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Structural characterization and prebiotic potential of polysaccharides from *Polygonatum sibiricum*

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ABSTRACT

Polygonatum sibiricum has been widely used due to its excellent biological activities. We prepared a novel polysaccharide from *P. sibiricum* (PSP) in this study. According a monosaccharide composition analysis, PSP was mainly composed of fructose and glucose with a molar percentage of 93.81:5.12. The main linkage types were identified as *α*-*D*-Glc*p*-1→ and →2-*β*-*D*-Fru*f*-1→. The molecular weight of PSP showed no significant change after simulated salivary and gastrointestinal digestion. However, PSP could be broken down by intestinal bacteria. Our findings revealed that PSP administration increased the abundance of probiotics such as *Bifidobacterium*. Furthermore, the results showed that gut microbes could utilize PSP to produce short-chain fatty acids including acetic acid, propionic acid, and butyric acid. Also, the PSP fermentation broth displayed an excellent scavenging effect on free radicals, including 2,2-diphenyl-1-picrylhydrazyl radical, superoxide radical, and hydroxyl radical. In summary, this study will help to promote the application of PSP as prebiotics in functional food and the medical industry.

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1. Introduction

Prebiotics is a non-digestible component that regulates the composition or activity of gut microbiota through the metabolism of gut microorganisms, thereby producing beneficial physiological effects on the host[1]. In recent years, the prebiotic potential of plant polysaccharides has received widespread attention, mainly including inulin, oligofructose, arabinoxylan, and various dietary fibers[2]. Prebiotics can specifically promote the growth of gut probiotics such as *Lactobacillus* and *Bifidobacterium*[1]. These prebiotics from plant sources can be fermented by gut microbiota in the colon[3-4], where the bacteria break down the polysaccharides into oligosaccharides, short-chain fatty acids (SCFAs), and other products, contributing to host's health.

Polygonatum sibiricum, belonging to Liliaceae, grows in China, Inner Mongolia, and Siberia. As a plant with medicinal and edible uses, *P. sibiricum* has a wealth of food uses. For example, the sprouts and rhizomes of *P. sibiricum* are used as vegetables in fried dishes or salads. Moreover, the rhizomes of *P. sibiricum* are also used to make herbal wine or boiled as a substitute for tea in daily life. In some regions, people add *P. sibiricum* rhizomes to cakes or seasonings as additives[5]. Furthermore, *P. sibiricum* can stimulate saliva and gastric juice secretion, protect the respiratory system, promote appetite, and enhance immunity[6]. *P. sibiricum* is mainly composed of steroidal saponins[7], flavonoids[8], alkaloids[9], lignans[8], amino acids[10], and polysaccharides[6]. In particular, the principal ingredient, i.e., *P. sibiricum* polysaccharides (PSP), have been reported to have

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hypoglycemic[11], anticancer[12], antioxidant[13], anti-inflammatory[14], and anti-osteoporosis activities[15].

Usually, the chemical structure, molecular weight, and biological activity of polysaccharides will change significantly after digestion in the gastrointestinal tract[16-17]. For instance, the digestive enzymes and pH changes in the gastrointestinal tract may impact the physicochemical properties of polysaccharides after the intake[18]. Nevertheless, for those polysaccharides with prebiotic effects, they are indigestible in the upper digestive tract, such as *Gracilaria rubra* polysaccharides (GRPS)[19] and *Lycium barbarum* polysaccharides (LBPS)[4]. So far, it remains unknown whether the digestive enzymes secreted by the host can break down PSP in the intestine. Also, it is not clear whether PSP can directly enter the bloodstream as a prototype or indirectly initiate its pharmacological effect in a way of microbial metabolites. In a previous study, PSP could be fermented by bacterial monoculture strains, promoting the growth of *Bifidobacterium* and *Lactobacillus*[20]. However, the digestive characteristics of PSP in the gastrointestinal tract and its effect on the composition of gut microbiota in the host are still required to be investigated.

This study aims to investigate the changes in physicochemical properties of PSP during the *in vitro* saliva-gastrointestinal digestion and subsequent human fecal fermentation. Using a water extraction and alcoholic precipitation method, we prepared PSP and investigated it physicochemical properties. Next, we studied the dynamic characteristics of PSP in simulated saliva-gastrointestinal digestion. And the effect of PSP on the fermentation of human gut microbiota and the production of their metabolites (SCFAs) were explored using an *in vitro* fermentation model. Also, we examined the antioxidant activity of PSP fermentation products.

2. Material and methods

2.1 Preparation of PSP

P. sibiricum was provided and authenticated by Professor Yong Yang in Chongqing Academy of Chinese Materia (Chongqing, China). In brief, 100 g of *P. sibiricum* powder was dissolved in distilled water at a ratio of 1:10 (*m*/*V*). The solution was precipitated by adding absolute ethanol to get a final concentration of 75% (*V*/*V*) and then let stand overnight at 4 °C to remove lipids, pigments, and small-molecule materials. After the centrifugation, the precipitate was collected and re-dissolved in distilled water, followed by the addition of 4% papain. After the hydrolysis at 50 °C for 10 h, the papain was inactivated at 90 °C. Then, the supernatant was collected by centrifugation at 4 000 × *g* for 15 min and deproteinized using Sevag reagent. After the centrifugation, the supernatant was precipitated by adding absolute ethanol to get a final concentration of 75% (*V*/*V*) and kept overnight at 4 °C. Next, the mixture was centrifuged, and the precipitate was collected and re-dissolved in distilled water. The PSP extract was freeze-dried in a freeze dryer (SP Virtis BenchTop Pro, Pennsylvania, USA) and stored at –80 °C for subsequent experiments.

2.2 Physicochemical properties of PSP

2.2.1 Ingredient analysis

Total protein content was measured with a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, MA, USA). Total carbohydrate consumption was determined by the phenol sulfate method, and the standard curve was drawn using glucose as a standard[21]. Reducing sugar content was determined by the 3,5-dinitrosalicylic acid (DNS) method[22]. Fructose content was determined by the resorcinol method[23]. The detailed experimental procedure was provided in the supplementary methods.

2.2.2 Molecular weight determination

The average molecular weight of the digested and fermented polysaccharide samples was determined by high-performance gel permeation chromatography (HPGPC) system collected with a 2707 autosampler and 2424 evaporative light scattering (ELS) Detector (Waters, MA, USA). A TSKgel GMPWXL column (7.8 mm × 300 mm, 13 μm) was used. The separation procedure was performed using ultrapure water as the mobile phase at a flow rate of 0.6 mL/min. Dextrans with different polymerization degrees (M_w = 5, 10, 12.6, 40, 60, 150, and 500 kDa) were used to establish the standard curve. Data were processed using Empower software.

2.2.3 Monosaccharide composition analysis

Five milligrams of PSP were dissolved in 2 mol/L trifluoroacetic acid and hydrolyzed at 120 °C for 2 h. Next, a small amount of methanol was added and distilled at 45 °C under reduced pressure to remove the trifluoroacetic acid. Then, 0.3 mol/L NaOH and 0.5 mol/L 3-methyl-1-phenyl-2-pyrazolin-5-one (PMP) were added, and the reaction was carried out at 70 °C for 30 min. After that, a hydrochloric acid solution was added to neutralize the reaction. The aqueous layer solution was extracted with chloroform, collected, and filtered through a 0.22 μm filter. The samples were used for monosaccharide composition analysis by high performance liquid chromatography (HPLC), and the detailed procedure was provided in supplementary methods.

2.2.4 Methylation analysis by gas chromatography-mass spectrometry (GC-MS)

In brief, 5 mg of PSP was dissolved in 2 mL DMSO. Subsequently, 50 mg of NaH was added, and the reaction was carried out at room temperature for 1 h under nitrogen protection. After that, 1 mL of CH_3I was added, and the reaction was performed at room temperature for 2 h. Then, the above reaction product was extracted with chloroform, and anhydrous Na_2SO_4 was added to the chloroform layer. Next, the product was hydrolyzed with 2 mol/L trifluoroacetic acid at 120 °C for 1.5 h, followed by the reduction with $NaBD_4$ and acetylation with acetic anhydride. The methylated PSP samples were analyzed by GC-MS with details in supplementary methods.

2.2.5 Fourier transform infrared spectrometer (FT-IR) analysis

For FT-IR analysis, 1 mg of PSP was ground with potassium bromide powder and pressed into 1 mm pellets. FT-IR spectra of 4 000–400 cm^{-1} were obtained on a iS50R FT-IR spectrometer (Thermo Nicolet, MA, USA).

2.2.6 Nuclear magnetic resonance (NMR) spectroscopy assay

The NMR analysis of PSP was performed using an AV-600 spectrometer (Bruker, Rheinstetten, Germany). Briefly, 50 mg of PSP was dissolved in D_2O and lyophilized to exchange ddH_2O. Then, PSP was redissolved in 0.6 mL of D_2O for NMR detection, and 1D and 2D NMR spectra were obtained. The 1D-NMR included ^1H NMR (600 MHz) and ^{13}C NMR (150 MHz), and the 2D NMR included ^1H-^1H correlation spectroscopy (COSY), heteronuclear single quantum correlation (HSQC), heteronuclear multiple bond coherence (HMBC), nuclear overhauser effect spectroscopy (NOESY), and total correlation spectroscopy (TOCSY). Data were analyzed by MestReNova 14.2.0 software.

2.3 Thin layer chromatography (TLC) analysis

TLC analysis of PSP was conducted on TLC Silica gel 60 F_{254} plates (Merck, Germany) with *n*-butanol-isopropanol-acetic acid-water (7:5:2:2, *V/V*) as the developing solvent. The spots on the TLC were detected by soaking 10% (*V/V*) H_2SO_4 in ethanol and heating at 120 °C for 5 min.

2.4 In vitro simulated digestion of PSP

The detailed experimental procedures for simulated digestion of saliva, gastric juice, and intestinal fluid were provided in supplementary methods.

2.5 In vitro fermentation of PSP with human fecal microbiota

2.5.1 Preparation of fecal suspension

The fresh fecal samples were obtained from 3 healthy volunteers who were free of digestive system diseases and received no antibiotic treatment within 3 months. The same mass of fresh feces from each volunteer was mixed, vortexed with sterile phosphate buffered saline (PBS) for 1–5 min in an ultra-clean table, and centrifuged at 500 × *g* for 5 min. Next, the supernatant was collected and transferred to a 50 mL sterile tube to obtain 20% fecal suspension.

2.5.2 Preparation of fermentation medium

The fermentation medium was prepared according to the reported method with some modifications[24]. In brief, 1 L fermentation medium consisted of 2 g peptone, 2 g yeast extract, 0.02 g hemin, 0.5 g *L*-cysteine, 0.5 g bile salts, 0.1 g NaCl, 0.01 g KH_2PO_4, 0.04 g K_2HPO_4, and 0.01 g $CaCl_2{\cdot}2H_2O$. The above ingredients were dissolved in ultrapure water and filtered through a 0.22 μm membrane for sterilization.

2.5.3 In vitro fermentation

For the *in vitro* fermentation, 6 mL of 20% fecal suspension was inoculated into a 54 mL medium with or without PSP (2.5 mg/mL). All tubes were incubated in a shaker (37 °C, 125 r/min) under an anaerobic environment (10% CO_2, 10% H_2, and 80% N_2). After the fermentation for 0, 3, 6, 12, 24, 36, and 48 h, 2 mL of medium were collected and placed on ice for 10 min to terminate the microorganism metabolism. After the fermentation, the medium was centrifuged at 13 000 × *g* for 3 min to remove the bacteria. Finally, the supernatant was stored at –80 °C for further analysis.

To determine the growth curve, we collected 200 μL fermentation broth from each experimental group at different time points and added it to a 96-well plate. The absorbance values at 600 nm were measured using a microplate reader. In addition, 2 mL fermentation broth was collected and centrifuged to detect the pH value using a pH meter. The contents of total carbohydrates, reducing sugars, and proteins in fermentation broth were assayed as those mentioned above. The molecular weight of PSP was determined by HPGPC and TLC analysis.

2.5.4 Determination of SCFAs by GC-MS

The SCFAs of fermentation broth were quantified by GC-MS with a DB-WAX column (30 m × 0.32 mm, 0.5 μm) (Agilent, CA, USA). The initial column temperature was set as follows: insulation at 50 °C for 1 min, followed by a temperature rise of 10 °C/min to 250 °C; insulation for 2 min with high purity helium (purity > 99.999%) as carrier gas (1.0 mL/min). The electron energy was 70 eV. The ion source and interface temperature were set to 230 and 250 °C, respectively. The solvent delay time was set to 6 min. The concentration of individual SCFAs was determined by constructing the calibration curves of acetic acid, propionic acid, butyric acid, and pentanoic acid standards.

2.6 Analysis of gut microbiota by 16S rDNA sequencing

Bacterial genomic DNA was extracted from each group after the fermentation for 48 h using a Fast DNA™ SPIN Kit (MP Biomedicals, CA, USA). Barcoded conventional primers (338F: 5′-ACTCCTACGGGAGGCAGCAG-3′; 806R: 5′-GACTACHVGGGTWTCTAAT-3′) were applied to amplify V3–V4 hypervariable regions of bacterial 16S rDNA gene. The PCR products were purified and sequenced on an MiSeq platform (Illumina, CA, USA). Sequences with a similarity above 97% were classified as operational taxonomic units (OTUs) using UCLUST (Version 1.2.22, http://www.drive5.com/uclust/downloads 1_2_22q.html). The sequence classification of each 16S rDNA gene was analyzed by UCLUST against the Greengene 13.5 16S rDNA database with a minimum threshold of 90% confidence. The α-diversity and β-diversity were calculated by Quantitative Insights Into Microbial Ecology (QIIME) based on sequencing reads and OTUs. In addition, we performed linear discriminant analysis effect size (LEfSe) to determine the differences in the dominant microbial taxa.

2.7 Antioxidant activity of PSP fermentation broth

The scavenging capacity of PSP fermentation broth against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, superoxide radicals, and hydroxyl radicals was measured as detailed in supplementary methods.

2.8 Statistical analysis

Data were expressed as means ± standard deviation (SD). Statistical analysis was carried out using GraphPad Prism 8.0 and IBM SPSS Statistics 25. One-way analysis of variance (ANOVA) followed by a Tukey's test was used to assess the difference among groups. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1 Physicochemical properties of PSP

We analyzed the physicochemical properties of PSP and the result showed that PSP was rich in total carbohydrates with low contents of reducing sugar, protein, and uronic acid (Table 1). The chromatogram of PSP appeared as a single symmetric peak (Fig. 1A), indicating that it is homogeneous based on the molecular weight distribution. The molecular weight of PSP was determined to be 3.06 kDa by HPGPC. Since the PMP derivatization method caused destruction to fructose structure, the peak of fructose did not show up in the HPLC chromatogram of PSP (Figs. 1B and C). Using a resorcinol method, the fructose content in PSP was detected to be 93.81%. And the remaining monosaccharide composition of PSP was displayed in Fig. 1C, containing glucose (5.12%), mannose (0.15%), galactose (0.50%), arabinose (0.21%), and galacturonic acid (0.21%) (Table 1). These data suggest that PSP was a heteropolysaccharide with fructose as the main monosaccharide.

Table 1
Physicochemical properties of PSP.

Index	Value
Main components (*m*/*m*)	
Total carbohydrate (%)	91.04 ± 6.76
Reducing sugar (%)	1.38 ± 0.05
Protein (%)	6.14 ± 0.36
Uronic acid (%)	2.81 ± 0.15
Monosaccharide molar ratio	
Fructose (%)	93.81
Glucose (%)	5.12
Mannose (%)	0.15
Galactose (%)	0.50
Arabinose (%)	0.21
Galacturonic acid (%)	0.21
M_w (kDa)	3.06

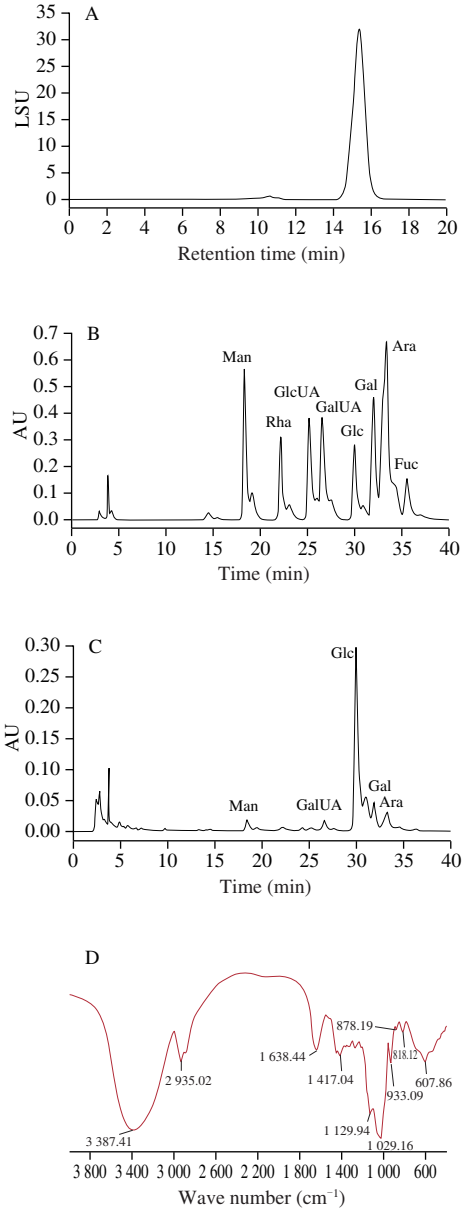


Fig. 1 Chromatogram of PSP by high-performance gel-permeation chromatography (HPGPC) and its monosaccharide composition using a PMP derivatization method. (A) HPGPC chromatogram of PSP. Chromatogram of PMP-derivatized (B) monosaccharide standard mixtures and (C) PSP by HPLC. Man, mannose; Rha, rhamnose; GlcUA, glucuronic acid; GalUA, galacturonic acid; Glc, glucose; Gal, galactose; Ara, arabinose, Fuc, fucose. (D) Fourier-transform infrared spectroscopy (FT-IR) spectra of PSP.

3.2 FT-IR spectrum analysis of PSP

Based on the FT-IR spectrum of PSP in Fig. 1D, the intense absorption peak at 3 387.41 cm^{-1} was mainly due to the stretching vibration of O–H, and 2 935.02 cm^{-1} was the absorption peak of C–H, both of which were the characteristic absorption peaks of polysaccharides. The absorption peaks at 1 638.44, 1 417.04, 1 129.94, and 1 029.16 cm^{-1} were separately attributed to the C=O, COOH, C–OH, C–O–C stretching vibrations, suggesting the presence of uronic acid and furanose ring (Fig. 1D). The absorption peak at 878.19 cm^{-1} was assigned to an α-type glycosidic bond. In addition, the peaks at 933.09 and 818.12 cm^{-1} were attributed to furanose rings with β-type glycosidic bonds (Fig. 1D).

3.3 Sugar residue of PSP by methylation analysis

To determine the type of glycosidic bonds in PSP, we performed the methylation analysis on partially methylated alditol acetates (PMAAs) of PSP by GC-MS. The result showed that PSP derivatives contained two PMAAs (Fig. S1A). The peak 1 in the total ion chromatogram (TIC) of PSP was identified to be terminal-*D*-glucopyranosyl (Fig. S1B and Table S1). The glycosidic bond type of fructose is unclear in the methylation analysis and will be deduced in conjunction with NMR analysis.

3.4 NMR analysis of PSP

The 1D and 2D NMR spectra were used to analyze the structure of PSP. The ^{1}H NMR spectrum of PSP was shown in Fig. 2A. As the ketose had no anomeric protons, only a single signal at δ 5.43 appeared in the anomeric proton region, which was assigned to H-1 of α-*D*-glucopyranose residues. Most of the proton signals were concentrated at δ 3.4–4.4. Our result indicates that PSP contained both α-configuration (δ > 5.0) and β-configuration (δ < 5.0), and the β-configuration was predominant, in agreement with the FT-IR analysis (Fig. 1D). In the ^{13}C NMR spectrum (Fig. 2B), most carbon signals were obtained from the region of δ 60–110. The signal observed in the anomeric carbon region at δ 103.6 was assigned to the typical peak of fructose residue (C-2). Hydrogen signals of fructose residues were assigned according to the ^{1}H-^{1}H COSY and HSQC spectra (Figs. 2C and D). The carbon signals at δ 60.43, 76.72, 74.33, 81.06, and 62.24 in the ^{13}C NMR spectrum (Fig. 2B) corresponded to C-1, C-3, C-4, C-5 and C-6 of the fructose residues, respectively. Additionally, the carbon signal of glucose residues was weak and not identified in the ^{13}C NMR spectrum, consistent with the low glucose content in PSP (Table 1). Finally, the ^{1}H and ^{13}C chemical shift signals of sugar residues were summarized in Table 2.

Table 2
^{1}H and ^{13}C NMR chemical shift of PSP in D_2O.

Glycosidic bond	Atom	Chemical shift δ					
		1	2	3	4	5	6
α-*D*-Glc*p*-1→	H	5.43	3.57	3.76			
	C	92.00	71.16	72.47			
→2-β-*D*-Fru*f*-1→	H	3.66/3.95		4.24	4.11	3.87	3.71
	C	60.43	103.66	76.73	74.33	81.06	62.24

The sequences and linkage sites of sugar residues in PSP were determined by the correlated peaks in the HMBC spectrum (Fig. 2E). The hydrogen signal H-1 (δ 3.66, 3.95) and the carbon signal C-2 (δ 103.66) of fructosyl residues had related signal peaks, indicating →2)-β-*D*-Fru*f*-(1→2)-β-*D*-Fru*f*-(1→ link existed in PSP. In comparison with methylation analysis and reported NMR spectra of glucose residues[25-26], the glucose residues were presumed to be bonded as α-*D*-Glc*p*-(1→2)-β-*D*-Fru*f*-(1→. Comprehensive analysis of monosaccharide composition, FT-IR, and NMR information analysis, we proposed the predicted structure of PSP, which was shown in Figs. 2F and S2.

3.5 Simulated in vitro digestion of PSP

As the first digestion site after food intake, the oral cavity secretes α-amylase that catalyzes the hydrolysis of α-1,4 glycosidic bonds[27]. To see whether saliva could break down PSP, we incubated PSP with a simulated saliva solution for 0−30 min. As shown in Fig. 3A, the reducing sugar contents in the digestion solution remained at 0.31 mg/mL during the whole digestion process. In parallel, HPGPC profiles of PSP were not changed after the digestion for different times (Fig. 3B). So did the TLC results, which showed that no new product was generated from the saliva digestion of PSP (Fig. 3C). Overall, we concluded that saliva failed to digest PSP, implying that the glycosidic bonds in PSP could not be hydrolyzed by α-amylase.

After initial digestion in the mouth, the food will be transported through the esophagus to the stomach for subsequent digestion. Thus, we incubated the PSP solution from saliva digestion with simulated gastric juice. As shown in Figs. 3D−F, the reducing sugar contents, HPGPC chromatograms of PSP, and TLC results were not changed during the whole digestion process. Taken together, PSP was not broken down in the gastric juice with an acidic condition (pH 3.0).

The small intestine is the leading site for nutrient digestion and absorption, so we investigated whether small intestine fluid could break down PSP. As shown in Figs. 3G and H, after the PSP solution from gastric juice digestion was incubated with simulated small intestinal digestion solution for different times, no significant changes were observed in the reducing sugar contents and HPGPC chromatograms of PSP. Noticeably, the TLC results demonstrated that a small number of monosaccharides were produced after the digestion of PSP by small intestinal fluid (Fig. 3I).

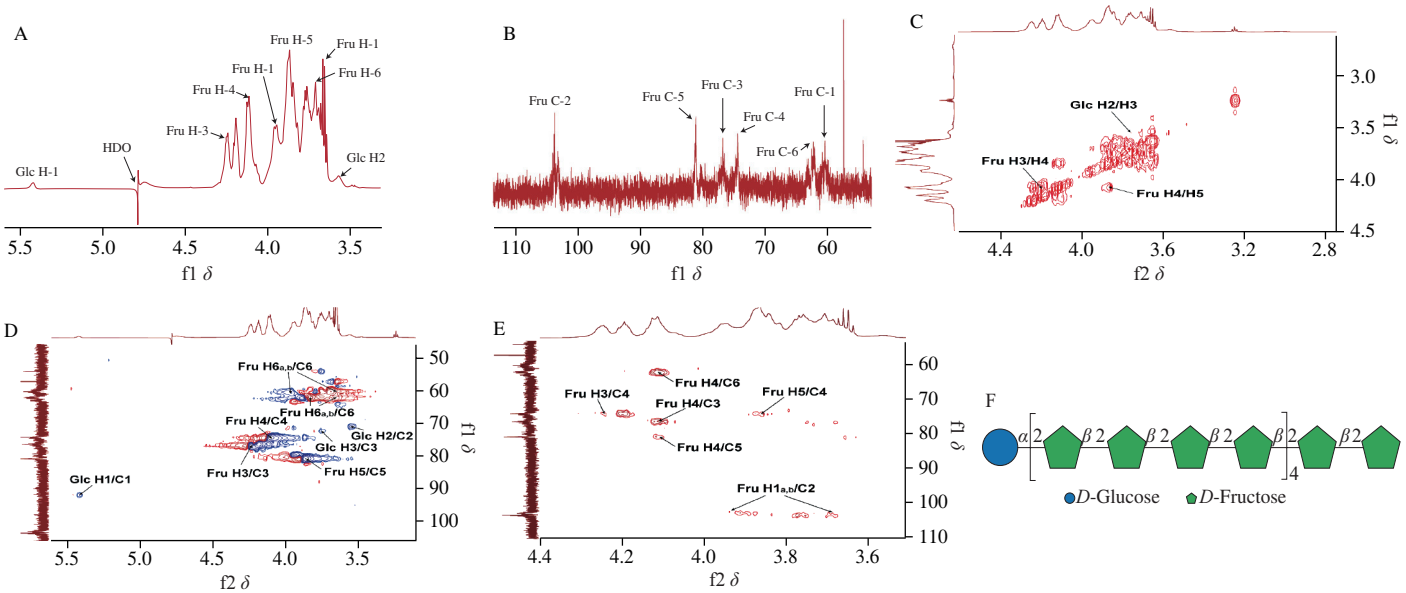


Fig. 2 Structural characterization of PSP by NMR spectroscopy. (A) ^{1}H NMR spectrum. (B) ^{13}C NMR spectrum. (C) ^{1}H-^{1}H correlation spectroscopy (COSY) spectrum. (D) Heteronuclear single quantum correlation (HSQC) spectrum. (E) Heteronuclear multiple bond correlation (HMBC) spectrum. (F) Proposed structure of PSP.

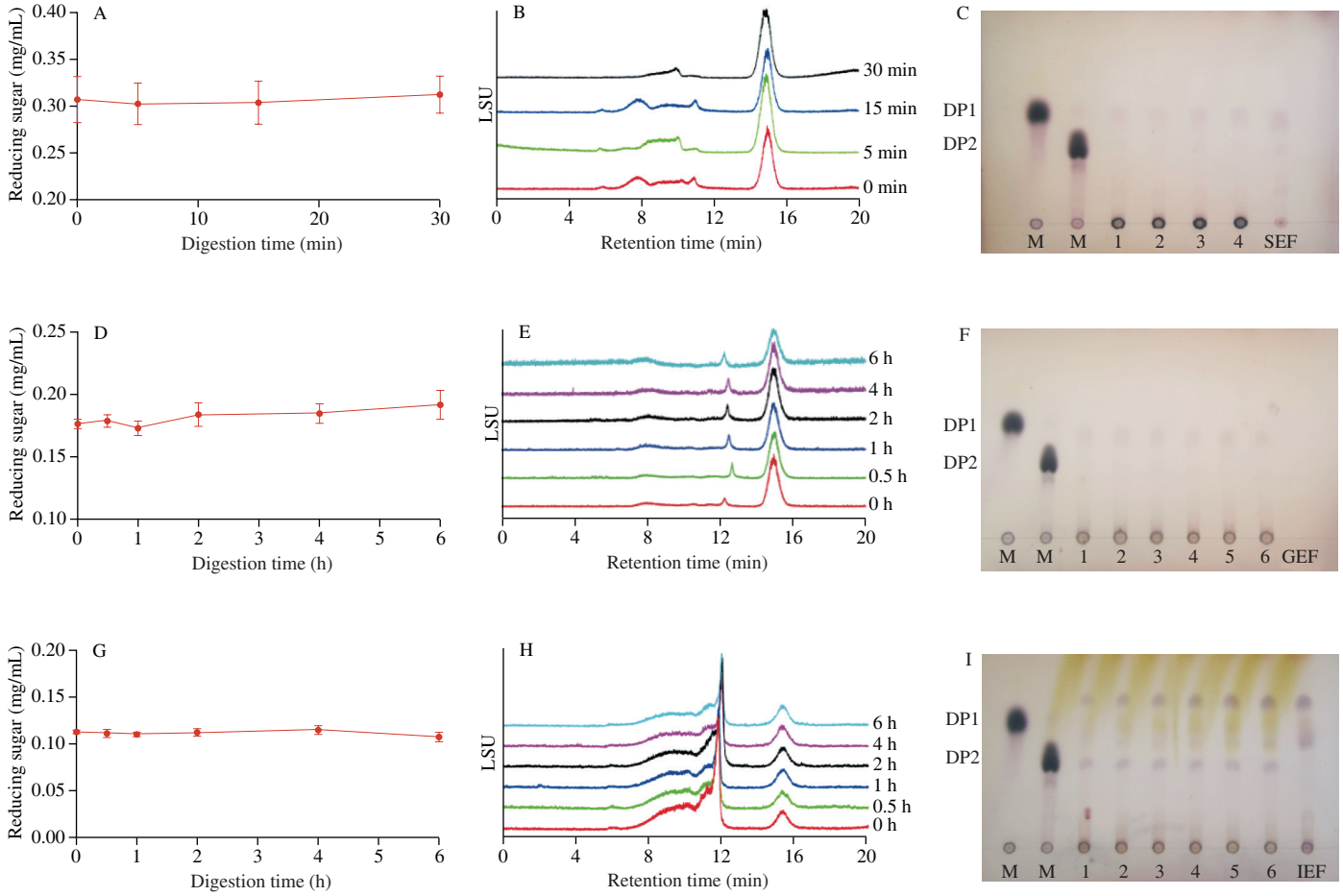


Fig. 3 Dynamic changes of reducing sugar content of PSP after simulated *in vitro* digestion in saliva, gastric juice, and small intestinal fluid. (A−C) Analysis of reducing sugar contents, HPGPC chromatograms, and TLC results after the simulated salicary digestion of PSP. (D−F) Analysis of reducing sugar contents, HPGPC chromatograms, and TLC results after the simulated gastric juice digestion of PSP. (G−I) Analysis of reducing sugar contents, HPGPC chromatograms , and TLC results after the simulated small intestinal fluid digestion of PSP. SEF, salivary electrolyte fluid. GEF, gastric electrolyte fluid. IEF, intestinal electrolyte fluid. DP1, glucose standard. DP2, maltose standard. Lane M, monosaccharide standard. Lane 1–4 in Fig. 3C, PSP samples after the digestion in simulated saliva for 0, 5, 15, and 30 min, respectively. Lane 1–6 in Fig. 3F, PSP samples after the digestion in simulated gastric juice for 0, 0.5, 1, 2, 4, and 6 h, respectively. Lane 1–6 in Fig. 3I, PSP samples after the digestion in simulated small intestinal fluid for 0, 0.5, 1, 2, 4, and 6 h, respectively. Data were presented as mean ± SD (n = 3).

3.6 In vitro fermentation of PSP

Considering that the gut microbiota can metabolize dietary polysaccharides through glycoside hydrolases[3,19], we next investigated the fermentation of PSP by human gut microbiota.

3.6.1 Analysis of bacterial growth curve, pH value, and protein content of PSP fermentation broth

We firstly examined the growth curve of gut microbiota in the fermentation broth. As indicated in Fig. 4A, the absorbance value ($OD_{600\ nm}$) of the negative control (NC) group rose rapidly at 3 h. Then, the bacteria reached a growth plateau at 36 h, and the absorbance value increased to 0.25. Compared to the NC group, the $OD_{600\ nm}$ of the PSP group got to 0.49 at 36 h and continued to rise to 0.51 at 48 h (Fig. 4A). The $OD_{600\ nm}$ of fermentation broth in the PSP group was consistently higher than the NC group from 6 h of fermentation.

Bacteria produce acidic metabolites (like SCFAs and lactic acid) in metabolizing polysaccharides, leading to the reduced intestinal pH and altered gut microbiota structure[28]. The result in Fig. 4B indicated that the pH value of the NC group decreased to 6.7 after the fermentation for 6 h and then remained stable during the subsequent fermentation. Comparatively, there was a significant reduction in pH in the PSP group during the fermentation, dropping to 4.4 at 48 h (Fig. 4B).

Additionally, we examined the total protein content in the fermentation broth, which displayed a decreasing trend during the fermentation in either the NC or PSP group (Fig. 4C). After the fermentation for 48 h, the protein content was 24.9% in the PSP group and 11.7% in the NC group.

3.6.2 Dynamic changes of total carbohydrates, reducing sugars and molecular weight

Further, we tested the dynamic changes of total carbohydrates in the fermentation broth. For the PSP group, the total carbohydrates decreased rapidly after the fermentation for 3–24 h and eventually reduced to 0.45 mg/mL at 48 h (Fig. 5A). Meanwhile, 84.9% of total carbohydrates were utilized by gut microbiota during the fermentation.

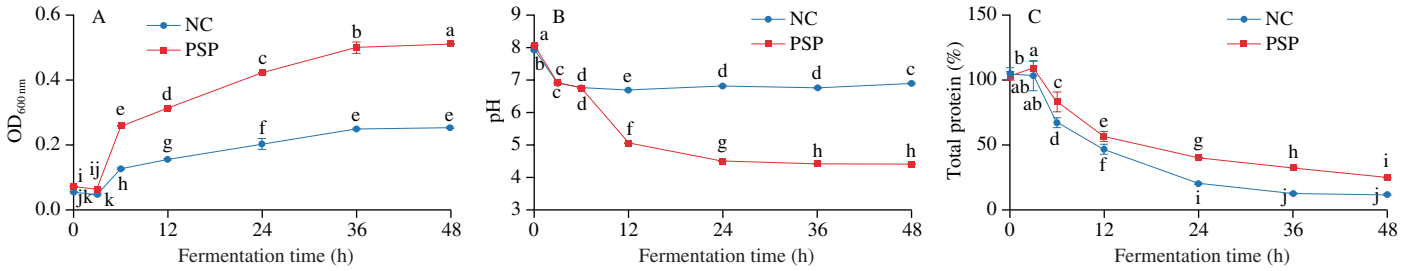


Fig. 4 Changes of bacterial growth curve, pH value, and protein content after human gut microbiota fermentation for 0−48 h. PSP was incubated with human gut microbiota for 48 h, and the fermentation broth was collected at different time points (0, 3, 6, 12, 24, 36, 48 h) for the analysis of (A) bacterial growth curve, (B) pH value, and (C) total proteins content. NC, negative control group; PSP, *P. sibiricum* polysaccharides group. Data were presented as mean ± SD (n = 3). Mean values with different letters are significantly different ($P < 0.05$).

Since glycosidases from gut microbiota can break the glycosidic bonds of polysaccharides and thus increase the production of reducing sugars[29], we compared the difference in reducing sugar contents of fermentation broth between the two experimental groups. As shown in Fig. 5B, there was no significant change in the sugar content of the NC group throughout the fermentation. In contrast, the reducing sugar content of the PSP group increased to 0.14 mg/mL at 48 h (Fig. 5B).

Due to the complexity of fermentation broth, the peak of the NC group occurred at 8–12 min, while the PSP peak was 15.3 min (Fig. 5C). Also, we analyzed the PSP in fermentation broth at different time points by HPGPC (Fig. 5D). It was found that the peak area of PSP gradually decreased with the increase of fermentation time. After the fermentation for 24 h, the HPGPC profiles suggest that the residues of PSP were extremely low, implying the decomposition of PSP by gut microbiota. Moreover, the TLC result confirmed that the total carbohydrates in the fermentation broth were significantly decreased during the fermentation (Fig. 5E).

3.7 *Effect of PSP on gut microbiota composition*

To evaluate the prebiotic potential of PSP, we assessed the effect of PSP on gut microbiota *in vitro* by 16S rDNA sequencing. By α-diversity analysis using Chao 1 and PD whole tree index, PSP enhanced the diversity of the gut microbial community (Figs. 6A and B). By β-diversity evaluation using principal component analysis (PCA) and nonmetric multidimensional scaling (NMDS), the gut microbiota samples were divided into 3 distinct taxa, indicating a good differentiation of gut microbiota among experimental groups (Figs. 6C and D).

To investigate the regulatory effect of PSP on specific gut bacteria, we analyzed the relative abundance changes of gut microbiota among experimental groups. At the phylum level, the gut microbiota of each group mainly consisted of Firmicutes, Bacteroidetes, and Proteobacteria, accounting for about 95% of the total bacterial community (Fig. 7A). And PSP significantly increased the proportions of Proteobacteria and Actinobacteria compared to the NC group ($P < 0.05$) (Fig. 7A). At the family level, PSP increased the abundances of Veillonellaceae, Enterobacteriaceae, and

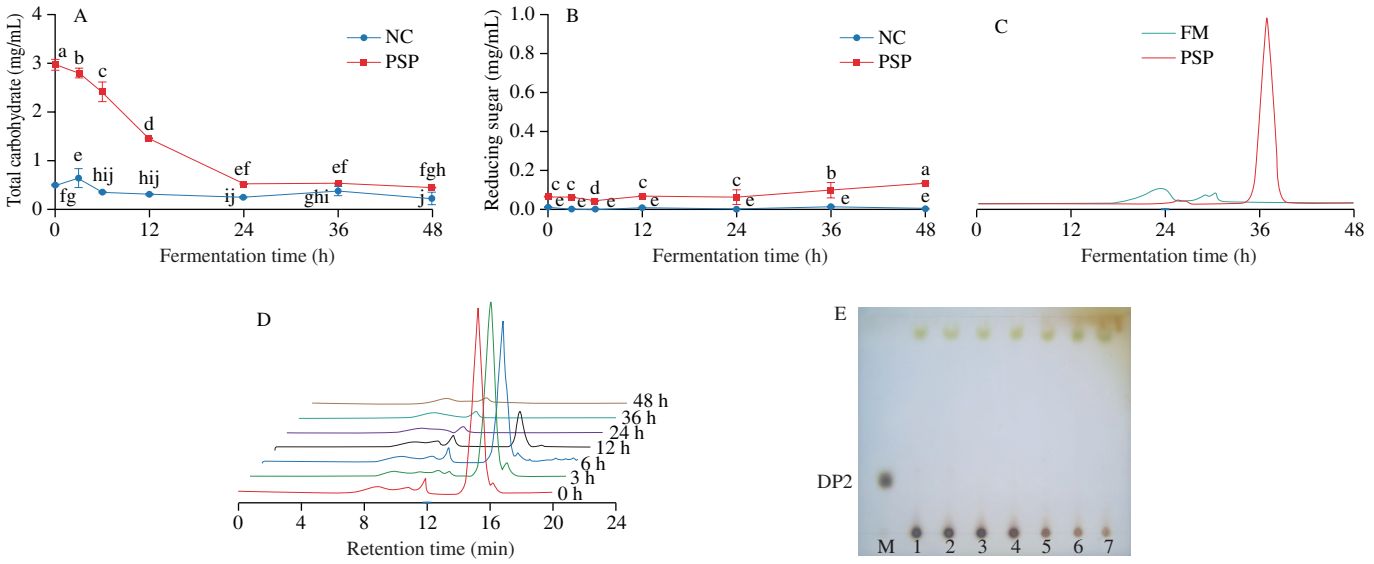


Fig. 5 Dynamic changes of total carbohydrates, reducing sugars, and molecular weight of PSP after *in vitro* fermentation for 0−48 h. PSP was incubated with human gut microbiota for 48 h, and the fermentation broth was collected at different time points (0, 3, 6, 12, 24, 36, 48 h) for the analysis of (A) total carbohydrates, (B) reducing sugars, (C) peak time of PSP, (D) peak area change, and (E) TLC result. DP2, maltose; Lane M, oligosaccharide standard; Lane 1−7 in Fig. 5E, PSP samples after the fermentation for different times. NC, negative control group; PSP, *P. sibiricum* polysaccharides group. Data were presented as mean ± SD (n = 3). Mean values with different letters are significantly different ($P < 0.05$).

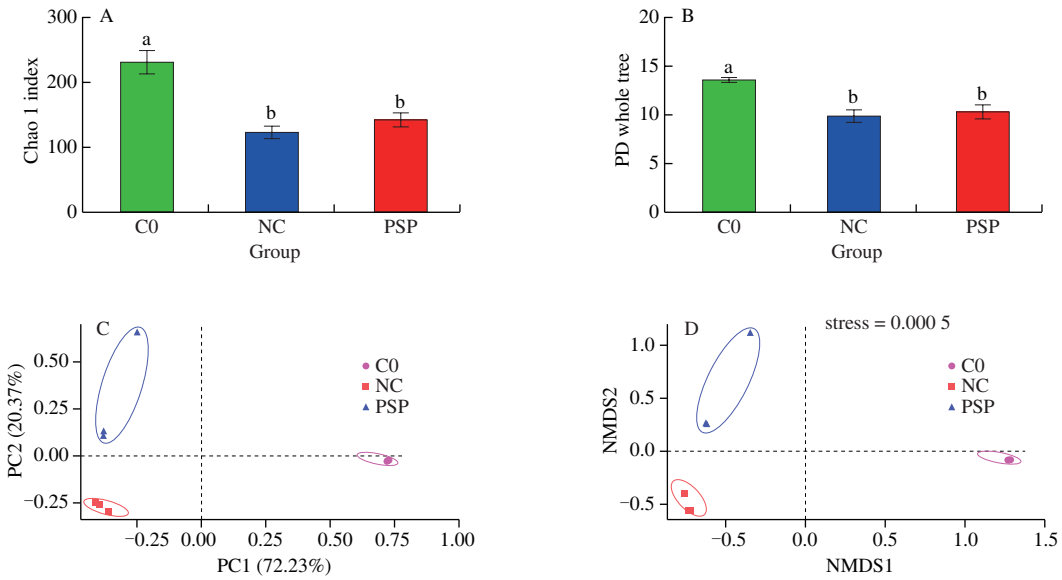


Fig. 6 Effect of PSP on diversities of human gut microbiota after *in vitro* fermentation for 48 h. (A−B) α-Diversity analysis of gut microbiota using Chao 1 index and PD whole tree. (C−D) β-Diversity analysis of gut microbiota by principal component analysis (PCA) and nonmetric multidimensional scaling (NMDS). Data were presented as mean ± SD (n = 3). C0, fecal fermentation group at 0 h; NC, i.e., control group, fecal fermentation without PSP for 48 h; PSP, i.e., PSP group, fecal fermentation with PSP for 48 h. Mean values with different letters are significantly different ($P < 0.05$).

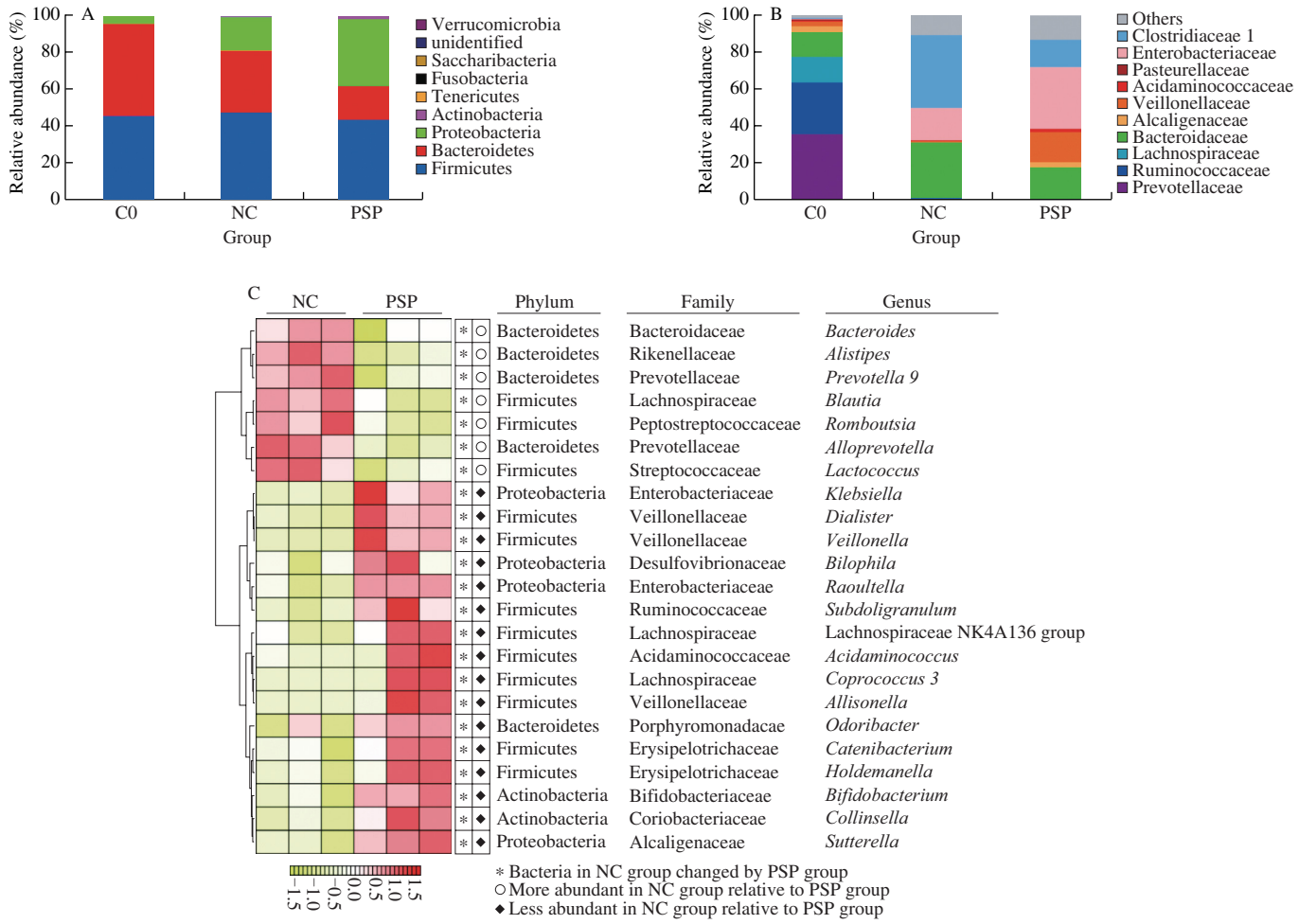


Fig. 7 Effect of PSP on abundances of human gut microbiota at phylum, family, and genus levels after fermentation for 48 h. (A) Microbial community changes at the phylum level using a bar plot. (B) Microbial community changes at the family level using a bar plot. (C) Abundance changes of representative bacteria at the genus level using a heatmap, in which the relative abundance of bacteria was proportional to the color alteration. C0, fecal fermentation at 0 h; NC, i.e., negative control group, fecal fermentation without PSP for 48 h; PSP, i.e., PSP group, fecal fermentation with PSP for 48 h.

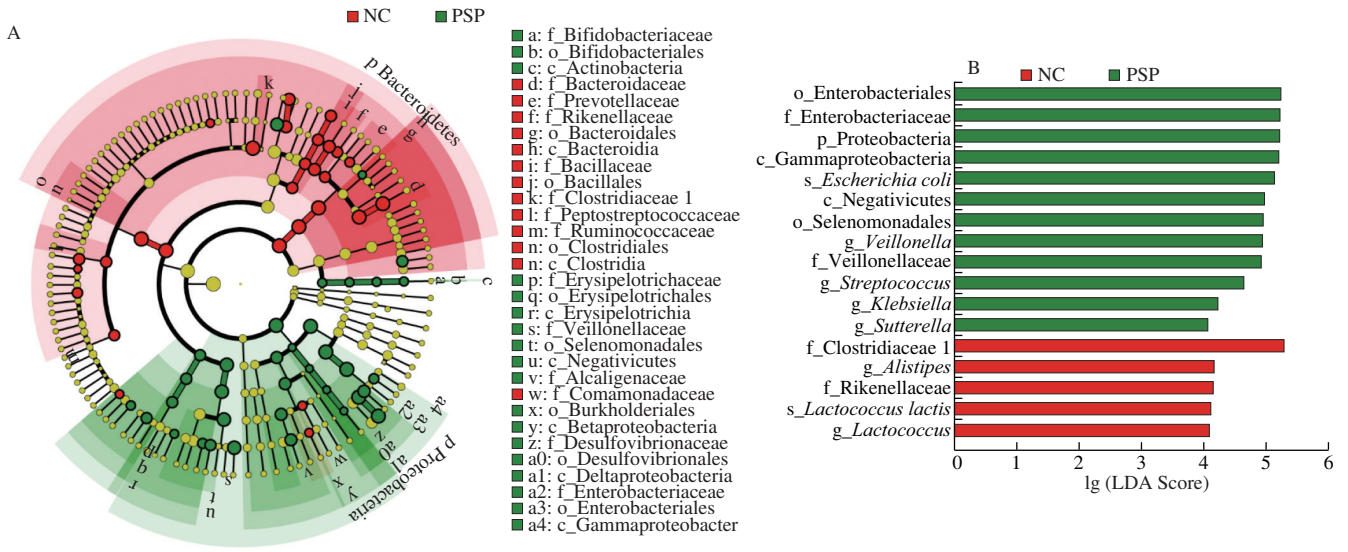


Fig. 8 Identification of most characteristic taxa between NC and PSP groups by linear discriminant analysis (LDA) effect size (LEfSe) assay. (A) Taxonomic abundance analysis on differentially enriched taxa using LEfSe. The circle size varies with the relative abundance of each taxon. (B) LDA scores above 4.0 indicated the most significant differences in bacteria taxa. The bar length of LDA represents the impact of significantly different species in each group.

Alcaligenaceae ($P < 0.05$, vs. NC group). In contrast, PSP deceased the abundance of Bacteroidaceae and Clostridiaceae ($P < 0.05$, vs. NC group) (Fig. 7B).

Next, we compared the relative abundance difference in intestinal flora between the NC and PSP groups at the genus level. As indicated in Fig. 7C, the 23 most variable bacterial genera were analyzed by hierarchical clustering heat map. Among them, several bacteria were increased in abundance by PSP (like *Sutterella* and *Bifidobacterium*), whereas some others were decreased (like *Bacteroides* and *Alistipes*) ($P < 0.05$, vs. NC group). After the fermentation for 48 h, the core microorganisms in the NC group were *Bacteroides* (29.7%), *Alistipes* (3.15%), and *Lactococcus* (3.09%) (Fig. S3). Besides, *Bacteroides* (17.2%), *Veillonella* (6.98%), *Klebsiella* (2.35%), and *Sutterella* (3.21%) dominated the PSP group (Fig. S3).

The LEfSe method was used to analyze the specific bacterial taxa in each experimental group. As shown in Fig. 8A, the Proteobacteria branch was the main bacteria in the PSP, and the characteristic bacteria in the NC group was Bacteroidetes, which plays an essential role in the fermentation of carbohydrate[30-31]. The LDA score histogram was plotted to analyze the dominant bacteria in both groups. As indicated in Fig. 8B, f_Clostridiaceae 1, g_*Alistipes*, f_Rikenellaceae, and g_*Lactococcus* were the predominant bacteria in the control group. Meanwhile, there were 12 dominant bacteria in the PSP group, such as o_Enterobacteriales, p_Proteobacteria, and c_Gammaproteobacteria.

3.8 *Effect of PSP on production of SCFAs and indole*

Since SCFAs and indole are principal bacterial metabolites after the polysaccharides intake, we detected the contents of both metabolites between the NC and PSP groups after the fermentation for 48 h. As shown in Fig. 9A, the levels of total SCFAs in both groups were increased during the fermentation process. The concentration of total SCFAs in the PSP group rose from (3.32 ± 0.14) μg/mL at 0 h to (98.21 ± 5.51) μg/mL at 48 h, much higher than that of the NC group ($P < 0.01$). The result indicates that PSP promoted the SCFAs production of gut microbiota.

Specifically, the concentration of acetic acid in the NC group was (12.24 ± 0.58) μg/mL at 36 h, while it was (56.22 ± 4.53) μg/mL in the PSP group (Fig. 9B). During the fermentation from 3 to 48 h, the level of acetic acid in the PSP group was remarkably higher than that in the NC group ($P < 0.01$) (Fig. 9B). So did the propionic acid, the level of which in the PSP group reached (34.36 ± 4.92) μg/mL at 36 h (Fig. 9C). Unlike the above two SCFAs, butyric acid and pentanoic acid were mainly produced in the late stage of gut microbiota fermentation (Figs. 9D and E). After the fermentation for 48 h, the contents of butyric acid and pentanoic acid in the PSP group were separately determined to be (23.82 ± 0.67) and (3.58 ± 0.35) μg/mL, significantly higher than those in the NC group (Figs. 9D and E). Additionally, we detected the content of indole by GC-MS. The result showed that no difference in indole concentration was observed between the NC and PSP groups at the initial fermentation stage (0−6 h). However, from 12 h after the fermentation, the indole level in the NC group was higher than that in the PSP group ($P < 0.01$) (Fig. 9F).

3.9 *Antioxidant activity of PSP fermentation broth*

Finally, we examined the scavenging effect of PSP fermentation broth on free radicals. Compared to the NC group, the fermentation with PSP enhanced the scavenging ability of gut microbiota against DPPH radical (Fig. 10A), and the maximum clearing capacity was up to 70.59% at 12 h. On the other hand, the superoxide radical scavenging rate of the PSP group was gradually increased and reached a peak at 24 h (26.76%) (Fig. 10B). In general, the PSP group had a much higher clearing capacity of superoxide radical than the NC group. It was interesting that the clearing capacity of the NC group against superoxide radical displayed a reduced trend during the fermentation process (Fig. 10B). Besides, the PSP group exhibited a comparatively better scavenging effect on hydroxyl radical than the NC group (Fig. 10C).

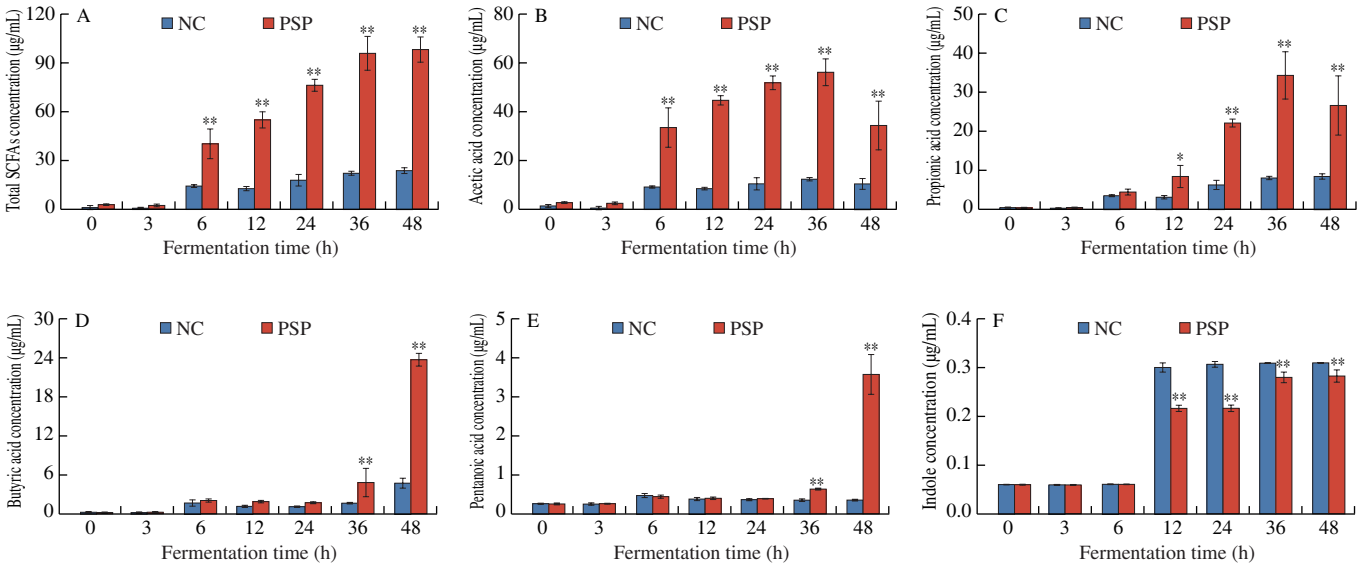


Fig. 9 Regulatory effect of PSP on production of SCFAs and indole during gut microbiota fermentation for 0−48 h. After the fermentation for different times (0, 3, 6, 12, 24, 36, 48 h), the broth was collected for the detection of bacterial metabolites, including (A) total SCFAs, (B) acetic acid, (C) propionic acid, (D) butyric acid, (E) pentanoic acid, and (F) indole. Data were presented as mean ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$, vs. NC group. NC, negative control group; PSP, *P. sibiricum* polysaccharides group.

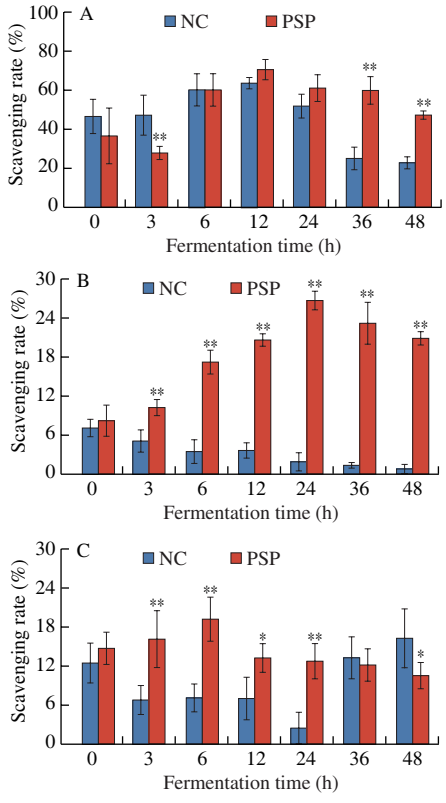


Fig. 10 Antioxidant activity of PSP fermentation broth. (A) DPPH radical scavenging activity. (B) Superoxide radical scavenging activity. (C) Hydroxyl radical scavenging activity. Data were presented as mean ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$, vs. NC group. NC, negative control group; PSP, *P. sibiricum* polysaccharides group.

4. Discussion

In this study, we prepared PSP from *P. sibiricum* by hot water extraction and alcoholic precipitation. The molecular weight of PSP was calculated to be 3.06 kDa, mainly composed of fructose and glucose (93.81:5.12, molar percentage). The data of structural analysis shows that PSP was a fructan linked with *α-D*-Glc*p*-1→ and →2-*β-D*-Fru*f*-1→. Xie et al.[32] obtained a neutral polysaccharide from *Polygonatum cyrtonema* Hua by hot water extraction at 80 °C. The molecular weight of PSP was 8.5 kDa and consisted of fructose and glucose (28:1). The monosaccharide type of PSP in our study was similar to the PSP prepared by Xie et al.[32]. Still, there were differences in the ratio of monosaccharide composition and glycosidic bond type, which may be caused by the different species of *P. sibiricum*. Li et al.[33] used the combination of hot water extraction and NaOH extraction to obtain an arabinogalactan from *P. sibiricum*, the monosaccharide composition was galactose, arabinose, and rhamnose (14:4:1), with a molecular weight of 141 kDa. Therefore, different extraction methods may be the main reason for the differences in polysaccharide structures. Altogether, there are differences in the structures of polysaccharides from *P. sibiricum*, reported in various papers, which may be due to different species of *P. sibiricum*, concoction processes, and extraction methods.

α-Amylase in saliva specifically cleaves the *α*-1,4-glycosidic bond and can break down most soluble digestible starches[34]. In this study, the simulated salivary digestion failed to decompose PSP, implying that PSP contained no *α*-1,4-glycosidic bonds (Figs. 3A–C). Though gastric juice can destroy the polysaccharide structure due to its strongly acidic environment and rich digestive enzymes[16,35], we found no significant changes in reducing sugar content and PSP molecular weight (Figs. 3D–F), indicating structural stability of PSP under the acidic environment. In addition, the intestinal digestion fluid had a little digestive effect on PSP (Figs. 3G–I), consistent with the *in vitro* digestion results of other plant polysaccharides[19]. These results elucidated that PSP could resist structural destruction by digestive enzymes or acidic pH in the upper gastrointestinal tract. Hence, we inferred that PSP might be metabolized by gut microorganisms in the cecum and colon.

Plant polysaccharides are excellent prebiotics for body health by regulating the structure of gut microbiota community[36]. Therefore, we further explored the effect of PSP on human intestinal bacteria by *in vitro* fermentation. The results show that PSP increased the *α*-diversity of gut microbiota (Figs. 6A and B). As an indicator of microbial community status, *α*-diversity is associated with bacterial productivity, function, and stability[37]. The 16S rDNA sequencing data suggest that PSP reduced the abundances of several bacteria, such as Bacteroidaceae and Prevotellaceae (Figs. 7B and C). Since PSP fermentation led to the production of SCFAs with a reduced pH value (Fig. 4B) and the growth of both bacteria was inhibited under an acidic environment[38], we deduced that the pH reduction by PSP fermentation should be responsible for the reduced abundances of Bacteroidaceae and Prevotellaceae. Similar findings were reported by Yan et al.[39] in diabetic mice with PSP treatment. On the other hand, the abundances of some bacteria were significantly increased by PSP fermentation, like *Coprococcus* and *Bifidobacterium* (Fig. 7C). It was found that *Coprococcus* was depleted in depression and positively correlated with dopamine metabolism[40]. Din et al.[41] confirmed that the probiotic *Bifidobacterium* could ameliorate colitis by upregulating the levels of super oxidase dismutase 1 (SOD1) and catalase (CAT) in colon tissues. Based on the above, these data suggest that PSP regulated intestinal microecology and had prebiotic potential to promote the host health.

Different from the upper digestive tract that secretes few glycosidases and cannot decompose indigestible polysaccharides, the gut microbiota expresses a variety of glycosidases for the effective metabolism of dietary polysaccharides[42]. In this study, after the anaerobic fermentation of PSP by human fecal bacteria for 48 h, the total carbohydrate content in the supernatant decreased to that of the NC group (Fig. 5A), accompanied by the promoted growth of gut microorganisms (Fig. 4A). It was suggested that the gut microbiota could utilize PSP as a substrate. A line of evidence has proved that intestinal bacteria can decompose polysaccharides to produce SCFAs, which are essential for body health[43-44]. Compared to the NC group, the metabolism of PSP by gut microbiota produced higher levels of acetic acid, propionic acid, butyric acid, and pentanoic acid (Fig. 9), illustrating that PSP had a facilitating effect on the production of SCFAs[19]. Among the four fatty acids, acetic acid was the major SCFA secreted by gut microbes, which is vital to reduce the expression of genes involved in gluconeogenesis and lipogenesis[45]. *Bifidobacterium* can metabolize oligofructose via the fructose-6-phosphate phosphoketolase pathway to produce lactate, acetate, formate, and minor succinate[46-47]. Besides, acetic acid helps prevent various diseases like constipation[48]. The other 3 SCFAs are also beneficial to the host. For instance, propionic acid reduces basal glucose uptake[49] and improves tissue insulin sensitivity[50]. In this research, PSP promoted the growth of *Veillonella*, which has been confirmed to utilize lactic acid as a substrate and produces propionic acid, acetic acid, carbon dioxide, and hydrogen[51]. Butyric acid is an energy source for colon cells and can effectively enhance intestinal barrier function[52]. Additionally, *Coprococcus* (family Lachnospiraceae), which belongs to the phylum Firmicutes, has been identified as a butyrate producer[53]. In addition, through the cross-feeding pathway of gut microbes, lactic and acetic acids produced by *Bifidobacterium* degrading oligofructose promote the growth of butyrate-producing colon bacteria[46-47]. Pentanoic acid can up-regulate tight junction proteins and keratin (KRT1), promoting tissue integrity and barrier function[54]. Additionally, PSP significantly redued the level of indole in the fermentation broth (Fig. 9F). Indole is a protein fermentation product and has several harmful effects on the body, such as systemic toxicity, nephrotoxicity, and carcinogenesis[55]. Taken together, the fermentation of PSP promoted the synthesis of SCFAs, indicating its potential protective effect on the intestine.

In this study, the scavenging effect of fermentation broth on free radicals in the PSP group was higher than those of the NC group (Fig. 10). We investigated this in terms of increased abundance of intestinal probiotics and altered polysaccharide structure. PSP administration increased the abundance of probiotic bacteria such as *Bifidobacterium* and *Coprococcus* (Fig. 7C). *In vivo* and *in vitro* studies have shown that probiotics exhibit antioxidant potential. Probiotics may modulate the redox status of the host via their metal ion chelating ability, antioxidant systems, regulating signaling pathways, enzyme producing reactive oxygen species (ROS), and intestinal microbiota. Folate, a metabolite of *Bifidobacterium*, has promising antioxidant activity. In addition, *Bifidobacterium* regulates the composition of intestinal microbiota and inhibits the overproliferation of harmful bacteria by producing SCFAs, which may contribute to decreasing oxidative stress[56-57]. So, we speculate that there is a connection between the increased antioxidant capacity of PSP fermentation broth and the increased abundance of probiotics. The antioxidant properties of polysaccharides depend on their molecular weight, glyoxylate percentage, monosaccharide composition, and glycosidic bonds[17,58-61]. It has been pointed out that the content of galacturonic acid plays a crucial role in determining the antioxidative activity of polysaccharides. The hydrogen atoms on the C–H bond of aldose rings or glyoxylates in polysaccharides can react with free radicals and exhibit an antioxidative effect[62]. PSP contains galacturonic acid (Table 1), which will be released once the intestinal bacteria break down the structure of PSP. This may be the reason for the high clearing capacity of PSP fermentation broth. In a previous study, Wang et al.[63] demonstrated that low-molecular weight polysaccharides exhibited higher antioxidant activities. Here, we confirmed that the scavenging effect of PSP fermentation broth on superoxide radical was gradually increased during the first 24 h of fermentation, in which the molecular weight of PSP was accordingly reduced due to bacterial decomposition (Fig. 10B). Thus, we presume that such increment in scavenging activity may also be due to the production of oligosaccharides released from PSP fermentation.

5. Conclusion

In summary, fructan polysaccharides with a molecular weight of 3.06 kDa was isolated from *P. sibiricum*. Using the simulated digestion and *in vitro* fermentation by human intestinal flora, we proved that saliva, gastric juice, and small intestinal digestion fluid failed to decompose PSP. In contrast, intestinal bacteria displayed the high metabolic activity of PSP. During the PSP fermentation, the diversity and abundance of gut microbiota were significantly affected, accompanied by the mass production of SCFAs, like acetic acid, propionic acid, butyric acid, and pentanoic acid. In addition, the PSP fermentation broth displayed a scavenging effect on free radicals. Our studies shed light on applying PSP as a potential prebiotic to the food and pharmaceutical industries.

Conflicts of interest

The authors have no conflict of interest to declare.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2022.9250184.

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