



Structural analysis and prebiotic activity of exopolysaccharide produced by probiotic strain *Bifidobacterium bifidum* EPS DA-LAIM

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Received: 3 October 2022 / Revised: 8 November 2022 / Accepted: 13 November 2022 / Published online: 1 December 2022
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Abstract

Exopolysaccharide (EPS)-producing *Bifidobacterium bifidum* EPS DA-LAIM was isolated from healthy human feces, the structure of purified EPS from the strain was analyzed, and its prebiotic activity was evaluated. The EPS from *B. bifidum* EPS DA-LAIM is a glucomannan-type heteropolysaccharide with a molecular weight of 407–1007 kDa, and its structure comprises 2-mannosyl, 6-mannosyl, and 2,6-mannosyl residues. The purified EPS promoted the growth of representative lactic acid bacteria and bifidobacterial strains. *Bifidobacterium bifidum* EPS DA-LAIM increased nitric oxide production in RAW 264.7 macrophage cells, indicating its immunostimulatory activity. *Bifidobacterium bifidum* EPS DA-LAIM also exhibited high gastrointestinal tract tolerance, gut adhesion ability, and antioxidant activity. These results suggest that EPS from *B. bifidum* EPS DA-LAIM is a potentially useful prebiotic material, and *B. bifidum* EPS DA-LAIM could be applied as a probiotic candidate.

Keywords Exopolysaccharide · *Bifidobacterium bifidum* · Prebiotics · Probiotics · Immunostimulatory activity

Introduction

The genus *Bifidobacterium* includes commensal microorganisms that are commonly found in the human gut (Hidalgo-Cantabrana et al., 2015). Bifidobacteria have been used as

probiotics because of their scientific relationship with the host's health benefits (Hill et al., 2014). Regarding intestinal microbial homeostasis, bifidobacteria exhibit antimicrobial activity against pathogens and inhibit or reduce cancer formation (Coakley et al., 2009).

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Exopolysaccharides (EPS) are extracellular layers of carbohydrates found in microorganisms and are polymers attached to the extracellular surface of the cell-forming slime (Hidalgo-Cantabrana et al., 2015). Some bacteria are covered with a layer of polysaccharides called the glycocalyx. Recently, demand for and interest in bacterial-derived EPS have been increasing due to their favorable effects on health issues (Amiri et al., 2019; Nachtigall et al., 2021). EPS production has been linked to the producer microorganism and its host through several physiological pathways (He and Bauer, 2014). EPS produced by bifidobacteria can cope with bacterial toxins and pathogens and have a beneficial effect on the host (Garrigues et al., 2010). EPS is a postbiotic secreted by lactic acid bacteria and bifidobacteria that can interact with host cells by acting as ligands and defend the host by aggregating with pathogens in the intestine (Castro-Bravo et al., 2018). Additionally, oxidative stress protection and anti-cancer properties of EPS have been discovered (Saadat et al., 2019).

EPS can be classified into two types depending on the composition of the repeating unit: homopolysaccharides (HoPS) and heteropolysaccharides (HePS) (Ryan et al., 2015). HoPS and HePS differ in whether they are composed of one type of monosaccharide or more than two types of monosaccharides (Ryan et al., 2015). Although previous studies have focused on EPS from lactic acid bacteria, only a few bifidobacterial polymers have been characterized and purified so far (Ruas-Madiedo et al., 2012). EPS described in bifidobacteria is the HePS type, and its main monosaccharide constituents are glucose, galactose, and rhamnose, which are the same as those identified in EPS produced by lactic acid bacteria (Hidalgo-Cantabrana et al., 2015). It is reported that there are two types of EPS, ropy type and non-ropy type, and EPS produced from the ropy type-producing *B. breve* provides resistance to acid and bile-salt (Fanning et al. 2012). Ropy type EPS-producing strains produce long filaments or strands due to the high viscosity of EPS when extended with an inoculation loop.

EPS is the polysaccharide produced by microorganisms in the largest amount, and it has the highest commercial potential due to its easy recovery from culture and low purification cost (Nachtigall et al., 2021). Previous studies on EPS have mainly focused on its industrial applications, including emulsification, texturization, sweetening, gelling, and water-binding capacity. However, researchers are currently focusing on the bioactive properties of EPS. Additionally, recent research has shown that EPS can improve health through its immunomodulatory, prebiotic, anti-inflammatory, antibi-film, and antioxidant effects (Amiri et al., 2019).

In this study, *Bifidobacterium bifidum*, which produces exopolysaccharides, especially ropy-type EPS, in high yield was isolated from human feces, and its prebiotic and immunostimulatory activities, as well as probiotic

properties such as acid tolerance, bile salt tolerance, pancreatin-tolerance, and gut adhesion ability, were evaluated.

Materials and methods

Bacterial strains and medium

Bifidobacterium bifidum EPS DA-LAIM, *B. bifidum* KCTC 13270BP, *B. bifidum* DLP1224, *Lactobacillus helveticus* DLP1181, *Lactiplantibacillus plantarum* DLP1110, *Limosilactobacillus fermentum* DLP1406, *Bifidobacterium bifidum* DLP1224, and *B. animalis* DLP1267 were cultivated in De Man, Rogosa, and Sharp medium (MRS, Oxoid, Hampshire, UK) (10 g protease peptone No. 3, 10 g beef extract, 5 g yeast extract, 20 g dextrose, 1 g poly-sorbate 80, 2 g ammonium citrate, 5 g sodium acetate, 0.1 g magnesium sulfate, 0.05 g manganese sulfate, 2 g dipotassium hydrogen phosphate, per liter) or BL medium (MB cell, Seoul, Korea) at 37 °C for 24 h under anaerobic conditions. For EPS production, mMRS medium (MRS medium without glucose) or BL medium was supplemented with sucrose, and the cells were incubated at 37 °C under anaerobic conditions. *B. bifidum* KCTC 13270BP was purchased from the Korean Collection for Type Cultures (Daejeon, Republic of Korea).

Escherichia coli was cultivated in tryptic soy broth (TSB, BD, Heidelberg, Germany) (17 g tryptone (pancreatic digest of casein), 3 g soytone (peptic digest of soybean), 2.5 g glucose, 5 g sodium chloride, 2.5 g dipotassium phosphate, pH 7.3, per l).

Isolation of *Bifidobacterium* strains

After collecting feces from healthy Korean volunteers, the feces were serially diluted using sterile 0.85%(w/v) NaCl, and then, 100 µL samples were spread onto a transgalactosylated oligosaccharide-mupirocin (TOS-MUP) selective agar plate (*Bifidobacterium* selective media)(MB cell, Seoul, Korea) (10 g tryptone, 1 g yeast extract, 3 g monopotassium phosphate, 4.8 g dipotassium phosphate, 3 g ammonium sulfate, 0.2 g magnesium sulfate heptahydrate, 0.5 g l-cysteine-HCl, 15 g sodium propionate, 10 g TOS, per liter), which was incubated at 37 °C for 48 h under anaerobic conditions. Colonies grown on TOS-MUP-selective agar plates were transferred to a new TOS-MUP-selective agar plate for pure culture, and 129 *Bifidobacterium* sp. candidate strains were selected. The design of this study was reviewed by the Public Institutional Review Board designated by the Ministry of Health and Welfare (IRB approval number: P01-202102-33-002).

Identification of *Bifidobacterium* strain

Genomic DNA was isolated from *Bifidobacterium* strains as previously described (Sharma et al., 2020). PCR amplification and nucleotide sequence analysis of the 16S rRNA gene were performed by Macrogen, Inc. (Seoul, Korea). Phylogenetic tree analysis was carried out as previously described (Kwon and Park, 2021).

Production of EPS from *B. bifidum* EPS DA-LAIM and removal of EPS

To produce EPS, a single colony of *B. bifidum* EPS DA-LAIM was inoculated into mMRS medium or BL medium supplemented with 2–10%(w/v) sucrose and incubated at 37 °C for 24–96 h under anaerobic conditions. EPS were removed from the cells by centrifugation at 11,000 × g for 20 min, and the cell pellet was washed with 0.85%(w/v) NaCl or PBS three times.

Crystal violet staining of EPS

Bifidobacterium bifidum EPS DA-LAIM was cultured at 37 °C for 96 h under anaerobic conditions in BL medium containing 5%(w/v) sucrose or 5%(w/v) glucose. Crystal violet staining of EPS was performed by exposing heat-fixed cells to crystal violet diluted with a half volume of distilled water for at least 1 min.

Scanning electron microscopy of *B. bifidum* EPS DA-LAIM

To prepare the EPS-containing *B. bifidum* EPS DA-LAIM sample, a bacterial colony was inoculated in mMRS broth with 10%(w/v) sucrose and incubated at 37 °C for 48–72 h under anaerobic conditions. To prepare EPS-removed *B. bifidum* EPS DA-LAIM samples, the culture was centrifuged at 11,000 × g for 20 min, and the pellet was washed three times with phosphate-buffered saline (PBS). The sample was centrifuged at 21,200 × g for 5 min for samples with or without EPS, fixed with 2.5%(v/v) glutaraldehyde for 8 h, and washed twice with 0.1 M phosphate buffer (pH 7.4) for 15 min each. For the second fixation, 1%(w/v) osmium tetroxide solution was used for 1 h and rinsed with 0.1 M phosphate buffer (pH 7.4) for 15 min. The morphology of *B. bifidum* EPS DA-LAIM was examined using a scanning electron microscope (SEM, H-7600, Hitachi, Tokyo, Japan) installed at Eulji University (Seongnam, Korea).

Purification of EPS

Bifidobacterium bifidum EPS DA-LAIM was cultured in mMRS broth supplemented with 10%(w/v) sucrose at 37 °C

for 48–72 h anaerobically. The culture was centrifuged at 11,000 × g at 4 °C for 20 min. Trichloroacetic acid was added to a final concentration of 10%(w/v), and the EPS was precipitated at 4 °C for 20 h. The solution was centrifuged at 11,000 × g at 4 °C for 20 min and filtered using a 0.45-µm SmartPor®-11PVDF syringe filter (Woongki Science Co., Ltd., Seoul, Korea). Two volumes of ice-cold 100% ethanol were added to the filtrate and incubated at 4 °C for 24 h. The precipitate was collected by centrifugation at 4 °C for 20 min at 11,000 × g, dissolved in distilled water, and dialyzed using dialysis-sack (molecular weight cut-off 12–14 kDa, Spectra/Por® membrane, Pre-wetted RC Tubing, Spectrum Laboratories, Inc., Broadwick Street, UK) for 24 h. Dialyzed EPS was then freeze-dried using a vacuum freeze-dryer (SUNILEYELA Co., Ltd., Seongnam, Korea).

Monosaccharide composition analysis by high-performance anion-exchange chromatography

EPS from *B. bifidum* EPS DA-LAIM was completely hydrolyzed to monosaccharides using 2 M trifluoroacetic acid (TFA) for 2 h. The residual acid was removed by flushing with N₂ gas, re-dissolving the dried precipitate in deionized water, and filtering through a 0.45 µm nylon syringe filter (GVS, Indianapolis, IN, USA). The filtered samples were analyzed using a high-performance anion-exchange chromatography (HPAEC) system (Dionex, Sunnyvale, CA, USA). A CarboPac PA-1 anion-exchange column and PA-1 guard column (Thermo Fisher Scientific, Waltham, MA, USA) were used to separate the monosaccharides. Twenty millimolar NaOH was used for isocratic separation of eluent (Hemmerich et al., 1994; Panagiotopoulos et al., 2001), and 120 mM NaOAc in 100 mM NaOH and 200 mM NaOH were used for residual washing and re-equilibrium processes before the next sample injection at 25 °C. Each monosaccharide used to identify the peak retention time was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Molecular weight analysis by high-performance size-exclusion chromatography

EPS from *B. bifidum* EPS DA-LAIM was dissolved in deionized water to make a 0.2%(w/v) solution, followed by filtering through a 0.45 µm nylon syringe filter (GVS). The filtered sample solution was analyzed using a high-performance size-exclusion chromatography (HPSEC) system (Ultimate 3000, Thermo Fisher Scientific) with a refractive index detector (RID, Thermo Fisher Scientific) at 50 °C. Shodex OHPak SB-804 and SB-802.5 HQ analytical columns (Shodex, Tokyo, Japan) were used for chromatographic separation in a column oven at 50 °C, and filtered deionized water (18 mΩ) was used as the column

eluent at 0.4 a flow rate (Alves et al., 2010). A standard curve was constructed using the P-82 pullulan standard (Shodex).

Methylation analysis for glycosidic linkage types by gas-chromatography mass-spectrometer

EPS from the *B. bifidum* EPS DA-LAIM sample was per-methylated using a NaOH/DMSO slurry with methyl iodide and separated into a dichloromethane layer for washing to obtain methyl derivatives. The *O*-methyl-derivative sample was fully hydrolyzed by 2 M trifluoroacetic acid (TFA), followed by the removal of the residual TFA by adding methanol and flushing with N₂ gas. The hydrolyzed residues were dissolved in NH₄OH and reduced with NaBD₄ to form linear sugars. The remaining hydroxyl groups were acetylated using acetic anhydride at 100 °C for 2.5 h and terminated by the addition of deionized water. Partially methylated alditol acetate (PMAAs) derivatives were separated into dichloromethane layers and dried by flushing with N₂ gas. The dried sample was re-dissolved in acetone as the final solvent for gas chromatography-mass spectrometry (GC-MS) analysis. The PMAAs were separated using a GC-MS system (Thermo Scientific™ ISQ 7000, Waltham, MA, USA) with a TraceGOLD TG-5MS GC column (Thermo Scientific™; 0.25 μm thickness; 0.25 mm ID; 30 m length). The analytical conditions was gradient mode: 50–300 °C at 10 °C/min with He as the carrier gas. Each PMAA peak was identified by referring to the PMAA mass spectral database of the Complex Carbohydrate Research Center (<https://glygen.ccrcc.uga.edu/ccrc/specdb/ms/pmaa/pframe.html>).

Acid-tolerance

The *Bifidobacterium* strain was cultured in MRS broth supplemented with 2%(w/v) sucrose and incubated at 37 °C for 24 h anaerobically. For both EPS-containing and EPS-free bacterial samples, an MRS medium containing 2%(w/v) sucrose with 1,000 units pepsin/ml (Sigma-Aldrich) at pH 3.0 was used. EPS-containing samples were inoculated at a final concentration of 1%(v/v) in the medium. The EPS-removed bacterial sample was centrifuged at 11,000 × g for 20 min, washed three times with 0.85%(w/v) NaCl, and resuspended in the medium. The samples were serially diluted and spread on MRS agar as a control (0 h). After 2 h incubation at 37 °C anaerobically, the EPS-containing sample and EPS-removed sample were serially diluted using 0.85%(w/v) NaCl and spread onto MRS agar as a sample (2 h). Agar plates were incubated at 37 °C for 48 h, and the viable cells were counted. Acid tolerance was calculated using Eq. (1).

$$\text{Acid-tolerance}(\%) = \frac{\log(\text{Viable cell number of sample})}{\log(\text{Viable cell number of control})} \times 100 \quad (1)$$

Bile-tolerance

The *Bifidobacterium* strain was cultured as described in the acid-tolerance section. For both EPS-containing and EPS-free bacterial samples, an MRS medium containing 2%(w/v) sucrose and 0.3%(w/v) Bacto O_xgall (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was used. The EPS-containing and EPS-free samples were treated as described in the acid-tolerance section. The samples were serially diluted and spread on MRS agar as a control (0 h). After 3 h incubation at 37 °C anaerobically, the EPS-containing sample and EPS-free sample were serially diluted using 0.85%(w/v) NaCl and spread onto MRS agar as a sample (3 h). Agar plates were incubated at 37 °C for 48 h anaerobically, and the viable cells were counted. Bile tolerance was calculated using Eq. (2).

$$\text{Bile-tolerance}(\%) = \frac{\log(\text{Viable cell number of sample})}{\log(\text{Viable cell number of control})} \times 100 \quad (2)$$

Pancreatin tolerance

The *Bifidobacterium* strain was cultured as described in the acid-tolerance section. For both EPS-containing and EPS-free bacterial samples, MRS broth supplemented with 2%(w/v) sucrose and 0.5% pancreatin (Sigma-Aldrich) was used. The EPS-containing and EPS-free samples were treated as described in the bile-tolerance section. Pancreatin-tolerance was calculated using Eq. (3).

$$\text{Pancreatin-tolerance}(\%) = \frac{\log(\text{Viable cell number of sample})}{\log(\text{Viable cell number of control})} \times 100 \quad (3)$$

DPPH radical scavenging activity

For the assay, a 0.2 mM DPPH solution dissolved in 1 ml ethanol was added to the bacterial cell pellet (5 × 10⁸ CFU) dissolved in 1 ml PBS. The mixed solution was allowed to react at 25 °C for 30 min in the dark, and the reaction solution was centrifuged at 13,500 × g for 3 min at 4 °C. The supernatant was added to each well of a 96-well plate, and the absorbance was measured at 517 nm. Ascorbic acid (0.1 mg/ml) was used as a positive control. The DPPH radical scavenging activity was calculated using Eq. (4).

$$\begin{aligned} & \text{DPPH scavenging activity}(\%) \\ & = \left(\frac{\text{ControlOD}_{517} - \text{SampleOD}_{517}}{\text{ControlOD}_{517}} \right) \times 100 \end{aligned} \quad (4)$$

Gut adhesion ability assay using Caco-2 cells

The *Bifidobacterium* strain was cultured in 5 ml of MRS medium supplemented with 2%(w/v) sucrose at 37 °C for 24 h. To prepare EPS-containing samples, bacterial cell pellet was obtained by centrifugation at 5,000×g for 3 min and resuspended in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (Corning) adjusted with pH 6.0. To prepare the EPS-free samples, the cell pellet was prepared by centrifuging 1 ml of the bacterial culture at 11,000×g for 20 min. The pellet was washed three times with Dulbecco's phosphate buffered saline (DPBS; Welgene, Gyeongsan, Korea) and resuspended in 1 ml Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (Corning) adjusted with pH 6.0. Cultivation of Caco-2 cells and treatment of bacterial cells were performed as described previously (Kwon and Park, 2021). The adhesion ability was calculated using Eq. (5).

$$\text{Adhesion ability}(\%) = \frac{\log(\text{Adhered bacteria cell number})}{\log(\text{Initial bacterial cell number})} \times 100 \quad (5)$$

Prebiotic index

One hundred microliters of mMRS broth or TSB broth without glucose supplemented with 2%(w/v) EPS was dispensed into a 96-well plate, and the cultures of, *Lactobacillus helveticus*, *Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum*, *B. bifidum*, and *B. animalis* were inoculated at a final concentration of 1%(w/v). For comparative experiments, the same experiment was conducted on mMRS broth or TSB broth without glucose with 2%(w/v) inulin, and the same experiments were conducted using a culture medium without a carbon source or with 2%(v/v) glucose as a control. The prebiotic index was calculated using Eq. (6):

$$\text{Prebiotic index} = \frac{\text{Optical density of probiotic culture at 600 nm}}{\text{Optical density of } E. coli \text{ culture at 600 nm}} \quad (6)$$

Determination of cytotoxicity on RAW 264.7 cells

The cytotoxicity and proliferation of *B. bifidum* EPS DA-LAIM cells and EPS in a RAW 264.7 murine macrophage cell line were evaluated by GeoVista. RAW 264.7 cells

were seeded into DMEM to make 1×10^5 cells per well and incubated at 37 °C with 5% CO₂. RAW 264.7 macrophage cells were treated with *B. bifidum* EPS DA-LAIM cells at 1.0×10^6 cells and treated with EPS at concentrations of 1, 10, 25, and 50 µg/ml. RAW 264.7 cells were treated with LPS at a final concentration of 1 µg/ml as a positive control. After sample treatment, cells were incubated at 37 °C with 5% CO₂ for 24 and 48 h. After 24 and 48 h of sample treatment, cell viability of RAW 264.7 cells was measured using the MTT assay.

Determination of nitric oxide production and expression of cytokines

To measure the nitric oxide (NO) production, RAW 264.7 cells were seeded at 5×10^5 cells per well in a 6-well plate and incubated at 37 °C with 5% CO₂ for 20 h, and RAW 264.7 cells were treated with *B. bifidum* EPS DA-LAIM and EPS with the same method as that used for the determination of cytotoxicity. RAW 264.7 cells treated with 1 µg/ml lipopolysaccharide (LPS) were used as a positive control. NO secretion and the expression of cytokines IL-1β and IL-6 were determined using Griess Reagent System (G2930, Promega, Madison, WI, USA), Mouse IL-1β ELISA Kit (ab100704, Abcam, Cambridge, UK), and Mouse IL-6 ELISA Kit (ab100712, Abcam), respectively.

Whole genome sequencing of *Bifidobacterium bifidum* EPS DA-LAIM

Whole-genome sequencing of *B. bifidum* EPS DA-LAIM was performed by CJ Bioscience Inc. (Seoul, Korea) using the Illumina MiSeq platform (Illumina, San Diego, CA, USA). Genomic sequences were screened for the presence of antibiotic resistance genes using ResFinder v.4.1 software and the Comprehensive Antibiotic Resistance Database (CARD) (<https://card.mcmaster.ca/>), and the resistance gene identifier software RGI v.5.2.0. ResFinder analysis was performed by default, and the RGI analysis criteria were perfect, strict, and loose hits. Virulence genes were retrieved using VirulenceFinder 2.0. An analytical database system was provided by the Center for Genomic Epidemiology (CGE). Homologous sequences to virulence genes of *Escherichia coli*, *Listeria*, *Staphylococcus aureus*, and *Enterococcus* were screened, and thresholds for percent identity (%ID) and minimum length were set at 90% and 60%, respectively.

Antibiotic susceptibility test

Antibiotic susceptibility was determined using an E-test strip (E-TEST®, bioMérieux, Marcy-l'Étoile, France), according to the manufacturer's instructions. The minimum inhibitory concentration (MIC) of the antibiotics was compared with the cut-off values for each antibiotic, guided by the European Food Safety Authority (EFSA, 2012).

Statistical analysis

All experiments were performed in triplicate, and the results are expressed as the mean \pm standard deviation (SD). Statistical significance of the experimental data was verified by one-way analysis of variance (ANOVA) using SPSS 25 (SPSS Inc., Chicago, IL, USA). Dunnett's test and Duncan's test were used to determine significant differences between multiple groups at $p < 0.05$.

Results and discussion

Isolation and identification of *B. bifidum* EPS DA-LAIM

To screen for EPS-producing bifidobacteria, 129 strains were isolated from human fecal samples. Among them, the strain EPS DA-LAIM was selected for this study because it has the highest EPS production rate and ropiness. Strain EPS DA-LAIM was identified as *Bifidobacterium bifidum* by phylogenetic tree analysis based on the nucleotide sequence of the 16S rRNA gene (Fig. S1).

Visualization of EPS

Because the selected *B. bifidum* EPS DA-LAIM strain produced significant quantities of EPS, the EPS was visible by crystal violet staining. *B. bifidum* EPS DA-LAIM cells and the EPS around the cells were stained purple with crystal violet, and the stained area was more obvious when the cells were cultured with sucrose supplementation than when cultured with glucose supplementation (Fig. 1A and B). *Bifidobacterium bifidum* DLP1224, an EPS-non-producing bacterium, was not stained around the cells (Fig. 1C). The culture of *B. bifidum* EPS DA-LAIM was highly viscous due to the ropy EPS, and three-layer separation of culture medium-EPS-bacteria cells was observed in the culture (Fig. 1D and E).

When the ropiness of the *B. bifidum* EPS DA-LAIM colony was checked, its length was determined to be 50 mm (Fig. 1F) and the freeze-dried EPS is shown in Fig. 1G.

Nakajima et al. (1992) reported that ropy-fermented milk-fed rats showed the highest ratio of high-density lipoprotein cholesterol/total cholesterol, indicating the important role of the extracellular material. When the culture broth of *Bifidobacterium bifidum* EPS DA-LAIM was observed using a scanning electron microscope, EPS were found to be particles on the cell surface (Fig. 1H). However, EPS was not found around the cells after centrifugation and washing, which is consistent with the results of crystal violet staining (Fig. 1I). Flemming and Wingender (2010) reported that polysaccharides are fine strands attached to the cell surface and form networks, which is similar to the results of this study.

Structural analysis of EPS from *B. bifidum* EPS DA-LAIM

The monosaccharide composition results (Fig. 2A) confirmed that *B. bifidum* EPS DA-LAIM mainly consisted of glucose and mannose, and small amounts of other monosaccharides (fucose, arabinose, glucosamine, and galactose) were also included in the EPS sample. The amount of mannose was greater than that of glucose, and the molar ratio of glucose to mannose was 0.26:1. EPS from *B. bifidum* EPS DA-LAIM had two main peaks, described as Mp1 (1007 kDa) and Mp2 (408 kDa), as shown in Fig. 2B. Other molecules in EPS, except for the two main peaks, mostly existed between 408 and 1007 kDa. These results suggest that EPS from *B. bifidum* EPS DA-LAIM is a glucomannan-type heteropolysaccharide with molecules of 408–1007 kDa. Methylation analysis showed that *B. bifidum* EPS DA-LAIM predominantly contained 2-mannosyl, 6-mannosyl, and 2,6-mannosyl residues (branching points) in its structure (Fig. 2C). Most glucose residues have a terminal glucose (t-Glc) form, which has a glycosidic linkage via only C₁ (anomeric carbon) except other carbons. *B. bifidum* EPS DA-LAIM has a highly branched structure with an oligo-2-mannosyl side chain on the 6-mannan backbone, similar to the glucomannan structure described in a previous study (Alves et al., 2010). Glucose residues were linked at the end of the mannosyl side chains, and galactose residues were expected to be linked to the mannose backbone or side chains (Daba et al., 2021). Figure 2D describes the proposed structure of EPS from *B. bifidum* EPS DA-LAIM according to the overall structural analysis results and a previous study.

Other reports show that EPS from *B. animalis* RH consists of glucose, mannose, galactose, arabinose, fructose, and rhamnose, which have pro-inflammatory effects. EPS from *B. longum* subsp. *longum* 35,624 consists of glucose, galactose, galacturonic acid, and 60-deoxy-talose and has an anti-inflammatory effect (Altmann et al., 2016). These EPS were not glucomannan-type and did not share a structure with EPS from *B. bifidum* EPS DA-LAIM.

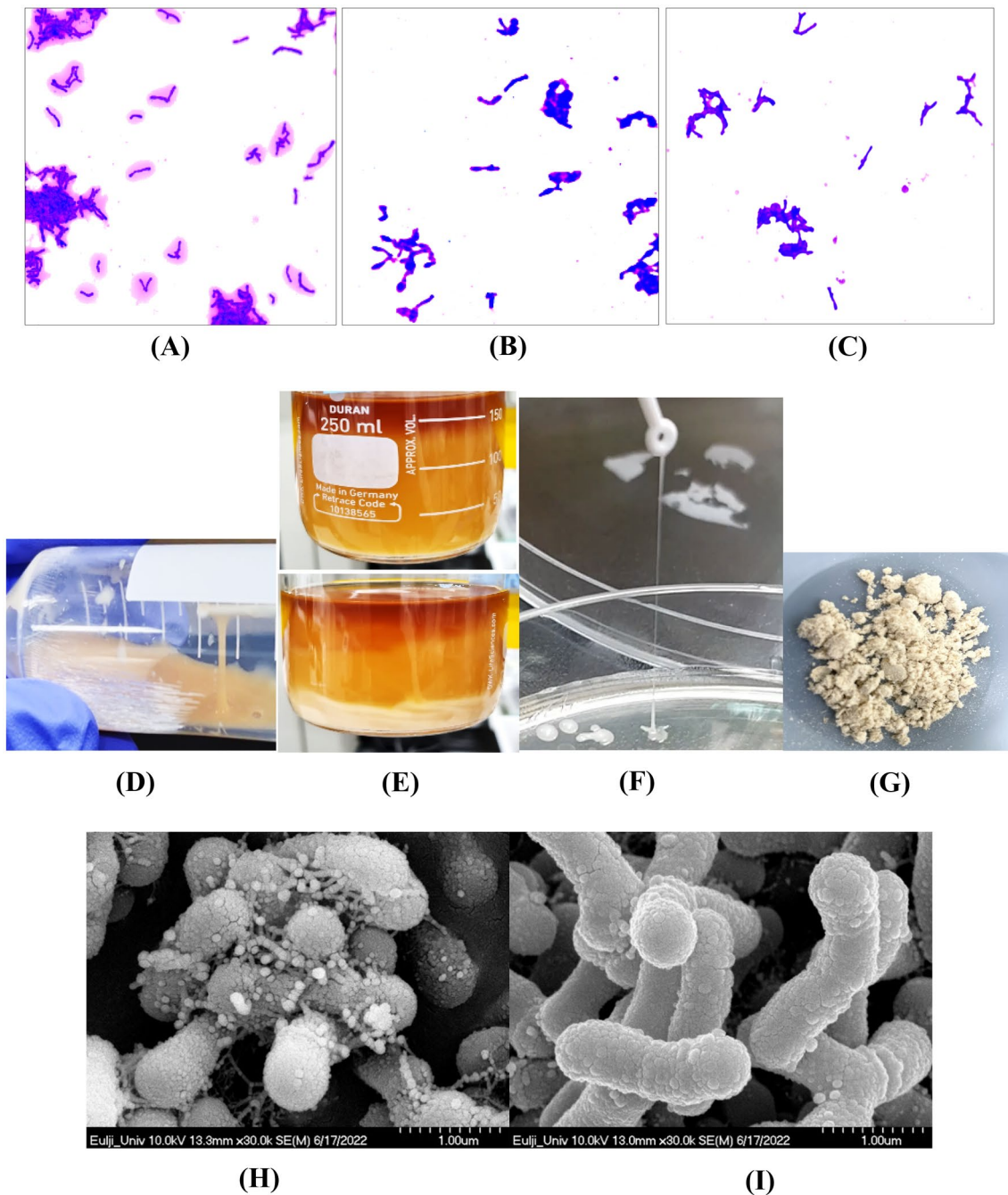


Fig. 1 Visualization of EPS produced by *B. bifidum* EPS DA-LAIM. **A** Crystal violet staining of *B. bifidum* EPS DA-LAIM cultured in BL+5% sucrose; **B** Crystal violet staining of *B. bifidum* EPS DA-LAIM cultured in BL+5% glucose; **C** Crystal violet staining of *B. bifidum* DLP1224 cultured in BL+5% sucrose; **D** Ropiness of *B. bifidum* EPS DA-LAIM culture broth; **E** three-layer separation of culture

medium-EPS-bacteria cells in the culture broth **F** Ropiness of EPS from *B. bifidum* EPS DA-LAIM colony; **G** Freeze-dried EPS powder; **H** Scanning electron microscope's image of the culture medium of *B. bifidum* EPS DA-LAIM; **I** Scanning electron microscope's image of *B. bifidum* EPS DA-LAIM treated by centrifugation and washing with PBS ($\times 10,000$)

Tolerance to Gastrointestinal Environment

Bifidobacterium bifidum EPS DA-LAIM cells showed higher tolerance to acid, bile salts, and pancreatin in the presence of EPS than in the absence of EPS (Fig. 3, Table S1). The

tolerances of *B. bifidum* EPS DA-LAIM to acid, bile salts and pancreatin were significantly higher than those of *B. bifidum* DLP1224, an EPS-non-producing strain or those of *B. bifidum* KCTC 13270BP, an EPS-producing patent strain. This result implies that EPS from *B. bifidum* EPS DA-LAIM

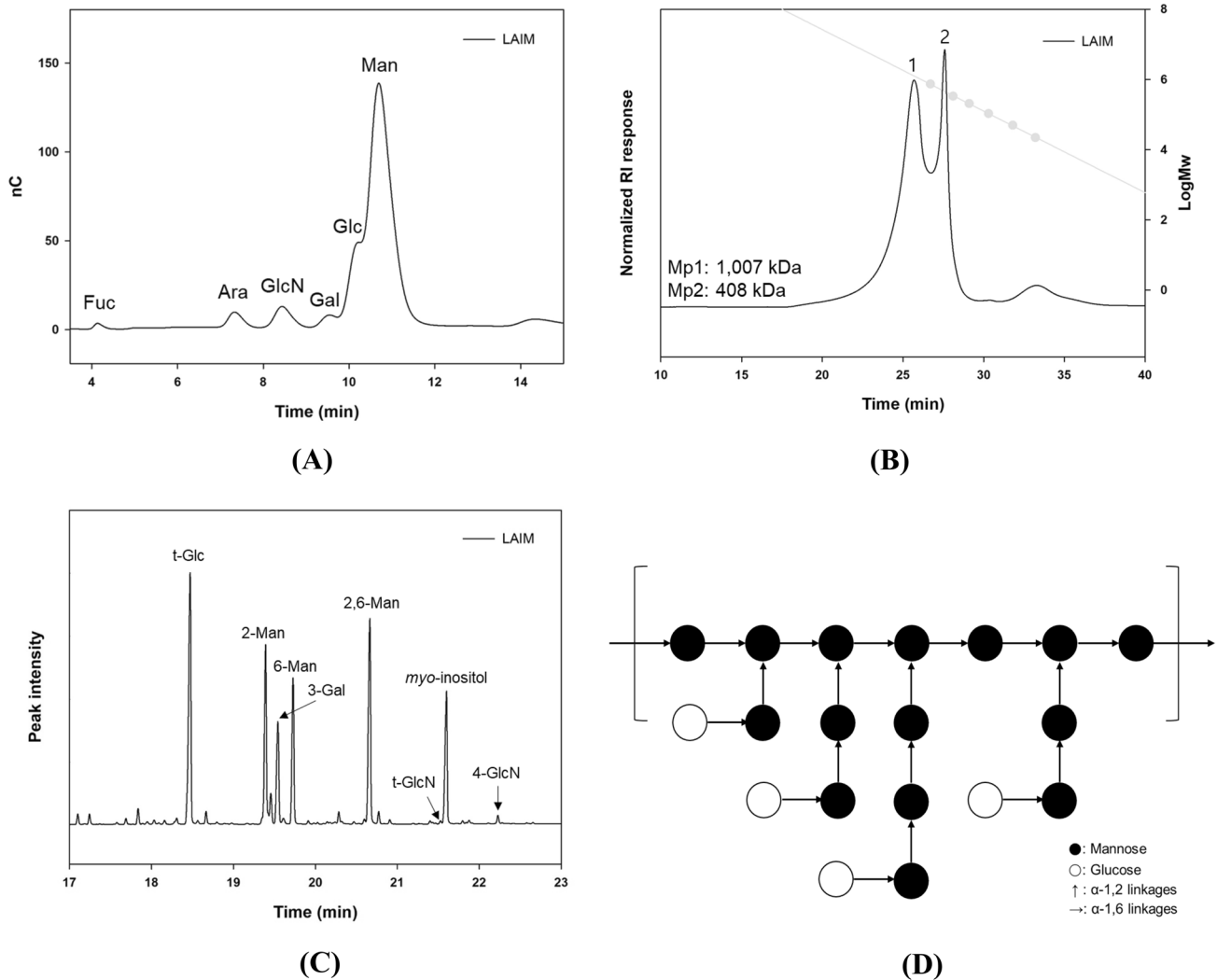


Fig. 2 Determination of the structure of EPS from *B. bifidum* EPS DA-LAIM. **A** Monosaccharide composition of EPS from *B. bifidum* EPS DA-LAIM. Fuc, fucose; Ara, arabinose; GlcN, glucosamine; Gal, galactose; Glc, glucose; Man; mannose. **B** Molecular weight distribution of EPS from *B. bifidum* EPS DA-LAIM. Grey symbols

were pullulan standard from P-20 to P-800. **C** Glycosyl linkage types of EPS from *B. bifidum* EPS DA-LAIM identified by mass spectra of PMAAs. *Myo*-inositol acted as the internal standard. **D** Schematic illustration of the proposed structure of EPS from *B. bifidum* EPS DA-LAIM

had a protective effect on *B. bifidum* EPS DA-LAIM cells in the gastrointestinal environment, and its protective effect was higher than those of EPS-producing *B. bifidum* KCTC 13270BP.

Tolerance to human gastric transit is an important selection criterion for probiotics. This bacterium may be able to better tolerate stomach acid and bile salts if it has an EPS protective layer (Brink et al., 2006). According to Takahashi et al. (2004), most strains under study showed survival rates of less than 1% at pH 3.0. Serafini et al. (2013) reported that the viabilities of *B. bifidum* PRL2010 after treatment with pH 3, 0.5% oxgall, and pancreatin were 16.2, 36.2, and 71.7%, respectively. This study indicated that the EPS-producing bifidobacterial strain, *B. bifidum* EPS DA-LAIM,

possesses higher tolerance to acid, bile salt, and pancreatin than EPS-non-producing bifidobacterial strains.

Gut adhesion ability by Caco-2 cell lines

The gut adhesion abilities of *B. bifidum* EPS DA-LAIM cells with EPS and without EPS were 73.8% and 71.6%, respectively, and EPS slightly promoted the gut adhesion activity (Fig. 4) The gut adhesion abilities of *B. bifidum* EPS DA-LAIM was similar to that of *Lactocaseibacillus rhamnosus* GG, representative probiotic strain. Gut adhesion of *B. bifidum* PRL2010, *B. bifidum* DSM20215, *B. bifidum* KCTC337, and *B. bifidum* KCTC5082 to HT-29 cells was lower than that of *B. bifidum* EPS DA-LAIM

Fig. 3 Gastrointestinal tract tolerance of *Bifidobacterium bifidum* strains. **A** Acid tolerance. **B** Bile-salt tolerance. **C** Pancreatin tolerance. LAIM (+EPS), EPS-containing *B. bifidum* EPS DA-LAIM culture; LAIM (-EPS), EPS-free *B. bifidum* EPS DA-LAIM cell pellet; 13270BP, *B. bifidum* KCTC 13270BP; DLP1224, *B. bifidum* DLP1224

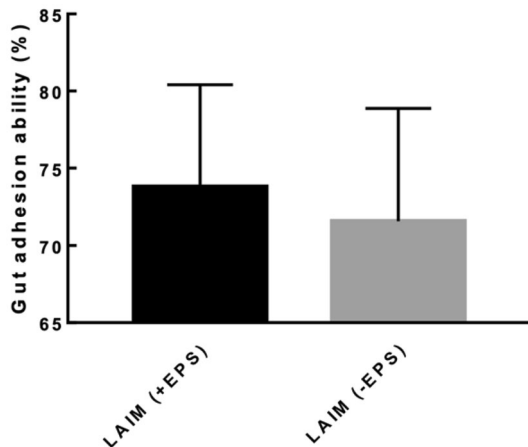
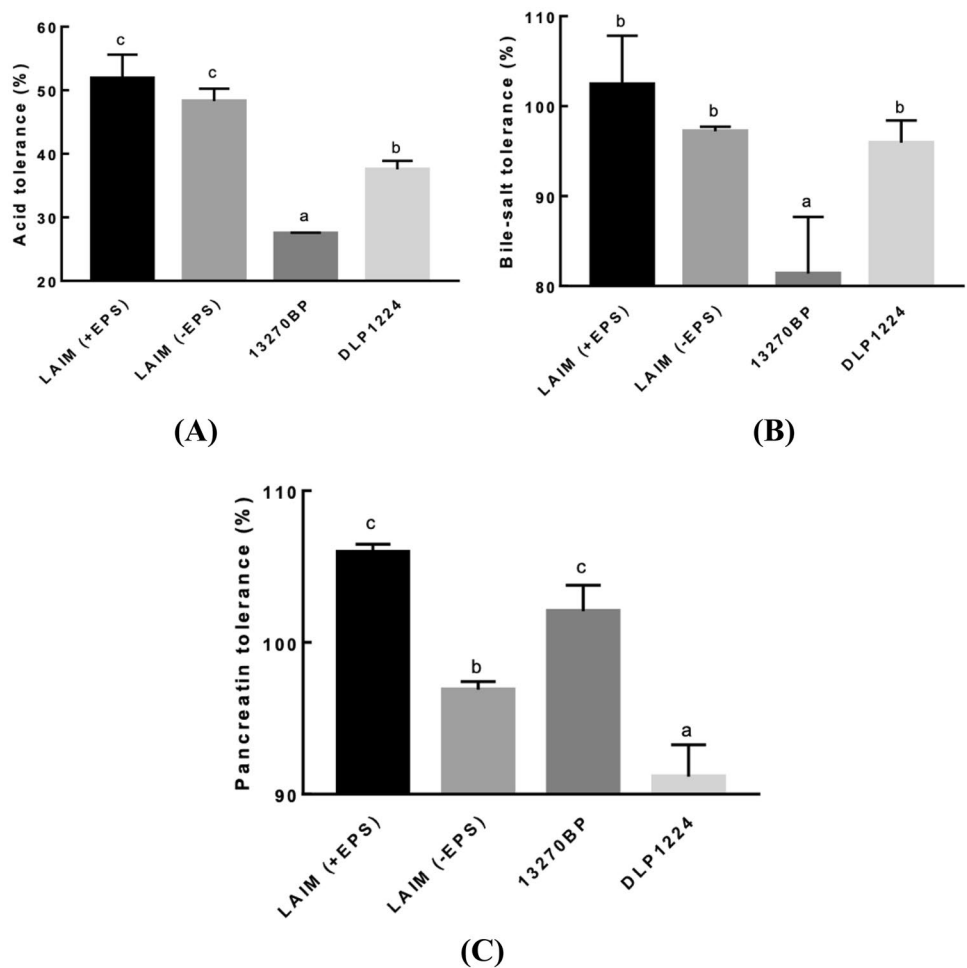


Fig. 4 Gut adhesion activity of *Bifidobacterium bifidum* strains. LAIM (+EPS), EPS-containing *B. bifidum* EPS DA-LAIM culture; LAIM (-EPS), EPS-free *B. bifidum* EPS DA-LAIM cell pellet

(González-Rodríguez et al., 2012; Serafini et al., 2013) (Table S2).

It is crucial for probiotic bacteria to adhere to the intestinal epithelium, because it encourages colonization and has immunomodulatory effects (Turchi et al., 2013). The components of bifidobacterial cells, including carbohydrates, extracellular proteins, lipoteichoic acids, and surface proteins, on the outer surface of the cell wall act as adhesive factors (González-Rodríguez et al., 2012).

Immunostimulant effects of EPS-producing *B. bifidum* EPS DA-LAIM

The viability of RAW 264.7 cells by treatment with *B. bifidum* EPS DA-LAIM cells and EPS from *B. bifidum* EPS DA-LAIM was higher than 120%, which showed no cytotoxicity of *B. bifidum* EPS DA-LAIM cells and EPS (Fig. 5A, B). The difference in cell viability between 24 and 48 h of incubation was negligible. Treatment with *B. bifidum* EPS DA-LAIM cells to RAW 264.7 cells promoted NO production, and it was proportional to the concentration of EPS on both 24 h and 48 h of incubation (Fig. 5C, D). This result

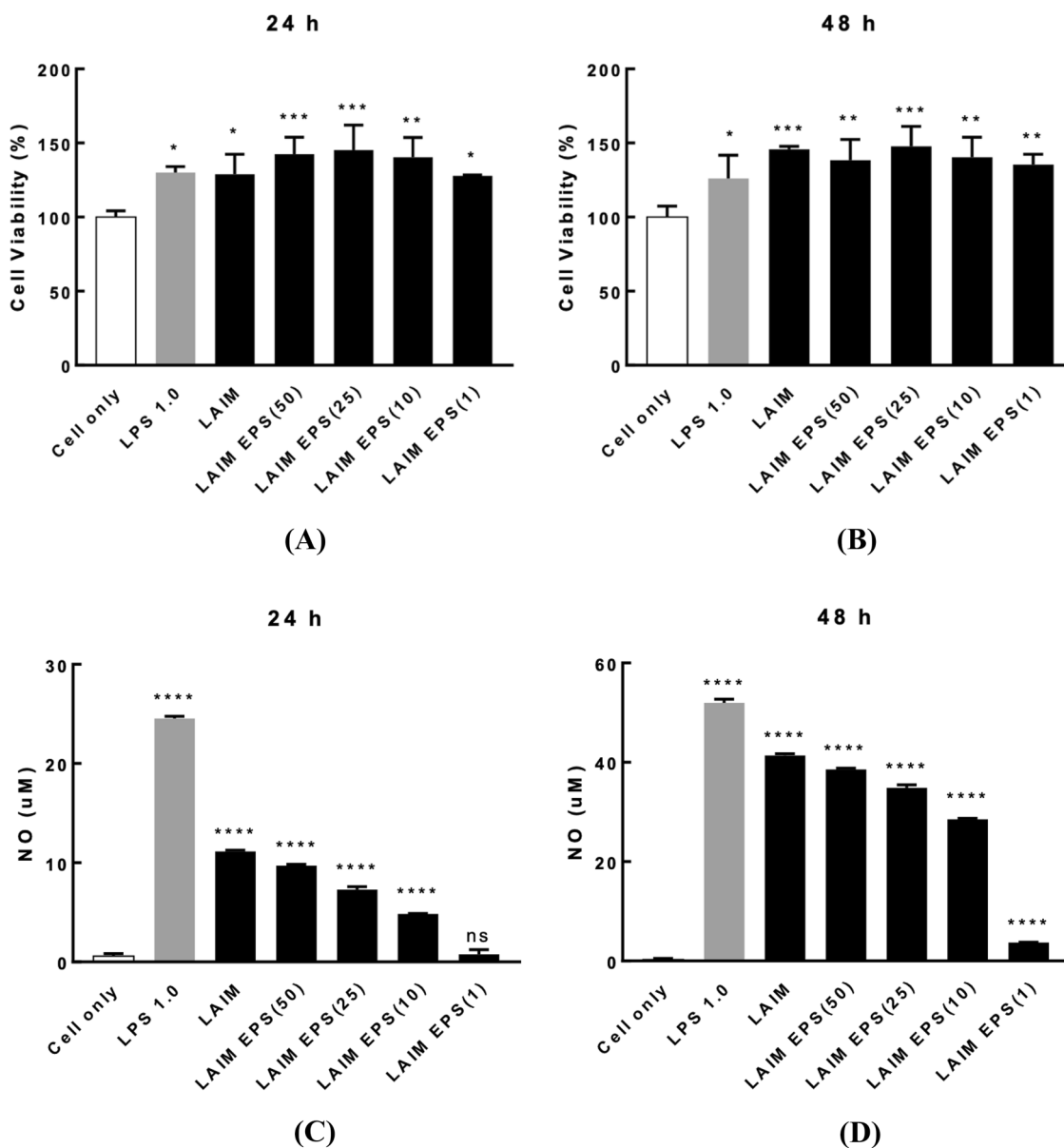


Fig. 5 RAW 264.7 cell viability and NO production by treatment of *B. bifidum* EPS DA-LAIM. **A** Cell viability by treatment of *B. bifidum* EPS DA-LAIM on 24 h of incubation. **B** Cell viability by treatment of *B. bifidum* EPS DA-LAIM on 48 h of incubation. **C** NO production by treatment of *B. bifidum* EPS DA-LAIM on 24 h of incubation. **D** NO production by treatment of *B. bifidum* EPS DA-LAIM on 48 h of incubation. Cell only, RAW 264.7 cells without sample treatment; LPS 1.0, treatment with 1.0 $\mu\text{g/ml}$ LPS; LAIM, *B.*

bifidum EPS DA-LAIM cells at a concentration of $1 \times 10^6/\text{ml}$ were used for treatment; LAIM EPS (50), 50 $\mu\text{g/ml}$ of EPS was used for treatment; LAIM EPS (25), EPS 25 $\mu\text{g/ml}$ of EPS was used for treatment; LAIM EPS (10), 10 $\mu\text{g/ml}$ of EPS was used for treatment; LAIM EPS (1), 1 $\mu\text{g/ml}$ of EPS was used for treatment. Asterisks show significant differences among groups at *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ ($n \geq 3$) using one-way ANOVA and Dunnett's test. ns, not significant

indicates that *B. bifidum* EPS DA-LAIM cells and EPS from *B. bifidum* EPS DA-LAIM had immunostimulant activity on RAW 264.7 cells, and EPS had a pivotal role in the immunostimulant activity of *B. bifidum* EPS DA-LAIM. When NO and cytokines are produced, the immune response is boosted in macrophages (Bogdan, 2001).

Cytokines are crucial for cell signaling and immune reactions. Interleukin (IL)-6 was expressed in RAW264.7 cells by treatment of *B. bifidum* EPS DA-LAIM as well as by treatment of EPS (Fig. S2a, b). IL-1 β was also expressed by treatment with *B. bifidum* EPS DA-LAIM as well as by treatment of EPS (Fig. S2c). IL1 β and IL-6 are proinflammatory cytokines secreted by mammalian macrophages upon

stimulation and activate different types of immune responses (Ayaz, 2018). Because IL1 β and IL-6 are regulated by NF- κ B, *B. bifidum* EPS DA-LAIM may enhance NF- κ B-mediated innate immunity (Vossenkämper et al., 2010).

DPPH radical scavenging activity

When the antioxidant ability was examined, the DPPH radical scavenging activity of *B. bifidum* EPS DA-LAIM cells (63.4%) was 1.5 times higher than that of 0.1 mg/ml ascorbic acid (43.2%) (Fig. S3). It is reported that DPPH scavenging activity of *B. longum* ATCC15708 is 41.6% and *L. acidophilus* ATCC4356 is 20.8%, which is lower than that of *B. bifidum* EPS DA-LAIM (Lin and Chang, 2000).

Prebiotic activity

When the prebiotic activity of purified EPS produced from *B. bifidum* EPS DA-LAIM was assessed using representative probiotic strains, EPS showed a high prebiotic index for all probiotic strains used in this study. The prebiotic index of the purified EPS produced by *B. bifidum* EPS DA-LAIM was higher than that of inulin, a representative prebiotic carbohydrate (Fig. 6). This result shows that EPS from *B. bifidum* EPS DA-LAIM had growth-promoting activity on probiotic strains, especially lactic acid bacteria, than inulin, indicating that EPS from *B. bifidum* EPS DA-LAIM is a promising prebiotic material.

Prebiotics are defined as ‘substrates that are selectively utilized by host microorganisms conferring a health benefit’ (Gibson et al., 2017). Previous studies have reported

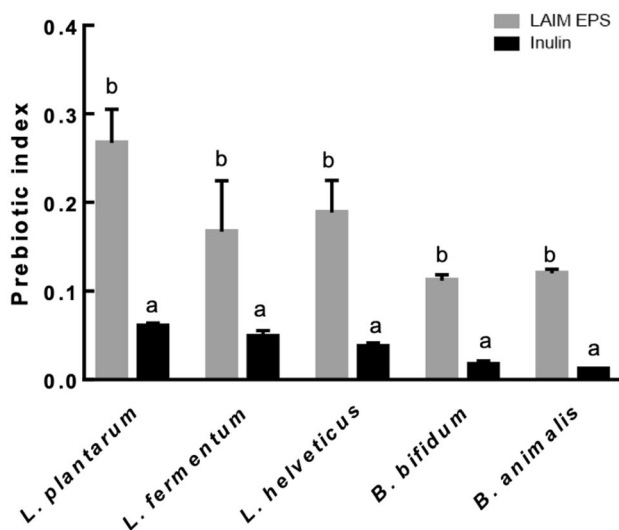


Fig. 6 Prebiotic effect of *B. bifidum* EPS DA-LAIM on probiotic strains. The black bar is the sample treated with inulin, and the gray bar shows the sample treated with EPS from *B. bifidum* EPS DA-LAIM

that EPS that reaches the intestinal level can resist gastric and intestinal degradation, with EPS acting as a substrate for bacterial growth in the intestinal tract. At the intestinal level, EPS act as a prebiotic that stimulates the growth of beneficial bacteria and the production of metabolites such as short-chain fatty acids, which play a key role in host health (Korcz et al., 2018).

Antibiotics susceptibility

When the antibiotic susceptibility of *B. bifidum* EPS DA-LAIM was tested using the E-test for nine antibiotics, the minimum inhibitory concentrations of all antibiotics tested were below the cut-off value, indicating that the sensitivity of *B. bifidum* EPS DA-LAIM to antibiotics was acceptable (Table S3). Potential probiotics must be safe to consume, and it is crucial to perform a minimal safety evaluation that includes antibiotic resistance to prevent clinical hazards. This result satisfied the criteria for antibiotic resistance in the Probiotic Safety Evaluation Guidelines for Functional Ingredients for Health Functional Food, published by the Korean Ministry of Food and Drug Safety in July 2021.

Analysis of the whole genome sequencing of *B. bifidum* EPS DA-LAIM

Whole genome sequencing was performed to identify antibiotic resistance (AMR) genes and virulence genes in the genome of *B. bifidum* EPS DA-LAIM. The whole genome sequence of *B. bifidum* EPS DA-LAIM consisted of nine contigs with a genome size of 2,236,819 bp, 1798 coding sequences, three rRNA genes, and 52 tRNA genes. The G + C content was 62.6%.

When AMR genes were analyzed using the RGI program, no perfect hits, three strict hits, and 100 loose hits were found. By filtering < 70% identity hits for matching CARD references, the AMR gene was obtained with one loose hit for elfamycin and two strict hits for mupirocin-like antibiotic and rifamycin (Table S4). ResFinder analysis did not identify a matching AMR gene. No virulence genes were found in the *B. bifidum* EPS DA-LAIM genome when the genome sequence was compared with the sequences of four well-known pathogens. Although elfamycin, mupirocin-like antibiotic, and rifamycin were detected as loose hits and strict hits, these antibiotics were not included in the list of important human and veterinary antibiotics in the EFSA guidelines (ESFA et al., 2018).

In this study, rropy-type EPS-producing *B. bifidum* EPS DA-LAIM was isolated and the structure of purified EPS from the strain was successfully analyzed. The EPS from *B. bifidum* EPS DA-LAIM are glucomannan-type heteropolysaccharides. The structure of EPS included mannose subunits linked with α -1,6 glycosidic linkage as a backbone

and α -1,2 glycosidic linkages as branches, which is different from that of glucomannan which is generally found in plants, whose backbone consists of β -1,4 glycosidic linkages of mannose. Purified EPS promoted the growth of health-beneficial probiotic strains; therefore, it can be used for the development of prebiotic materials. EPS-producing *B. bifidum* EPS DA-LAIM showed acid, bile salt, and pancreatin tolerance properties, and EPS itself improved gastrointestinal tract tolerance. *Bifidobacterium bifidum* EPS DA-LAIM also showed high antioxidant activity, gut adhesion ability, and immunostimulatory activity in macrophages, and its antibiotic susceptibility satisfied the criteria for antibiotic resistance guided by the Korean Ministry of Food and Drug Safety. Because of the gastrointestinal tract tolerance, high gut adhesion ability, immunostimulatory properties, and acceptable antibiotic susceptibility, *B. bifidum* EPS DA-LAIM is a potential probiotic candidate, and the mechanism of immunostimulatory activity on macrophages must be elucidated.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10068-022-01213-w>.

Declarations

Conflicts of interest The authors declare no conflicts of interest.

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