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Pea albumin extracted from pea (*Pisum sativum* L.) seed protects mice from high fat diet-induced obesity by modulating lipid metabolism and gut microbiota

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ABSTRACT

Plant protein has been reported to play a key role in the prevention of obesity and associated complications. It is unknown whether pea albumin may exert anit-obesity and obesity-associated metabolic disorders alleviation. Pea albumin was isolated from pea seed (*Pisum sativum* L.) and determined its functional role for anti-obesity and metabolic disorders alleviation in high fat diet (HFD)-fed mice. Pea albumin administration reduced body weight gain, improved glucose tolerance and insulin sensitivity, reduced inflammatory cytokines secretion, and alleviated hepatic steatosis in HFD-fed mice. Interestingly, pea albumin inhibited lipid accumulation in 3T3-L1 cells *in vitro* and modulated lipid metabolism by upregulating critical proteins implicated in lipolysis and fatty acid oxidation, and downregulating lipogenesis *in vivo*. Moreover, pea albumin restored gut microbial composition to normal fat diet condition and selectively promoted the growth of beneficial gut bacteria (*Akkermansia*, *Parabacteroides* etc.). Collectively, the data demonstrated that pea albumin protects mice from HFD-induced obesity and associated metabolic disorders.

1. Introduction

Obesity is increasing across the globe and has become one of most prevalent public health problem in the past decade (Muller, Bluher, Tschop, & DiMarchi, 2022; Tian et al., 2021). Obesity, a metabolic disorder caused by a variety of factors (unhealthy lifestyles, individual genetic variants and energy-balance dysregulation), is associated with substantial health risks, such as insulin resistance, type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD), and cardiovascular disease, hypertension, cancers and so on (Hajer, van Haeften, & Visseren, 2008; Lim, Jung, Joo Suh, Choi, & Kim, 2022), which are leading causes of morbidity and mortality worldwide (Hajer et al., 2008). Thus, global obesity prevention is an urgent public health and has been advocated for decades (Chan & Woo, 2010; W. Wang et al., 2019). Although several drugs have been used to fight obesity and overweight, the number of obesity and obesity-associated comorbidities continue to grow. Additionally, anti-obesity medications may cause serious side like anxiety, depression, suicide and cardiovascular risk, etc (Rodgers, Tschop, &

Abbreviations: AA, amino acids; ALT, alanine aminotransferase; As, arsenic; AST, aspartate aminotransferase; ATGL, adipose triglyceride lipase; BCA, bicinchoninic acid protein; BW, body weight; Cd, cadmium; C/EBPα, CCAAT/enhancer binding protein α; Cr, chromium; Cyto C, cytochrome *C*; ELSIA, enzyme linked immunosorbent assay; epiWAT, epididymal WAT; FASN, fatty acid synthase; FBG, fasted blood glucose; FBS, fetal bovine serum; NEFA, nonestesterified fatty acid; FXR, farnesoid X receptor; HDL-C, high density lipoprotein cholesterol; HE, hematoxylin and eosin; HFD, high fat diet; Hg, mercury; HPLC, high-performance liquid chromatography; HSL, hormone-sensitive lipase; 3- IBMX, isobutyl-methylxanthine; ingWAT, inguinal WAT; IRS1, insulin receptor substrate 1; ITT, insulin tolerance test; LDA, linear discriminant analysis; LDL-C, low density lipoprotein cholesterol; LEfSe, linear discriminant analysis effect size; mesWAT, mesenteric WAT; NAFLD, non-alcoholic fatty liver disease; ND, normal-fat diet; NMR, nuclear magnetic resonance; OGTT, oral glucose tolerance test; OTUs, operational taxonomic units; P, pea albumin; Pb, lead; PBS, phosphate balanced solution; PCA, Principal component analysis; PPARγ, peroxisome proliferators-activated receptor γ; PVDF, polyvinylidene fluoride membranes; SCD1, steroyl-CoA-desaturase-1; T2D, type 2 diabetes; T-CHO, total cholesterol; TG, triglycerides; WAT, white adipose tissues.

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Wilding, 2012), resulting in a problem in continuous administration (Bessesen & Van Gaal, 2018; Muller et al., 2022). Accordingly, new strategies for the treatment of obesity and associated metabolic disorders are greatly needed (Shi & Burn, 2004). Recently, plant bioactive protein have been considered as one of safe strategies for obesity prevention and obesity-associated metabolic disorders alleviation (Lee et al., 2016).

A large amount of evidence has implicated that high fat diet (HFD) consumption leads to gut microbiota dysbiosis which contribute to the onset and development of obesity and obesity-associated chronic disease (Cani et al., 2019; Miyamoto et al., 2019; Murphy, Velazquez, & Herbert, 2015; Turnbaugh, Backhed, Fulton, & Gordon, 2008; Turnbaugh et al., 2009). Strategies that aim at restoring gut microbiota in obese individuals have been proposed as interventions for prevention and treatment of obesity and associated metabolic disorders (Miyamoto et al., 2019; Tian et al., 2021). *Akkermansia*, commensal bacteria belonging to the *Verrucomicrobia* phylum which inhabits gastrointestinal tract, has been proved to reverse HFD-induced obesity, diabetes, insulin resistance, and other cardio-metabolic disorders in mice and humans (Ansaldo et al., 2019; Cani & de Vos, 2017; Dao et al., 2016; Everard et al., 2013). Furthermore, a recent study revealed that *Parabacteroides goldsteinii* could be used to prevent and treat obesity and associated metabolic disorders in mice (Wu et al., 2019). Additionally, *Parabacteroides distasonis* has been shown to alleviate obesity and associated dysfunctions in mice via activating farnesoid X receptor (FXR) signaling by secondary bile acid, and activating intestinal gluconeogenesis by succinate (K. Wang et al., 2019). Of note, bioactive extracts protect from HFD-induced obesity, insulin resistance and intestinal inflammation in association with enhanced abundance of *Akkermansia* or *Parabacteroides* in gut (Dao et al., 2016; Do et al., 2021; S. Li, Wang, Liu, & He, 2020; Sarma et al., 2017; H. Wang et al., 2018; Wu et al., 2019). The above statements indicate that bioactive extracts may be used to prevent and treat obesity and obesity-associated disease through modulating composition and functions of gut microbiota.

Accumulating evidence from epidemiologic and intervention trials indicate that consumption of some plant proteins may exert antioxidant, antihypertensive, anti-inflammatory effects, induce muscle thickness promotion, alleviate lipid metabolism disorders, lower cholesterol, and modulate intestinal bacteria composition (Ge et al., 2020; Lee et al., 2016; Lu, He, Zhang, & Bing, 2020; Morita et al., 1997; Rigamonti et al., 2010; Tong et al., 2021). Recently, pea protein with a well-balanced amino acids (AA) profile is being consumed widely due to its wide biological and pharmacological activities including colitis alleviation, T2D prevention, cardiovascular diseases prevention, anticarcinogenic, antihypertensive, hypocholesterolemic, muscle thickness promotion, and gut microbiota modulation (Aranda-Olmedo, Ruiz, Peinado, & Rubio, 2017; Babault et al., 2015; Duranti, 2006; Leterme, 2002; Utrilla et al., 2015). Pea albumin as one of four major groups of pea protein (globulin, albumin, prolamin, and glutelin) accounts for approximately 18–25% of total pea protein and is rich in sulfur containing AA and other essential AA (Croy, Hoque, Gatehouse, & Boulter, 1984; Ge et al., 2020; Lu et al., 2020; Rubio et al., 2014). Pea albumin have been claimed to exert potential health benefits like colitis alleviation, anticarcinogenic, antihypertensive, hypoglycemic, hypocholesterolemic, etc (Babault et al., 2015; Duranti, 2006; Leterme, 2002; Utrilla et al., 2015). In this regard, we hypothesized that pea albumin could have a positive effect on all these functions and be beneficial for obesity prevention.

To date, despite beneficial effects of pea protein on treatment of obesity and associated metabolic disease have been demonstrated. However, beneficial effects of pea albumin on anti-obesity and obesity-associated disease alleviation have not been examined and underlying mechanisms are largely unknown. In the present study, we isolated pea albumin from pea seed (*Pisum sativum* L.) to determine effects of pea albumin on body weight (BW), fat mass, inflammatory cytokines, glucose homeostasis, insulin sensitivity, lipid metabolism in adipose tissue, and gut microbiota composition.

2. Materials and methods

2.1. Reagents

Phosphate balanced solution (PBS, #10010-001) were purchased from Gibco (Rockville, MD, USA). Primary antibodies against fatty acid synthase (FASN, #3189S), adipose triglyceride lipase (ATGL, #2138S), hormone-sensitive lipase (HSL, #4107S), cytochrome *C* (Cycto C, #11940S), CCAAT/enhancer binding protein α (C/EBPα, #8178S), and peroxisome proliferators-activated receptor γ (PPARγ, #2435) were obtained from Cell Signaling Technology (Danvers, MA, USA). Primary antibodies against insulin receptor substrate 1 (IRS1, AF7299), phosphor (p)-IRS1 (Ser302, AI621) and p-IRS1 (Ser307, AF5845) were purchased from Beyotime Biotechnology (Haimen, China). Antibody against β-actin (sc-47778) was purchased from Santa Cruz Biotechnology (San Diego, CA, USA). Peroxidase-conjugated secondary antibodies against rabbit and mouse and enhanced chemiluminescence kit were obtained from Huaxingbio Biotechnology Co. (Beijing, China). The bicinchoninic acid protein (BCA) assay kit was purchased from Huaxingbio Biotechnology Co. (Beijing, China). Insulin (128-100) was obtained from Cell Applications (San Diego, CA, USA). Enzyme linked immunosorbent assay (ELSIA) kits for Serum IL-1α (KET7012) and TNF-α (KET7012) were purchased from Abbkine Scientific Co., Ltd. (Wuhan, China). Unless stated, all other chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA).

2.2. Animals and diets

All the animal experimental procedures were approved by the Institutional Animal Care and Use Committee of China Agricultural University (AW82102202-5-1). Four-week-old C57BL/6 male mice were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China) and raised in a controlled environment (24 ± 2 °C, 50 ± 5% humidity, 12 h dark-light cycle) with free access to food and drinking water. After a 1-wk adaptation period, mice were fed a normal fat diet (ND, 10% energy from fat, D12450J, Research diet, New Brunswick, USA) or a HFD (60% energy from fat, D12492, Research diet, New Brunswick, USA) and randomly divided into one of six groups: ND group mice (n = 9) were fed a ND and received PBS by oral gavage daily; ND_P group mice (n = 9) were fed a ND and received 1.5 g/kg BW pea albumin by oral gavage daily; HFD fed mice respectively received an oral gavage of an equivalent volume of sterile PBS (HFD group), 0.375 (HFD_P1 group), 0.75 (HFD_P2 group) or 1.50 (HFD_P3 group) g/kg BW pea albumin. Detailed program of pea albumin administration throughout the experiment period is presented in **Figure S1A**. BW and food intake were measured once-weekly through an 8-week trial. The composition of the diets is illustrated in **Table S1**. Body change curve (% of initial BW) and cumulative food intake were calculated.

2.3. Cell culture and treatment

The 3T3-L1 cells were maintained in DMEM with 10% fetal bovine serum (FBS) and were induced to differentiate into adipocytes as previously described (Chen et al., 2018). Briefly, cells were cultured in induction medium containing 10 μg/mL insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-methylxanthine (IBMX) for 2 days after reaching 100% confluence. The induction medium was replaced with differentiation medium containing 10 μg/mL insulin for 2 days. Thereafter, differentiated cells were cultured in a medium containing 10% FBS for another 3 days. Pea albumin at dose of 100, 200, 400, 800 or 1600 μg/mL were added during the whole differentiation period.

2.4. Preparation and composition analysis of pea albumin

Pea seed (*Pisum sativum* L.) was provided by Yantai Shuangta Food Co., Ltd. (Yantai, Shandong, China). The pea albumin was prepared

according to previous description (Rubio et al., 2014). Pea albumin were characterized by SDS-PAGE (**Figure S1B**) to monitor the electrophoretic pattern flowing the previous method (Mudgil, Kamal, Yuen, & Maqsood, 2018). Composition analysis of pea albumin was carried out by using diverse analytical methods listed in **Table S2**.

2.5. Body composition measures

At week 8, mice placed in a restraint cylinder were performed to measure fat mass by using nuclear magnetic resonance (NMR) analyzer (MesoQMR23-060H, NIUMAG, Suzhou, China). NMR analysis was used to assess absolute fat mass and lean mass based on total BW, and data were used to determine percentage of fat mass (fat mass/total body mass × 100).

2.6. Lee index

Mice were weighted, and anal-tonasal distance were measured using ruler to calculate the Lee index as an indirect reflection of body composition according to previous described (Sun et al., 2021).

2.7. Oral glucose tolerance test

Oral glucose tolerance test (OGTT) was performed after overnight fasting 1 week before the end of the experiment as previously described (B. Wang et al., 2021). Mice in each group received a solution of 50% glucose load (2 g/kg BW) by oral gavage (K. Wang et al., 2019). Blood collected from the tail vein was used to measure blood glucose concentration at 0, 30, 60, 90 and 120 min after oral glucose administration by using a One Touch glucometer (Accu-Chek; Roche, Meylan, France).

2.8. Insulin tolerance test

Insulin tolerance test (ITT) was performed as an index of insulin sensitivity by intravenous injection of insulin (0.8 U/kg BW) in mice after 4 h food withdrawal as previously described (Carper, Coue, Laurens, Langin, & Moro, 2020). Blood was collected from tip of the tail vein and analyzed blood glucose levels using a One Touch glucometer (Accu-Chek; Roche, Meylan, France) at 0, 30, 60, 90 and 120 min after injection of insulin.

2.9. Serum biochemical analysis

At end of experiment, blood was collected from the retro-orbital sinus of each mouse. Serum was assayed for triglycerides (TG, A110-1-1), total cholesterol (T-CHO, A111-1-1), nonestesterified fatty acid (NEFA, A042-2-1), high density lipoprotein cholesterol (HDL-C, A112-1-1), low density lipoprotein cholesterol (LDL-C, A113-1-1), alanine aminotransferase (ALT, C009-2-1) and aspartate aminotransferase (AST, C010-2-1) using kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) as previous described (Chen et al., 2021). Levels of serum leptin (PL700) and insulin (PI608) levels were measured using commercially available kits from Beyotime Biotechnology (Haimen, China) according to the manufacturer's instructions. TG content of mature adipocytes was measured using the same kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.10. Detection of IL-1α and TNF-α

Serum IL-1α and TNF-α were detected using ELISA kits according to the manufacturer's instructions.

2.11. Tissue sampling

After mice were euthanized with CO_2, liver, spleen, kidney, white adipose tissues (WAT) [epididymal WAT (epiWAT), inguinal WAT (ingWAT) and mesenteric WAT (mesWAT)] were precisely dissected, collected, weighted, immersed in liquid nitrogen, and then stored at −80 °C for further analysis. Additionally, samples of liver, epiWAT, and ingWAT were either immediately resected and fixed in 4% paraformalin solution for histopathological examination as previously described (Do et al., 2021).

2.12. Histopathological analyses

Liver, epiWAT, and ingWAT embedded in paraffin were cut into 4 μm thickness sections using a handy microtome (Thermo Fisher 456 Scientific, CA, USA) and further stained with hematoxylin and eosin (HE) by standard methods. The slices were photographed using a fluorescence microscope (Olympus IX50) linked to the NIS-ELEMENTS F3.2 software. Area and diameter of adipocytes from at least six samples per group were analyzed using the Image-pro plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

2.13. Oil red O staining

Cultured cells washed with PBS and then were fixed with 4% paraformalin for 15 min at room temperature. The cells were stained with Oil red O solution for 30 min. The stained cells were viewed with a microscope.

2.14. Hepatic lipid analysis

Cellular architecture and lipid accumulation of liver were examined by HE staining according to the previous method (Chen et al., 2021). The amount TG and T-CHO in liver were determined with the same kits for serum TG and T-CHO analysis.

2.15. Western blot analysis

Total proteins from epiWAT tissue were isolated using RIPA lysis buffer (10 mM Tris-HCl, pH 7.4; 150 mM NaCl; 10 mM EDTA; 1% NP-40; 0.1% SDS; 1.0 mM PMSF; 1.0 mM Na_3VO_4; 1.0 mM NaF) supplemented with protease and phosphatase inhibitors. The concentration of protein was quantified by a BCA protein assay kit (Applygen Technologies). Identical amounts of proteins (50 μg) were separated on SDS-PAGE gels, transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA), and blocked with 5% skim milk for 1 h at room temperature. After incubation with a primary antibody overnight at 4 °C, the membranes were washed with buffer and incubated with an HRP-conjugated secondary antibody at room temperature for 1 h. The protein bands were incubated with an enhanced chemiluminescence kit and visualized using ImageQuant LAS 4000 mini system (GE Healthcare, USA). Band density was measured by using the Image J software (NIH, Bethesda, MD, USA). β-actin was used as a loading control.

2.16. Gut microbial analysis

Gut microbiota DNA was extracted from colon content collected in a sterile environment using FastDNA ® SPIN kit (MP Biomedicals, Solon, USA) according to manufacturer's recommendations. The 16S rDNA V3-V4 region of the bacterial gene was amplified with universal primers 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT). The amplicons products were pooled equal concentration and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, USA) for high-throughput sequencing at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Chimeras were filtered using USEARCH, and the remaining sequences were grouped to generate operational taxonomic units (OTUs). The 16S rRNA gene sequence data were processed using linear discriminant analysis effect size (LEfSe). LEfSe differences among biological groups were tested for significance using a nonparametric factorial Kruskal−Wallis

sum-rank test followed by Wilcoxon rank-sum test. Principal component analysis (PCA) plot was created using the R software based on Bray-Curtis dissimilarity.

2.17. Statistical analysis

All results are presented as means ± SEMs. Multiple comparisons among groups were evaluated using one-way analysis of variance (ANOVA) followed by the Duncan's multiple comparison method with the SAS software, version 9.1 (SAS Institute Inc., Cary, North Carolina, USA). $P < 0.05$ was considered as statistical significance.

3. Results

3.1. Pea albumin reduced HFD-induced obesity and suppressed inflammatory cytokines

To evaluate the metabolic effects of pea albumin on obesity, mice were fed with a ND or HFD with PBS or pea albumin by oral gavage daily for 8 weeks. Compared with mice in ND group, HFD induced ($P < 0.05$) obesity which was characterized by BW changes (Fig. 1**A**), images of mice (Fig. 1**C**) and Lee index (Fig. 1**D**), while pea albumin dose-dependent suppressed ($P < 0.05$) BW gain (Fig. 1**A**) and improved ($P < 0.05$) Lee index (Fig. 1**D**) without dose-dependent effects in HFD-induced mice. The cumulative food intake for 56 days had no differences among HFD fed mice (Fig. 1**B**). Food intake and energy intake relative to BW were lower ($P < 0.05$) in HFD fed mice when compared with ND group and ND_P group (**Table S3**). No differences on food intake and energy intake relative to BW among HFD, HFD_P1, and HFD_P2 groups. Additionally, relative food intake and relative energy intake of ND group and ND_P group mice were higher ($P < 0.05$) than HFD group (**Table S3**). Further analysis on serum lipid profiles demonstrated that HFD resulted in higher ($P < 0.05$) concentration of TG (Fig. 1**E**), T-CHO (Fig. 1**G**), and HDL-C (Fig. 1**H**) compared with mice in ND group, while pea albumin oral administration markedly reduced ($P < 0.05$) the serum levels of TG (Fig. 1**E**) and T-CHO (Fig. 1**G**). Pea albumin (1.5 g/kg BW) oral administration significantly ($P < 0.05$) reduced NEFA concentration in serum when compared with HFD group (Fig. 1**F**). Additionally, no difference ($P > 0.05$) was found among groups on LDL-C concentration in serum (Fig. 1**I**). Of note, no differences were observed between ND group and ND_P group. Generally, pea albumin oral administration had a dose-dependent effect on BW and serum TG concentration. Mice in the HFD group had higher ($P < 0.05$) serum levels of IL1-α and TNF-α relative to the ND and ND_P group (Fig. 1**J, K**). Interestingly, HFD fed mice orally administrated with 0.75 or 1.5 g/kg BW pea albumin significantly suppressed ($P < 0.05$) the increase of IL1-α and TNF-α in serum when compared with HFD group (Fig. 1**J, K**). Taken together, these data indicated that pea albumin alleviated HFD-induced obesity and suppressed inflammatory cytokines in mice, and effects of the high dose were greater than those of the low dose.

3.2. Pea albumin prevented HFD-induced adipocyte hypertrophy

To investigate the effects of pea albumin on adipocyte hypertrophy, the body composition, major WAT, and organs index were assessed. NMR analysis showed that HFD feeding lead to increasement on percentage of fat mass by 88.6% compared to the mice feeding ND (Fig. 2**A**). Consistent with this finding, we observed enlarge and increase ($P < 0.05$) weights (Fig. 2**C, D, and F**) and morphology (Fig. 2**B, E**) of ingWAT, epiWAT and mesWAT. Interestingly, pea albumin effectively ($P < 0.05$) reduced the percentage of fat mass (Fig. 2**A**), weight of epiWAT (Fig. 2**B, C**), weight of ingWAT (Fig. 2**B, D**), and weight of mesWAT (Fig. 2**E, F**) with a dose-dependent effects. Additionally, mice fed HFD had a lower ($P < 0.05$) liver index than mice feeding ND, but no change was observed in HFD fed mice with or without pea albumin oral administration. Furthermore, no differences on kidney and spleen were founded among the six groups. Collectively, these results showed that HFD induced adipocyte hypertrophy was suppressed by pea albumin in a dose-dependent manners.

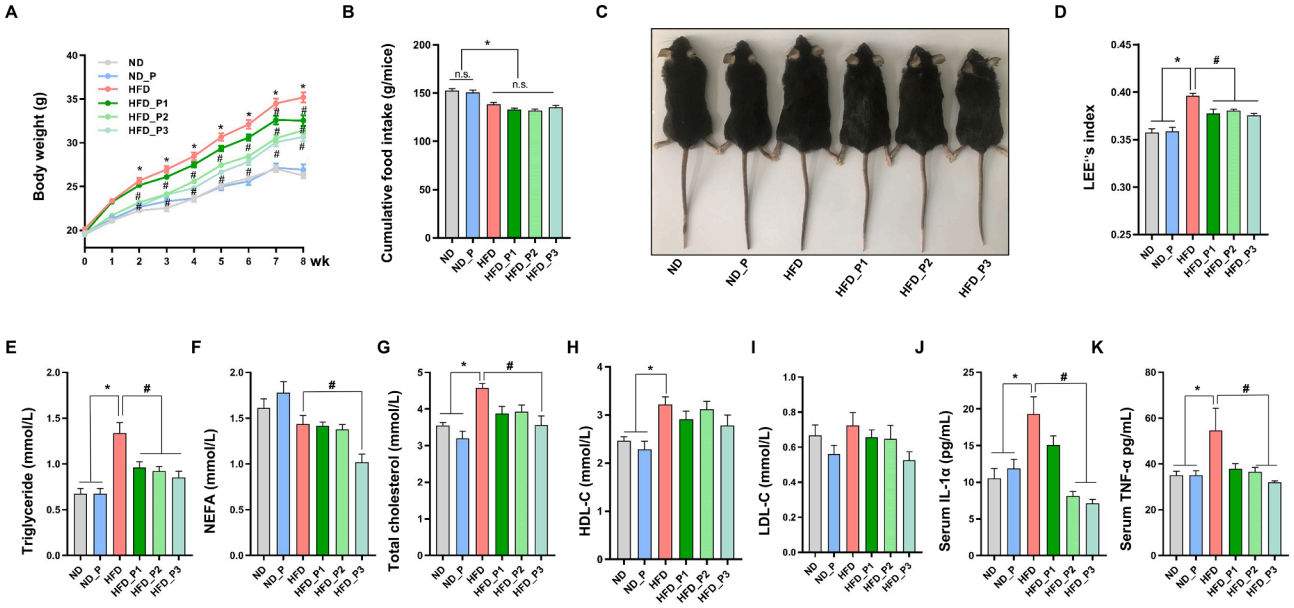

Fig. 1. Pea albumin reduces high-fat diet (HFD)-induced obesity in mice. (A) Body weight change curve. (B) Cumulative food intake. (C) Representative photographs of mice. (D) Lee index. (E-K) Serum levels of TG, NEFA, T-CHO, LDL-C, HDL-C, IL-1α and TNF-α of mice. Data are means ± SEMs, n = 9/group. * $P < 0.05$, HFD vs. ND or ND_P; # $P < 0.05$, HFD vs. HFD_P1, HFD_P2 or HFD_P3. ND_P, 1.5 g/kg body weight pea albumin by oral gavage; HFD_P1, 0.375 g/kg body weight pea albumin by oral gavage; HFD_P2, 0.75 g/kg body weight pea albumin by oral gavage; HFD_P3, 1.5 g/kg body weight pea albumin by oral gavage; TG, triglycerides; NEFA, nonestesterified fatty acid; T-CHO, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; IL-1α, Interleukin-1α; TNF-α, Tumor necrosis factor-α; HFD, high fat diet; ND, normal-fat diet.

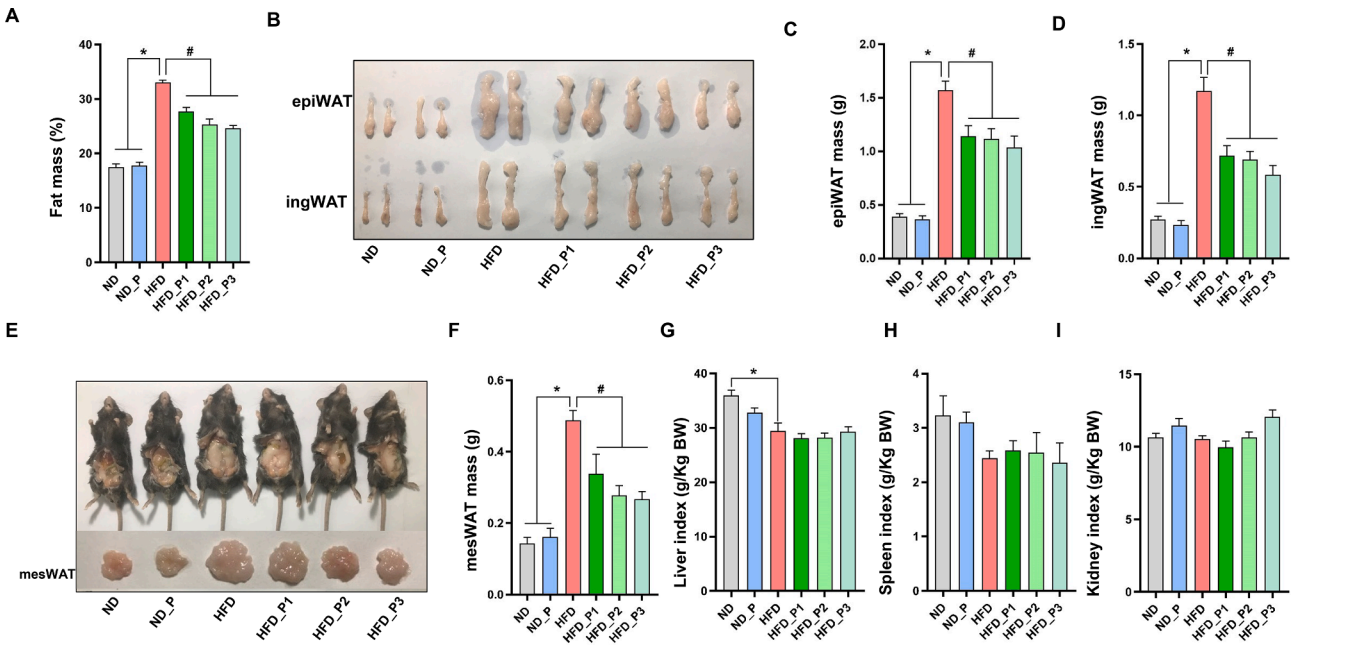

Fig. 2. Pea albumin prevents HFD-induced adipocytes hypertrophy. (A) Fat mass percentage of mice. (B) Representative images of epiWAT and ingWAT depots. (C) Weights of epiWAT. (D) Weights of ingWAT. (D) Representative anatomical image (top panel) and mesWAT (lower panel) of mice. (F) Weights of mesWAT. (G-I) Organ index of liver, spleen, and kidney. Data are means ± SEMs, (n = 9/group). * $P < 0.05$, HFD vs. ND or ND_P; # $P < 0.05$, HFD vs. HFD_P1, HFD_P2 or HFD_P3. ingWAT: inguinal white adipose tissue; epiWAT: epididymal white adipose tissue; mesWAT: mesenteric white adipose tissue. ND_P, 1.5 g/kg body weight pea albumin by oral gavage; HFD_P1, 0.375 g/kg body weight pea albumin by oral gavage; HFD_P2, 0.75 g/kg body weight pea albumin by oral gavage; HFD_P3, 1.5 g/kg body weight pea albumin by oral gavage; HFD, high fat diet; ND, normal-fat diet.

3.3. Pea albumin improved HFD-induced change of lipid metabolism in WAT

To further dissect the mechanism underlying of pea albumin on reducing HFD-induced obesity, we measured the diameter and area of adipocytes in ingWAT and epiWAT after staining with HE. Consistent with increased adipocyte hypertrophy (Fig. 3**A-F**), HFD feeding increased ($P < 0.05$) average area and diameter of epiWAT and ingWAT adipocytes (Fig. 3**A, B**) when compared with mice in ND group. Interestingly, mice treated with HFD plus different dose of pea albumin remarkably reduced ($P < 0.05$) the average area and diameter of epiWAT and ingWAT adipocytes in a dose-dependent manners compared to mice in HFD group. Next, we sought to assess lipid metabolism in WAT by Western blot analysis. Results in Fig. 3**C, D** revealed that HFD feeding significantly upregulated ($P < 0.05$) the protein abundance of FASN, C/EBPα, PPARγ, p-IRS1(Ser302) and p-IRS1(Ser302) in epiWAT (Fig. 3**C, D**) compared with mice in ND group, while pea albumin oral administration markedly reduced the protein abundance of FASN, C/EBPα p-IRS1(Ser302) and p-IRS1(Ser302) but not PPARγ abundance (Fig. 3**C, D**). Additionally, mice fed with HFD reduced ($P < 0.05$) the protein abundance of ATGL, HSL, and Cyto C in epiWAT (Fig. 3**C, D**) when compared with the ND group, while HFD fed mice supplemented with pea albumin by oral gavage markedly ($P < 0.05$) suppressed these proteins abundance (Fig. 3**C and 3D**). Next, 3 T3-L1 cells were used to identify the functional role of pea albumin in lipid metabolism *in vitro*. Cells were induced to differentiation with or without pea albumin supplementation. Interestingly, pea albumin treatment (100, 200, 400, 800 or 1600 μg/mL) reduced the intracellular accumulation of TG, as shown by intracellular TG detection (Fig. 3**E**) and the Oil red O staining (Fig. 3**F**). These results indicated the functional role of pea albumin in improving HFD-induced change of lipid metabolism in WAT.

3.4. Pea albumin attenuated HFD-induced insulin resistance and hepatic steatosis

Obesity-associated chronic diseases such as insulin resistance and hepatic steatosis were also evaluated in our study. Compared to that in the ND group, HFD feeding reduced ($P < 0.05$) the glucose tolerance (Fig. 4**A**) and insulin sensitivity (Fig. 4**B**) with a larger area under OGTT and ITT. Consistently, HFD feeding enhanced ($P < 0.05$) fasted blood glucose (FBG, Fig. 4**C**), insulin (Fig. 4**D**), and leptin (Fig. 4**E**) compared to ND fed mice. Of note, HFD fed mice received pea albumin enhanced ($P < 0.05$) glucose tolerance (Fig. 4**A**) and insulin sensitivity **(**Fig. 4**B)** with a smaller area under OGTT (Fig. 4**A**) and ITT (Fig. 4**B**). Additionally, HFD fed mice treated with pea albumin showed reductions ($P < 0.05$) in levels of FBG (Fig. 4**C**), insulin (Fig. 4**D**), and leptin (Fig. 4**E**) in a dose-dependent manners. Mice fed HFD resulted in higher phosphorylation of IRS1 (Ser302) and IRS1 (Ser307) in liver (Fig. 4**F**) compared with mice fed ND, while pea albumin (0.75 or 1.5 g/kg BW) oral administration markedly reversed the enhanced phosphorylation of IRS1 (Ser302) and IRS1 (Ser307).

Next, we further evaluated the effects of pea albumin on hepatic steatosis. The livers of mice after feeding HFD for 8 weeks displayed intrahepatic TG deposition showed by the HE staining (Fig. 4**G**). Consistently with this finding, mice fed HFD had enhanced liver TG content (Fig. 4**H**) and serum ALT activity (Fig. 4**J**), which indicated hepatic steatosis and damage to liver. Notably, pea albumin oral administration in HFD fed mice apparently reduced ($P < 0.05$) intrahepatic TG deposition (Fig. 4**G, H**), serum ALT level, and serum AST level (Fig. 4**K**). No differences on liver T-CHO content were observed among groups (Fig. 4**I**). Collectively, treatment with pea albumin resulted in improvement on insulin resistance and hepatic steatosis.

3.5. Pea albumin alleviated HFD-induced microbiota dysbiosis

To confirm whether the alleviation of pea albumin on HFD induced obesity was attributed to change of gut microbiota composition, high

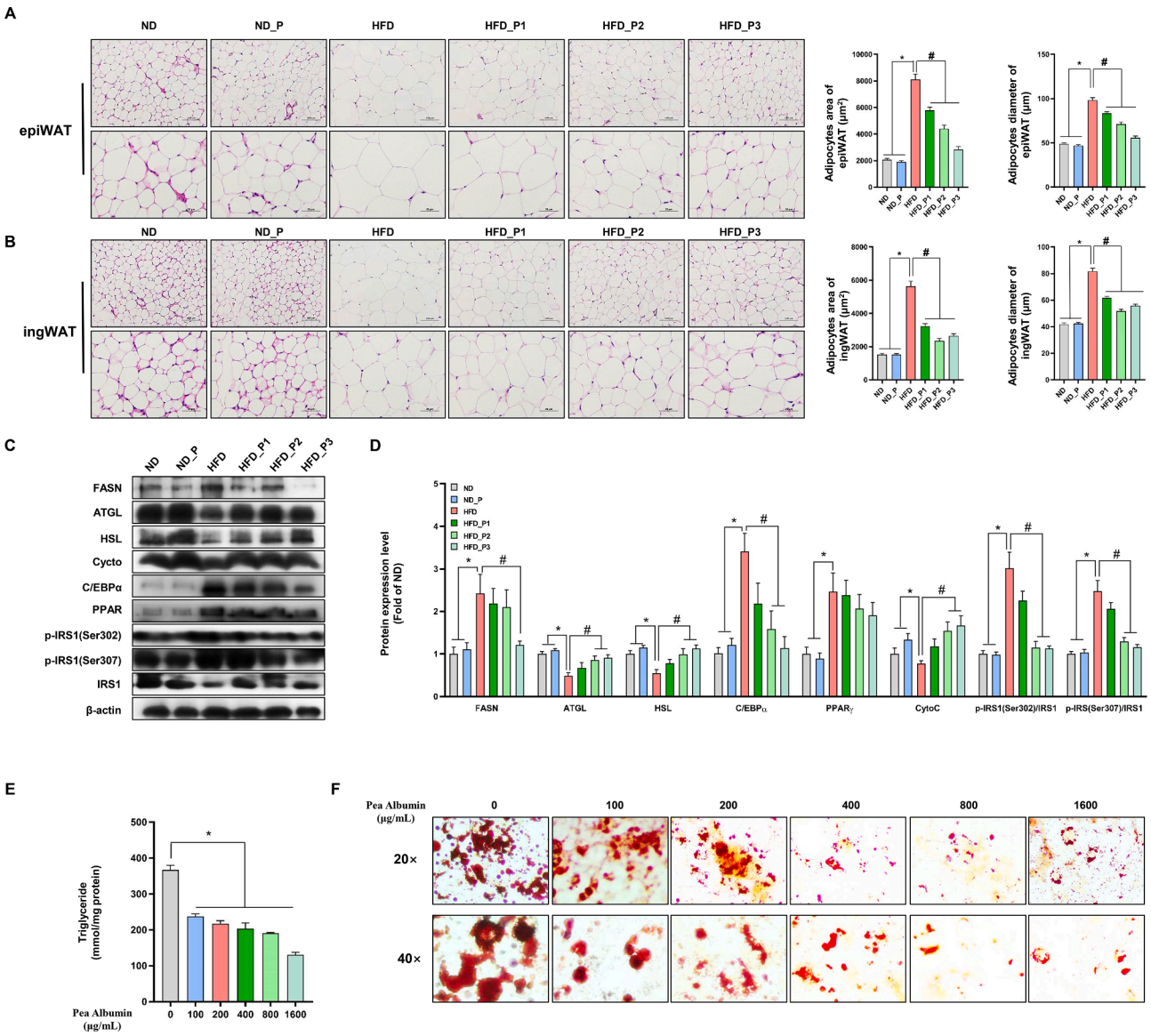

Fig. 3. Pea albumin prevents high fat diet-induced change of lipid metabolism in white adipose tissue. (A) Representative images, average areas, and diameters of epiWAT. (B) Representative images, average areas, and diameters of ingWAT. (C) Representative western blots of markers for lipogenesis (FASN), lipolysis (ATGL, HSL), ipid oxidation (CytoC), adipocyte differentiation (C/EBPα, PPARγ), p-IRS1(Ser302), and p-IRS1(Ser307) were measured in visceral fat depots (epiWAT). (D) The statistical analysis of protein abundance in Fig. 3C. The scale bars for HE staining are 50 μm (top panel) and 100 μm (lower panel). (E) TAG contents in 3 T3-L1 cells. (F) Oil red O staining. Data are means ± SEMs, n = 9/group. * $P < 0.05$, HFD vs. ND or ND_P; # $P < 0.05$, HFD vs. HFD_P1, HFD_P2 or HFD_P3. ingWAT: inguinal white adipose tissue; epiWAT: epididymal white adipose tissue; ATGL: adipose triglyceride lipase; C/EBPα; CCAAT/enhancer binding protein α; CytoC; cytochrome *C*; FASN; fatty acid synthase; HSL; hormone-sensitive lipase; PPARγ; peroxisome proliferators-activated receptor γ; IRS1, insulin receptor substrate 1; ND_P, 1.5 g/kg body weight pea albumin by oral gavage; HFD_P1, 0.375 g/kg body weight pea albumin by oral gavage; HFD_P2, 0.75 g/kg body weight pea albumin by oral gavage; HFD_P3, 1.5 g/kg body weight pea albumin by oral gavage; HFD, high fat diet; ND, normal-fat diet. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

throughput gene-sequencing of the V3-V4 region of 16S rDNA genes from the feces of mice were performed. As shown in Fig. 5**A, B**, α-diversity showed by sobs (community richness) and shannoneven (community evenness) index were significantly diminished ($P < 0.05$) by HFD feeding compared to ND fed mice, which were restored ($P < 0.05$) by pea albumin oral administration (Fig. 5**A, B**). β-diversity analyses by PCA based on Bray-Curtis distance displayed a marked separation of gut microbial structure between ND and HFD groups ($R^2 = 0.527$, $P < 0.001$, Fig. 5**C**). According to PCA and PERMANOVA ($R^2 = 0.527$, $P < 0.001$), HFD fed mice with pea albumin oral administration shaped partially overlapping clusters in the ordination plot close to ND group, suggesting that the modulatory role of pea albumin on gut microbiota structure of HFD fed mice (Fig. 5**C**). As illustrated in Fig. 5**D**, *Firmicutes*, *Bacteroidetes*, and *Desulfobacterota* were predominant in the gut microbiota at the phylum level. Furthermore, ration of F/B was evaluated by HFD feeding compared with ND fed mice (**Figure S2A**), while pea albumin oral administration reduced the ration of F/B and evaluated proportion of phylum *Bacteroidetes*, *Desulfobacterota* and *Verrucomicrobia* (**Figure S2A**). At the genus level, HFD feeding evaluated the relative abundance of *Lactobacillus*, *Enterorhabdus*, *Lactococcus*, and *Staphylococcus* (Fig. 5**E, Figure S3A-D**), and reduced the relative abundance of *unclassified_f_Lachnospiraceae*, *norank_f_Muribaculaceae*, *norank_f_Oscillospiraceae*, *Faecalibaculum*, *Mucispirillum*, *Bacteroides*, *Akkermansia*, and *Parabacteroides* (Fig. 5**E, Figure S3A-D**). Of note, these values tended to be restored to those in mice fed ND through intervention of pea albumin (Fig. 5**E**). Next, LEfSe analysis was performed to

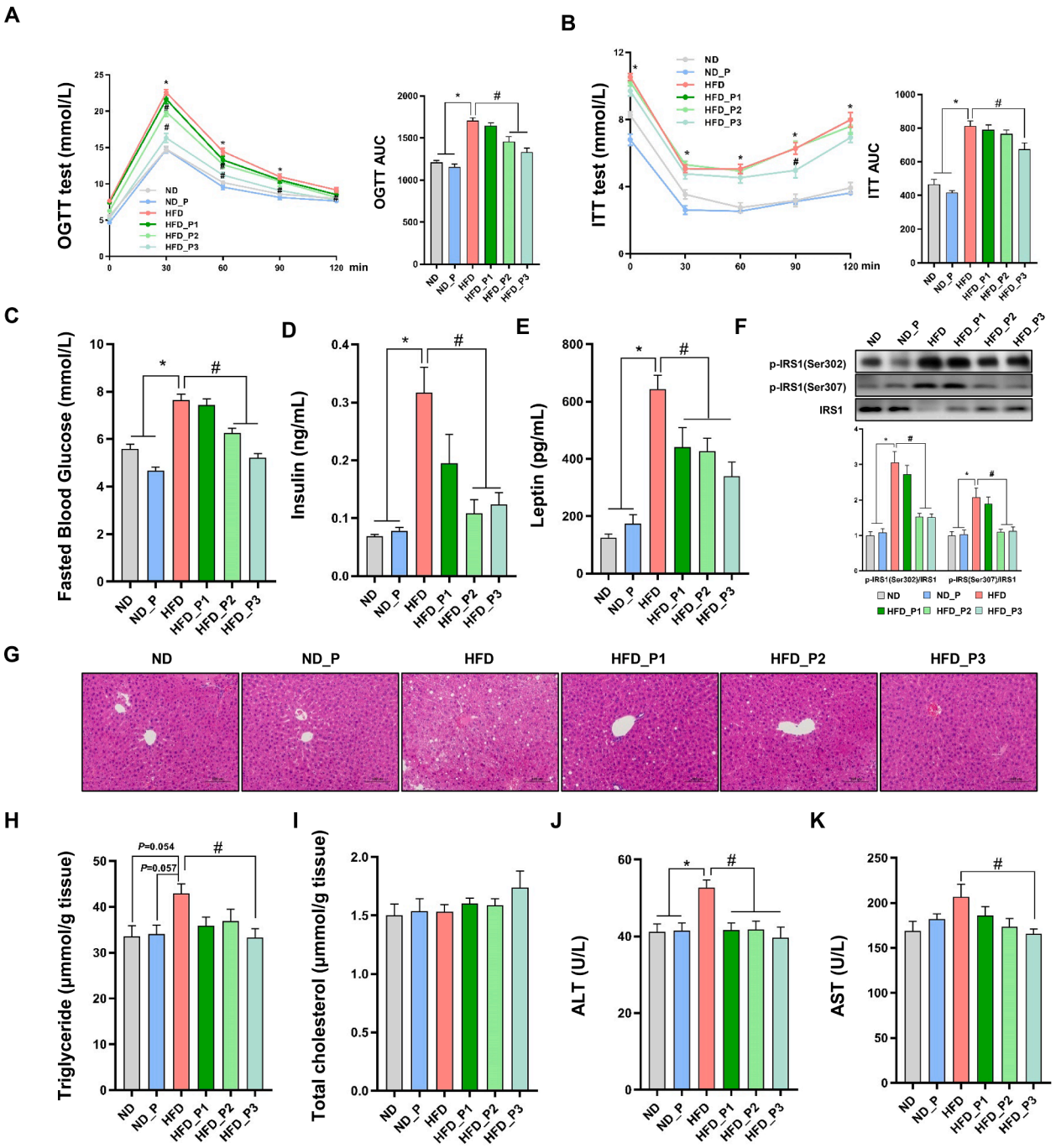

Fig. 4. Insulin resistance and hepatic steatosis were alleviated by pea albumin in HFD-fed mice. (A) Blood glucose profile and AUC measured during an OGTT. (B) Blood glucose profile and AUC measured during an ITT. (C) Fasted blood glucose levels in mice. (D, E) Insulin and leptin level in mice. (F) Protein level of p-IRS (Ser 302) and p-IRS (Ser307) in liver. (G) Representative images of H&E staining of the liver section. (H) Hepatic triglycerides. (I) Hepatic total cholesterol. (J) Serum alanine aminotransferase (ALT) level. (K) Serum Aspartate aminotransferase (AST) level. Data are means ± SEMs, (n = 9/group). * $P < 0.05$, HFD vs. ND or ND_P; # $P < 0.05$, HFD vs. HFD_P1, HFD_P2 or HFD_P3. AUG, area under the curve; OGTT, oral glucose tolerance test; ITT, insulin tolerance test; IRS1, insulin receptor substrate 1; ND_P, 1.5 g/kg body weight pea albumin by oral gavage; HFD_P1, 0.375 g/kg body weight pea albumin by oral gavage; HFD_P2, 0.75 g/kg body weight pea albumin by oral gavage; HFD_P3, 1.5 g/kg body weight pea albumin by oral gavage; HFD, high fat diet; ND, normal-fat diet.

determine differentially abundant fecal bacterial taxa in HFD fed mice in response to pea albumin oral administration. As shown in Fig. 5**F**, we found five feature bacterial genera particularly including *Akkermansia* and *Parabacteroides* were enriched by high dose of pea albumin oral administration, while other twelve feature bacterial genera in HFD fed mice. Furthermore, Spearman correlation analysis was performed to understand the correlation coefficients between differentially enriched microbes and obesity-associated traits (BW, fat mass, FBG, TG, T-CHO, insulin, etc.). Results in Fig. 5**G** indicated that *Akkermansia* displayed a strong negative correlation with BW ($P < 0.05$), fat mass ($P < 0.05$), FBG ($P < 0.05$), T-CHO ($P < 0.05$), and TG ($P < 0.01$). and *Parabacteroides* showed a strong negative correlation with BW ($P < 0.001$), fat mass ($P < 0.01$), weight of ingWAT ($P < 0.05$), Lee index ($P < 0.05$), FBG ($P < 0.01$), and T-CHO ($P < 0.05$). Above all, these data suggested that pea albumin modulated the gut microbiota in HFD-fed mice and that enhanced *Akkermansia* and *Parabacteroides* abundance which play a

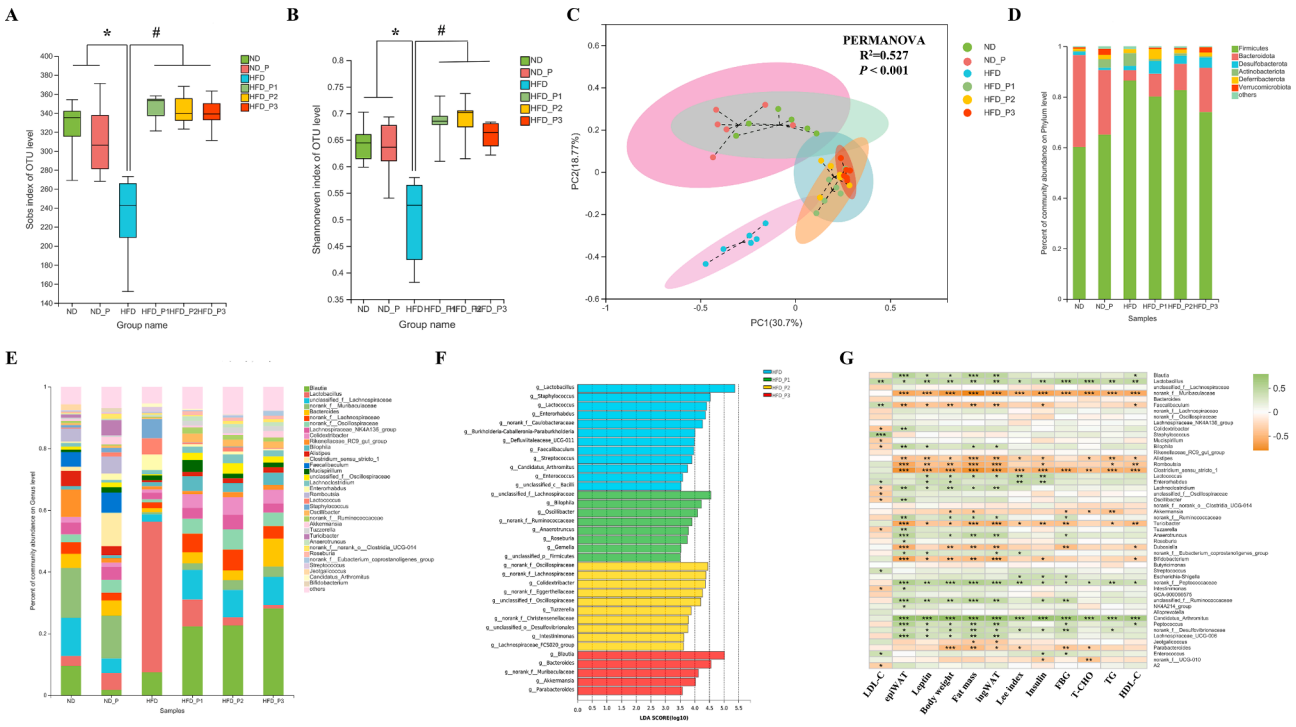

Fig. 5. Effects of pea albumin on gut microbial composition of mice. (A) Sobs index. (B) Simpson index. (C) Relative abundance of gut microbiota at phylum level. (D) Relative abundance of gut microbiota at genus level. (E) Principal coordinate analysis (PCoA) plots of the six groups assessed by PERMANOVA. (F) Analysis of differences in the microbial taxa with linear discriminant analysis (LDA) score 3.5 or greater from phylum to genus levels in gut microbiota communities under different treatments (LEfSe difference in dominant microorganisms under different treatments. (G) Spearman correlation between gut top 50 genera and growth development or serum biochemical parameters. The green color denotes a positive correlation, while orange color denotes a negative correlation. The intensity of the color is proportional to the strength of Spearman correlation. Data are means ± SEMs, n = 6/group. * $P < 0.05$, HFD vs. ND or ND_P; # $P < 0.05$, HFD vs. HFD_P1, HFD_P2 or HFD_P3; ND_P, 1.5 g/kg body weight pea albumin by oral gavage; HFD_P1, 0.375 g/kg body weight pea albumin by oral gavage; HFD_P2, 0.75 g/kg body weight pea albumin by oral gavage; HFD_P3, 1.5 g/kg body weight pea albumin by oral gavage; HFD, high fat diet; ND, normal-fat diet. TG, triglycerides; T-CHO, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; ingWAT: inguinal white adipose tissue; epiWAT: epididymal white adipose tissue; FBG, fasted blood glucose. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

critical role in alleviating HFD-induced obesity.

4. Discussion

Obesity, a complex multifactorial disease that is characterized by BW gain, adipocyte hypertrophy, metabolic disorders, and insulin resistance, and gut microbiota dysbiosis, has been one of most prevalent public health problem and socioeconomic issues. Anti-obesity medications are limited because of high cost, tolerance and serious side effects. New strategies for the treatment or prevention of obesity and associated metabolic disorders are greatly needed. In the present study, we isolated and prepared pea albumin from pea seed. Pea albumin oral administration prevented HFD-induced BW gain, glucose intolerance, insulin resistance, inflammatory cytokines secretion, adipocyte hypertrophy and hepatic steatosis by modulating lipid metabolism and gut microbiota composition.

Four major bands on SDS-PAGE were identified as lipoxygenase (~100 kDa,), pea albumin 2 (~24 kDa), pea albumin 1(~15 kDa) and trypsin inhibitors (~10 kDa) based on previous description (Park, Kim, & Baik, 2010; Yang et al., 2020). As shown in **Table S3**, total protein content (79.70%), lysine (8.07%), threonine (4.73%), arginine (4.58%), valine (3.43%), glutamate (12.18%) and aspartate (9.42%) were measured and similar with previous reports (Croy et al., 1984; Rubio et al., 2014). Furthermore, concentrations of heavy metals, such as lead (Pb), arsenic (As), cadmium (Cd), chromium (Cr), and mercury (Hg) were very low and listed in **Table S3**.

Growing evidence demonstrated that pea protein plays an important role in colitis alleviation, T2D alleviation, cardiovascular diseases prevention, anticarcinogenic, antihypertensive, hypocholesterolemic, muscle thickness promotion, and gut microbiota modulation (Babault et al., 2015; Duranti, 2006; Leterme, 2002; H. Li et al., 2011; Utrilla et al., 2015). However, pea albumin with wide biological and pharmacological activities have not been examined the effects on HFD induced-obesity, obesity-associated disease, and gut microbiota dysbiosis (Bibi, de Sousa Moraes, Lebow, & Zhu, 2017). To investigate effects of pea albumin on obesity, obesity-associated disease, and gut microbiota, HFD fed mice were supplemented with 0.375, 0.75 or 1.5 g/kg BW pea albumin. Our results found that HFD fed mice supplemented with pea albumin reduced BW gain, Lee index, serum TG, serum NEFA, and serum T-CHO, as well as fat mass. Recent study demonstrated that pea protein could reduce serum T-CHO by altering cecal metabolites involved in arginine/histidine pathway, primary bile acid biosynthesis, and short-chain fatty acids production (Tong et al., 2021). Additionally, *in vitro* assay demonstrated that pea albumin hydrolysates significantly reduced relative lipid accumulation in 3T3-L1 cells when compared with lact-albumin hydrolysate treatment (Ruiz, Olias, Clemente, & Rubio, 2020). Furthermore, pea albumin administration significantly reduced mRNA expression of pro-inflammatory cytokines and TLRs, and restored gut microbiota (Utrilla et al., 2015), which indicating a potential role of pea albumin on alleviating inflammation induced by obesity. In the present study, enhanced serum IL-1α and TNF-α in mice fed HFD were observed when compared with mice fed ND. Interestingly, enhanced inflammatory cytokines were significantly abrogated by pea albumin supplementation, indicating a functional role of pea albumin in ameliorating the HFD-induced inflammation.

Consistent with the effects described above on adipocyte

hypertrophy. Our subsequent results indicated that the diameter and area of adipocytes of ingWAT and epiWAT in HFD fed mice were enlarged compared with control group. Pea albumin supplementation remarkably reduced the area and diameter of epiWAT and ingWAT adipocytes in a dose-dependent manners. To understand the underlying mechanism responsible for beneficial effects on adipocytes size, we performed Western blot to analyze the critical proteins implicated in lipogenesis (FASN), lipolysis (ATGL, HSL), lipid oxidation (Cyto C), and adipocyte differentiation (C/EBPα, PPARγ). Interestingly, abundances of proteins for lipolysis and fatty acid oxidation were markedly augmented by pea albumin oral administration, indicating a critical role for pea albumin in ameliorating the HFD-induced adipocyte hypertrophy. These beneficial effects of pea albumin on WAT lipid metabolism were similar with soy protein or soy protein hydrolase which have been extensively reported to improve insulin resistance and lipid levels by activating PPAR pathways and by reducing FAS, steroyl-CoA-desaturase-1 (SCD1), and delta-5 and delta-6 desaturases expression (Ahnen, Jonnalagadda, & Slavin, 2019; Mezei et al., 2003; Mullen, Brown, Osborne, & Shay, 2004; Tovar et al., 2005).

Glucose intolerance, insulin resistance and hepatic steatosis commonly accompanied by obesity and associated metabolic disorders. Glucose tolerance and insulin sensitivity with a larger area under OGTT and ITT than control. Consistent with previous studies (Liu et al., 2015), HFD feeding led to elevated level of FBG, insulin and leptin, when compared with ND group mice, while pea albumin oral administration reversed those alteration. Furthermore, HFD feeding led to elevated phosphorylation of IRS1 (Ser302) and IRS1 (Ser307) which are necessary for the inhibition of the IR/IRS-1 interaction and result in insulin resistance (Tsukumo et al., 2007; Werner, Lee, Hansen, Yuan, & Shoelson, 2004). Interestingly, the enhanced phosphorylation of IRS1 (Ser302) and IRS1 (Ser307) were reversed by the pea albumin administration which is consistent previous studies (Gao, Song, Du, & Mao, 2018; Ren, Sun, Li, Gao, & Zhang, 2022). Additionally, HE staining of liver tissue indicated the presence of hepatocyte ballooning and fat accumulation in the liver of mice fed HFD as confirmed by elevated TG concentration in liver. Pea albumin oral administration apparently improved glucose tolerance and insulin sensitivity, and reduced serum level of FB, insulin, and leptin. Also, pea albumin reduced activities of serum ALT and AST and decreased the level of TG in liver of mice fed HFD. Notably, HFD-induced liver steatosis was rescued by pea albumin oral administration, therefore reducing liver injury and associated histological alterations. Few population studies have examined the beneficial effects of pea albumin on glucose homeostasis, insulin sensitivity, and hepatic steatosis, so the underlying mechanism has not been elucidated. Restoration of gut microbiota may contribute to the beneficial effects of pea albumin.

Gut microbiota dysbiosis and its contribution to the onset and development of obesity and obesity-associated chronic disease have promoted active search for novel therapeutic interventions by targeting the gut microbiota (Cani & de Vos, 2017; Everard et al., 2013; Miyamoto et al., 2019). HFD consumption profoundly leads to gut microbiota dysbiosis revealed by α-diversity (Sobs index and Shannoeven index) which is consistent with previous studies (Do et al., 2021; H. Wang et al., 2018; Wu et al., 2019). Furthermore, PCA analysis (β-diversity) confirmed that successive HFD feeding change microbiota community structure, while pea albumin shaped partially overlapping clusters in the ordination plot close to ND group. Correspondingly, our subsequent analysis demonstrated that pea albumin oral administration markedly modulated gut microbiota composition from phylum to genus. Accumulating studies revealed an increase in *Firmicutes* and a reduction in *Bacteroidetes* abundance (elevated F/B ration) in phylum level in obese individuals, consistent with our finding (Tian et al., 2021; K. Wang et al., 2019; Zhao et al., 2017), while pea albumin especially evaluated proportion of phylum *Bacteroidetes* and *Verrucomicrobi* (**Figure S3B**). At genus level, relative abundance of *Lactobacillus, Enterorhabdus, Lactococcus, and Staphylococcus* were enhanced and relative abundance of unclassified_f_*Lachnospiraceae*, norank_f_*Muribaculaceae*, norank_f_*Oscillospiraceae*, *Faecalibaculum*, *Mucispirillum*, *Bacteroides*, *Akkermansia*, and *Parabacteroides* were reduced in HFD fed mice. These alterations were weakened by pea albumin supplementation. Of note, *Akkermansia* belonging to the *Verrucomicrobi phylum*, characterized as next-generation beneficial microbe involved in BW gain loss, blood glucose homeostasis, adipose tissue metabolism, and gut microbiota (Cani & de Vos, 2017; Dao et al., 2016; Everard et al., 2013), displayed a strong negative correlation with obesity traits (BW gain and fat mass) and metabolic syndromes. Furthermore, the abundance of *Lactobacillus* enriched by HFD were markedly diminished by pea albumin which is in line with previous study and indicates a high-calorie resulting in significant enrichment of *Lactobacillus* (Tian et al., 2021; J. Wang & Jia, 2016; Wu et al., 2019). Conversely, some previous studies indicated that the abundance of *Lactobacillus* present a negative correlation with obesity traits (S. Li et al., 2020; H. Wang et al., 2018). The abundance of *Lactobacillus* should be at normal levels to maintain a health state. Otherwise, *Parabacteroides* show beneficial effects on lowering BW, reducing weights of WAT, and metabolic disorder alleviation based on LEfSe analysis and Spearman correlation analysis, these findings are agreement with previous reports (K. Wang et al., 2019; Wu et al., 2019). Thus, these data indicate that pea albumin protects mice from obesity and associated metabolic disorders by restoring gut microbiota and selectively enhanced the relative abundance of beneficial intestinal bacteria (*Akkermansia*, *Parabacteroides* etc.).

5. Conclusion

In conclusion, we isolated the pea albumin from pea seed and determined its anti-obesity and obesity-associated metabolism disorder alleviation in HFD fed mice. Our results indicate that pea albumin negatively modulated lipid accumulation via suppressing adipogenesis and promoting fatty acid oxidation. Furthermore, pea albumin oral administration corrected the gut microbiota dysbiosis by modulating the gut microbiota composition, especially, selectively enhancing the relative abundance of beneficial intestinal bacteria (*Akkermansia*, *Parabacteroides* etc.). These new findings provide fundamental information regarding the functional role of pea albumin (Fig. 6), and make pea albumin as a potential candidate for treating obesity and associated metabolic syndromes. However, which and how pea albumin components improve insulin sensitivity and hepatic steatosis, which still needs more deep proof-to-concept works using proteomics and transcriptomics *in vivo* and *in vitro* studies. In future, it would be important to identify the specific ones of pea albumin for further exploit as an agent with anti-obesity properties.

6. Ethics Statement

All the animal experimental procedures were approved by the Institutional Animal Care and Use Committee of China Agricultural University (AW82102202-5-1).

CRediT authorship contribution statement

Ning Liu: Writing – original draft, Methodology, Investigation, Data curation, Conceptualization, Writing – review & editing, Funding acquisition. **Zhuan Song:** Writing – original draft, Methodology, Investigation, Data curation. **Wenhua Jin:** Formal analysis. **Yue Yang:** Resources. **Shiqiang Sun:** Resources. **Yiquan Zhang:** Resources. **Shucheng Zhang:** Resources. **Siyuan Liu:** Resources. **Fazheng Ren:** Conceptualization, Writing – review & editing, Funding acquisition. **Pengjie Wang:** Conceptualization, Writing – review & editing, Funding acquisition.

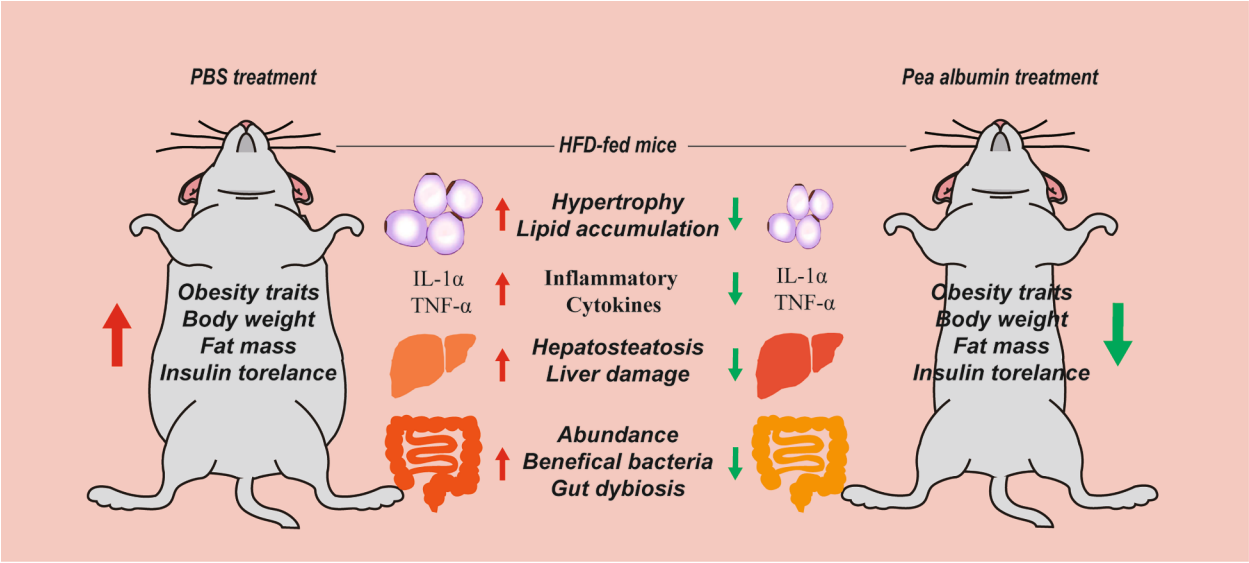

Fig. 6. The proposed anti-obesity mechanism of pea albumin in high fat diet fed mice.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2022.105234.

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