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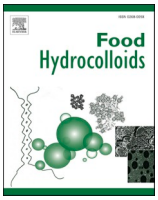

Enzymatic modification of pomelo pectins for shaping the gut microbiota to a healthy pattern

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ABSTRACT

This study investigated the impact of enzymatic hydrolyzation on the characteristics, composition, and *in vitro* fermentation patterns of pectins extracted from pomelo (*Citrus maxima*) by thermal treatments. Pectins were obtained using subcritical water extraction (SWE) and hot water extraction with chelator assistance, and subsequently, enzymatically modified using pectinase. The investigation covered constituent monosaccharide composition, molecular weight (Mw) distribution, and other structural parameters of the pectins. The primary monosaccharide present in the extracted pomelo pectins was uronic acid (UA), accompanied by relatively low levels of other neutral monosaccharides. The findings indicate that chelating agents coupled with thermal treatment allowed for the successful extraction of low degree of methylation (DM) pectins from pomelo peel. Enzymatic hydrolyzation reduced the DM and Mw of pectin, while maintaining a nearly unchanged monosaccharide mole ratio compared to the parental pectins. *In vitro* fermentation models were employed to compare the fermentation patterns of pectin before and after enzymatic modification. Enzymatic modification not only heightened the fermentability of pectins but also altered UA utilization patterns, expediting the production of short-chain fatty acids (SCFAs) during *in vitro* fermentation. Moreover, enzymatic hydrolyzation induced changes in microbial composition, elevating the relative abundance of potential probiotics such as *Bacteroides* while suppressing potential pathogens like *Escherichia-Shigella*. In conclusion, the study posits that enzymatic modification of pectins into low Mw and low DM fragments enhances fermentability and beneficially modifies the microbiota of fermented digesta into a potentially healthy pattern.

1. Introduction

Pectin is a main type of soluble fiber present in the cell wall of pomelo (Gamonpilas, Buathongjan, Kirdsawasd, et al., 2021). In a prior study on pomelo (*Citrus grandis*) peel pectins, we observed that both chelator assistance and subcritical water treatment led to an increased pectin yield (Wang et al., 2023). Chelators function by binding to metal ions, such as calcium, which may be present in the plant material. This binding causes the calcium to dissociate from the gel it is bound to, facilitating the dissolution of pectin. Subcritical water extraction (SWE) represents an eco-friendly and efficient technology for extracting plant polysaccharides, utilizing liquid-state water under elevated temperatures (100–374 °C) and pressures (0.1–22.1 MPa) (Liew, Teoh, Tan, Yusoff, & Ngoh, 2018).

Chelator-assisted extraction resulted in an increase in pectin molecular weight (Mw) and a decrease in the degree of methyl esterification (DM). Conversely, subcritical water treatment exhibited the opposite effect. Utilizing citric acid for extraction allowed us to obtain pectin with a low rhamnogalacturonan-I (RG-I) and high homogalacturonan (HG) domain from the discarded pomelo peels (Wang et al., 2023). The chemical structural patterns of the obtained pectins displayed variations due to the distinct treatment methods applied (Methacanon, Krongsin, & Gamonpilas, 2014).

The structure of pectin is highly complex, comprising a blend of linear and branched polysaccharide chains. In our previous work, the HG domain of pomelo pectins ranged from approximately 70-90%,

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while the RG-I domain constituted 3.5–10.2% (Wang et al., 2023). RG-I typically features branched side chains like arabinan, galactan, and/or arabinogalactan (Niu et al., 2023). Pectinases play a pivotal role as invaluable tools for modifying pectin to achieve desired properties (Duvetter et al., 2009). Pectinases, a diverse group of complex enzymes, can be categorized based on their specific activities. For instance, polygalacturonase and pectin lyase degrade pectin samples into smaller molecules, such as pectin fragments, oligosaccharides, and monosaccharides, while pectinesterase facilitates the de-esterification of pectin (Duvetter et al., 2009; Jayani, Saxena, & Gupta, 2005). The modification of pectin's physical and chemical characteristics subsequently tailors its functionality and properties. Particularly, pectin rich in RG-I domains, low-Mw homogalacturonan, and low-methyl esterified pectin have exhibited beneficial prebiotic properties (Gulfi, Arrigoni, & Amadò, 2007; Tian et al., 2017; Zhao et al., 2021). Therefore, the hydrolyzed fragments of pectinase may possess specific characteristics that could provide promoted health benefits.

The health benefits are partly determined by the fermentability of the pectic fragments. The extent of pectin fermentation influences regulatory effects on microbial composition and the generation of microbial metabolites (Jonathan et al., 2012). Various complex pectic polysaccharides have been studied in *vitro* fermentation to imply the process of microbial fermentation that may occur in the gut (Dongowski, Lorenz, & Proll, 2002; Tian et al., 2017; Zhao et al., 2021). In the present study, the selected pectins were obtained using chelator assistance with hot water and subcritical water extraction. Pectinase was used to modify the polymers, and structural changes during enzymatic hydrolysis were monitored. An *in vitro* fermentation model was established to evaluate the fermentation properties of the obtained pectic fractions, and the correlation between pectin structural properties, metabolites, and gut microbiota was analyzed.

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 the dialysis bag (8000–14000 Da) were purchased from Beijing Solarbio Science and Technology Co. Ltd (Beijing, China). The pectinase (CAS: 9032-75-1, enzyme activity at 30000 U/g, derived from *Aspergillus niger*, complex enzymes that mainly contain pectin polygalacturonase, pectinesterase, and pectin lyase) was purchased from Shanghai Macklin Biochemical Technology Co., Ltd (Shanghai, China). Enzyme activity definition: The unit of pectinase activity, denoted as 1 U (unit), is defined as the amount of enzyme required to generate 1 micromole of glucose-equivalent substances by decomposing pectin within 1 min, under the conditions of 50 °C and pH 4.8. The standards of short-chain fatty acids (SCFAs) were purchased from Aladdin Biochemical Technology Co. Ltd (Shanghai, China). The monosaccharide standards and dextran standards were purchased from Sigma-Aldrich Chemical Co., Inc. (WI, USA). The commercial fructooligosaccharides (FOS) with a purity at 95% were purchased from Quantum Hi-Tech (Guangdong) Biological Co., Ltd. (Jiangmen, China). This product is characterized by sucrose-derived trisaccharides, tetrasaccharides, and pentasaccharides, formed through β-(1 → 2) glycosidic bonds linking one to three fructose units to the fructose residues of sucrose. The standard pectins (Amid CU-L and Classic CU-L series) with different DM values were obtained from Herbstreith & Fox Co., Inc. (Neuenbürg, Germany). All other chemical reagents used were analytical purity.

2.2. Extraction and modification of pomelo pectins

The extraction of pectins from fresh pomelo peels (including flavedo and albedo parts) were described previously (Wang et al., 2023). EDTA-2Na assisted hot water extracted pectin (*E*-HWP) and citric acid assisted subcritical water extracted pectin (CA-SWP) were selected for enzymatic modification due to their difference in molecular weight (Mw) and fermentation patterns. The yields of CA-SWP and *E*-HWP were calculated based on the weight of dried pectin and dried pomelo peel.

For further enzyme treatment, CA-SWP and *E*-HWP was dissolved into acetic acid-sodium acetate buffer solution (50 mM, pH 4.5) to achieve a concentration of 5 mg/mL. The pectinase (30000 U/g) was added into the corresponding mixed solution to achieve the enzyme activity at 2.5 U/mL. Pectinases were used to individually treat CA-SWP and *E*-HWP through two stages (I and II) with treatment durations of 0.5 and 2.5 h at 25 °C, respectively (Fig. 1). This process aimed to obtain distinct pectin fragments (E1 and E2-CA-SWP and E1 and E2-E-HWP). Subsequently, the hydrolyzed solution was heated at 90 °C for 15 min to denature and inactivate the pectinases. Finally, the solution was filtered and dialyzed by dialysis bag (8000–14000 Da) at 4 °C for 48 h to remove monosaccharides, oligosaccharides, and low molecules pectin fragments. The final pectinase treated solution of CA-SWP and *E*-HWP was dried by freezing vacuum to get their enzymatically hydrolyzed pectin samples. The yield of E1 & E2 (%, w/w) = the weight of modified pectin samples/the weight of unmodified pectin × 100%.

2.3. Molecular weight (Mw) distribution

The Mw analysis was conducted using high performance size exclusion chromatography (HPSEC) with TSK-GEL on a TSKgel Guard-column SuperSW guard column (6 mm ID × 40 mm), followed by 4000, 3000 and 2500 TSKgel Super AW series columns (6 mm ID × 150 mm), all connected to an RI detector (RefractoMax520, Thermo Fisher, MA, USA) (Ai et al., 2022; Wang et al., 2023). The mobile phase was a solution of $NaNO_3$ at a concentration of 0.2 M, passing through tandem column operating at a temperature of 55 °C with a flow rate of 0.6 mL/min for pectins, and a temperature of 60 °C with a flow rate of 0.45 mL/min for fermented digesta, respectively. The standard curve was obtained by various dextran standards (Mw of 10, 40, 70, 500 and 2000 kDa) (Wang et al., 2023).

2.4. Constitute monosaccharide content and composition

The constitute monosaccharide content and composition were analyzed as described previously (Luo et al., 2024; Wang et al., 2023). 10 mg samples and 1 mg of inositol (internal standard) were mixed and hydrolyzed in 72% (w/w) H_2SO_4 (1 mL) 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 (Shimadzu G2010Plus, Kyoto, Japan) (Englyst & Cummings, 1984). The hydrolyzed samples were oxidized by heating with *p*-hydroxydiphenyl and $Na_2B_4O_7$. The resulting product was then reacted with 98.3% (w/w) H_2SO_4 in order to form purple chromophore. The uronic acid (UA) content of the samples was quantified at 520 nm with D-galacturonic acid as a standard (Blumenkrantz & Asboe-Hansen, 1973). Total carbohydrate contents were calculated by summing all determined monosaccharides, including rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl), mannose (Man), galactose (Gal), glucose (Glc) and UA.

2.5. FT-IR spectroscopy and degree of methylation (DM)

The dried pectin's KBr tablets were subjected to Fourier transform infrared (FT-IR) spectrometry 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 with a salt-to-sample proportion of 90:10. The DM of the samples was determined by referencing an established standard curve with *I* and DM values. *I* was calculated as $I = A_{1745}/(A_{1745} + A1_{630}) \times 100\%$, where *I* represents the percentage of ester-based peak area. A_{1745} and A_{1630} represent the absorption peak area of the methyl

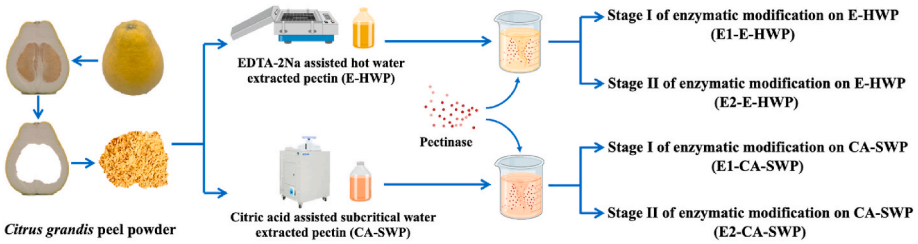

Fig. 1. Extraction and enzymatic modification processing of pomelo pectins.

ester group and the carboxylate absorption peak area, respectively, as reported in previous studies (Yu et al., 2021). The standard curve was established using pectins with different DM (3, 20, 37, 55, 62.8 and 70.5%).

2.6. *In vitro fermentation experiments*

2.6.1. *In vitro fermentation*

The *in vitro* fermentation was conducted with some modifications based on previously published method (Wang et al., 2023). Briefly, fresh fecal samples were collected from eight healthy donors, consisting of five males and three females aged 20–24, who had not received any antibiotics treatment or had any digestive-related illnesses within the past three months. The research was approved by IRB of Jinan University (Approval: JNUKY-2023-0070). The collected fecal matters were homogenized, centrifuged and diluted to a concentration of 0.1 g/mL using phosphate-buffered saline (PBS) (10 mM, pH 7.4).

The fermentation basal nutrient medium was prepared as described previously (Wang et al., 2023). The *in vitro* fermentation was conducted using 5 mL anaerobic fermentation tubes. The CA-SWP and *E*-HWP, and their enzymatically hydrolyzed pectin samples (E1/E2-CA-SWP and E1/E2-CA-SWP) were dissolved in sterilized medium to a concentration of 10 mg/mL. FOS were used as the positive control, and the basal nutrient growth medium without adding any else carbon source was used as the blank control (CON). The fermentation medium was inoculated with 10% of the total volume of diluted feces supernatant. The tubes were sealed and incubated at 37 °C (100 rpm) in a vibrating incubator for 0, 3, 6, 9, 12 and 24 h.

2.6.2. *Determination of gas production and pH values*

The gas production (mL) was measured using a piston type sterile needle inserted through a rubber plug into tubes without touching the medium. The pH values of fermented samples were determined using a 962244 detector (PHS–3C, Shanghai Leici Apparatus Corp., Shanghai, China) under 25 °C.

2.6.3. *Determination of SCFAs*

Samples were supplemented with diethylacetic acid as an internal standard. The mixed solution of 2 μL was injected into a 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) (Tan et al., 2023). The standards curve was generated using acetic, propionic, butyric, isobutyric, valeric and isovaleric acid.

2.6.4. *Microbiota shifts during in vitro fermentation*

The genomic DNA was extracted with a stool genomic DNA extraction kit (Solarbio, CHN) according to the operation instructions. The concentration of the DNA was determined by agarose gel electrophoresis and NanoDrop 2000 (Thermo Fisher, USA). The amplification of 16 S rRNA gene of V3–V4 region from bacteria was amplified by 343 F: 5′-TACGGRAGGCAGCAG-3′ and 798 R: 5′-AGGGTATCTAATCCT-3′ universal primer set. The primer sequences were removed from the raw data sequences by Cutadapt (version 1.14). The qualified paired-end raw data were subjected to quality control analysis including quality filtering, denoising, merging, and chimera removal by DADA2 according to default parameters in QIIME 2 (2020.11) (Quast et al., 2013). The resulting representative sequences and amplicon sequence variants (ASVs) abundance tables were obtained. Further, the representative sequences for each ASV were selected by QIIME 2 and annotated by mapping to SILVA database (version 138) (Tan et al., 2023).

2.7. *Statistic analysis*

The statistical analysis of the data was carried out by SPSS 21 software. Visualization of the data was performed by GraphPad Prism 9.0, Origin 2021 and Excel 2016 software. Significant differences at $p < 0.05$ were determined with ANOVA tests followed by Duncan tests.

3. Results

3.1. *Structural characteristics of pomelo pectins*

The main monosaccharide among these pectin samples was UA (≥85 mol%), and the sum of the other monosaccharides (Rha, Fuc, Ara, Xyl, Man, Gal, and Glc) were all below 7 mol% (Table 1). Compared with *E*-HWP, a significant ($p < 0.05$) decrease in UA (84.9 mol% vs 89.3 mol%) and a significant ($p < 0.05$) increase in Ara (7.4 mol% vs 4.8 mol%) and (Gal 2.9 mol% vs 2.1 mol%) were observed in its hydrolyzed pectin sample E2-E-HWP. No significant differences were observed in the mole ratio of monosaccharides for CA-SWP and its two enzymatically hydrolyzed samples. The total carbohydrate content of the pectic samples decreased with the increase of enzymatic incubation time: CA-SWP (98.1%) > E1-CA-SWP (92.4%) > E2-CA-SWP (81.9%) and *E*-HWP (92.9%) > E1-E-HWP (88.8%) > E2-E-HWP (77.3%). Simultaneously, in the enzymatic hydrolyzation stages I and II, a declining trend in DM value was evident: CA-SWP (21.0) > E1-CA-SWP (14.7) > E2-CA-SWP (10.1); *E*-HWP (15.8) > E1-E-HWP (11.4) > E2-E-HWP (8.6) (Table 1). The Mw of *E*-HWP (238 kDa) was significantly ($p < 0.05$) higher than CA-SWP (82 kDa). Both E1 and E2 pectin fragments exhibited a significantly reduction in Mw, compared to CA-SWP and *E*-HWP ($p < 0.05$) (Table 1 and Fig. 2A).

3.2. *FT-IR pattern of pomelo pectins*

The FT-IR characteristic peaks of extracted pectin CA-SWP and *E*-HWP and their enzymatically hydrolyzed samples are shown in Fig. 2B. The peak near 2940 cm^{-1} was attributed to the stretching vibration of *C*–H (Zhao et al., 2021). All pectin samples showed an absorption peak at 1745 cm^{-1}, which was caused by the stretching vibration of the ester C═O bond, and the peak at 1630 cm^{-1} was due to the stretching vibration of the carboxyl C═O bond in pectin (Yu et al., 2021). The peak intensity at 1745 cm^{-1} in CA-SWP and *E*-HWP were greater than their enzymatically hydrolyzed samples, correspondingly. The peak near 1100 cm^{-1} was caused by the stretching vibration of C–O–C (Wang et al., 2022).

Table 1
Characteristics, constituent monosaccharide composition and structural patterns of pomelo pectins.

Item	CA-SWP	E1-CA-SWP	E2-CA-SWP	*E*-HWP	E1-E-HWP	E2-E-HWP
Characteristics						
Yield (%, w/w)	14.5 ± 1.4	91.7 ± 1.1	83.24 ± 0.8	10.5 ± 2.1	93.2 ± 1.6	85.5 ± 0.8
Mw (kDa)	82 ± 8^{b}	39 ± 6^{d}	14 ± 3^{e}	238 ± 11^{a}	49 ± 5^{c}	13 ± 5^{e}
Total carbohydrate content (%, w/w)	98.1 ± 2.4^{a}	92.4 ± 4.0^{b}	81.9 ± 2.8^{c}	92.9 ± 4.4^{ab}	88.8 ± 3.8^{b}	77.3 ± 3.1^{c}
Monosaccharide composition (mol%)						
Rha	1.1 ± 0.1^{ab}	0.9 ± 0.0^{b}	1.0 ± 0.0^{ab}	0.9 ± 0.0^{ab}	0.9 ± 0.2^{b}	1.2 ± 0.1^{a}
Fuc	0.1 ± 0.0^{bc}	0.1 ± 0.0^{c}	0.1 ± 0.0^{bc}	0.1 ± 0.0^{bc}	0.1 ± 0.0^{b}	0.2 ± 0.1^{a}
Ara	1.4 ± 0.5^{c}	1.0 ± 0.2^{d}	1.1 ± 0.3^{cd}	4.8 ± 0.6^{b}	4.8 ± 0.5^{b}	7.4 ± 0.3^{a}
Xyl	0.6 ± 0.1^{a}	0.6 ± 0.1^{ab}	0.5 ± 0.2^{ab}	0.3 ± 0.1^{c}	0.5 ± 0.2^{ab}	0.4 ± 0.1^{bc}
Man	0.8 ± 0.0^{bc}	0.6 ± 0.2^{d}	0.6 ± 0.1^{cd}	0.8 ± 0.3^{b}	0.8 ± 0.0^{b}	1.0 ± 0.1^{a}
Gal	1.9 ± 0.1^{c}	2.0 ± 0.1^{bc}	1.5 ± 0.1^{d}	2.1 ± 0.2^{bc}	2.1 ± 0.2^{b}	2.9 ± 0.3^{a}
Glc	1.5 ± 0.1^{c}	2.0 ± 0.2^{a}	1.5 ± 0.1^{c}	1.7 ± 0.3^{b}	1.7 ± 0.1^{b}	2.1 ± 0.2^{a}
UA	92.6 ± 1.4^{a}	92.9 ± 3.1^{a}	93.7 ± 2.0^{a}	89.3 ± 3.0^{a}	89.1 ± 2.5^{a}	84.9 ± 1.8^{b}
Structural patterns						
DM	21.0 ± 1.3^{a}	14.7 ± 1.2^{b}	10.1 ± 0.2^{cd}	15.8 ± 1.0^{b}	11.4 ± 1.5^{c}	8.6 ± 0.6^{d}
Rha/UA (10^{-3})	11.9	9.7	10.7	10.1	10.1	14.1
(Ara + Gal)/Rha	3.0	3.3	2.6	7.7	7.7	8.6
UA/(Fuc + Xyl + Rha + Ara + Gal)	18.2	20.2	22.3	10.9	10.6	7.0

Note: DM: degree of methyl esterification. 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 mole ratio. The different superscript letters indicate significant difference ($p < 0.05$).

3.3. Dynamic changes of pH, gas production and pectin utilization during fermentation

During the 24 h of *in vitro* fermentation, the pH of pectin supplemented groups decreased, while the pH of CON group exhibited minor fluctuations around 6.0 (Fig. 3A). The pH value in CA-SWP group (pH 5.2) was significantly lower than in *E*-HWP (pH 5.7) at the end of fermentation. Compared with *E*-HWP, the pH value of both E1-E-HWP (pH 5.4) and E2-E-HWP (pH 5.5) significantly ($p < 0.05$) decreased after 24 h *in vitro* fermentation. Interestingly, when compared with CA-SWP group, the pH value of E1-CA-SWP group (pH 5.5) and E2-CA-SWP group (pH 5.4) were significantly ($p < 0.05$) higher after 24 h fermentation.

As shown in Fig. 3B, the gas production volume of *E*-HWP was lower than those of CA-SWP during the fermentation. After 24 h of *in vitro* fermentation, significant differences ($p < 0.05$) in gas production were observed, with the order: E2-E-HWP (2.9 mL) > E1-E-HWP (2.4 mL) > *E*-HWP (1.5 mL). The gas production of CA-SWP (3.1 mL) was significantly ($p < 0.05$) higher than E1-CA-SWP group (2.5 mL) and E2-CA-SWP group (2.7 mL) after 24 h.

In general, the total carbohydrate and UA content in all pectin samples exhibited a significant downtrend as fermentation time progressed (Fig. 3C and **D**). After 24 h of *in vitro* fermentation, the carbohydrate contents of E1-E-HWP group (1.9 mg/mL) and E2-E-HWP group (2.0 mg/mL) were significantly lower compared with *E*-HWP group (3.9 mg/mL). There was non-significant difference in the residual carbohydrate content between CA-SWP (1.2 mg/mL), E1-CA-SWP (1.0 mg/mL) and E2-CA-SWP (0.9 mg/mL) after 24 h (Fig. 3C). After 24 h of *in vitro* fermentation, the leftover UA in E1-E-HWP group (1.8 mg/mL) was significantly ($p < 0.05$) lower compared to that in *E*-HWP group (3.8 mg/mL). Compared with CA-SWP group (1.1 mg/mL), the UA content in E2-CA-SWP group (0.7 mg/mL) were significantly lower after 24 h of fermentation (Fig. 3D). The utilization rate of total carbohydrate and UA was defined by dividing the average consumption of each sample at a specific stage by the starting content of that same stage. In 0–12 h ranges, the utilization rates of both total carbohydrate and UA for *E*-HWP group were lower than that for CA-SWP group, until they equalized in 12–24 h. An initially rising utilization rates of UA and total carbohydrates for CA-SWP group and its enzymatically hydrolyzed samples were observed in 0–9 h ranges, and then a falling utilization rates in 9–24 h ranges. In 9–12 h, CA-SWP group reached a top UA utilization rate of 57.9%, while E1-CA-SWP and E2-CA-SWP hit their maxima of 59.6% and 60.2% in 6–9 h, respectively. As fermentation progresses, the utilization rates of carbohydrates and UA for *E*-HWP group and its enzymatically hydrolyzed samples tend to increase.

3.4. Degradation of pectin Mw during in vitro fermentation

The main population of molecules present in the parental pectins shifted towards a lower Mw with fermentation progressed (Fig. 4). The Mw of CA-SWP and its enzymatically modified samples decreased over time and was barely detectable after 24 h fermentation (Fig. 4A-C). Compared to CA-SWP and its enzymatically modified samples, the Mw of *E*-HWP and its enzymatically modified samples correspondingly reduced at a lower extent (Fig. 4D-F). Compared with untreated pomelo pectin (CA-SWP or *E*-HWP), more extensive degradations were observed for its enzymatically hydrolyzed samples during the *in vitro* fermentation.

3.5. Utilization of neutral monosaccharides during in vitro fermentation

Significant degradations of the main neutral sugars were found for all pomelo pectins after 24 h of fermentation (Fig. 5). Gal was the most abundant monosaccharide in CA-SWP and its enzymatically hydrolyzed samples (Fig. 5A-C), while Ara was the most abundant monosaccharide in *E*-HWP and its enzymatically hydrolyzed samples (Fig. 5D-F). The Rha/UA ratio increased with fermentation progressed in E1-CA-SWP and E2-CA-SWP, while a decrease was observed at 12 h in CA-SWP (Fig. 5C). Additionally, there was a decrease in the (Ara + Gal)/Rha ratio during the first 9 h of fermentation across all groups (Fig. 5D). As shown in Fig. 5E, The UA/(Fuc + Gal + Ara + Rha + Xyl) values increased in *E*-HWP and its enzymatically hydrolyzed samples over time, with a turning point seen at 12 h for E1-E-HWP and E2-E-HWP. For CA-SWP and its enzymatically hydrolyzed samples, this value rose in the first 6 h of fermentation before declining.

3.6. SCFA production

In all the pomelo pectin supplemented groups, the SCFA production increased with fermentation time (Fig. 6). Acetic acid is the most dominant SCFA (Fig. 6A), followed by propionic acid and butyric acid. Significant ($p < 0.05$) higher levels of acetic acid and total SCFAs (Fig. 6G) were found in CA-SWP group than in both E1-CA-SWP and E2-CA-SWP after 24 h of fermentation. On the contrary, after 24 h fermentation of E1-E-HWP and E2-E-HWP with lower Mw and DM, the concentrations of acetic acid, propionic acid (Fig. 6B) and total SCFAs in

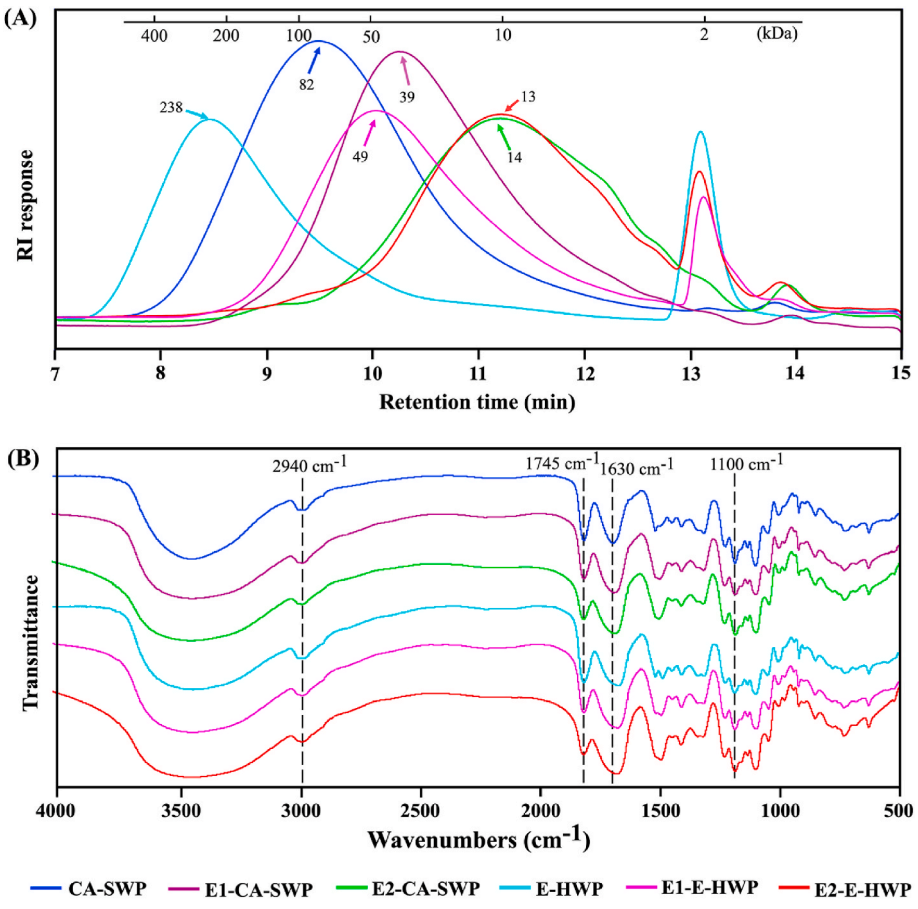

Fig. 2. The Mw distribution (A) and FT-IR spectra (B) of pomelo pectin CA-SWP and *E*-HWP, and their enzymatically hydrolyzed pectic fractions.

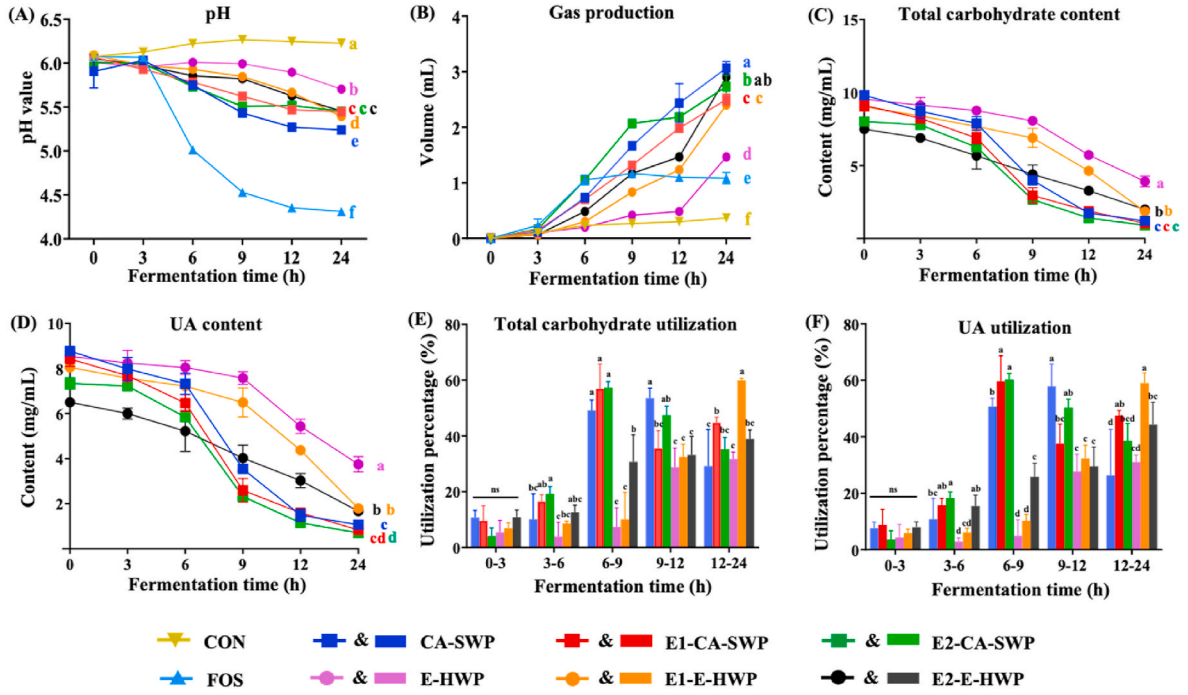

Fig. 3. Preliminary assessment of pomelo pectins during *in vitro* fermentation using human inoculum. Dynamic changes of pH value (A), total gas production (B), total carbohydrate content (C), UA content (D), utilization rate of UA (E) and total carbohydrates (F) during fermentation. Values are means ± SD (n = 3). The different superscript letters indicate significant differences ($p < 0.05$) between groups, while same letters indicate no significance.

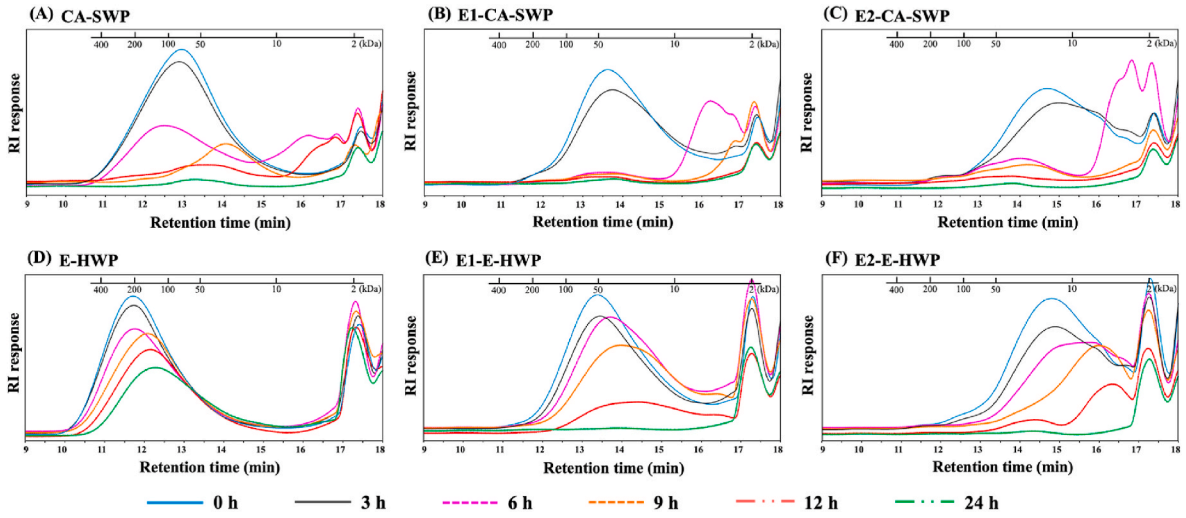

Fig. 4. Mw degradation of pectins during i*n vitro* fermentation. HPSEC profiles of CA-SWP (A), E1-CA-SWP (B), E2-CA-SWP (C), *E*-HWP (D), E1-E-HWP (E) and E2-E–HWP (F) at different fermentation time points.

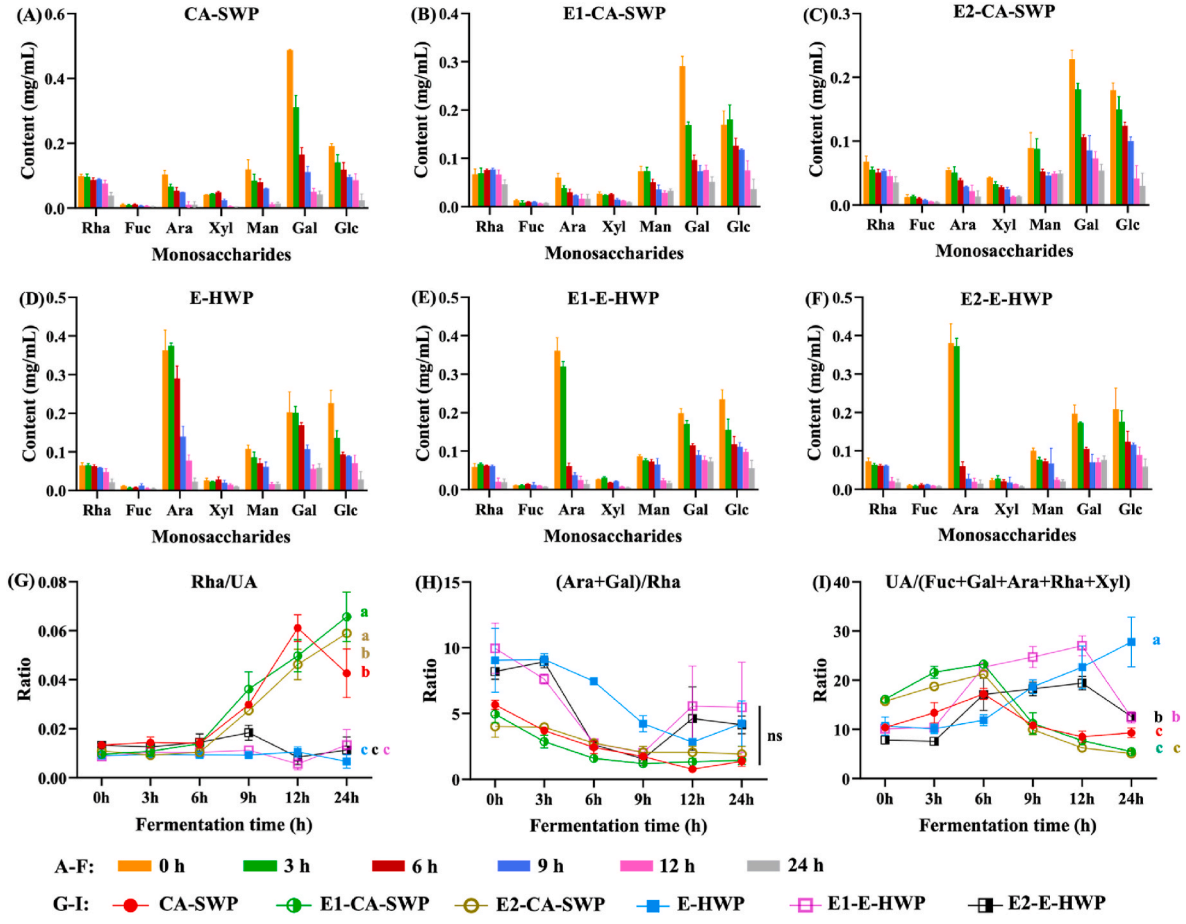

Fig. 5. Dynamic changes on neutral monosaccharides and structural patterns of pectins during *in vitro* fermentation. Monosaccharides contents of CA-SWP (A), E1-CA-SWP (B), E2-CA-SWP (C), *E*-HWP (D), E1-E-HWP (E) and E2-E-HWP (F) at different fermentation time points. Rha/UA: the contribution of RG-I domain to the entire pectin (C), (Ara + Gal)/Rha: the degree of RG-I branching (D), UA/(Fuc + Gal + Ara + Rha + Xyl): the linearity of pectin (E), calculating by mole ratio.

the two groups were significantly ($p < 0.05$) higher compared to the group using their parental pectin *E*-HWP. CA-SWP group showed higher ($p < 0.05$) concentrations of the upper three main SCFA and total SCFAs since 6 h of fermentation compared with *E*-HWP group. No significant difference was found between E1-CA-SWP and E1-E-HWP for total SCFAs and all individual SCFA except butyric acid (Fig. 6C) after 24 h of fermentation. Higher ($p < 0.05$) levels of acetic acid, butyric acid and total SCFAs were found in E2-CA-SWP than in E2-E-HWP. The ratio of total SCFAs/BCFAs increased with fermentation progressed in all pectin supplemented groups (Fig. 6 I).

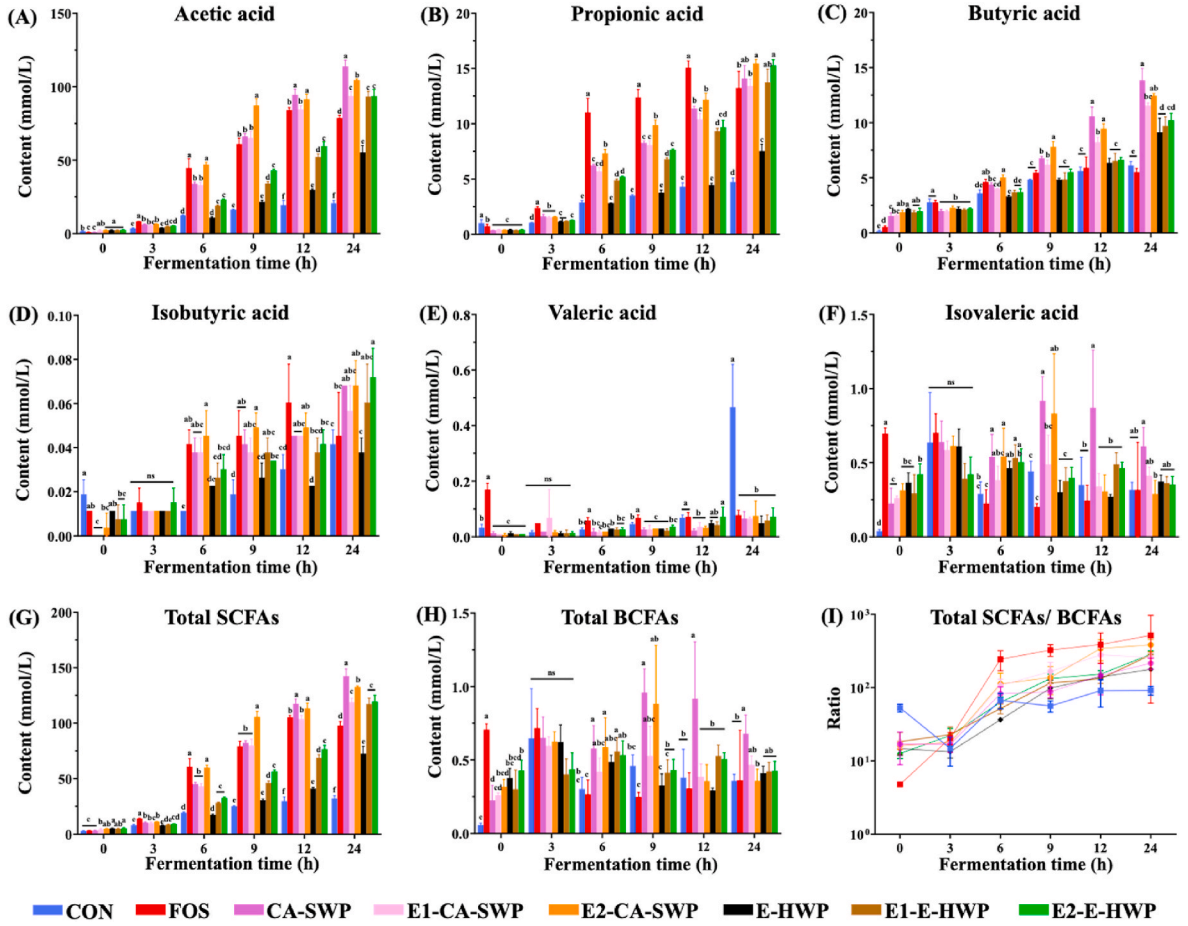

Fig. 6. Dynamic changes of SFCA production during *in vitro* fermentation of pomelo pectins. Acetic acid (A), propionic acid (B), butyric acid (C), isobutyrate acid (D), valeric acid (E) and isovaleric acid (F). The total SCFAs was the sum of the six individual SCFA (G), and total BCFAs was summed by 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 24 h, while same letters indicate no significance.

3.7. Bacterial diversity

After 24 h *in vitro* fermentation, a significant ($p < 0.05$) decrease in the Chao index was observed in CA-SWP (169.4), *E*-HWP (182.6), and their enzymatically hydrolyzed samples when compared to CON (231.2) (Fig. 7A). There was non-significant difference in the Chao index between E1-CA-SWP (170.5), E2-CA-SWP (163.5), and CA-SWP. *E*-HWP had a significantly ($p < 0.05$) higher Chao index than its enzymatically hydrolyzed samples. The Simpson index (Fig. 7B) of all pectins were significantly ($p < 0.05$) higher than that of CON (0.91) after 24 h *in vitro* fermentation. There was non-significant difference in the Simpson index among CA-SWP (0.96) and its enzymatically hydrolyzed samples. In addition, the E1-E-HWP (0.92) had a significant ($p < 0.05$) decrease in the Simpson index compared to *E*-HWP (0.94).

The PCoA plots captured ~80% of variation in microbial communities among the ASVs across all fermented digesta, with PCoA 1, PCoA 2 and PCoA 3 explaining 37.71%, 22.68% and 19.78%, respectively (Fig. 7C). The pectin supplemented samples clustered separately from the controls. Samples in CA-SWP, E1-CA-SWP, and E2-CA-SWP groups clustered closely, and samples in E1-E-HWP and E2-E-HWP groups clustered separately from *E*-HWP. Generally, the intergroup differences of CA-SWP and its enzymatically hydrolyzed samples were smaller than *E*-HWP and its hydrolyzation samples.

3.8. Microbiota composition

After 24 h *in vitro* fermentation, the microbial communities in the fermented digesta of various pectin and CON groups were mainly composed of Bacteroidota, Firmicutes, Proteobacteria and Fusobacteriota phylum (Fig. 7D). Compared with CA-SWP, the relative abundance of Fusobacteriota decreased in its enzymatic hydrolyzed samples, while the relative abundance of other three main phyla did not show significant alteration. Furthermore, compared to *E*-HWP group (22.9%), the relative abundance of Bacteroidota was significantly ($p < 0.05$) higher in E1-E-HWP group (39.5%) and E2-E-HWP group (47.2%).

The top 30 relative abundance of microbiota at genus level in all fermented digesta are show in the heat map (Fig. 7E). The addition of pectins significantly changed the microbial composition of the fermentation digesta compared with the control group after 24 h of *in vitro* fermentation. The addition of pomelo pectins all promoted the relative abundance of the *Faecalibacterium* in the fermentation digesta, while inhibited *Fusobacterium*, *Escherichia-Shigella*, and *Sutterella* (Table 2). Most of pectins except *E*-HWP (18.2 %) decreased the relatice abundance of *Lachnoclostridium* in fermented digesta when compared with CON (5.4%). The relative abundance of *Bacteroides* in CA-SWP group (23.3%) was significantly ($p < 0.05$) higher than in E1-CA-SWP group (18.6%) and E2-CA-SWP group (15.9%). While the relative abundance of *Bacteroides* in *E*-HWP group (20.6%) was significantly ($p < 0.05$) lower than in E1-E-HWP group (37.5%) and E2-E-HWP group (43.5%) (Table 2). Compared with CON group (1.6%), the relative abundance of *Prevotella* increased in CA-SWP group and its enzymatically hydrolyzed samples supplemented groups. Higher relative abundance of *Prevotella* was present in E1-CA-SWP group (34.1 %) and E2-CA-SWP group (37.3 %) than in CA-SWP group (28.1%).

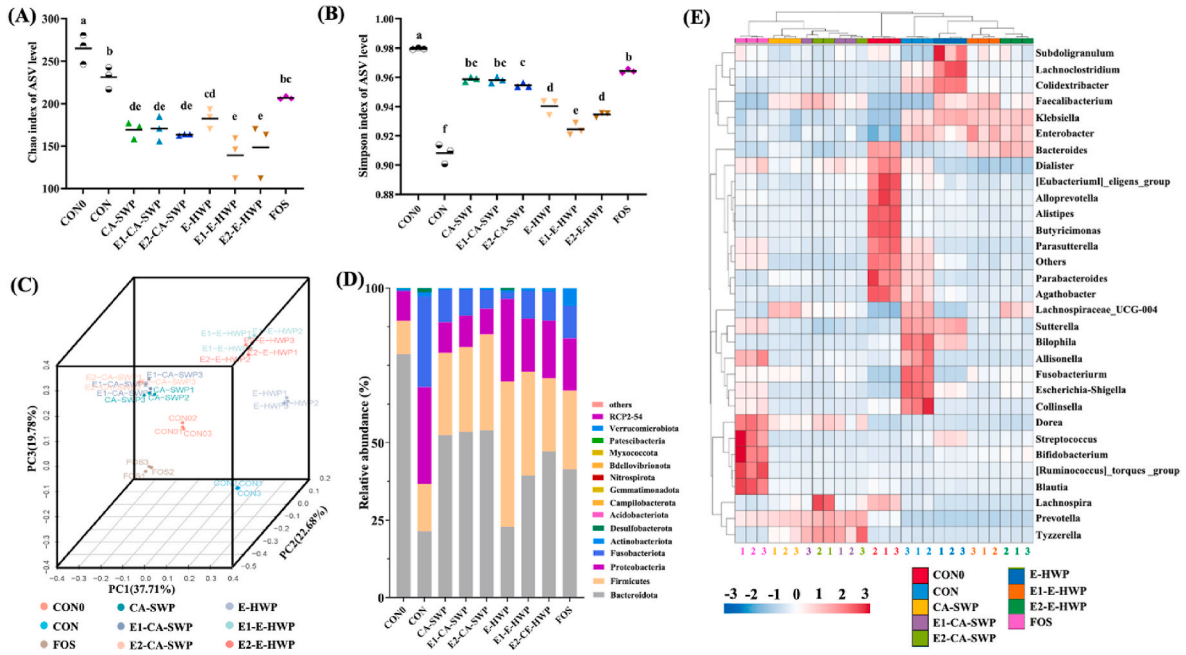

Fig. 7. Differential modulation of microbiota composition by pomelo pectins. Chao indexes (A), Simpson indexes (B) and principal coordinate analysis (C) of gut microbiota after 24 h of *in vitro* fermentation and CON at 0 h. The bacterial taxonomic profiling of gut microbiota at the phylum level (D), and the heatmap analysis (E) of the relative abundances at the genus level after 24 h of *in vitro* fermentation.

Table 2
Influence of pectins on relative abundance (%) of microbial taxa at the genus level after 24 h of *in vitro* fermentation.

Phylum	Genus	CON	CA-SWP	E1-CA-SWP	E2-CA-SWP	*E*-HWP	E1-E-HWP	E2-E-HWP
Bacteroidota	*Bacteroides*	17.6 ± 0.6^{de}	23.3 ± 0.7^{c}	18.6 ± 1.2^{de}	15.9 ± 2.4^{e}	20.6 ± 1.3^{cd}	37.5 ± 0.8^{b}	43.5 ± 2.9^{a}
	Prevotella	1.6 ± 0.2^{c}	28.1 ± 1.1^{b}	34.1 ± 3.8^{a}	37.3 ± 2.2^{a}	1.0 ± 0.1^{c}	1.3 ± 0.2^{c}	2.8 ± 0.0^{c}
Firmicutes	*Faecalibacterium*	2.8 ± 0.1^{d}	21.3 ± 0.6^{bc}	22.7 ± 5.9^{abc}	25.8 ± 5.2^{ab}	24.2 ± 4.0^{abc}	28.9 ± 2.6^{a}	17.9 ± 3.5^{c}
	Lachnoclostridium	5.4 ± 0.6^{b}	1.4 ± 0.1^{c}	1.2 ± 0.0^{c}	1.0 ± 0.1^{c}	18.2 ± 1.2^{a}	2.2 ± 0.4^{c}	2.7 ± 0.2^{c}
Fusobacteriota	*Fusobacterium*	29.4 ± 1.1^{a}	10.7 ± 0.2^{b}	8.5 ± 1.5^{bc}	6.3 ± 2.1^{c}	2.1 ± 0.2^{d}	9.1 ± 2.1^{b}	9.4 ± 0.2^{b}
Proteobacteria	*Klebsiella*	8.4 ± 0.2^{c}	3.2 ± 1.9^{d}	3.6 ± 0.6^{d}	3.0 ± 0.5^{d}	12.1 ± 0.3^{a}	10.1 ± 0.7^{b}	10.3 ± 0.4^{b}
	Escherichia-Shigella	9.6 ± 0.3^{a}	1.2 ± 0.2^{d}	1.4 ± 0.3^{d}	1.2 ± 0.2^{d}	3.3 ± 0.3^{b}	1.4 ± 0.2^{d}	2.7 ± 0.3^{c}
	Sutterella	11.2 ± 0.3^{a}	4.8 ± 0.2^{b}	4.4 ± 0.2^{b}	3.6 ± 0.4^{c}	10.5 ± 0.6^{a}	4.9 ± 0.4^{b}	5.0 ± 0.3^{b}

3.9. Correlation analysis

The correlations among the top 20 bacterial genera in fermented digesta, SCFA levels and structural parameters (monosaccharides, Mw, and DM) of the respective pectins are shown in Fig. 8. UA content was positively ($p < 0.05$) correlated with the relative abundance of *Blautia* and *Parasutterella*. Compared to Gal and Xyl, Ara and Glc content showed generally opposite correlations with the bacterial genera. Mw and DM of pectin were positively ($p < 0.05$) correlated with the relative abundance of *Colidextribacter* and *Sutterella*, but negatively ($p < 0.05$) correlated with the *Prevotella* level (Fig. 8A). The concentration of acetic, butyric acid, and total SCFAs exhibited a significant ($p < 0.05$) positive correlation with the relative abundance of *Blautia*, *Parabacteroides*, and Lachnospiraceae_*UCG-004*. Conversely, SCFAs concentrations, particularly acetic and butyric acids, were negatively ($p < 0.05$) correlated with *Escherichia-Shigella*, *Bilophila*, and *Klebsiella* (Fig. 8B). Gal level was positively ($p < 0.05$) correlated with the acetic acid and butyric acid concentration, while the Ara and Glc content had significant ($p < 0.05$) negative correlations with the two SCFAs. UA level, DM and Mw value were negatively ($p < 0.05$) correlated with the production of propionic acid (Fig. 8C).

4. Discussion

Chelating agents coupled with thermal treatment allowed for the successful extraction of low DM pectins from pomelo peel. In this study, we obtained two populations of low DM pectin with different Mw distributions. Previous studies have demonstrated that acidic extraction processes typically lead to the production of high DM pectin, while alkaline extraction procedures often result in pectin with a low DM value (Methacanon et al., 2014; Wandee, Uttapap, & Mischnick, 2019). The low DM of the pomelo pectins CA-SWP likely resulted from the cleavage of methyl groups from the galacturonan chains at a high temperature and a high citric acid concentration (Yilmaz-Turan et al., 2023). Extraction with EDTA and the long extraction time could also promote the hydrolysis of methyl ester, which could explain the low DM of *E*-HWP (Mota et al., 2020).

Pectinase was used to degrade the two pomelo pectins to a different extent for better gut microbiota modulating potential. Under the action of pectinase (Duvetter et al., 2009), which mainly includes

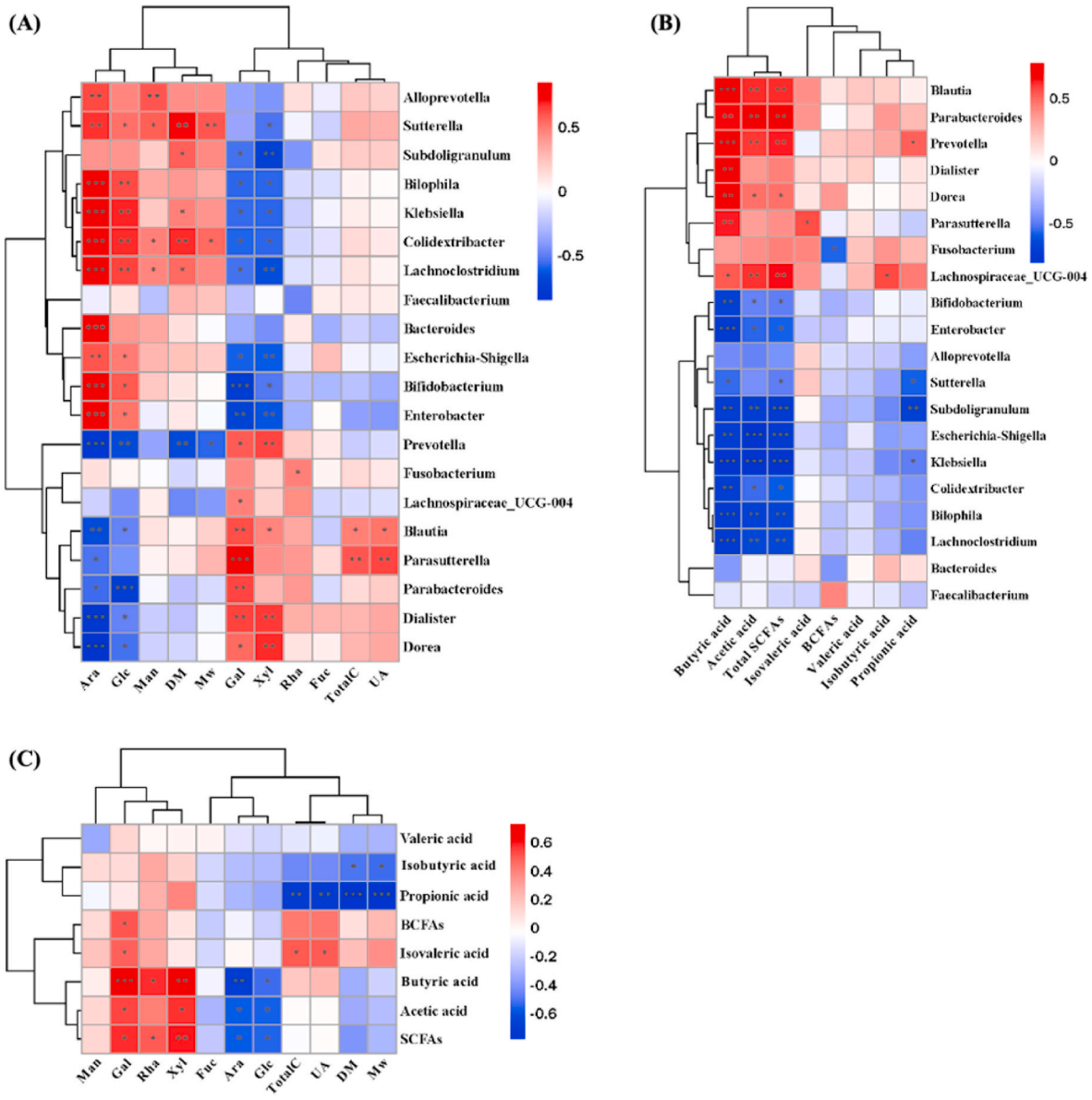

Fig. 8. Correlation analysis among bacterial genera, structural parameters and SCFA levels. Correlation between bacterial genera and structural parameters (A). Correlation between bacterial genera and SCFAs production (B). Correlation between SCFAs concentration and structural parameters (C). Positive correlations are represented by red color and negative correlations by blue color. Color intensity is proportional to the correlation coefficients (Spearman correlation). Significant correlations are indicated by *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$).

polygalacturonase, pectin lyases, and pectinesterase in the present study, pectin fragments with lower DM values and lower Mw were expectedly obtained. Beyond the alteration of Mw and DM, the monosaccharide composition in the mole ratio of hydrolyzed samples was not significantly altered when compared with parental pectins. Our results partly indicate that the pectinase released the pectin backbone constituent monosaccharides and the branch constituent monosaccharides at a similar speed, or the released fragments composed of a similar monosaccharide molar ratio as the parental pectins.

The obtained pomelo pectins were further subjected to microbial utilization. The degradation of Mw and reduction of residual monosaccharide levels to varying degrees during *in vitro* fermentation indicate sustained and effective degradation and utilization of pectins by gut microbiota. Due to the complex structure, a series of enzymes secreted by the gut microbiota contribute to the deconstruction of pectins. These enzymes may originate from a bacterial consortium or only a single organism, and there could be a synergistic or competitive relationship among the gut microbiota (Cao et al., 2023; Ndeh & Gilbert, 2018). The less efficient enzymatic degradation and microbial utilization of *E*-HWP could be partly explained by the longer time needed to achieve a similar extent of degradation compared to the pectins with lower Mw. Consistently, we also found that enzymatic hydrolysis of pomelo pectins enhanced the fermentability of carbohydrates during *in vitro* fermentation compared with the parental pectins. In agreement with our data, fragments obtained by enzymatic modification of apple pectin, konjac glucomannan, and blackberry polysaccharides exhibited higher fermentability (Li et al., 2022; Mao et al., 2021; Zhao et al., 2021). When the same amounts of carbohydrates were used, a higher number of molecules are present in the enzymatically modified samples, which could facilitate degradation by sufficient amounts of microbial enzymes during fermentation.

Pomelo pectins were efficiently consumed by gut microbiota, and, in turn, these pectins selectively promoted the growth of specific strains. Both CA-SWP and *E*-HWP tended to increase the relative abundance of

Bacteroides in the fermentation digesta, consistent with previous studies on HG, apple pectin, and pomelo pectins (Wang et al., 2022; Zhao et al., 2021). The enzymatically tailored fragments with lower Mw and DM properties had a more pronounced promotion effect on the growth of *Bacteroides* compared to *E*-HWP. *Bacteroides* is considered a bacterium with potential beneficial effects, possessing a variety of carbohydrate-utilizing enzymes that enable effective utilization of pectin and the production of a substantial amount of SCFAs through fermentation (Wexler, 2007). *Prevotella*, a symbiotic bacterium in the gut involved in the breakdown of polysaccharides (Precup & Vodnar, 2019), exhibited similar growth promotion effects in response to pomelo pectin CA-SWP and its enzymatically modified samples. However, contrary to our findings on the correlation of *Prevotella* growth and constituent monosaccharides, a previous study reported that the growth of *Prevotella* was positively correlated with Ara content and negatively correlated with Xyl content (Calvete-Torre et al., 2022). This difference may be partly caused by the varied microbial community for fermentation. Compared to CA-SWP and its enzymatically tailored samples, the lower relative abundance of *Prevotella* in *E*-HWP and its enzymatically degraded samples could be partly ascribed to the higher degree of RG-I branching and lower level of linearity (Table 1) which might limit the enzymatic release of sugars for the growth of *Prevotella*. *Faecalibacterium*, considered a next-generation probiotic with benefits for gut health, showed a significant increase in relative abundance with pomelo pectin supplementation. Similar promoting effects were observed for pectooligosaccharides obtained from lemon peel, which substantially increased beneficial species like *Faecalibacterium prausnitzii* (Míguez, Vila, Venema, Parajó, & Alonso, 2020). Research has revealed that pomelo peel-derived pectins demonstrate selective prebiotic effects, promoting the growth of beneficial microbes and SCFA-generating bacteria (Gamonpilas, Buathongjan, Kirdsawasd, et al., 2021; Gamonpilas, Buathongjan, Sangwan, et al., 2021). Besides the promoting effect on bacterial growth, the addition of pomelo pectins significantly suppressed the relative abundance of potential pathogens. *Escherichia--Shigella* is commonly considered pro-inflammatory and is a major pathogen associated with infectious diarrhea (Abu & Syeda, 2014). In line with our findings, it has been reported that, during *in vitro* fermentation, pectin derived from okra demonstrated a low relative abundance of pathogenic bacteria, specifically *Escherichia-Shigella* (Guo et al., 2023).

During fermentation, various microbial metabolites are produced, altering the intestinal environment and interacting with host intestinal cells to mediate a series of physiological effects (Cao et al., 2023). Higher levels of the main microbial metabolites, SCFAs, crucial for human health, were produced during the fermentation of pomelo pectins with lower Mw and DM in the present study. This could be partly attributed to the efficient degradation of low Mw and DM pectins by microbial enzymes, allowing abundant easy-accessible carbohydrates to promote SCFA production. Higher Mw and DM of pectin may hinder microbial enzyme degradation, resulting in a lower utilization rate of carbohydrates and lower SCFA production (Tian et al., 2016; Zhao et al., 2021). Acetic acid was the most predominant SCFA produced by pomelo pectin fermentation, consistent with previous studies on pectins from various sources (Ai et al., 2022; Tian et al., 2016). Microbial-derived acetic acid reduced inflammation and improved epithelial barrier function (Hosmer, McEwan, & Kappler, 2023). While many bacteria can tolerate acetic acid reasonably well, acetic acid accumulation can impair growth of *Escherichia coli* and other bacteria (Pinhal, Ropers, Geiselmann, & de Jong, 2019). In the present study, the production of butyric acid is primarily attributed to the genus *Faecalibacterium* (Duncan, Barcenilla, Stewart, Pryde, & Flint, 2002). The increase in butyric acid content during gut microbiota fermentation may also be due to bacterial cross-feeding. Evidences have shown that some butyrate producers can use exogenous acetic acid for butyric acid production (Duncan et al., 2004; Rios-Covian, Gueimonde, Duncan, Flint, & de los Reyes-Gavilan, 2015). The exact mechanism underlying the relationship between pathogens *Escherichia-Shigella* and *Klebsiella*, and SCFAs is not yet fully understood. Still, they may compete with other gut bacteria for nutrients and resources necessary for SCFA production, thus inhibiting the overall level of SCFAs produced (den Besten et al., 2013).

5. Conclusions

Enzymatic degradation of pomelo pectins resulted in the expected production of pectin fragments with lower DM and lower Mw. This enzymatic treatment not only enhanced fermentability but also altered the UA utilization model of pectins, leading to an improvement in SCFA levels during fermentation. Enzymatically tailored pectins demonstrated a significant impact on the microbial composition of fermentation digesta, fostering the growth of potential probiotics like *Bacteroides* while concurrently suppressing the proliferation of pathogens such as *Escherichia-Shigella*. Overall, the enzymatic modification of pectin using pectinase to obtain pectins with low Mw and DM proves to be an effective method for shaping the gut microbiota into a healthier pattern.

CRediT authorship contribution statement

Yuxin Wang: Writing – original draft, Validation, Methodology, Investigation. **Jiaxin Liu:** Visualization, Validation, Methodology. **Yi Long:** Visualization, Writing – review & editing. **Hong Yao:** Writing – review & editing, Validation, Methodology. **Henk A. Schols:** Writing – review & editing. **Paul de Vos:** Writing – review & editing. **Weibin Bai:** Writing – review & editing, Supervision. **Lingmin Tian:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Abbreviations

Mw	molecular weight
DM	degree of methylation
CA-SWP	citric acid assisted subcritical water extracted pectin
E1-CA-SWP	first stage of enzymatic modification on CA-SWP
E2-CA-SWP	second stage of enzymatic modification on CA-SWP
E-HWP	EDTA-2Na assisted hot water extracted pectin
E1-E-HWP	first stage of enzymatic modification on *E*-HWP
E2-E-HWP	second stage of enzymatic modification on *E*-HWP
FT-IR	Fourier transform-infrared spectrometry
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

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