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Gypenosides improve the intestinal microbiota of non-alcoholic fatty liver in mice and alleviate its progression

Xiaoqing Huang[a,c,d], Wenfan Chen[a], Changsheng Yan[b,c], Renzhi Yang[a,c,d], Qiongyun Chen[b,c], Hongzhi Xu[b,c,**], Yuanpeng Huang[a,d,*]

[a] Department of Traditional Chinese Medicine, Zhongshan Hospital, Xiamen University, Xiamen, 361004 Fujian PR China
[b] Department of Gastroenterology, Zhongshan Hospital, Xiamen University, Xiamen, 361004 Fujian PR China
[c] Institute for Microbial Ecology, Xiamen University, Xiamen, 361005 Fujian PR China
[d] Department of Traditional Chinese Medicine, School of Medicine, Xiamen University, Xiamen 361102, Fujian Province, PR China

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ABSTRACT

Gypenosides (GP) are a type of traditional Chinese medicine (TCM) extracted from plants and commonly applied for treatment of metabolic diseases. This study aims to explore the effects of GP extracts on alleviating non-alcoholic fatty liver disease (NAFLD). In this experiment, C57BL/6 J mice were randomly assigned into normal diet control (ND), HFHC (high-fat and high-cholesterol) and HFHC + GP (GP) groups. Mice in HFHC group were fed HFHC diet combined with fructose drinking water for 12 weeks to induce the animal model of NAFLD, followed by ordinary drinking water until the end of the experiment. In the HFHC + GP group, mice were fed HFHC diet combined with fructose drinking water for 12 weeks, followed by GP-containing drinking water till the end. Mouse body weight was measured weekly. After animal procedures, mouse liver and serum samples were collected. It is shown that GP administration reduced body weight, enhanced the sensitivity to insulin resistance (IR) and decreased serum levels of ALT, AST and TG in NAFLD mice. In addition, GP treatment alleviated steatohepatitis, and downregulated ACC1, PPARγ, CD36, APOC3 and MTTP levels in mice fed with HFHC diet. Furthermore, GP treatment markedly improved intestinal microbiota, and reduced relative abundance ratio of Firmicutes / Bacteroidetes in the feces of NAFLD mice. Our results suggested that GP alleviated NAFLD in mice through improving intestinal microbiota.

1. Introduction

NAFLD is a type of chronic liver disease caused by non-alcoholic factors. The incidence of NAFLD in Western countries is about 30%–40%, which is 15%–20% in females [1]. The prevalence of NAFLD in Chinese urban population is about 15% [2]. NAFLD is the risk factor to increase susceptibilities to type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), and chronic kidney disease [3]. In addition, approximately 20% NAFLD patients would progress to non-alcoholic steatohepatitis and even hepatic carcinoma [4]. The pathogenesis of NAFLD is mainly attributed to IR. Once IR occurs, insulin inhibits lipolysis of adipose tissues and hepatic gluconeogenesis. Moreover, insulin promotes steatohepatitis by stimulating de novo synthesis of liver lipids, eventually progressing to NAFLD [5]. In recent years, the incidence of NAFLD is on the rise, and seriously endangers human health [6]. Effective drugs targeting NAFLD are lacked and it is urgent to clarify the pathogenesis of NAFLD [7].

GP are perennial vines widely grown in Asian, and their leaves have been processed in food and tea [8]. Previous studies have demonstrated that GP are beneficial to treatment of obesity [9], CVD [10], T2DM [11] and cancers [12,13]. It is reported that GP administration reduces body weight, liver weight and blood cholesterol levels in ob/ob mice by activating AMPK [14]. Liu et al. showed that GP can stimulate activities of brown fat cells and promote the conversion of white fat to brown fat [15]. Therefore, GP may be promising drugs utilized for NALFD treatment.

Intestinal microbiota is of significance in the progression of NAFLD [16]. By analyzing the composition of fecal flora in genetically obese mice, elevated ratio of Firmicutes / Bacteroidetes is identified to be related to obesity [17]. Another study reported that transplanting cold microbiota into sterile mice can enhance the insulin sensitivity of the host and cold tolerance through inducing the conversion of white fat to

* Corresponding author at: Department of Traditional Chinese Medicine, Zhongshan Hospital, Xiamen University, Xiamen, 361004 Fujian PR China.
** Corresponding author at: Department of Gastroenterology, Zhongshan Hospital, Xiamen University, Xiamen, 361004 Fujian PR China.
E-mail addresses: xuhongzhi@xmu.edu.cn (H. Xu), huangyp998@xmu.edu.cn (Y. Huang).

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brown fat. Cold microbiota transplantation also reduces fat accumulation and increases energy consumption [18].

Through literature review, GP are believed to promote fat metabolism, which is closely linked to intestinal microbiota. Based on these findings, we proposed that GP may be beneficial to NALFD treatment through improving intestinal microbiota, which was specifically explored in this paper.

2. Materials and methods

2.1. Materials and diet

GP (purity > 80%, determined by HPLC) were offered by Shanxi Baicaocui Biological Technology, 190306 (Supplement Fig. 1). HFHC diet was provided by Research Diets Inc, D12079B and fructose was from Merck Life Science (Shanghai) Co. Ltd, F0127.

2.2. Animals and groups

Twenty-five six-week-old C57BL/6 J mice were provided by Xiamen University Experimental Animal Center (18070480730001). Animal procedures were examined by Medical Ethics Committee of Xiamen University. Every 5 mice were housed in one polycarbonate cage in an environment of 25 ± 1 °C, 40–60% humidity and 12 h/12 h dark/light cycle. After one-week habituation, 5 mice were fed normal diet and water, and the remaining were fed HFHC diet and fructose water (0.02 g/ml) for 12 weeks to induce the in vivo NAFLD model [19]. Afterwards, these 20 mice were assigned into two groups with HFHC diet and GP-containing water (300 mg/kg) or normal drinking water for 6 weeks. All diets were preserved at 4°C and replaced every three days. Body weight of each mouse was weekly recorded. At last, mice were fasting for 12 h and anesthetized by 7% chloral hydrate (0.05 mL/10 g). Blood, abdominal fat, liver and intestinal contents of mice were collected.

2.3. Insulin tolerance test (ITT) and intra-peritoneal glucose tolerance test (IPGTT)

At the 18th week of animal procedures, mice were fasting for 6 h and intraperitoneally injected with 0.75 unit/kg insulin (Eli Lilly) for performing ITT. On the next day, mice were fasting for 18 h and intraperitoneally injected with 2.0 g/kg glucose (Sigma-Aldrich) for performing IPGTT. Glucose levels were determined at 0, 15, 30, 60 and 120 h after injection, respectively, using a portable blood glucose meter (ACCU-CHEK Performa, Roche Diabetes Care, Germany, 79527612734). Glucose concentration - time point plots were depicted to calculate AUC (area under curves) of ITT and IPGTT [20].

2.4. Determination of AST, ALT, TG and HOMA-IR in serum

Blood samples were collected from mouse eyeballs and placed at room temperature (RT) for 1 h. After centrifugation at 3500 rpm for 10 min, serum samples were collected for determination of TG, ALT and AST levels using commercial kits (Nanjing Jiancheng Bioengineering Institute, A110-1, Solarbio Science & Technology Co., Ltd, Beijing, BC1555 and BC1565). Plasma insulin level was determined by ELISA (ALPCO, 80-INSMSU-E01). HOMA-IR was finally calculated based on the following formula: HOMA-IR = Fasting insulin concentration (mU/L) × fasting blood sugar (FBS, mmol/L) / 22.5 [21].

2.5. Histopathological and Oil-Red-O examination of liver tissues

Mouse hepatic lobules were immersed in 4% paraformaldehyde, paraffin embedded, sliced and dehydrated. Sections were subjected to H &E staining and observed for steatosis under a microscope (magnification 200×). Steatotic liver tissues were pathologically examined for evaluating their histopathological grade.

For Oil-Red-O examination, liver tissues were immersed in 4% paraformaldehyde for 10 min, washed with PBS for three times and immersed in 60% isopropanol for 10 s. Subsequently, tissues were dyed with 0.3% oil-red solution for 1 min, washed in 60% isopropanol and PBS, and finally observed under a microscope (magnification 200×).

2.6. RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA in mouse liver tissues was extracted using TRIzol reagent (Life Technologies, CA, USA) and the concentration and qualification were measured on NanoDrop 2000 Spectrophotometer. Qualified RNA was reversely transcribed into cDNA using the PrimeScript™RT reagent Kit with gDNA Eraser (Takara, Tokyo, Japan) and amplified using TB Green™ Premix Ex Taq™ (Takara, Tokyo, Japan). Relative levels were calculated using $2^{-\Delta\Delta CT}$ method. Prime sequences were listed in Supplement Table 1.

2.7. Immunohistochemistry (IHC)

IHC examinations were carried out on 3 mm thick sections. Primary antibodies for IHC were unmasked in 10 mM sodium citrate buffer (pH 6.0) at 90 °C for 30 min. Sections were immersed in 0.03% hydrogen peroxide for 10 min at RT to inactivate endogenous peroxidase. They were immersed in blocking serum [PBS containing 0.04% bovine serum albumin (A2153, Sigma-Aldrich, Shanghai, China) and 0.5% normal goat serum (X0907, Dako Corporation, Carpinteria, CA, USA)] for 30 min at RT. Subsequently, sections were reacted with primary antibodies [anti-CD36, anti-PPARγ, anti-SOD and anti-ACC1 (18836-1-AP, Proteintech, USA)] overnight at 4 °C. Sections were then washed three times for 5 min in PBS. Non-specific was antibodies were blocked with 0.5% casein and 5% normal serum for 30 min at RT. Finally, IHC staining was developed using diaminobenzidine substrate, and sections were counterstained with hematoxylin.

2.8. 16S rDNA bioinformatics analysis and statistics

The genomic DNA of mouse fecal sample was extracted using a DNA extraction kit (Qiagen, Germantown, MD USA). The extracted DNA was subjected to PCR amplification for V3-V4 variable region of 16S ribosomal RNA gene in bacteria. Primers used for 16S rDNA bioinformatics were as follows: 338F: 5′-ACTCCTACGGGAGGCAGCAG-3′ and 806R: 5′-GGACTACHVGGGTWTCTAAT-3′. Biterminal sequencing was conducted based on the standard protocols on the Illumina MiSeq platform in triplicate. Raw data were submitted to the SILVA (SSU and LSU databases 128) database. OTU (Operational Taxonomic Units) analysis under 97% similar level, multivariate analysis and significant test were performed [22].

2.9. Statistical processing

SPSS 18.0 was used for data analyses and GraphPad Prism 8.0 was used for figure editing. Data were expressed as mean ± standard deviation. Differences between two groups were analyzed by variance analysis. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. GP alleviate body weight, liver weight and improve IR in NAFLD mice

Mouse body weight was weekly recorded and liver tissues were harvested and weighed after sacrifice. After the 18-week experimental period, changes in body weight of each group were depicted (Fig. 1A). Mice in HFHC group started to gain body weight at the 4th week, which was markedly heavier at the 18th week relative to that in ND group (Fig. 1B). Body weight in GP group was reduced relative to HFHC group

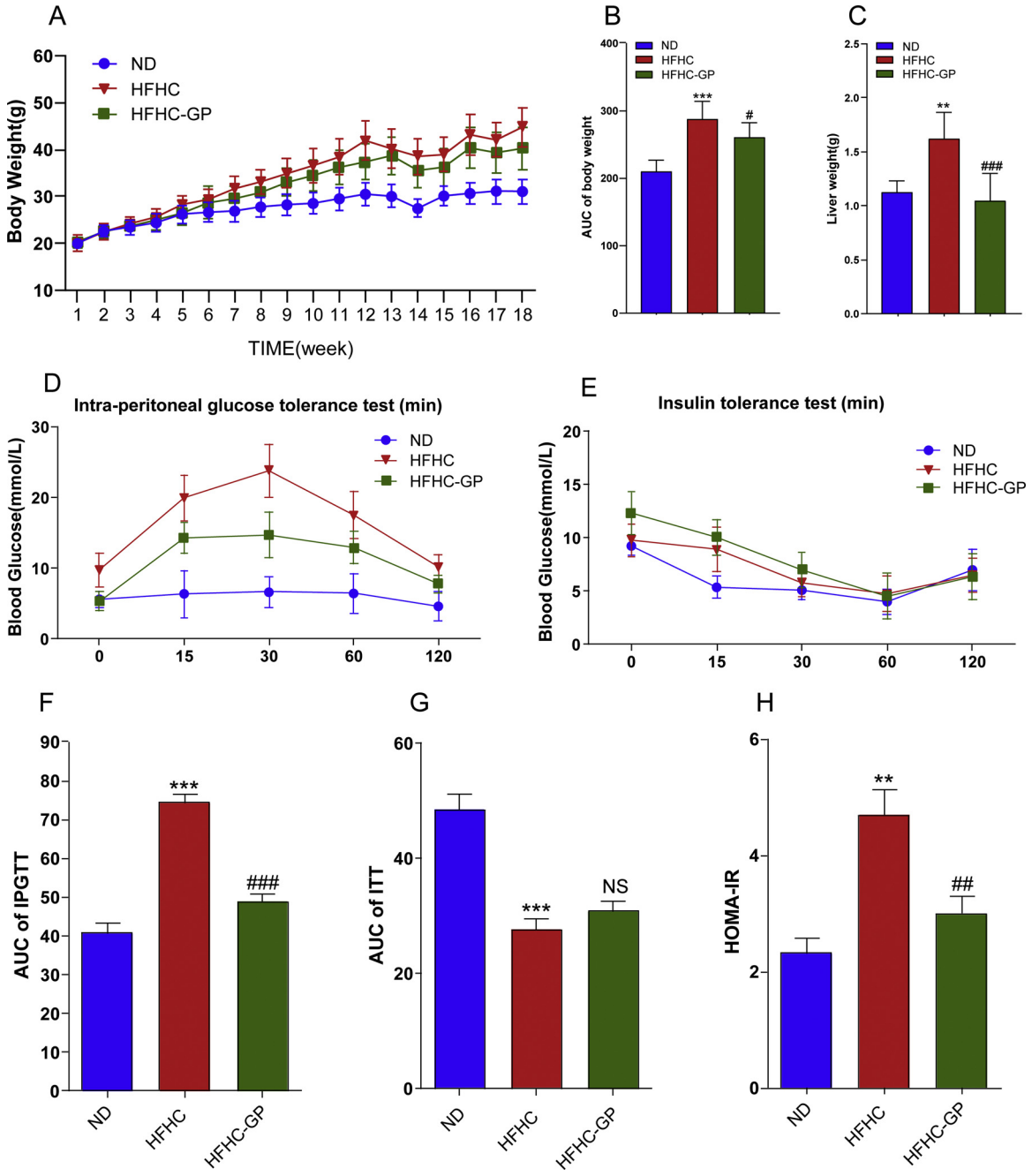

Fig. 1. The effect of GP on body weight, glucose tolerance and insulin sensitivity. A. Body weight change of mice. B. Quantification of the area under the curve (AUC) from the body weight. C. Liver weight of mice. D. Effect of GP on the glucose tolerance was determined by intraperitoneal glucose tolerance test (IPGTT). E. Effect of GP on the glucose tolerance was determined by insulin tolerance test (ITT). F. Quantification of the area under the curve (AUC) from the IPGTT. G. Quantification of the area under the curve (AUC) from the ITT. H. Homeostasis model assessment-insulin resistance (HOMA-IR). Statistical analyses were conducted with one-way ANOVA. *P < 0.05, ***P < 0.001 versus ND; ##P < 0.01, ###P < 0.001 versus HFHC.

(Fig. 1B). Mouse liver weight was larger in HFHC group than ND group and GP group (Fig. 1C). To assess the influence of GP on NAFLD-induced changes in glucose levels, FBS was determined at five time points. FBS in GP group was similar to that in ND group, and markedly lower than that in HFHC group (Fig. 1F). However, ITT was similar among each group (Fig. 1G). HFHC diet enhanced HOMA-IR in mice, which was reduced by GP treatment (Fig. 1H). The above data demonstrated the ability of GP to reduce NAFLD-induced body weight and liver weight gain, as well as to enhance insulin sensitivity.

3.2. GP alleviate hyperlipidemia in NALFD mice

HFHC feeding markedly enhanced serum levels of ALT, AST and TG in mice, and were further reversed to the normal levels by GP treatment (Fig. 2A–C). Hence, GP were capable of alleviating NALFD-induced hyperlipidemia.

3.3. GP alleviate HFHC feeding-induced pathological lesions in mouse liver and intestines

H&E and Oil-Red-O staining revealed severe steatohepatitis and neutrophil inflammatory aggregation in mouse liver tissues of HFHC

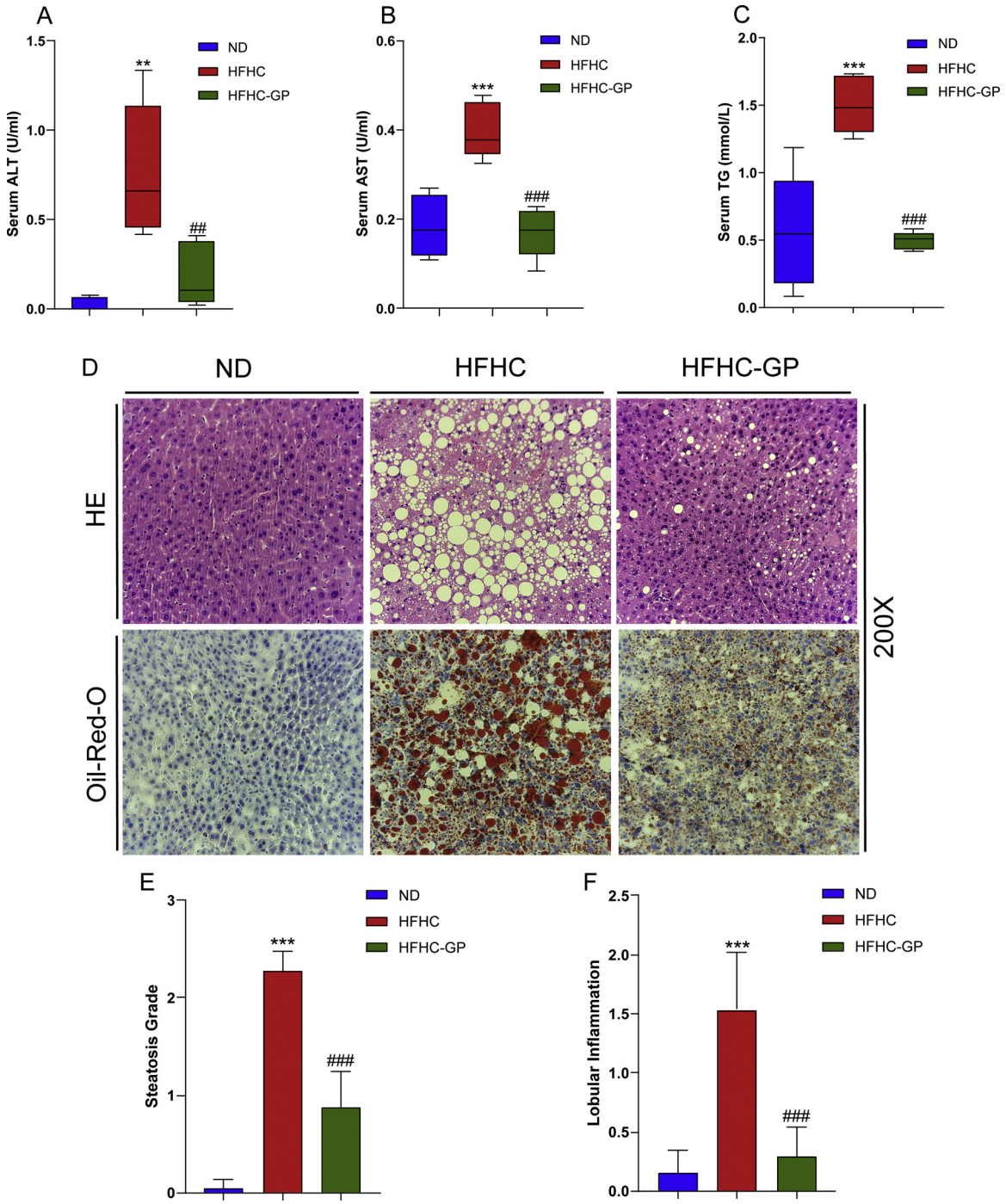

Fig. 2. The effect of GP on serum levels of ALT, AST, TG and liver pathological lesions. The effect of GP on serum ALT (A), AST (B) and TG (C). Representative images of liver section stained with H&E (magnification: 200×) and Oil-red-O (magnification: 200×) (D). Liver steatosis grade examined in H&E (E), and liver lobular inflammation examined in Oil-red-O. $^{**}P < 0.01$, $^{***}P < 0.001$ versus ND; $\#\#P < 0.01$, $\#\#\#P < 0.001$ versus HFHC.

group (Fig. 2D). GP treatment markedly alleviated these pathological lesions (Fig. 2E, F). In addition, GP improved colonic lymphitis caused by HFHC diet (Supplement Fig. 2).

3.4. GP downregulate key genes related to lipid metabolism

QRT-PCR and IHC were conducted to determine the influence of GP on expressions of key genes associated with lipid metabolism. The mRNA levels of ACC1, PPARγ, CD36, APOC3 and MTTP were markedly upregulated after HFHC feeding, while ACC2 level remained unchangeable. GP treatment reversed the upregulated lipid metabolism-associated genes (Fig. 3A). IHC also revealed the same trends that GP treatment reduced positive expressions of CD36, ACC1, PPARγ and SOD1 in mice of HFHC group (Fig. 3B).

3.5. GP enhance intestinal microbiota diversity in NAFLD mice

The α diversity is reflected by the Shannon index [23]. In the Shannon dilution curve, the curve of each sample tended to be smooth, indicating that the sequencing depth was sufficient to reveal the biodiversity in the sample (Fig. 4A). Venn diagrams depicted much more diversities in ND group than those in HFHC and GP group, which was

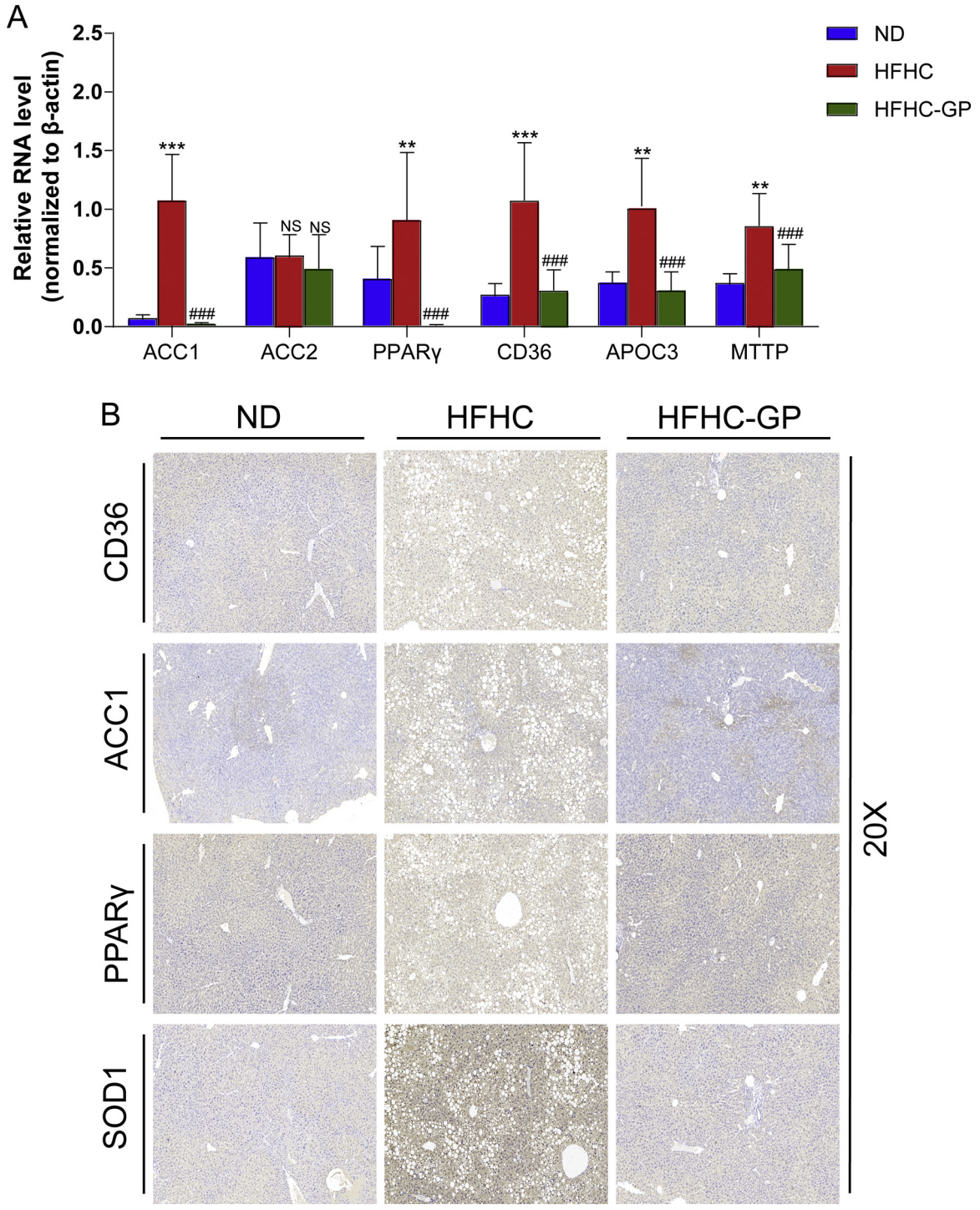

Fig. 3. Expressions of lipid metabolites in liver tissues. The mRNA expressions of lipogenesis-related genes including ACC1, ACC2, PPARγ, CD36, APOC3 and MTTP normalized to β-actin (A). Protein levels of CD36, ACC1, PPARγ and SOD1 were verified by IHC in liver tissues (B). $^{**}P < 0.01$, $^{***}P < 0.001$ versus ND; $^{###}P < 0.001$ versus HFHC.

the lowest in HFHC group (Fig. 4B). Unweighted UniFrac principal coordinates analysis (PCoA) illustrated a significant difference in the distribution of intestinal microbiota among the three groups. Particularly, intestinal microbiota was much more abundant in GP group than that in HFHC group, and was similar to that in ND group (Fig. 4C).

3.6. GP alleviate HFHC-induced intestinal microbiota disorder

Firmicutes and Bacteroidetes have relative abundances of approximately 90%, presenting absolute advantage in all intestinal microbiota (Fig. 4D). The ratio of Firmicutes / Bacteroidetes has been considered to be a key indicator reflecting microbiota changes. It is positively correlated with obesity and other metabolic diseases [24,25].In our experiment, the abundance of Firmicutes was similar among the three group. However, the abundance of Bacteroidetes was much lower in HFHC group relative to ND (Fig. 4E) and GP group (Fig. 4F). The ratio of Firmicutes / Bacteroidetes markedly increased in HFHC group, whereas it decreased in GP group (Fig. 4G). Long-term

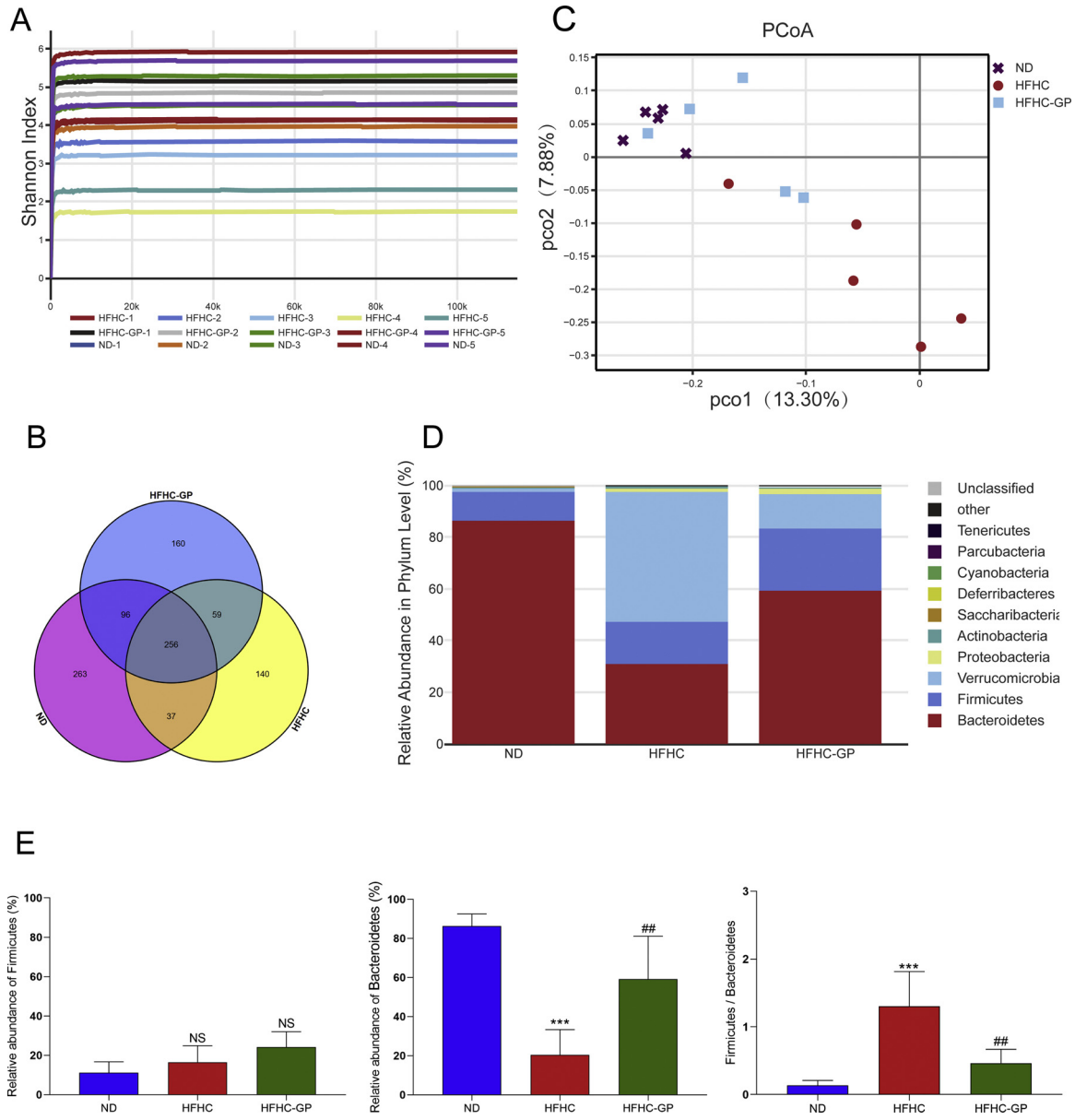

Fig. 4. The effect of GP on diversity and abundance of gut microbiota. Shannon index value indicated the diversity of the samples (A); OTU Venn analysis of samples of different groups (B); PCoA of the between samples distances (beta diversity) computed using unweighted unifrac distance (C). Relative abundance analysis in phylum level (D). Relative abundance of Bacteroidetes and Firmicutes at the phylum level (E). $***P < 0.001$ versus ND; $##P < 0.01$ versus HFHC.

HFHC diet resulted in the dysregulated Firmicutes / Bacteroidetes, which was improved by GP treatment.

Subsequently, the heatmap uncovered abundances of 52 key flora at genus level (Fig. 5A). Totally, three kinds of flora exhibited pronounced differences, namely Rikenellaceae_RC9_gut_group, Fissicatena and Akkermansia. In particular, the latter two showed higher abundances in HFHC group, which were reversed in GP group (Fig. 5B, C). Moreover, HFHC feeding enhanced the abundance of Rikenellaceae in mouse intestine, while no significant difference was discovered (Supplement Fig. 3). The above results indicated that GP can restore HFHC-induced dysbiosis of the intestinal microbiota and maintain the intestinal health. To compare the differences in metabolic pathways between the HFHC and GP groups, KEGG analysis was performed. Significant differences in metabolic pathways such as energy metabolism, glycan biosynthesis and metabolism were seen between GP and HFHC group (Fig. 5D).

4. Discussion

Hepatic steatosis is not only a prerequisite for the development of NAFLD, but also a key event involving in metabolic dysfunction. Therefore, improvement of steatosis and lipid metabolism disorder are priorities in the treatment course. Effective drugs targeting steatosis are lacked currently [26,27]. GP, as TCM, contain a variety of biologically active ingredients. Among them, saponins can reduce steatohepatitis by autophagy regulation [28] and carbon tetrachloride (ccl4)-induced liver fibrosis in rats [29]. Flavone can induce hepatocyte death [30]. Polysaccharide is one of the main components of GP with anti-oxidation capacity, which is able to regulate immune activity and alleviate liver damage [31]. Hence, GP are believed to alleviate NAFLD. In this paper, GP effectively reduced body weight and improved steatohepatitis in NAFLD mice. In addition, IR and decreased serum levels of AST, ALT and TG in NAFLD mice were alleviated by GP treatment. Moreover, GP could remarkably improve pathological lesions in liver tissues owing to HFHC diet (Fig. 6).

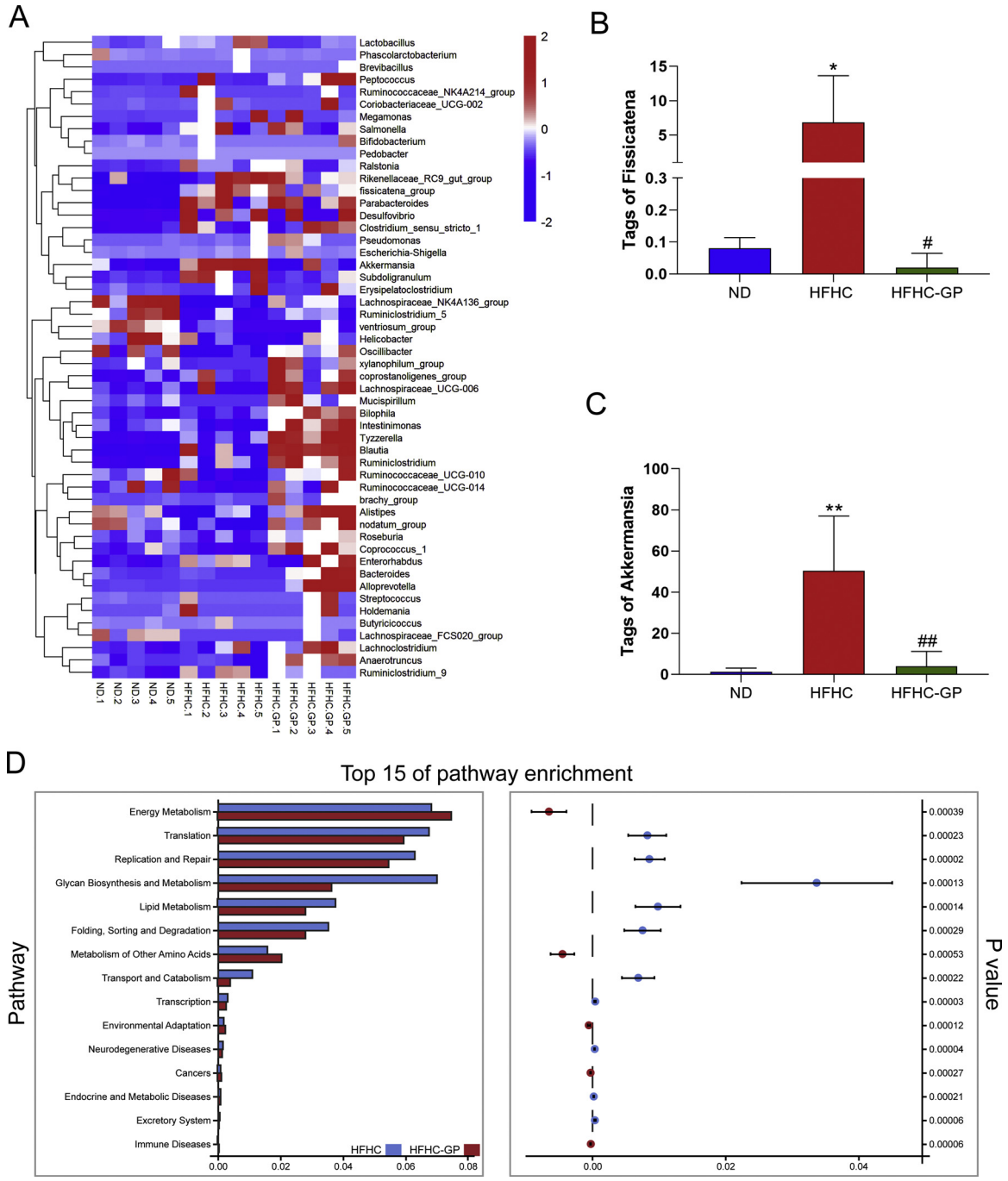

Fig. 5. Intestinal microbiota analysis at the genus level. Heatmap of the abundance of 52 key species in response to GP treatment (A). Relative abundance of Fissicatena (B) and Akkermansia (C) in the ND, HFHC and HFHC-GP groups. Pathway enrichment analysis of the HFHC and HFHC-GP groups (D). The y-axis indicated differential pathways, and x-axis indicated the abundance of differential pathway. In the right part, the y-axis was *P* value and x-axis was CI of pathway abundance. $*P < 0.05$, $**P < 0.01$ versus ND; $\#P < 0.05$, $\#\#P < 0.01$ versus HFHC.

For de novo lipogenesis (DNL), ACC1 and ACC2 are two crucial enzymes participating in this process. ACC1, a cytosolic enzyme, participates in the rate-limiting step in DNL. ACC2, a mitochondrial membrane associated enzyme, could generate malonyl-CoA primarily, and inhibit CPT-1 and transports long chain fatty acid transport into mitochondria [32,33]. Therefore, ACC can increase liver fat deposition and lead to steatohepatitis. Fatty acids can be oxidized in mitochondria and peroxisomes. SOD activates the antioxidant pathway to cause excess ROS, leading to increase in mitochondria and peroxidase β-oxidation [34]. High levels of free fatty acid (FFA) activate PPARγ via the liver X receptor and pregnane X receptor, which in turn upregulate CD36 level in hepatocytes [35]. Meanwhile, the liver esterifies the ingested FFA into triacylglycerol, and the latter forms VLDL and releases it into blood. VLDL transports the lipid from liver to peripheral tissues, where MTTP contributes triglycerides to synthesize chylomicrons. APOC3 is an important component of VLDL, and both MTTP and APOC3 activation lead to lipid deposition [36–38]. Our findings showed upregulation of ACC1, PPARγ, CD36, MTTP and APOC3 in mice of HFHC group, which were reversed by GP treatment. IHC analysis further confirmed their positive expressions in mouse liver tissues. Therefore, GP could alleviate NALFD-induced pathological state of NAFLD through regulating key genes associated with liver lipid

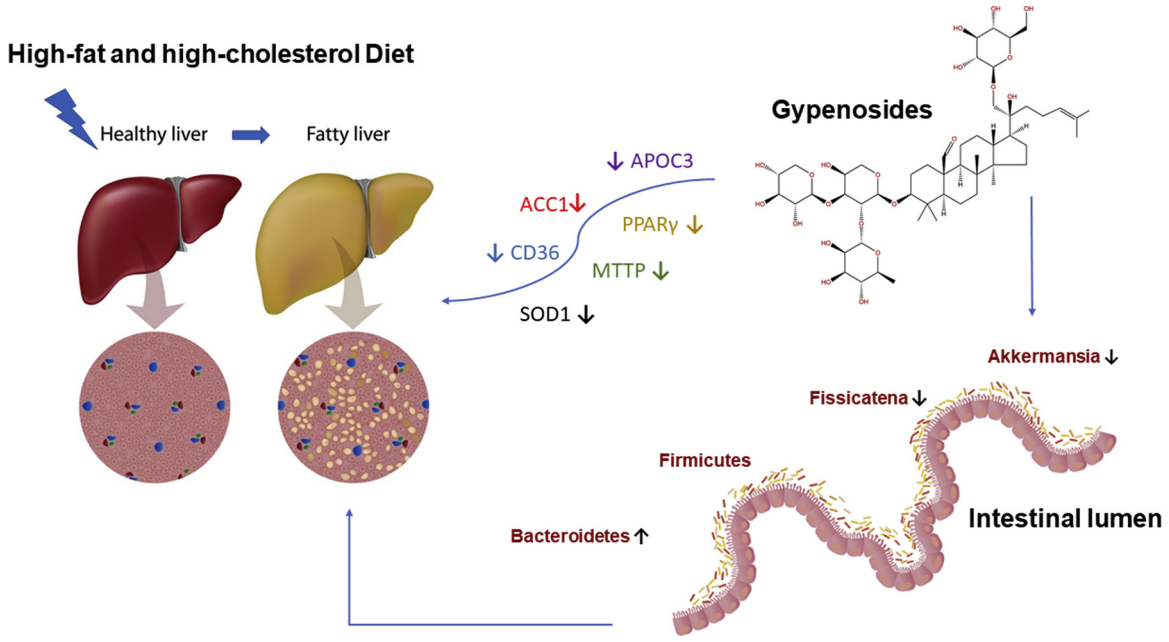

Fig. 6. Schematic representation of the proposed mechanism of Gypenosides in NAFLD.
HFHC was used to induce the model of the NAFLD in mice. Gypenosides reduced HFHC-induced secretion of hepatic lipid factors ACC1, PPARγ, CD36, APOC3 and MTTP expression levels. Gypenosides decreased relative abundance of Fissicatena and Akkermansia, but *increased* relative abundance of Bacteroidetes.

metabolism.

Abundant microorganisms are present in intestines, exhibiting fundamental roles in the body [39]. It is reported that intestinal microbial disorders are one of the important factors leading to obesity [40]. In this paper, 16 s rDNA analysis revealed that the bacterial biodiversity of mice in HFHC group was markedly lower than that of the ND group, and it was higher in GP group than that of HFHC group. No significant changes in the composition of intestinal microbiota were identified among the three groups. Bacteroides and Firmicutes are the most abundant microbial species in the host microbiota. Bacteroides is involved in the absorption and degradation of host polysaccharides, exerting a dominant role in the intestinal ecosystem [41]. A recent study showed that the ratio of Firmicutes / Bacteroidetes is elevated in intestines of obese adults and children [42]. Our findings uncovered that GP treatment reduced relative abundance ratio of Firmicutes / Bacteroidetes in the feces of NAFLD mice.

Akkermansia is ascertained as a human intestinal mucin-degrading bacterium, accounting for 3–5% of the microbial population in healthy humans [43]. A relevant study found that the abundance of Akkermansia is negatively correlated with body weight and obesity in mice and humans [44]. A clinical study has shown that Fissicatena is highly abundant in the intestinal tract of cirrhosis patients and positively correlated with the production of SCFAs [45]. Our results illustrated the promotive effects of GP on abundances of Akkermansia and Fissicatena in intestines of NAFLD mice. In addition, KEGG enrichment analysis showed that intestinal microbiota in GP-fed mice were mainly enriched in metabolic pathways, including energy metabolism, glycan biosynthesis and metabolism. The above studies showed that GP can significantly improve intestinal microbiota, thereby alleviating the progression of NAFLD.

5. Conclusions

GP alleviate NAFLD-induced body weight gain, IR and upregulated key genes associated with lipid metabolism. Besides, GP improve NAFLD through enhancing the abundance of intestinal microbiota.

Authorship

Yuanpeng Huang, Hongzhi Xu designed and supervised the project. Xiaoqing Huang, Wenfan Chen, Renzhi Yang and Qiongyun Chen performed the experiments, collected and analyzed the data. Xiaoqing Huang and Changsheng Yan wrote the paper.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2019.109258.

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