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Monosaccharide composition influence and immunomodulatory effects of probiotic exopolysaccharides

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ABSTRACT

Exopolysaccharides (EPSs) are metabolites of probiotics that have gained wide interest recently. A strain of *Lactobacillus reuteri* Mh-001 with high exopolysaccharide (EPS) production ability was isolated, identified, and were used to investigate the anti-inflammatory effects of the EPSs. Among the three unpurified EPSs, RAW246.7 murine macrophages treated with 5 ppm of EPS 1 revealed the lowest tumour necrosis factor α (TNF-α) secretion (325.32 ± 51.10 pg/ug DNA). The second lowest TNF- α secretion occurred with EPS 2 (701.12 ± 86.108 pg/ug DNA) from Mh-002. EPSs 4, 5, and 6 were further purified from EPS 1. Cells treated with 1 ppm of EPS 4 had the lowest TNF-α secretion of all (209.20 ± 84.34 pg/ug DNA). The monosaccharide components, EPS 4 and EPS 1, had the highest galactose content (45 ± 2.75% and 39 ± 2.75%, respectively). The monosaccharide percentages (galactose > rhamnose > glucose) were related to the anti-inflammatory activity of the EPSs. The galactose content of EPSs enhanced their anti-inflammatory effects on the macrophages. These data indicate that EPS possesses beneficial physiological effects such as anti-inflammatory properties, and the monosaccharide content of the EPS was the factor influencing the anti-inflammatory properties.

1. Introduction

The probiotic effects arise not only from microorganisms and bacterial wall components, but also from metabolites such as peptides and exopolysaccharides (EPS) produced during fermentation. EPSs from lactic acid bacteria have gained more attention owing to their health benefits. Lactic acid bacteria, food-grade organisms, are 'generally-recognized-as-safe', produce EPSs that are potentially useful as additives to improve texture and prevent synaeresis of fermented milk products. Additionally, some EPSs produced by lactic acid bacteria could confer health benefits to the consumer.

EPSs occur in two forms depending on where they are produced by the bacterial cell. Capsular polysaccharides and slime polysaccharide are included, where the polymer is closely and loosely associated with the bacterial surface, respectively. Since some bacteria release capsular polysaccharide material at the periphery of the capsule, it may be difficult to discern [1]. Protection against toxic compounds, bacteriophages, osmotic stress, bacterial desiccation are thought for EPS's function, and to permit adhesion to solid surfaces and biofilm formation [2].

EPSs are glycopolymers present on the surface of many bacteria. Due to the ability of these polymers to confer desirable sensorial attributes to the products, lactic acid bacteria producing EPS are traditionally used for the manufacturing of fermented dairy products, such as increasing viscosity or firmness, or improving its texture and stability [3].

As to EPS-producing bacteria, it is not clear whether EPS favour or prevent the transitory colonisation of the gut [4]. However, the total bifidobacteria population of the intestinal contents of rats significantly increased resulting in modification of the bacterial population and metabolic activity of the intestinal microbiota after oral administration of EPS-producing bifidobacteria in milk suspensions [5]. Purified polymers found in human fermented faecal matter had a similar effect [6]. EPS isolated from *Lactobacillus* and *Bifidobacterium* counteracted the effects of bacterial toxins and enteropathogens, providing potential benefits to the host [7–9]. In addition, other health benefits imparted by probiotics are attributed to their EPSs as is the case for the immunomodulatory capability of some of the biopolymers [10,11].

Pro-inflammatory cytokine Tumour necrosis factor-α (TNF-α) induces the production of terminal inflammatory mediators, such as pro-inflammatory interleukins and eicosanoid from inflammatory cells [12]. Crohn's disease treatment has been non-specific anti-inflammatory agents or the administration of immunosuppressive [13]. Recent strategies for Crohn's disease treatment include immune activation attenuation in the gastrointestinal tract and therapeutic agents treatment including IL-10, probiotics, and pro-inflammatory cytokines (such as TNF-α) neutralization by antibodies [14,15]. Many cell

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types including T cells and macrophages/monocytes produce IL-10 and TNF-α [16]. Microbial metabolites (such as polysaccharide) activate macrophages. The activated macrophages kill the bacteria by secrete cytokines to modulate immunity, phagocytosis, and present bacterial antigens to helper T cells [17].

The aims of this study were to screen and identify probiotic species producing EPSs. We determined the EPS molecular weights, purified the EPSs by anion-exchange chromatography, and determine the monosaccharide composition of different crude EPSs and purified EPSs. Additionally, we determined the TNF-α concentrations produced by murine RAW 264.7 macrophages treated with various concentrations of EPSs. Finally, we clarified the relationship between the chemical composition of monosaccharides and the immunomodulatory effects of the EPSs.

2. Materials and methods

2.1. Screening and identification

We collected fermented vegetable samples (20 g) from a traditional market in Kaohsiung, Taiwan, were suspended in 100 mL sterile water, followed the method of Nguyen et al. [18]. We used serial dilution and the spread-plate method for obtaining the different microorganisms. We used de Man, Rogosa, and Sharpe medium (MRS), Trypticase Soy-Yeast Extract Broth (TPY), and the streak-plate method to isolate colonies in the original inoculants. All the bacteria were grown aerobically in 100 mL of MRS broth [19] rotated at 120 revolutions/min at 37 °C. For the identification to species, we used the Gram stain, catalase test, and motility test for the preliminary identification of the bacteria. In addition, we use the Triple Sugar-Iron Agar Test (producing orange coloured medium) and eosin-methylene blue agar (producing purple coloured colonies on the agar). Strain Mh-001 was identified based on morphological, physiological characteristics, and 16S rDNA sequence analysis. The full length 16S rDNA gene sequence was amplified by polymerase chain reaction (PCR), and primers 16sF1 [5′ TACCCGTGCAG AAGCG 3′] and 16sR1[5′ TACCCGTGCAGAAGCG 3′]. Sequence analysis was performed by the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic analysis was performed with a MEGA version 4.1 program by neighbor joining (NJ)/maximum parsimony (MP) [20].

2.2. EPS extraction and detection

For pre-cultured medium, 1% bacteria was inoculated into Fresh MRS (100 mL) medium and cultivated for 48 h, and cell number was counted in Petroff-Hausser counting chamber (Fisher Scientific, Waltham, MA, USA) after 2 days of incubation using centrifuged (20 min at 6000 ×*g*) 1-mL culture samples. Two volumes of cold (4 °C) ethanol were added to one volume of supernatant, and the mixtures were stored at 4 °C overnight. Precipitates were collected by centrifugation for 5 min at 6000 ×*g*, resuspended in one volume of demineralized water, precipitated with two volumes of cold ethanol and centrifugation, and dried the pellets at 55 °C. EPS was determined by measuring total carbohydrate content of dried pellets.

Phenol-sulphuric acid method was used to determine the total amount of carbohydrate in the EPS [21]. A four-fold volume of double distilled water were used to dissolve the pellets and filtered by a 0.22-μm^2 filter (Millipore, Tokyo, Japan), and 25 mL, 80% phenol was added to 1-mL sample, which was washed rapidly in 2.5 mL of 98% H_2SO_4 after 10 min. The absorbance of the samples was measured at a wavelength of 490 nm, with a glucose solution as a standard.

2.3. Purification of EPS

We used the previously published extraction method of Tallon et al. [22] to extract the EPSs. After the freezing dry process, we use eight-fold deionized water to dissolve the EPSs, which were then filtered through a 0.22-μm filter and collected. We used anion exchange chromatography to purify negative net charge of EPS with an anion-exchange, sepharose gel-filled glass column (quaternary methyl ammonium Accel plus column, 1.5 m × 20 cm (GE Healthcare). The column was eluted with 0.05 M NH_4HCO_3, pH 8.0 at a 2 mL/min flow rate, and stepped gradient of 0.15 M to 1 M (0.15 M → 0.2 M → 0.3 M → 0.4 M → 0.5 M → 1 M) NaCl was followed in the same buffer. The purified EPSs were monitored spectrophotometrically at a wavelength of 490 nm. The fraction collector (Frac 900, GE Healthcare) collected one sample tube of EPS every 3 min.

2.4. TNF-α production by RAW264.7 macrophages

We cultured murine macrophage RAW264.7 cells in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% foetal calf serum and antibiotics (SAFC Biosciences, Lenexa, KS; 10% FCS-RPMI 1640) at 37 °C in a 5% CO_2 humidified incubator for the following in vitro experiments modified from Komatsu et al. [23]. 5×10^4 cells/well were grown in 12-well plates and treated with EPS at different concentrations for 24 h for the determination of cell growth. The cells were washed twice with fresh RPMI medium and then treated 1000 ng/mL of lipopolysaccharide (LPS) for 3 h to induce cell inflammation. At the indicated times, the media and cells were separated. TNF-α levels were measured in the media, and the cells were examined for viability using trypan blue (0.4% in PBS) exclusion staining and counted in a haemocytometer.

2.5. TNF-α measurement

TNF-α activity was measured in the media (produced according to Section 2.4) using L929 mouse fibroblasts in vitro cytotoxicity assay [23]. Briefly, L929 cells (Riken Cell Bank, Tsukuba, Japan) were plated in 50 μL of 10% foetal calf serum-supplemented RPMI 1640 in a 96-well flat-bottom plate (Iwaki Glass, Tokyo, Japan) at 1.5×10^4 cells/well and incubated for 24 h at 37 °C. TNF-α and recombinant murine TNF-α (GIBCO BRL, Gaithersburg, MD, USA) was used in a 25-μL aliquot of the serially diluted medium as a standard and of actinomycin D (Sigma Chemical Company, ST Louis, MO, USA) 25 μL, 4 μg/mL were added to the wells and incubated for 24 h. We used 0.2% crystal violet (Wako Pure Chemical Industries, Tokyo, Japan) in 2% ethanol to stain the viable cells. The absorbance of the well in a microplate reader was measured at a wavelength of 595 nm (Bio-Rad Laboratories, Richmond, CA, USA). Total DNA detection of RAW264.7 followed the method of Schreiber [24] by detecting wavelength 570 nm.

2.6. Monosaccharide analysis

2.6.1. EPS acid hydrolysis

Each fraction containing 1 mg of EPS was dialyzed, freeze dried and dissolved in 1000 μL of distilled water. To hydrolyse the EPS, 1 mL of 4 M trifluoroacetic acid (TFA) was added; the tube was filled with N_2 for 20 s and then heated at 110 °C for 4 h. Next, 250 μL of 3 N KOH was added to precipitate the TFA, followed by freeze drying for 2 days. TFA was removed by extraction with 1 mL of methanol (five times). Nitrogen then used to evaporate the methanol to dryness, leaving the EPS hydrolysate, which was redissolved in 1 mL of methanol and stored at −20 °C until use.

2.6.2. EPS derivation

The EPS acid hydrolysate (40 μL) was placed into a 60 °C water bath to evaporate the methanol and 40 μL of 0.3 N NaOH was added. Then, 40 μL of 0.5 M PMP (1-phenyl-3-methyl-5-pyrazolone) was added, and the tubes were placed in a water bath at 70 °C for 90 min. Next, they were placed into the cold at 4 °C for 10 min and then 60 μL of 0.3 N HCl was added to stop the reaction. Next, 1 mL of chloroform was added to extract the free PMP (three times). Nitrogen was then used to dry the

Table 1
Exopolysaccharide (EPS) production of 10 bacterial strains[a].

Bacterial strain	EPS production (mg/L)
Lactobacillus reuteri Mh-001	7275.86 ± 114.94
Lactobacillus fermentum Mh-002	4613.70 ± 51.87
Enterococcus faecalis Mh-003	1737.98 ± 22.23
Pediococcus pentosaceus Mh-004	3890.22 ± 64.5
Lactobacillus casei Mh-005	2968.26 ± 181.9
Lactobacillus casei Mh-006	2460.48 ± 167.8
Lactobacillus coryniformis Mh-007	2848 ± 108.00
Lactobacillus coryniformis Mh-008	4558.42 ± 306.34
Bifidobacterium longum BCRC14634	2753.22 ± 163.50
Lactobacillus acidophilus BCRC 14079	3230.51 ± 36.11

[a] EPS production was determined by quantifying the carbohydrate content as D-glucose equivalents of triplicate evaluation by using the phenol-sulphuric acid method.

EPS derivative, which was dissolved in 0.5 mL of double-distilled water and filtered through a 0.22-μm pore size filter.

2.6.3. EPS monosaccharide analysis

The monosaccharide composition was analysed using high performance liquid chromatography (HPCL) with a C-18 reverse phase column and an ultraviolet/visual wavelength detector set to a wavelength of 250 nm (Hitachi, Osaka, Japan). The mobile phase consisted of acetonitrile in distilled water (10%) and HCl (0.01%) and eluted at 1.0 mL min^{-1} flow rate. For peak identification, we used external standard containing arabinose, fructose, glucose, galactose, xylose, and rhamnose.

2.7. Statistical analysis

Data were analysed using one-way analysis of variance with SigmaStat software (Systat Software Inc., Point Richmond, CA, USA). p values <0.05 represent statistically significant differences between the compared data sets.

3. Results

3.1. Screening and identification of probiotic bacteria

From the fermented vegetable samples, ten bacterial strains were isolated. Eight strains were identified by 16S rDNA sequence, and the carbohydrate content in supernatant broth is listed in Table 1, includes two BCRC strains. However, strain *Lactobacillus reuteri* Mh-001 was selected as its highest EPS production capability (7275.86 ± 114.94) for the followed study.

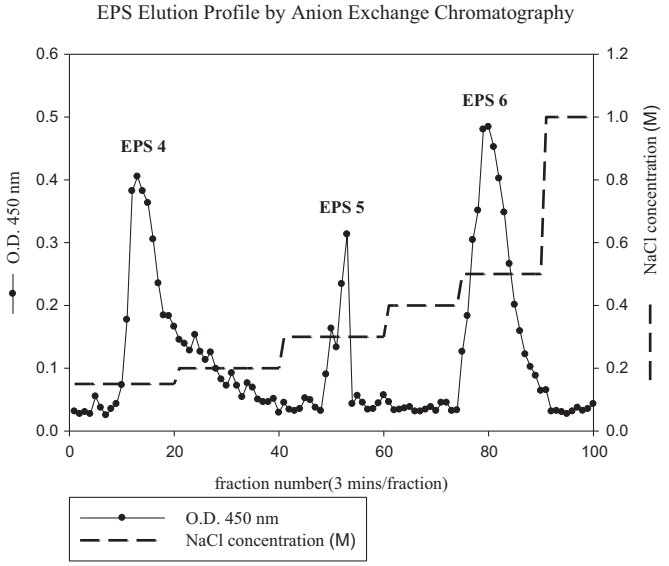

Fig. 2. Exopolysaccharide (EPS) elution profile by DEAE-sepharose anion exchange chromatography. The first peak is EPS 4; the second peak is EPS 5 and the third peak is EPS 6.

Colonies of strain Mh-001 were gram-positive, rod-shaped when observed under the phase-contrast microscope (Nikon Eclipse 80i, Japan). By using API-50 CHL kits, the strain was tentatively identified as *Lactobacillus* sp. The 16S rDNA genes partially sequenced (GenBank accession No. CP000705.1) exhibited high similarity (99.00%) with *Lactobacillus reuteri* DSM 20016 complete genome. The phylogenetic analysis (Fig. 1) shows that strain Mh-001 had the closest relationship with *Lactobacillus reuteri* and identified as *Lactobacillus reuteri*, and named *Lactobacillus reuteri* Mh-001. The second high EPS production *L. fermentum* Mh-002, and the lowest EPS production *Enterococcus faecalis* Mh-003 were identified by using the same methods as the reference species.

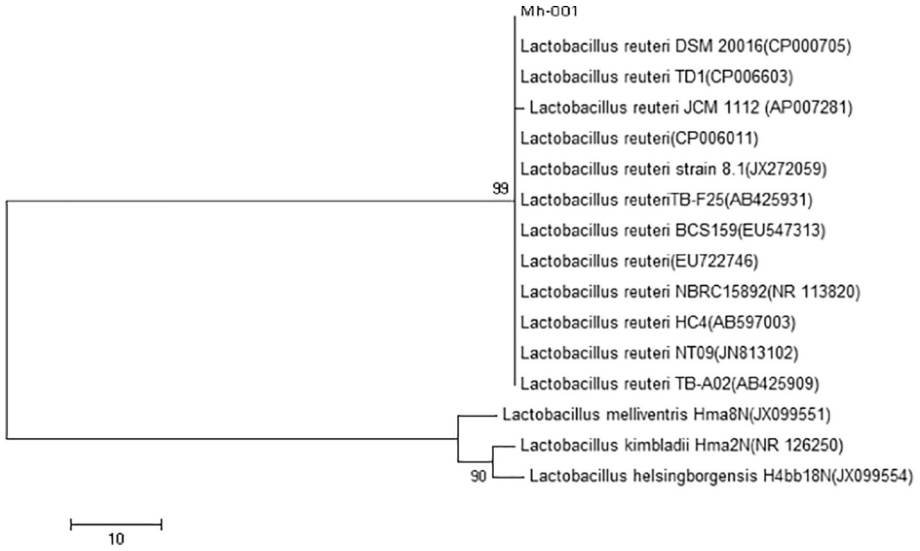

Fig. 1. Phylogenetic neighbor joining tree obtained with the 16S rDNA sequences of strain Mh-001 and members of related bacteria. Numbers at branch points are bootstrap values for a parsimony-based analysis.

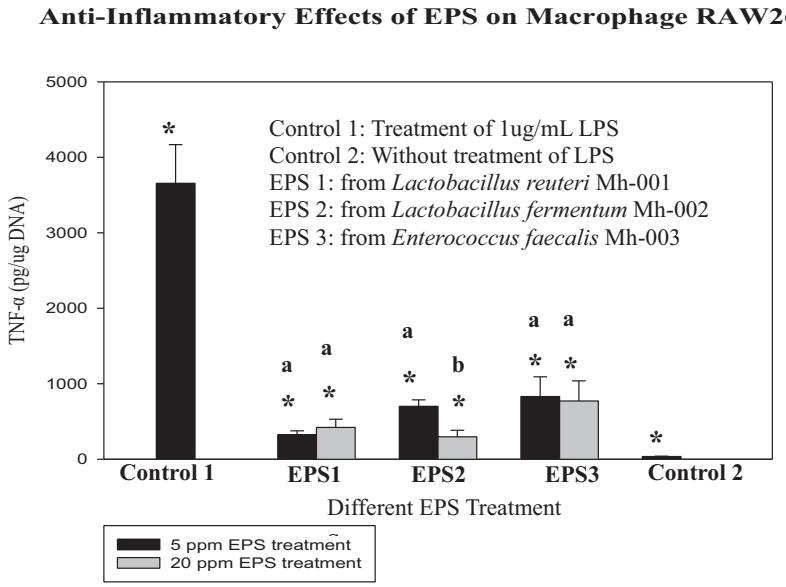

Fig. 3. Anti-inflammatory effects of exopolysaccharide (EPS) treatment on macrophage RAW264.7 cells. The vertical axis is tumour necrosis factor α (TNF-α) (pg/μg of DNA). Control 1: Treatment with 1 μg/mL of lipopolysaccharide (LPS). Control 2: No LPS treatment. EPS 1 source: *Lactobacillus reuteri* Mh-001, EPS 2 source: *Lactobacillus fermentum* Mh-002, EPS 3 source: *Enterococcus faecalis* Mh-003. Bars show means of triplicates ± SD. *p < 0.05 compared with the control group 1 TNF-α (pg/μg DNA). Means in the same bar (the same EPS with different concentration) with different letters are significantly different, p < 0.05.

3.2. Extraction and purification of EPS

EPS extraction and detection were performed according to the method of Wu [25]. Crude EPS was collected from the bacteria by centrifugation and precipitation with 95% ethanol (3,1 ethanol: EPS volume). DEAE sepharose anion exchange-chromatography (GE) was done using a 26-mm diameter, 600-mm length glass column (GE), and a sodium chloride gradient. The gradient concentrations were 0.15 M → 0.2 M → 0.3 M → 0.4 M → 0.5 M → 1.0 M. The EPS in each concentration was eluted in 100 fractions as shown in Fig. 2. Fractions 10–21 were collected as EPS 4; fractions 49–54 were collected as EPS 5; fractions 76–92 were collected as EPS 6. All of the above fractions equalled the

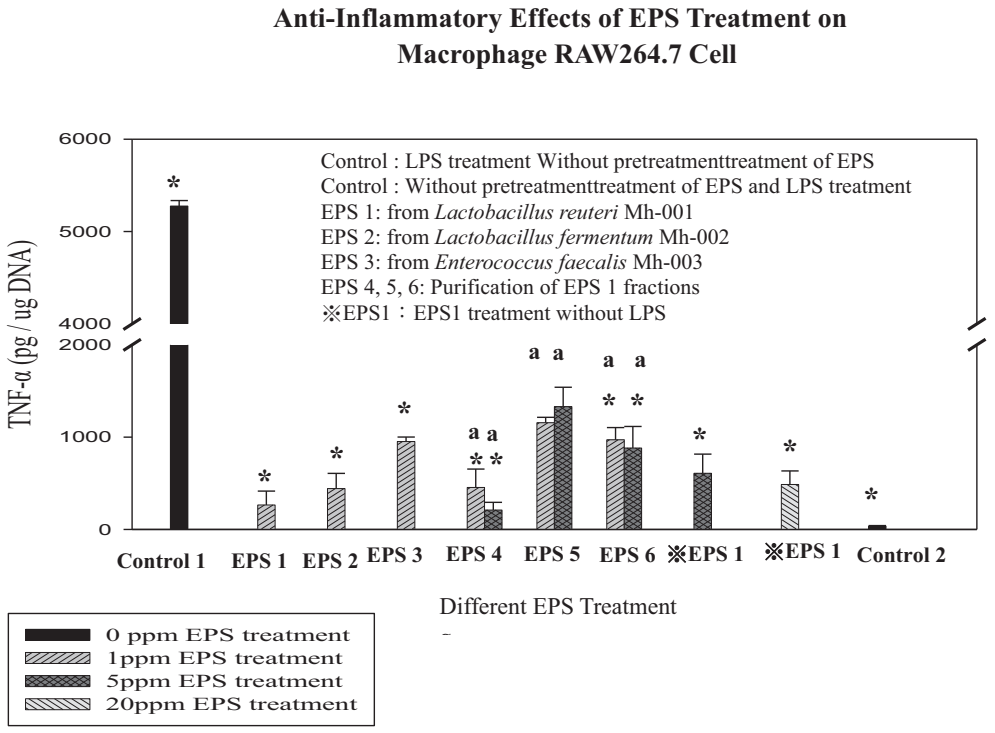

Fig. 4. Anti-inflammatory effects of exopolysaccharide (EPS) treatment on macrophage RAW264.7 cells. Cell Control: No EPS treatment. EPS 1 from *Lactobacillus reuteri* Mh-001, EPS 2 from *Lactobacillus fermentum* Mh-002, and EPS 3 from *Enterococcus faecalis* Mh-003. EPS 4, EPS 5, and EPS 6 were purified from EPS 1 fractions †EPS1 is EPS 1 treatment without the lipopolysaccharide (LPS) treatment. Bars show means of triplicates ± SD. *p < 0.05 compared with the control group 1 tumour necrosis factor α (TNF-α, pg/μg of DNA). Means in the same bar (the same EPS with different concentration) with different letters are significantly different, p < 0.05.

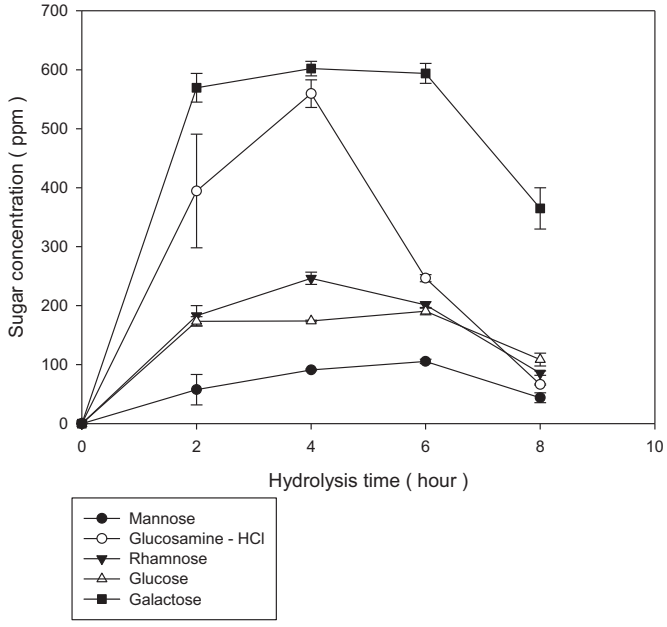

Fig. 5. Monosaccharide composition (1 mg/mL exopolysaccharide, EPS) subjected to treatment with 4 M trifluoroacetic acid (TFA) at 110 °C for various hydrolysis times (2, 4, 6, and 8 h).

total carbohydrate concentration determined by the phenol-sulphuric method. The rest of the fractions were collected for the detection of TNF-α production.

3.3. Secretion of TNF-α by RAW264.7 cells after EPS and LPS treatment

Fig. 3 shows the secretion of TNF-α by RAW264.7 macrophages after 5 or 20 ppm (μg/mL) crude EPS pre-treatment for 24 h and 1 μg/mL LPS treatment for 3 h. Control group 1 received LPS treatment to induce inflammation, but no EPS pre-treatment; the TNF-α level was 3656 ± 511.74 pg/μg of DNA. Control group 2 received no EPS or LPS treatment, and the TNF-α level was 36.29 ± 4.47 pg/μg of DNA. The EPS 1 group was pretreated with EPS and received LPS to induce inflammation. The source of the EPS 1 was from *L. reuteri* Mh-001 cultivated in MRS medium. The TNF-α level was 325.33 ± 51.10 pg/μg of DNA at 20 ppm EPS 1 pre-treatment. Similarly, the TNF-α level was 421.84 ± 108.45 pg/μg of DNA at 5 ppm EPS 1 pre-treatment. The EPS 2 and EPS 3 groups were treated similarly to the EPS 1 group, but the source of EPS 2 was Mh-002. The TNF-α level was 338.29 ± 124.53 pg/μg of DNA at 20 ppm EPS 2 pre-treatment. At 5 ppm of EPS 2 pre-treatment, the TNF-α level was 296.46 ± 87.14 pg/μg of DNA. The source of EPS 3 was Mh-003. The TNF-α level was 830.88 ± 260.33 pg/μg of DNA at 20 ppm EPS 3 pre-treatment while it was 771.34 ± 265.95 pg/μg of DNA at 5 ppm EPS3 pre-treatment.

Fig. 4 shows the secretion of TNF-α from the cells after 1 or 5 ppm of EPS pre-treatment with EPS1–EPS6 for 24 h followed by treatment with 1 μg/mL of LPS for 3 h. The TNF-α level of control group 1 was 5275.87 ± 59.48 pg/μg of DNA. The lowest TNF-α level among the seven EPS groups was for 5 ppm pretreatment with EPS 4 (209.20 ± 84.34 pg/μg of DNA) followed by pretreatment with 1 ppm of EPS 1 (263.75 ± 151.48 pg/μg of DNA), 1 ppm of EPS 2 (442.42 ± 164.08 pg/μg of DNA), and 1 ppm of EPS 3 (951.89 ± 49.03 pg/μg of DNA). Pre-treatment with 1 ppm of purified EPS 5 or EPS 6 resulted in higher TNF-α levels (1155.34 ± 58.52 pg/μg of DNA and 971.14 ± 131.97 pg/μg of DNA, respectively). Pre-treatment with 5 ppm EPS 1 without LPS, designated "EPS1" in Fig. 4, resulted in a TNF-α level of 608.44 ± 207.31 pg/μg of DNA. For EPS 1 pre-treatment with 1 ppm, the TNF-α was 486.87 ± 145.42 pg/μg of DNA. The bars show the mean of triplicate samples ± SD ($p < 0.05$) as compared with the control group 1. Means in the same bar with different letters are significantly different, $p < 0.05$ (Fig. 4).

3.4. Optimization of EPS acid hydrolysis and monosaccharide production

Fig. 5 shows the monosaccharide composition of 1 ppm of EPS hydrolysed with 4 M TFA at 110 °C for various hydrolysis times (2, 4, 6, and 8 h). The EPSs were composed of galactose, glucosamine, rhamnose, glucose, and galactose. Among the four hydrolysis times, 4 h of acid hydrolysis yielded the greatest concentration of monosaccharides including galactose (602 ± 12 ppm), glucosamine (560 ± 23 ppm), and rhamnose (246 ± 10 ppm) followed by glucose (174 ± 1 ppm) and galactose (91 ± 1 ppm).

3.5. EPS monosaccharide composition

Fig. 6 shows the HPLC profile of six monosaccharide standard peaks, and the retention times of mannose (10.66 min), glucosamine

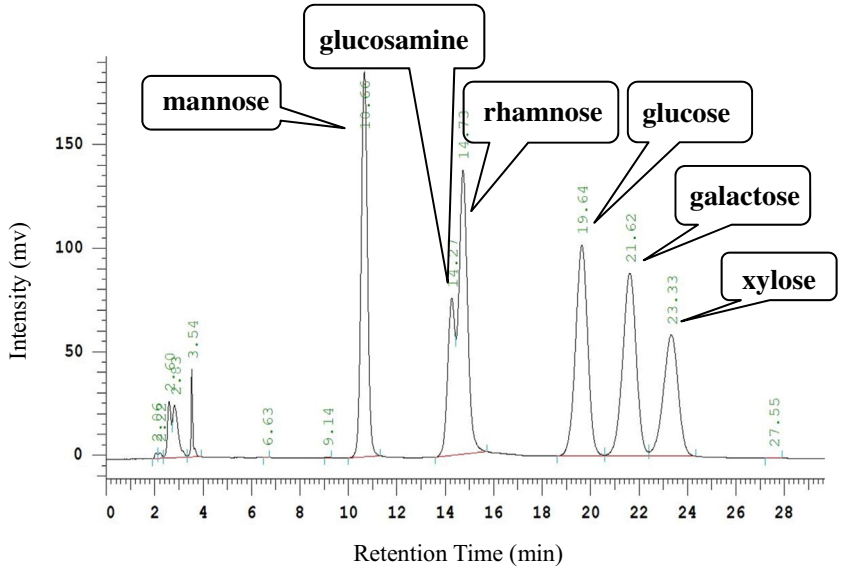

Fig. 6. The elution profile of the saccharide standard (includes xylose, galactose, glucose, rhamnose, mannose, and glucosamine) after acid derivation by high-performance liquid chromatography with a C-18 reverse-phase column.

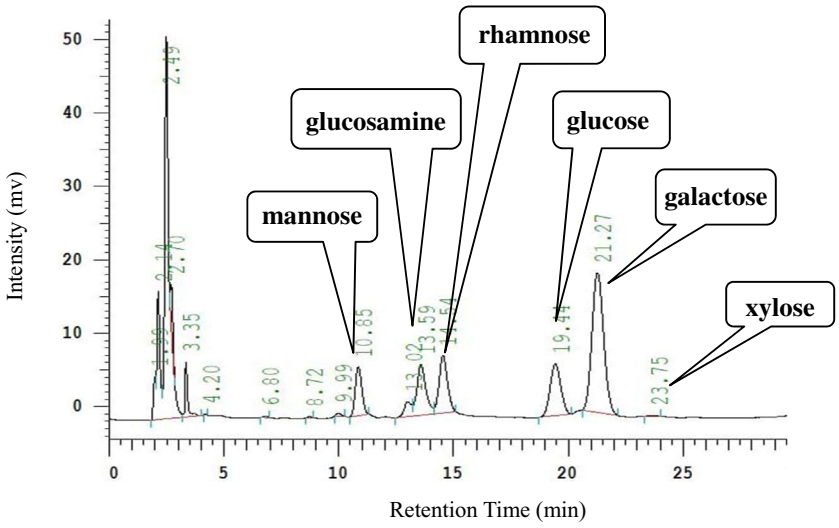

Fig. 7. The elution profile of exopolysaccharide (EPS) 1 after acid hydrolysis and derivation by high-performance liquid chromatography with a C-18 reverse-phase column.

(14.27 min), rhamnose (14.73 min), glucose (19.64 min), galactose (21.62 min), and xylose (23.33 min). Figs. 7 and 8 show the EPS 1 and EPS 4 HPLC profiles after acid hydrolysis. Table 1 shows the monosaccharide composition and percentages for the different EPSs based on HPLC peak integrals from the standard curve. Among all the monosaccharide percentages of the EPSs, EPS 4 had the highest galactose percentage ($45 \pm 2.7\%$) and a high rhamnose level ($13 \pm 0.2\%$) while xylose was undetectable. EPS 1 had high galactose ($39 \pm 0.2\%$) and rhamnose ($15 \pm 0.2\%$) percentages but a low glucose percentage ($16 \pm 0.1\%$).

4. Discussion

EPS 1, EPS 2, and EPS 3 obtained from the probiotics screened from newborns' faeces were used to investigate the anti-inflammatory effects of the EPSs. EPSs 4, 5, and 6 were further purified from EPS 1. Cells treated with 1 ppm of EPS 4 had the lowest TNF-α secretion of all. The monosaccharide percentages (galactose > rhamnose > glucose) were related to the anti-inflammatory activity of the EPSs. The galactose content of EPSs enhanced their anti-inflammatory effects on the macrophages. EPSs from *Bifidobacterium longum* are useful as mild immune modulators of macrophages (Wu et al., 2010). This contributes to their capacity to prevent or abate gastrointestinal infections and even some food-spoilage microbes [25]. Of all the samples tested in the current study, 5 ppm of EPS 4 had the greatest anti-inflammatory effect on RAW246.7 macrophages. Additionally, at 1 ppm, the original EPS1 had a better anti-inflammatory effect than any of the EPSs purified from it (EPSs 4, 5, and 6). At 5 ppm of EPS 4 had the greatest anti-inflammatory effect among all the samples. Theoretically, food molecules after purification should be more effective than the crude food as anti-inflammatory molecules but purified EPS did not conform to this theory. Lopez et al. [26] showed that purified EPS from *Bifidobacterium* sp. stimulated the proliferation of peripheral blood mononuclear cells. Furthermore, neutral and high molar mass EPS minimized the immune response, whereas acidic and smaller polymers drew forth the increase of immune response. Li et al. [27] demonstrated the same results for antioxidants. They indicated that purified EPSs (EPS 1, 2, 3) from *Lactobacillus helveticus* MB 2-1 had similar molecular weights of 2×10^5 Da and was composed of galactose, glucose, and mannose. Its antioxidant activities decreased in the order of crude EPS > EPS 3 > EPS 2 > EPS 1. Liu et al. extracted three exopolysaccharides from *P. umbellatus* and resolved their structures and relative bioactivities like immunomodulatory effect and DNA damage protecting activity [28].

Polysaccharides acid degradation depends on the conformation, and from one polysaccharide to another, the resistance stress to hydrolysis is

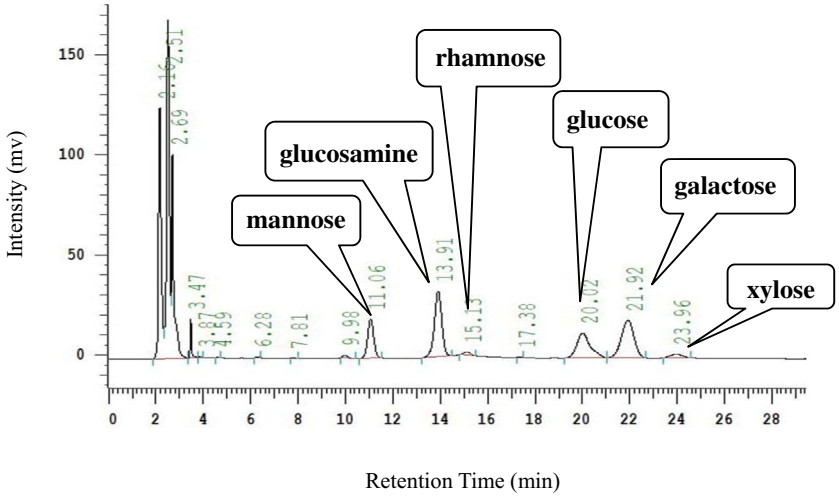

Fig. 8. The elution profile of exopolysaccharide (EPS) 4 after acid hydrolysis and derivation by high-performance liquid chromatography with a C-18 reverse-phase column.

Table 2
Monosaccharide composition and percentages of different exopolysaccharides (EPS).

EPSs	Mannose	Glucosamine	Rhamnose	Glucose	Galactose	Xylose
EPS 1	6 ± 0.2%	24 ± 0.1%	15 ± 0.2%	16 ± 0.1%	39 ± 0.2%	UD
EPS 2	42 ± 1%	2 ± 0.1%	4 ± 0.1%	16 ± 0.3%	22 ± 0.4%	15 ± 0.3%
EPS 3	28 ± 1%	35 ± 1%	3 ± 0.1%	16 ± 0.3%	8 ± 0.1%	10 ± 0.1%
EPS 4	20 ± 1.7%	20 ± 1.4%	13 ± 0.2%	2 ± 1.5%	45 ± 2.7%	UD
EPS 5	19 ± 1.2%	UD	23 ± 0.8%	58 ± 2.2%	UD	UD
EPS 6	49 ± 2.1%	5 ± 0.2%	5 ± 0.1%	39 ± 1.3%	2 ± 0.05%	UD

UD = undetected (percentage < 0.1%), and the values show means of triplicates ± SD.

altered. Therefore, acidic hydrolysis optimization is necessary for the accurate analysis. In our study, TFA, a proper acid for degrading polysaccharides, was chosen to hydrolyse the probiotic EPSs into component monosaccharides. By changing the hydrolysis times, hydrolytic temperatures, and TFA concentrations, the EPSs were investigated to achieve complete hydrolysis. In this experiment, the EPS sample was hydrolysed for different times with 4 M TFA at 110 °C, labeled with PMP for component monosaccharides, and analysed by HPLC.

Table 2, Figs. 3 and 4 show the galactose, rhamnose, and glucosamine content of EPS enhanced the anti-inflammatory effect on macrophages, and the glucose content might suppress the effect. The impact factor of monosaccharide content to the anti-inflammatory effect decreased in the order of high galactose > high rhamnose > high glucosamine. Das et al. [29] revealed α-D-glucan from *Lactobacillus plantarum* DM5 increased the growth of probiotic bacteria but did not support the growth of non-probiotic bacteria. From the matching of monosaccharide content to anti-inflammatory effects of the Mh-001, Mh-002, and Mh-003 EPSs, we suggest that the EPSs produced by the probiotic strains would not be glucan, but would more likely be galactan or rhamnan.

In conclusion, we screened faeces for probiotics using biochemical and biological methods. We isolated and identified *Lactobacillus reuteri* Mh-001, *Lactobacillus fermentum* Mh-002, and *Enterococcus faecalis* Mh-003. "Miao et al. [30] reported that the EPS of *Lactobacillus reuteri* SK24.003 was produced from sucrose and was composed exclusively of glucose. Additionally, it exhibited an α (1 → 4) backbone with an α (1 → 6) branch at every fourth residue. These results suggest that the novel α-glucan produced by *L. reuteri* SK 24.00 could be broadly used in the food and material fields. Notararigo et al. [31] revealed that β-D-glucan from *Pediococcus parvulus* 2.6 or *Lactococcus lactis* MX 9000 activated macrophages had an anti-inflammatory effect. Velasco et al. [32] clarified α-phosphoglucomutase and α-UDP-glucose pyrophosphorylase might be the bottlenecks for β-D-glucan biosynthesis.

Among all of the EPS samples tested, 1 ppm of EPS 4 had the best anti-inflammatory activity on RAW236.7 macrophages. Among the EPS samples tested at 5 ppm, the original EPS 1 had better anti-inflammatory effects than EPS 4, EPS 5, and EPS6, which were purified from EPS 1. For all the EPSs, we found that higher galactose and rhamnose contents enhanced their anti-inflammatory effects on the macrophages. Unlike Liu et al., they concluded their EPS from *Polyporus umbellatus* were mainly composed of mannose, along with galactose and glucose with different molar ratio [28]. This is not total the same of our result, due to the different metabolite composition between bacteria and mold. The data indicate that EPSs from probiotics possessed beneficial physiological effects, such as anti-inflammatory properties. Moreover, the monosaccharide composition of the EPS is the factor that influences the anti-inflammatory activity. These findings are of significance in the discovery of new functional foods and the development of new agricultural products.

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Competing interests

The authors declare that they have no competing interests.

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