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Depolymerized RG-I enriched pectin from citrus segment membrane modulates gut microbiota, increases SCFAs production, promotes the growth of *Bifidobacterium spp.*, *Lactobacillus spp. and Faecalibaculum spp.*

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Abstract:

Rhamnogalacturonan-I (RG-I) enriched pectin (WRP) was recovered from citrus processing water by sequential acid and alkaline treatment in a previous study. RG-I enriched pectin was proposed as a potential supplement for functional food and pharmaceutical development. However, previous studies illustrated favorable modulations of gut microbiota by RG-I enriched pectin were based on *in vitro*, changes in the overall microbial structure and whether there is a structure-dependent way in modulation of gut microbiota remain largely enigmatic. In the present study, modulations of gut microbiota by commercial pectin (CP), WRP and its depolymerized fraction (DWRP) with different RG-I content and Mw were compared *in vivo*. Revealed by 16s rRNA high-throughput sequencing, WRP and DWRP mainly composed of RG-I, modulated the gut microbiota in a positive way. WRP significantly increased the abundance of prebiotic such as *Bifidobacterium* spp., *Lactobacillus spp.*, while DWRP increased SCFAs producers including species in Ruminococcaceae family. By maintaining more balanced gut microbiota composition and enriching some SCFA producers, dietary WRP and DWRP also elevated SCFAs content in the colon. Collectively, our findings offer new insight into structure-activity correlation of citrus pectin and provide impetus towards the development of RG-I enriched pectin with small molecular weight for specific use in health-promoting prebiotics ingredient and therapeutic.

Keywords: RG-I; citrus pectin; gut microbiota; SCFAs; prebiotic

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1. Introduction

Canned citrus segments, as an instant and delicious fruit product, enjoying a great popularity all over the world with an annual trade value of almost $900 million (source: UN Comtrade). China accounts for nearly 70% canned citrus segments on the international market, is the largest citrus planting and harvesting country in the world [1]. However, up to one million pounds of solid waste and effluent water were produced in citrus canning processing factories annually, latter of which was actually segment membrane solution, contains high organic substances (polysaccharides principally), representing both an economic and an environmental challenge [2]. Our previous study recovered pectin with higher RG-I content from basic water during the segment membrane removal process, occurring in citrus canning factories [2].

Pectin is mainly composed of structurally distinct regions including homogalacturonan (HG), rhamnogalacturonan (RG-I), rhamnogalacturnan (RG-II) [3]. HG, whose backbone consisted of α-1,4 linked galacturonic acid that is partially methyl-esterified at C-6 and *O*-acetylated in positions 2 and 3, occupying about 65% of commercial pectin. RG-I, accounting for 20-35% of commercial pectin, is based on a backbone being formed from a repeating disaccharide of [→2)-α-L-Rha*p*-(1→4)-α-D-GalA*p*-(1→] residues with neutral side chains attached to the O-4 position and sometimes the O-3 position of α-L-Rhap backbone units [4]. Extraction of commercial pectin uniformly aims at maintaining more HG content for best quality control and better application in the food industry as a gelling agent, thickening agent, stabilizer, emulsifier, and color-protecting agent[5-7]. However, during

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the harsh extraction condition, RG-I region is mostly destroyed [8], which is gaining attention increasingly because of its bioactivity including anti-galectin-3 activity (a lectin associated with cancer progression and metastasis) [7], antitumor activity [9], immunomodulation ability [10], and prebiotic activity [11, 12]. Structure features including RG-I content, neutral sugar content, neutral side chains length and variable linking types, and molecular weight (Mw), determined to some extent the suitability of specific application of the pectin per se [5, 13].

The structural diversity of pectin and the increasing concern in RG-I region arouse more people rethinking the relationship between pectin's structure and function recently. Gut microbiota plays fundamental roles in modulation of host's metabolism, nutrition and immunity [14, 15]. Gut microbiota impairment is tightly linked to various disease and metabolic disorders including inflammation bowel diseases [16, 17], diabetes [18, 19], non-alcoholic fatty liver disease (NAFLD) [20], hypertension [21], obesity [22, 23], and cardiovascular disease [23, 24]. Restoring the disrupted gut microbiota through personalized colonization of prebiotics represents an effective strategy for the management of gut microbiota-related diseases [25, 26]. Accumulating evidence illustrated that non-digestible carbohydrates in daily diet can alleviate and treat disease through modulating the gut microbiota composition [27]. Therefore, elucidating the effect of pectin as a dietary supplement on gut microbiota would be significantly beneficial to clarify its functional mechanisms. RG-I region is highly complex part of pectin and is reported to be potentially used as prebiotics and gastrointestinal drug delivery microcapsules [28, 29]. Previous studies reported that citrus pectin has *in vitro*

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prebiotic activity by stimulating the growth of *Bifidobacterium bifidum*, *Lactobacillus paracasei*, *Bacteroides plebeius*, and *Ruminococcus gnavus* during *in vitro* fermentation [12, 13, 30]. Besides, the favourable changes in microbiota composition after pectin supplementation depended greatly on RG-I content, neutral sugars composition, degree of esterification and branching [13]. However, the structure-function relationship of pectin has few been evaluated *in vivo* before.

Considering the remarkable non-digestibility of citrus pectin in the upper gastrointestinal tract [12, 31, 32], we investigated the relationship between structure of pectin and modulation of gut microbiota *in vivo*. In our study, the basic structure and chain conformation of CP, WRP, and DWRP were investigated by NMR, SEC-MALLS and AFM. C57BL/6J mice were administrated with CP, WRP and DWRP at a dosage of 100mg/kg/day, their effects on gut microbiota composition and SCFAs were studied by 16S rRNA and gas chromatography (GC). The diversity and composition of gut microbiota were compared at the phylum, class, family and genus levels. Meanwhile, the spatial structure difference of gut microbiota was also involved. Our study indicates distinct modulations of gut microbiota by different pectin and provides theoretical foundations for developing RG-I enriched pectin as a health-promoting and therapeutic prebiotics ingredient.

2 Materials and Methods

2.1 Material and preparation of pectin

Citrus segments material (*Citrus unshiu Marc.*) recovered from canning processing basic water was provided by a citrus fruit canning factory in China. The

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material was washed with 95% (v/v) food grade ethanol for 2-3 times to desalt, the pectin was precipited and oven-dried at 55℃ for 24-36h, which was abbreviated as WRP. DWRP was degraded from WRP based on the metal-free Fenton reaction, relying on H_2O_2/ascorbic acid, adapted from a previous study [33]. Briefly, the reaction conditions were 200 mM H_2O_2, 20 mM ascorbic acid and temperature at 45℃. The WRP (5000mg) starting material was dissolved in 1000 mL ultrapure water, H_2O_2 and ascorbic acid was then added with mixing and the reaction was maintained at 45℃ for 30min. The depolymerized products were desalted by dialysis using a 500 Da cut-off membrane for 72h under flowing water, concentrated and subsequently lyophilized to obtain refined samples for further study.

2.2 Structural analysis of pectin

2.2.1 Primary structure of CP, WRP, and DWRP

Primary structure of the three pectin was studied by HPLC, FTIR, and NMR. Monosaccharide standards, 1-phenyl-3-methyl-5-pyrazolone (PMP), D_2O and commercial pectin from citrus peel (abbreviated as CP) were all purchased from Sigma-Aldrich (Shanghai, China). All other used chemicals were of analytical grade. Chemical compositions of CP, WRP and DWRP (Table 1) were determined according to previous described methods with modifications [34]. FTIR and NMR analysis were conducted based on the method of a previous report [8].

2.2.2 Chain conformation of CP, WRP, and DWRP

Chain conformation of the three pectin was studied by SEC-MALLS and AFM. For size exclusion chromatography with multi-angle laser light scattering

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(SEC-MALLS) analysis, pectin was dissolved in 0.2M NaCl solution at a concentration of 5mg/mL. 50μL of solution was injected through a sample loop after filtering through a syringe-filter (pore size of member was 0.22μm). The molar mass and root mean square (RMS) radius of gyration was determined through high-performance (HP) size exclusion chromatography (SEC) equipped with multi-angle laser light scattering (MALLS) (Wyatt Dawn Heleos-II, USA) and RI detector at 25 ℃. Isocratic elution with 0.2M NaCl solution at a flow rate of 0.5mL/min was performed on combined columns including Shodex OH SB-G (pre-column), Shodex SB-806 HQ and Shodex SB-804 HQ (Showa Denko KK, Japan). The molar mass was calculated based on the dn/dc value of 0.0880mL/g.

For atomic force microscope (AFM) analysis, pectin was dissolved in ultrapure water at a concentration of 1mg/mL with continuous stirring for 2 h incubating at 60°C. The stock solutions were next diluted by sodium dodecyl sulphate (SDS) solution, obtaining a mixed solution containing pectin and SDS both at a concentration of 10 μg/mL. The diluted solutions were then stirred for 24h and filtered through a syringe-filter (pore size of member was 0.22μm). After the samples were ready, 10μL pectin solution was moved to three freshly cleaved mica substrates using micropipette respectively. Then three mica substrates were air-dried and observed by AFM (XE-70, Park Scientific Instruments, Suwon, Korea) using taping mode in air at room temperature (humidity: 50%-60%). The probe is a classical silicon cantilever (Appnano AN-NSC10) with a spring constant of 37N/M and a resonance frequency of approximately 300 kHz. Nanoscope Analysis software was

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used for image manipulation.

2.3 Animals and experimental design

All procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University School of Medicine. 40 C57BL/6J male mice (SPF, 6-8 weeks old, IACUC-20180917-02) were purchased from Zhejiang Chinese Medical University Laboratory Animal Research Center. The mice were kept under the specific-pathogen-free conditions in a 12-hour light/dark cycle with free access to food and sterile drinking water (DW) in a temperature-controlled room (21°C±2°C). Before starting the experiments, the mice were housed 25 per cage and exchanged multiple times to make fecal microbiomes homogeneous. After one week of acclimatization, the mice were randomly divided into four groups: CD group, CD-CP group, CD-WRP group and CD-DWRP group (10 mice per group, 5 mice per cage) and fed for 9 weeks with standard chow diet (Rodent diet, SHOBREE, Jiangsu Synergy Pharmaceutical Biological Engineering Co, Ltd, Nanjing China). Mice were supplemented daily with 200μL of sterile water (vehicle), CP (100mg/kg·d), WRP (100mg/kg·d) or DWRP (100mg/kg·d) respectively via intragastric gavage. The compositions and energy densities of the diets are listed in Table S2. The body weight was measured weekly, food intake was daily recorded. At the time indicated, the mice were fasting for 12 hours and anaesthetised, the whole blood was collected from the orbital plexus. Then, the mice were sacrificed. Epididymal white adipose tissues, liver, small intestine, caecum and colon samples were removed and weighed. Meanwhile, caecal contents were collected in Eppendorf tubes and immediately

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stored at -80℃ for subsequent analysis. The intestinal tissue index was calculated using the following formula: intestinal tissue index = intestinal tissue weight/body weight.

2.4 Biochemical analysis and cytokine measurements of serum

Serum was isolated by centrifugation (4℃, 12,000g, 10min). Serum total cholesterol and triacylglycerol concentrations were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. TNF-α, LPS and insulin levels were then quantified using commercial ELISA kits (Cloud-clone Crop, USA).

2.5 16S rDNA analysis

Five samples of each group were selected randomly for 16S rRNA analysis. DNA was extracted from the caecal 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') [35]. The 5' ends of the primers were tagged with specific barcodes 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

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

Samples were sequenced on an Illumina MiSeq platform according to the manufacturer's recommendations, provided by LC-Bio. Paired-end reads was assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH. Quality filtering on the raw tags were performed under specific filtering conditions to obtain the high-quality clean tags according to the FastQC (V 0.10.1). Chimeric sequences were filtered using Verseach software (v2.3.4). Sequences with $\geqslant$ 27% similarity were assigned to the same operational taxonomic units (OTUs) by Verseach (v2.3.4). Representative sequences were chosen for each OTU, and taxonomic data were then assigned to each representative sequence using the RDP (Ribosomal Database Project) classifier. The differences of the dominant species in different groups, multiple sequence alignment were conducted using the PyNAST software to study phylogenetic relationship of different OTUs. OTUs abundance information were normalized using a standard of sequence number corresponding to the sample with the least sequences. Alpha diversity is applied in analyzing complexity of species

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diversity for a sample through 4 indices, including Chao1, Shannon, Simpson and Observed species. All this indices in our samples were calculated with QIIME (Version 1.8.0). Beta diversity analysis was used to evaluate 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).

2.6 Caecal and colonic short-chain fatty acids

Short-chain fatty acids (SCFAs), including acetate, propionate and butyrate, were measured in caecal and colonic samples using an external standard method described by Wu T R et al [36] with minor modifications. Briefly, caecal contents (70mg) of each animal were suspended in 700μL 0.01M of PBS, and mixed intermittently on a vortex mixer for 10 min and then centrifuged at 12,000g for 5min at 4℃. The supernatant were acidified with an equal volume of 0.1M H_2SO_4 and extracted with 800μL of ethyl ether. The contents of SCFAs were measured in the organic phase using a gas chromatograph (Agilent Technologies, Stockport, UK) equipped with a 30m×0.25mm×0.25μm HP-INNOWax column (No. 19091N-133; Agilent Technologies, USA) and flame ionization detector (Agilent Technologies). The determination program for SCFAs was as follows: the temperatures of injector and detector were 240℃ and the column temperature was 200℃. Split injection (20:1). The initial column temperature was 110℃ and maintained for 5 min, thereafter increasing at a rate of 20℃/min until reaching 240℃, which was held there for 5 min.

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The flow rates of N_2 (carrier gas), H_2 (make-up gas), and air were 20, 15 and 150 mL min^{-1}, respectively. SCFAs of all samples were quantified by comparing peak areas with those of chemical standards.

2.8 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 followed by the non-parametric Kruskal-Wallis test with Dunnett's multiple comparisons test. Significance was set at $p < 0.05$.

3 Results and discussion

3.1 Pectin structure analysis

CP, WRP and DWRP are mainly composed of galacturonic acid, rhamnose, galactan, arabinan, glucuronic acid, fucose, glucose (Table 1), in different proportions. CP has the highest HG content (55.22%), followed by DWRP (42.29%) and WRP (25.03%). Conversely, WRP has the highest RG-I content (70.44%), followed by DWRP (56.29%) and CP (35.77%). WRP has the highest degree of branching with a Rha:[Gal+Ara] ratio of around 1:20, the branches dominated by arabinan, while CP and DWRP has lower arabinan. Despite the differences in HG/RGI content, CP and WRP both have similarly large Mw of around 500 kDa (Table S1), while DWRP has low Mw of 12.1 kDa (Fig. S1). According to FTIR (Fig. 1A), both CP and DWRP gave an obvious absorbance at 1745 cm^{-1} (COO-R) and 1635 cm^{-1} (COO-), only the WRP showed an sole absorption peak at 1635 cm^{-1}, confirming that WRP had the highest RG-I content. Detailed structural information

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about the proton environment of CP, WRP and DWRP was shown by ^{1}H NMR (Fig. 1B). Five major signals were assigned to the proton in the D-galacturonic acid: H-1, 5.03 ppm; H-2, 3.66 ppm; H-3, 3.93 ppm; H-4, 4.07 ppm and H-5, 4.35ppm, respectively [37]. In the anomeric region, the signal at 5.03 ppm was attributed to the H-1 of rhamnose [9], which was obvious in both spectra of WRP and DWRP. Besides, the signals between 5.07 and 5.20 ppm were attributed to the H-1 of different types of arabinan [8]. Therefore, CP, DWRP and WRP were shown to have successively larger signals for arabinan, consistent with the monosaccharide composition. Besides, the spectra from WRP and DWRP showed additional peaks that were hard to assign, probably owning to the presence of larger Ara, Gal, and Rha amounts, nearly equal to that of GalA.

SEC is most appropriate for macromolecules with large Mw. Conformation plots of CP and WRP in 0.2M aqueous NaCl solution are calculated from the slope between RMS radius and molar mass (Fig. 2A). The slope value of linear fitting for CP and WRP was 0.12 and 0.05 respectively, predicting branched conformation consistent with the anomalous SEC phenomenon of plots bending upward at the low molar mass region [38, 39]. Mark-Houwink-Sakurada equation (MHS, $[\eta]=KMw^{\alpha}$) was used to infer the chain conformation of macromolecules [40]. Value for CP and WRP was $[\eta]=0.22Mw^{0.61}$ (mL/g)、$[\eta]=0.58Mw^{0.45}$ (mL/g) respectively (Fig. 2B). According to the literature, values of α ranging from 0-0.3, 0.5-0.8, 1 and 1.8-2 correspond to chain conformation is spherical, random coil, semi-rigid chain and rod chain respectively [38, 41]. CP and WRP had α values of 0.61 and 0.45 respectively, indicating a higher

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branched chain structure of WRP.

AFM was used to imagine the molecule shapes of CP, WRP and DWRP (Fig. 2). The micrographs show that chain-like structure is characteristic for all the pectins. WRP appears significantly more branched than CP and DWRP, with interlaced structure of a network. CP and DWRP were shown to have successively higher linear and sparse branch compared to WRP, consistent with the chromatograms of the chain conformation. The expected diameters of single polysaccharide strands, imaged by AFM and adopting helical conformations, range from 0.5 to 0.8 nm [42]. The diameters of CP, WRP and DWRP were calculated as 0.58 nm, 0.75 nm and 1 nm respectively, suggesting that DWRP is probably slightly aggregated.

3.2 Effects of pectin with different RG-I content on the structure of gut microbiota community

RG-I region can be partially degraded by specific gut microbes including including *Bacteroide thetaiotaomicron*, *Bifidobacterium Longum*, [43]. Nevertheless, the effect of RG-I on the composition and diversity of the gut microbiota *in vivo* is unknown. We investigated and compared the effects of pectin differing in RG-I content, Mw and conformation on the gut microbiota profile in mice.

C57BL/6J mice underwent a nine-week dietary treatment consisting of conventional chow supplemented with the three pectin preparations. High-throughput sequencing was adopted to characterize the diversity of caecal microbiota at the end of the nine week intervention. Surprisingly, the caecal gut community richness and diversity after the interventions with large Mw pectins (CD-CP and CD-WRP) were

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not significantly different from the standard chow (CD), as demonstrated by Chao1, Shannon and Simpson (Table S3). The CD-DWRP significantly decrease the richness and diversity of caecal microbiota. In accordance with our observation, oral administration of Lentinula edodes-derived polysaccharides also significantly decreases diversity and the amount of OTUs (Operation Taxonomic Units) in caecal microbiota [44]. As for RG-I enriched pectin (Fig S3.), we tentatively put forward that this was probably owing to the relative enrichment effects of some beneficial bacteria while the destructive bacterial was reduced because of its antibacterial effects, which was proposed by the previous studies [30, 45-47]. Nevertheless, the exact explanation needs further analysis of detail on specific microbial species.

Based on clustering analysis (Fig S2), supplementation with pectins led to change in the cluster of microbial groups compared to standard chow. The groups were further analysed and compared using principal component analysis (PCA) and 3D-principal coordinate analysis (3D-PCoA). As shown by PCA, the three pectin changed the structure of the gut microbiota dissimilarly (Figure 3). With a greater distinction in segregation, 3D-PCoA depicted that CD-WRP group showed a more pronounced microbiota structural shift than that of CD-CP and CD-DWRP groups all along the first, second, and third principle (Figure 3b), compared to CD alone. This suggests a strong effect of arabinan-rich RG-I pectin on the microbiome. A similar microbiota community modulation pattern was observed in response to CD-CP and CD-DWRP (Figure 3b). They both have lower RG-I content and branching. Overall, the results suggest that structure (Mw, branching) and composition

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(RG-I/HG/arabinose content) of pectin are important factors in the modulation of gut microbiota in C57BL/6J mice.

Bacterial populations of all four diet groups were then compared at the phylum, class and genus levels (Fig. 4). At the phylum level, the dominant bacterial communities were Firmicutes and Bacteroidetes (Fig. 4A). The Bacteroidetes group is a major group responsible for polysaccharide degradation, while the Firmicutes group possess smaller number of polysaccharides-degrading enzymes [48]. In addition, WRP and DWRP were found to have a differing effect on the abundance of Actinobacteria (Fig. 4A). WRP decreased the abundance of Actinobacteria significantly, which was increased by DWRP and even higher than the CD group.

At the class level, the dominant bacterial were classified into Clostridia, Bacteroidia, Deltaproteobacteria and Actinobacteria, with a less proportion of Erysipelotrichia, Bacilli and Epsilonproteobacteria (Fig. 4B). At the genus level, the dominant bacterial were classified into *Porphyromonadaceae*, *Desulfovibrio*, *Lachnospiraceae*, *Lachnoclostridium*, *Bacteroides*, *Ruminococcaceae*, *Lactobacillus* and *Oscillospira* (Fig. 4C). To a lesser proportion, *Butyrivibrio*, *Ruminococcus*, *Acetatifactor*, *Olsenella* and *Allobaculum* were also found in different proportions in these four groups (Fig. 4C).

3.3 Key phylotypes of gut microbiota modulated by supplementation of chow with WRP and DWRP

To identify the specific bacteria that were modulated in response to different pectin, the linear discriminant analysis (LDA) effect size (LEFse) analysis was

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performed. The taxonomic cladogram and LDA score obtained from LEFse analysis identified and visualized the modulatory effect of CP, WRP and DWRP on caecal microbiota (Fig 5, Fig 6 and Fig S4). At the genus level, a pairwise comparison between the caecal microbiota of the CD-CP, CD-WRP and CD-DWRP groups displayed that, WRP treatment significantly promoted the growth of *Bacteroides spp.* and its next-generation such as *Bacteroides caecimuris, Bacteroides_ovatus*, Ruminococcaceae family, especially its next-generation such as *Ruminococcus spp., Butyricicoccus spp.* in caecal microbiota of C57BL/6J mice, while DWRP significantly increase the abundance of *Bifidobacterium spp., Lactobacillus spp.*, *Faecalibaculum spp.*, *Faecalibaculum rodentium,* and *Bacteroides thetaiotaomicron*. (Fig 6).

Specifically, DWRP treatment increased the amount of *Bifidobacterium spp.* and *Lactobacillus spp.* by about 3 to 8 fold compared to other three groups (Table 2). The amount of *Faecalibaculum spp.* in CD-DWRP group was increased by 17 to 35 fold compared to other two pectin treated groups. Similarly, the amount of *Ruminococcus spp.* in CD-WRP group was also significantly increased by 5 fold compared to the CD group. As for the family level, WRP treatment significantly promoted the growth of Ruminococcaceae family, Desulfovibrionaceae family and Bacteroidales family, while the DWRP treatment significantly increased the abundance of Clostridiales family, Erysipelotrichaceae family and Coriobacteriaceae family (Fig. 5 and Fig 6). More specifically, the amount of Ruminococcaceae family was increased by 16 fold in CD-WRP group compared to the CD group (Table 2). As for the class level, the CP

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and DWRP showed significant increase in the abundance of Deltaproteobacteria and Actinobacteria, Erysipelotrichia respectively, when the CD-WRP group showed insignificant decreased abundance of them (Fig. 4). However, at the phylum level, only the Actinobacteria was discovered to be increased by DWRP, no significant change in the phylum structure of caecal microbiota was found after CP or WRP intervention (Fig 4 and Fig S4). WRP and DWRP have a more specific and potentially beneficial effect on the caecal microbiota compared to CP, though they resulted in discrepant modulations of gut microbiota.

Ruminococcaceae family is one of autochthonous and benign species that reside in the caecum and the colon [49]. As short chain fatty acids (SCFAs) producer, it has been clarified to be responsible for the degradation of various polysaccharides [50-53]. In the CD-WRP group, Ruminococcaceae family was significantly increased, whose population size is inversely correlated with increased intestinal permeability [54], high blood triglycerides [55] and obesity [53]. Among the Ruminococcaceae family, *Ruminococcus spp.* and *Butyricicoccus spp.* were significantly enriched. *Ruminococcus spp.*, whose fermentation metabolites is acetate, was also reported to be enriched by oral administration of established prebiotics like inulin [56]. *Butyricicoccus spp.*, with butyrate-producing activity, has been reported to be a beneficial bacterial that can suppress inflammatory related diseases [57]. Members in *Bacteroides* spp. can utilize nearly all of the major plant glycans including the most complex RG-I and RG-II region [58, 59]. Especially, the *Bacteroides thetaiotaomicron* was elucidated to have a large RGI-PUL (polysaccharide utilization location) and

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galacturan-PUL thus can utilize RG-I backbone and galacturan side chains [58]. Furthermore, 60% of the *Bacteroides* spp. are capable of degrading arabinan side chains in RG-I region [60]. Therefore, the average abundance of *Bacteroides* spp. is successively higher in the caecal samples from mice supplemented with CP, WRP and DWRP with sequentially higher RG-I and arabinan side chain content (Table 2). *Desulfovibrio* spp. was decreased in obese host [61, 62], *Clostridium_XlVa* spp. was enriched in the healthy mice compared to tumor-bearing mice [63]. Since dietary WRP significantly increased the amount of *Desulfovibrio* spp. and *Clostridium_XlVa* spp., further studies are subsequently preferred to explore whether oral administration of WRP could beneficial for ameliorating obesity or colon cancer.

As the main prebiotics, *Bifidobacterium* and *Lactobacillus* are both crucial for the maintenance of healthy homeostasis [64]. DWRP significantly enriched *Bifidobacterium* and *Lactobacillus*, which was consistent to the previous research where the pectin fraction (with low Mw of 3000-4000Da) degraded from the parent citrus pectin showed much better prebiotic activity [32]. This could be due to increased solubility or accessibility of pectic backbone (due to less branches) to microbial degrading enzymes. Besides, *Faecalibaculum rodentium*, as potentially important specie, was also significantly enriched by DWRP (Table 2). It has higher fermentation ability, especially for butyrate production, is hypothesized as the main replacer of *Lactobacillus* and *Bifidobacterium* between the early and late stages of life, along with a shift from lactate metabolism to increased SCFAs production and carbohydrate metabolism [65].

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Totally, oral administration of DWRP rather than CP or WRP enriched the amount of prebiotic microbiota. In another previous study, six pectic oligosaccharides standing for specific substructure within pectin and the parent polysaccharides were evaluated for the fermentation properties [66]. Neutral sugar fractions was discovered to lead to an increase in *Bifidobacterium* populations and higher organic acid yields. Besides, arabinan, galactan, oligoarabinosides and oligogalactosides were the most selective substrates for bifidobacteria. Collectively, pectin with low Mw and higher neutral sugars content better promotes growth of beneficial bacteria [66, 67]. Therefore, the prebiotic activity of DWRP is probably owing to its high RG-I content and low Mw.

3.4 Effects of citrus pectin with different RG-I content on SCFAs

SCFAs are end products of fermentation of dietary fibres by specific anaerobic intestinal microbiota [68, 69]. Accumulating evidence suggests that SCFAs plays a crucial and favourable role in host physiology and energy homeostasis [70, 71]. Acetate, propionate and butyrate are the most abundant components of SCFAs (constitute >95% of the SCFA content), while formate, caproate and valerate are present in substantially lower amounts and make up the remaining <5% [72].

In the current study, the level of total SCFAs was found higher in both cecum and feces of CD-WRP and CD-DWRP group compared with the CD group, while that of CD-CP group was higher in cecum but slightly lower in feces (Fig. 7). It was in line with that some polysaccharides was reported to increase SCFAs production both *in vivo* and *in* vitro [73-76], indicating that the gut-derived SCFAs together with gut

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microbiota modulation may contribute to the beneficial effects of WRP and DWRP.

The CD-CP group contained significantly higher concentrations of acetate (30.32±3.23 μmol/g) and total SCFAs (41.49±12.24 μmol/g) compared to the CD group with acetate concentration of 12.51±2.46 μmol/g and total SCFAs concentration of 26.68±4.52 μmol/g in the caecal (Fig. 7A, Table S4), while this trend was surprisingly reverse in the colon (Fig. 7B). The production of acetate and butyrate were reported being promoted by the fermentation of galacturonic acid and xylose, while the production of propionate being promoted by arabinose and glucose fermentation [77]. Collectively, the increase of acetate and butyrate in cecum might be due to the fermentation of GalA in CP. Interestingly, CD-WRP and CD-DWRP group showed no significant concentration increase in neither total SCFAs nor any kind of SCFAs in the caecal content, while the CD-WRP group significantly increased the concentrations of total SCFAs (28.35±4.65 μmol/g) and acetate (20.57±2.09 μmol/g) compared to the CD group with total SCFAs concentration of 20.43±2.09 μmol/g and acetate concentration of 13.69±1.58 μmol/g in the colonic content (Fig. 7B, Table S5). The slight increase production of propionate in CD-WRP and CD-DWRP was mainly owing to the fermentation of relatively higher content of Rha and other neutral sugars in RG-I [13], which was also proved by the strong correlation between *Bacteroides* spp. and propionate content in caecum (Fig. S6). However, the SCFAs in the colonic content seemed to be successively not affected by CP and DWRP, which may be owing to the amount of CP and DWRP reaching the colon is lower.

Therefore, we tentatively presumed that there were a sequential degradative

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model of pectin enriched with different region in the lower digestive tract, which was consistent with what was previously pointed out for complex glycan and arabinogalactan depolymerisation [59]. It's possible that the RG-I enriched pectin with large Mw (WRP as a typical representative), arabinose and galacturonan side chains in RG-I region were firstly fermented in the cecum, the remaining RG-I fraction was then transferred to the colon and fermented subsequently; for the HG enriched pectin with high Mw and limited side chains content (CP as a typical representative), cecum was the main fermentation place, few could reach the colon; for pectin with relatively higher RG-I content and lower Mw (DWRP as a typical representative), partly could be fermented in cecum, while some could reach the colon.

3.5 Growth performance and biochemical parameters in response to dietary WRP and DWRP supplementation

After a 9-week of intervention, three pectin intervention groups all had a lower body weight and body weight gain than the CD group (Fig. 8B). Additionally, weight gain of CD-WRP group was the lowest. As indicated in previous study, dietary polysaccharides with a prebiotic effect was capable of decreasing the body weight as well as food intake of experimental mice [69, 78]. Here dietary CP, WRP and DWRP were found to significantly decrease the body weight gain while no significant change in the food intake was discovered during four mice groups (Fig. S5). Differing from the fucoidan which was reported to lose weight by promoting satiety [79], both CD-WRP and CD-DWRP had similar average food intake with CD group, therefore weight gain decrease was not owing to energy intake reduction. Besides, pectin can

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delay gastric emptying, slow the intestinal transport, affects the mixing of food and digestive enzymes, therefore affects the digestion and absorption of carbohydrates and fats [80]. Given that characteristic bacterial in lean host such as *Desulfovibrio* spp., *Bifidobacterium* spp. and *Clostridium_XlVa* spp. were largely enriched in WRP and DWRP, besides elevated concentrations of SCFAs could regulate energy homeostasis, the weight loss in these two group could be partly owing to that WRP and DWRP both stimulates beneficial gut microbiota especially for SCFA-producing ones and increases levels of total SCFAs in the colon.

Considering there is a tight correlation between SCFAs and mediation of inflammation and energy metabolism [69], the levels of lipid and inflammatory cytokines from all mice were analysed (Table S6). Significant decrease of inflammatory cytokines levels including lipopolysaccharides (LPS) and Tumor Necrosis Factor-α (TNF-α) were found in both CD-WRP and CD-DWRP group (Table S6). The discrepancy occurred here between three groups was probably owing to the discriminating modulation of gut microbiota resulting from different structural features (Fig. 3B; Fig. 4). Since when LPS is absorbed and enters into circulation system, the inflammatory response of the host will be triggered [81]. Therefore, taken together, the prebiotic activity by RG-I enriched DWRP in C57BL/6J mice is mainly mediated by marked structure modulation of the gut microbiota, which includes inhibiting a wide range of intestinal microbes and enriching some SCFA producers, and at least in part, by elevating SCFAs levels in the colon as well as reducing serum LPS levels.

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4. Conclusion

By enriching the amount of prebiotic bacteria including *Bifidobacterium* spp., *Lactobacillus spp.*, and SCFAs producing bacteria： *Faecalibacterium spp*., dietary DWRP shows best potential prebiotic effect. Whereas dietary WRP mainly enriched SCFAs producing bacteria including Ruminococcaceae family, especially *Ruminococcus spp., Butyricicoccus spp.* as well as *Bacteroides* spp. which is mainly responsible for arabinan side chains degradation. WRP and DWRP modulate the gut microbiota beneficial for the host in a structure-dependent path. Collectively, high RG-I content, low Mw will benefit the intestinal microbial ecology more.Besides, CD-WRP group showed the most pronounced caecal microbiota structural shift compared to the CD group, demonstrating a strong effect of arabinan-rich RG-I pectin on the modulation of caecal microbiota. Besides, based on the comparison of specific SCFAs in caecum and colon, we tentatively hypothesize that WRP were probably being sequentially utilized in the caecum and colon, while CP were mainly fermented in the caecum. By providing new insights into the well-admitted beneficial effects of RG-I enriched pectin in positive modulation of gut microbiota, our results rationalize that the RG-I enriched pectin with low Mw could be commercially used as a novel prebiotic substrate through manipulating the gut microbiota.

Conflicts of interest

There are no conflicts of interest to declare.

Author contributions

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G.Z.M. designed experiments, performed the animal studies and statistical analysis and wrote the manuscript, S.G.C. and X.Q.Y. provided the funding and insightful suggestions to the work. S.L. helped to take care of the animals and performed the OGTT. C. O. and R.J.L. improved the language of this manuscript. All authors read and approved the final manuscript.

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Figure captions:

Fig. 1. Primary structure of CP, WRP and DWRP. (A) FTIR spectra of the three pectin; (B) The ^{1}H NMR spectrum of the three pectin.

Fig. 2. Chain conformation of CP, WRP, and DWRP. Conformation plots of CP and WRP in 0.2 M aqueous NaCl solution at 25 ºC. (A) The relationship between Rg and Mw, (B) Mark-Houwink-Sakurada equation; Representative topographical AFM imagines of (C) CP, (D) WRP and (E) DWRP

Fig. 3. Response of the cecal and colonic gut microbiota structure to CP, WRP and DWRP treatment. PCA score plot of cecal (A), 3D-PCoA of the cecal (B)

Fig. 4. Structural composition of gut microbiota among all mice groups. Cecal (a) microbiota in CD, CD-CP, CD-WRP and CD-DWRP groups at phylum level; cecal (b) microbiota in CD, CD-CP, CD-WRP and CD-DWRP groups at class level; cecal (c) microbiota in CD, CD-CP, CD-WRP and CD-DWRP groups at genus level.

Fig. 5. The taxonomic cladogram obtained from LEFse analysis of gut microbiota in different groups. Taxonomic cladogram of cecal microbiota in CD, CD-CP, CD-WRP and CD-DWRP groups

Fig. 6. The LDA score obtained from LEFse analysis of gut microbiota in different groups. A LDA effect size of more than 2.5 was used as a threshold for the LEFse analysis.

Fig. 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. *P<0.05; ns, not significant.

Fig. 8. Growth performance (A) and weight gain (B) of mice in response to dietary CP, WRP and

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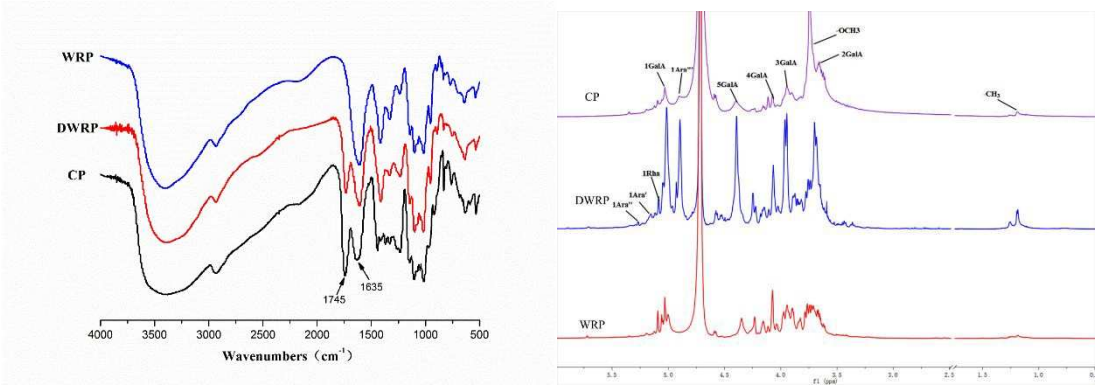
DWRP. *P<0.05.



(A) (B)

Fig. 1

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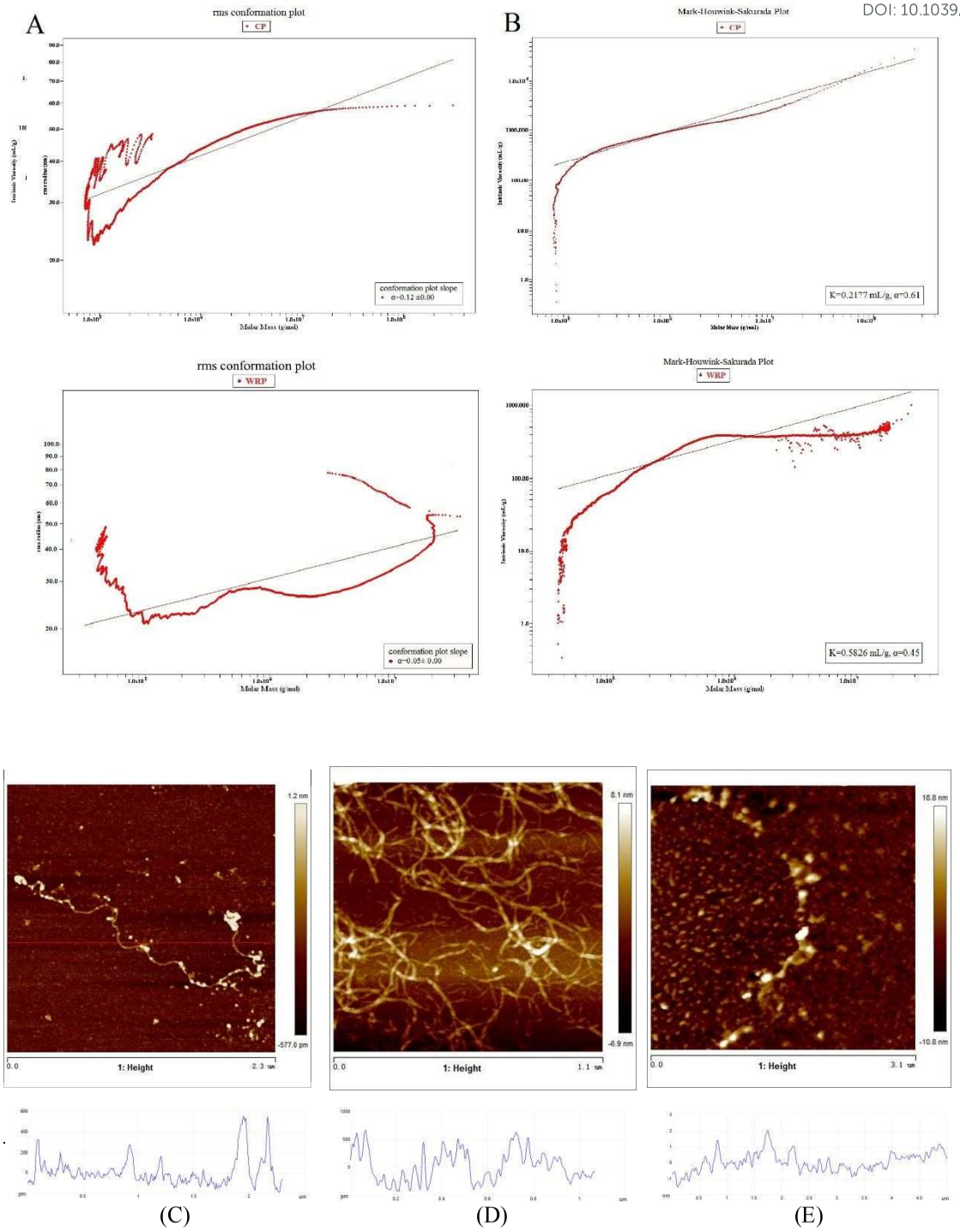

Fig. 2

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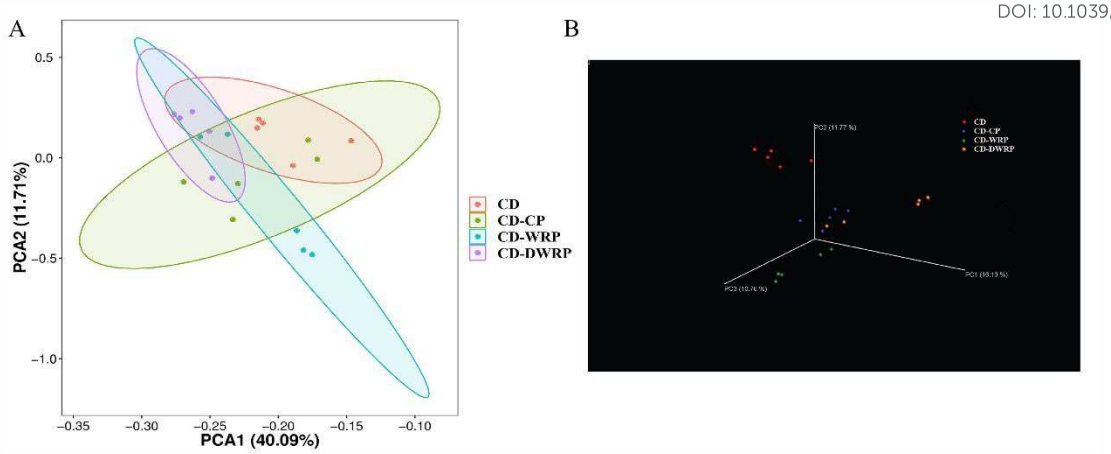

Fig. 3

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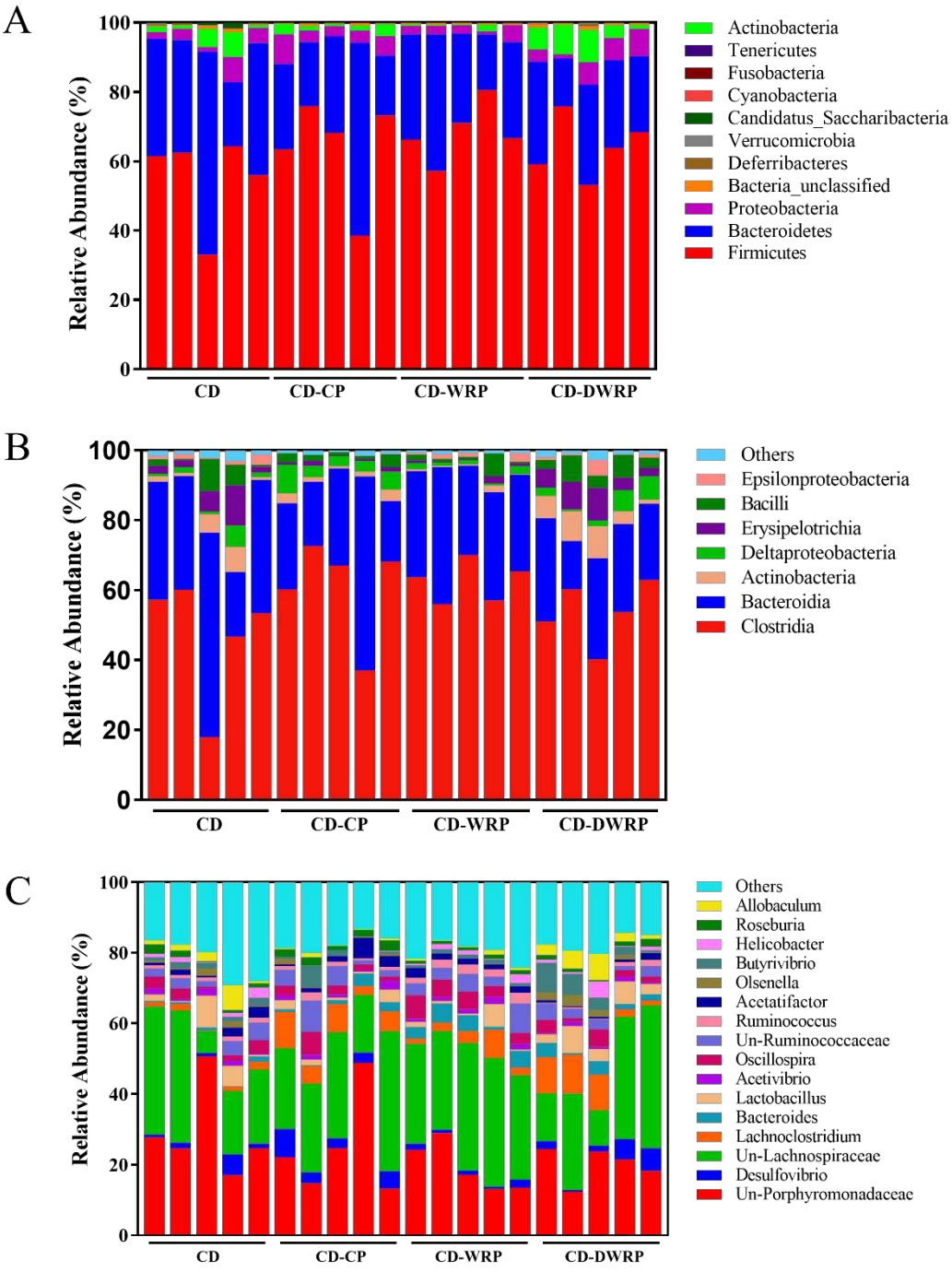

Fig. 4

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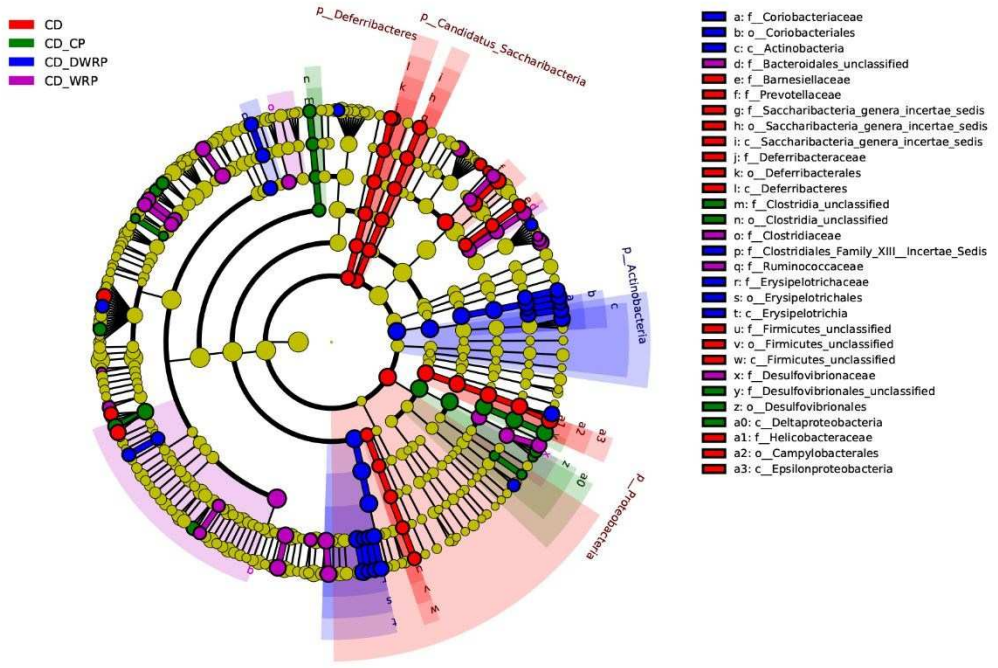

Fig. 5

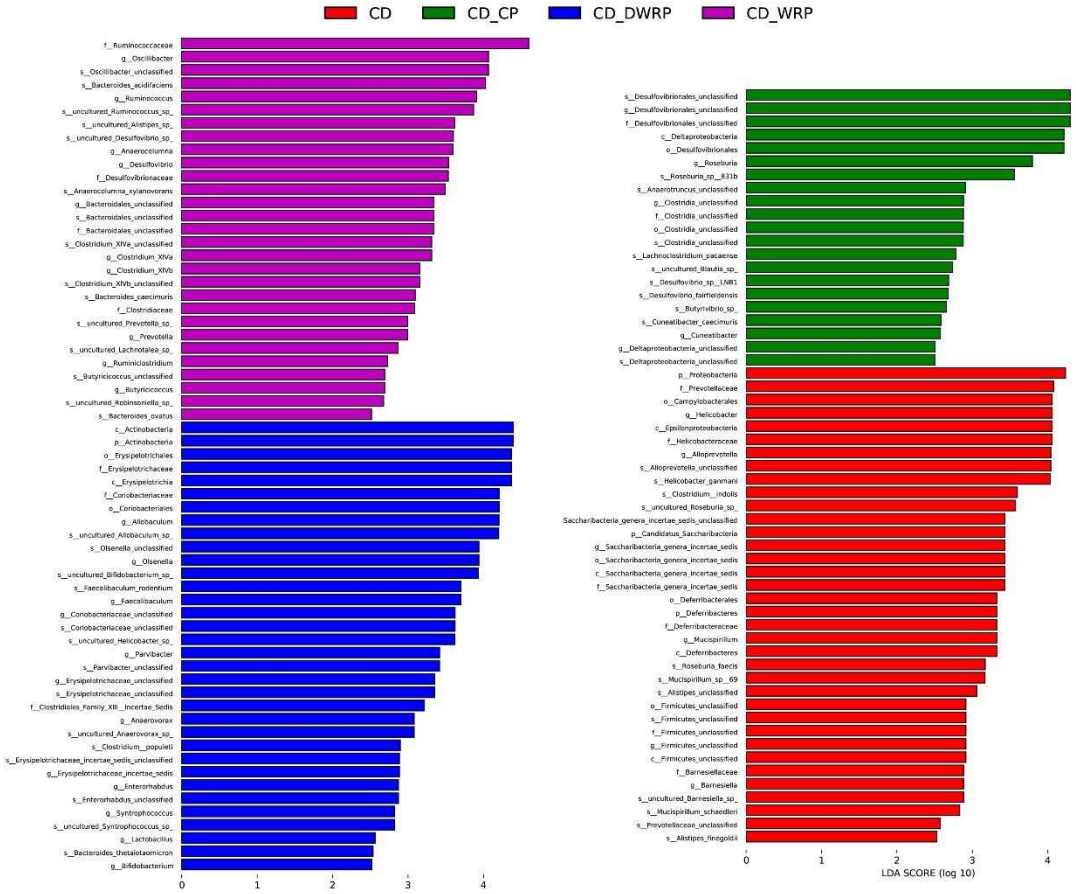

Fig. 6

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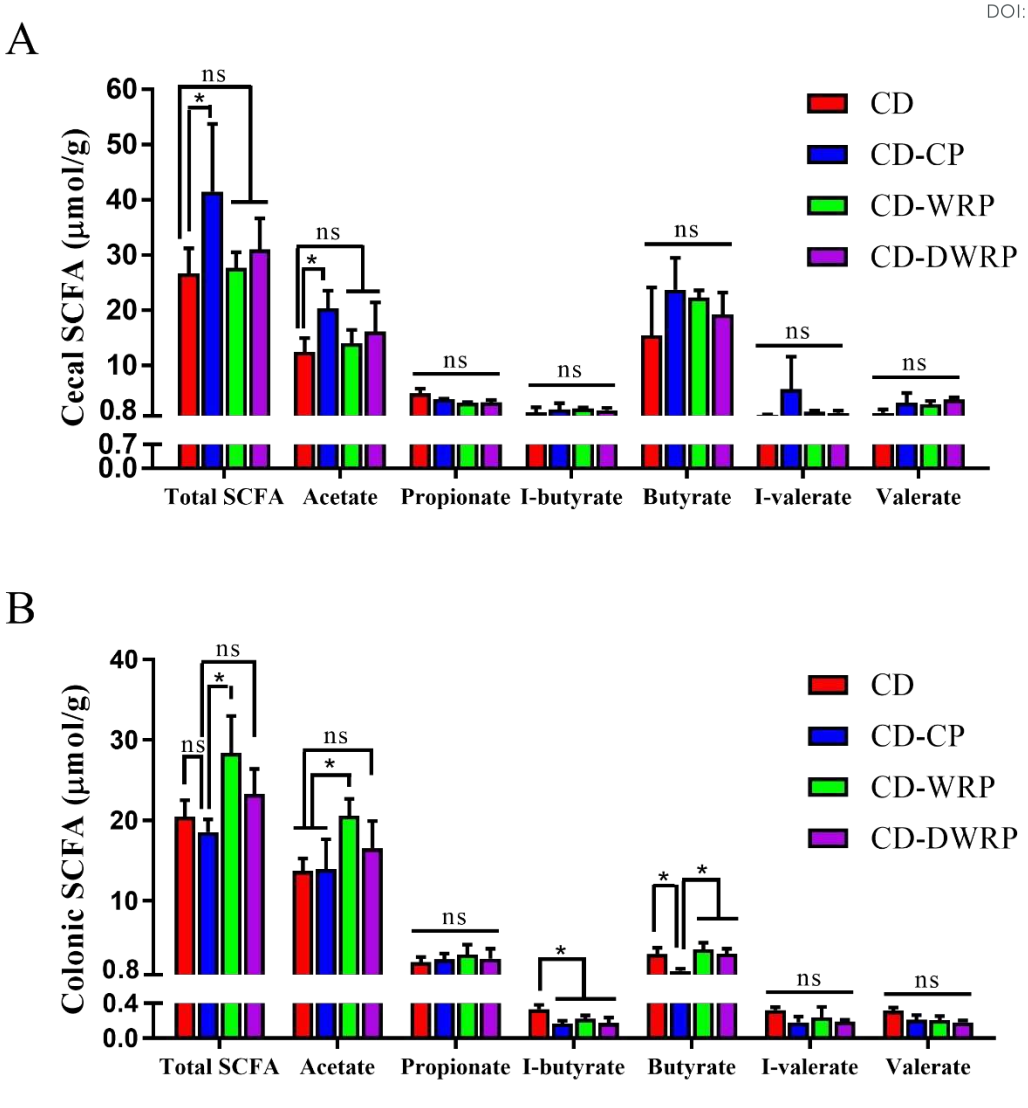

Fig. 7

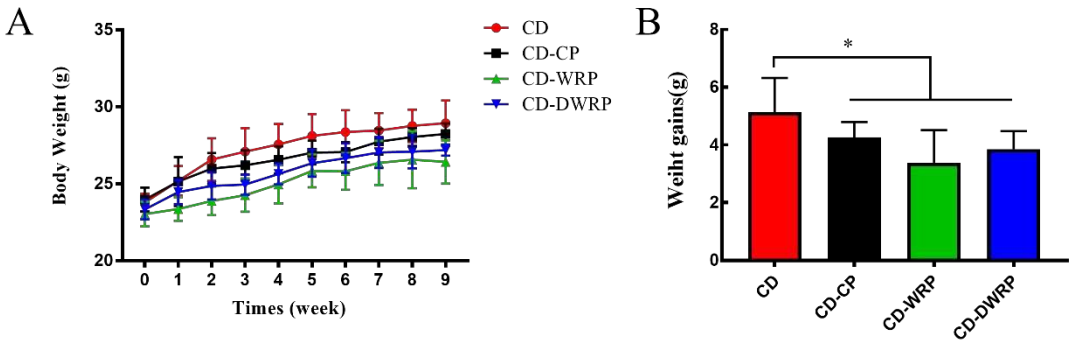

Fig. 8

Table 1. Monosaccharide compositions of CP, WRP and DWRP

Pectin	Molar ratio of monosaccharide								HG (%)	RG-I (%)	HG/RG-I	Rha:(Gal+Ara)
	Man	Rha	GluA	GalA	Glu	Gal	Ara	Fuc				
CP	0	4.45±0.38	0.37±0.02	56.99±0.69	10.15±0.03	13.03±0.12	13.84±0.14	0.20±0.02	52.55±1.07	35.77±1.02	1.47±0.07	1:6
WRP	2.16±0.17	3.61±0.16	0.25±0.01	28.64±0.85	0.75±0.06	14.27±0.16	48.94±0.81	1.37±0.16	25.03±0.98	70.44±1.22	0.36±0.02	1:20
DWRP	0	6.64±0.08	0.18±0.02	48.93±1.01	0.46±0.05	9.43±0.52	33.58±0.83	0.07±0.02	42.29±1.08	56.29±1.50	0.75±0.04	1:6

The molar percentage of homogalacturonan(HG) and rhamnogalacturonan of type I (RG-I) were calculated as the following formula:

HG (%)=GalA(mol%)-Rha (mol%)

RG-I (%)≈ 2Rha(mol%)+Ara(mol%)+Gal(mol%)

The Rha:(Gal+Ara) ratio stands for the degree of the side chain branching.

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Table 2. The abundance of key phylotypes of gut microbiota modulated by dietary CP, WRP and DWRP in CD-fed mice

Key Phylotypes	CD	CD-CP	CD-WRP	CD-DWRP
g_*Bifidobacterium*	0.87±1.32 ^{ab}	0.44±0.33 ^{a}	0.26±0.37 ^{a}	2.09±1.83 ^{b}
g_*Lactobacillus*	1.64±2.27 ^{a}	1.70±1.15 ^{a}	2.34±2.03 ^{a}	4.46±2.03 ^{b}
g_ *Faecalibaculum*	0.37±0.36 ^{a}	0.06±0.05 ^{a}	0.03±0.02 ^{a}	1.05±0.67 ^{b}
g_*Bacteroides*	0.55±0.53 ^{a}	1.62±1.34 ^{ab}	2.85±2.14 ^{b}	2.27±1.49 ^{ab}
g_*Ruminococcus*	1.12±0.22 ^{a}	1.21±0.69 ^{a}	2.34±0.57 ^{b}	0.72±0.64 ^{a}
f_Ruminococcaceae	1.10±0.61 ^{a}	12.68±7.17 ^{bc}	17.58±3.32 ^{c}	9.97±2.41 ^{b}
g_*Clostridium_XlVa*	0.12±0.12 ^{a}	0.31±0.30 ^{ab}	0.48±0.30 ^{b}	0.17±0.16 ^{ab}
g_*Desulfovibrio*	0.24±0.17 ^{a}	0.14±0.06 ^{a}	0.78±0.34 ^{b}	0.21±0.11 ^{a}

Data representing relative abundance (the percentage of bacteria) of the key phylotypes of gut microbiota are expressed as the mean ± SD (n=6). The means with different superscript 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

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Graphical abstract:

Rhamnogalacturonan-I (RG-I) pectin (WRP) with high neutral sugar side chain was recovered from citrus segment membrane. The present study reveals that WRP can stimulate the growth of beneficial microbiome, improving SCFA content. Besides, the effect could be enhanced by free-radical depolymerizing of WRP into DWRP.