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Bifidogenic properties of polysaccharides isolated from mushroom *Lentinula edodes* and enhanced immunostimulatory activities through *Bifidobacterial* fermentation

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Short title: Bifidobacterial fermentation and immunoactivity of mushroom polysaccharides

Abstract

Polysaccharides from *Lentinula edodes* mushrooms (LePS) are well-known for their immunomodulatory activities, which may have a functional connection with probiotic fermentation in the gut microbiota. This study was to evaluate the bifidogenic properties and impact of *Bifidobacterial* fermentation on the LePS molecules and immunoactivities. Two LePS fractions, LePS-40 (3.97×10^7 Da) and LePS-80 (1.43×10^5 Da) with differences in molecular weight (MW) and chemical composition were isolated from the mushroom hot-water extract and fermented with a *Bifidobacterium breve* strain. The higher-MW fraction LePS-40 was more significantly consumed and utilized by the bacteria for growth and acetic acid production during the fermentation, due probably to its higher total carbohydrate content and glucose-abundant composition. The *Bifidobacterial* fermentation caused a notable MW reduction of both LePS fractions and, more interestingly, also led to a higher immunostimulatory activity in RAW 264.7 macrophage cells. Further assessment of the separated fractions by ultrafiltration of the fermentation liquid (digesta) suggested that the immunoactivity was mainly attributed to the partially degraded LePS instead of the small-molecule metabolite products derived from the *Bifidobacterial* fermentation. In summary, monosaccharide composition was a more significant determinant than MW on the bifidogenic properties of LePS and the immunomodulatory activities after fermentation.

Keywords: *Lentinula edodes* mushroom; Polysaccharides; *Bifidobacterial* fermentation; Short chain fatty acid; Immunomodulatory activity.

1. Introduction

Probiotics, prebiotics and their synbiotic combinations are increasingly recognized as effective dietary and therapeutic ingredients for maintaining a balanced intestinal microbiota which is essential for human health. *Bifidobacterium*, one of the most important probiotic genera, is found in many probiotic health products and can grow anaerobically across a wide pH range (4.5–8.5) (Chen *et al*., 2019). Its beneficial effects include inhibiting harmful bacteria by lowering the intestinal pH through the production of short-chain fatty acids (SCFAs) (Ashida *et al*., 2009). *Bifidobacteria* also secrete various enzymes and proteins that are involved in carbohydrate metabolism, such as glycosyl hydrolases (GHs) and carbohydrate-binding proteins (Kelly *et al*., 2021; Larsbrink *et al*., 2014). These enzymes and protein molecules are essential for the *Bifidobacteria* to utilize dietary fibers and non-digestible carbohydrates from various sources. Bifidogenic food ingredients can be utilized by *Bifidobacteria* as nutrients, such as the carbohydrate fibers as the carbon source, to support the growth of *Bifidobacteria* and production of SCFAs (Kang *et al*., 2022). *B. breve* is one of the most common *Bifidobacterium* species in probiotic products and also in the human gut (Sharma *et al*., 2021). Its genome for carbohydrate metabolism has been fully characterized, revealing several genes that encode carbohydrate transport systems and enzymes *(Ameri et al*., 2022).

Previous studies from our lab have shown that the prebiotic or bifidogenic effects and metabolic outcomes of some fungal and plant polysaccharides in *Bifidobacterial* cultures were dependent on both the structures of polysaccharides and the *Bifidobacterial* species. For example, the exopolysaccharide (EPS) of a medicinal fungus, a glucomannan from konjac plant (KGM) was only slightly fermented and utilized for the bacterial growth of different

Bifidobacterium species (Song *et al*., 2018), while arabinoxylan could only be well utilized by *B. longum* (Song *et al*., 2020). A more recent study by Li *et al*. (2021) has shown that the fermentation of an EPS from a medicinal fungus in two *Bifidobacterial* cultures, *B. breve* and *B. longum*, led to notably enhanced anti-inflammatory activity due probably to partial degradation of the high-molecular-weight (MW) EPS. Another study by Zhang *et al*. (2018) detected significant changes in the physicochemical properties of polysaccharides from an Asparagus vegetable caused by *Lactobacillus plantarum* fermentation together with a notable enhancement of antioxidant and immunomodulatory activities.

Polysaccharides from edible and medicinal fungi or mushrooms have numerous bioactivities such as immunomodulatory (Zhao *et al*., 2020), anti-inflammatory (Yu *et al*., 2023), and anti-tumor (Xu *et al*., 2022) and attracted wide interest for nutraceutical and therapeutic applications. Most bioactive polysaccharides are resistant to the human digestive enzymes in the upper gut and are most often metabolized by gut bacteria in the large intestine. Therefore, their health benefits to the human host may have a close association with their metabolism by gut bacteria as revealed by many research studies (Jayachandran *et al*., 2017). *Lentinula edodes*, generally called Xianggu in Chinese and Shiitake in Japanese, is one of the most popular edible mushroom species worldwide for its favorable flavor, aroma, and high nutritional and medicinal value (Gaitán-Hernández *et al*., 2019). Various polysaccharides including β-glucans and heteropolysaccharides have been isolated from *L. edodes* mushrooms, and shown antitumor, immunomodulatory, microbiome-regulating, and other beneficial effects (Sheng *et al*., 2021). In a previous study from our lab (Li *et al*., 2023a), an high-MW PS fraction LePS-40 composed mainly of β-glucans was extracted from *L. edodes*

mushrooms by autoclaving-ultrasonication and showed significant immunostimulatory activities in macrophage cell culture.

This study was aimed to assess the bifidogenic effects of high- and low-MW PS fractions (LePS) extracted from the *L. edodes* mushroom and the changes of LePS molecular properties and immunoactivity caused by *Bifidobacterial* fermentation. Based on previous studies, two PS fractions (LePS-40 and LePS-80) were isolated from the mushroom hot-water extract and fermented with *B. breve* in a liquid medium. The bacterial growth, SCFA production, and changes in the LePS molecules were analyzed during the fermentation. The immunostimulatory activity of the fermentation liquid or digesta from the bacterial fermentation of LePS was assessed in RAW 264.7 cell culture.

2. Materials and methods

2.1. Chemicals and biochemicals

B. breve (CICC6079) was attained from China Centre of Industrial Culture Collection (CICC). For cell culture, Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were procured from Thermo Fisher Scientific, a reputable supplier based in Waltham, MA, United States. To maintain proper pH and osmotic balance during experiments, phosphate-buffered saline (PBS) was obtained from Macklin, a well-known provider of laboratory chemicals. Additionally, a range of essential reagents such as penicillin, streptomycin, lipopolysaccharide (LPS), sulphanilamide, N-1-naphthyl ethylenediamine dihydrochloride, and phosphoric acid were acquired from Sigma-Aldrich (St. Louis, MO, USA). Monosaccharide and SCFA standards were from Aladdin Chemical Reagent Co., Ltd (Shanghai, China). Inulin

was from Sigma (DP 36, MW 3463 Da). All other chemical reagents were of AR grade or higher grade and obtained from Aladdin Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Preparation of *L. edodes* polysaccharides

The dry *L. edodes* mushrooms in their fruit body form were procured from Zhejiang Fangge Pharmaceutical Co., Ltd., located in Qingyuan County, Zhejiang, China. Upon acquisition, these mushrooms were preserved in vacuum-sealed plastic bags at an ambient temperature ranging from 20 to 25 °C until they were ready for use. To extract the polysaccharides known as LePS, the dried mushrooms were first ground into a fine powder using an electric milling device (800Y, Platinum Ou Hardware products Co., LTD, China). The mushroom powder was subsequently sieved through an 850 μm mesh to ensure uniform particle size. The next step involved the defatting of the mushroom powder using ethanol in a weight-to-volume ratio of 1:8. After this solvent was removed, the remaining solid residue was dried in an oven (Memmert oven, UM200, Hong Kong) at a consistent temperature of 50 °C until it reached a constant weight. The defatted powder was macerated in distilled water (1:30 w/v) for 30 minutes and then autoclaved at 121 °C for 60 minutes. After cooling to room temperature, the solid-liquid mixture was centrifuged (48,380×g, 45 min, 4 °C) (Beckmann Coulter, the avanti J-26 XPI), and the supernatant was mixed with ethanol 40% (v/v) and precipitate was kept at 4 °C for overnight. The precipitate was separated by centrifugation (12,430×g, 20 min, 4 °C) as the crude LePS-40 fraction, and the supernatant was further precipitated at 80% (v/v) ethanol to attain LePS-80. The crude PS fractions were redissolved in distilled water, and freeze-dried and then deproteinated by the Sevag method, and dialyzed for 48 hours through a 3500 Da-MWCO

membrane. The PS solution was freeze-dried to obtain the partially purified PS fractions, LePS-40 and LePS-80, respectively. More details for the extraction, isolation and purification of polysaccharides from the *L. edodes* mushroom can be found in a previous report (Li *et al*., 2023a).

2.3. *Bifidobacterial* fermentation of polysaccharides

The *B. breve* strain was chosen for this study based on a previous study (Li *et al*., 2021) and preliminary experiments with five *Bifidobacterial* species. The *B. breve* culture was maintained in starch-free Reinforced Clostridium Medium (RCM) as described previously (Li *et al*., 2021). The PS fractions LePS-40 and LePS-80 were tested in comparison with inulin (as a prebiotic polysaccharide) and glucose (as the standard carbon source). All these carbohydrates were dissolved in RCM liquid medium at 5 g/L final concentration and sterilized by autoclaving at 121 °C for 15 min. A starter culture of *B. breve* was prepared by incubating the bacterial strain from a stock culture in RCM broth for about 18 h. The starter culture was then inoculated at 2% (v/v) into 10 mL centrifuge tubes each being filled with 5 mL of RCM containing one of the carbohydrates or no carbon source as the control. The culture tubes were placed together with anaerobic sachets in airtight jars and incubated at 37 °C with shaking at 200 rpm for 48 h, during which time culture tubes were taken at selected time intervals, 0, 4, 8, 12, 24, and 48 h for analysis and assessment of bacterial growth. The optical density (OD) of fermentation liquid was measured at 600 nm with a spectrophotometer for quantification of the bacterial concentration and growth. The bacterial

suspension was centrifuged (16090×g, 10 min, 4 °C) and the supernatant was collected as the *digesta* for analysis of soluble components and for assessment of immunoactivity.

2.4. Fractionation of digesta

The liquid digesta collected from the *Bifidobacterial* fermentation was separated into two MW fractions through a 5 kDa MWCO ultrafiltration (UF) membrane using Amicon® Ultra centrifugal tubes (Millipore Amicon Ultra, Germany) at 4000×g for 30 min at 4 °C. The filtrate was collected as the lower MW fraction (<5 kDa). The retentate was washed repeatedly with Milli-Q water and centrifuged in the UF tubes to ensure the elimination of smaller molecules. The final retentate, containing the higher MW components (>5 kDa), was diluted to the original volume to be used for the subsequent immunological activity tests in cell culture.

2.5. Analysis of *Bifidobacterial* fermentation broth (digesta)

2.5.1. Bacterial cell viability

The viability of bacterial cultures grown on various carbon sources was evaluated by measuring the final concentration and colony-forming units (CFU) of bacteria in the culture medium. For determination of CFU, the bacterial suspension was subjected to stepwise dilution with 0.85% (w/v) saline solution in the range of 10^{-1} to 10^{-10}. Subsequently, the diluted suspension was seeded onto a solid RCM agar plate and incubated anaerobically at 37 °C for 48 h. The number of viable colonies on the plate was counted and the range of 30 to 300 colonies was recorded as valid counts.

2.5.2. Medium pH and SCFAs

The pH of the culture medium was measured before and after bacterial fermentation using a pH meter. SCFAs in the bacterial culture medium were analyzed by gas chromatography (GC) following a documented method with minor modifications (Song et al., 2018). Briefly, the pH of the bacterial culture liquid was adjusted to 2-3 using 1.0 M HCl, and then centrifuged at 2790 ×g for 20 min at 4 °C. The supernatant was supplemented with 2-ethylbutyric acid as an internal standard at a final concentration of 1 mM prior to injection into the GC system. GC analysis was conducted using an Agilent 7980B GC system equipped with a flame ionization detector (FID) (Agilent Technologies Inc., USA) and a fused-silica capillary column (dimension 30 × 0.32 mm) coated with a free fatty acid phase of 0.25 film thickness (DB-FFAP 123-3232, Agilent Technologies Inc.). Nitrogen gas was used as the mobile phase at a flow rate of 0.6 mL/min. The oven temperature was initially set at 80 °C and maintained for 2 minutes, and then was gradually raised at a 6 °C/min to 180 °C and held for 4 minutes. The detector temperature was set at 220 °C, and the sample liquid (1 μL) was injected at 200 °C. Several SCFA standards (Aladdin®, Shanghai, China) including acetic acid (A116165), propionic acid (P110443), n-butyric acid (B110439), i-butyric acid (I103521), n-valeric acid (V108269), and i-valeric acid (I108280) were used for identification and quantification purposes.

2.6. Analysis of LePS and its changes during *Bifidobacterial* fermentation

2.6.1. Chemical composition of LePS

As reported previously (Song et al., 2018), the total carbohydrate content of LePS was determined using the Anthrone test. This test involves hydrolysis of the polysaccharide samples at 100 °C in the presence of anthrone agent, generating a yellow-green coloration. The absorbance of the sample solution was measured at 620 nm with a spectrophotometer that was calibrated using glucose. The protein content was determined by the Lowry method, which involves the reaction of copper ions with an alkaline medium at a temperature of 100 °C to produce a blue color. The absorbance was measured at 750 nm and calibrated to total protein concentration with bovine serum albumin as a reference.

2.6.2. Monosaccharide composition

The analysis of monosaccharide composition was conducted using the sophisticated technique of 1-phenyl-3-methyl-5-pyrazolone-high-performance liquid chromatography (PMP-HPLC). This method utilized a UV detector in conjunction with a ZORBAX Eclipse XDB-C18 column, specifically designed for high-resolution separation in analytical applications (Agilent, 5 m, 4.6 mm × 250 mm, Santa Clara, CA, USA) as previously documented (Li *et al*., 2023a). The LePS and digesta samples were hydrolyzed with 4 M trifluoroacetic acid (TFA) at 110 °C for 4 hours. Following hydrolysis, the resulting hydrolysate underwent a derivatization process, which involved the addition of 50 µL of 0.3 M sodium hydroxide (NaOH) and 50 µL of 0.5 M PMP, before being extracted using chloroform to isolate the desired components. Digesta without TFA treatment was also analyzed for the free monosaccharides generated from fermentative metabolism and degradation of the LePS. ZORBAX Eclipse XDB-C18 column (Agilent, 5 m, 4.6 mm 250 mm, Santa Clara, CA, USA) was used to examine the derivatized hydrolysate. Two

mobile phase solutions denoted A and B were used. Mobile phase A was composed of an aqueous solution of sodium phosphate buffer (0.05 M pH 6.9) containing acetonitrile at 85:15 volume ratio; mobile phase B was composed of a sodium phosphate buffer (0.05 M, pH 6.9) containing acetonitrile at 60:40 volume ratio. The HPLC analysis was performed at three-time intervals, 0, 10, and 30 minutes with 0%, 15%, and 25% of mobile phase B at a flow rate of 1 mL/min. The sample peaks were detected at a wavelength of 250 nm, and the identify and concentration of monosaccharides were determined with the standard chromatograms of pure monosaccharides (Sigma, St. Louis, MO, USA).

2.6.3. Molecular weight

The molecular weight (MW) of original LePS and fermented samples was determined using high-pressure gel permeation chromatography (HPGPC) coupled with refractive index (RI) and multi-angle laser light scattering (MALLS) detection, as described previously (Li *et al*., 2023a). The sample solution at volume of 100 μL was injected into the apparatus and was eluted with water at a rate of 0.6 mL/min. Breeze V3.3 software was used to compute the MW value using a calibration curve prepared with dextran MW standards ranging from 1.0 to 670 kDa (Sigma, St. Louis, MO, USA). The weight-average molecular weight (M_w) of the polysaccharide fractions was determined using a *dn/dc* value of 0.138 mL/g. Data collection and analysis were performed using the web-based program Astra (version 6.1.7, Wyatt Technologies Co., Santa Barbara, CA, USA).

2.6.4. Fourier transform infrared (FT-IR) spectroscopy

Each of the LePS samples was grounded together with KBr (1:100 mg) and pressed into a thin disk. The disk was scanned in the 4000-400 cm^{-1} range on an FT-IR spectrometer.

2.7. *In vitro* immunoactivity assays

2.7.1. Cell culture and LePS treatment

The immunoactivity tests were performed on RAW 264.7 murine macrophage cell line (InvivoGen, San Diego, CA, USA) as reported previously (Li *et al*., 2023a). The RAW 264.7 cell culture was maintained in DMEM supplemented with 10% FBS at 37 °C in a humidified 5%-CO2 environment. Cells in the logarithmic phase were treated with the LePS and the *bifidobacterial* digesta samples at various concentrations (dilution ratios 1:5, 1:10 and 1:20 in the cell culture medium) for 24 hours. Lipopolysaccharide (LPS) (200 ng/mL) was tested as a positive control and untreated cell culture was used as a negative control.

2.7.2. Macrophage proliferation assay

The proliferation activity was assessed by MTS test. At the logarithmic phase of culture, the RAW 264.7 cells were plated at 1×10^5 cells/mL in each well of a 96-well plate and incubated for 24 hours. Then, the cells were washed twice with PBS and then incubated with 100 μL of the test samples at various concentrations. MTS solution (20 μL) was added to each well and incubated for another 3.75 hours. The formazan absorbance was measured at 492 nm with a microplate reader. Cell proliferation rate was represented as the percentage increase in absorbance between treated and untreated control cells.

2.7.3. Nitric oxide (NO) analysis

The stimulation of NO production in immune cells is a common measure of immunoactivity. NO concentration in the cell culture was determined with the Griess assay as reported previously (Li et al., 2021). The cells in logarithmic growth phase were seeded into 96-well plates (100 μL) at a final concentration of 5×10^5 cells/mL and incubated for 24 h and then treated with LePS and digesta samples or LPS at selected concentrations. After incubation for 24 h, cells were treated with Griess reagent, followed by absorbance measurement at 540 nm. To ensure accurate quantification, the measured absorbance values were converted to NO concentrations through a calibration curve established with sodium nitrite, which served as a standard reference for comparison.

2.7.4. Phagocytic activity assay

Neutral red uptake was used to evaluate phagocytic activity as reported previously (Gong et al., 2022). In brief, the cells in the logarithmic phase (after cultured for ~24 hours) were planted at a density of 5×10^5 cells per well in a 96-well plate and exposed to the LePS and digesta samples for 24 hours. The supernatant was discarded and incubated for 20 minutes, followed by the addition of 100 uL of culture medium containing 0.01% neutral red to each well. The culture wells were rinsed repeatedly with PBS to remove the unabsorbed neutral red, followed by addition of 100 L of cell lysis solution (1:1 mixture of ethanol and pure acetic acid) to each well. After lysis treatment for two hours at 4 ^{o}C in a refrigerator, the absorbance at 540 nm was measured with a plate reader.

2.7.5. Analysis of cytokines by ELISA

As reported previously, the cells at the logarithmic phase (after incubation for ~24 hours) were plated in 96-well plates at a density of 1.0×10^5 cells per well (Li *et al*., 2023a). After incubation for 24 hours, the liquid medium was removed and treated with the LePS, digesta samples or LPS (IL-6 and TNF-α) for 24 hours. The treated culture liquid in the plate wells was collected and centrifuged, and the cytokine levels of IL-6 and TNF-α in the supernatant were detected with ELISA kits (JingMei Biotechnology, Jiangsu, China).

2.8. Statistical analysis

All experiments conducted were performed in triplicate to ensure the reliability and validity of results. The results from these experiments are presented as the mean values plus/minus standard deviation (SD). The significance of treatment effects was determined by a two-way analysis of variance (ANOVA) using SPSS software (version 13.0, Chicago, IL, USA) for statistical assessments. Furthermore, all figures presented in this paper were created using Prism 9.0. $p < 0.05$ was statistically significant.

3. Results and discussion

3.1. Composition and properties of LePS fractions

Table 1 displays the total sugar content, protein content, and monosaccharide composition of LePS. Notably, LePS-40 exhibited a significantly higher MW of 3.97×10^7 Da compared to 1.43×10^5 Da for LePS80. In terms of chemical composition, LePS-40 had a higher carbohydrate and lower protein content than LePS-80. As both LePS fractions contained a significant portion of protein after Sevag deproteinization, they can be regarded as

polysaccharide-protein complexes. Moreover, both LePS fractions contained glucose, galactose, and mannose, although in different ratios. Predominantly, LePS-40 consisted of glucose, suggesting a β-glucan-rich polysaccharide, consistent with previous findings (Li *et al*., 2023a). In contrast, LePS-80 had a substantial presence of both glucose and mannose, with a minor proportion of galactose. Similarly, a previous study has also shown that glucose, galactose, and mannose were frequently observed in the hetero-PS from *L. edodes* in numerous studies (Zhang *et al*., 2011).

Table 1. Molecular weight and chemical composition of crude *Lentinula edodes* polysaccharides (LePS) extracted by different ethanol concentrations.

LePS	MW (Da)	Total carbohyd. (wt%)	Total protein (wt%)	Monosacch. molar ratio		
				Glc	Gal	Man
LePS-40	$3.97 \times 10^{7\,b}$ (±2.34 %)	61.34 ± 1.73^{b}	12.45 ± 0.85^{a}	14.16	1.66	1
LePS-80	$1.43 \times 10^{5\,a}$ (±3.42%)	48.27 ± 0.60^{a}	24.66 ± 1.21^{b}	5.05	0.45	1

Yield values are presented as mean ± SD, n = 3. Different letters (a, b, c) indicate significant differences in the columns ($p < 0.05$).

The FT-IR spectra of LePS are presented in **Fig. 1**. The two PS fractions LePS-40 and LePS-80 exhibited all similar spectral characteristic peaks, due probably to their similar glycosidic bonds and monosaccharide constituents. The strong and broad absorbance near 3350 cm^{-1} can be ascribed to the stretching vibration of O−H in the molecular structure, while the weak absorption at 2910 cm^{-1} is attributed to the stretching vibration of C-H (Zhang *et al*., 2023). The peak at 1640 cm^{-1} may arise from the -OH bending vibration or stretching vibration of the C=O bond in proteins or polysaccharide-protein complexes (Zhang *et al*., 2023). The low

absorbance indicates a lower protein content in LePS-40. The characteristic peak at 1015 cm^{-1} corresponds to the stretching vibration of C-O-C and the presence of pyranoside in the molecule (Jiang *et al*., 2023). Additionally, the peak at 905 cm^{-1} indicates the presence of the β-glycosidic bond, a critical feature that may affect the biological functionality of these polysaccharides (Li *et al*., 2023a).

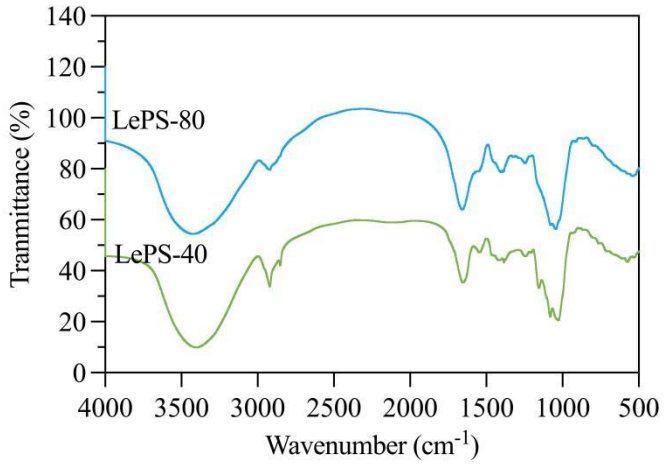

Fig. 1. FTIR spectra of LePS-40 and LePS-80.

3.2. Effects of LePS on *Bifidobacterial* growth and acid production

Fig. 2 illustrates the culture dynamics of *B. breve* over a 48-hour period in Reinforced Clostridial Medium (RCM) with the addition of four distinct carbon sources and a control without a carbon source. Colony-forming unit (CFU) counts, serving as a proxy for cell proliferation and viability, escalated over time across all groups. Notably, the group utilizing glucose exhibited the highest CFU counts, while the control group had the lowest (**Fig. 2A**). The cultures containing LePS-40 showed a significantly higher viability than those with inulin and LePS-80, suggesting differential substrate utilization. Another important indicator for anaerobic fermentation of polysaccharides is the pH decreases due to acid production. As

shown in **Fig. 2B**, the initial pH values of the medium were approximately 5.6 and decreased gradually during the fermentation period. At 48 hours, the pH value in the medium containing glucose decreased to 3.97, which was the lowest among the different treatments. The pH value in the medium containing LePS-40 and LePS-80 respectively decreased to 4.25 and 4.51, while the pH value in the medium containing inulin and no carbon source (control group) respectively decreased to 4.65 and 4.94. The pH value of the medium containing LePS-40 was significantly higher ($p < 0.05$) than that in the presence of LePS-80, which was consistent with the bacterial growth results.

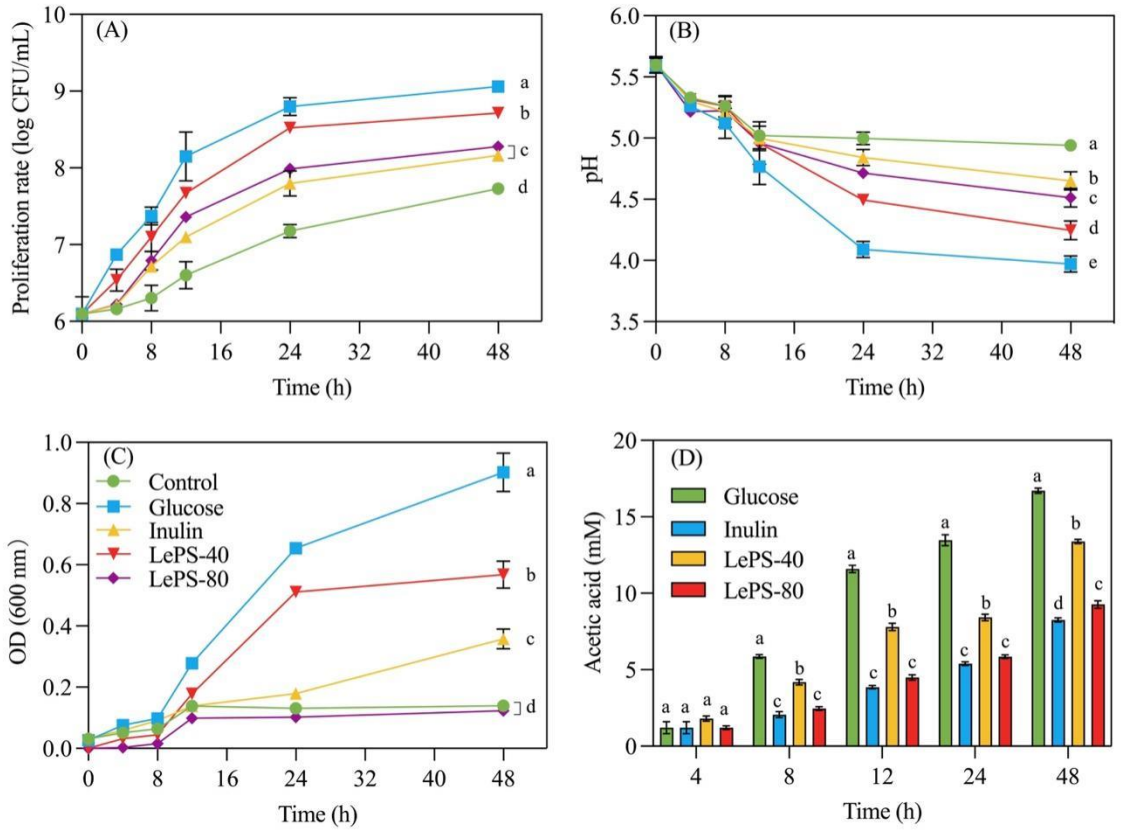

Fig. 2. Changes in (A) bacterial concentration, (B) medium pH, (C) proliferation rate, and (D) acetic acid production during *B. breve* fermentation in RCM containing different carbon sources and no carbon source. Different letters a, b… represent statistically significant differences ($p < 0.05$).

As a measure of bacterial concentration and growth, the OD (**Fig. 2C**) of glucose and LePS-40 culture group showed a rapid increase in the first 24 hours and a slow increase in the remaining culture period. In the LePS-80 and control group without a carbon source, the OD showed only a small increase over the whole culture period and the OD in the inulin group was only slightly higher efficiency.

SCFAs including acetate, propionate, and butyrate, are major end metabolite products of the *Bifidobacterial* fermentation of polysaccharides. SCFAs produced from gut bacterial fermentation also make important contributions to human functions such as intestinal barrier function, epithelial cell proliferation, immune responses (Li *et al*., 2023b). Acetic acid was found to be the main SCFA produced during the *B. breve* bacterial fermentation, consistent with previous research findings (Song *et al*., 2018). As shown in **Fig 2D**, the acetic acid concentration gradually increased during the 48-hour fermentation period and was much higher in glucose and LePS-40 group and the lowest in control. The relative level of acetic acid production with different was in agreement with that of pH decrease (**Fig. 2B**).

Therefore, these results indicated that LePS-40 was more active to support or promote the *B. breve* growth, proliferation and acid production. All these four culture characteristics have shown consistently that LePS-40 was well fermented by the *B. breve*, indicating its high bifidogenic potential.

3.3. Consumption of LePS by *B. breve* fermentation

The total carbohydrate concentration in *B. breve* culture with different carbon sources (glucose, LePS-40, LePS-80, and inulin) was measured to assess the consumption of LePS by

B. breve (**Fig. 3**). After 48-hour fermentation, the medium containing glucose had the highest carbohydrate consumption rate (84%), followed by LePS-40 (74%), LePS-80 (62%), and inulin (18%). The LePS-40 fraction was consumed at a much faster pace than LePS-80. The high consumption rate of LePS-40 by *B. breve* may be attributed, at least in part, to its much higher total carbohydrate content and a highly abundant glucose content compared with LePS-80 as shown in **Table 1**, which could be inferred that the glycosidic bond of LePS-40 is more easily depolymerized and utilized by this *B. breve*. In general, the structural characteristics of a polysaccharide such as composition, molecular weight, glycosidic linkage are major factors affecting its consumption and metabolism by *Bifidobacterial* fermentation (Wei *et al*., 2022). The implications of these consumption patterns are significant, considering the role of *Bifidobacteria* in gastrointestinal health and the potential prebiotic nature of LePS.

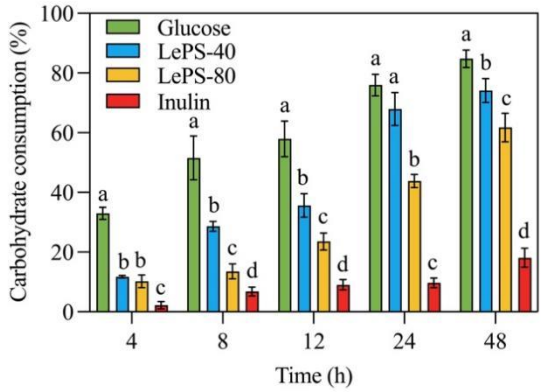

Fig. 3. Changes in carbohydrate consumption during *in vitro* fermentation. Different letters represent statistically significant differences ($p < 0.05$).

3.4. Changes of LePS molecular properties during fermentation

As shown in **Table 2,** the molecular weight (MW) of LePS-40 and LePS-80 during fermentation, decreased gradually and constantly throughout the 48-hour fermentation. In comparison, the MW of LePS-40 decreased much more dramatically, about 385-fold from

374 1.03×10^7 to 2.67×10^4 than that of LePS-80, about 14-fold from 2.85×10^5 to 2.00×10^4. The
375 results confirmed the degradation of LePS during *Bifidobacterial* fermentation. The more
376 rapid and dramatic degradation was consistent with the much higher consumption rate of
377 LePS-40 than LePS-80.

378 **Table 2.** Molecular weight changes of LePS-40 and LePS-80 during the bacterial fermentation.

Time (h)	LePS-40	LePS-80
0	1.03×10^7 (±0.57%)	2.85×10^5 (±0.67%)
4	9.91×10^6 (±6.96%)	2.19×10^5 (±0.67%)
8	6.88×10^6 (±1.46%)	1.88×10^5 (±0.37%)
12	3.59×10^6 (±1.21%)	1.64×10^5 (±7.06%)
24	3.71×10^4 (±5.67%)	1.05×10^5 (±1.27%)
48	2.67×10^4 (±5.56%)	2.00×10^4 (±5.08%)

379 **Fig. 4** shows the alterations in monosaccharide composition of LePS-40 and LePS-80 during
380 *B. breve* fermentation. Throughout the fermentation, both the free and residual glucose levels
381 of polysaccharides in the LePS-40 and LePS-80 groups both decreased to varying degrees,
382 while the residual glucose content decreased significantly. In contrast to glucose, the residual
383 amount of the other two sugars mannose and galactose decreased but their free sugar content
384 increased. This was probably attributed to a higher uptake and consumption rate of glucose
385 than the other two sugar constituents. The glucose content in LePS-40 was much higher than
386 that in LePS-80 ($p < 0.05$), so that the decreasing trend of free glucose content in the LePS-40
387 group was relatively flat. As previously established (Pokusaeva et al., 2011), *Bifidobacteria*
388 are saccharolytic and able to metabolize complex carbohydrates into monosaccharides via the
389 'bifid shunt' pathway, which allows the *Bifidobacteria* to produce energy from carbohydrates

more effectively than other fermentative pathways. In a more recent study, experiments were performed on the utilization of different sugars by a *B. longum* strain, finding that glucose was a more favorable than other sugars for the bacterial growth (Duboux *et al*., 2022). The high uptake and utilization of glucose by *B. breve* may also be the reason why LePS-40 is more beneficial for its growth than LePS-80, as LePS-40 contains more glucose than LePS-80.

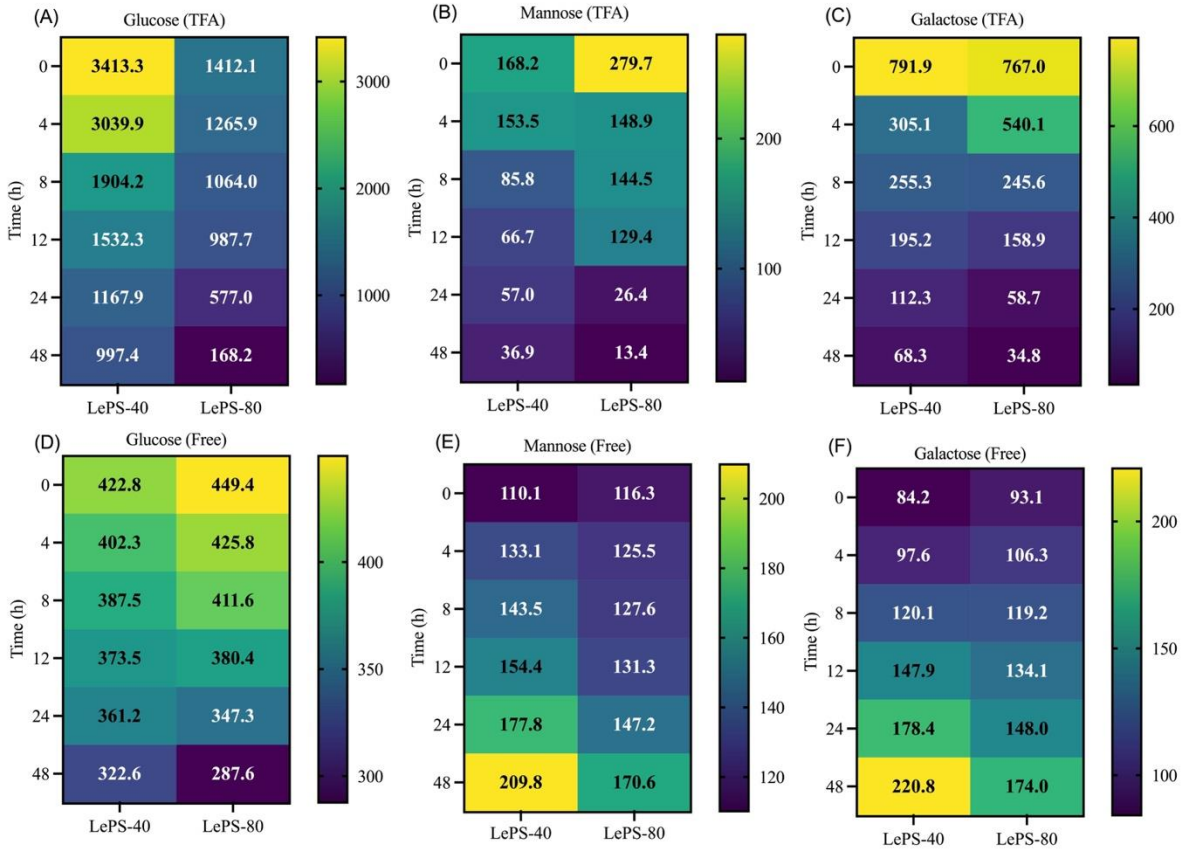

Fig. 4. Heat map of (A), (B), (C) total monosaccharide and (D), (E), (F) free monosaccharide composition from LePS-40 during *Bifidobacterial* fermentation at different time point.

3.5. Immunoactivities of LePS-40 and LePS-80 during fermentation

The RAW264.7 macrophage cell culture was treated with the digesta samples taken from *Bifidobacterial* fermented LePS at selected time points to test their influence on the cell

viability and immune responses. According to MTS assay, the viability of RAW264.7 cells was not significantly affected by any of the LePS samples before or after the *Bifidobacterial* fermentation (**Fig. 5**), as the cell viability remained over 80% after exposure to the LePS samples, regardless of the different concentrations. Similarly, fungal polysaccharides has been considered negligible cytotoxicity when the cell activity was maintained above 70% after treatment (Li *et al*., 2020). With different proportions at a fixed culture time, the cell viability was reduced most obviously in the presence of digesta samples at 1:5 dilution, which may probably attributed to the relatively high concentration of organic acids to cause a negative effect on cell viability.

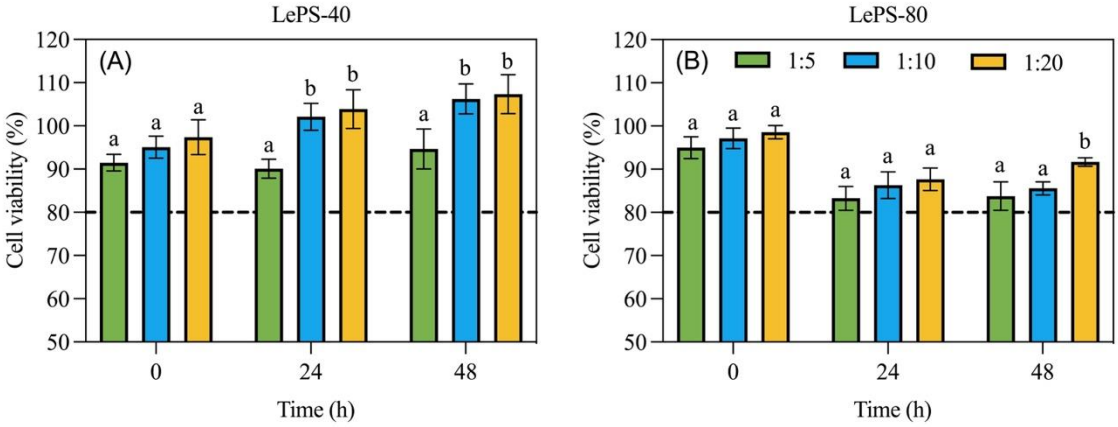

Fig. 5 Effect of LePS on RAW264.7 cell viability (dashed line = 80% viability level). Different letters represent statistically significant differences ($p < 0.05$).

As a characteristic response of immune cells, NO is a substance made by immune cells, which helps to wake up the immune system and protect us from germs. The NO production showed an increase with the fermentation time from 0 to 24 and then 48 h for both LePS fractions (**Fig. 6**). In addition, the increase in NO was concentration-dependent both before and after

418 the probiotic fermentation. Overall, the level of NO production was significantly higher of
419 LePS-40 group than that of LePS-80 group ($p < 0.05$) (Supplementary Fig. S2A).

420 IL-6 and TNF-α are two important cytokines in that regulate immune responses stimulated by
421 various antigens. TNF-α can induce macrophage-dependent immune responses, while IL-6
422 can induce antibody responses by activating B cells (Wang & He, 2020). As shown in **Fig. 6**,
423 compared with the control group, the LePS-40 and LePS-80 significantly increased the IL-6
424 and TNF-α secretion of RAW 264.7 cells in a concentration-dependent and fermentation
425 time-dependent manner. By comparison of levels at 48 hours of fermentation in each group,
426 the TNF-α production was significantly higher of LePS-40 group than that of LePS-80 group
427 though there was no significant difference in the IL-6 production (Supplementary Fig.
428 S2B&C).

429 Another valid indicator of the immunomodulatory action of natural polysaccharides is
430 macrophage phagocytosis. As shown in **Fig. 6**, the pinocytic capacity of RAW 264.7 cells
431 was increased by LePS-40 group and LePS-80 group compared to the control group ($p < 0.05$)
432 (Supplementary Fig. S2D), and more significantly by LePS-40 group. The stimulation effect
433 was also increased with the fermentation time.

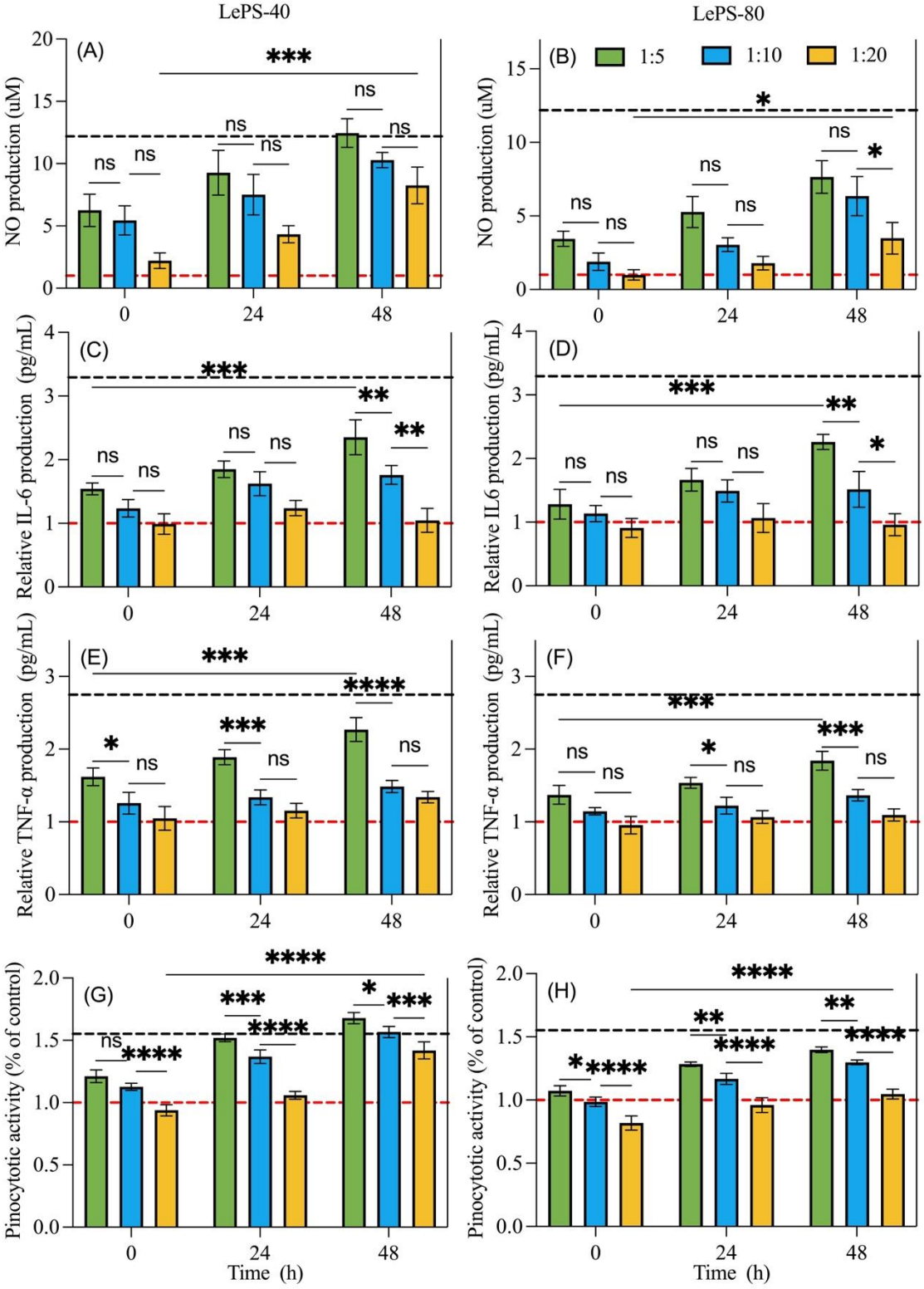

Fig. 6. Effects of different *Lentinus edodes* polysaccharides (LePS) fractions on NO production (A, B), cytokines (IL-6 & TNF-α) (C&D, E&F) and phagocytosis (G, H) of RAW 264.7 macrophages before and after fermentation. The black line refers to LPS, the red line refers to control; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.6. Identification of the active molecular fractions of LePS-40 digesta

For further identification of the active molecular fractions, the *Bifidobacterial* digesta was separated by a 5 kDa UF membrane into two fractions, LePS-40-L of MW < 5 kDa and LePS-40-H of MW > 5 kDa. As shown in **Fig. 7,** the lower MW digesta fraction showed little or even negative effect, and the higher MW fraction had a notable promoting effect on both immunomodulatory markers at a very low concentration of 10 μg/mL. Therefore, the results suggest that the immunostimulatory effects of digesta from *Bifidobacterium*-fermented LePS-40 was mainly attributed to the partially degraded LePS rather than the lower MW metabolite products. Similarly, the anti-inflammatory effect of digesta from *Bifidobacterium*-fermented EPS was also attributed mainly to the high MW fraction containing the partially degraded EPS (Li *et al*., 2021).

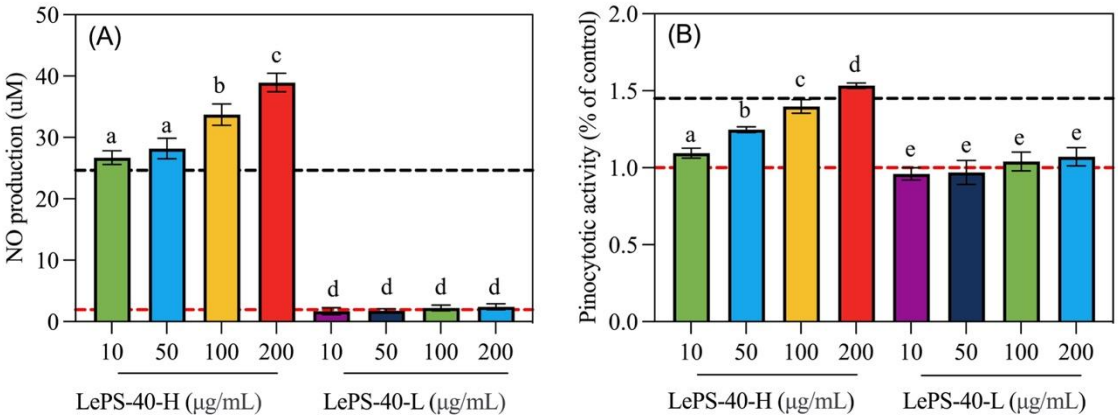

Fig. 7. Effect of different concentrations LePS-40 digesta on (A) NO production and (B) phagocytosis of RAW264.7 cells, respectively. The values are presented as mean ± SD, n = 6. (The black line refers to LPS, the red line refers to control)

3.7. Comparison with previous studies on *Bifidobacterial* fermentation of polysaccharides

Polysaccharide is the main component of dietary fiber, which can significantly affect human nutrition and health through the fermentation of intestinal microbiota, especially *Bifidobacterium*. *Bifidobacterium* is one of the most important probiotic species in promoting gut health. Previous studies have found that different sources and types of polysaccharides exhibit significantly different metabolism outcomes from *Bifidobacterial* fermentation. For example, an exopolysaccharide (EPS) from a medicinal fungus Cs-HK1 and a plant PS konjac glucomannan (KGM) were only slightly consumed and utilized by *B. breve* and a few other *Bifidobacterial* species, leading to small increases in the bacterial growth (OD increase) and SCFA production (Song *et al*., 2018). In contrast arabinoxylan (AX) was well fermented by a *B. longum* strain and utilized as a sole carbon source for the bacterial growth, even more favorable than glucose (Song et al., 2020). In present study, LePS-40 was also significantly consumed by the *B. breve* bacteria fermentation leading to substantial increases in biomass and SCFA production. LePS has been identified as a β-glucan-rich polysaccharide with a (1 → 3)-β-D-glucan backbone (Li *et al*., 2023a). Studies have shown that the strong immunological activity of mushroom polysaccharides can be attributed to β-glucan (Sheng *et al*., 2022) and polysaccharide-protein complex (Yin *et al*., 2021). Lentinan, a β-glucan originated from *L. edodes* is one of the most famous mushroom polysaccharides with proven and potent immunomodulatory activity (Murphy *et al*., 2022). Another study has demonstrated that a water-soluble, highly branched β-glucan isolated from the spore of *G. lucidum* exhibited significant immune-enhancing effects in dinitrochlorobenzene-induced delayed-type ear swelling in mice (Wang *et al*., 2017). In the fermentation process, *B. breve*

could secrete glycoside hydrolases, such as β-glucosidase and β-galactosidase, which cleaved specifically the glycosidic bonds linking the LePS polysaccharide chain (Pokusaeva *et al*., 2011). The enzyme hydrolysis of the LePS may lead to lower MW fragments and the exposure of new functional groups, to enhance the molecular interaction with the receptors of immune cells (Li et al., 2023a). This enhanced interaction may lead to a more robust activation of the immune response, as a reasonable explanation for the enhanced immunostimulatory activity after the fermentation of LePS. The monosaccharide composition of LePS-40 was abundant of glucose with a molar ratio of 84.2%, while the molar ratio of glucose was only about 35.1% in Cs-HK1 EPS and 34.0% in KGM (Song et al., 2018). In another study (Li *et al*., 2021), *Bifidobacterial* fermentation of an EPS from the Cs-HK1 fungus composed of about 61.2% glucose also increased the growth and SCFA production more significantly than another EPS fraction extracted by Song *et al*. (2018). Therefore, it could be inferred that the significant differences in the fermentation results with the above-mentioned polysaccharides may be mainly attributed to differences in their molecular structure and chemical composition.

4. Conclusions

In our present study, the two polysaccharide fractions (LePS-40 and LePS-80) extracted from *L. edodes* mushroom could be well fermented in pure culture of *B. breve*. The carbohydrate content or polysaccharide portion of these fractions appeared to be primarily utilized by *Bifidobacteria* for growth and acid production. Our findings suggest that the structure and

composition of LePS fractions, rather than molecular weight, are the key factors determining the fermentability and bifidogenic activity. Notably, the immunostimulatory activity of LePS-40 was significantly enhanced after Bifidobacterial fermentation. The findings highlight the potential of LePS-40 as a favorable substrate for *Bifidobacterial* proliferation and for development of novel synbiotics through *Bifidobacterial* fermentation. The fermented polysaccharides and other fermentation products may have potential applications in modulating immune responses and preventing or treating inflammatory diseases. However, the scope of present study was limited by the use of a single *B. breve* strain, and further research is needed to isolate the LePS fractions and more systematically assess their structural changes during the fermentation.

CRediT authorship contribution statement

Lin Xi Huang: Methodology, Investigation, Visualization, Writing - original draft. Jun Hui LI: Methodology. Fang Ting GU: Methodology. Yan Yu ZHU: Methodology. Zi Chen ZHAO: Methodology. Jian Yong Wu: Conceptualization, Project administration, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors claim that there are no known competing financial stakes or personal relationship that could appear to have influenced the research reported in this paper.

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Data availability

Data will be made available on request.

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Supplementary data

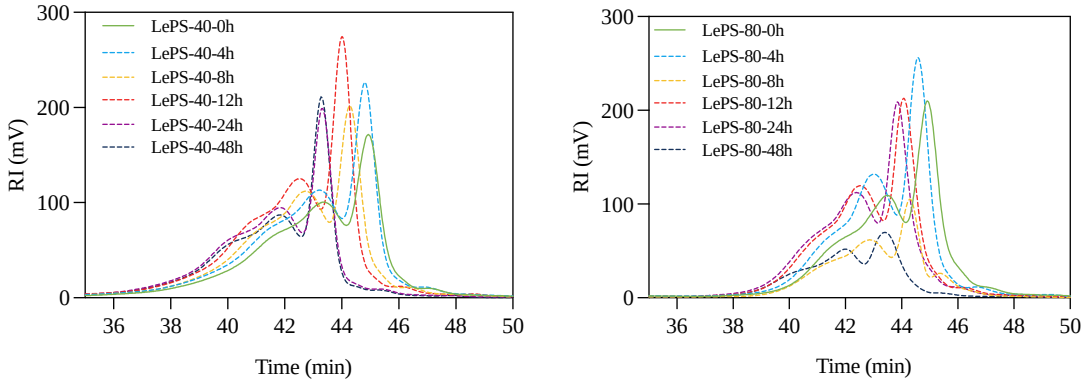

Figure S1. The GPC profile of LePS-40 during fermentation at different time point.

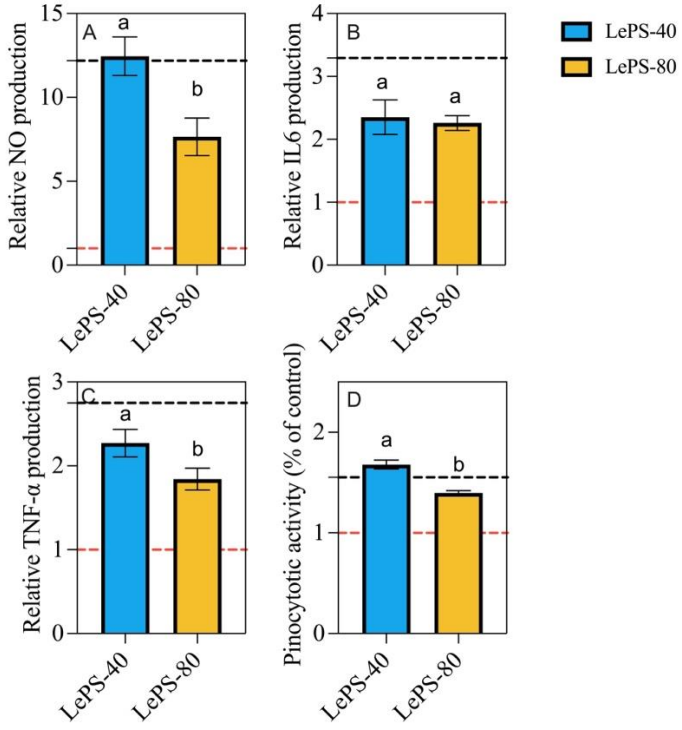

Figure S2. Comparison of different *Lentinus edodes* polysaccharides (LePS) fractions after fermented 48 h on NO production (A), IL-6 (B), TNF-α (C) and phagocytosis (D) of RAW 264.7 macrophages before and after fermentation.