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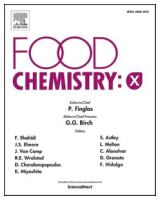

Simulated digestion, dynamic changes during fecal fermentation and effects on gut microbiota of *Avicennia marina* (Forssk.) Vierh. fruit non-starch polysaccharides

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D-Glucose (PubChem CID: 5793)
3-Methyl-1-phenyl-2-pyrazolin-5-one (PubChem CID: 4021)
3,5-Dinitrosalicylic acid (PubChem CID: 5251731)
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Propionic acid (PubChem CID: 1032)

ABSTRACT

Grey mangrove (*Avicennia marina* (Forssk.) Vierh.) fruit is a traditional folk medicine and health food consumed in many countries. In this study, its polysaccharides (AMFPs) were obtained and analyzed by chemical and instrumental methods, with the results indicating that AMFPs consisted of galactose, galacturonic acid, arabinose, and rhamnose in a molar ratio of 4.99:3.15:5.38:1.15. The dynamic changes in AMFPs during the digestion and fecal fermentation processes were then investigated. The results confirmed that AMFPs were not depolymerized by gastric acid and various digestive enzymes. During fermentation, 56.05 % of the AMFPs were utilized by gut microbiota. Galacturonic acid, galactose, and arabinose from AMFPs, were mostly consumed by gut microbiota. AMFPs obviously decreased harmful bacteria and increased some beneficial microbiota, including *Megasphaera*, *Mistuokella*, *Prevotella*, and *Megamonas*. Furthermore, AMFPs obviously increased the levels of various short-chain fatty acids. These findings suggest that AMFPs have potential prebiotic applications for improving gut health.

1. Introduction

For decades, an increasing number of studies have suggested that the gut microbiota has a remarkable ability to impact human health, including maintaining intestinal barrier integrity, regulating the immune system, adjusting metabolism, promoting vitamin synthesis, and inhibiting intestinal pathogens (Schroeder & Bäckhed, 2016). Changes of gut microbial structure are closely related to host physiology and pathology, and numerous studies have shown that various serious diseases, such as colon cancer, inflammatory bowel disease, type 2 diabetes, and obesity, are closely associated with the dysbiosis of gut microbiota (Schroeder & Bäckhed, 2016). Thus, promoting probiotics and maintaining microbial diversity and microecological balance may be an important way to regulate the health of the host. Diet plays an important role in modulating gut microbiota and therefore improving health status (Attaye, Pinto-Sietsma, Herrema, & Nieuwdorp, 2020), and digestion-resistant carbohydrates such as certain polysaccharides can reach the large intestine and be utilized by the microbiota. Numerous studies have demonstrated that the gut microbiota composition may be affected by polysaccharide fermentation together with its fermentative

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metabolites, especially short-chain fatty acids (SCFAs) to promote human health (Tang, Ding, Sun, Liu, Kan, & Jin, 2019). Therefore, some polysaccharides may have a high development and application potential as prebiotics to improve human health.

Avicennia marina (Forssk.) Vierh., a grey or white mangrove tree, belongs to the family Acanthaceae, which is widely grown in subtropical and tropical regions (ElDohaji, Hamoda, Hamdy, & Soliman, 2020). This plant is not only an ecologically important mangrove tree species but also a traditional folk medicine used to treat ulcers, abscesses, and rheumatism in some Asian countries, such as China (Bibi et al., 2019). Previous studies have confirmed the presence of various classes of phytochemicals in *A. marina*, such as polysaccharides, free fatty acids, flavonoids, glycosides, tannins, hydrocarbons and triterpenes (Yi et al., 2020). They are shown to have antidiabetic, anti-inflammatory, antiviral and anticancer activities (Behbahani, Zadeh, & Mohabatkar, 2013; ElDohaji, Hamoda, Hamdy, & Soliman, 2020). In particular, *A. marina* fruits are used as a traditional folk medicine and a food in some countries, such as China, Australia, Egypt, and New Zealand (Budiyanto et al., 2022; ElDohaji, Hamoda, Hamdy, & Soliman, 2020). Moreover, based on modern people's healthy food habits, these fruits have also become increasingly popular because of their nutritional properties. Polysaccharides are one of the main bioactive compounds in fruits and have received considerable attention (Yuan & Zhao, 2017). However, to the best of our knowledge, few reports have focused on *A. marina* fruit polysaccharides (AMFPs), especially their changes and functions after oral administration. For the potential development of natural food and medicine, comprehensive research on AMFPs is needed. Thus, in the present study, polysaccharides were obtained from *A. marina* fruits, and their chemical compositions were determined. Furthermore, the digestion behavior and fermentation ability of the AMFPs were assessed. The findings of this study will be helpful for further studying the bioavailability of the AMFPs and the potential beneficial effects of polysaccharides on human health by modulating the gut microbial ecosystem.

2. Materials and methods

2.1. Materials and chemicals

A. marina (Forssk.) Vierh. fruits were collected from Beihai (Guangxi, China). Monosaccharide standards, such as mannose (Man), ribose (Rib), arabinose (Ara), and fucose (Fuc), and bovine serum albumin (BSA), were obtained from Aladdin Chemical Reagent Co., Ltd. (Shanghai, China). 3, 5-Dinitrosalicylic acid (DNS) was obtained from Shanghai Solarbio Bioscience & Technology Co., Ltd (Shanghai, China). Lipase (Lot No. SLBL2143V, 41.62 ± 5.62 U/mg determined using triacetin), α-amylase (Lot No. BCCB0496, 30.80 ± 6.32 U/mg), pancreatin (Lot No. SLBV6830, activities of lipase, amylase, protease in pancreatin were 59.844 ± 10.18, 116.10 ± 12.73, and 135.93 ± 2.11 U/mg, respectively), pepsin (Lot No. BCBR2017V, 301.40 ± 23.98 U/mg), 3-methyl-1-phenyl-2-pyrazolin-5-one (PMP), glucose (Glc), galactose (Gal), galacturonic acid (GalA), glucuronic acid (GlcA), and rhamnose (Rha) were provided by Sigma Chemical Co. (St. Louis, MO, USA). Coomassie brilliant blue G-250, fructooligosaccharide (FOS), trypsin (Lot No. M54318066, 357.35 ± 5.53 U/mL), xylose (Xyl), and amyloglucosidase from *Aspergillus niger* (Lot No. C10778757, > 100,000 U/mL) were purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). Other commercially obtained chemicals, such as NaCl, $NaHCO_3$ and NaOH, were of analytical grade.

2.2. Preparation of the AMFPs

A. marina (Forssk.) Vierh. fruits were washed, peeled, dried in a hot air oven at 60 °C, and smashed by a grinder. The fruit powder was extracted twice by hot water with a ratio of water to material of 20:1 (mL/g) at 95 °C for 3 h. The supernatant was collected by centrifugation at 4816×*g* for 15 min and then hydrolyzed by 0.05 % (w/w) of α-amylase at 50 °C for 4 h and 0.05 % (v/v) of amyloglucosidase (pH 4.5) at 40 °C to remove starch. The hydrolysis with the amyloglucosidase was continued until the solution did not turn blue with iodine solution. The hydrolysate was then deproteinized by the Sevag method (Supporting Information–Methods). After centrifugation at 4816×*g* for 15 min, the supernatant was precipitated with a final concentration of 75 % ethanol (v/v). The precipitates obtained after centrifugation were dissolved in deionized water, concentrated to about 25 % of the original volume and lyophilized. Finally, AMFPs were obtained for further analysis.

2.3. Determination of chemical compositions

The total carbohydrate, protein, uronic acid, and reducing sugar contents of the AMFPs were analyzed by the modified phenol–sulfuric acid method using Ara as a standard, the Coomassie Brilliant Blue G-250 staining method using BSA as a standard, the m-hydroxydiphenyl method using GalA as a standard, and the DNS colorimetry using Glc as a standard, respectively (Supporting Information–Methods) (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956; Miller, 1959; Yuan et al., 2015).

2.4. Analysis of molecular weight (M_w)

The M_w distribution of the AMFPs was analyzed by HPLC (Shimadzu Prominence LC-2030C 3D) according to our previous study (Yuan et al., 2015). The apparatus was equipped with a refractive index detector and a Shodex OH-pak SB-804 HQ column (8 × 300 mm). The mobile phase and its flow rate were 0.1 mol/L NaCl solution and 0.5 mL/min, respectively.

2.5. Determination of the monosaccharide composition

An HPLC apparatus (Shimadzu Prominence LC-2030C 3D) equipped with a diode array detector and an Agilent Zorbax Eclipse Plus C18 column (4.6 × 250 mm) was used to analyze the monosaccharide composition as described in our previous report (Yuan et al., 2015). For the analysis of free monosaccharides released after the simulated digestion and fermentation, the AMFP samples during digestion were directly derived by PMP. To analyze the monosaccharide composition of the AMFPs and possible changes, AMFP samples before and after fermentation were hydrolyzed and then derived by PMP.

2.6. FT-IR spectrometric and NMR analysis

AMFPs were mixed with dried potassium bromide powder, ground, pressed into pellets, and analyzed by a Nicolet iS50 Fourier transform infrared spectroscopy (FT-IR) spectrometer (Thermo Fisher Scientific, Wilmington, MA, USA).

A 500 MHz Bruker Avance DRX 500 spectrometer was used to analyze the ^{1}H and ^{13}C NMR spectra of the AMFPs at 300.0 K. The sample was dissolved in deuterium oxide (D_2O) with 99.9 % D at 20–40 mg/ml. The number of scans for the ^{1}H and ^{13}C NMR was 512 and 4000, respectively.

2.7. Simulated salivary, gastric, and small intestinal digestion of the AMFPs

The methods described by Rui et al. (2019) with minor modifications were used to investigate AMFP digestion in artificial saliva, gastric fluid, and intestinal juice. Briefly, 100 mL of the simulated salivary medium at pH 7.0 was prepared by deionized water containing α-amylase (200.0 mg), mucin (100.0 mg), KCl (15.0 mg), and NaCl (12.0 mg). One hundred milliliters of gastric juice at pH 2.0 was prepared with deionized water containing KCl (110.0 mg), NaCl (310.0 mg), $CaCl_2$ (25.0 mg),

$NaHCO_3$ (60.0 mg), gastric lipase (25.0 mg), pepsin (23.6 mg), and 1.0 mol/L CH_3COONa solution (1.0 mL). An intestinal electrolyte solution containing 0.33 g/L $CaCl_2$, 0.65 g/L KCl, and 5.4 g/L NaCl was prepared for preparing the simulated intestinal juice. The small intestinal juice at pH 7.0 mainly consisted of 7 % pancreatin (w/v), 0.032 ‰ trypsin (w/v) and 4 % bile salt (w/v).

For the salivary digestion of the AMFPs, 8 mg/mL AMFP solution was added to the prepared salivary medium and stirred at 37 °C for 2 h. The reaction mixture was removed at different time points, and boiled for 10 min to inactivate amylase for further analysis (Zhao, Qin, Guan, Zheng, Liu, & Zhao, 2018). For gastric digestion, AMFP solution was added to the prepared gastric juice and reacted at 37 °C for 6 h. The solution was removed at different time points and boiled for 10 min to inactivate enzymes for further analysis. To investigate the small intestinal digestion of the AMFPs, the reaction mixture of AMFPs and gastric juice was adjusted to neutral, added to the intestinal juice, removed at different time points, and boiled for 10 min to inactivate enzymes for further analysis.

2.8. In vitro fermentation of the AMFPs

The *in vitro* fermentation of the AMFPs was performed by using a previously described method (Ma et al., 2022), with minor modifications. The experiments were performed under the approval of Guangxi University of Chinese Medicine. Briefly, fresh fecal samples were collected from 4 healthy volunteers who agreed to participate in the study and provided informed written consent. Notably, these volunteers had not taken antibiotics in the preceding 3 months. Feces were collected immediately in a sterile plastic container after defecation on the day of inoculation. Fecal samples with the same weight were evenly dispersed in autoclaved physiological saline and centrifuged to produce a fecal suspension without large and insoluble particles. The fecal suspension was inoculated into basal nutrient medium containing AMFPs and incubated at 37 °C for 24 h using an Anaero Pack system. The system is effective both for establishing the anaerobic condition and for the growth of anaerobic bacteria. Furthermore, it is easy to setup, which make it an excellent alternative to the anaerobic chamber maintained by sparging O_2-free N_2/CO_2 gas. The basal nutrient growth medium and FOS were used as the blank and positive control, respectively. Fermentations were performed in triplicate for each group, and the samples were removed at different time points of fermentation for further analysis.

2.9. Determination of gut microbes

The microbial DNA from all of the samples was extracted according to the method described in a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). Then, the V3–V4 region of the bacterial 16S rDNA pyrosequencing was amplified using the primers 5′-CCTACGGGNGGCWGCAG-3′ (forward) and 5′-GGACTACHVGGGTATCTAAT-3′ (reverse). An Illumina NovaSeq PE250 platform (Illumina, San Diego, CA, USA) was used to sequence the prepared libraries.

2.10. Assays of pH and SCFAs

The pH of the fermented samples was measured by a pH meter (FiveEasy Plus, Mettler Toledo, China). For the determination of SCFAs, fermented sample was added to an equal volume of 2-ethylbutyric acid and centrifuged at 8228×*g* for 5 min. The supernatant was filtrated by a 0.22-μm filter and measured using a DB-FFAP column (0.25 μm × 0.25 mm × 30 m, J &W Scientific, Agilent) on a GC–MS system (7890B-5977B, Agilent Technologies, Palo Alto, CA, USA). The analysis conditions were as follows: helium flow rate of 1 mL/min, initial oven temperature of 100 °C, and temperature increase to 180 °C at a rate of 5 °C/min and hold for 4 min.

2.11. Statistical analysis

All experiments were performed in triplicate, and the data are shown as the means ± SD. One-way analysis of variance (ANOVA) followed by Duncan's test using SPSS software version 26.0 (SPSS Inc., Chicago, USA) was used for the statistical analysis. *P* values less than 0.05 (i.e., $^*P < 0.05$) represent the statistical significance of data. The alpha-diversities, including Chao1, ACE, Shannon, and Simpson, were calculated using QIIME (version 1.9.1). The principal component analysis (PCA) and clustering analysis were performed by an R project Vegan package (version 2.5.3).

3. Results and discussion

3.1. Physicochemical properties of the AMFPs

The AMFPs were obtained *via* a series of steps, including hot water extraction, alcohol precipitation, removal of starch and deproteinization. The contents of total carbohydrate, uronic acid and protein of the AMFPs were calculated as 43.14 ± 5.53 %, 39.13 ± 2.70 %, and 2.38 ± 0.05 %, respectively. Ara was chosen as a standard to determine the content of total carbohydrate because its content was the highest among the monosaccharides in the AMFPs. The measured content of total carbohydrate was low because AMFPs contained other monosaccharides and the absorbance of various monosaccharides obviously differed under the same conditions (Dubois et al., 1956). Four main fractions (F1-F4) were observed in the AMFPs and their M_ws were determined to be 557.28, 101.76, 22.73 and 3.68 kDa, respectively (Fig. 1a). The monosaccharide composition of the AMFPs included Gal, GalA, Ara, and Rha in a molar ratio of 4.99:3.15:5.38:1.15. In addition, the AMFPs contained a trace amount of Glc, Man, and GlcA (Fig. 1b). According to these results, the AMFPs did not contain starch. As shown in Fig. 1c, the bands at 2938 cm^{-1} and 3382 cm^{-1} could be assigned to the C—H and O—H stretching vibrations in the sugar ring, respectively, while the bands at 1746 cm^{-1} and 1614 cm^{-1} were due to the carbonyl asymmetric stretching vibration (Yuan et al., 2015), indicating the presence of uronic acid in the AMFPs, which was consistent with the monosaccharide composition. Some water-soluble non-starch polysaccharides with different monosaccharide compositions were reported from various edible and medicinal plants in previous studies (Anwar, Birch, Ding, & Bekhit, 2022). They were mainly composed of Ara, Gal, Glc, Man, Rha, Xyl, GalA, GlcA, fructose (Fru), and Fuc. For example, in 2015, non-starch polysaccharides were extracted from the roots of American ginseng by Guo et al. and determined to consist of Rha, Ara, Gal, Glc and GalA, with a weight ratio of 1:4:8:8:50 (Guo et al., 2015). The monosaccharide types are similar to those of the AMFPs. However, AMFP had the highest content of Ara and contained only minute amounts of Glc. The monosaccharide compositions could suggest the special structures of polysaccharides, which may contribute to their various biological activities.

The ^1H and ^{13}C NMR spectra of the AMFPs were shown in Fig. 1d. The ^1H NMR signals of the AMFPs were broad and overlapped, which may be due to their high M_w. The chemical shift at approximately 1.27 ppm was assigned to the methyl proton of the Rha residue (Shakhmatov, Toukach, & Makarova, 2020). The signals at approximately 2.1 ppm indicated that some sugar residues of the AMFPs were *O*-acetylated (Yuan et al., 2020). Studies have indicated that acetyl groups in polysaccharides may be essential for their biological activity (Yuan et al., 2020). The signals of various ring protons of sugar residues were observed at 3.6–4.3 ppm. The AMFPs were mainly composed of six kinds of α and β-configurations of sugar residues, according to the signals of the anomeric protons at approximately 5.17 ppm, 5.11 ppm, 4.98 ppm, 4.93 ppm, 4.48 ppm, and 4.41 ppm. The ^{13}C NMR spectrum of the AMFPs also showed weak and overlapping signals because of their high M_w, although the number of scans reached 4000. The signals of methyl carbons of the acetyl groups and Rha residues observed in the ^1H NMR

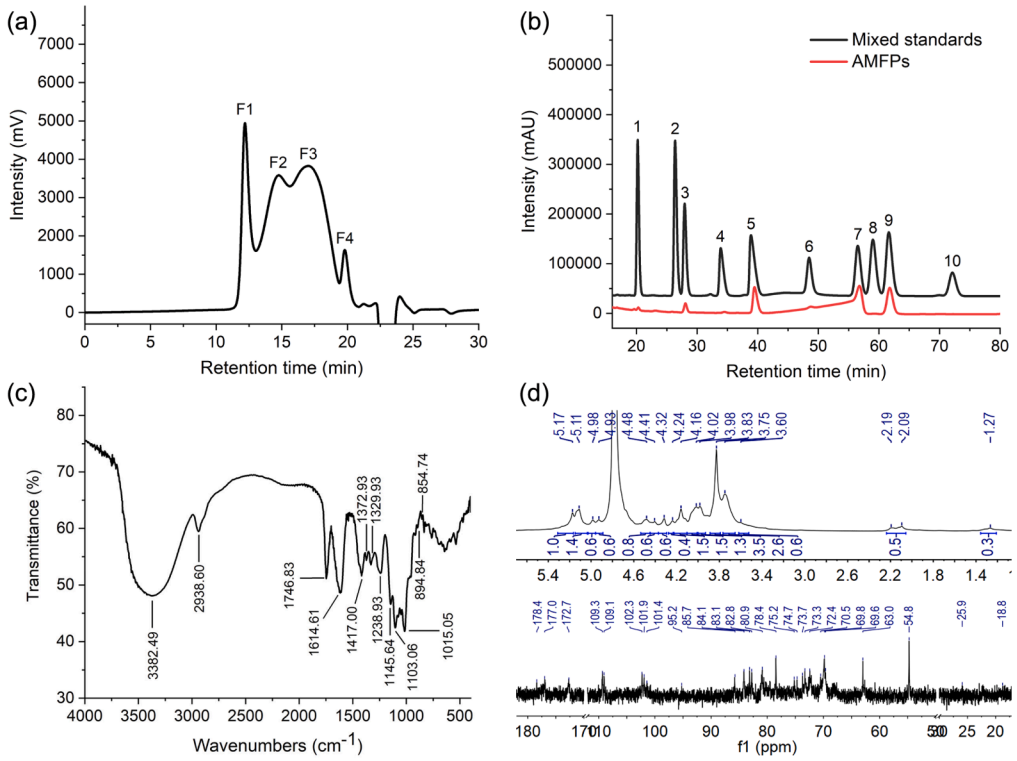

Fig. 1. Physicochemical properties of the AMFPs. Molecular weight of the AMFPs (a); monosaccharide composition of standards and AMFPs (b) (peaks followed by the orders: 1, Man; 2, Rib; 3, Rha; 4, GlcA; 5, GalA; 6, Glc; 7, Gal; 8, Xyl; 9, Ara; 10, Fuc); FT-IR spectrum of AMFPs (c); ^{1}H and ^{13}C NMR spectra (d).

spectrum were at approximately 26.0 and 18.8 ppm, respectively (Shakhmatov et al., 2020). The signals at 54.8 ppm indicated that some sugar residues of the AMFPs were *O*-methylated. The chemical shifts at δ 63.0 ppm may be due to the unsubstituted C-6 signals of Gal. The chemical shifts of the other carbons of the sugar rings were at approximately 69.6–85.7 ppm. The signals at approximately 95.2 ppm, 101.4 ppm, 101.9 ppm, and 102.3 ppm could be attributed to the anomeric carbons of the Gal, GalA, and Rha residues, and that from 109.1–109.3 ppm may be due to the anomeric carbons of Ara*f* (Košťálová & Hromádková, 2019). The signals at approximately 172.7–178.4 ppm could be assigned to the carbonyl groups of GalA (Yuan et al., 2020). According to the ^{1}H and ^{13}C NMR spectra, the AMFPs had a high purity because no impurity signals could be observed. In detail, no signal appeared in the region of 7–10 ppm of the ^{1}H NMR spectrum of the AMFPs (Fig. S1), which further confirmed that very little protein existed in the AMFPs (Huang & Mohanty, 2010). The proteins in the AMFPs could be removed well after deproteinization by the Sevag method and ethanol precipitation, which is consistent with other studies (Ding et al., 2019; Guo et al., 2015).

3.2. *Possible changes in the AMFPs after simulated saliva and gastrointestinal digestion*

Studies have shown that polysaccharides remain unchanged under simulated digestion conditions (Ding et al., 2019). However, enzymes, salts and pH may result in polysaccharide hydrolyzation during digestion (Wu et al., 2021). Therefore, the possible changes in AMFPs during digestion were investigated in this study. According to the composition and physicochemical properties of AMFP, we hypothesized that AMFPs could resist the saliva and gastrointestinal digestion. To verify our hypothesis, we used an extended period of digestions, i.e., 2 h of oral phase, 6 h of gastric phase and then additional 6 h of intestinal phase. The potential small changes in polysaccharides during digestion may be detected in an extended period of digestions. The M_w changes before and after salivary digestion showed that there were no changes in the M_w of AMFPs at different digestion times (Fig. S2a). Moreover, the contents of the total carbohydrates and reducing sugars remained stable after 2 h of salivary digestion ($P > 0.05$) (Table S1), and no free monosaccharides were produced from the AMFPs after salivary digestion (Fig. S3a).

The simulated gastric fluids prepared in this study were based on available physiological data of gastric juice from human. No changes in the AMFP were found after the simulated salivary digestion. Therefore, 8 mg/mL AMFP solution prepared by deionized water was fed into the gastric phase for the simulated gastric digestion. According to the chromatograms, the retention time and peak area of AMFPs were constant during the simulated gastrointestinal digestion process from 0 to 6 h (Fig. S2c-f). No changes in total carbohydrate and reducing sugar contents were observed during this process (Table S1). Moreover, the free monosaccharides produced from the AMFPs during gastrointestinal digestion were further determined, and the results are shown in Fig. S3b and c. No free monosaccharides were released from AMFPs in the gastrointestinal digestive juice, which suggested that the simulated gastrointestinal fluid had no degradative impact on AMFP. Therefore, combining the results of changes in M_w, total carbohydrates, reducing sugars and free monosaccharide, we confirmed that AMFPs could resist digestion and safely reach the large intestine. Although the static *in vitro* digestion model has limitations to mimic the complex dynamics of the digestion process or the physiological interactions with the host (Brodkorb et al., 2019), it can be useful and reliable to reveal the digestibility of AMFPs. Using the similar *in vitro* digestion model for investigation of polysaccharides with an extended period of digestions, some polysaccharides from other sources, such as *Crassostrea gigas* (Ma, Jiang, & Zeng, 2021), and Fuzhuan brick tea (Chen et al., 2018), also have been found to be anti-digestive stability.

Starch polysaccharides from plant tissues can be easily hydrolyzed by amylase in the oral cavity and gastrointestinal tract, thereby producing large amounts of Glc (Shim, Lee, Hong, Kim, & Lee, 2020). The produced Glc can be directly absorbed and utilized by the human body. Therefore, polysaccharides containing high levels of starch that can be degraded by amylase and gastric acid may not be suitable for

development as prebiotics. In this study, starch was removed from the AMFPs by amylase and amyloglucosidase. No changes in AMFPs observed during *in vitro* digestion process probably because AMFPs are not sensitive to the acidic environment, and humans lack AMFP-degrading enzymes. Based on the above preliminary results, the AMFPs had the characteristics of a potential prebiotic, and their probiotic effects were further investigated by *in vitro* fermentation model in this study.

3.3. Changes in the AMFPs after in vitro fermentation

3.3.1. Change in the molecular weight (M_w) of the AMFPs

After passing through the oral and gastrointestinal tract, digested or undigested food further moves into the colon and may be utilized by the intestinal microflora. HPLC is an effective method for analyzing the changes in polysaccharides in food during fermentation by microflora and is widely used by researchers to investigate the metabolism of polysaccharides (Chen et al., 2018; Ding et al., 2019). Therefore, the possible changes in the M_w of the AMFPs were first analyzed by HPLC before and after fermentation (Fig. 2a and b). Before fermentation, the peak with a retention time of approximately 22.0 min was from the substance in the culture medium. After 6 h of fermentation, the peak areas of F1, F2 and F3 of the AMFPs decreased obviously from 28.53×10^5 to 16.37×10^5 (Table S2), suggesting that the AMFPs were utilized quickly by gut microbiota. At the same time, the peak area at approximately 21.0 min increased from 18.46×10^5 to 21.06×10^5 during the first 6 h of fermentation, indicating that part of the AMFPs was degraded to low M_w oligosaccharide fragments by gut microbiota. The content of AMFPs decreased gradually, and the area of small molecule substances continued to increase in a time-dependent manner within 12 h. After that, the area at approximately 21.0 min decreased from 22.12×10^5 to 14.37×10^5, which illustrated that the fragments with low M_w were consumed by gut microbiota. These phenomena were also observed in some other studies on other heteropolysaccharides (Chen et al., 2018; Ma et al., 2022).

3.3.2. Changes in total carbohydrates, reducing sugars, and monosaccharides

Gut microbes can secrete many glycoside hydrolases to depolymerize polysaccharides, which results in a reduction in total carbohydrates. Therefore, the change in residual carbohydrates may be used as an indicator of the extent of carbohydrate fermentation. Fig. S4 shows the carbohydrate consumption of AMFPs during fermentation. The residual carbohydrates of the fermentation culture steadily decreased from 100 % to 43.95 % after 24 h of fermentation, indicating that 56.05 % of AMFPs could be consumed by gut microbiota. The utilization rate was similar to that of polysaccharides from *Crassostrea gigas* (57.88 %) (Ma, Jiang, & Zeng, 2021), but lower than that of Fuzhuan brick tea polysaccharides (75.90 %) (Chen et al., 2018). The different utilization rates of polysaccharides by gut microbiota may be due to the differences in their structures and in the bacterial community (Ndeh & Gilbert, 2018).

Studies have shown that the glycosidic bonds of polysaccharides can be hydrolyzed by gut microorganisms and release many reducing ends. In our present study, the reducing sugar contents rapidly increased during the first 12 h of fermentation, indicating that a mass of glycosidic bonds were broken down by gut microbiota (Fig. S4). Subsequently, the reducing sugar content decreased rapidly, which suggested that the produced reducing sugars were consumed by gut microbiota during the rest of fermentation.

Residual monosaccharides represent the remaining monosaccharides in AMFP after utilization by microorganisms at different time points, which can reflect the utilization of AMFP by microorganisms. Free monosaccharides are the released monosaccharides from AMFP during the fermentation process, which can reflect the utilization degree of different monosaccharides by microorganisms. The results of residual monosaccharides at different time points are shown in Table 1. The molar ratios of all the monosaccharides except Rha markedly decreased over time ($P < 0.05$). The main monosaccharides of the AMFPs, such as Ara, Gal, and GalA, were mostly consumed by fecal bacteria after 24 h, among which the utilization rate of GalA was the highest. As shown in Table S3, several free monosaccharides were detected at the beginning of the fermentation, which may be due to the free monosaccharides in the broth solution. A study by Ma et al. (Ma et al., 2022) showed that free monosaccharides, such as Glc, existed in the fermentation broth. Glc and Man disappeared after 6 h of fermentation, and Gal could not be detected after 12 h of fermentation, which illustrated that free monosaccharides were utilized by microorganisms. In addition, the molar ratio of GalA increased in a time-dependent manner ($P < 0.05$), which indicated that the production rate of GalA from the AMFPs after degradation was higher than the utilization rate of GalA by microorganisms. Uronic acid may have a significant influence on the activity of polysaccharides, and carboxy reduction may significantly weaken their activity (Zhao et al., 2018). According to the results of previous studies

Table 1
Concentration of residual monosaccharides at different time points during fermentation *in vitro*.

Fermentation time (h)	Concentration of residual monosaccharides (mg/mL)			
	Rha	GalA	Gal	Ara
0	0.407 ± 0.055 ^{a}	0.730 ± 0.094 ^{a}	0.953 ± 0.136 ^{a}	1.151 ± 0.156 ^{a}
6	0.435 ± 0.009 ^{a}	0.718 ± 0.033 ^{a}	0.638 ± 0.007^{b}	1.004 ± 0.032 ^{a}
12	0.431 ± 0.014 ^{a}	0.621 ± 0.016^{b}	0.474 ± 0.020^{c}	0.643 ± 0.030^{b}
24	0.448 ± 0.003 ^{a}	0.254 ± 0.006^{c}	0.415 ± 0.003^{c}	0.466 ± 0.005^{c}

Mean values in the same column with different letters indicate significant difference ($P < 0.05$).

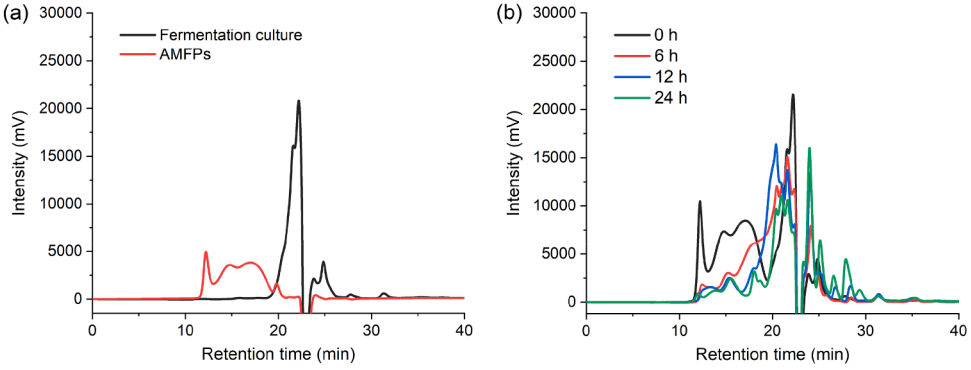

Fig. 2. Changes in molecular weight of the AMFPs during fermentation.

and our present study, polysaccharides rich in GalA could be easily utilized by beneficial bacteria (Ding et al., 2019; Li et al., 2018). The effects of GalA on the fermentation of AMFPs and the mechanisms warrant further investigation.

3.3.3. Compositional changes in the gut microbiota

The structure of the microbiota is strongly influenced by food intake, particularly polysaccharide administration, which further influences host health (Flint, Duncan, Scott, & Louis, 2015). The α-diversity results, including the Ace, Chao1, Simpson and Shannon indices, are shown in Fig. 3a. The index values showed that the richness in the AMFP or FOS group was lower than that in the blank group. Meanwhile, the AMFP group exhibited a higher richness than the FOS group. Supplementation with FOS and AMFPs might tend to promote the proliferation of some bacteria. The growth and competitiveness of the dominant bacteria may lead to the decrease and even disappearance of other bacteria, and hence result in the reduction of bacterial diversity (Rui et al., 2019). PCA is an efficient nonlinear dimension reduction method used to evaluate the β-diversity among samples, which is based on all detected operational taxonomic unit (OTUs). As illustrated in Fig. 3b, the data from samples in the same group were near each other and kept away from those of other treatments. The first two components accounted for 98.91 % of the differences among the three groups, with the first principal component level accounting for 88.00 %. A similar result was also obtained from the multivariate analysis based on the Bray-Curtis method (Fig. 3c). The above results suggested that the microbiota community in the AMFP group was different from those in the blank and FOS groups.

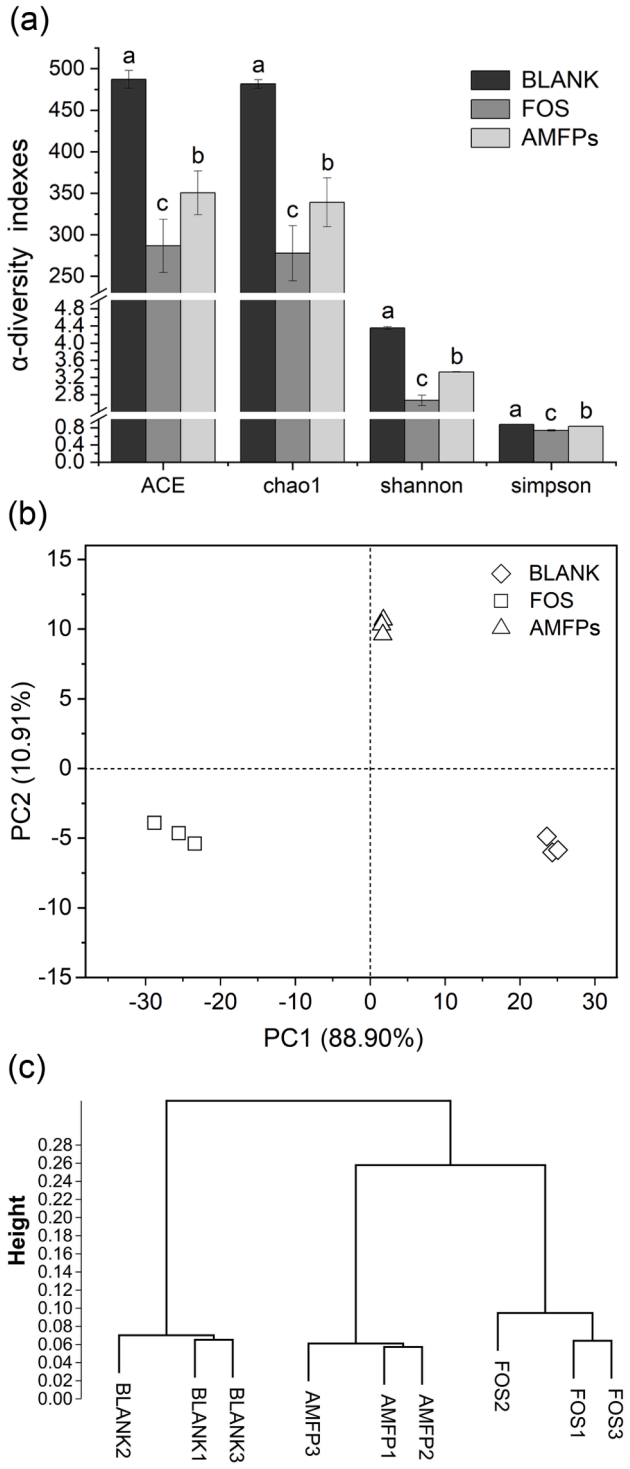

Fig. 3. Composition of gut microbiota: (a) α-diversity indices of the microbiota, (b) PCA of the gut microbiota at the OTU level, and (c) multivariate analysis of variance from matrix scores based on the Bray-Curtis method.

Bacteria in the large intestine, including Bacteroidetes and Firmicutes, can have an important role in fermenting non-digestible carbohydrates and thus benefit host health. The data in Fig. 4a show that Actinobacteria, Proteobacteria, Firmicutes, Fusobacteria, and Bacteroidetes were the predominant bacterial phyla in the blank, FOS and AMFP groups. The levels of Firmicutes in the AMFP and FOS groups were significantly increased and obviously higher than those in the blank group. However, the FOS group showed a reduced relative abundance of Bacteroidetes, which is the same as previous results (Wu et al., 2021). The main reason may be that the acidic conditions after fermentation may benefit the growth of some bacteria belonging to Firmicutes and inhibit the proliferation of some bacteria belonging to Bacteroidetes. Some bacterial pathogens, such as *Escherichia coli*, *Salmonella*, and *Shigella*, belong to the Proteobacteria phylum and can disturb the balance of the gut microbiota community and trigger metabolic and immune disorders (Shin, Whon, & Bae, 2015). In this study, the relative abundances of Fusobacteria and Proteobacteria were obviously reduced by the AMFP and FOS treatment and were lower than those in the blank group.

Accumulating evidence indicates that *Bifidobacterium*, *Megamonas*, *Megasphaera*, and *Prevotella_9* are abundant under conditions with high contents of polysaccharides and are positively associated with human health (Chen et al., 2018; do., 2019; Kovatcheva-Datchary et al., 2015). *Mitsuokella* has been reported to produce SCFAs by utilizing carbohydrates, and to inhibit the growth and invasion of *Salmonella* (Levine, Louis, Thomson, & Flint, 2012). *Bifidobacterium* is also an important probiotic that encodes several genes involved in the degradation and utilization of plant-derived polysaccharides (Ndeh & Gilbert, 2018). The genus *Collinsella* is known to correlate with the reduced risk of some diseases, including cancer, obesity, and inflammatory bowel diseases (Pérez-Burillo, Pastoriza, Jiménez-Hernández, D'Auria, Francino, & Rufián-Henares, 2018). Hence, a high population of *Collinsella* is desired for human health. In our present study, *Bacteroides*, *Escherichia-Shigella*, *Klebsiella*, *Lachnoclostridium*, and *Megasphaera* were the core microbiota in the blank group, while *Megasphaera*, *Megamonas*, *Mitsuokella*, *Lactobacillus* and *Bifidobacterium* were the representative bacterial genera in the FOS group (Fig. 4b). The relative abundances of *Bifidobacterium*, *Collinsella*, *Megamonas*, *Megasphaera*, *Mitsuokella*, and *Prevotella_9* were obviously enhanced by the AMFP treatment and higher than those in the blank group (Table S4). Furthermore, *Collinsella* was boosted during the AMFP anaerobic fermentation process. At the same time, *Escherichia-Shigella*, *Fusobacterium* and *Klebsiella*, regarded as representative harmful bacteria, dramatically declined after the fermentation of FOS or AMFPs. Altogether, AMFPs were found to modulate the microbiota structure, resulting in the proliferation of beneficial bacteria and the growth inhibition of pathogens. The findings corresponded with that of previous studies, in which non-starch polysaccharides from natural sources were

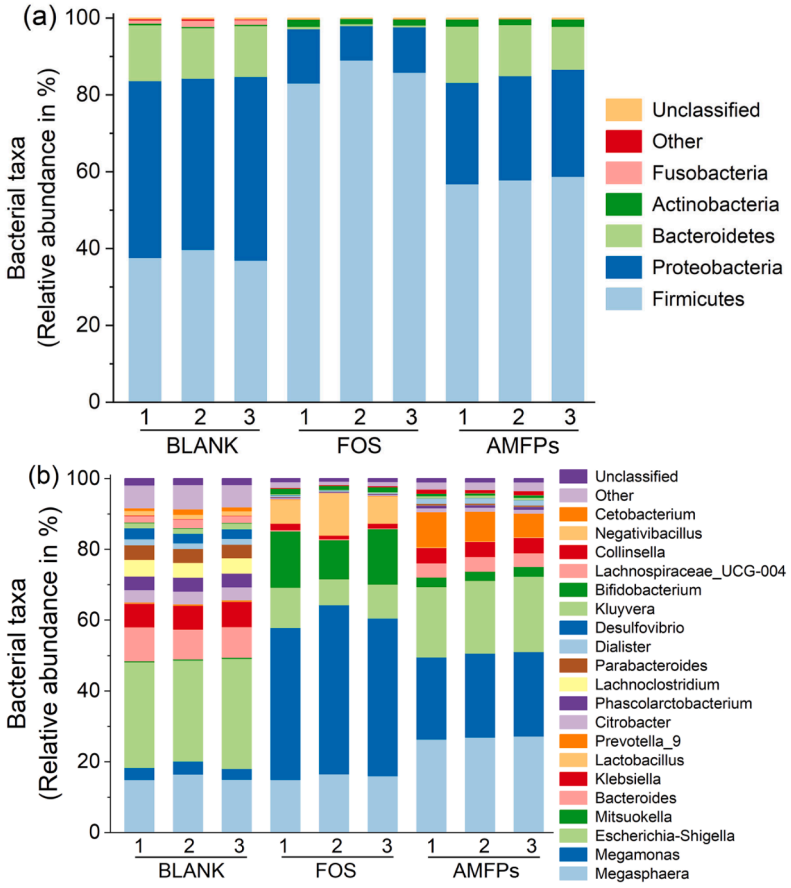

Fig. 4. Changes in the microbial community at the phylum (a) and genus (b) levels after 24 h of fermentation.

found to promote the growth of some beneficial bacteria, and inhibit the proliferation of enteropathogenic bacteria determined at both the phylum and genus levels (Chen et al., 2018; Ma et al., 2021). These non-starch polysaccharides have the potential to be novel prebiotics for improving human health.

3.3.4. *Changes in pH value and SCFA production*

The pH changes of the fermentation culture during fermentation are summarized in Fig. 5a. At the beginning of fermentation, the pH values of the fermentation culture were approximately 7.0 in various groups. The pH value of the solution in the AMFP group rapidly decreased during the initial 6 h fermentation, slightly decreased during the following 6 h fermentation, and then slightly increased for the remaining time. The pH of the AMFP fermentation culture decreased from 7.05 to 5.18 after 24 h of fermentation, which was obviously lower than that in the blank group at the same time point. AMFPs had an obvious influence on the gut microenvironment, although the effect was weaker than that of FOS. A weakly acidic environment has been reported to be due to some metabolites, such as SCFAs, produced by microbiota (Flint et al., 2015).

SCFAs, including acetate, propionate, i-butyrate, *n*-butyrate, i-valeric and n-valeric acids, are the main final products of some polysaccharides fermented by gut microbiota. An increasing number of studies have confirmed that SCFAs possess a range of health benefits, such as modulating the immune response, preventing carcinogenesis, and regulating metabolic disorders (Agus, Clément, & Sokol, 2020). Therefore, the content of SCFAs during fermentation was detected by GC–MS and the results are listed in Fig. 5b-i. Acetic, propionate, *n*-butyric and n-valeric acids were the main fermentation products in each group; however, their contents differed in the various groups. In the positive control group, the fermentation of FOS significantly increased the levels of acetic acid and propionic acid during the 12 h, which is consistent with that from other reports (Chen et al., 2018; Ma et al., 2021; Wu et al., 2021). After 12 h, the levels of acetic acid and propionic acid decreased, and those of *n*-butyric and *n*-valeric acid significantly increased, indicating that there are conversions from acetic acid to other SCFAs, which is consistent with those from other studies (Zeyneb et al., 2021). Acetic acid was also the dominant SCFA in the AMFP group, followed by propionic acid. The levels of acetic and propionic acid in the AMFP group during fermentation were enhanced from 1.306 mM to 8.600 mM and 0.206 mM to 4.486 mM, respectively. Previous studies indicated that fermentation of polysaccharides containing a high proportion of GalA by gut microbiota gave rise to a relatively higher level of acetic acid (Ding et al., 2019; Zhao, Bi, Yi, Wu, Ma, Li, 2021). The high amounts of acetic and propionic acid may be due to the relatively high abundance of *Megasphaera*, *Megamonas* and *Prevotella* (Xu, Zhu, Li, & Sun, 2020). Notably, the *n*-butyric acid and *n*-valeric acid in the AMFP group showed a higher level than those in the blank group at 24 h of fermentation. As one of the important SCFAs, butyric acid can provide energy for the colonic epithelium and presents anti-inflammatory activity. *n*-Valeric acid has been reported as a novel therapeutic target for treating diseases, such as radiation injuries, cancer and colitis (Lordan, Thapa, Ross, & Cotter, 2020). Some species from the *Megasphaera* genus are designated producers of *n*-butyric and *n*-valeric acids (Fu, Liu, Zhu, Mou, & Kong, 2019; Zhao et al., 2021), which is consistent with the gut microbiota results. The content of total SCFAs in the AMFP group was 19.299 mmol/L after 24 h of fermentation, which was lower than that in the FOS group (21.697 mmol/L), but obviously higher than that in the blank group (12.033 mmol/L). In summary, AMFPs fermented by the fecal microbiota led to an increase in individual and total SCFAs, which may be beneficial to human health.

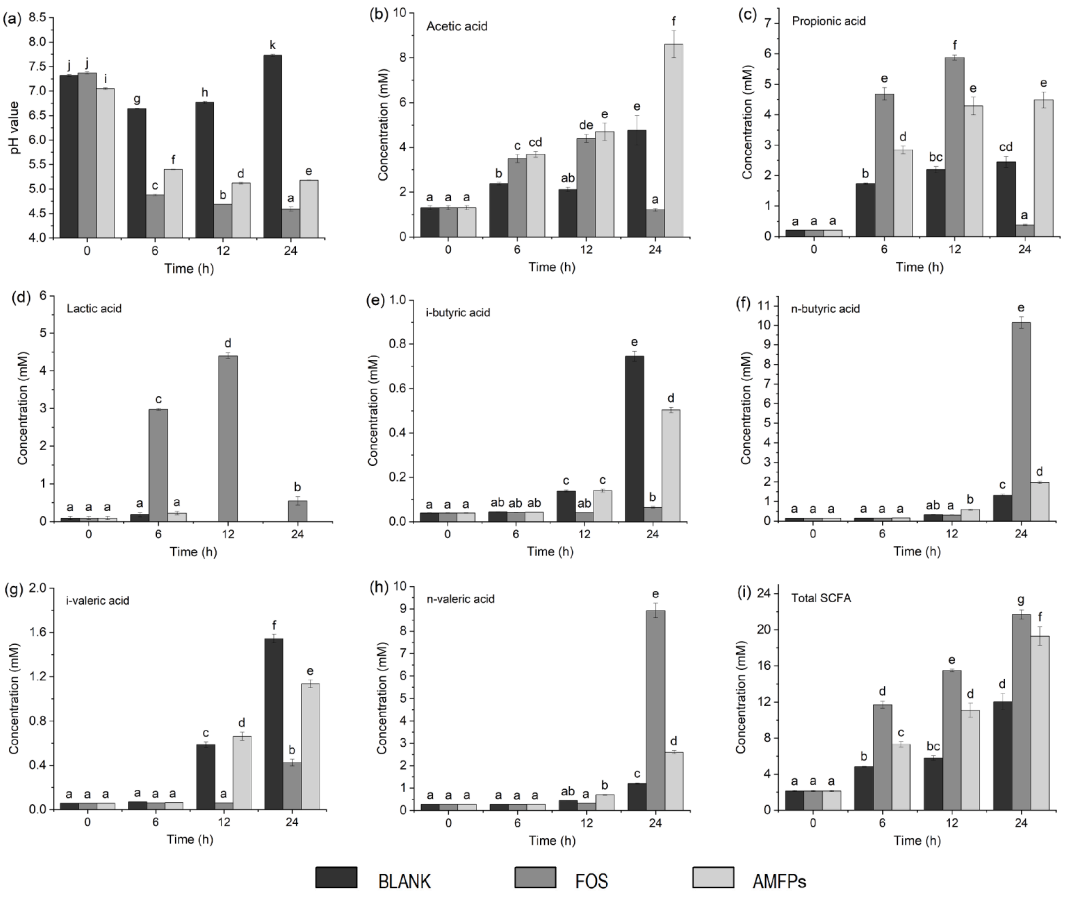

Fig. 5. Dynamic changes in pH value and SCFA levels during fermentation. Different letters indicate significant differences ($P < 0.05$) among different groups.

4. Conclusions

The non-starch polysaccharide AMFPs with high purity were prepared by hot water extraction, and alcohol precipitation, after starch removal and deproteinization. The AMFPs consisted of four polysaccharide fractions and four monosaccharides (Ara, Gal, GalA, and Rha). Instrumental methods such as FT-IR and NMR confirmed the structural features and high purity of the AMFPs. AMFPs could hardly be hydrolyzed during the simulated salivary and gastrointestinal digestion conditions, but could be utilized by gut microbiota. According to the residual carbohydrates of the fermentation culture, 56.05 % of the AMFPs was consumed by the gut microbiota. The monosaccharides Ara, Gal, and GalA produced from AMFPs were mostly utilized by gut microbiota, and the utilization rate of GalA was the highest. AMFPs can regulate the composition of the microbial community. The relative abundance of beneficial bacteria, such as *Megasphaera*, *Megamonas*, *Prevotella_9*, *Mitsuokella*, *Collinsella*, and *Bifidobacterium*, was elevated, while that of enteropathogenic bacteria, including *Escherichia-Shigella*, *Fusobacterium* and *Klebsiella* was inhibited. Furthermore, the concentration of SCFAs, especially acetic and propionic acids, was obviously enhanced by the AMFP treatment. These findings suggested that AMFPs have potential benefits by modulating intestinal health, and may be a new potential prebiotic for use in the food and pharmaceutical industries. In the future, *in vivo* digestion and fermentation characteristics of AMFPs need to be studied using animal models to lay the foundation for application of the AMFPs as a prebiotic.

CRediT authorship contribution statement

Qingxia Yuan: Conceptualization, Investigation, Writing – original draft, Formal analysis, Methodology, Funding acquisition. **Kunling Lv:** Investigation, Writing – original draft, Data curation. **Jinwen Huang:** Investigation. **Shujing Sun:** Investigation. **Ziyu Fang:** Investigation. **Hongjie Tan:** Investigation. **Hong Li:** Investigation. **Dan Chen:** Investigation. **Longyan Zhao:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition. **Chenghai Gao:** Conceptualization, Supervision, Funding acquisition. **Yonghong Liu:** Conceptualization, Supervision, Funding acquisition.

Declaration of Competing Interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2022.100475.

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