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Highly Branched RG-I Domain Enrichment are Indispensable for Pectin Mitigating Against High-Fat Diet-Induced Obesity

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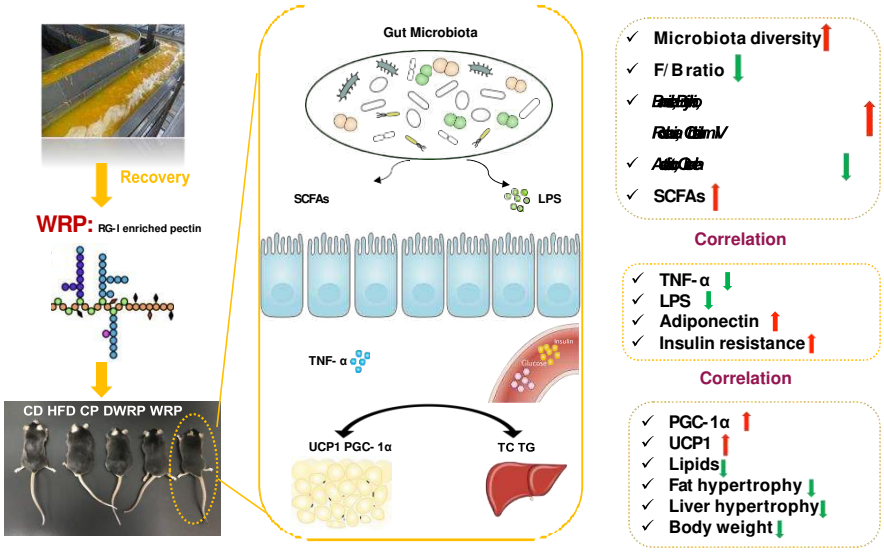
Keywords: RG-I, Molecular weight, Side chain, Pectin, High-fat diet, Obesity, Gut microbiota

Abstract

Obesity is associated with gut microbiome dysbiosis. Our previous research has shown that highly branched RG-I enriched pectin (WRP, 531.5 kDa, 70.44% RG-I, Rha:(Gal+Ara)=20) and its oligosaccharide with less branched (DWRP, 12.1 kDa, 50.29% RG-I, Rha:(Gal+Ara)=6) are potential prebiotics. The present study is conducted to uncover the impact by which the content, molecular size and branch degrees of RG-I on the inhibiting effect of high-fat diet (HFD)-induced obesity. The commercial pectin (CP, 496.2 kDa, 35.77% RG-I, Rha:(Gal+Ara)=6), WRP and DWRP were orally administered to HFD-fed C57BL/6J mice (100mg kg^{-1} d^{-1}) to determine their individual effects on obesity. WRP significantly prevented bodyweight gain, insulin resistance, and inflammatory responses in HFD-fed mice. No obvious anti-obesity effect was observed in either CP or DWRP supplementation. Mechanistic study revealed that CP and DWRP could not enhance the diversity of gut microbiota, while WRP treatment positively modulated the gut microbiota of obese mice by increasing the abundance of *Butyrivibrio*, *Roseburia*, *Barnesiella*, *Flavonifractor, Acetivibrio*, and *Clostridium* cluster IV. Furthermore, the WRP significantly promoted browning of white adipose tissue in HFD-fed mice, while CP and DWRP did not. WRP can attenuate the HFD-induced obesity by modulation of gut microbiota and lipid metabolism. Highly branched RG-I domain enrichment are essential for pectin mitigating against the HFD-induced obesity.

Graphical abstract:



Introduction

The prevention of obesity is a challenge of global proposition. Evidence has shown that obesity is associated with reduced gut bacterial diversity or altered proportions of bacterial species [1-4]. Consumption of plant polysaccharides revealed a significant and positive effect on adiposity-induced lipid metabolic disorders and gut microbiota dysbiosis [5-8]. Among them, pectin and derived-oligosaccharides are good candidate modulators of obesity due to their fermentation potential by various probiotic microorganisms to modulate the obesity due to their complex structure that fermented by various of probiotics [9-11].

Pectin is a complex heteropolysaccharide which consists of structurally distinct domains including homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan type I (RG-I), rhamnogalacturonan type II (RG-II), arabinan, and arabinogalactan. RG-I is comprised of a backbone being formed from a repeating

66 disaccharide of [→2)-α-L-Rha*p*-(1→4)-α-D-GalA*p*-(1→] residues with Ara and Gal
67 residues attached to the *O*-4 or *O*-3 position of α-L-Rha*p* backbone units [12]. It is usually
68 removed from commercial pectin preparations by hot acid treatment as it is considered
69 a hinder for pectin gelling. However, accumulating evidence has illustrated that pectin
70 containing RG-I regions from various sources can modulate the composition of obesity-
71 related intestinal microbiota and increase the production of butyrate, which is a
72 dominant protective agent against obesity [13-17]. Recent findings suggested has been
73 reported that polysaccharide utilization loci (PULs) of gut bacteria activated by
74 different RG-I domains can recruit a myriad of glycoside hydrolases (GHs) and
75 polysaccharides lyases (PLs) for metabolism of RG-I pectin molecules [18-19]. Thus, RG-I
76 is hypothesized to contribute significantly to bacterial fermentation in the colon leading
77 to favorable changes in gut microbiota composition [20-21]. Particularly, Khodaei and his
78 colleagues have confirmed that potato RG-I pectin stimulated the growth of
79 *Lactobacillus spp.* and *Bifidobacterium spp*. Reduction in these two species are
80 proposed biomarker of gut dysbiosis and found to be decreased under high-fat diet
81 (HFD) conditions [22]. A recent research also indicated the pectin containing RG-I can
82 modulate the composition of obesity-related gut microbiota and upregulate the
83 production of butyrate—a dominant protective agent against obesity [23]. Moreover,
84 apple pectin rich in RG-I strongly promoted *Bifidobacterium*, *Bacteroides*, and
85 *Lactobacillus* in HFD-fed mice colon, subsequently producing short-chain fatty acids
86 (SCFAs) which limits the secretion of proinflammatory cytokines and alleviated the

obesity caused inflammation [24]. However, up to now the impact of RG-I content in pectin on the HFD-induced obesity is still unclear.

Apart from the RG-I content, another factor affected the bioactivity of the RG-I pectin is molecular size [25]. A recent study on citrus pectic oligosaccharides containing RG-I with a molecular weight of 3~4 kDa have shown hypocholesterolemic effects on HFD-fed mice by modulating specific gut bacterial groups [10]. Besisdes, the report from Gómez et al. has shown pectic oligosaccharides (5.9~22.8 kDa) containing relative high content RG-I (37.65%) caused better shifts prebiotic properties than high Mw pectin (51.4~82 kDa), confirming the essential of molecular size in functional properties of RG-I pectin [26]. However, most of the research merely focused on the preparation and probiotic effect of pectic oligosaccharides primarily consists of HG, less studies were conducted on RG-I enriched oligosaccharides [11, 26-27]. Furthermore, a greater proportion of side chains in RG-I pectin was confirmed that can promote the growth of *Bacteroides* species [28-30]. The arabino/galacto-oligosaccharides derived from the side chains of RG-I were proved to be more fermented by *Bifidobacterium* than those from backbone of RG-I [22]. These observations indicate that the neutral sugar branching chains may have a great impact on the gut microbial composition improvement of RG-I enriched oligosaccharides. Since the neutral sugar side chains were degraded significantly during the pectic oligosaccharide preparation process. There is also necessary to take the branching degrees into account for assessment the RG-I oligosaccharides' beneficial effects in the gut microbiota.

In our previous study, RG-I enriched oligosaccharides (DWRP, 50.29% RG-I content, 12.1 kDa, Rha: (Gal+Ara)=1:6) degraded from citrus canning processing basic water recovered pectin (WRP, 70.44% RG-I, 531.5 kDa, Rha: (Gal+Ara)=1:20) were obtained by metal-free Fenton reaction. DWRP can significantly enriched *Bifidobacterium and Lactobacillus* populations, and WRP can improve the *Bacteroides*, *Desulfovibrio* and Ruminococcaceae in mice [29]. The results evidenced both the highly branched RG-I enriched pectin with large Mw and RG-I oligosaccharides with less branching degree can modulate the gut microbiota. However, these effects on gut microbe of obesity mouse is still unclear.

Therefore, the main aim of this study was to uncover the contribution by which RG-I content, molecular size and branching degrees of pectin to the alleviation of HFD-induced obesity and obesity-induce microbiota dysbiosis shaping. The RG-I enriched pectin recovered (WRP), its degradation products (DWRP) and commercial HG dominated pectin (CP, 496.2 kDa, 35.77% RG-I, Rha:(Gal+Ara)=6) were selected in this study. First, investigating the effects of CP, WRP, and DWRP treatment on obesity and obesity-induced metabolic disorders in HFD-fed mice. Then, the contribution of pectin on gut microbiota composition and SCFAs were studied by 16S rRNA and gas chromatography (GC). Moreover, qRT-PCR analysis and immunohistochemistry were also used to analyze the expression of genes and proteins related to brown-like adipocyte formation, respectively.

2. Experimental Section

2.1 Preparation of Pectin

Rhamnogalacturonan-I (RG-I)-enriched pectin (WRP) and its degradation products (DWRP) was recovered from citrus (*Citrus unshiu Marc.*) processing water by sequential acid and alkaline treatments in a previous study [31]. WRP (Mw=531.5 kDa) was recovered from the citrus segments material and was previously reported to have 70.44% of RG-I content with high degree of side chain branching (Rha: (Gal+Ara)=1:20), while its depolymerized fraction DWRP (Mw=12.1 kDa, 56.29% RG-I content) with less side chain branching (Rha: (Gal+Ara)=1:6). Commercial pectin (CP) was bought from Sigma-Aldrich (Shanghai, China). and is mainly composed of HG (52.55%) with an average Mw of 496.2 kDa and low degree of side chain branching (Rha: (Gal+Ara)=1:6) was used in the present study.

2.2 Animal Experiments

Fifty C57BL/6J male mice (SPF, 6-8 weeks old, IACUC-20180917-02) were kept under specific-pathogen-free conditions in a 12-hour light/dark cycle with free access to standard chow diet (CD; 12% of energy from fat; Rodent diet, SHOBREE, Jiangsu Synergy Pharmaceutical Biological Engineering Co, Ltd, Nanjing China) and sterile drinking water in a temperature-controlled room (21 °C±2 °C). After an accommodation period of 1 week, the mice were randomly divided into five groups (10 mice/group) and were fed for 8 weeks with CD, high fat diet (HFD, 60% of energy from fat; Research Diets D12492, Opensource Diets, USA), HFD with 100mg/kg CP (HFD-CP), HFD with 100mg/kg WRP (HFD-WRP), HFD with 100mg/kg DWRP (HFD-DWRP). A certain amount of pectin according to the dosages of 100mg/kg was

dissolved in 200 μL distilled water and administrated orally via intragastric gavage once per day. The compositions and energy densities of the diets are listed in **Table S1**. Body weight and food intake were measured weekly.

The oral glucose tolerance test (OGTT) was performed three days before sacrifice. Overnight-fasted mice were administrated with glucose solution (2 g/kg body weight, 66% solution) by oral gavage, then blood glucose was measured from tail vein blood at 0, 30, 60, 90, 120 min using test strips (ACCU-CHEK Performa) and a portable glucose meter (Roche Diagnostics, Shanghai, China). The blood glucose level before glucose administration represented the fasting glucose concentration. Incremental area under the curve (AUC) was calculated using the trapezoidal method.

Mice were fasting for 12 hours, anaesthetised and sacrificed by cervical dislocation after 9 weeks. Blood and tissues were collected and stored at -80 °C until further use. All procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University School of Medicine.

2.3 Biochemical analysis and cytokine measurements of serum

Serum was isolated by centrifugation (4 °C, 12,000g, 10 min). Serum total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Serum TNF-α, IL-6, LPS, Insulin and adiponectin protein levels were then quantified using commercial ELISA kits (Cloud-clone Crop, USA)

following the manufacturer's instruction.

2.4 Liver and epididymal fat histology

The fresh liver, inguinal white adipose tissue (iWAT) and epididymis WAT (eWAT) were isolated and fixed with 4% neutral formalin solution at room temperature for 48 h. After dehydration, eWAT, iWAT and liver were clarified in benzene and embedded in low melting point paraffin wax. Sections (3 nm thick) were cut and stained with haematoxylin and eosin (H&E staining) for light microscopic examination. All of these assays were performed in a blinded manner.

2.5 Immunohistochemistry staining

The paraffin sections of iWAT were subjected to deparaffination, antigen retrieval, endogenous peroxidase activity blocking. Thereafter, slides were incubated with UCP1 primary antibody (Santacruz Biotechnology Inc., USA) and horseradish peroxidase (HRP)-conjugated secondary antibody. After 3, 3-diaminobenzidine (DAB) immunostaining, harris hematoxylin counterstaining, dehydration and coverslipping, the sections were observed in DS-Ri1-U3 Nikon digital imaging system and the positive integral optical density (IOD) of UCP1 in the immunohistochemical pictures was analyzed with the Image J software (National Institute of Health, MD, USA).

2.6 RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted from eWAT, iWAT, and BAT using TRIzol reagent (Invitrogen, CA, USA), which was then used to synthesize cDNA with PrimeScript RT reagent Kit with gDNA Eraser (Takara, Beijing, China). Quantitative real-time PCR was performed using SYBR Green Master Mix (Applied biosystems, CA, USA), 96-

well plates and an Applied Biosystems QuantStudio 3 Real-Time PCR instrument (Life Technologies, Singapore). qPCR was performed for 40 cycles with following programs: 50 °C for 2 min, 95 °C for 1s, 60 °C for 40s. Relative quantification was done based on the $2^{-\Delta\Delta CT}$ method. Expression was normalized to the housekeeping gene.

2.7 16S rRNA gene analysis

Cecal samples were collected and used for the bacterial 16S rRNA sequencing. Five samples of each group were selected randomly for 16S rRNA analysis. DNA was extracted from the cecal solid contents of mice by using the E.Z.N.A. ®Stool DNA Kit (D4015, Omega, Inc., USA) according to manufacturer's instructions. The total DNA was eluted in 50 μL of Elution buffer and stored at -80 °C until measurement in the PCR by LC-Bio Technology Co., Ltd.The V3-V4 region of the prokaryotic (bacterial and archaeal) small-subunit (16S) rRNA gene was amplified with slightly modified versions of primers 338F (5'-ACTCCTACGGGAGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') [32]. The 5' ends of the primers were tagged with specific barcods per sample and sequencing universal primers.

The PCR products were purified by AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantified by Qubit (Invitrogen, USA). The amplicon pools were prepared for sequencing and the size and quantity of the amplicon library were assessed on Agilent 2100 Bioanalyzer (Agilent, USA) and with the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA), respectively. PhiX Control library (v3) (Illumina) was combined with the amplicon library (expected

215 at 30%). The libraries were sequenced either on 300PE MiSeq runs and one library was
216 sequenced with both protocols using the standard Illumina sequencing primers,
217 eliminating the need for a third (or fourth) index read.

218 Samples were sequenced on an Illumina MiSeq platform according to the
219 manufacturer's recommendations, provided by LC-Bio. Paired-end reads was assigned
220 to samples based on their unique barcode and truncated by cutting off the barcode and
221 primer sequence. Paired-end reads were merged using FLASH. Quality filtering on the
222 raw tags were performed under specific filtering conditions to obtain the high-quality
223 clean tags according to the FastQC (V 0.10.1). Chimeric sequences were filtered using
224 Verseach software (v2.3.4). Sequences with ≥97% similarity were assigned to the
225 same operational taxonomic units (OTUs) by Verseach (v2.3.4). Representative
226 sequences were chosen for each OTU, and taxonomic data were then assigned to each
227 representative sequence using the RDP (Ribosomal Database Project) classifier. The
228 differences of the dominant species in different groups, multiple sequence alignment
229 was conducted using the PyNAST software to study phylogenetic relationship of
230 different OTUs. OTUs abundance information were normalized using a standard of
231 sequence number corresponding to the sample with the least sequences. Alpha diversity
232 is applied in analyzing complexity of species diversity for a sample through 4 indices,
233 including Chao1, Shannon, Simpson and Observed species. All indices of samples were
234 calculated with QIIME (Version 1.8.0). Beta diversity analysis was used to evaluate
235 differences of samples in species complexity. Beta diversity were calculated by

principle co-ordinates analysis (PCoA) and cluster analysis by QIIME software (Version 1.8.0). The spearman's rho nonparametric correlations between the gut microbiota and heal-related indexes were determined using R packages (V2.15.3).

Alpha diversity indexes, relative abundance of phyla, principal component analysis (PCA) and linear discriminant analysis (LDA) effect size (LEFse) analysis were assessed.

2.8 Ceacal and colonic short-chain fatty acids

Production of SCFA in the ceca and feces of mice was analysed using a 7890A GC (Agilent Technologies, Stockport, UK) using a slightly modified method [6]. Detailed description of these methods is described in a previous study.

2.9 Statistical Analysis

Data were expressed as means ± SD. Statistical analysis was performed using GraphPad Prism V.7.04 (GraphPad Software, USA). One-way analysis of variance (ANOVA) for multiple comparisons was conducted, followed by the non-parametric Kruskal–Wallis test with Dunnett's multiple comparisons test.[33] Significance was set at $p < 0.05$.

3. Results

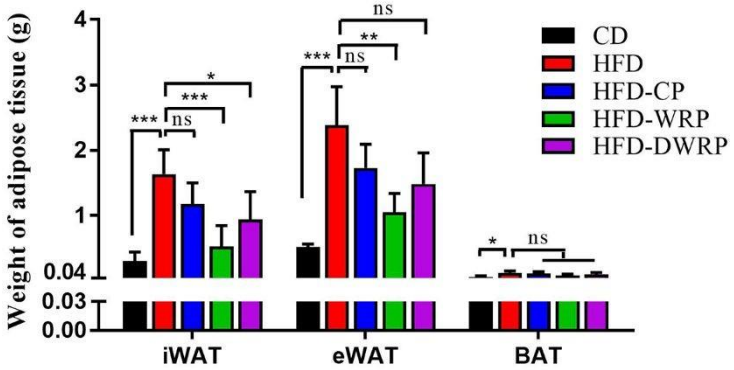
3.1 WRP Prevented Body Weight Gain in HFD-induced Obese Mice

To test the effects of pectin supplementation on body weight, we fed mice with HFD with or without pectin supplementation for 8 weeks. Compared with the CD, mice fed an HFD showed a significant and sustained increase body weight (260%) (**Figure 1**). Notably, WRP supplementation dramatically prevent the body weight gain caused

by HFD ($p < 0.001$, **Figure 1B&C**). However, no significant improvement in weight gain was observed in the HFD-CP and HFD-DWRP groups. As shown in **Figure 1 C~E**, HFD significantly induced the weight gain of liver, kidney, inguinal white adipose tissue and epididymal white adipose tissue of mice. In parallel with weight gain caused by HFD (**Figure 1E**), the weight gain of white adipose tissue and visceral fat of HFD fed mice was prevented decreased observably when intervened by WRP (iWAT, $p < 0.001$; eWAt, $p < 0.01$). Besides, WRP apparently reduced macrosteatosis, hepatocyte ballooning in the livers of obese mice. The liver and fat tissue morphology in HFD-CP and HFD-DWRP groups was the same as in the HFD. The fat tissue morphology failed to maintained in HFD-CP and HFD-DWRP group (**Figure 2**).

Some reports suggested that fucoidan was reported to affected appetite regulation and subsequent control of body weight [34]. There was no significant difference in food intake between groups (see on **Figure S1**), indicating that the mitigating effects of WRP

E



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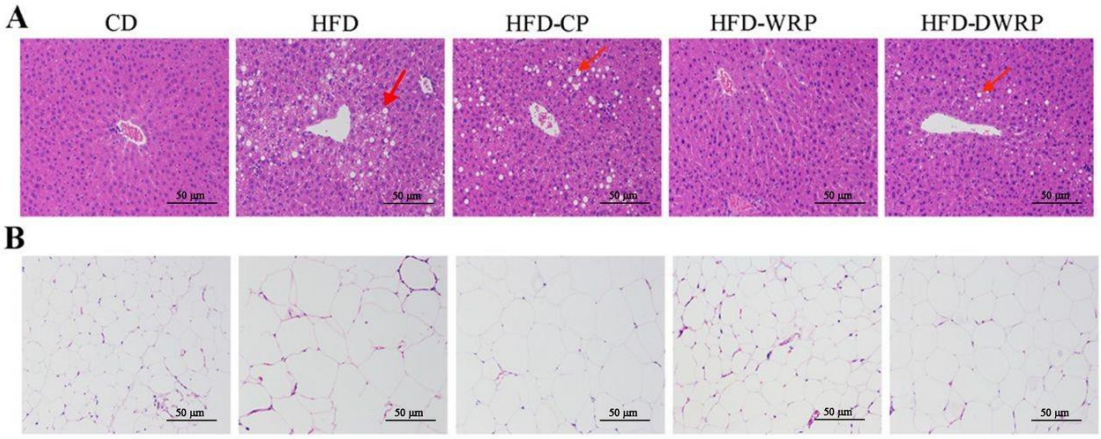
272 **Figure 1.** Whole body and tissue weight fed on conventional chow (CD) and high-fat diet (HFD)
273 for 8 weeks. A: Growth curve of mice in different groups; B : The weight gain of mice in each
274 group after 8 weeks of feeding; C~E: shows the weight of liver, kidney, inguinal white adipose
275 tissue, epididymal white adipose tissue and brown adipose tissue of mice, respectively. (Data are
277 presented as means ± SD (n=8 mice per group). *p < 0.05; **p < 0.01; ***p < 0.001; ****p <
278 0.0001; ns, not significant.

279 3.2 WRP Alleviated HFD-induced Hyperlipidemia, Hyperglycemia, and
280 Inflammatory Responses

281 As shown in **Table 1**, the serum level of total triacylglycerol (TG), total
282 cholesterol (TC), low density lipoprotein cholesterol (LDL-C) and free fatty acids (FFA)

283 in mice were negatively controlled and the high-density lipoprotein cholesterol (HDL-
284 C), adiponectin and HDL-C/LDL-C content positively controlled by WRP treatment.[35]
285 Pro-inflammatory cytokines have been shown systemic inflammation but also insulin
286 resistance [36]. And bacterial lipopolysaccharide (LPS) is an early factor in the triggering
287 of metabolic diseases induced by obesity [37]. The adipose tissues of obese animals and
288 humans secreted considerable level of pro-inflammatory cytokines and LPS compared
289 with lean individuals [36, 38]. In the present study, supplementation of WRP and DWRP
290 significantly controlled the level of serum LPS and TNF-α in HFD-fed mice. Further,
291 to determine the effect of different pectins on glucose homeostasis and insulin
292 sensitivity, OGTT and fasting insulin test were performed. As shown in **Figure 3**, HFD
293 treatment impeded the glucose utilization ability as the levels of fasting blood glucose
294 ($p < 0.0001$) and insulin ($p < 0.01$) were significantly increased compared to CD group.
295 Nevertheless, WPR intervened HFD-fed mice exhibited lower glucose levels at all time
296 points up to 120 min after oral glucose challenge and reduced AUC glycemic response.
297 Moreover, WPR and DWPR supplementation lowered the plasma levels of glucose and
298 insulin compared with the HFD group (**Figure 3D**). Together, WRP effectively
299 alleviated the dyslipidemia of induced by HFD through negatively control of blood
300 lipid and proinflammatory factors content; on the other hand, WRP improved the
301 glucose intolerance and insulin sensitivity.



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303 **Figure 2.** Histological assessment of livers (A) and epididymal white adipose tissue (B) in HFD-
304 induced obesity mice. (H&E stain, 200×magnification)

305 3.3 WRP Promotes Browning of White Adipocytes in HFD-induced Mice

306 The average cell size of iWAT in HFD group was significant larger than CD group
307 (**Figure 4A**). WRP supplementation the size of iWAT significantly lower than HFD
308 group, but no obvious difference in size of iWAT was observed in both HFD- CP and
309 HFD-DWRP groups. Under some stimulation (cold condition or β-3 adrenergic
310 agonist), the content of mitochondria in WATs increased dramatically and enhances
311 thermogenic properties. This process was called "browning", and brown-like
312 adipocytes expressed large amounts of uncoupling protein 1 (UCP1) to enhance energy
313 expenditure in WATs [39]. As expected, the immunohistochemistry staining results
314 revealed that the expression level of UCP1 protein in iWAT was remarkably
315 upregulated in HFD-WRP group compared to HFD group (**Figure 4A**). Consistent with
316 these changes, qPCR analysis confirmed that WRP increased the mRNA level of UCP1
317 in iWAT (5.65-fold *v.s.* HFD group, $p < 0.001$) (**Figure 4B**). In addition,
318 supplementation of WRP also remarkably increased the expression of some

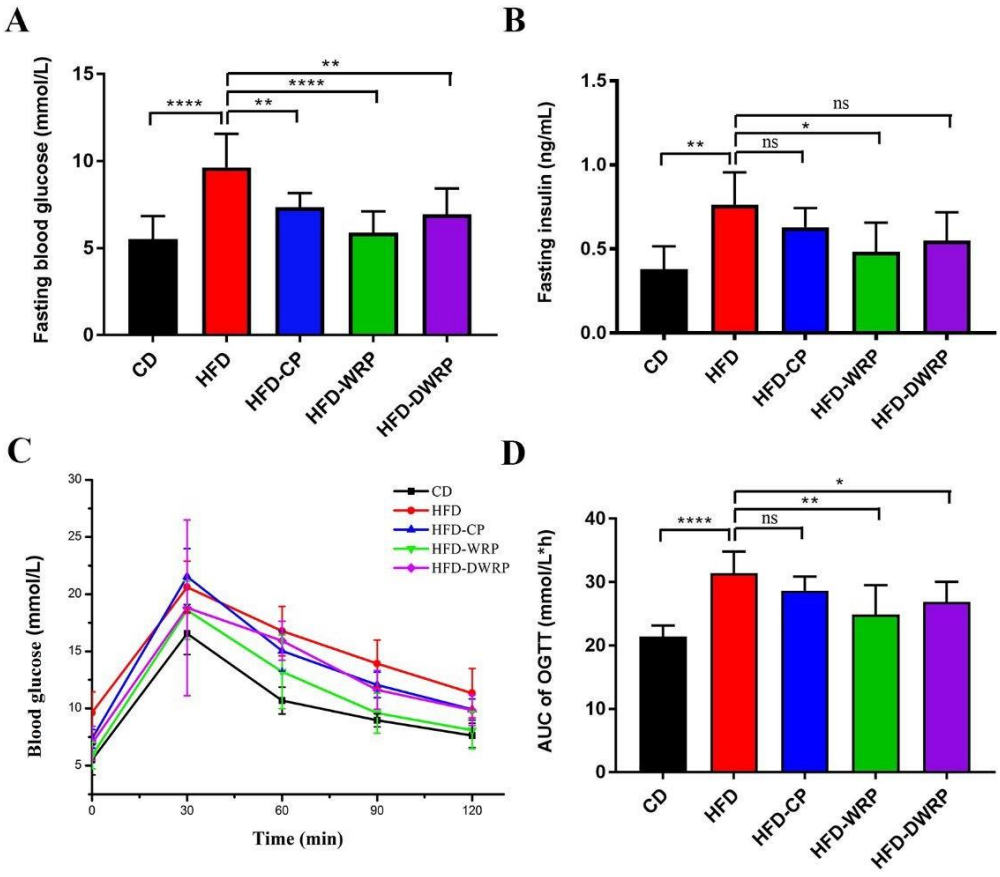
319 thermogenic genes and beige adipocyte-selective markers in iWAT, such as PRDM16

320 ($p < 0.0001$), PGC-1α ($p < 0.01$), ERRα ($p < 0.05$), mtTFA ($p < 0.05$), Tmem26 ($p <$

321 0.05), CD137 ($p < 0.01$), and Cidea ($p < 0.01$) (**Figure 4B&C**). These findings

322 demonstrated that supplementation of WRP stimulates browning of iWAT and

323 increased adaptive thermogenesis in HFD-fed mice.



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325 **Figure 3.** Effects of CP, WRP and DWRP on the development of insulin resistance in HFD-fed

326 mice. (A) Fasting blood glucose; (B) fasting insulin; (C) blood glucose; and (D) AUC of OGTT are

327 shown. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns, not significant.)

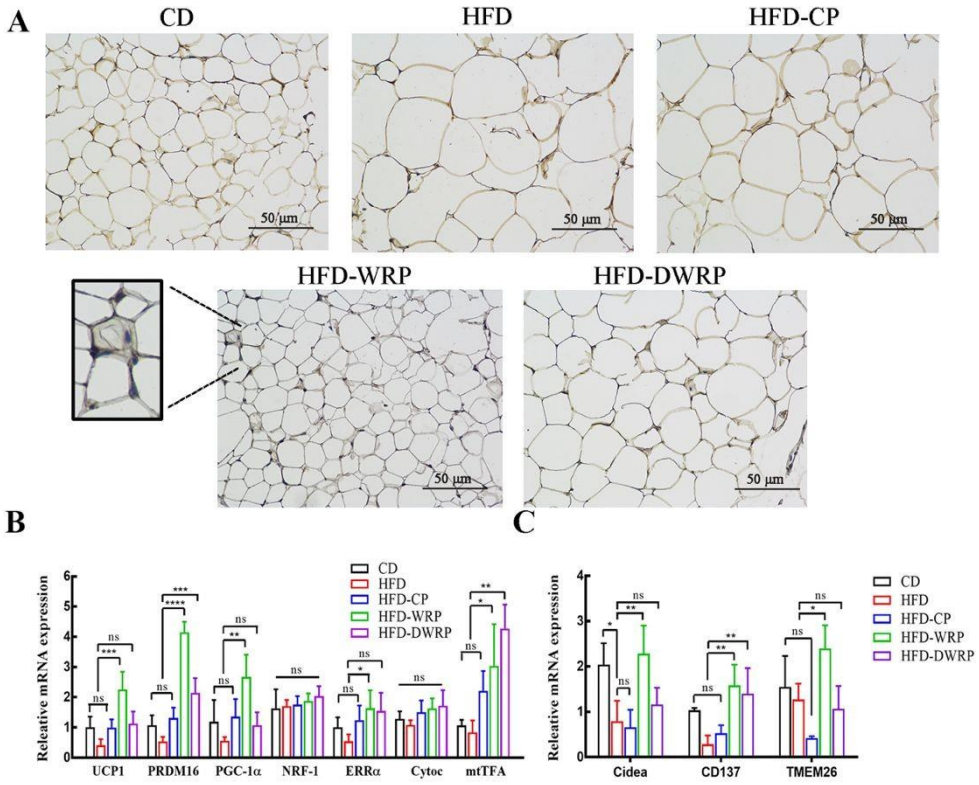


Figure 4. RG-I enriched pectin promoted thermogenesis and browning in iWAT of HFD-fed C57BL/6J mice. (A) Immunohistochemistry for UCP1 protein in iWAT of HFD-fed C57BL/6J mice (magnification 200 times). (B) Thermogenic genes and (C) beige adipocyte-selective markers in iWAT. Relative expression of UCP1, PRDM16, PGC-1α, Nuclear respiratory factor-1 (NRF-1), estrogen-related receptor α (ERRα), Cytochrome c (Cytoc), mitochondrial transcription factor A (mtTFA), Cidea, CD137 and TMEM26 was assessed by qRT-PCR and was Compared to the HFD group. Results are expressed as mean± SD (n ≥ 5). ns, not significant. (*)(**)(***)p < 0.05, 0.01, 0.001, Compared to the HFD group based on one-way analysis of variance (ANOVA) with Duncan's range tests

3.4 WRP Prevents HFD-induced Gut Dysbiosis in Mice

Sequencing analysis of caecal samples from WRP-intervened mice produced an average of 1179±156 observed species compared to the HFD group (960±51) (see on **Table S3**). Next, α-diversity analysis was performed to determine the community richness and diversity. Significant differences in the richness (Chao estimator) and

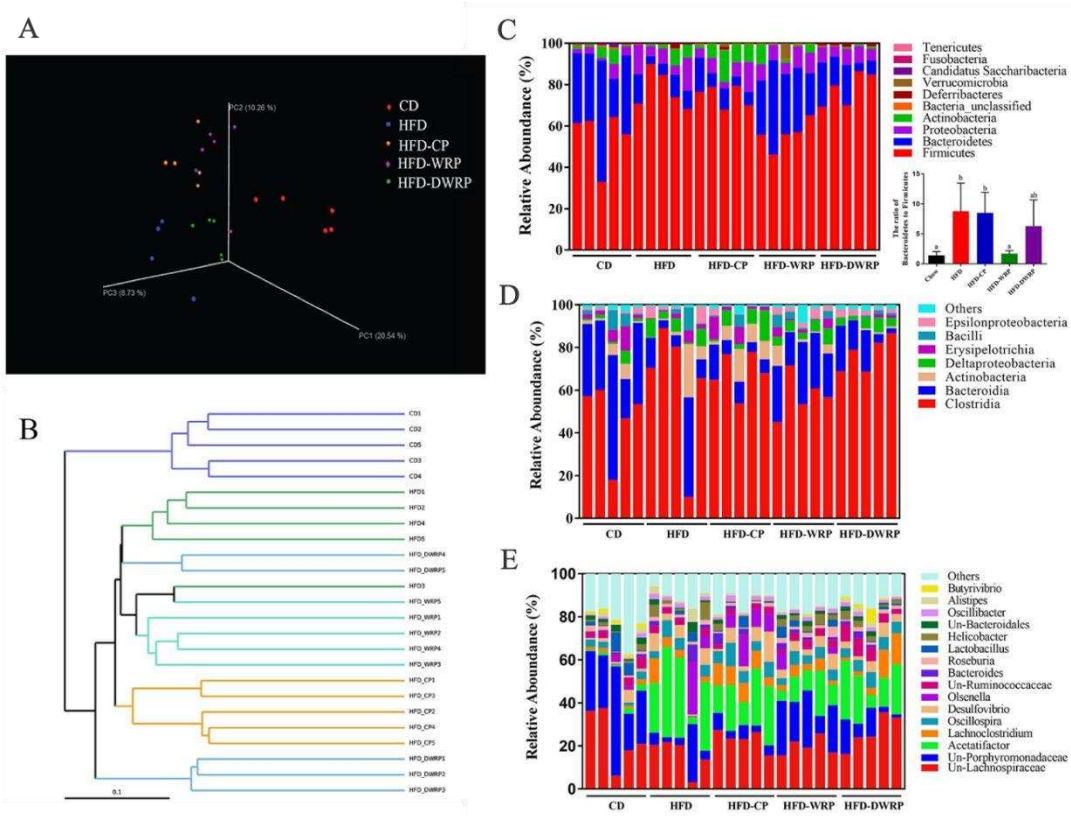
diversity index (Shannon and Simpson index) were detected with the HFD and HFD-WRP groups. In addition, both CP and DWRP shown no remarkable effect on the gut microbiota richness. WRP mitigated the phenomenon of extremely reduced gut microbiota species richness and diversity caused by HFD (see on **Table S3**). The β-diversity analysis based on principal coordinate analysis (PCoA) plots of weighted UniFrac distance and UPGMA showed significant separation was observed between HFD-WRP and HFD groups, and there was also a clear dividing line among the CD, HFD and HFD-WRP groups (**Figure 5A&B**). Collectively, these results indicated that WRP alleviate intensively the gut microbiota dysbiosis in HFD-fed mice compared to CP and DWRP.

At the phylum level (**Figure 5C**), HFD markedly increased the Firmicutes/Bacteroidetes (F/B) ratio to 8.78 compared to 1.44 in CD group. WRP supplementation reduced F/B ratio to 1.72 in obese mice which comparable to that of CD group, while HFD-CP and HFD-DWRP groups with a ratio of 8.52 and 6.30 respectively (**Figure 5C, Table S4**). The relative abundance of Proteobacteria in HFD-fed mice was significantly higher. Nevertheless, Proteobacteria level showed an inconspicuous decline under the intervention of CP, WRP and DWRP. At class level (**Figure 5D**), the Bacteroidia (Bacteroidetes phylum) comprised 36.27%±14.43%, 15.72%±12.05%, 8.83%±4.73%, 23.49%±5.41% and 12.16%±7.12% of gut microbiota in CD, HFD, HFD-CP, HFD-WRP and HFD-DWRP groups. WRP significantly enhanced the abundance of Bacteroidia in the caecum of HFD-fed mice ($p < 0.05$, see

364 on **Table S4**). In contrast, WRP reversed the increase tendency on the abundance of
365 Clostridia (Firmicutes phylum) caused by HFD. Additionally, predominant bacteria in
366 the caecum of mice were Lachnospiraceae (Firmicutes phylum) and
367 Porphyromonadaceae (Bacteroidetes phylum) at family level (see on **Figure S2**). On
368 **Table S4**, the relative abundance of Lachnospiraceae in CD, HFD, and HFD-WRP
369 group were 40.38%±7.23%, 61.98%±8.26%, and 46.42%±7.85%, respectively. On the
370 other side, the relative abundance of Porphyromonadaceae in CD, HFD, and HFD-WRP
371 group were 23.80%±4.64%, 4.14%±1.43%, and 15.92%±6.78%. The results based on
372 the class and family level also explained the decrease of F/B ratio with WRP
373 supplementation. At the genus level, *Un-Lachnospiraceae*, and *Acetatifactor* were
374 predominant bacteria (average relative abundance above 10%), the rest consisted of
375 *Lachnoclostridium, Acetatifactor, Olsenella, Lactobacillus, Butyrivibrio, Alistipes,*
376 *Desulfovibrio,* and *Bacteroides* with an average relative abundance below 5% (**Figure**
377 **5E**). HFD feeding significantly increased the obesity-related bacteria in the cecum of
378 mice, such as *Acetatifactor* and *Olsenella,* while WRP significantly declined the
379 relative abundance of these bacteria (**Figure 4E**, **Table S4**). In addition, the obesity
380 negative-related bacteria (*Barnesiella* and *Butyrivibrio*) were enriched under the
381 intervention of WRP.

382 **Figure 6** shown pathogenic taxa Firmicutes, Clostridia, Clostridiales,
383 Lachospiraceae, *Acetatifactor*. and *Desulfovibrio* were higher in HFD group compared
384 to CD group, while CD group enriched the beneficial phylotypes Bacteroidates,

385 *Bifidobacterium*, *Butyrivibrio*, Porphyromonadaceae, *Alloprevotella*, *Anaerostipes*,
386 and *Anaerotaenia* were enriched in CD group. After treating with CP, there were only
387 2 significant different OUT units. Notably, 19 remarked different OUT units were
388 observed in HFD-WRP group (LDA score threshold > 3 were listed). **Figure 6C**
389 presented the dominate bacteria taxon in the caecum of HFD-fed mice intervened by
390 WRP. Specifically, WRP supplementation significantly increase the abundance of
391 Bacteroidia, *Bacteroidales*, *Barnesiella*, *Butyrivibrio*, *Roseburia*,
392 Prophyromonadaceae, *Flavonifractor, Acetivibrio,* and *Clostridium* cluster IV, while
393 HFD-DWRP group enriched the obesity-related *Butyrivibrio* and *Mucispirillum* genus.
394 The gut dysbiosis induced by HFD was effectively modulated after treatment of WRP,
395 which was due to the more complex RG-I domain stimulating the growth of intestinal
396 microorganisms.
397397



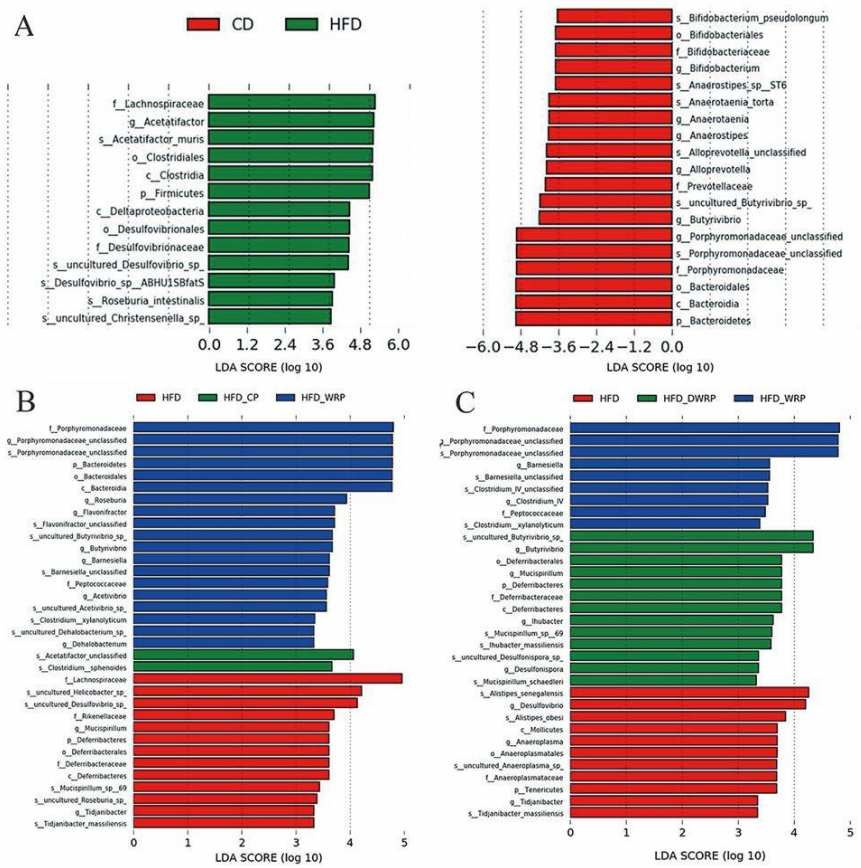
398398

399 **Figure 5.** Structural composition of gut microbiota. (A) PCoA plot of cecal microbiota in HFD-fed

400 mice based on weighted UniFrac metric. (B) UPGMA analysis of cecal microbiota of HFD-fed mice.

401 Cecal microbiota in CD, HFD, HFD-CP, HFD-WRP and HFD-DWRP groups at phylum (C), class

402 (D) and genus (E) level



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404 **Figure 6.** LEfSe comparison of gut microbiota. (A) The LDA score between CD and HFD groups,
405 with LDA score > ±3.6; (B) The LDA score between CD, HFD-WRP and HFD-CP groups, with
406 LDA score > 3.3; (C) The LDA score between CD, HFD-WRP and HFD-DWRP groups, with LDA
407 score > 3.3

408 3.5 WRP Treatment Promotes Generation of SCFAs in HFD-induce Mice

409 The gut microbiota was modulated by WRP supplementations in HFD-fed mice,
410 thus, we investigated the effect of WRP on SCFAs-microbial metabolites [40]. HFD
411 significantly inhibited total SCFAs, acetate and propionate in obese mice caecum but
412 had no effect on butyrate generation (**Figure 7**). CP, WRP and DWRP treatment
413 remarkably prevented the suppression of ceacal total SCFAs, acetate and valerate. The

of acetate and butyrate, while the production of propionate being promoted by arabinose and glucose fermentation [41]. The increase production of propionate in HFD-WRP and HFD-DWRP may be due to the higher content of arabinose and rhamnose in RG-I pectin compared to CP [20]. Compared with CD group, HFD significantly decreased the acetate, butyrate and valerate levels in the colon feces except propionate. Notably, only HFD-WRP group contained significantly higher concentrations of all kinds of SCFAs in obese mice colon compared to HFD group. However, no significant improvement of these SCFAs was observed in both HFD-CP and HFD-DWRP groups.

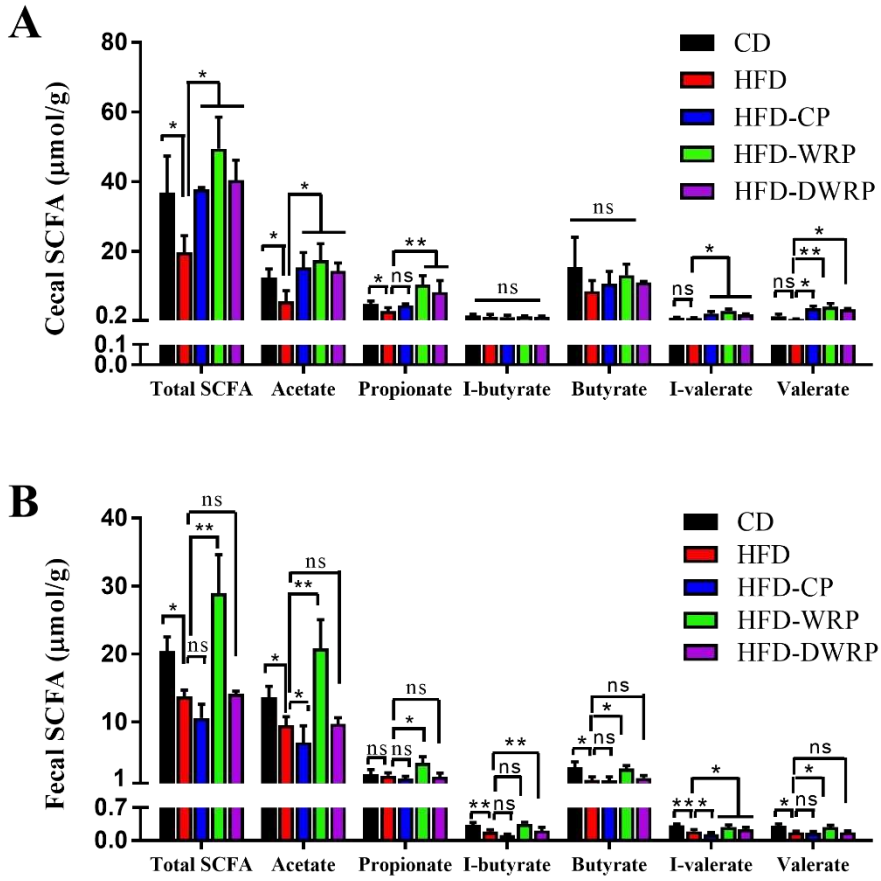
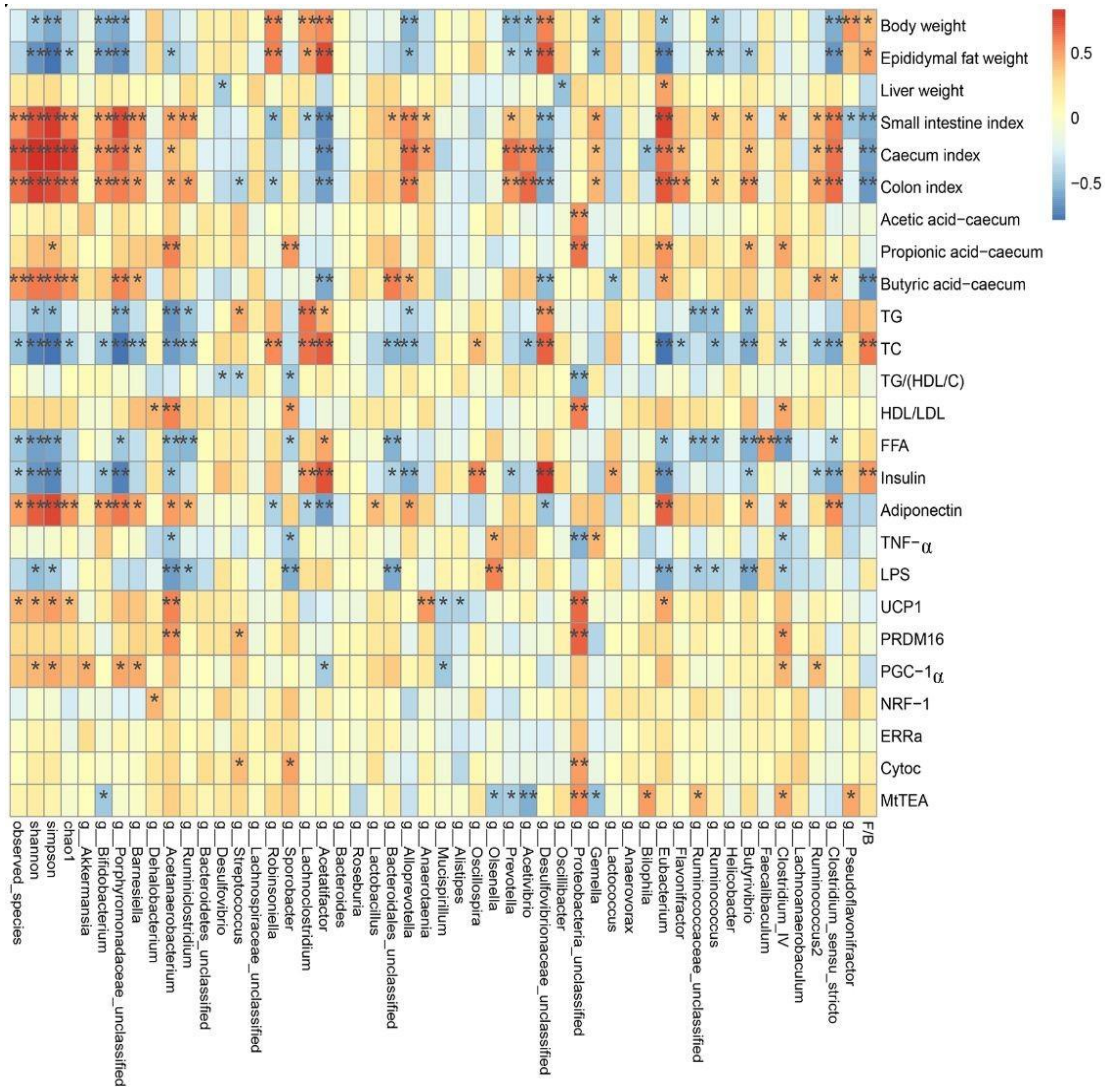


Figure 7. The concentration (μmol/g) of acetic, propionate, butyrate, I-butyrate, valerate, and I-valerate in the cecal contents (A) and colon feces (B) of pectin treated group and chow diet group.

3.6 Correlation of gut microbiota with obesity-related indexes

The Spearman's correlation analysis was performed to clarify the correlation among the microbiota and obesity-related indexes (**Figure 8**). *Pseudoflavonifrator*, *Desulfovibrionaceae*, *Acetatifactor*, *Lachnoclostridium*, and *Robinsoniella* were strongly positively correlated with body weight, epididymal fat weight, TG, TC, and insulin, whereas they were strongly negatively correlated with the gut tissue index ($p < 0.01$ or $p < 0.05$), suggesting that they may be the most significant genera for the development of obesity. In addition, the Simpson index, Bifidobacterium, Prorphyromonadaceae, *Acetanaerobacterium*, *Alloprevotella*, *Prevotella*, *Acetivibrio*, *Acetatifactor*, *Prevotella*, *Gemella*, *Eubacterium*, *Ruminococcus*, *Butyrivibrio*, and *Clostridium_sensu_stricto* were highly positively correlated with the gut tissue index, while they were highly negatively correlated with body weight, epididymal fat weight, TG, total cholesterol and LPS ($p < 0.01$ or $p < 0.05$), indicating that they may play the most important role in the obesity.



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440 **Figure 8.** Heatmap of Sperman's correlation between cecal microbiota and health-related indexes.
441 The indexes of α diversity including observed species; the Shannon, Simpson, and Chao1 indexes;
442 The colors range from blue (negative correlation) to red (positive correlation). Significant
443 correlations are marked by *p < 0.05 and **p < 0.01

444 4. Discussion

445 4.1 RG-I Content, Molecular Weight and Branching Chains are Key Factors 446 of Pectin to Prevent Obesity

447 In our previous research, we found WRP and DWRP had a positive modulation of
448 gut prebiotic microbiota, which stimulated our interests in the potential anti-obesogenic
449 effects of RG-I enriched pectin and its oligosaccharides [29, 31]. However, the gut

450 microbiota of mice changed dramatically under the condition of HFD feeding, and in
451 turn might affect the ferment of pectin in the microbiota. In the present study, we first
452 found that WRP with a high molecular size (531.5 kDa) and RG-I content (70.44%)
453 mitigated against the HFD-induced body weight gain, adipocyte hypertrophy, fatty
454 liver, and hyperlipidemia without suppressing the food intake. On the contrary, no
455 significant decrease in body weight gain was discovered after oral administration of CP
456 (rich in HG domain) and DWRP (depolymerized from WRP). This indicates that the
457 content and molecular structure of RG-I enriched pectin are important for its biological
458 function. Bacterial species characteristic of lean hosts, such as *Butyrivibrio*, *Roseburia*,
459 Prophyromonadaceae, *Barnesiella*, *Flavonifractor*, *Acetivibrio*, and *Clostridium*
460 cluster IV were largely enriched in HFD-WRP group. The anti-obesogenic effects
461 would be explained by the production of SCFAs which could regulate energy
462 homeostasis. Meanwhile, CP and DWRP supplementation barely motivated the
463 enrichment of beneficial bacteria or the diversity of gut microbiota in obese mice.

464 Notably, data published by our group exactly had shown convincingly that CP
465 intervention has not potentially beneficial effect on the gut microbiota of the chow diet
466 feeding mice [29]. We tentatively linked the reason to easy accessibility of pectic
467 backbone (due to less RG-I branches) to microbial degrading enzymes and single
468 monosaccharide composition, which activated much less microbiota species into the
469 fermentation of CP. Multiple species from Bacteroidetes phylum harbored very broad
470 potential to utilize the RG-I domain from pectin [19]. As described by Li el at., RG-I

pectin purified from *Fructus Mori* promoted the growth of *Bacteroides thetaiotaomicron,* a dominant gut bacteria strain shown to be to beneficial the intestinal mucosa of human [42]. The intricate RG-I pectin comprised complex side-chain components (arabinan, galactan, and arabinogalactan) contributed significantly to the gut microbiota fermentation and favourable changes in microbiota composition [18, 28]. Therefore, the high RG-I domain content contributed significantly to the capacity of pectin in HFD-induced obesity modulation.

As highlighted by others, pectic oligosaccharides better promoted growth of beneficial bacteria and had the potential to reduce metabolic conditions such as obesity [11, 27, 43]. In here, WRP supplementation mitigated the gut microbiota of HFD-fed mice dramatically. Paradoxically, no modulation effect of gut microbiota was observed in HFD-DWRP group (**Figure. 6**). Specifically, many reports have confirmed that a greater proportion of Ara and Gal in RG-I pectin promoted the growth of *Bacteroides* species [28-30]. Compared to WRP (Rha: (Gal+Ara) ratio of around 1:20), DWRP (Rha: (Gal+Ara)=1:6) had much less degree of Ara and Gla side chains, so large Mw WRP might better regulate the gut microbiota of obese mice [44]. It's also worth noting that the fermentation effect of DWRP in obese mice gut may be different from pectic oligosaccharides which mainly composed of GalA studied in most articles. Concretely, *Barnesiella*, *Clostridium* cluster IV which negatively correlated to obesity were restored in HFD-WRP group, while HFD-DWRP group enriched the *Mucispirillum* genus (**Figure 6**). Since the increased levels of *Mucispirillum* was closely correlated

with obesity, which suggested that no anti-obesity effect was observed in HFD-DWRP group may due to the *Mucispirillum* induced gut dysbiosis [45]. This could also give a clue as to why DWRP could not inhibit the HFD-induced body weight gain: the low branching degree structural RG-I domain and low Mw of DWRP led a halfway rectification of gut dysbiosis in obese mice.

Our results showed that highly branched RG-I enriched pectin with large molecular size is a strong candidate in prevent of HFD-induced obesity.

4.2 WRP alleviates the HFD-induced Obesity via regulation gut dysbiosis and adipocytes thermogenesis

In this study, HFD caused an increase in the Firmicutes/Bacteroidetes ratio (F/B), which was reported to be related to obesity [46]. Whereas WRP supplementation reduced the F/B ratio remarkably (**Table 1**, **Figure 5**), since the capacity to utilize RG-I was widespread among colonic Bacteroidetes but relatively uncommon among Firmicutes [20]. Furthermore, we observed changes in the level of Lachospiraceae, *Acetatifactor*, *Desulfovibrio*, *Olsenella*, *Alistipes*, *Mucispirillum* were higher in HFD group. Bacteroidales, *Butyrivibrio*, *Roseburia*, Prophyromonadaceae, *Barnesiella*, *Flavonifractor*, *Acetivibrio*, and *Clostridium* cluster IV, which were restored with WRP supplementation. With regard to the Firmicutes phylum, HFD produced an increase in Lachnospiraceae, which is a potent Firmicutes family related to the regulation of immune and obesity [47]. Here, we observed changes in the level of Lachospiraceae was significant higher in HFD group, but HFD-WRP group presented lower level of Lachnospiraceae. At a genus level, WRP supplementation presented a decrease in

514 *Acetatifactor* which was significantly increased by HFD. As described by several
515 authors, *Acetatifactor* was found abundant in the intestine of obese mouse and has been
516 related to arthritis progression in mice [1, 48-49]. Specially, key members of luminal
517 *Clostridium* cluster IV were known to be butyrate producers, which are closely
518 associated with both obesity and weight loss clearly [50-51]. The presence *Clostridia*
519 species from clusters IV of has consistently been shown leads to the increase of
520 regulatory T cells which secrete the anti-inflammatory cytokine IL-10 [52]. In line with
521 this, WRP supplementation led to enhanced *Clostridium* cluster IV enrichment, which
522 was positively related to butyrate in caecum and negatively related to TNF-α ($p < 0.05$)
523 and LPS ($p < 0.05$) (**Figure 8**) .

524 The most remarkable differences caused by WRP was in the Bacteroidetes phylum.
525 Consistently, we also found that WRP led to enhanced Bacteroidales abundance, which
526 was positively related to butyrate in caecum and gut index ($p < 0.05$), and negatively
527 correlated with the body weight gain, TC ($p < 0.01$), insulin ($p < 0.05$), and LPS levels
528 ($p < 0.01$). Furthermore, HFD caused by a decrease in the abundance of the
529 Porphyromonadaceae family and, in particular, the *Barnesiella* genus which has been
530 proved to positively impact on reducing the pro-inflammatory milieu in the gut [53]. Chiu
531 et al. has reported higher levels of *Barnesiella* in lean individuals compared to obese
532 patients [54]. These variables were consistently confirmed by us, WRP elevated the levels
533 of *Barnesiella* and Porphyromonadaceae which were negatively correlated with body
534 weight and TC ($p < 0.05$), positively correlated with gut index ($p < 0.05$). In contrast,

535 an increase in *Alistipes* genus of HFD-fed mice was observed in this study. Gram-
536 negative *Alistipes* was the foremost noteworthy taxon related to obesity and was found
537 to be expanded beneath HFD conditions [55].

538 In addition, we have also detected an enrichment of *Roseburia*. The *Roseburia*
539 (Actinobacteria phylum) was a typical SCFAs-producing bacterium within the intestine
540 that can anaerobically degrade polysaccharides such as arabinoxylan into SCFAs,
541 which protected against HFD-induced obesity by activating the release of gut hormone
542 and enhancing intestinal barrier function [45, 56]. Several reports pointed out that the
543 content of acetic acid and propionic acid were associated with an increased abundance
544 of *Olsenella* in pectin or fructo-oligosaccharides-fed mice [57-58]. However, As
545 highlighted by some researchers, *Olsenella* could be a target bacterial flora for obesity
546 since it was enriched considerably in HFD-fed mice [48, 59-60]. Based on our findings, the
547 abundance of *Olsenella* was enriched by HFD while it reduced to the level close to the
548 CD group after WRP supplementation. Our results exhibit high level of *Acetivibrio* in
549 HFD-WRP group and negatively correlated to body weight ($p < 0.01$), TC ($p < 0.05$).
550 Data published by Yang et al. shown maize-derived feruloylated polysaccharides
551 significantly increased *Acetivibrio* and controlled the weight gain induced by HFD [61].
552 One hypothesis is that multiple glycoside hydrolase secreted from *Acetivibrio*
553 hydrolyzed arabinose side chain of WRP and stimulated SCFAs production, as cecal
554 butyrate level positively correlated with *Acetivibrio* [62].

555 Previous research has illustrated that HFD triggered inflammatory responses,

556 defecting gut barrier integrity, and increasing the cytokines [55]. In our model, WRP
557 treatment significantly decreased the level of serum LPS and TNF-α in HFD-fed mice.
558 Specifically, the LPS-producing microbiota such as *Desulfovibrio* and *Mucispirillum*
559 became abundant after HFD feeding [48]. Notably, *Mucispirillum* has been described as
560 a mucus-associated bacterium that bursts during inflammation in HFD-induced obese
561 mouse model [63]. *Desulfovibrio* is one kind of genus belonging to Desulfovibrionaceae
562 and Proteobacteria, positively related to obesity-induced inflammation [64]. Moreover,
563 the correlation analysis further verified that the significant correlation between
564 *Desulfovibrio* and these obesity-related indexes (**Figure 8**).

565 On the other hand, butyrate and other short chain fatty acids are known to inhibit
566 fat accumulation and adipocyte dysfunction [65]. Here, we found that WRP intervention
567 significantly enriched the fecal and ceacal butyrate. Moreover, the correlation analysis
568 verified the positive association of microbiota diversity with ceacal butyrate. Several
569 studies reported that *Barnesiella*, *Roseburia*, *Clostridium* cluster IV, and *Butyrivibrio*
570 which are highly associated with the production of SCFAs, especially the synthesis of
571 butyrate [45]. Moreover, suppression HFD-induced body weight gain of WRP could
572 related to SCFAs secreted by relevant microbes (**Figure S2**). In line with this, it has
573 been revealed that WRP treatment enriched these microbes and prevented the HFD-
574 induced gut dysbiosis in mice.

575 Interestingly, a crucial analysis revealed that SCFAs can stimulated the white
576 adipose tissue browning [66]. Consistently, we also found WRP supplementation reduced

the white adipose tissue's weight, upregulated UCP1 expression, a specific protein uncouples respiration from ATP synthesis and generates heat (**Figure 4**). Moreover, WRP showed potential in fat browning by activating PGC-1α, a master regulator of mitochondrial biogenesis [67]. Compared with the HFD group, gene expressions of the beige adipocyte-selective markers including UCP1, TMEM26, CD137, and Cidea were markedly increased in HFD-WRP group (**Figure 4B&C**). The above results indicated that the supplementation of WRP triggered the browning of iWAT in HFD-fed mice.

Overall, we found that WRP can attenuate HFD-induced obesity, inflammation and gut dysbiosis through combined effects of the white adipose browning and gut microbiota modulation, while the CP (HG dominated pectin) and DWRP (less branched RG-I oligosaccharide) have only limited effect on resistance to HFD-induced obesity. Our findings convinced that highly branched RG-I domain enrichment are essential for pectin mitigating against the HFD-induced obesity.

Abbreviations : BAT: Brown adipose tissue; CD: Normal chow diet; DWRP: depolymerized fraction of RG-I enriched pectin from citrus segment membrane; HDL: High density lipoprotein; HFD: High fat diet; OTU: Operational taxonomic units; OGTT: Oral glucose tolerance test; PGC-1α: Peroxisome proliferators-activated receptor-γ coactivator 1-alpha; RG-I: Rhamnogalacturonan-I; LDL: Low density lipoprotein; LEfSe: Linear discriminant analysis effect size; TG: Total triacylglycerol; TC: Total cholesterol; TNF-α: Tumor necrosis factor-α; UCP1: Uncoupling protein 1;

599 WAT: White adipose tissue; WRP: RG-I enriched pectin from citrus segment

600 membrane.

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604

605 Ethics statement

606 The animal study was reviewed and approved by Zhejiang Chinese Medicine

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612 **Author contributions**

613 S.G.C. conceived the study; K. Z. wrote the manuscript; G.Z.M. designed 614 experiments, performed the animal studies and statistical analysis; D.M.W. contributed 615 to sample preparation; C.X.Y. helped to perform the animal studies; H. X. helped to 616 revise the manuscript; X.Q.Y., R. J. L. and C.O. interpreted the data; S.G.C. critically

617 revised the manuscript. All authors read and approved the final manuscript. 618

Supporting information

619 The following are available online. Table S1: The compositions and energy 620 densities of the diets; Table S2: The specific formula of the high-fat diet; Table S3: 621 Diversity and richness of cecal microbiota in CP, WRP and DWRP supplements alter 622 the diversity; Table S4: The abundance of key phylotypes of gut microbiota in HFD-

623 fed C57BL/6J mice; Table S5: PCR primers used in this study; Figure S1: Weekly food

624 intake (A) and average food intake (B) of mice in response to dietary CP, WRP and 625

DWRP; Figure S2: LEfSe comparison of cecal microbiota. LDA score of cecal

626 microbiota between the CD, HFD, HFD-CP, HFD-WRP and HFD-DWRP groups.

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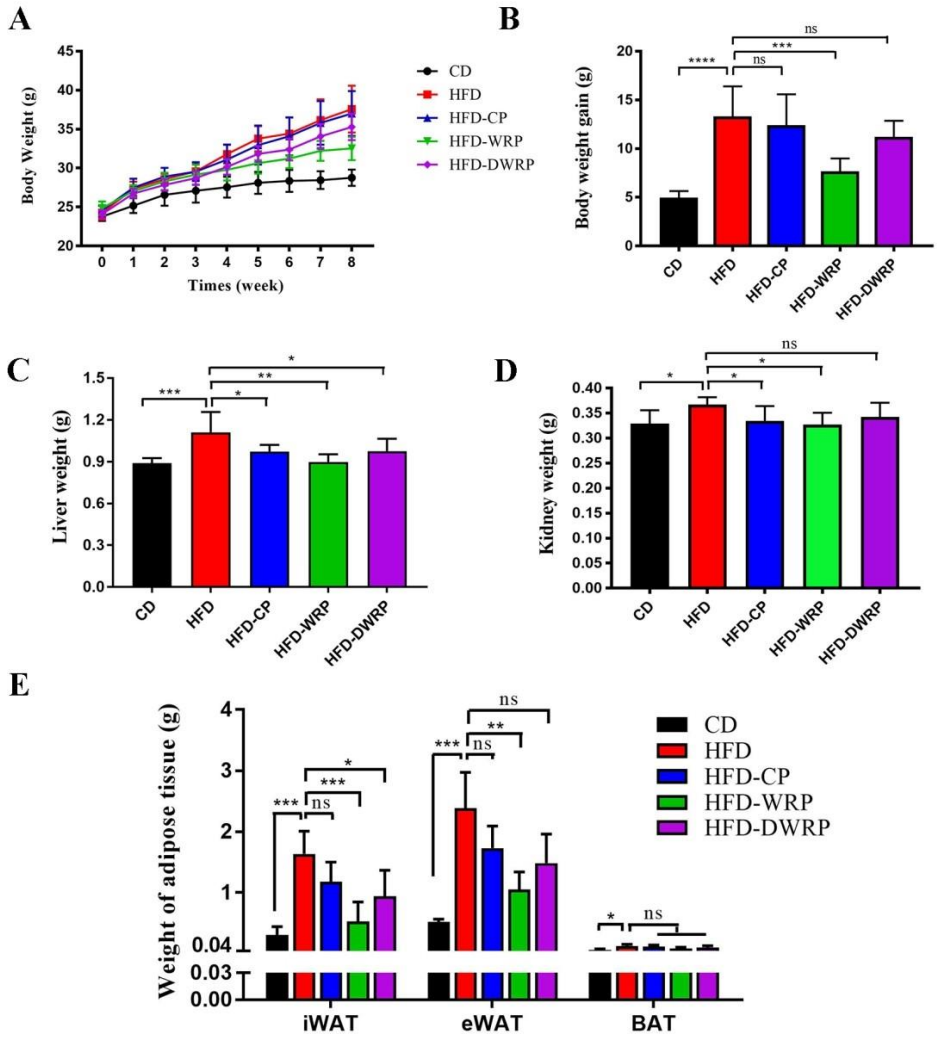
Tables

Table 1 Effects of RG-I pectin on serum biochemical parameters in C57BL/6 Mice

Groups	CD	HFD	HFD-CP	HFD-WRP	HFD-DWRP
TC (mmol/L)	4.99±0.65 ^{a}	9.66±0.75 ^{d}	8.58±0.76 ^{c}	5.28±0.42 ^{a}	6.43±0.56 ^{b}
TG (mmol/L)	1.15±0.27 ^{a}	1.49±0.11 ^{b}	1.41±0.21 ^{ab}	1.26±0.30 ^{ab}	1.27±0.15 ^{ab}
HDL-C (mmol/L)	1.52±0.27 ^{a}	2.10±0.37 ^{ab}	2.12±1.02 ^{bc}	2.65±0.62 ^{c}	2.81±1.47 ^{c}
LDL-C (mmol/L)	1.99±0.45 ^{a}	4.00±1.78 ^{b}	2.94±0.39 ^{a}	2.01±0.54 ^{a}	2.29±0.77 ^{a}
FFA (mmol/L)	1.27±0.43 ^{ab}	1.72±0.12 ^{b}	1.57±0.47 ^{b}	1.33±0.33 ^{ab}	1.20±0.44 ^{a}
Insulin (ng/mL)	0.38±0.12 ^{a}	0.76±0.27 ^{c}	0.61±0.19 ^{bc}	0.50±0.20 ^{ab}	0.56±0.15 ^{ab}
LPS (EU/mL)	0.44±0.02 ^{ab}	0.54±0.05 ^{c}	0.54±0.14 ^{bc}	0.41±0.07 ^{a}	0.39±0.07 ^{a}
TNF-α (ng/mL)	1.29±0.55 ^{bc}	1.58±0.35 ^{c}	0.91±0.39 ^{ab}	0.68±0.19 ^{ab}	0.52±0.12 ^{a}
Adiponectin (ug/mL)	51.03±12.95 ^{d}	27.14±3.62 ^{a}	31.52±3.54 ^{ab}	47.77±9.45 ^{cd}	38.07±4.36 ^{bc}
TG/HDL-C	0.74±0.24 ^{a}	0.75±0.14 ^{a}	1.33±1.25 ^{a}	0.48±0.05 ^{a}	0.55±0.19 ^{a}
HDL-C/LDL-C	0.78±0.11 ^{a}	0.57±0.22 ^{a}	0.63±0.33 ^{a}	1.38±0.43 ^{b}	1.54±1.19 ^{b}

The means with different superscript letters (a, b, and c) represent statistically significant results ($p < 0.05$) based on one-way analysis of variance (ANOVA) with Duncan's range tests, whereas means labeled with the same superscript correspond to results that show no statistically significant differences.


A
Body Weight (g)
Times (week)
CD
HFD
HFD-CP
HFD-WRP
HFD-DWRP
B
Body weight gain (g)
C
Liver weight (g)
D
Kidney weight (g)
E
Weight of adipose tissue (g)
iWAT
eWAT
BAT

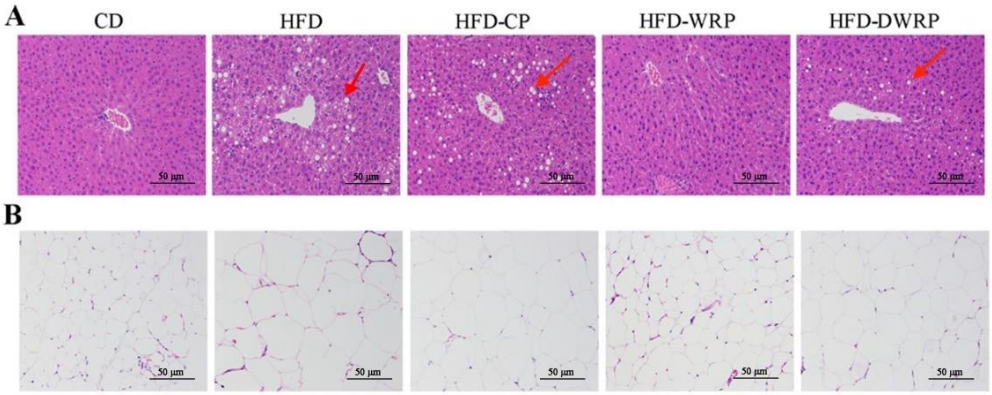
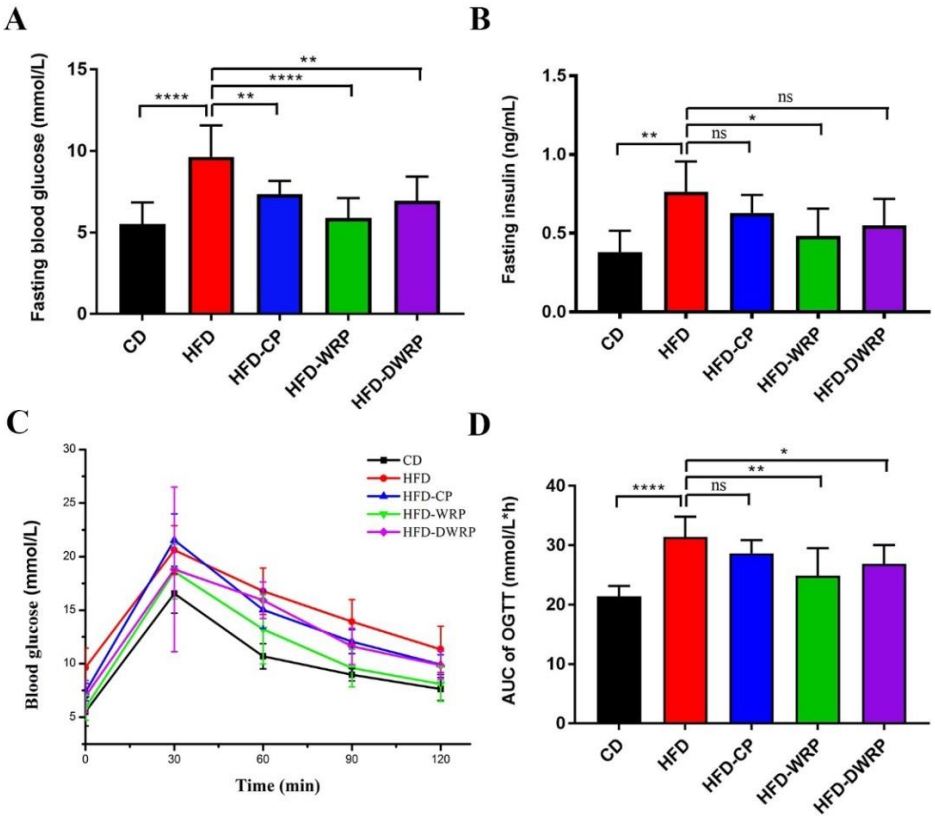


Figure 2. Histological assessment of livers (A) and epididymal white adipose tissue (B) in HFD-induced obesity mice (H&E stain, 200□magnification)


A
Fasting blood glucose (mmol/L)
15
10
5
0
****
**
****
**
CD
HFD
HFD-CP
HFD-WRP
HFD-DWRP
B
Fasting insulin (ng/mL)
1.5
1.0
0.5
0.0
**
ns
*
ns
CD
HFD
HFD-CP
HFD-WRP
HFD-DWRP
C
Blood glucose (mmol/L)
30
25
20
15
10
5
0
30
60
90
120
Time (min)
CD
HFD
HFD-CP
HFD-WRP
HFD-DWRP
D
AUC of OGTT (mmol/L*h)
40
30
20
10
0
****
**
ns
*
CD
HFD
HFD-CP
HFD-WRP
HFD-DWRP

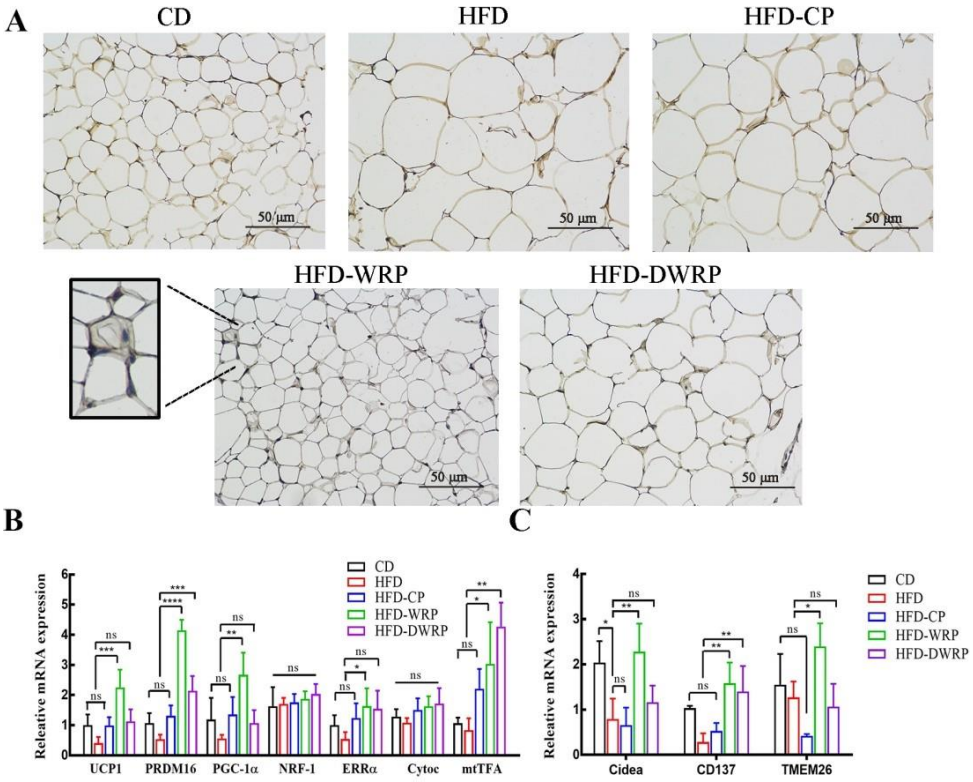


Figure 4. RG-I enriched pectin promoted thermogenesis and browning in iWAT of HFD-fed C57BL/6J mice. (A) Immunohistochemistry for UCP1 protein in iWAT of HFD-fed C57BL/6J mice (magnification 200 times). (B) Thermogenic genes and (C) beige adipocyte-selective markers in iWAT. Relative expression of UCP1, PRDM16, PGC-1α, Nuclear respiratory factor-1 (NRF-1), estrogen-related receptor α (ERRα), Cytochrome c (Cytoc), mitochondrial transcription factor A (mtTFA), Cidea, CD137 and TMEM26 was assessed by qRT-PCR and was Compared to the HFD group. Results are expressed as mean± SD (n≥5). ns, not significant,; (*)(**)(***)$p < 0.05$, 0.01, 0.001, Compared to the HFD group based on one-way analysis of variance (ANOVA) with Duncan's range tests

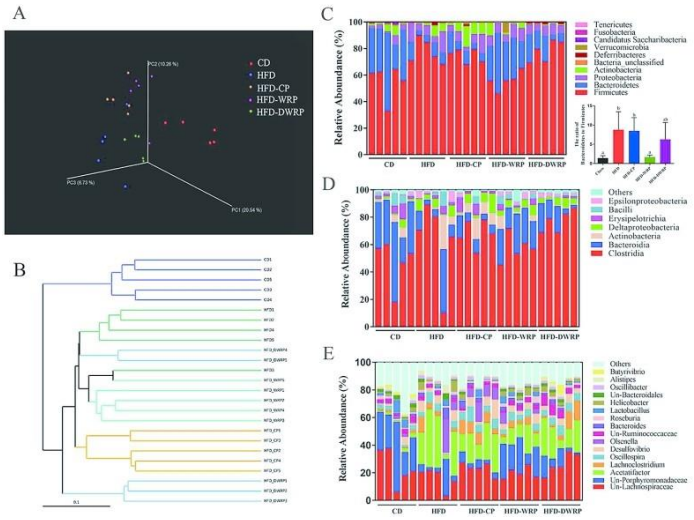


Figure 5. Structural composition of gut microbiota. (A) PCoA plot of cecal microbiota in HFD-fed mice based on weighted UniFrac metric. (B) UPGMA analysis of cecal microbiota of HFD-fed mice. Cecal microbiota in CD, HFD, HFD-CP, HFD-WRP and HFD-DWRP groups at phylum (C), class (D) and genus (E) level

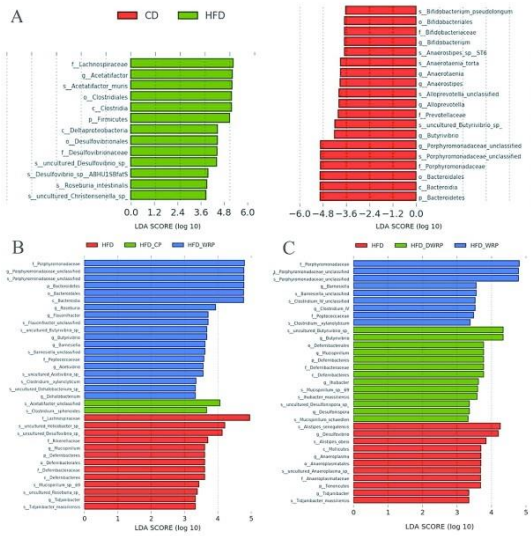


Figure 6. LEfSe comparison of gut microbiota. (A) The LDA score between CD and HFD groups, with LDA score > ±3.6; (B) The LDA score between CD、HFD-WRP and HFD-CP groups, with LDA score＞3.3; (C) The LDA score between CD、HFD-WRP and HFD-DWRP groups, with LDA score＞3.3.

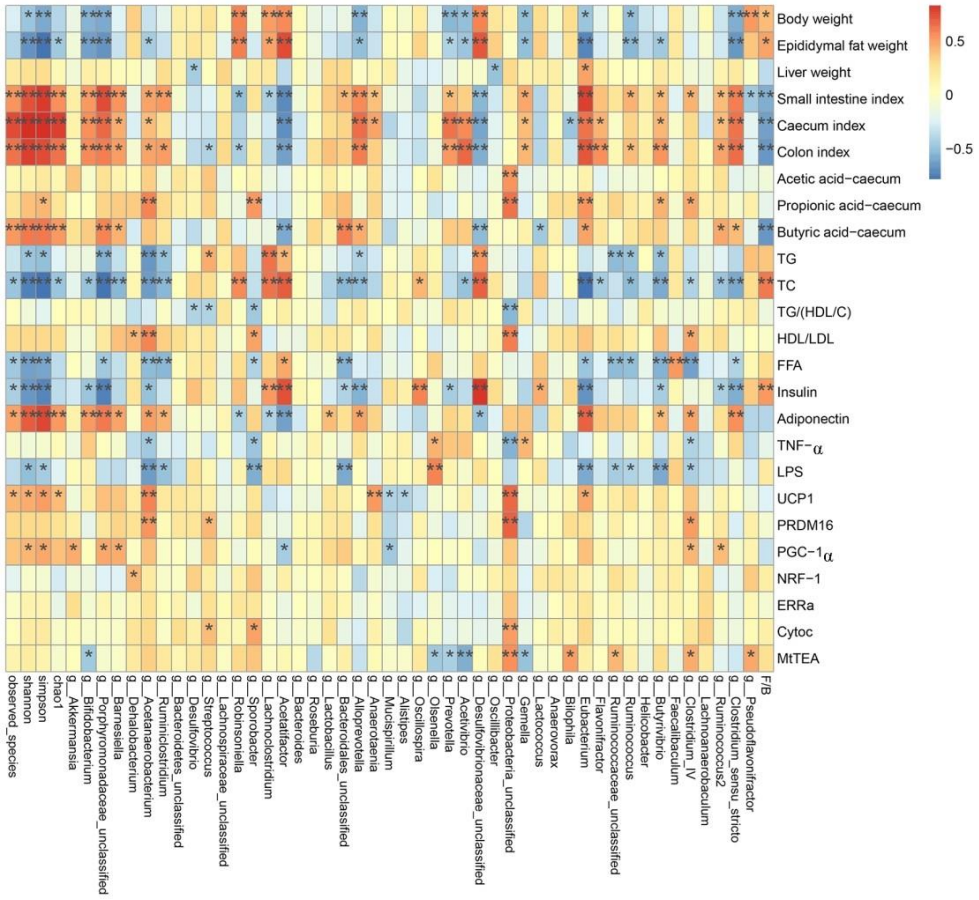


Figure 7. Heatmap of Sperman's correlation between cecal microbiota and health-related indexes. The indexes of α diversity including observed species; the Shannon, Simpson, and Chao1 indexes; The colors range from blue (negative correlation) to red (positive correlation). Significant correlations are marked by *p < 0.05 and **p < 0.01