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Food Hydrocolloids

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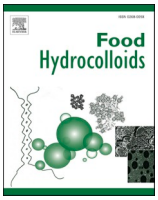
https://doi.org/10.1016/j.foodhyd.2023.108755

Food Hydrocolloids 141 (2023) 108755

Contents lists available at ScienceDirect

Food Hydrocolloids

journal homepage: www.elsevier.com/locate/foodhyd



Effects of thermal treatments on the extraction and *in vitro* fermentation patterns of pectins from pomelo (*Citrus grandis*)

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ARTICLE INFO

Keywords:
Pomelo pectin
Subcritical water
Chelator-assisted extraction
Structural characterization
In vitro fermentation
Dietary fiber

ABSTRACT

Pomelo peel, a by-product of fruit processing, is an excellent source of pectin. We aimed to study the effects of thermal treatments on the extraction and *in vitro* fermentation patterns of pectins from pomelo peel. We used subcritical water extraction and hot water extraction, with or without chelator (citric acid or EDTA-2Na) assistance to obtain the pectins. The pectin fractions were characterized by their constituent monosaccharide composition and molecular weight (Mw) distribution. The degree of methyl esterification of pomelo pectins was determined using FT-IR spectroscopy. We also compared the fermentation patterns of the pectin using an *in vitro* simulated human gut fermentation model, monitored Mw changes during fermentation by high performance size exclusion chromatography (HPSEC), and analyzed short-chain fatty acid (SCFA) using gas chromatograph. Our results showed that both chelator assistance and subcritical water treatment increased the pectin yield. Chelator-assisted extraction increased the pectin Mw and decreased the degree of methyl esterification (DM) whereas, subcritical water treatment had the opposite effect. Pectin with low rhamnogalacturonan-I (RG-I) and high homogalacturonan (HG) domain can be obtained from the used pomelo peels by citric acid-assisted extraction. Although gut microbiota intensively utilized uronic acid (UA) of pomelo during early fermentation stages, constituent neutral monosaccharides were consumed even faster than UA. Pomelo pectins enhanced the relative abundance of *Bacteroides* and *Prevotella 9*, and stimulated the production of SCFA, particularly acetic acid. Generally, pomelo pectin could be considered a new source for modulating gut microbiota towards a healthy pattern in functional foods.

1. Introduction

The pomelo (*Citrus grandis*) is widely cultivated in southwest China, Southeast Asia, and other Asian countries due to its high yield, ease of preservation, and plant characteristics (Xiao, Ye, Zhou, & Zhao, 2021). This fruit contains not only pectin but also other polysaccharides, phenolic compounds, flavonoids, vitamins, and other nutrients (Chen, Hu, Yao, & Liang, 2016). However, the pomelo peel contains a large amount of insoluble dietary fiber, making it difficult to chew for consumption. Additionally, naringin's bitter taste negatively impacts the edible value of pomelo peel. Therefore, it is essential to explore a novel, high-efficient, and environmentally friendly extraction method for the easily bioavailable pectin from pomelo peel.

The structure, extraction methods, applications, and potential beneficial effects on health of pomelo pectin have garnered significant attention (Xiao et al., 2021). The main chain of pectin primarily consists of homogalacturonan (HG ≈ 65%) made up of galacturonic acid (GalA). The rhamnogalacturonan I (RG-I ≈ 20–35%) skeleton is formed by the repeating disaccharide unit (GalA alternately combined with Rhamnose residues) (Mohnen, 2008). Hot water extraction (HWE) is commonly

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https://doi.org/10.1016/j.foodhyd.2023.108755
Received 15 December 2022; Received in revised form 1 April 2023; Accepted 6 April 2023
Available online 11 April 2023

used for plant polysaccharides due to its convenience and environmental friendliness compared to other methods. Chelators can assist in hot water extraction to increase pectin yield (Koh, Xu, & Wicker, 2018; Manikandan & Lens, 2022). Subcritical water extraction (SWE) is a green and efficient technology for extracting organic compounds such as polysaccharides using liquid-state water under high temperature (100–374 °C) and high pressure (0.1–22.1 MPa) (Liew, Teoh, Tan, Yusoff, & Ngoh, 2018). It offers the advantage of rapid, efficient extraction and is associated with high yields, quality, and purity. Additionally, the modification of the molecular structure by subcritical water may enhance the bioactivity of active substances (Zhang, Wen, Zhang, Duan, & Ma, 2020).

Pectins are considered prebiotics because they are nondigestible but can be utilized by gut microorganisms to produce metabolites that regulate the composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host (Holscher, 2017; Thomas, Suzuki, & Zhao, 2015). Additionally, pectins are highly valued polysaccharides with health effects. Pectins with different structures and/or compositions have been found to be potentially effective in protecting against and preventing gastrointestinal issues, reducing cholesterol, and regulating immunity (Cui, Wang, Zhao, Zhou, & Zheng, 2022; Zaid, Mishra, Wahid, & Sakinah, 2019; Zhu et al., 2022). The health-promoting features of pectin include its fermentability and the corresponding changes in intestinal microbial composition (Kang et al., 2022). In the gut, pectin is utilized by gut microbiota and fermented into microbial metabolites such as short-chain fatty acids (SCFA) (Holscher, 2017). Pectins can support proper modulation of gut microbiota. For example, pectin from *Cucumis metuliferus* peels was found to increase the abundance of beneficial bacteria like *Bifidobacterium* (Zhu et al., 2022), and soy and citrus pectin increased the relative abundance of *Prevotella* in pig colonic digesta (Tian et al., 2017). Sugar beet pectin also stimulated members of the genus *Lactobacillus* during cecal fermentation in rats (Tian et al., 2016).

In the present study, different extraction methods were compared to obtain pectin from pomelo peels. The pectin fractions were characterized by their constituent monosaccharide composition and molecular weight (Mw) distribution. We compared the fermentation patterns of the pectin using an *in vitro* simulated human gut fermentation model. This knowledge could be used to determine and recommend efficient utilization of pectin and corresponding regulation of intestinal microbiota by different extraction methods.

2. Materials and methods

2.1. Materials and reagents

The fresh pomelos *Citrus maxima* cv. (Burm.) Merr. cv. Shatian Yu were purchased from Guangxi province, China. The chelators citric acid, EDTA-2Na and dialysis bag were purchased from Beijing Solarbio Science and Technology Co. Ltd (Beijing, CHN). The monosaccharide standards and dextran standards were acquired from Sigma-Aldrich Chemical Co., Inc. (WI, USA). SCFA standards were obtained from Aladdin Biochemical Technology Co. (Shanghai, CHN). All other chemicals and reagents were of analytical grade.

2.2. Extraction of pectin from pomelo peels

To extract the pectin, fresh pomelo peels were dried at 50 °C to a constant weight and the fragments became hard and fragile. The peels were then ground into a powder and sifted through an 80-mesh screen. The ratio of water to material was 20:1 (v/w dried powder). The mixed solutions for the extraction of SWP were gathered under an automatic pressure steam sterilizer (GI54TW, Zealway instrument, Inc., USA). The program was set to rise from room temperature to 0.15 Mpa at 127 °C in 35 min and maintained the condition for 20 min, then relieved to atmospheric pressure in 35 min. The treatment condition for HWP was 85 °C for 1.5 h. We defined the six groups of pomelo pectins (PPs) as follows: W-SWP/HWP (subcritical/hot water-extracted pectin), CA-SWP/HWP (subcritical/hot water citric acid-extracted pectin, with a citric acid solution of 5% w/v), and E-SWP/HWP (subcritical/hot water EDTA-2Na-extracted pectin, with an EDTA-2Na solution of 1% w/v) (Fig. 1). The supernatant was collected and the pH was adjusted to 7.0 at 25 °C. Next, four volumes of anhydrous ethanol were added to the solutions, which were then kept at 4 °C for 12 h. The resulting deposition was separated by filtration and dissolved in ultrapure water. Dialysis was conducted using a dialysis bag (8–14 kDa) at 4 °C for 48 h, and then the samples were dried at low temperature under vacuum. The extraction yield was calculated as follows: Extraction Yield (%) = W1/W2 × 100%, where W1 and W2 are the weight of dried pectin samples and the weight of dry pomelo peel powder, respectively.

2.3. In vitro simulation of human colonic fermentation

The *in vitro* simulated gut fermentation was conducted using a modified version of a published method (Ai et al., 2022; Liu et al., 2022). Fresh fecal samples were obtained from six healthy donors (three males and three females, aged 20–24), who had not experienced any digestive or antibiotic treatment for at least three months. The feces were collected and homogenized in sterile centrifuge tubes filled with phosphate-buffered saline (pH = 7.4). The fecal supernatant was collected after centrifugation at 500×*g* for 10 min, and the fluid was diluted to a concentration of 0.1 g/mL. All further procedures were performed within 1 h of dilution.

The fermentation basal nutrient medium (1000 mL) was prepared by adding NaCl (4.5 g), K_2HPO_4 (2.5 g), $CaCl_2{\cdot}2H_2O$ (0.45 g), $MgSO_4{\cdot}7H_2O$ (0.5 g), $FeSO_4{\cdot}7H_2O$ (0.005 g), ox bile (0.05 g), cysteine (0.4 g), bactopeptone (3.0 g), casein (3.0 g), 1% Resazurin indicator (1 mL), haemin (0.01 g), para-aminobenzoic acid (0.05 g), D-biotin (0.002 g), vitamin B-12 (0.0005 g), menadione (0.001 g), pantothenate (0.01 g), nicotinamide (0.005 g), and thiamine (0.004 g). *In vitro* fermentation was conducted in 5 mL anaerobic fermentation tubes. W/CA/E-HWP and W/CA/E-SWP were dissolved in sterilized medium to a concentration of 10 mg/mL. Fructo-oligosaccharides (FOS) were used as the positive control, and the basal nutrient growth medium without any carbon source was used as the blank control (CON). The fermentation medium was inoculated with 10% of the total volume of diluted fecal supernatant. The tubes were sealed and incubated at 37 °C (100 rpm) in a vibrating incubator for 0, 6, 12, 24, and 48 h.

2.4. Physiochemical analyses

Molecular weight distribution. The samples (5 mg) were dissolved in 1 mL ultrapure water and passed through 0.22 μm filter membrane to remove impurity. The Mw of PPs were determined by high performance size exclusion chromatography (HPSEC) with TSK-GEL columns connected in series of TSK-Gel guard column (TSKgel Guardcolumn SuperSW, 6 mm ID × 40 mm), 4000, 3000 and 2500 Super AW (6 mm × 150 mm) series coupled to RI detector (RefractoMax520, Thermo Fisher, MA, USA). The $NaNO_3$ solution was used as mobile phase at 55 °C column environment and a concentration of 0.2M with 0.6 mL/min speed. Each 20 μL pectin solution was injected into the system for analysis, and the standard curve was obtained by various dextran standards (Mw of 10, 40, 70, 500 and 2000 kDa) (Jermendi et al., 2023; Voragen, Schols, De Vries, & Pilnik, 1982).

Constituent monosaccharide content and composition. Samples (10 mg) containing 1 mg of inositol as internal standard were pre-hydrolyzed in 72% (w/w) H_2SO_4 at 30 °C for 1 h followed by 1 M H_2SO_4 at 100 °C for 3 h. The monosaccharides released were derivatized into their alditol acetates and determined by gas–liquid chromatography (Englyst & Cummings, 1984). The uronic acid (UA) content of the samples was quantified using the p-hydroxydiphenyl colorimetry method at 520 nm with D-galacturonic acid as a standard substance. The detailed

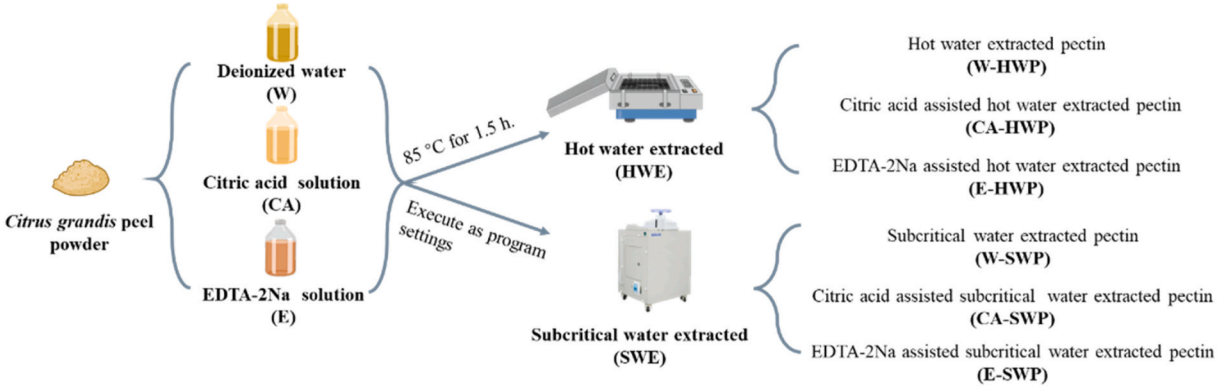

Fig. 1. Extraction processing and grouping of pomelo pectins.

determination conditions were the same as described in a publication (Blumenkrantz & Asboe-Hansen, 1973). Total contents of carbohydrate were calculated by summing all determined monosaccharides, including rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl), mannose (Man), galactose (Gal), glucose (Glc) and UA.

FT-IR spectroscopy and degree of methyl esterification. FT-IR spectrometry was used to record the dried pectin's KBr tablettings using the Nicolet iS50+iN10 spectrometer (Thermo Fisher Scientific, MA, USA) with a resolution of 4 cm^{-1}, cumulative scan of 32 and a wavelength range of 4000–500 cm^{-1}. KBr discs were prepared using a 90:10 salt: sample proportion. The DM of samples was determined by calculating from established standard curve with I and DM value. $I = A_{1745}/(A_{1745} + A_{1630}) \times 100\%$, where I is the percentage of ester based peak area. The A_{1745} and A_{1630} are the absorption peak area of methyl ester group and carboxylate absorption peak area, respectively (Filippov & Kohn, 1975; Zhao et al., 2021). The standard cure was established by various standards of DM pectin (3, 20, 37, 55, 62.8 and 70.5%).

Total polyphenols, protein content, pH *and gas production.* The content of proteins and total polyphenols were determined according to BCA assay (Smith et al., 1985) and Folin-Ciocalteu colorimetric method (Singleton & Rossi, 1965), respectively. The pH values of fermented samples were measured by 962244 detector (PHS–3C, Shanghai Leici Apparatus Corp., Shanghai, CHN). The gas production (mL) was recorded from the specific scale value by inserting piston type sterile needle through the rubber plug into tubes without contacting medium.

SCFA content and composition. The diethylacetic acid was added into samples as internal standard (Tornero-Martinez et al., 2019). 2 μL of mixed solution was injected into gas chromatograph (Shimadzu G2010Plus, Kyoto, JPN) equipped with a DB-FFAP column (30 m × 0.53 mm × 1.00 μm) (Agilent Technologies Inc., Calif. USA) and a flame ionization detector (FID) (Gao et al., 2020). The standards curve was obtained by acetic, propionic, butyric, isobutyric, valeric and isovaleric acid.

2.5. Analysis of microbiota after in vitro fermentation

Samples collected from the *in vitro* fermentation after 48 h, and the ones from CON group at 0 h were analyzed for microbiota composition. DNA was extracted from the samples using a DNA Extraction Kit (Omega Bio-Tek, USA). The DNA concentration of the samples was determined using the Qubit dsDNA HS Assay Kit and Qubit 4.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Oregon, USA), and the quality was assessed by 1% agarose gel electrophoresis. The full-length 16S rRNA gene was amplified from the genomic DNA using the universal primer set 27F: AGRGTTTGATYNTGGCTCAG and 1492R: TASGGHTACCTTGTTASGACTT. The multiplexed sequencing was conducted using both forward and reverse 16S primers tailed with sample-specific PacBio barcode sequences. The clean reads were obtained by using UCHIME algorithm (v8.1) to detect and remove chimera sequences. Sequences with similarity ≥97% were clustered into same operational taxonomic unit (OTU) by USEARCH (v10.0). All selected representative reads were annotated and performed based on the Naive Bayes classifier in QIIME2 using the SILVA database (Quast et al., 2013) (release 132) with a confidence threshold of 70%.

2.6. Statistical analysis

Statistical data were analyzed by SPSS 21 software. GraphPad Prism 9.0, Origin 2021, Excel 2016 were used for graphics presentation. Significant differences at $p < 0.05$ were determined with ANOVA tests followed by Duncan's multi-range tests.

3. Results and discussion

3.1. Physicochemical properties of the pomelo pectins

The yield of pectin was significantly higher when chelator assistance and subcritical water treatments were used, as compared to thermal water extraction (Table 1). The top three yields of pectin were observed in CA-HWP (15.4 ± 0.6%), CA-SWP (14.5 ± 0.3%), and E-HWP (13.5 ± 0.3%). The reason for the higher yield with chelators may be due to the presence of high valence ions, which promote complete dissolution of pectin (Guo, Duan, Wang, & Huang, 2014; Zheng et al., 2016). The high temperature and pressure during subcritical water extraction promote solubilization, while high temperature weakens hydrogen bonds and increases extraction efficiency (Wang, Chen, & Lü, 2014). The lower amounts of protein and total phenols in chelator-assisted groups compared to the corresponding water-extracted group (Table 1) was mainly due to the increased solubilization of pectin in the former groups.

The Mw of pectin extracted from pomelo peels was decreased by subcritical water treatment, while chelator assistance increased the Mw (Table 1). The Mw distributions of the pectins extracted using different treatments were determined using HPSEC (Fig. 2A), and the results showed that the Mw ranged from 56.3 kDa (E-HWP) to 220.4 kDa (W-SWP) for the thermally extracted pectins. The higher Mw of the HWP group compared to the SWP group may be due to the promotion of pectin hydrolysis by the high temperature and pressure during subcritical water extraction (Wang et al., 2014). Compared to the water-extracted group, the chelator-assisted extractions using citric acid and EDTA showed higher Mw, which may be explained by the pectin polymerization during production process using chelators like citric acid, and the dissolution of high Mw pectin by chelator (CDTA) extraction (Kurita, Fujiwara, & Yamazaki, 2008; Qin, Liu, Lv, & Wang, 2020).

3.2. Carbohydrate contents and constituent monosaccharides

Table 1 shows that the carbohydrate and UA contents of the pomelo

Table 1
Physicochemical properties, constituent monosaccharide composition and structural patterns of the pomelo pectins.

Item	W-HWP	CA-HWP	E-HWP	W-SWP	CA-SWP	E-SWP
Physical properties						
Yield (w/w %)	4.7 ± 0.8^{e}	15.4 ± 0.6^{a}	10.5 ± 1.4^{d}	12.9 ± 0.4^{c}	14.5 ± 0.3^{ab}	13.5 ± 0.3^{bc}
DM (%)	19.0 ± 1.0^{c}	10.5 ± 1.5^{e}	15.1 ± 2.3^{d}	30.5 ± 0.8^{a}	19.4 ± 0.6^{c}	24.8 ± 1.8^{b}
Mw (kDa)	134.0 ± 13.2^{c}	188.5 ± 4.1^{b}	220.4 ± 24.3^{a}	56.3 ± 1.7^{e}	88.4 ± 1.9^{d}	137.1 ± 2.8^{c}
Chemical composition						
Total carbohydrate (%)	78.8 ± 9.1^{b}	91.4 ± 3.0^{a}	88.7 ± 1.3^{a}	90.1 ± 1.7^{a}	95.2 ± 2.1^{a}	90.4 ± 2.6^{a}
Total UA (%)	70.3 ± 7.4^{c}	86.2 ± 2.8^{ab}	82.8 ± 2.5^{ab}	80.1 ± 0.6^{ab}	89.7 ± 2.0^{a}	77.1 ± 2.8^{bc}
Protein (%)	9.0 ± 0.4^{a}	5.1 ± 0.4^{c}	6.1 ± 1.0^{c}	7.7 ± 0.7^{b}	2.9 ± 0.2^{d}	6.0 ± 1.0^{c}
Total phenols (%)	0.27 ± 0.01^{a}	0.05 ± 0.01^{e}	0.13 ± 0.00^{c}	0.20 ± 0.01^{b}	0.04 ± 0.01^{e}	0.08 ± 0.01^{d}
Monosaccharide content/molar ratio (%)						
Rha	0.5/0.8	0.4/0.5	0.4/0.5	0.5/0.7	0.5/0.6	0.5/0.6
Fuc	0.1/0.1	t/t	t/t	t/0.1	t/t	t/0.1
Ara	2.6/4.2	2.6/3.7	2.4/3.5	5.3/7.4	0.3/0.4	7.4/10.2
Xyl	0.7/1.1	0.1/0.2	0.2/0.3	0.3/0.4	0.4/0.5	0.4/0.6
Man	1.1/1.5	0.2/0.2	0.5/0.6	1.0/1.1	0.5/0.5	0.8/0.9
Gal	2.1/2.8	1.4/1.6	1.4/1.7	1.6/1.8	2.2/2.5	1.9/2.2
Glc	1.4/1.9	0.5/0.6	1.0/1.2	1.2/1.5	1.6/1.8	2.4/2.7
UA	70.3/87.7	86.2/93.2	82.8/92.3	80.1/87.0	89.7/93.6	77.1/82.7
Structural patterns						
HG (%)	69.8	85.8	82.4	79.6	89.2	76.6
RG-I (%)	5.8	4.8	4.6	7.9	3.5	10.2
Rha/UA (10^{-3})	8.9	5.5	5.9	7.6	6.6	7.4
(Ara + Gal)/Rha	9.0	10.4	9.4	14.0	4.7	20.2
UA/(Fuc + Xyl + Rha + Ara + Gal)	9.8	15.4	15.5	8.4	23.0	6.1

Note: DM: degree of methyl esterification. The monosaccharide composition of pectins were presented in two ways: monosaccharide content and molar ratio, respectively. HG (%) = UA - Rha, and RG-I (%) = UA – HG + Rha + Ara + Gal, which were calculated by monosaccharide content. The contribution of RG-I domain to the entire pectin was calculated by Rha/UA; the degree of RG-I branching was calculated by (Ara + Gal)/Rha; and the linearity of pectin was calculated by UA/(Fuc + Gal + Ara + Rha + Xyl); which were calculated by molar ratio. t: trace amount, <0.05%. The different superscript letters indicate significant difference ($p < 0.05$).

pectins were all above 77% and 70%, respectively. The monosaccharide content and molar ratio in Table 1 indicate that the main composition of PPs was UA (mostly galacturonic acid) and an increase in the total carbohydrate content was observed after subcritical water extraction. Notably, the highest total carbohydrate and UA contents were found in CA-SWP at 95.2% and 89.7%, respectively, compared to other groups. The contents of other constituent monosaccharides were much less than UA, with molar ratios ranging from a minimum of 0.02% (Fuc, CA-SWP) to a maximum of 10.2% (Ara, E-SWP). The ratio of HG in all groups was above 65%, indicating that HG was the main domain of PPs. These results suggest that both HWP and SWP pectin were mostly composed of UA (82.7–93.6% mol) and small amounts of neutral sugars. Similar proportions of UA and neutral sugars can be found in Pomelo (*Citrus maxima*) pectin extracted using inorganic acids (nitric and hydrochloric acid) (Methacanon, Krongsin, & Gamonpilas, 2014). The contribution of the RG-I region to the entire pectin varied between a minimum of 0.0055 (CA-HWP) and a maximum of 0.0089 (W-HWP). The degree of RG-I branching varied between a minimum of 4.7 (CA-SWP) and a maximum of 20.2 (E-SWP). CA-SWP had the highest degree of linearity at 23.0, with the lowest RG-I branching at 4.7 compared to other groups. Generally, pectin with low RG-I and high HG domain can be obtained from the used pomelo peels by citric acid-assisted extraction.

3.3. FT-IR analysis

The structure of thermally treated pectin samples was further characterized using FT-IR spectroscopy (Fig. 2B). The characteristic absorption of O–H bond can be found at a stretched intense peak around 3450 cm^{-1}, and the bands around 2935 cm^{-1} were due to the stretching vibrations of C–H (Cui et al., 2020). Fig. 2B shows the spectra in the wavenumber range where pectin characteristic bands are typically detected (1700-600 cm^{-1}). A significant band of methyl-esterified carboxyl groups (1753 cm^{-1}) was observed in each group (Cui et al., 2020). Furthermore, a strong and weak absorption peak around 1624 $cm^{-1}$and 1440 cm^{-1} were attributed to C═O and COO- stretching vibrations, respectively (Liew et al., 2018). The absorption peak area of methyl ester group and carboxylate absorption peak area were used to calculate the DM of pectins. All pectin groups had low degrees of methyl esterification (<50%). In general, the DM of SWP were higher than HWP, which indicates that DM of PPs can be increased by subcritical extraction. Moreover, the DM of W-HWP (19.0%) were higher than CA/E-HWP (10.5% and 15.1%) and the same condition was observed in SWP. The results indicate that the use of chelators decreased the DM values of the PPs, and citric acid-assisted extraction led to a greater reduction in DM values than EDTA-assisted extraction. In general, the spectra between the studied samples were similar and had the characteristic absorption patterns of pectin, while significant differences in the intensity and absorption areas were observed, which were reflected in the DM values.

3.4. Dynamic changes of pH, gas production, and carbohydrate contents during in vitro fermentation

As shown in Fig. 3(A-B), the control group (CON) maintained a relatively smooth pH value around 6.5. In contrast, the pH value in pomelo pectin groups decreased at different rates during the 48-h fermentation period, although the final pH was significantly higher than in FOS ($p < 0.05$). A sharp decrease in pH was observed in the FOS group, which is consistent with our previous finding that high amount of lactic acid together with SCFA were produced during FOS fermentation (Ai et al., 2022). Gas production gradually increased during fermentation in all groups except for CON, which stopped increasing at 12 h. The end of pomelo pectin fermentation gas production sequence decreased in the following order: W-SWP (4.06 mL) > E-SWP (3.86 mL) > E-HWP (3.40 mL) > CA-SWP (3.20 mL) > CA-HWP (3.16 mL) > W-HWP (3.12 mL).

The carbohydrates including UA were intensively utilized by the gut microbiota within 24 h of fermentation (Fig. 3C and **D**). The consumption of total carbohydrates from the initial value until after 24 h of *in vitro* fermentation can be sorted as follows: W-SWP (8.42 mg/mL)> CA-SWP (7.76 mg/mL)> W-HWP (6.79 mg/mL)> E-SWP (6.11 mg/mL)> CA-HWP (5.81 mg/mL)> E-HWP (4.45 mg/mL). The favorable microbial consumption of total carbohydrates was found in pectins with low Mw induced by subcritical water extraction, which is in agreement with a previous study (Zhao et al., 2021). The utilized UA after 24 h of fermentation was highest in W-SWP (7.68 mg/mL), followed by CA-SWP (7.31 mg/mL), W-HWP (5.89 mg/mL), CA-HWP (5.39 mg/mL), E-SWP (4.66 mg/mL), and E-HWP (3.96 mg/mL). The limited UA consumption for E-HWP group could be partly due to the low DM (15.1%, Table 1)

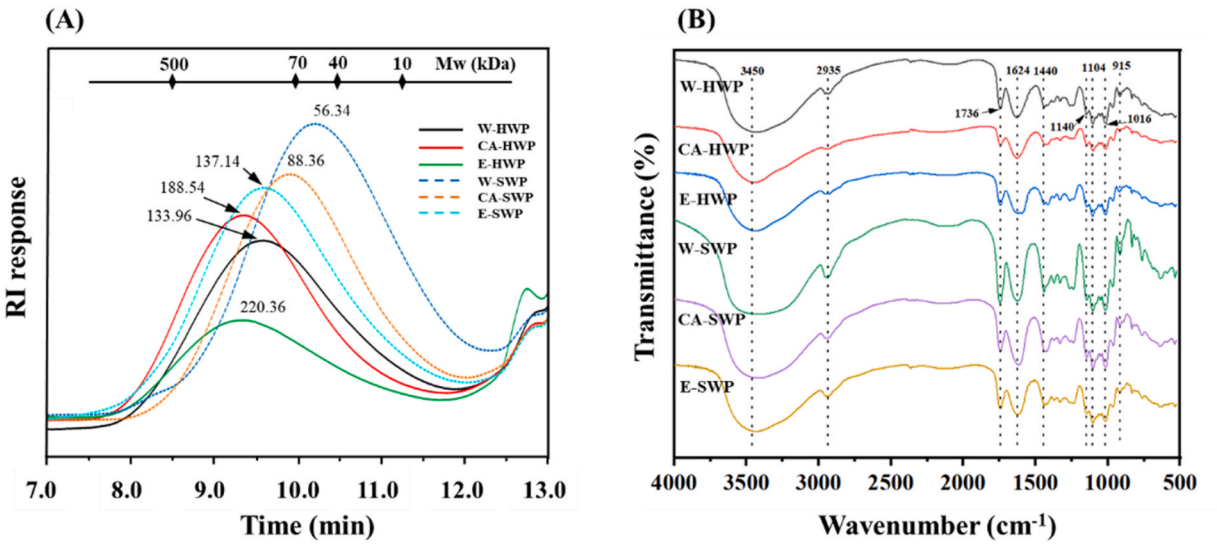

Fig. 2. Mw distribution and FT-IR spectrum of PPs. HPSEC profiles (A), FT-IR spectra (B) of thermal extracted pectins from pomelo with/without chelators assistance.

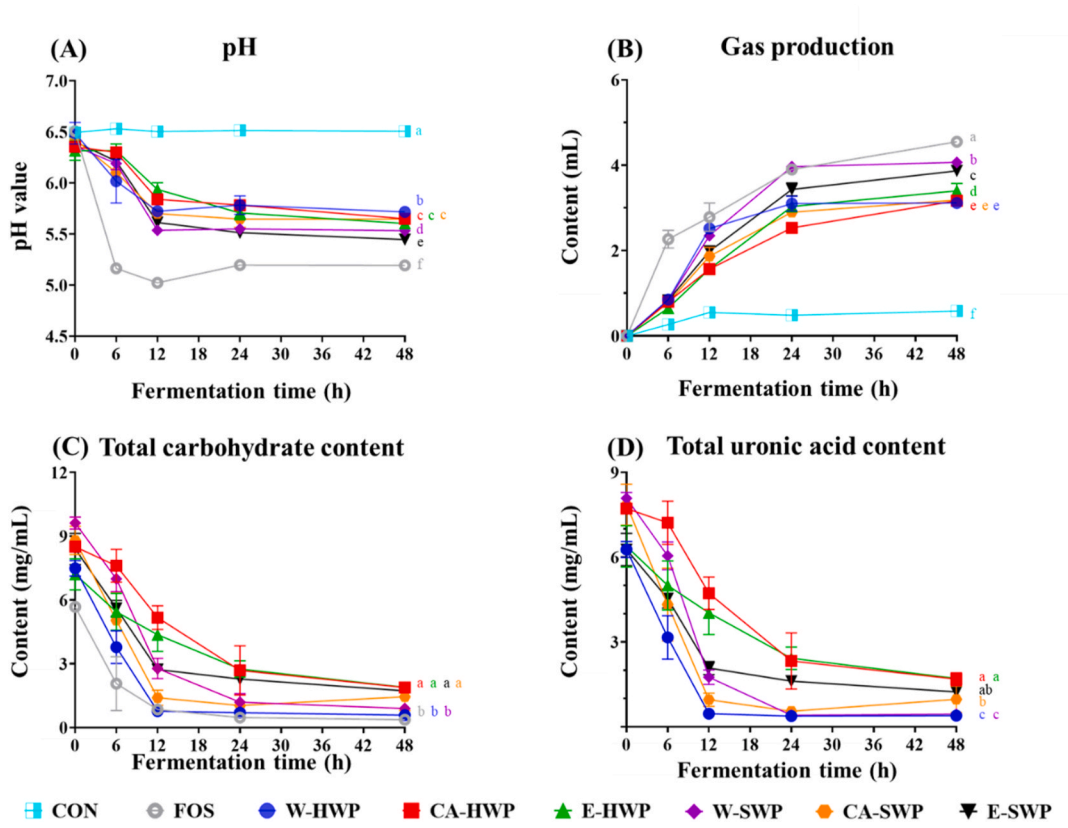

Fig. 3. Preliminary assessment of PPs during *in vitro* fermentation. Dynamic changes of pH value (A), total gas production (B), total carbohydrate content (C) and total UA content (D) of thermal extracted pectins from pomelo with/without chelators assistance at different times during *in vitro* fermentation. Values are means ± SD (n = 3) The different superscript letters indicate significant differences ($p < 0.05$) between different groups at 48 h, while same letters indicate no significance.

and high Mw (220.4 kDa, Table 1), which facilitated the formation of strong gel via calcium ions present in the fermentation medium. Our results are in contrast to previous findings that low DM pectin was fermented faster than high DM pectins *in vivo* and *in vitro* (Dongowski, Lorenz, & Proll, 2002; Tian et al., 2016; Tian et al., 2017). It could be attributed to the influences of other structural patterns, such as Mw, distribution of methyl-esters, ratio of HG, linearity, and degree of branching.

3.5. Dynamic changes of Mw and monosaccharides composition

As depicted in Fig. 4, all pomelo pectins underwent significant degradation by the gut microbiota after 24 h of fermentation. The Mw populations at various time points can be utilized to monitor the fermentability and the utilization speed of polysaccharides by gut microbiota (Wu et al., 2022). The limited degradation of E-HWP after 6 h of fermentation could be mainly ascribed to the high Mw and low DM as

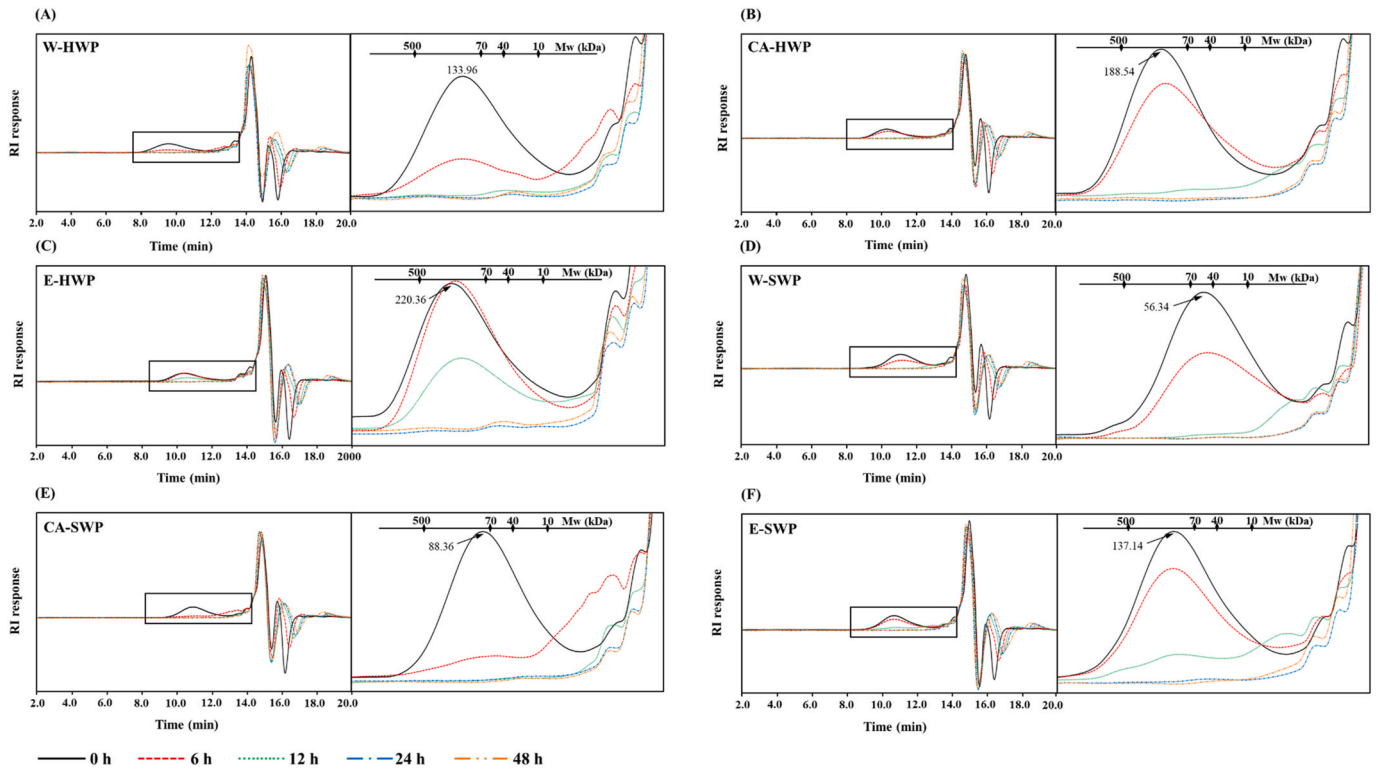

Fig. 4. Mw degradation of PPs during *in vitro* fermentation. HPSEC profiles on Mw degradation of W-HWP (A), CA-HWP (B), E-HWP (C), W-SWP (D), CA-SWP (E) and E-SWP (F) groups' dynamic changes during *in vitro* fermentation.

explained in the upper part.

As shown in Fig. 5(A-F), a gradual decrease in Ara, Gal, and Man over time was observed in all the groups during fermentation, indicating that these monosaccharides were effectively utilized by the gut microbiota. Rha/UA increased in all groups from 0 to 24 h and decreased thereafter (Fig. 5G), indicating that the main domain HG of pectin was preferably utilized compared to RG-I during the first 24 h of fermentation. However, the turning point at 24 h can be attributed to the effective fermentation of RG-I after the preferential utilization of HG by the gut microbiota. The decreasing ratio of (Ara + Gal)/Rha suggests that the branches of RG-I were preferentially degraded by gut microbiota, in comparison to the RG-I backbone, which is in agreement with our previous data (Ai et al., 2022). The linearity of pectin (measured as UA/(Fuc + Gal + Ara + Rha + Xyl)) in the CA/E-HWP groups increased significantly during the early stages of fermentation. This suggests that the gut microbiota preferred to consume the neutral monosaccharides rather than the galacturonic acid present in the pectin during early fermentation stages. This preference could be due to the relatively high Mw and low DM of CA/E-HWP which has high calcium ions binding capacity, limiting the accessibility for microbial enzymes. Conversely, a decrease in linearity values indicates the preferred consumption of galacturonic acid by gut microbiota.

3.6. Modulation of gut microbiota by pomelo pectins

Bacteroidota, Firmicutes, Proteobacteria, and Fusobacteriota had a higher relative abundance compared to other phyla and were determined to be the dominant bacteria in pectin-fermented groups (Fig. 6A). When compared to CON-48h, a decrease in the relative abundance of Proteobacteria and Fusobacteriota, and an increase in the relative abundance of Firmicutes and Bacteroidota were observed in all pomelo pectin supplemented groups.

The Firmicutes phylum, which includes the *Lactococcus*, *Phascolarctobacterium*, *Lachnospira*, and *Faecalibacterium* genera, are commonly found in the gut of healthy individuals and may be potential beneficial bacteria (Hu et al., 2019; Sun et al., 2022). As shown in Fig. 6B, compared to CON-48h, *Faecalibacterium* and *Phascolarctobacterium* increased in all sample groups after 48 h of *in vitro* fermentation, while E-HWP and CA-SWP had a significant promotion effect on *Faecalibacterium* and *Phascolarctobacterium*, respectively. Furthermore, a significant increase in the relative abundance of *Lactococcus* and *Lachnospira* was observed in W/E-SWP and CA-HWP/SWP, respectively. Polysaccharides can be utilized by Bacteroidota as the main energy source (Ferreira-Lazarte, Kachrimanidou, Villamiel, Rastall, & Moreno, 2018). The Bacteroidota phylum, which includes the *Bacteroides*, *Prevotella 9*, and *Parabacteroides* genera, can benefit the host by preventing potential pathogens that may colonize and infect the gut (Han et al., 2020; Wu et al., 2022). The relative abundance of *Bacteroides* and *Prevotella 9* increased in all the pomelo pectin supplemented groups compared to CON-48h group. In agreement to our data, a significant increase of *Bacteroides* by citrus pectins has been reported before (Dongowski et al., 2002). In a previous study, we also observed that the relative abundance of *Prevotella* in pig gut was significantly increased by pectin supplementation (Tian et al., 2017).

The heat map in Fig. 6C indicated the relative abundance of top 20 bacteria at genus level. The HWP and SWP showed different effects on bacterial profiles. Compared to other pectin-fermented groups, W/E-SWP had a relatively poorer inhibition on *Escherichia-Shigella*, while promoting a higher relative abundance of *Lactococcus*. The CA-HWP group had high relative abundances of *Lachnospira* and *Enterobacter*, whereas the E-HWP group had a high abundance of *Faecalibacterium*. The CA-SWP group had high relative abundances of *Enterococcus*, *Phascolarctobacterium*, and *Pantoea*, while the W-HWP group had increased relative abundances of *Sutterella* and *Lachnospiraceae UCG 004*. These results suggest that supplementation with pomelo pectins could alter the microbial composition and relative abundance, which could potentially be beneficial for maintaining intestinal homeostasis.

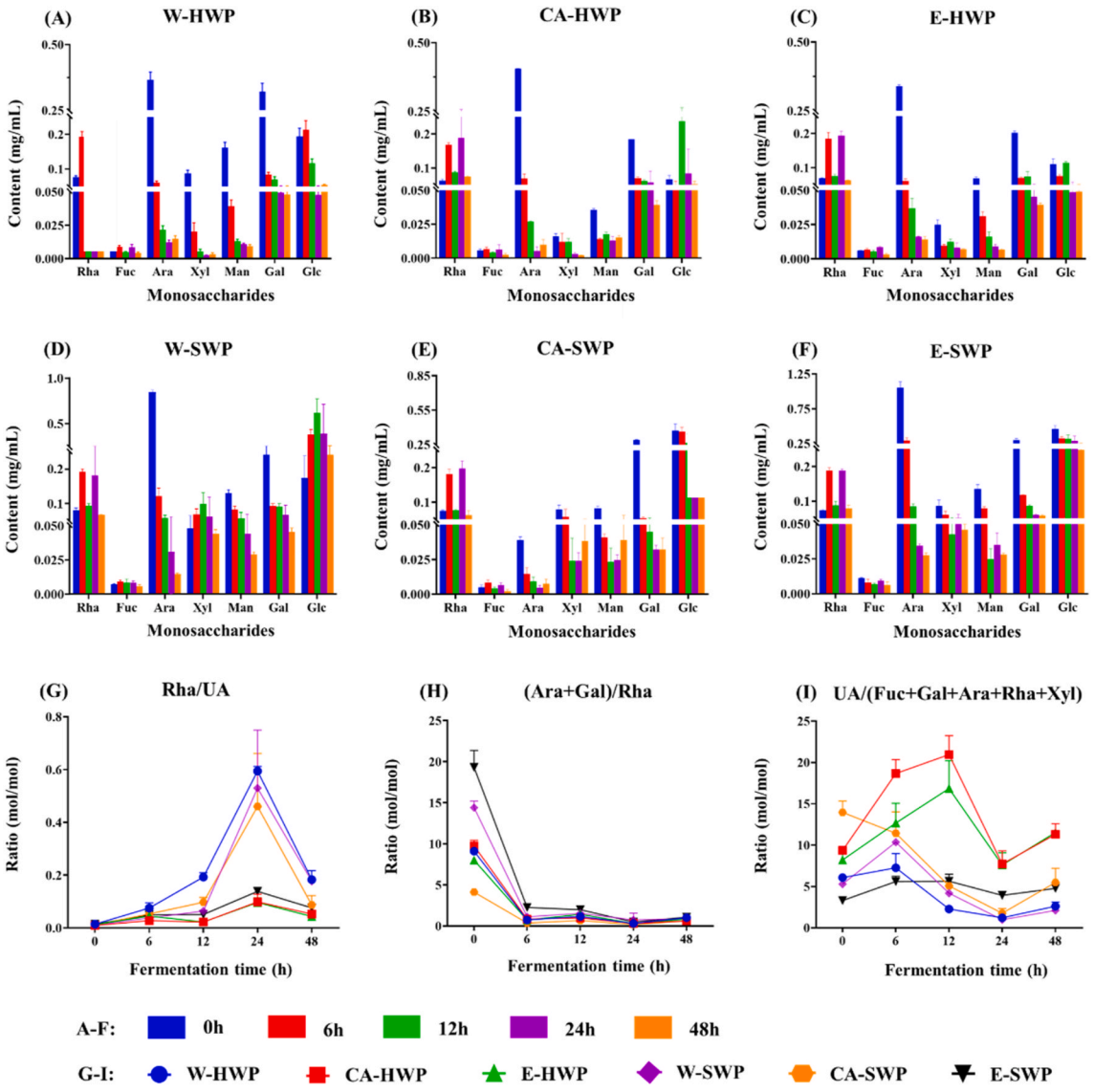

Fig. 5. Dynamic changes on monosaccharides composition and structures of PPs during *in vitro* fermentation. Dynamic changes on monosaccharides contents of W-HWP (A), CA-HWP (B), E-HWP (C), W-SWP (D), CA-SWP (E) and E-SWP (F) during *in vitro* fermentation at different time points. Rha/UA: the contribution of RG-I domain to the entire pectin (G), (Ara + Gal)/Rha: the degree of RG-I branching (H), UA/(Fuc + Gal + Ara + Rha + Xyl): the linearity of pectin (I), calculating by molar ratio.

3.7. Dynamic changes of SCFA production

As shown in Fig. 7, the contents of all types of SCFA increased after 24 h of *in vitro* fermentation, which is consistent with the effective utilization of carbohydrates by gut microbiota (Fig. 3C and **D**). However, the total SCFA content decreased at 48 h compared to 24 h, which could be explained by the carbohydrate shortage and the consumption of SCFA by gut microbiota (Sun & O'Riordan, 2013. Acetate and propionate are both effective anti-inflammatory mediators (Kang et al., 2022). Compared to FOS, there was a significant ($p < 0.05$) increase in the concentration of acetic acid in pectin groups, especially CA-SWP, which may be correlated with the higher relative abundance of *Bacteroides* that produces acetic acid during fermentation (Sun & O'Riordan, 2013. Citrus pectins have been suggested to be a good source for acetate production (Tian et al., 2016). All pectin groups had higher propionic acid content than the control, particularly W-HWP in the pectin groups. Butyrate can increase the expression of tight junction proteins, thereby reducing potential gut permeability and inflammation associated with a leaky gut (Bang et al., 2018). All pectin groups had significantly ($p <$ 0.05) increased butyrate acid content, particularly W-SWP, which can be attributed to the *Lachnoclostridium*, *Ruminococcaceae*, and *Faecalibacterium* genera that produce butyrate. The pH value decreased significantly ($p < 0.05$) in FOS group than others after 48 h *in vitro* fermentation (Fig. 3A), which may be conducted to high relative abundance of *Bifidobacterium* (Fig. 6F), a genus that can ferment carbohydrate and produce lactic acid which is more acidic than other SCFAs (Valdes-Varela, Ruas-Madiedo, & Gueimonde, 2017).

The isobutyric acid and isovaleric acid are the metabolism of branched-chain amino acids as the final product of protein catabolism that utilized by gut microbiota (Ratajczak et al., 2021). The ratio of total SCFAs/total branched short-chain fatty acid (BCFAs, sum of isobutyric acid and isovaleric acid, Fig. 7H) can be used to assess the overall fermentation pattern during different time points. Pomelo pectin groups had combination use of carbohydrate and proteins as the energy sources and carbohydrates were preferred (Koecher et al., 2014; Sun & O'Riordan, 2013. As shown in Fig. 7I, the microbiota's consumption of carbohydrates over time results in a shift in gut microbiota from carbohydrate fermentation to partial protein utilization. The total

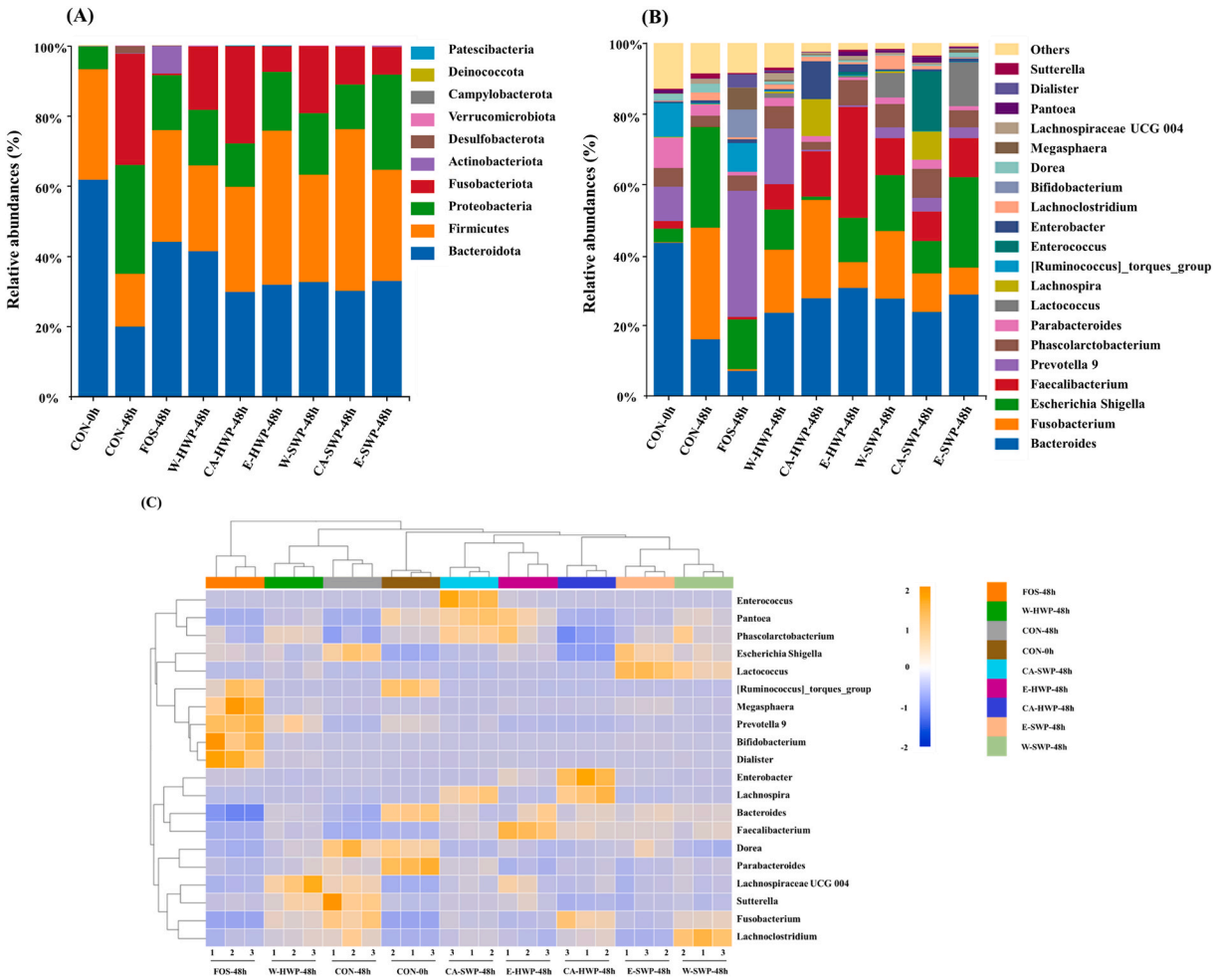

Fig. 6. Comparison of PPs on microbiota composition after *in vitro* fermentation. The bacterial taxonomic profiling of gut microbiota at the phylum level (A), the genus level (B), and the heatmap analysis (C) of the relative abundances at the genus level for all groups after 48 h *in vitro* fermentation. Different colors indicate different bacteria in the phylum or genus. CON-0h: control group after 0 h of fermentation. Group-name-48 h: the corresponding group after 48 h of fermentation.

SCFAs/total BCFAs ratio of E-HWP was significantly ($p < 0.05$) higher than the other groups at 24h and 48 h, which can be attributed to its slower degradation and utilization by gut microbiota due to its high Mw of 220.4 kDa (Table 1).

Previous research has shown that pectins with low Mw have favorable fermentation properties, resulting in high production of SCFAs and an abundant population of SCFA-producing genera (Zhao et al., 2021). Subcritical water extraction not only significantly increases the yield, but also alters the pectin structure by degrading the Mw and increasing the percentage of HG in the resulting products. These changes increase the fermentability and prebiotic effects of the corresponding pectins. All pomelo pectins showed great potential for improving the gut microbiota community and producing beneficial prebiotic effects, especially E-HWP and CA-SWP, which had a relatively high abundance of *Bacteroides* and *Faecalibacterium*. The manipulation of pectic substances on gut microbiota metabolites is complicated, and the link between structural specificity of pectin and fermentability need further exploration.

4. Conclusion

Pomelo peel, a by-product of fruit processing, is an excellent source of pectin. The chemical structure of pomelo pectins highly depends on the extraction methods used. Our results indicate that chelator-assisted extraction increased the pectin Mw and decreased the DM of pectin, whereas subcritical water treatment had the opposite effect. Pectin with low RG-I and high HG domain can be obtained by citric acid-assisted extraction. Pomelo pectins enhanced the relative abundance of *Bacteroides* and *Prevotella 9*, and stimulated the production of SCFA, particularly acetic acid. Subcritical water extraction could promote the fermentability and prebiotic potential of obtained pectin. Generally, pomelo pectin could be considered a new source for modulating gut microbiota towards a healthy pattern in functional foods.

Funding

This work was supported by the International Science and Technology Cooperation Project of Guangdong (2021A0505030043), the Foundation of Key Laboratory of South China Agricultural Plant Molecular Analysis and Genetic Improvement, South China Botanical Garden, Chinese Academy of Sciences, the Pearl River Talent Plan (2017GC010387), and the (2019TQ05N770). Youth Science and Technology Innovation Talent of Guangdong TeZhi Plan

Author contributions

Yuxin Wang: Methodology, Investigation, Validation, Writing - Original Draft. **Jiaxin Liu**: Methodology, Validation, Visualization. **Li Chen:** Methodology, Validation. **Shuang Jin**: Review & Editing. **Can An**: Review & Editing. **Long Chen**: Writing - Review & Editing. **Bao Yang**: Writing - Review & Editing. **Henk A. Schols**: Writing - Review & Editing. **Paul de Vos**: Writing - Review & Editing. **Weibin Bai:** Supervision, Writing - Review & Editing**. Lingmin Tian**: Conceptualization,

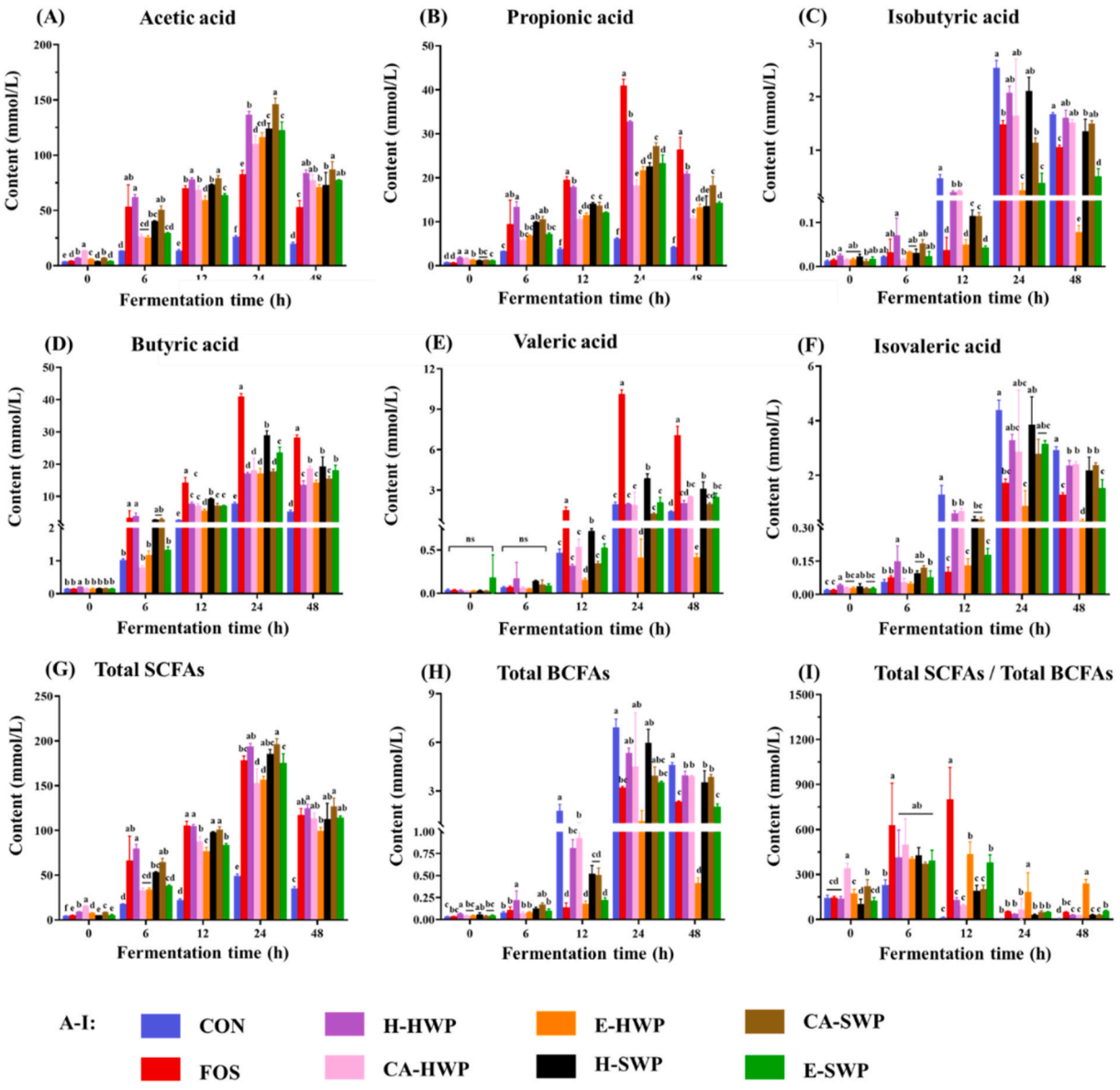

Fig. 7. Dynamic changes of PPs' SFCAs production during *in vitro* fermentation. Dynamic changes of all groups on SCFAs contents (production or consumption) at different time points during *in vitro* fermentation: acetic acid (A), propionic acid (B), isobutyric acid (C), butyrate acid (D), valeric acid (E) and isovaleric acid (F). The total SCFAs was calculated by sum of the six short chain fatty acids (G), and total BCFAs was summed by isobutyric and isovaleric acids (H). Values are means ± SD (n = 3) The different superscript letters indicate significant differences ($p < 0.05$) between different groups at 48 h, while same letters indicate no significance.

Supervision, Writing - Review & Editing.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Abbreviations

PPs	pomelo pectins
HWP	hot water extraction pectin
SWP	subcritical water extraction pectin
W/CA/E-HWP	hot water only extraction/critic acid assisted extraction/EDTA-2Na assisted extraction pectin
W/CA/E-SWP	subcritical water only extraction/citric acid assisted extraction/EDTA-2Na assisted extraction pectin
FT-IR	Fourier transform-infrared spectral
Mw	molecular weight
HPSEC	high performance size exclusion chromatography
Rha	rhamnose
Fuc	fucose
Ara	arabinose
Xyl	xylose
Man	mannose
Gal	galactose
Glc	glucose
GalA	galacturonic acid
UA	uronic acid
CON	blank control
FOS	fructo-oligosaccharides
SCFAs	short-chain fatty acids
BCFAs	branched short-chain fatty acids
HG	homogalacturonan

RG-I rhamnogalacturonan I
DM Degree of methyl esterification

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