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Effects of enzymatic treatment on the in vitro digestion and fermentation patterns of mulberry fruit juice: A focus on carbohydrates

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Abstract:	The aim of this study was to investigate the effects of enzymatic treatments (pectinase, pectin lyase, and cellulase) on the in vitro digestion and fermentation characteristics of whole mulberry fruit juice. The analysis focused on changes in carbohydrate properties within the black mulberry fruit matrix during simulated digestion and fermentation. Human fecal microbiota were collected and introduced to the fruit matrix to monitor the fate of both soluble and insoluble polysaccharides during fermentation. The results revealed that enzymatic treatments enhanced the solubilization of carbohydrates from mulberry fruits, with pectinase showing the most significant effect. Throughout the process of in vitro digestion, there was a gradual increase in the percentage of solubilized carbohydrates from the mulberry juice substrate. The digested suspensions underwent dialysis to remove degradation fragments, and a lower quantity of carbohydrate in the enzyme-treated groups compared to the control. Polysaccharide populations with varying molecular weights (Mw) were obtained from the soluble fractions of mulberry residues for subsequent fermentation. An increase in Mw of soluble polysaccharides was detected by HPSEC during fermentation in certain cases. The gut microbiota demonstrated the ability to convert specific insoluble fractions into soluble components, which were subsequently subjected to microbial utilization. Enzymatic treatments during mulberry juice preparation can potentially positively impact health by influencing gut microbiota and short-chain fatty acid (SCFA) modulations. Enzymes could serve as valuable tools for producing functional fruit and vegetable juices, with the need to specify processing conditions for specific raw materials remaining a subject of further investigation.
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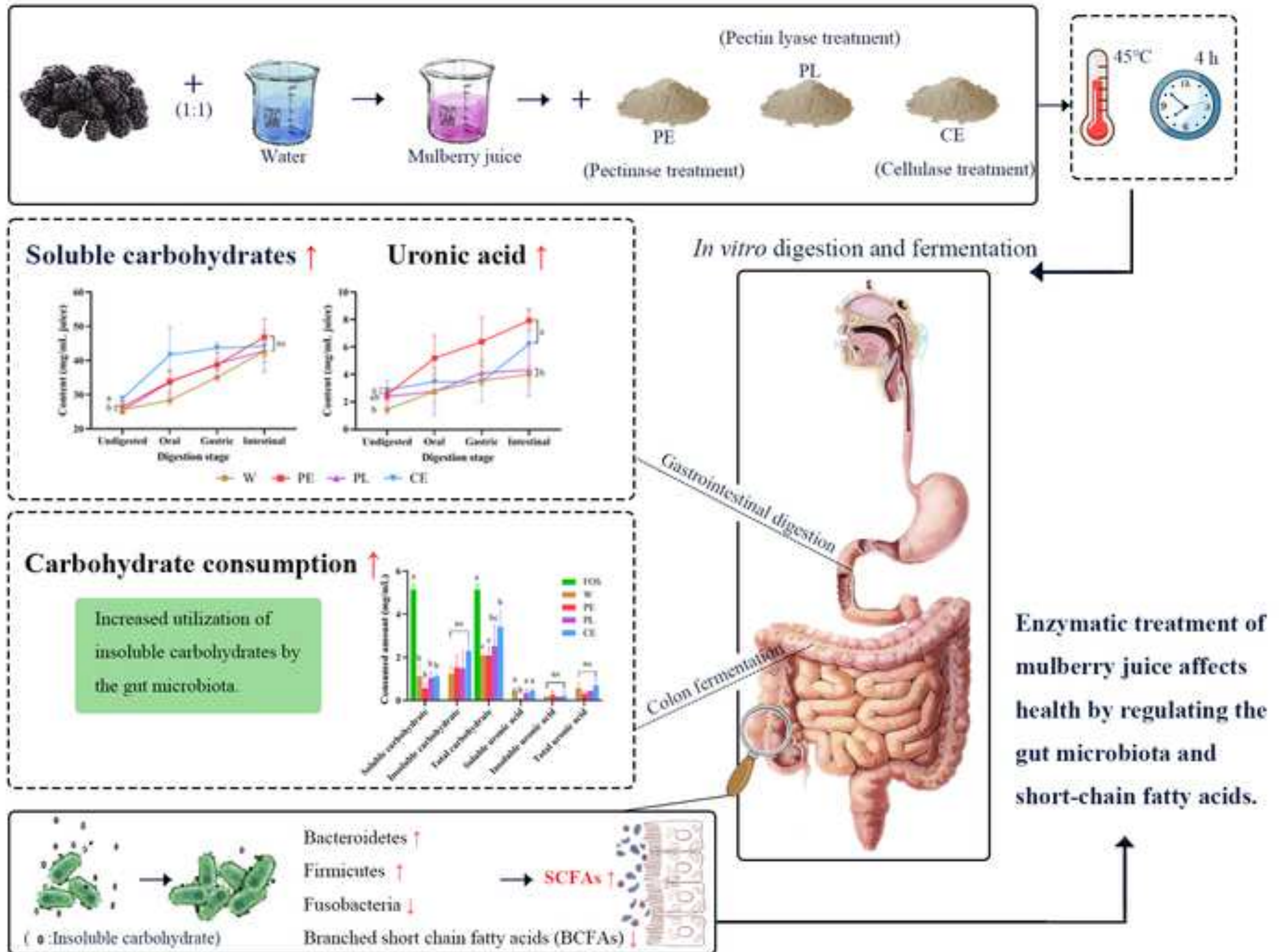

1 **Effects of enzymatic treatment on the *in vitro* digestion and fermentation patterns of**
2 **mulberry fruit juice: A focus on carbohydrates**

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ABSTRACT:

The aim of this study was to investigate the effects of enzymatic treatments (pectinase, pectin lyase, and cellulase) on the *in vitro* digestion and fermentation characteristics of whole mulberry fruit juice. The analysis focused on changes in carbohydrate properties within the black mulberry fruit matrix during simulated digestion and fermentation. Human fecal microbiota were collected and introduced to the fruit matrix to monitor the fate of both soluble and insoluble polysaccharides during fermentation. The results revealed that enzymatic treatments enhanced the solubilization of carbohydrates from mulberry fruits, with pectinase showing the most significant effect. Throughout the process of *in vitro* digestion, there was a gradual increase in the percentage of solubilized carbohydrates from the mulberry juice substrate. The digested suspensions underwent dialysis to remove degradation fragments, and a lower quantity of carbohydrate in the enzyme-treated groups compared to the control. Polysaccharide populations with varying molecular weights (Mw) were obtained from the soluble fractions of mulberry residues for subsequent fermentation. An increase in Mw of soluble polysaccharides was detected by HPSEC during fermentation in certain cases. The gut microbiota demonstrated the ability to convert specific insoluble fractions into soluble components, which were subsequently subjected to microbial utilization. Enzymatic treatments during mulberry juice preparation can potentially positively impact health by influencing gut microbiota and short-chain fatty acid (SCFA) modulations. Enzymes could serve as valuable tools for producing functional fruit and vegetable juices, with the need to specify processing conditions for specific raw materials remaining a subject of further investigation.

Keywords: mulberry juice, enzymatic treatment, *in vitro* digestion, fermentation pattern, gut microbiota

47 **1. Introduction**

48 Fruit juices are rich in nutritious components, making them important for human health and
49 commercially valuable (Sharma, Patel, & Sugandha, 2017). However, the presence of insoluble
50 substances often leads to natural turbidity in fruit juices, affecting their overall quality (Ribeiro,
51 Henrique, Oliveira, Macedo, & Fleuri, 2010). Plant cell walls, which consist of complex structural
52 polysaccharides, lignin, and proteins, contribute to cell stability and resistance to the removal of
53 intracellular components (Gligor et al., 2019). In recent years, macerating enzymes such as
54 pectinases, cellulases, and hemicellulases have been widely used in fruit juice production to
55 enhance juice recovery, increase total soluble solids, improve clarity, and reduce viscosity and
56 turbidity (Ribeiro et al., 2010; Sharma et al., 2017). These enzymes play a crucial role in breaking
57 down the cell wall matrix, allowing the release of bioactive molecules such as polyphenols, and
58 polysaccharides from the fruit pulp into the juice (Das, Nadar, & Rathod, 2021).

59 Dietary fiber is abundant in fruit juices, but it cannot be digested by the body's enzyme system.
60 However, upon reaching the colon, it can be fermented by colonic bacteria, leading to the
61 production of beneficial short-chain fatty acids (SCFAs) that promote health (Byrne, Chambers,
62 Morrison, & Frost, 2015). The physicochemical properties of dietary fiber play a significant role
63 in their fermentation process in the colon (Vaz Rezende, Lima, & Veloso Naves, 2021). Soluble
64 fibers and oligosaccharides are more readily fermented compared to insoluble ones (Vaz Rezende
65 et al., 2021). Therefore, using enzyme preparations to deconstruct the cell walls of fruits and
66 release easily fermentable dietary fiber fractions into the juice holds promise in maximizing their
67 utilization.

68 Mulberries (*Morus* spp.), species of deciduous flora, are known for their rapid growth and
69 widespread distribution across temperate, subtropical, and tropical regions (Wen et al., 2019).

70 Black mulberry fruit contains a variety of functional substances, including flavonoids,
71 anthocyanins, and polysaccharides (Yuan & Zhao, 2017). The mulberry polysaccharides possess
72 numerous biological activities, such as antioxidant, anti-obesity, anti-diabetic, anti-inflammatory,
73 hepatoprotective, and prebiotic effects (Ai et al., 2021). The structural characteristics of mulberry
74 polysaccharides, such as molecular weight (Mw) and monosaccharide composition, have been
75 shown to significantly influence their biological activities (Ai et al., 2021). Our previous study on
76 black mulberry fruit demonstrated that enzymatic treatments improved the solubilization of
77 polysaccharides from cell wall, and modified the population and chemical structure of soluble
78 polysaccharides, resulting in different fermentation patterns (Ai et al., 2022). However, the
79 properties of whole pulp juice digestion and human fecal fermentation with respect to the effects
80 of altered carbohydrate structure remain unclear.

81 Hence, the effect of dietary fiber modification of mulberry fruit based on enzyme preparations
82 on the digestive and fermentation characteristics of the whole mulberry juice was investigated.
83 Different enzyme preparations were used to extract mulberry juice and the release of carbohydrate
84 was compared. Dynamic changes of carbohydrates in mulberry juice were evaluated during the *in*
85 *vitro* simulated process of digestion and fermentation. In addition, this study concentrated at how
86 mulberry juice digested affected human fecal microbiota and SCFAs synthesis.

87 2. Material and methods

88 *2.1. Materials and reagents*

89 Fresh black mulberry fruits (*Morus nigra* L.) were sourced from a local indigenous store in
90 Shantou, China. Food-grade enzyme preparations were obtained from Xiasheng Industrial Group
91 Co., Ltd. (Ningxia, China), including pectinase (containing polygalacturonase, lyase, and pectin
92 esterase) and pectin lyase (a sub-category of pectinases) derived from *Aspergillus niger*, and

93 cellulase (containing endoglucosidase) from *Trichoderma reesei*. The enzymes were further
94 diluted or dissolved using a sodium acetate buffer (100 mM, pH 5.0) to create working solutions
95 with enzyme activity of 3000 U/mL for pectinase, 500 U/mL for pectin lyase, and 1000 U/mL for
96 cellulase. Macklin Biochemical Co., Ltd. (Shanghai, China) provided α-amylase, pepsin, trypsin,
97 bile salts, and SCFAs standards. All other reagents used were of analytical grade.

98 *2.2. Enzymatic treatment of mulberry fruit juice*

99 Fresh mulberry fruits (100 g) were mixed with distilled water (1:1, w/v) and crushed to obtain
100 cloudy mulberry juice. Pectinase, pectin lyase, and cellulase enzymes were used to pretreat the
101 juice, while a control group without enzymatic treatment was also included. The enzyme dosage
102 was set at 0.3% (v/w) of the fresh fruit weight, and the treatment conditions involved incubation
103 at 45°C for 4 h with a pH of 4.5. Following enzymatic treatment, all groups were subjected to a
104 10-min treatment in a boiling water bath to deactivate the enzymes. The four groups were labeled
105 as follows: W (without enzymatic treatment), PE (pectinase treatment), PL (pectin lyase treatment),
106 and CE (cellulase treatment).

107 *2.3. In vitro simulated saliva-gastrointestinal digestion*

108 The *in vitro* saliva-gastrointestinal digestion of cloudy mulberry juice was simulated
109 following previously published methods with minor modifications (Brodkorb et al., 2019; Liu et
110 al., 2022). Simulated saliva fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal
111 fluid (SIF) were prepared according to the procedures described previously (Brodkorb et al., 2019).
112 Briefly, 100 mL of mulberry juice was mixed with 750 mg of *α*-amylase (20 U/mg), 0.5 mL of
113 $CaCl_2(H_2O)_2$ (0.3 M), and SSF (pH=7.0, 1:1, v/v), and then incubated for 2 min in a water bath
114 shaker at 37 °C. Following salivary digestion, 150 mL of oral digest was combined with 200 mg
115 of pepsin (3000 U/mg), 0.075 mL of $CaCl_2(H_2O)_2$ (0.3 M), and SGF (pH=2.0, 1:1, v/v) and

116 incubated for 2 h in a water bath shaker at 37 °C. Finally, 150 mL of gastric digest was mixed with
117 120 mg of trypsin (250 U/mg), 45 g of bile salts and 0.3 mL of $CaCl_2(H_2O)_2$ (0.3 M), and SIF
118 (pH=7.0, 1:1, v/v), and then incubated in a water bath shaker at 37 °C for 3 h. Samples were
119 collected at different digestion stages.

120 *2.4. In vitro fecal fermentation*

121 Samples of mulberry juice digesta, obtained after *in vitro* simulated small intestine digestion,
122 were collected and subjected to heat treatment in a water bath at 100°C for 10 min to inactivate
123 the enzymes. The digesta was then dialyzed using a 3.0 kDa Mw cutoff, followed by freeze-drying
124 for further use in fermentation studies. *In vitro* human fecal fermentation, including medium
125 preparation, was conducted following a previously published method (Yang et al., 2021; Wang et
126 al., 2023). A microbial inoculum was prepared by combining equal amounts of fecal samples from
127 five healthy donors (3 males and 2 females) with ages ranging from 22 to 25 years and an average
128 BMI of 26.5 kg/m^2 (ranging from 23.1 to 35.3). The donors had not taken antibiotics or
129 experienced digestive illnesses in the past three months. Donors were fully informed that their
130 fecal samples would be used solely for experimental purposes, and their privacy was strictly
131 protected throughout the study. This study was approved by Investigational Review Board (IRB)
132 of the Jinan University (JNUKY-2023-0072).

133 *In vitro* fermentation was carried out in capped 4-mL anaerobic fermentation flasks. A mixture
134 of 0.4 mL of fecal slurry and 3.6 mL of baseline nutritional growth medium, which included the
135 residues from 20 mL of small intestine-digested sample after dialysis, was prepared. Fructo-
136 oligosaccharide (FOS) was used as the positive control, while the basal nutrient growth medium
137 without a carbon source served as the blank control (Con). Fermentation was conducted in a
138 vibrating incubator at 37°C and 100 rpm. Fermented samples were collected at 0, 6, 12, 24, and

139 36 h for further analysis.

140

141 *2.5. Molecular weight (Mw) distribution*

142 The molecular size distribution of the fermentation digesta was determined using the
143 methodology outlined in our prior study with modification (Wang et al., 2023). Briefly, high-
144 performance size exclusion chromatography (HPSEC) was performed utilizing a sequence of three
145 TSK-Gel columns (4000-3000-2500 SuperAW; 150 × 6 mm) connected in series, employing an
146 Ultimate 3000 HPLC system (Dionex, Sunnyvale, CA, USA). A mobile phase of 0.2 M $NaNO_3$
147 was employed, with a flow rate of 0.45 mL/min. The elution temperature was maintained at 60 °C,
148 and the injection volume was set at 20 μL. The fermentation digesta was collected and subjected
149 to analysis to assess the molecular size distribution of polysaccharides at various fermentation time
150 points (0, 6, 12, 24, 36 h).

151 *2.6. Analyses of neutral monosaccharides and uronic acid content*

152 After centrifuging the digestion or fermentation sample for 15 min at 4°C and 10500×g, the
153 supernatant (soluble) and precipitate (insoluble) were collected and freeze-dried, respectively. The
154 monosaccharide composition of the obtained fractions was analyzed following the previously
155 described methods (Ai et al., 2022; Wang et al., 2023). Briefly, the samples were pre-hydrolyzed
156 with 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 released
157 monosaccharides were converted into their alditol acetates and analyzed using gas chromatography
158 with a DB-255 column (15 m × 0.53 mm × 1 μm, Agilent Technologies Inc., CA, USA), with
159 inositol as the internal standard. The uronic acid (UA) content of the samples was quantified at
160 520 nm using the p-hydroxydiphenyl colorimetric method, with D-galacturonic acid as the
161 standard substance. The total carbohydrate contents were calculated by summing all determined

162 monosaccharides, including rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl),
163 mannose (Man), galactose (Gal), glucose (Glc), and UA. The total carbohydrate/UA determined
164 in the supernatant and precipitate were considered as soluble and insoluble carbohydrate/UA,
165 respectively.

166 *2.7. Analysis of gut microbiota*

167 Following 36 h of fermentation, the genomic DNA of each fermented group was extracted
168 with the assistance of DNA Extraction Kit (DP812, Beijing, China) according to the
169 manufacturer's instructions. After that, PCR amplification for the full-length 16S bacterial rRNA
170 genes was carried out using the forward primer 27F (5'-AGRGTTTGATYNTGGCTCAG-3') and
171 the reverse primer 1492R (5'-TASGGHTACCTTGTTASGACTT-3'). The PacBio Sequel II
172 platform (Biomarker Technologies, Beijing, China) was used for library creation, sequencing, and
173 data analysis. SMRT Link (version 8.0) was used to correct the original subreads and create
174 Circular Consensus Sequencing (CCS) reads. CCS readings from various samples were recognized
175 using barcode sequences. After chimeras were eliminated, high-quality CCS readings were
176 obtained (UCHIME algorithm, v8.1). Operational taxonomic units (OUT) clustering: sequences
177 were clustered at the 97% similarity level using USEARCH (version 10.0) with a conservative
178 threshold of 0.005% for OTU filtration. The OTU taxonomy annotation was conducted in QIIME2
179 (Version 2020.6) using the SILVA database (release 132) with a confidence level of 70%.

180 *2.8. Analysis of SCFAs content*

181 SCFAs was determined using the technique described previously (Tian et al., 2016). A gas
182 chromatograph (Shimadzu G2010Plus, Kyoto, Japan) with a DB-FFAP column (30 m × 0.53 mm
183 × 1.00 μm, Agilent Technologies Inc., Calif., USA) was used. Acetic, propionic, butyric, isobutyric,
184 valeric, and isovaleric acids were used as standards.

2.9. Statistical analysis

All experimental groups were designed with three replicates of the experiment. Graphic presentation was conducted using GraphPad Prism 8.0 software. All statistical analyzes were performed with SPSS 26.0 statistical software. All data were expressed as mean values ± standard deviation, $p<0.05$ was considered statistically significant.

3. Results and discussion

3.1. Effect of enzymatic treatment on carbohydrates in mulberry fruit juice

The carbohydrates found in black mulberry fruits (*M. nigra*) primarily consisted of Rha, Fuc, Ara, Xyl, Man, Gal, Glc, and UA (Table 1), aligning with previous research (Ai et al., 2021; Ai et al., 2022). Among these, Glc was the predominant unit, potentially present partly as free monomer rather than exclusively as polysaccharides, considering the substantial presence of monosaccharides in the cell fluid of mulberry fruit (Gundogdu, Muradoglu, Gazioglu Sensoy, & Yilmaz, 2011). Carbohydrate content analysis served as an initial assessment of the hydrolytic efficiency of different enzymes on the mulberry fruit cell wall, releasing various carbohydrate forms including monomers, oligomers, and polymers (Ribeiro et al., 2010; Sharma et al., 2017).

The soluble carbohydrate contents of the samples (**Table 1**) ranged from 7.82 to 14.86 mg/mL juice (W<CE<PL<PE). UA content was quantified to estimate the release of pectic polysaccharides from mulberry fruit, as mulberry fruit polysaccharides are predominantly composed of pectin rich in GalA (Ai et al., 2021; He et al., 2018). As presented in **Table 1**, insoluble UA in the samples varied from 1.81 to 5.64 mg/mL juice (PE<PL<CE<W). Despite cellulase displaying relatively lower efficiency in releasing GalA (CE group), it solubilized a higher amount of Glc compared to other groups (**Table 1**). These findings indicate that the used enzymatic treatments enhanced the solubilization of carbohydrates from black mulberry fruit.

Notably, the PE group exhibited significantly higher soluble total carbohydrate and UA levels ($p < 0.05$) compared to the W group. Overall, the results highlight that, among the used enzymes, pectinase exhibited the most effective carbohydrate solubilization from black mulberry fruit, followed by pectin lyase.

Table 1. Carbohydrate contents of fractions obtained from mulberry juice after enzymatic treatments.

Parameter	W	PE	PL	CE
	(mg/mL juice)			
Soluble carbohydrates	7.82±0.43^{c}	14.86±1.14^{a}	12.05±0.75^{b}	11.46±0.34^{b}
Rha	0.12±0^{a}	0.18±0.07^{a}	0.16±0.06^{a}	0.22±0.07^{a}
Fuc	0±0^{b}	0.01±0^{a}	0.02±0.01^{a}	0.01±0^{ab}
Ara	0.03±0^{b}	0.16±0.05^{a}	0.11±0.06^{ab}	0.11±0.04^{ab}
Xyl	0.02±0^{b}	0.07±0.05^{ab}	0.10±0.07^{ab}	0.19±0.05^{a}
Man	0.73±0.02^{ab}	0.92±0.10^{a}	0.62±0.11^{b}	0.51±0.16^{b}
Gal	0.07±0^{a}	0.11±0.04^{a}	0.09±0.03^{a}	0.14±0.03^{a}
Glc	5.40±0.13^{c}	8.14±0.67^{ab}	7.11±0.57^{b}	8.74±0.65^{a}
UA	1.43±0.27^{b}	5.26±0.83^{a}	3.85±0.70^{a}	1.54±0.73^{b}
Insoluble carbohydrates	17.40±0.23^{a}	10.36±0.56^{c}	13.17±0.25^{b}	13.76±0.85^{b}
Rha	0.29±0.05^{a}	0.23±0.02^{ab}	0.25±0.01^{ab}	0.19±0.03^{b}
Fuc	0.03±0.01^{a}	0.02±0.01^{a}	0.02±0^{a}	0.02±0^{a}
Ara	0.30±0.05^{a}	0.17±0^{b}	0.23±0.01^{b}	0.22±0.01^{b}
Xyl	0.44±0.07^{a}	0.39±0.02^{a}	0.36±0^{ab}	0.28±0.02^{b}
Man	1.08±0.16^{bc}	0.89±0.03^{c}	1.18±0.06^{ab}	1.30±0.04^{a}
Gal	0.29±0^{a}	0.25±0.03^{ab}	0.28±0.02^{ab}	0.23±0.03^{b}
Glc	9.34±0.55^{a}	6.60±0.27^{bc}	7.63±0.07^{b}	6.00±0.82^{c}
UA	5.64±0.71^{a}	1.81±0.28^{c}	3.22±0.21^{b}	5.53±0.07^{a}

Note: The soluble carbohydrate/monomers of W group and the insoluble carbohydrate/monomers of all groups were determined. For the PE, PL, and CE groups, the soluble carbohydrate/monomers were calculated by subtracting the insoluble fraction from the sum of soluble and insoluble fractions in the W group, under the assumption of equal totals across all groups. Values are means ± SD (n = 3) and data in horizontal with different lowercase letters indicate significant differences ($p<0.05$).

3.2. Changes of carbohydrates during simulated digestion

The *in vitro* digestion model facilitates the simulation of carbohydrate transport in mulberry

223 juice through the human digestive tract. Throughout the process of *in vitro* digestion, there is a
224 gradual increase in the percentage of solubilized carbohydrates and uronic acid from the black
225 mulberry juice substrate (**Fig. 1A** and **1B**). This observation is consistent with a previous study
226 that reported a rise in total soluble carbohydrates in blackberry fruit following digestion in the
227 small intestine (Dou, Chen, Huang, & Fu, 2022). The solubilization of carbohydrates and uronic
228 acid from the mulberry matrix post-gastrointestinal digestion can be attributed in part to the low
229 pH in the stomach and enzymatic treatments (Yuan et al., 2020). A portion of the mulberry
230 polysaccharides undergoes degradation through glycosidic bond cleavage, releasing reducing
231 saccharides. This observation is consistent with a previous study on pumpkin polysaccharides (Yu
232 et al., 2019). After digestion, the levels of soluble uronic acid in the PE and PL groups were
233 significantly higher than those in the W group ($p<0.05$). The difference in soluble carbohydrates
234 and uronic acid between the W group and the enzyme-treated groups decreased during the
235 simulated *in vitro* digestion, indicating that enzymatic treatment accelerated carbohydrate release
236 prior to digestion.

237 Since the solubilized carbohydrates encompassed both monomers and oligomers, as well as
238 polymers, the extent of branching and linearity within the soluble portion could not
239 comprehensively explain their patterns. As an alternative approach, we examined the attributes of
240 the insoluble components, primarily consisting of polymers (**Fig. 1C-1E**). Conspicuously, the
241 application of pectinase and pectin lyase led to a significant increase ($p<0.05$) in the contribution
242 of rhamnogalacturonan I (RG-I) to the insoluble pectin backbone (**Fig. 1C**) and a reduction
243 ($p<0.05$) in the linearity of insoluble pectin (**Fig. 1E**) in the undigested samples. This outcome
244 indicates a preference for the solubilization and degradation of homogalacturonan (HG) and HG
245 region of pectins compared to the hairy region or branches of RG-I in the mulberry cell wall. A

246 similar rationale could be extended to elucidate the elevation ($p<0.05$) in RG-I contribution (**Fig. 1C**) and the decline ($p<0.05$) in insoluble pectin linearity (**Fig. 1E**) after intestinal digestion for the PE group. Regarding the degree of RG-I branching within the insoluble pectin for all groups, no discernible variation due to enzymatic treatment was observed (**Fig. 1D**). Nonetheless, following intestinal digestion, the degree of RG-I branching in insoluble pectin for the PE and PL groups displayed significant reductions compared to the W group. These findings imply that the treatments involving pectinase and pectin lyase during juice preparation facilitated the solubilization of pectin branches in the form of polymers and/or oligomers from mulberry substrates during simulated digestion. These polymers and oligomers, potentially induced by enzymes during juice preparation, might have been retained within the substrates and subsequently released during digestion. The observed distinctions highlight that enzymatic treatments during juice preparation deconstructed the cell wall of mulberry, consequently influencing the digestive attributes of carbohydrate fractions in mulberry juice.

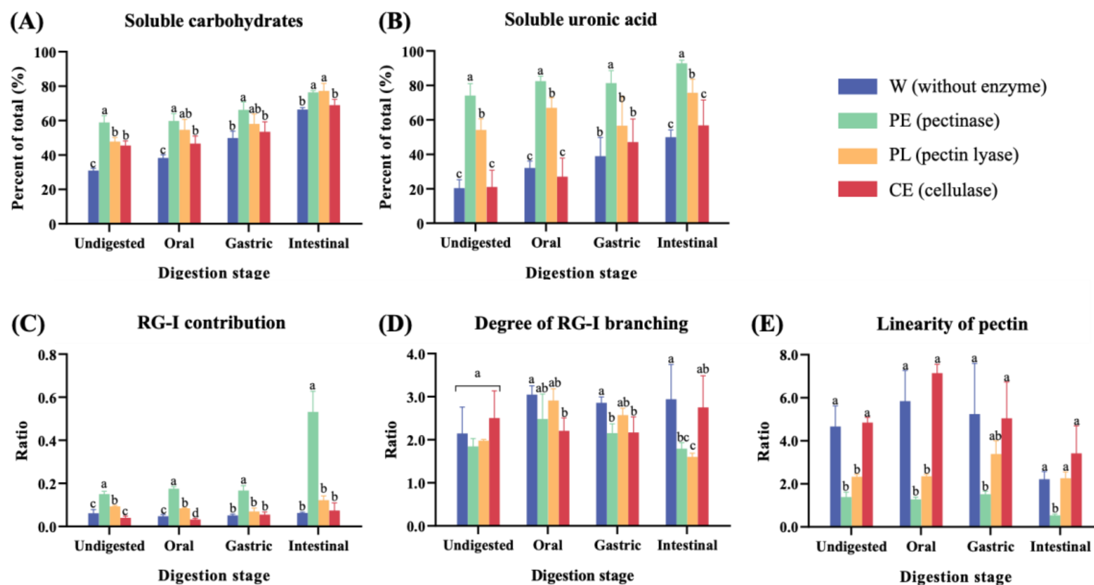


Figure 1. Dynamic changes in carbohydrates of mulberry juice during *in vitro* digestion. (A) soluble carbohydrate content; (B) soluble uronic acid content; (C) the contribution of RG-I domain to the entire insoluble pectin backbone which was calculated by Rha/UA (mol/mol); (D) the degree of RG-I branching of insoluble pectin which was calculated by (Ara+Gal)/Rha (mol/mol); (E) the

264 linearity of insoluble pectin which was calculated by UA/(Fuc+Gal+Ara+Rha+Xyl) (mol/mol);
265 Values are means ± SD (n = 3) and data at each digestion stage with different lowercase letters
266 indicate significant differences ($p<0.05$).
267

268 *3.3. Characterization of mulberry juice residues after simulated digestion and absorption*

269 The digested black mulberry matrix, encompassing both soluble and insoluble components,
270 underwent dialysis (3.0 kDa molecular weight cutoff) to simulate absorption and subsequent *in*
271 *vitro* simulated fermentation. Dialysis stands as an effective method for eliminating inorganic salts,
272 monosaccharides, oligosaccharides, and other low molecular weight compounds (Shi, 2016), as
273 well as phenols and flavonoids, from the mixture digested in the small intestine (Maduwanthi &
274 Marapana, 2021). The dialyzed mulberry juice digesta predominantly contained polysaccharides,
275 which serve as the primary substrates for fermentation by gut microbiota. Despite using the same
276 amount of mulberry for digestion, varying quantities of residues were obtained from the groups
277 after dialysis for subsequent *in vitro* fermentation (**Table 2**). A lower quantity of total carbohydrate
278 and UA was observed in the enzyme-treated groups compared to the W group. This could be
279 attributed to enzymatic degradation of carbohydrates and dialysis of the resulting degradation
280 fragments. Among the enzymes used, pectinase exhibited the strongest impact on the
281 fragmentation of cell wall polysaccharides (**Table 2**) due to the high abundance of pectins in
282 mulberry (Ai et al., 2021; Ai et al., 2022). Although pectinase released higher proportions of
283 carbohydrate and UA from the mulberry matrix (**Fig. 1A** and **1B**), there was a smaller quantity of
284 soluble carbohydrate and UA remaining for fermentation in the PE group compared to the other
285 groups (**Table 2**). This could be partially attributed to the fact that the solubilized carbohydrates
286 facilitated by pectinase were primarily small molecules, which were subsequently removed during
287 dialysis. A similar rationale could be applied to explain the lower levels of insoluble carbohydrates
288 and certain constituent monosaccharides present in the enzyme-treated groups compared to the

289 group without enzymatic treatments.

290 The soluble residues from black mulberry intended for fermentation were characterized for
291 molecular weight (Mw) using HPSEC (0 h, **Fig. 2**). The mean Mw of the primary polysaccharide
292 population ranked as follows: PL>PE>CE. Although a similar amount of soluble carbohydrate was
293 present in the W group compared to the PL and CE groups (**Table 2**), no concentrated polymer
294 population was observed in the W group. Since no oligosaccharides were detected using HPAEC
295 (data not shown), this could be partly explained by the fact that the polysaccharides in the W group
296 were evenly distributed with different Mw, albeit in low amounts, as indicated by the low RI
297 response (**Fig. 2A**).

298

299 **Table 2.** Carbohydrate contents of soluble residues from black mulberry juice after *in vitro*
300 simulated digestion and absorption.

Parameter	**W**	**PE**	**PL**	**CE**
	(mg/mL juice)			
Soluble carbohydrates	4.38±0.21^{a}	3.21±0.15^{b}	4.65±0.06^{a}	4.54±0.22^{a}
Rha	0.54±0.15^{ab}	0.52±0.14^{b}	0.81±0.02^{ab}	0.90±0.23^{a}
Fuc	0.15±0.06^{b}	0.25±0.05^{ab}	0.39±0.05^{a}	0.38±0.09^{a}
Ara	0.35±0.01^{a}	0.40±0.05^{a}	0.39±0.03^{a}	0.36±0.05^{a}
Xyl	0.31±0.04^{a}	0.23±0.02^{b}	0.18±0.03^{b}	0.20±0.03^{b}
Man	0.52±0.05^{ab}	0.46±0.01^{b}	0.60±0.06^{a}	0.43±0.04^{b}
Gal	0.23±0.04^{b}	0.28±0.05^{ab}	0.36±0.04^{a}	0.30±0.07^{ab}
Glc	1.21±0.04^{a}	0.65±0.09^{c}	1.04±0.09^{b}	1.01±0.06^{b}
UA	1.07±0.14^{a}	0.42±0.07^{b}	0.89±0.15^{a}	0.97±0.02^{a}
Insoluble carbohydrates	9.31±0.15^{a}	4.32±0.27^{c}	6.04±0.11^{b}	6.61±0.47^{b}
Rha	1.47±0.18^{a}	0.78±0.05^{c}	1.09±0.15^{b}	0.53±0.11^{c}
Fuc	0.59±0.12^{a}	0.12±0.04^{b}	0.23±0.02^{b}	0.09±0.04^{b}
Ara	0.63±0.11^{a}	0.13±0.02^{b}	0.58±0.11^{a}	0.46±0.02^{a}
Xyl	0.54±0.02^{b}	0.34±0.03^{c}	0.60±0.01^{a}	0.55±0.03^{ab}
Man	0.69±0.11^{a}	0.20±0.03^{c}	0.39±0.04^{b}	0.56±0.08^{a}
Gal	0.55±0.06^{a}	0.16±0.02^{c}	0.28±0^{b}	0.33±0.03^{b}
Glc	1.72±0.11^{a}	1.49±0.21^{a}	1.52±0.08^{a}	1.43±0.08^{a}

UA	3.13±0.23^{a}	1.11±0.10^{b}	1.34±0.29^{b}	2.66±0.33^{a}
Total carbohydrates	13.69±0.32^{a}	7.53±0.42^{c}	10.69±0.06^{b}	11.15±0.31^{b}
Rha	2.01±0.27^{a}	1.30±0.10^{b}	1.90±0.15^{a}	1.43±0.12^{b}
Fuc	0.73±0.15^{a}	0.37±0.07^{c}	0.62±0.04^{ab}	0.48±0.10^{bc}
Ara	0.99±0.11^{a}	0.52±0.05^{b}	0.96±0.09^{a}	0.81±0.06^{a}
Xyl	0.85±0.05^{a}	0.57±0.03^{c}	0.78±0.02^{ab}	0.75±0.03^{b}
Man	1.20±0.06^{a}	0.66±0.02^{c}	0.99±0.04^{b}	0.99±0.06^{b}
Gal	0.78±0.10^{a}	0.44±0.03^{c}	0.64±0.04^{ab}	0.63±0.04^{b}
Glc	2.93±0.14^{a}	2.14±0.14^{c}	2.55±0.02^{b}	2.43±0.07^{b}
UA	4.21±0.32^{a}	1.53±0.17^{c}	2.24±0.35^{b}	3.63±0.32^{a}

301 Note: The total carbohydrate/monomers were calculated by adding up the soluble and insoluble
302 fractions which were determined. Values are means ± SD (n = 3) and data in horizontal with
303 different lowercase letters indicate significant differences (p<0.05).
304

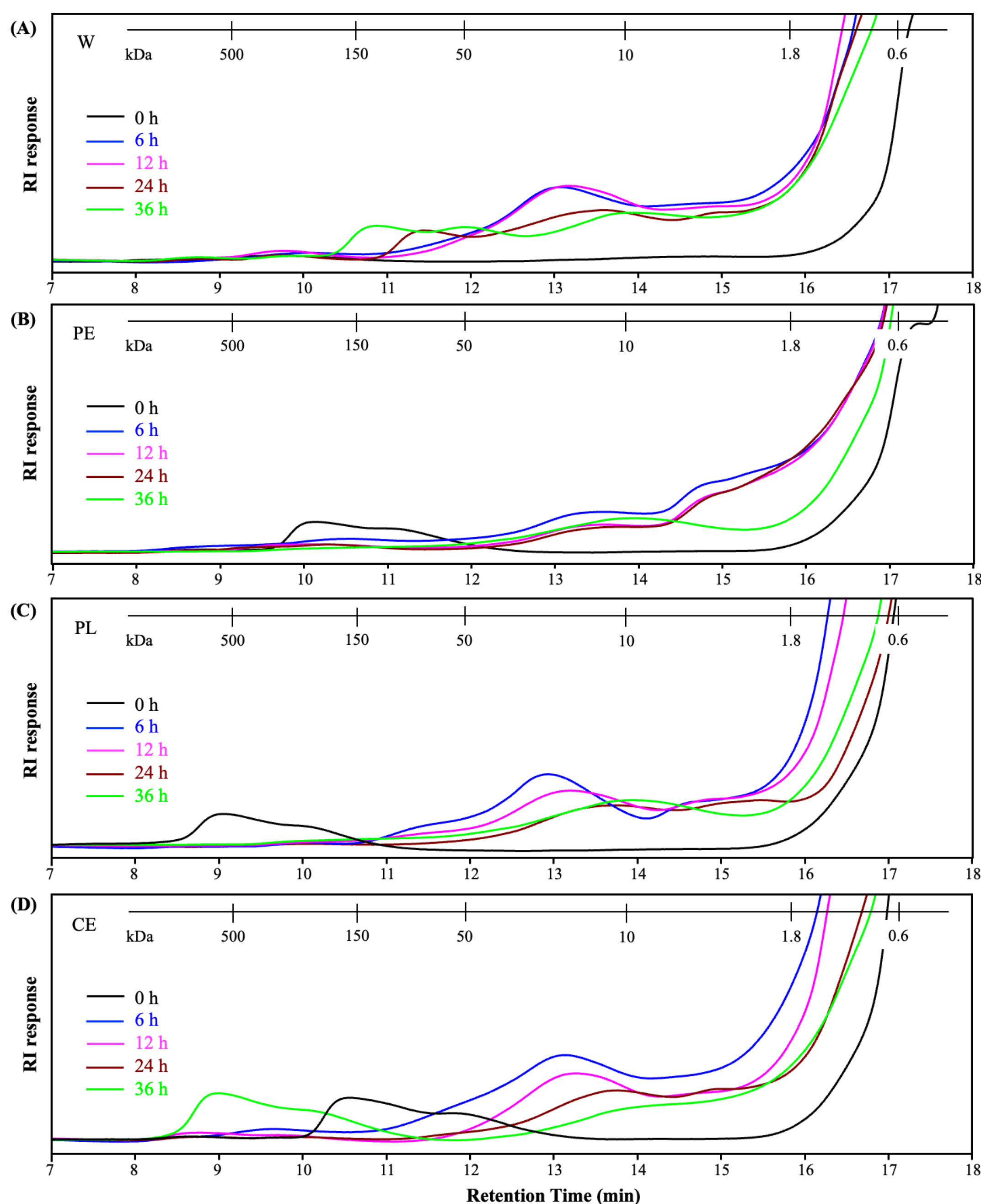
305 *3.4. Changes of carbohydrates during simulated fermentation*

306 The variations in molecular weight (Mw) of the samples during *in vitro* fermentation are
307 shown in **Fig. 2**. The Mw of the main polysaccharide population in the PE group (**Fig. 2B**) and PL
308 group (**Fig. 2C**) generally decreased as fermentation time increased. This suggests that the
309 polysaccharides were progressively broken down into smaller fragments by the human fecal
310 microbiota. However, the Mw of the predominant polysaccharide population in the soluble fraction
311 of the W group and CE group increased from 12 h to 36 h (**Fig. 2A**) and from 24 h to 36 h (**Fig.
312 2D**), respectively. These findings contrast with our previous results where soluble mulberry
313 polysaccharides experienced significant degradation after 24 h of *in vitro* fermentation (Ai et al.,
314 2022). This discrepancy could be attributed in part to the high Mw polysaccharides being
315 solubilized from the insoluble residue of the mulberry matrix in the W and CE groups during
316 fermentation. The gut microbiota exhibited the capability to transform certain insoluble fractions
317 into soluble components, which were subsequently subjected to fermentation and utilization
318 (Ramasamy, Venema, Schols, & Gruppen, 2014). The solubilized polysaccharides in the W and
319 CE groups during fermentation could be relatively more resistant to microbial enzymes compared

320 to those solubilized before fermentation. Alternatively, it is possible that the rate of solubilization

321 of polymers from the insoluble part could outpace the microbial utilization of soluble

322 polysaccharides.



323

324 **Figure 2.** Dynamic Mw changes of soluble residues from black mulberry juice during in vitro
325 fermentation. HPSEC was used to characterize the fermentation broth containing soluble residues
326 obtained after *in vitro* simulated digestion and absorption. (A) without enzymatic treatment, W;
327 (B) pectinase treatment, PE; (C) pectin lyase treatment, PL; and (D) cellulase treatment, CE.

Although polysaccharides might have been solubilized from insoluble residues, the consumption of soluble polysaccharides gradually increased with the duration of fermentation (**Fig. 3**). After 36 h of in vitro fermentation, the PE group exhibited lower levels of consumed soluble carbohydrate (**Fig. 3A**) and UA (**Fig. 3E**) compared to the other groups. This could be attributed to the cleavage of most pectinase-accessible glycan linkages during enzymatic treatment, rendering the soluble residue in the PE group more resistant to microbial enzymes during fermentation than in the other groups. In comparison to the other groups, the lower levels of consumed soluble rhamnose and galactose in the W group (**Fig. 3B** and **3D**) could be attributed to a higher quantity of these monosaccharides being solubilized from the insoluble parts (**Fig. 3G** and **3I**), thereby mitigating the reduction of soluble ones. The gut microbiota gradually utilized mulberry carbohydrates, with greater consumption of the soluble fraction compared to the insoluble part (**Fig. 3A** *vs* **3F**). Considering the potential solubilization of carbohydrates from the insoluble matrix (**Fig. 3F-3J**), the net disparity of soluble polysaccharides in contribution could be more pronounced. This preferential fermentation discrepancy between soluble and insoluble fiber aligns with observations from our previous study on chicory root pulp pectin (Ramasamy, Venema, Schols, & Gruppen, 2014).

In addition to solubility, various other factors, including molecular weight, degree of polymerization and esterification, and the presence of branches in the molecule, can influence the capacity of gut microbiota to metabolize non-starch polysaccharides (Hamaker & Tuncil, 2014; Tian et al., 2016; Tian et al., 2019). To gain a deeper understanding of changes in polysaccharide composition and relevant structural properties of both soluble and insoluble fractions, the RG-I contribution (mol%) and relevant molar ratios (**Fig. 4**) were calculated based on the molar proportions of monosaccharides (Kyomugasho et al., 2018; Wang et al., 2023). Although there was

351 only moderate variability in the proportion of RG-I between the soluble samples after the same
352 fermentation time, there was a significant decrease in RG-I contribution observed from 0 h to 6 h
353 for the enzyme-treated groups (**Fig. 4A**). Similarly, the insoluble RG-I was also preferentially
354 utilized by gut microbiota, as indicated by the decreasing RG-I contribution values at the early
355 stages of fermentation (**Fig. 4E**).

356 The Rha/UA values were close to 1.0 (0 h, **Fig. 4B**), indicating that the soluble polysaccharide
357 in the PE, PL, and CE groups mainly consisted of RG-I. The decrease in Rha/UA values during
358 fermentation could be partly explained by the fact that rhamnose released from the RG-I backbone
359 by microbial enzymes was consumed at a higher rate compared to the released uronic acid (Fig.
360 4B and 4F). A higher degree of RG-I branching signifies that the RG-I domains possess more
361 and/or longer side chains (Wang et al., 2023). The branching chains of black mulberry pectin were
362 easily degraded by microbial enzymes during fermentation (Ai et al., 2022). The lower degree of
363 RG-I branching could be attributed to the removal and/or shortening of side chains by added
364 pectinase and endogenous pectinase during juice preparation (**Fig. 4C** and **4G**). The increased RG-
365 I branching values could be partly due to the fact that the short galactose side chains provided
366 resistance to microbial RG-hydrolase cleaving the RG-backbone (Sengkhamparn et al., 2009). The
367 higher linearity levels of the soluble part in W and the insoluble fraction in CE at 0 h of
368 fermentation were mainly due to the removal of UA by pectinase and pectin lyase. The increased
369 linearity of pectin indicated that the RG-I region of pectins from black mulberry was more
370 accessible than the HG region to the microbial enzymes secreted during fermentation (**Fig. 4D** and
371 **4H**).
372
373

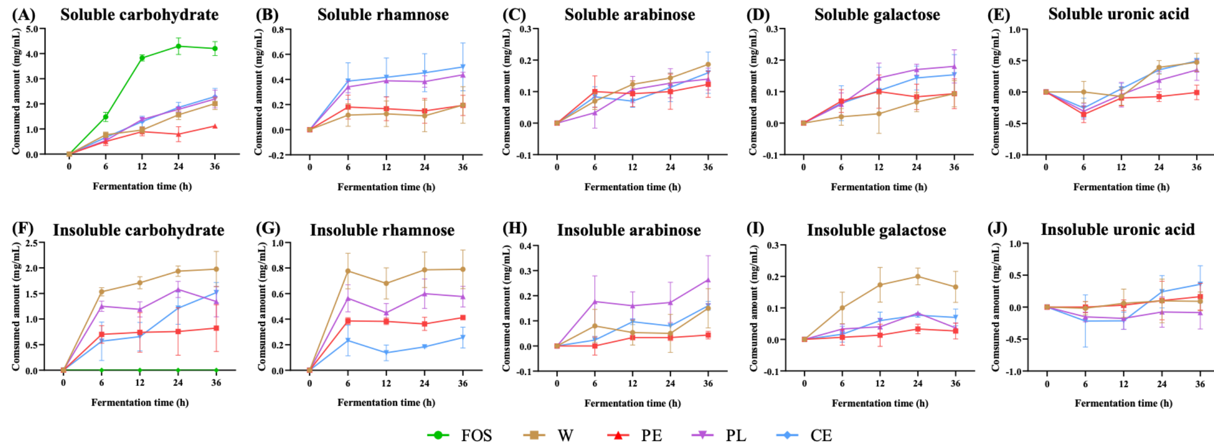


Figure 3. Consumed carbohydrates during *in vitro* fermentation of residues from black mulberry juice after simulated digestion and absorption. The consumed carbohydrates from residues include soluble carbohydrate (A), soluble rhamnose (B), soluble arabinose (C), soluble galactose (D), soluble uronic acid (E), insoluble carbohydrate (F), insoluble rhamnose (G), insoluble arabinose (H), insoluble galactose (I), and insoluble uronic acid (J).

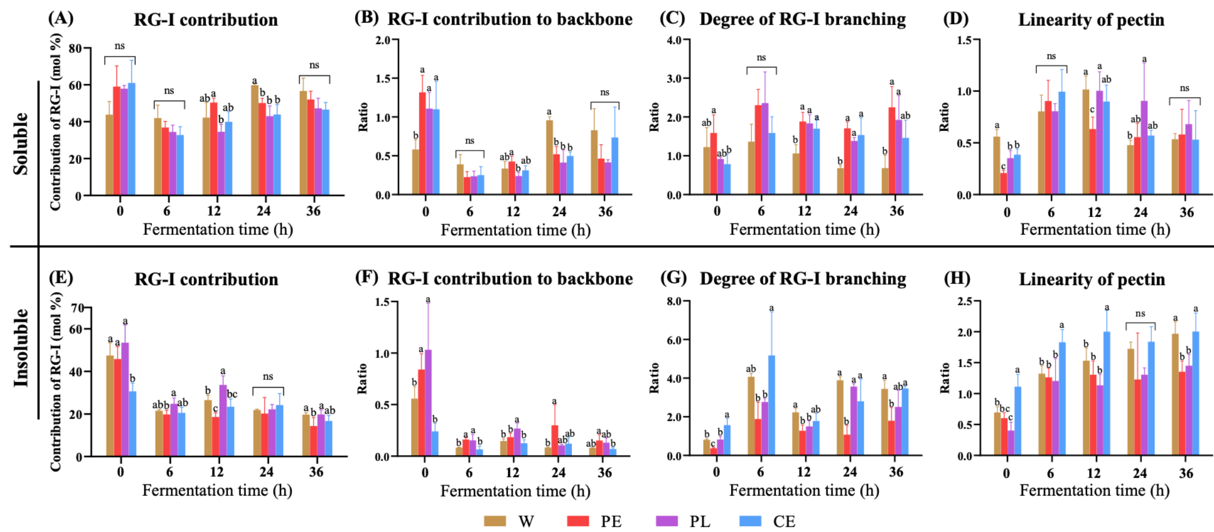


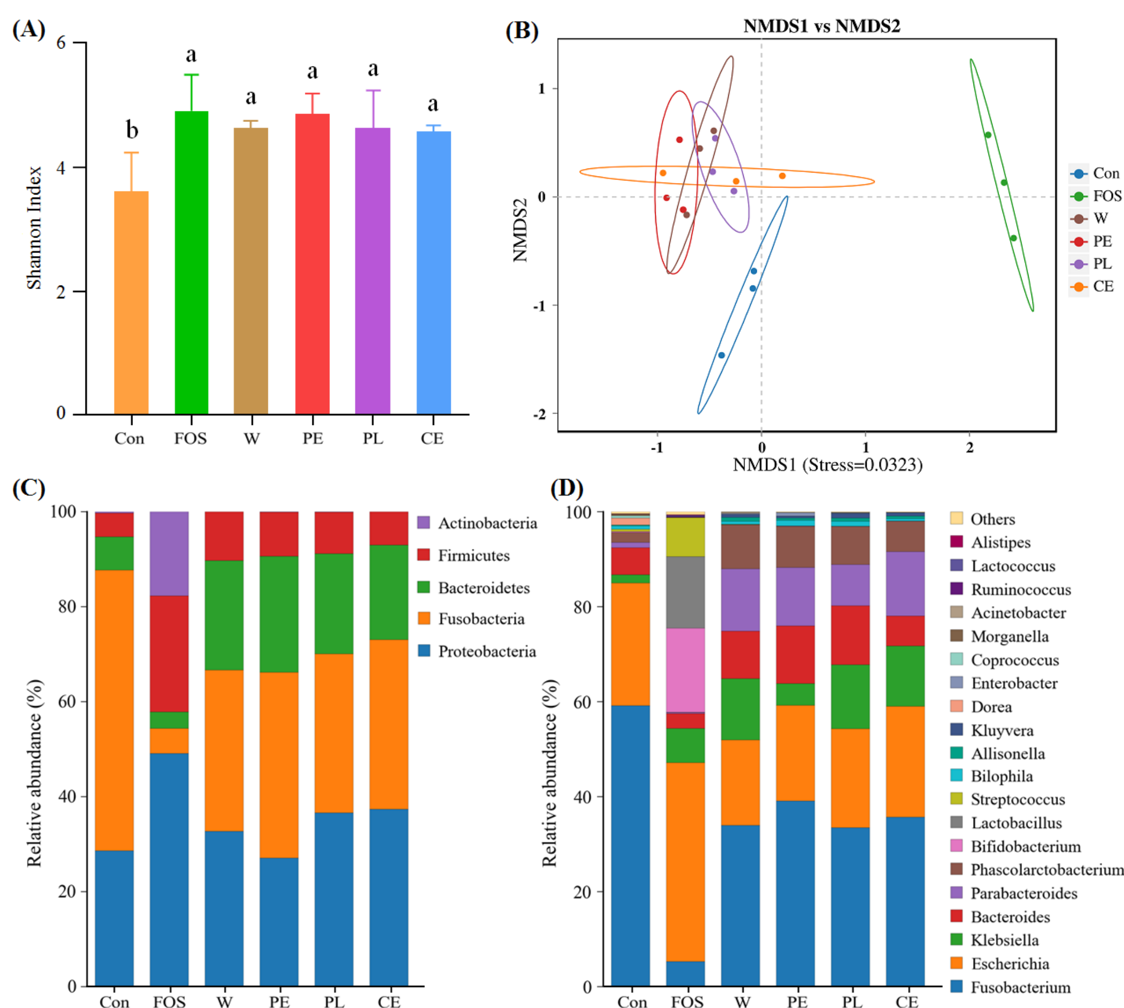
Figure 4. Dynamic changes in compositional and structural properties of residues from black mulberry juice during *in vitro* fermentation. The data were calculated based on the molar proportions of monosaccharides. Contribution of RG-I to the soluble (A) and insoluble (E) fractions was calculated as 2Rha+Gal+Ara (mol%); the contribution of RG-I domain to the soluble (B) and insoluble (F) pectin backbone was calculated by Rha/UA (mol/mol); the degree of RG-I branching of soluble (C) and insoluble pectin (G) was calculated by (Ara+Gal)/Rha (mol/mol); the linearity of soluble (D) and insoluble (H) pectin was calculated by UA/(Fuc+Gal+Ara+Rha+Xyl) (mol/mol); Values are means ± SD (n = 3) and data at each fermentation timepoint with different lowercase letters indicate significant differences (p<0.05). "ns" stands for no statistically significant difference.

3.5. Effect of mulberry juice on gut microbial composition

The diverse digestion patterns and subsequent fermentation characteristics of mulberry juice have led to varied fates of the carbohydrates introduced through enzymatic treatment. These distinct polysaccharide populations, available for gut microbiota consumption, have consequently influenced the composition of the gut microbial community. The results showed that the Shannon diversity indices of the PE, PL, CE, W, and FOS groups were significantly higher than those of the Con group ($p<0.05$, **Fig. 5A**), indicating that the supplementation of mulberry juice fraction or FOS increased microbial diversity. The NMDS plot showed distinct differences in gut microbiota between the mulberry juice groups and the Con and FOS groups, while the four mulberry juice groups exhibited similar microbial community structures (**Fig. 5B**). These findings suggest that supplementation of mulberry juice, rich in dietary fiber, can significantly shape the microbial community, which is consistent with previous studies (Hamaker & Tuncil, 2014; Vaz Rezende et al., 2021).

Figure 5C shows that, after 36 h of fermentation, the dominant phyla identified in all fermented groups were Proteobacteria, Fusobacteria, Bacteroidetes, and Firmicutes, with a high abundance of Actinobacteria observed in the FOS group. Compared to the Con group, the FOS group exhibited a significant decrease in the relative abundance of Bacteroidetes and a substantial increase in the relative abundance of Firmicutes, consistent with previous findings (Li et al., 2020; Ai et al., 2022). A significant decrease in the relative abundance of the opportunistic pathogen Fusobacteria (Brennan & Garrett, 2019) was observed in the mulberry juice groups (Con>PE>CE>W>PL). Fermentation of mulberry juice digesta led to an increase in the relative abundance of Bacteroidetes (PE>W>PL>CE>Con) and Firmicutes (W>PE>PL>CE>Con). Bacteroidetes is known to be a dominant bacterial phylum in the human gut, capable of

416 catabolizing a wide range of complex food carbohydrates (Tuncil et al., 2017; Wu et al., 2021).
417 Firmicutes possesses a diverse array of enzymes that efficiently hydrolyze carbohydrates,
418 facilitating the conversion of polysaccharides into SCFAs, thereby promoting positive health
419 effects in the host (Sheridan et al., 2016). The higher abundance of Bacteroidetes and Firmicutes
420 observed in this study suggests that the polysaccharide components were the primary substrate for
421 mulberry juice fermentation.
422



423
424 **Figure 5.** The α- and β- diversity index analysis and bacterial taxonomic profiling analysis of
425 species of fermentation broth (36 h) from different treatments. (A): Shannon index, (B): NMDS
426 analysis based on weighted unifrac analysis, (C): the relative abundance of bacterial community
427 at the phylum level, (D): the relative abundance of bacterial community at the genus level.
428 Significant variances are denoted by different lowercase letters in the same figure (p<0.05).

Changes in gut microbiota at the genus level are shown in **Fig. 5D**. Compared to the Con, mulberry juice digesta effectively reduced the relative abundance of gram-negative anaerobic bacterium *Fusobacterium* (PL<W<CE<PE<Con) which has been linked to the occurrence of colorectal cancer (Zhou, Chen, Yao, & Hu, 2018). *Escherichia coli* is the most prevalent strain in the human microbiota, and isolates have probiotic, commensal, and pathogenic functions in the host (Gatsios, Kim, & Crawford, 2021). When mulberry juice groups were compared to Con group, the relative abundance of *Escherichia* seemed to be lower (Con>CE>PL>PE>W). All groups that were incubated with mulberry juice digesta increased the relative abundance of *Bacteroides* (PL>PE>W>CE>Con), *Parabacteroides* (CE>W>PE>PL>Con), and *Phascolarctobacterium* (W>PE>PL>CE>Con) in comparison with the Con group. The modulating effects of mulberry juice digesta on gut microbiota is in agreement with the data of mulberry fruit polysaccharide fermentation in terms of changes in the relative abundance of gut microbiota (Ai et al., 2022). This suggests that the primary substrates involved in the control of the gut microbiota were undigested soluble polysaccharides in mulberry juice digesta. Mulberry juice, on the other hand, encouraged the development of several gut microbiota (such as *Parabacteroides* and *Phascolarctobacterium*), which were not identified or at low levels during mulberry fruit polysaccharide fermentation (Ai et al., 2022). This may be related to the existence of other bioactive substances in the juice, such as binding polyphenols, which could also be biotransformed directly to further influence the intestinal microbiota (Aravind, Wichienchot, Tsao, Ramakrishnan, & Chakkaravarthi, 2021). The different bacterial profiles among the enzyme-treated groups could be partly attributed to the fact that enzymatic treatment altered the physicochemical properties of dietary fibers, which in turn affected the fermentation characteristics.

452 *3.6. Dynamic changes of SCFAs during fermentation*

453 SCFAs are essential metabolites produced by microbiota in the gut during the utilization of
454 dietary fiber, and they play a crucial role in maintaining host health. As shown in **Fig. 6**, the total
455 SCFA content in the mulberry juice groups ranged from 29.7 to 32.6 mmol/L (CE>PL>W>PE,
456 p>0.05) after 36 h of fecal fermentation, which was significantly higher than the Con group
457 (15.7±0.9 mmol/L) (p<0.05). Among the mulberry juice groups, no significant differences were
458 observed in the levels of acetic acid and butyric acid, except for the propionic acid content, which
459 was significantly higher in the CE group compared to the PE group (p<0.05) (**Fig. 6A-C**). The
460 higher concentrations of acetic and propionic acids in the mulberry juice groups may be attributed
461 to the higher relative abundance of *Bacteroides*, which has the ability to produce acetic and
462 propionic acids from carbohydrates (Gomez, Gullon, Yanez, Schols, & Alonso, 2016; Wu et al.,
463 2021). The increased production of acetic acid in the FOS group could be mainly attributed to the
464 fermentation by Bifidobacterium and Lactobacillus (Li et al., 2020), while the lower content of
465 propionic acid may be linked to the lower relative abundance of *Bacteroides* (**Fig. 5D**).
466 Furthermore, propionic and butyric acids are known as end products of *Phascolarctobacterium*
467 fermentation (Wu et al., 2021), which could partly explain the elevated concentrations of propionic
468 and butyric acids in the mulberry juice groups. Additionally, the fermentation of galactose and
469 galacturonic acid from polysaccharides in mulberry juice may also contribute to the production of
470 butyric acid (Li et al., 2020).

471 Only small amounts of iso-butyric, valeric, and iso-valeric acids were detected in the
472 fermentation broth of all samples (**Fig. 6D-E**). Isobutyric acid and isovaleric acid, known as
473 branched-chain short-chain fatty acids (BCFAs), are end products derived from the degradation of
474 branched-chain amino acids (valine, leucine, and isoleucine) by the gut microbiota (Smith, &

Macfarlane, 1997). After 36 h of fermentation, the total BCFA levels were significantly lower in the mulberry juice groups compared to the Con group ($p<0.05$) (**Fig. 6H**), indicating that mulberry juice consumption inhibited protein fermentation, which can be beneficial for gut health. As fermentation advanced, the ratios of total SCFAs to total BCFAs (**Fig. 6I**), used as an indicator of the preferred utilization of carbohydrate and protein by gut microbiota (Ai et al., 2022; Wang et al., 2023), exhibited a gradual increase. The decrease in the proportion of BCFAs with the increasing total SCFAs therefore suggests that the high SCFA concentrations are mainly due to the availability of carbohydrates during the 36 h of fermentation. These results imply that enzyme-treated mulberry juice has the potential to enhance the production of SCFAs, which could offer health benefits to the host.

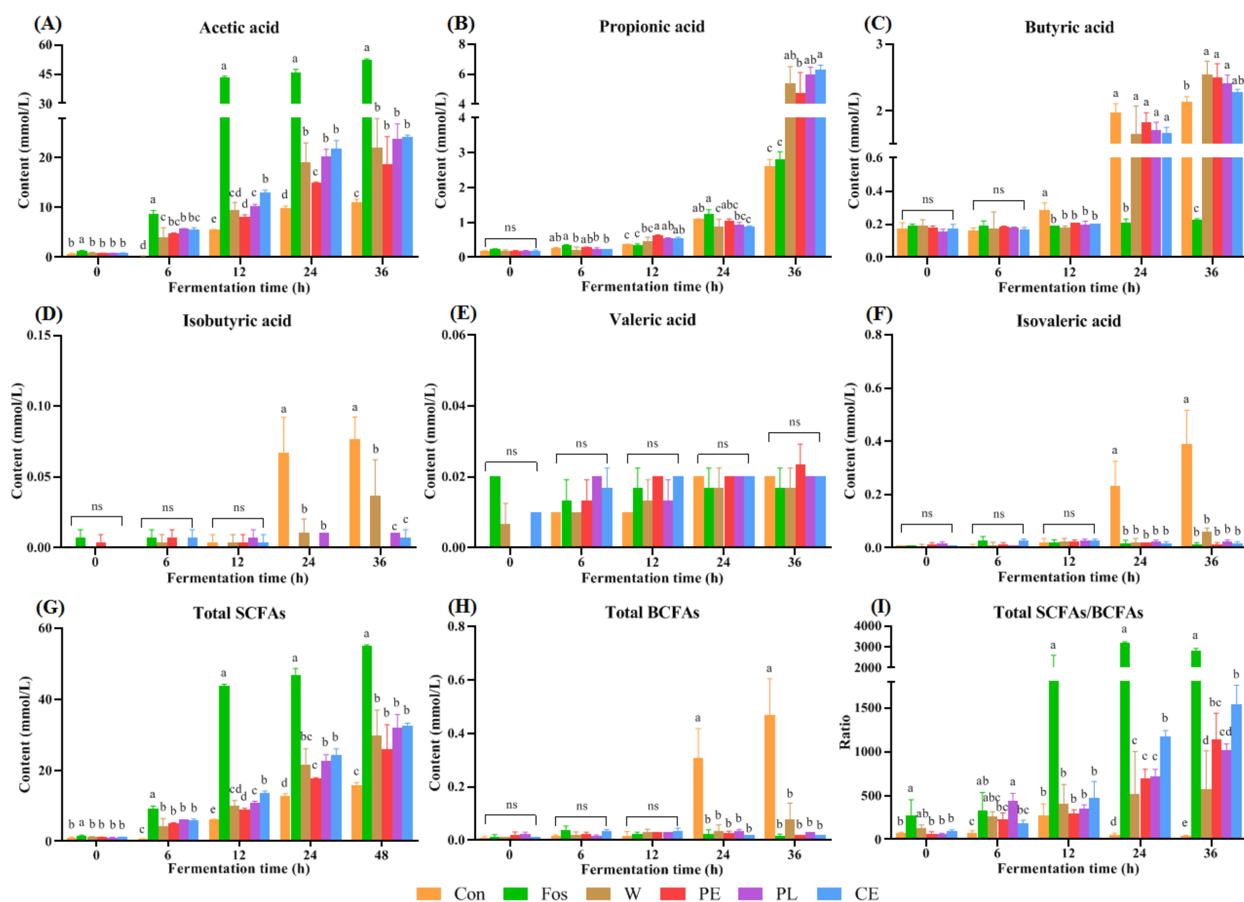


Figure 6. Changes in SCFAs during *in vitro* fermentation. Total SCFAs denotes the sum of acetic acid, propionic acid, butyrate acid, valeric acid, iso-butyric acid and iso-valeric acid.

488 whereas total BCFAs denotes the sum of iso-butyric acid and iso-valeric acid. A significant
489 difference (p<0.05) is shown by the distinct lowercase letters in the figure. "ns" stands for no
490 statistically significant difference. Values are means ± SD (n = 3).
491

492 4. Conclusions

493 Enzymatic treatments were beneficial to the solubilization of carbohydrates from mulberry
494 fruit into the juice. The digestion and fermentation patterns in terms of carbohydrates were
495 consequently affected by the enzymatic treatments. These distinct polysaccharide populations,
496 available for gut microbiota consumption, have consequently influenced the composition of the
497 gut microbial community. Our results suggested that enzymatic treatments during mulberry juice
498 preparation can have a positive impact on health by influencing gut microbiota and SCFAs
499 modulations. Enzymes are potential tools for the production of functional fruit and vegetable juice,
500 and it remains to specify the processing conditions for certain raw material.

501

502 Declaration of competing interest

503 The authors declare no competing financial interest.

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514 Methodology, Investigation, Validation, Writing - Original Draft. Yuxin Wang: Methodology,
515 Investigation. Songen Wang: Methodology, Investigation. Henk A. Schols: Writing - Review &
516 Editing. Hauke Smidt: Writing - Review & Editing. Maurizio Battino: Writing - Review & Editing.
517 Weibin Bai: Supervision, Writing - Review & Editing. Lingmin Tian: Conceptualization,
518 Supervision, Writing - Review & Editing.
519

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