2$FC $\geq$ |1| and p-value < 0.05) with a total of 828 genes whereas less genes (n = 717) showed differences in gene expression between HFPS and HFD groups (Fig. 3B, C). When mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway Database, these genes showed enrichment in a series of metabolic signaling pathways, especially those related to fatty acid metabolism and lipid metabolism (Fig. 3D). Of note, PPAR signaling was the most significantly enriched signaling pathway in HFPS mice (Fig. 3D). Twenty-nine genes that were enriched in the PPAR signaling pathway were mainly involved in lipid metabolism; including lipid degradation (Rxrg, Pparg, Cd36, Acbp, Lpl, Fabp1, Fabp2, Acs1, Acs3, Acs4, Slc27a1, Slc27a2, Slc27a4), lipid biosynthesis (Me1, Scd1), lipid storage (Plin2, Scd1), and lipid transport (Ppara, Lpl, ApoB).

Fig. 6. PS changed the gut microbiota composition in HFD mice. (A) Major contributors (LDA scores $>3.5$) at differentiating among treatment groups were found by linear discriminant analysis effect size (LEfSe) analysis on taxa from family to OTU levels (n = 6). (B) OTUs with LDA scores $>3.5$ and significantly different across the 3 diet treatments as calculated by Kruskal–Wallis H test based on relative abundances (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001. CON, standard control diet; HFD, standard high-fat diet; HFPS, HFD supplemented with PS; PS, polysaccharides from Platycodon grandiflorus; LDA, linear discriminant analysis; OTU, operational taxonomic unit.
Plin4, Plin5), lipid transport (Pltp, Apoa2, Apoa5), β-oxidation (Cpt1α, Cpt2, Acadm, Acaa1b, Acox1, Ehhadh), and cholesterol metabolism (Hmgcs2, Cyp7a1, Cyp8b1) (Fig. 4A). This was consistent with the results of real-time quantitative PCR where several key genes of lipid metabolism in the PPAR signaling pathway were regulated based on diet, especially the most significantly upregulated PPARγ (Fig. 4B). Overall, these results suggest that PS affects the expression of genes related to lipid metabolism and the activation of PPAR signaling may contribute to the impacts of PS on obesity development.

4.3. PS prevented HFD-induced gut dysbiosis in mice

Previous studies have shown that interactions between diet and gut microbiota are crucial modulators in the development of obesity and diabetic phenotypes [5]. We therefore investigated the effects of PS on gut microbiota composition by amplicon sequencing of the bacterial 16S rRNA gene (V3–V4 region) in fecal samples from CON, HFD and HFPS mice. A total of 442,923,850 base pairs were sequenced, producing 1,066,901 reads after quality-filtering and chimera checking. Reads were clustered at 97% identity, producing 449 OTUs overall. The specific number of OTUs shared between each treatment group, and unique to each group, is shown in Supplementary Fig. 1. The rarefaction curves and Pan analysis showed similar curves among samples with ends tending to be flat, indicating comparable and enough sequence depth across all samples (Supplementary Fig. 2).

An important feature of obesity is a reduced diversity of gut microbiota [14]. The increased Shannon index and decreased Simpson index of the gut microbiota both confirm that HFD significantly reduced the gut microbiota diversity compared to CON mice (Fig. 5A, B). While both indices in HFPS group were not significantly different to those of the CON mice, though the Simpson index was not displaying a significant difference between HFPS and HFD groups (significant difference only observed in Shannon index) (Fig. 5A, B). Additionally, the decreased Shannon even index of the HFPS mice (contrasting to the HFD mice) indicated an evaluated evenness in alpha diversity, although it was still significantly different to CON group (Fig. 5C). Overall, HFPS mice had increased alpha diversity of the gut microbiota compared to HFD mice. Principal coordinate analysis (PCoA) showed overall differences in the gut bacterial composition in mice across the three groups (Fig. 5D, E). These differences can be further visualized by the relative abundance of

Fig. 7. PS increased the SCFAs production in the gut of HFD mice. Concentrations of (A) acetate, (B) propionate, (C) butyrate and (D) valerate in cecal content (n = 9). Labeled means without a common letter differ, P < 0.05. CON, standard control diet; HFD, standard high-fat diet; HFPS, HFD supplemented with PS; PS, polysaccharides from Platycodon grandiflorus.
bacterial composition at the genus level (Supplementary Figure 3).

The results of the LEfSe analysis on taxa from family to OTU levels found major contributors (LDA scores >3.5) differed among treatment groups (Fig. 6A, B). We found that Lachnospiraceae were dominant in the gut microbiota of all samples, although the number and the type of dominant OTUs varied across diet groups (Fig. 6B). HFD induced communities were dominated by 9 OTUs from Lachnospiraceae (>45% relative abundance) and had moderate dominance by Bacteroides and 4 OTUs from Oscillospiraceae (Fig. 6B). CON communities showed a lower dominance with a significant increase of Muribaculaceae and Faecalibaculum, and a decrease of Bacteroides and Lachnospiraceae (Fig. 6B). HFPS communities were largely dominated by four other OTUs of Lachnospiraceae (>35% relative abundance). Overall, the major differences in microbial composition between HFD and CON mice, mainly driven by a reduction in the dominance of Muribaculaceae and Faecalibaculum and an increase of Lachnospiraceae, Oscillospiraceae and Dubostella in HFD, were significantly mitigated in HFPS mice (Fig. 6B). Of note, HFPS mice had a significantly greater proportion of Akkermansia compared to both HFD and CON mice (Fig. 6B).

Many members from the genera Akkermansia, Faecalibaculum and the family Muribaculaceae are known for their ability to produce SCFAs, hence, four major SCFAs in mice feces were analyzed and we found that HFPS mice had significantly increased the concentrations of acetate, propionate and butyrate in their feces compared to HFD mice (Fig. 7).

4.4. The beneficial effects of PS on HFD mice depended on the gut microbiota

To further investigate whether PS-ameliorated HFD induced obesity depends on the gut microbiota, we eliminated the microbiota using broad-spectrum antibiotics in experiment 2 (Fig. 8A). The amount of stool-extracted DNA was around 100-fold lower in the antibiotic-treated mice compared to mice in experiment 1 (Supplementary table 3), indicating a significant depletion of gut microbiota. Several variables were measured including body weight (Fig. 8B), body weight gain (Fig. 8C), average energy intake (Fig. 8D), cell size (Fig. 9A, E), weight of white
adipose tissue (Fig. 9D), and fat accumulation in liver tissue (Fig. 9B, C), and none of which showed significant difference in these gut microbiota-depleted HFD mice either fed with PS (A-HFPS) or not (A-HFD). Moreover, A-HFPS mice showed no significant differences in plasma TC, TG, HDL and LDL levels compared to A-HFD mice (Table 2). These results suggested that the function of PS on diet-induced obesity was dependent on the role of the gut microbiota.

4.5. PS promoted the growth of gut bacteria and the production of SCFAs in vitro

In the above in vivo model, we found HFPS mice had a greater abundance of Akkermansia in their gut microbiota, compared to HFD and CON mice. Further, we performed an anaerobic fermentation experiment to explore the effects of PS on the growth and metabolic activity of gut bacteria in vitro, especially Akkermansia. The growth curves of the total bacteria and Akkermansia of both groups (Bac vs. PS+) showed similar patterns (Fig. 10 A and B, respectively), whereas PS promoted proliferation of the gut bacteria. At 24 h, the

Table 2
Serum biochemical analysis for A-HFD and A-HFPS mice.

<table>
<thead>
<tr>
<th>Serum Parameter</th>
<th>A-HFD</th>
<th>A-HFPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (mg/dL)</td>
<td>31.11 ± 2.65a</td>
<td>31.22 ± 1.95a</td>
</tr>
<tr>
<td>AST (mg/dL)</td>
<td>124.22 ± 8.88a</td>
<td>123.00 ± 5.99a</td>
</tr>
<tr>
<td>TP (mmol/L)</td>
<td>6.27 ± 0.11a</td>
<td>6.28 ± 0.13a</td>
</tr>
<tr>
<td>GLU (mmol/L)</td>
<td>7.73 ± 0.43a</td>
<td>7.92 ± 0.37a</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>0.25 ± 0.01a</td>
<td>0.27 ± 0.01a</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>3.85 ± 0.16a</td>
<td>3.83 ± 0.15a</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>3.18 ± 0.17a</td>
<td>3.16 ± 0.12a</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>0.79 ± 0.06a</td>
<td>0.80 ± 0.05a</td>
</tr>
<tr>
<td>AST/ALT</td>
<td>4.08 ± 0.26a</td>
<td>4.00 ± 0.20a</td>
</tr>
<tr>
<td>LDL/HDL</td>
<td>0.25 ± 0.02a</td>
<td>0.25 ± 0.01a</td>
</tr>
</tbody>
</table>

Data shown are mean ± SEM of 9 mice per group. One-way ANOVA, P < 0.05. Labeled means without a common letter differ, A-HFD, standard high-fat diet; HFPS, HFD supplemented with PS; A-HFD, HFD with free access to sterile drinking water added with an antibiotic cocktail; A-HFPS, HFPS with free access to sterile drinking water added with an antibiotic cocktail; PS, polysaccharides from Platycodon grandiflorus; H&E, hematoxylin and eosin.
average number of *Akkermansia* in the PS+\(\text{Bac}\) group \(10^{7.7}\) was about 10 times that of the \(\text{Bac}\) group \(10^{6.73}\) (Fig. 10 B). In addition, we also studied the effect of PS on the production of SCFAs by gut microbiota during *in vitro* anaerobic fermentation. The results are shown in Fig. 10 C, D and E. Overall, the concentrations of acetate, propionate and butyrate were increased with the prolongation of PS anaerobic fermentation time. In the fermentation system without PS, the bacteria were also able to produce low concentrations of SCFAs, while the addition of PS significantly increased the production of all three SCFAs.

5. Discussion

PG is a rich source of polysaccharides, with a higher concentration compared to most common vegetables. Our previous study described the beneficial effects of PS in PG on obesity, which were attributed to changes in the gut microbiota [24]. Previous *in vivo* studies focused on the saponin components in PG [29,30], ignoring the role of PS. There were some *in vitro* studies indicating immunity-enhancing and antioxidant properties of PS [17,21,23], however, their effects had not been confirmed *in vivo*.

In this study, we found that PS effectively alleviated HFD-induced...
weight gain, excessive fat accumulation in adipose and liver tissues, and hyperlipidemia in mice. Additionally, we showed that PS can suppress the HFD-induced changes in the composition, structure, and metabolic function of the gut microbiota, especially increasing the production of SCFAs. Our microbiota-depleted mouse model results further support the hypothesis that the effects of PS on obesity are dependent on the function of gut microbiota. There are concerns about the validity of antibiotic-induced microbiota-depleted animal models [31,32], due to the impact of antibiotics on host energy metabolism and the potential to induce resistance to diet-induced obesity, particularly with high-dose exposure. However, in this study, we used a low-dose long-term antibiotic exposure, and observed more fat accumulation in both A-HFD and A-HFPS mice than in the control group. Thus, the similar body weight of A-HFD and A-HFPS mice in this study is unlikely to be the result of the antibiotic treatment or the absence of gut microbiota inducing weaker fat metabolism. Combined, our results suggested that the function of PS on diet-induced obesity was dependent on the role of the gut microbiota. Overall, our findings highlight the beneficial role of PS in combating diet-induced obesity and the crucial role played by the gut microbiota.

A major impact of PS on gut microbial composition is the significant increase in the abundance of Muribaculaceae, Faecalibaculum and Akkermansia. Muribaculaceae, previously known as S24-7, a family of bacteria within the order Bacteroidales [33]. This finding aligns with our previous study that found PG significantly increased the proportion of Faecalibaculum and several OTUs from S24-7 in HFD gut communities [24]. Muribaculaceae has been recently identified as a major mucus-monosaccharides forager, competing with pathogens that rely on mucus-derived nutrients, making it an ecological gatekeeper in healthy guts [34].

Our study found that PS can promote the growth of Akkermansia and increase the production of SCFAs (acetate, propionate, and butyrate) both in vivo and in vitro. Akkermansia, first identified in human feces in 2004 [35] is associated with mucus health and has been found to have prebiotic functions including alleviating obesity and metabolic disorders caused by HFD [36,37], improving colitis [38], enhancing intestinal immunity and intestinal barrier [39], and exhibiting anticancer [40] and anti-aging [41] effects. Akkermansia colonizes the intestinal mucus layer and is a typical intestinal mucosal resident bacterium. It degrades the intestinal mucus to produce acetate and butyrate while promoting mucus secretion and mucosal immunity, thus strengthening the intestinal barrier [39]. Obesity-induced imbalance in the gut microbiota are usually followed by intestinal barrier dysfunction with increased intestinal permeability, resulting in systematic inflammation and reinforced metabolic disorder [42,43]. We found an increase in Akkermansia, Muribaculaceae and SCFAs (produced by the bacteria) therefore further studies on the effect of PG on the intestinal mucus layer and intestinal barrier function would be useful.

Diet-induced obesity involves changes in the expression of genes related to various signaling pathways [44]. Our comprehensive analysis of transcriptome differences between treatment groups showed that PS had effects on many genes associated with obesity, particularly in the PPAR signaling pathway. PPAR is a sensor for changes in fatty acid levels and their derivatives, which can alter gene transcription in response to ligand binding [45]. This makes it a key factor in maintaining physiological homeostasis, including lipid and carbohydrate metabolism, and is therefore an important target for addressing metabolic disorders.

In line with this, our study found that PS reduced adipose and hepatic fat accumulation and lowered plasma levels of TC, TG, HDL, and LDL in HFPS mice, in agreement with the changes of genes in the PPAR signaling pathway. Additionally, some genes in the PPAR signaling pathway were also regulated by AMPK, such as Parg, Cd36, Lpl, Scd1 and Cpt1, which partly explains previous findings that PG or its extracts could alter the expression of genes in the AMPK signaling pathway [46, 47].

The crosstalk between PPAR signaling and the gut microbiota has been widely studied [48,49], with some bacteria found to regulate the PPAR signal pathway and alleviate HFD-induced obesity in mice [50]. Butyrate-producing microbes have also been shown to impact PPAR-γ signaling and shift the energy metabolism of colonic epithelial cells toward β-oxidation [51]. Our study found that PS increased the proportion of several SCFAs-producing gut bacteria and elevated the concentrations of acetate, propionate, and butyrate in feces, providing a possible explanation for the changes in PPAR signaling. However, further studies are needed to verify this relationship.

In conclusion, our results suggest that PS has the potential to mitigate HFD-induced obesity by modifying the changes in gut microbiota composition and function caused by HFDs. Our study provides a foundation for further investigation of the underlying mechanisms of the beneficial effects of PS and highlights the potential of PS as a functional food. Future experiments are required to fully understand the causal link between PS-induced changes in obesity-related phenotypes and the gut microbiota, as well as which compounds in PS modulate the gut microbiota.

CRediT authorship contribution statement

Study design: W. Ke, X. Huang, X. Hu and F. Chen; Lab work: W. Ke; Data visualization: W. Ke and D. A. Yang; Data analysis and interpretation: W. Ke and D. A. Yang; Writing: W. Ke, D. A. Yang and K. J. Flay; Reviewing and Editing: D. A. Yang, C. Li, X. Huang and K. J. Flay; Funding acquisition: W. Ke and D. A. Yang.

Declaration of Competing Interest

We, the authors of the research study titled’ Polysaccharides from Plantcodon grandiflorus attenuates high-fat diet induced obesity in mice through targeting gut microbiota’ declare the following potential conflicts of interest: The authors declare that there are no conflicts of interest.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopharma.2023.115318.

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