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Partial characterization and immunostimulatory activity of exopolysaccharides from *Lactobacillus rhamnosus* KF5

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ABSTRACT

Lactobacillus rhamnosus KF5, a strain newly isolated from the faeces of a healthy human volunteer, has been shown to produce the exopolysaccharides (EPS) in skim milk. Two EPS fractions were separated from the fermented skim milk by removing proteins, ethanol precipitation, anion-exchange and gel permeation chromatography. Fraction S1, with a low average molecular weight of 1.36×10^4 Da, was composed of glucose, arabinose, glucosamine, galactosamine and galactose in an approximate molar ratio of 2.03:1.29:1.25:0.72:0.61. Such monosaccharide composition of the exopolysaccharide by lactic acid bacteria has not been reported so far, and S1 is likely to be an unusual type of microbial EPS. Whilst Fraction S2, with a high average molecular weight of 1.23×10^6 Da, contained rhamnose, glucose and galactose in a molar ratio of approximately 1.73:1.47:1.00. Both of EPS fractions could significantly stimulate splenocyte proliferation in vitro, indicating two EPS fractions had the potential immunomodulatory activity.

1. Introduction

Microbial exopolysaccharides (EPS) are synthesized by a wide variety of bacteria in the natural ecological environments, mainly involved in the prevention of desiccation, protection against toxic and/or environmental stresses and adherence to surfaces (De Vuyst, De Vin, Vaningelgem & Degeest, 2001; Jolly, Vincent, Duboc & Neeser, 2002; Sutherland, 1999). Lactic acid bacteria (LAB) are Generally Regarded as Safe (GRAS) organisms, therefore the EPS-producing LAB strains can be directly applied to fermented dairy products (e.g. yoghurt and cheese) with highly stable properties in the rheology, texture and mouthfeel (Bouzar, Cerning & Desmazeaud, 1997; Dabour, Kheadr, Fliss & LaPointe, 2005; Dabour, Kheadr, Benhamou, Fliss & LaPointe, 2006; Hassan, 2008; Hess, Roberts & Ziegler, 1997; Purohit, Hassan, Bhatia, Zhang & Dwivedi, 2009). Moreover, LAB EPS may also provide beneficial functions to human health, including antitumor activities (Shiomi, Sasaki, Murofushi & Aibara, 1982), antimutagenicity (Sreekumar & Hosono, 1998; Tsuda, Hara & Miyamoto, 2008), anti-ulcer (Nagaoka, Hashimoto, Watanabe, Yokokura & Mori, 1994), anti-inflammatory properties (Guzmán, Gato, Lamela, Freire-Garabal & Calleja, 2003; Nowak et al., 2012) and immune-modulating activities (Ciszek-Lenda, Nowak, Śróttek, Gamian & Marcinkiewicz, 2011; Kitazawa, Itoh, Tomioka, Mizugaki & Yamaguchi, 1996; Kitazawa et al., 2000; Liu et al., 2011; Sato et al., 2004; Yasuda, Serata & Sako, 2008). The other health promoting effects of LAB EPS like antihypertensive (Ai et al., 2008) and cholesterol-lowering activities (Maeda, Zhu, Omura, Suzuki & Kitamura, 2004) were also reported. Although a variety of physiological functions of LAB EPS were reported, the immunomodulatory activities of LAB EPS are drawing much attention. The phosphopolysaccharide from *L. delbrueckii* ssp. *bulgaricus* OLL1073R-1 exhibited mitogenic activities to stimulate lymphocyte proliferation and induce interferon-γ (Makino et al., 2006), whereas the heteropolysaccharides synthesized by *Lactobacillus casei* Shirota, having big sizes or high molecular weights, acted as suppressors of the immune response (Yasuda et al., 2008). The different immunomodulatory activities of EPS by lactic acid bacteria were related to the physicochemical properties and structural characteristics of their EPS.

Lactobacillus rhamnosus strain KF5 was newly isolated from a healthy human volunteer, and previous studies indicated strain KF5 was able to produce exopolysaccharides in skim milk. The crude EPS could be partly utilized by human intestine bacteria as fermentable substrates in fecal slurry batch cultures, and increase the

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levels of short-chain fatty acids (SCFA) in vitro, exhibiting the putative prebiotic properties. In addition, the splenocyte proliferation assay in vitro revealed the crude EPS could efficiently stimulate the splenocyte proliferation, suggesting the crude EPS exhibited potential immunological activities. Based on the preliminary studies, it is a greatly significant study on the exopolysaccharides produced by *L. rhamnosus* KF5.

The aims of this work were to isolate and purify the exopolysaccharides produced by *L. rhamnosus* KF5, and to investigate the characteristics of EPS fractions and their splenocyte proliferation activity in vitro.

2. Materials and methods

2.1. Strain and culture conditions

L. rhamnosus strain KF5 was isolated from the faeces of a healthy human volunteer. It was identified as *L. rhamnosus* based on the physiological characteristics and 16S rDNA sequences analysis. This microorganism was deposited in China General Microbiological Culture Collection Center (CGMCC No. 6430). Stock culture was maintained at −80 °C in MRS (Merck, Germany) broth with 20% (v/v) glycerol, and was propagated twice in MRS broth at 37 °C for 16–18 h, prior to the experiments.

The medium used for *L. rhamnosus* KF5 to produce EPS was 12% reconstituted skim milk supplemented with 1.0% glucose, sterilized at 118 °C for 15 min. Batch fermentation was performed in a 5.0 L fermenter (BioG-Micom, Biotron, Korea) at 32 °C for 30 h under anaerobic conditions.

2.2. Extraction and purification of exopolysaccharides

The crude EPS were extracted by the procedure described by Ai et al. (2008), with slight modifications. Briefly, the fermented milk was first heated in boiling water, and centrifuged to remove bacteria cells and coagulated proteins. Trichloroacetic acid (TCA) was then added to the cooled supernatant to a final concentration of 7% (w/v) and kept at 4 °C for 10 h. After precipitated proteins being removed by centrifugation, EPS in the supernatant were precipitated by slowly adding three volumes of prechilled 95% ethanol. The polysaccharides were collected by centrifugation and redissolved in distilled water, dialyzed extensively against distilled water. The retenate was lyophilized and then white crude polysaccharides were obtained.

The crude EPS were first purified on a DEAE-Sepharose Fast Flow (GE, USA) column (D 2.6 cm × 30 cm), equilibrated with 50 mM Tris–HCl buffer (pH 7.60) and were initially eluted with 1.5 column volume buffer at a flow rate of 3.0 mL/min, and then eluted with 50 mM Tris–HCl buffer (pH 7.60) with a linear NaCl gradient of 0.2–1.2 M, and 6 mL per tube were collected with a fraction collector. The carbohydrate content in the eluent was determined by the phenol–sulfuric acid method (DuBois, Gilles, Hamilton, Rebers & Smith, 1956). One polysaccharide fraction named S1 was obtained by the elution of Tris–HCl buffer, and the other fraction named S2 was eluted by a linear gradient of NaCl concentration then collected. They were dialyzed against deionized water for 3 d at 4 °C, with several changes of water and lyophilized. The two fractions were further purified on a Sepharose CL-6B gel column (D 1.6 cm × 100 cm) respectively, and eluted with 0.05 M Tris–HCl buffer (pH 7.60) with 0.15 M NaCl at a flow rate of 0.275 mL/min, and 1.5 mL of each tube were collected. The eluent containing EPS was detected, collected, dialyzed against the deionized water for 3 d at 4 °C and then lyophilized.

2.3. Chemical compositions analysis

Total carbohydrate content was determined by phenol–sulfuric acid method, using glucose as a standard (DuBois et al., 1956). The protein content was analyzed by Bradford method (1976), using bovine serum albumin as a standard. The uronic acid content was determined according to m-hydroxydiphenyl colorimetric method (Filisetti-Cozzi & Carpita, 1991).

2.4. Measurement of average molecular weight

The purity and the average molecular weight of purified EPS fractions were determined by high-performance size-exclusion chromatography (HPSCE). The analysis was performed on Agilent 1260 HPLC system (Agilent, USA), equipped with two gel permeation chromatography columns, a PL aquagel-OH column (ID 7.5 × 300 mm, 8 μm) (Agilent, USA) and TSK G3000 PWxl column (ID 7.8 × 300 mm, 10 μm) (Tosoh, Japan) connected in series. 20 μL of the purified fraction of 0.5 mg/mL was loaded, and eluted with 0.1 M sodium nitrate buffer at a flow rate of 0.9 mL/min. The eluent was monitored with a refractive index detector (RID) (Agilent, G1329B). Pullulan polysaccharide calibration kit (Agilent technologies, USA) of Mw 5900, 9600, 21,100, 47,100, 107,000, 200,000 708,000 and 1330,000 Da was used to calibrate the average molecular weights. All samples were filtered through a 0.22 μm membrane before injection.

2.5. Monosaccharide composition analysis

The monosaccharide composition analysis was performed by high performance anion exchange chromatography (HPAEC) (ICS5000, Dionex, USA) (Quigley & Englyst, 1994). The polysaccharides were first hydrolyzed with 4 M trifluoroacetic acid (TFA) at 121 °C for 2 h in a sealed hydrolysis bottle. After the excessive TFA was removed by evaporation under a stream of N_2, the EPS hydrolysate was dissolved in deionized water, filtered through 0.45 μm nylon filter and then analyzed on Carbo Pac PA20 column (ID 3 mm × 150 mm) (Dionex, USA) with pulsed amperometric detection (PAD) using gradient elution procedure of H_2O–250 mM NaOH–1.0 M NaAc as mobile phase. The column was eluted at a flow rate of 0.5 mL/min and the injection volume of sample was 20 μL.

2.6. Infrared spectra of EPS

The IR spectrum of the polysaccharide was determined using a Fourier transform infrared (FT-IR) spectrophotometer (Nicolet Nexus 470, NICOLET, USA). The purified polysaccharide was ground with potassium bromide (KBr) powder and pressed into pellets for FT-IR measurement in the frequency range of 4000–400 cm^{-1}, at a resolution of 4 cm^{-1}.

2.7. Splenic lymphocyte proliferation assay

BALB/c mice (aged 6–8 weeks and weighing 19–21 g) were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. The animals were sacrificed by CO_2 anoxia, and the spleens were collected. Single-cell suspensions of spleen lymphocytes were prepared as previously described by Kaspers, Lillehoj, Jenkins, and Pharr (1994). Splenocytes in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal calf serum (Sigma) were seeded into 96-well flat-bottomed microplate at a concentration of 1×10^6 cells/mL. They were then stimulated by EPS at final concentrations of 10, 100 and 1000 μg/mL, using 50 μL of concanavalin A (ConA, 5 μg/mL, Sigma) as a positive control and complete RPMI-1640 medium as a negative control. The cells were cultured for 72 h at 37 °C in a humidified 5% CO_2 atmosphere.

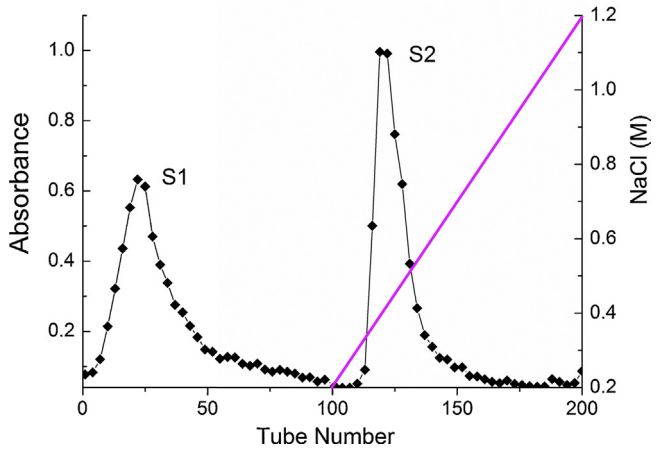

Fig. 1. Elution profile of exopolysaccharides produced by strain *L. rhamnosus* KF5 on DEAE–sepharose fast flow column.

Splenic lymphocyte proliferation was assayed by the conventional ^{3}H- thymidine method (^{3}H- TdR method) (Gillis, Crabtree & Smith, 1979). After incubation, 20 μL ^{3}H- TdR (370 kBq/mL, Shanghai Institute of Nuclear Research, China) was added into each well. The cells were pulsed with ^{3}H- TdR for 8 h at 37 °C and harvested onto glass fiber filters and measured in a liquid scintillation counter (Packard, USA). Results were expressed in counts per minutes (CPM). The proliferation activity (%) was defined and calculated as follow: proliferation activity (%) = (CPM $_{sample}$ − CPM $_{control}$)/CPM $_{control}$ × 100. Data were analyzed by one-way analysis of variance (ANOVA) and the Student's *t* test. Differences were considered statistically significant for $^{*}p < 0.05$ versus ConA.

3. Results and discussion

3.1. Preparation of exopolysaccharides

L. rhamnosus KF5 formed smooth, humid and ropy colonies on MRS agar plate after incubation anaerobically at 37 °C for 48 h, producing a long string up to 40 cm when the colonies were picked up with an inoculation loop.

After *L. rhamnosus* KF5 was incubated in 12% sterile skim milk supplemented with 1.0% glucose for 30 h in a fermentor without stirring, the fermented milk was obtained with a slimy appearance and a little whey syneresis. The polysaccharides were prepared from the fermented milk by removing the bacteria cells and proteins, followed by centrifugation, ethanol precipitation, dialysis and lyophilization. Finally, about 130 mg/L fluffy and white exopolysaccharides were obtained.

The crude polysaccharides were then isolated by ion-exchange chromatography and two fractions were obtained (Fig. 1). The two fractions S1 and S2 were then further purified by gel filtration chromatography, respectively. There was one single and relatively symmetrical peak appeared on the elution profile for each fraction, indicating each fraction of EPS obtained by ion-exchange chromatography was homogeneous. The protein content of the purified EPS was also detected by Bradford method and no protein was detected for S1 and S2. Besides, the uronic acid content of EPS fractions was determined by m-hydroxydipheny1 colorimetric method, and neither S1 nor S2 contained the uronic acid. The carbohydrate content of both S1 and S2 was up to 85%, calibrated with glucose as a standard.

3.2. Average molecular weight of EPS

The chromatograms from HPSEC analysis of EPS fractions were shown in Fig. 2, and there was only one symmetrical peak in each chromatogram, which further proved that fraction S1 and S2 were homogeneous. The weight average molecular weight (Mw) of S2 (1.23×10^{6} Da) was much higher than that of S1 (1.36×10^{4} Da). The molecular weight distribution of S1 and S2 were in agreement with the results of LAB EPS (from 10^{4} to 6×10^{6} Da) reported previously (De Vuyst et al., 2001; Hassan, 2008).

3.3. Monosaccharide composition of EPS

The monosaccharide compositions of S1 and S2 were analyzed after acid hydrolysis by HPAEC. As shown in Fig. 3, the results indicated that both of exopolysaccharide fractions produced by *L. rhamnosus* KF5 belonged to the heteropolysaccharide. Fraction S1, the low-molecular weight EPS, was composed of glucose (Glc), arabinose (Ara), glucosamine (GlcN), galactosamine (GalN) and galactose (Gal) in an approximate molar ratio of 2.03:1.29:1.25:0.72:0.61. S1 was rich in GlcN (37%), Glc (30.33%), Ara (15.6%) and GalN (15.6%), as well as Gal (5.71%). Such monosaccharide composition of the exopolysaccharide by lactic acid bacteria has not been reported so far, and the predominant component, glucosamine, up to 37% of the EPS, was hardly reported in the LAB EPS. The results strongly indicated that S1 was likely to be an unusual type of LAB EPS family. Exopolysaccharides containing amino sugars produced by lactic acid bacteria were reported in previous literatures. For instance, the polysaccharides produced by *L. rhamnosus* GG (ATCC 53103) grown in HYLA (enzymatically hydrolyzed lactose) was composed of GlcN (16%), Gal (75%) and Rha (9%) (Landersjö, Yang, Huttunen & Widmalm, 2002). The capsular polysaccharide (CPS) and the slime-polysaccharide (SPS) produced by *L. rhamnosus* JAAS8 grown in MRS broth or semi-defined medium consisted of different sugars. The CPS was composed of galactose and *N*-acetylglucosamine (GlcNAc) with a molar ratio of 5:1, but the SPS contained Gal, Glc and GlcNAc in a molar ratio of 4:1:1 (Yang et al., 2010). Marshall, Cowie, and Moreton (1995) also reported the polysaccharides produced by *Lc. lactis* subsp. *cremoris* LC330 in cremoris defined medium (CDM) contained Glc, Rha, Gal and

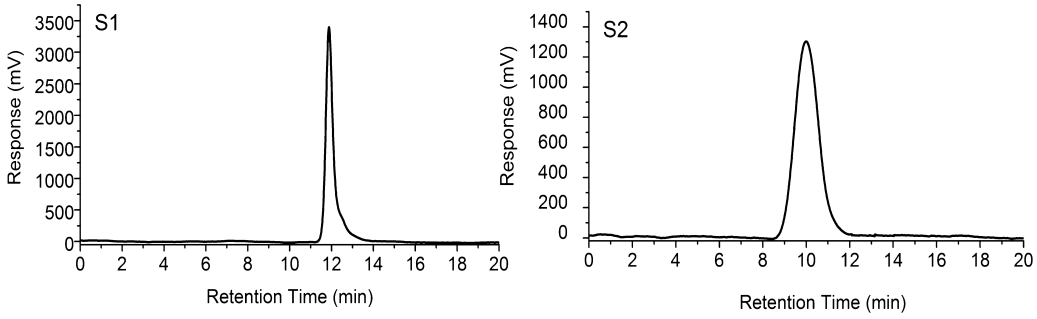

Fig. 2. High-performance size-exclusion chromatograms of EPS fractions (S1 and S2).

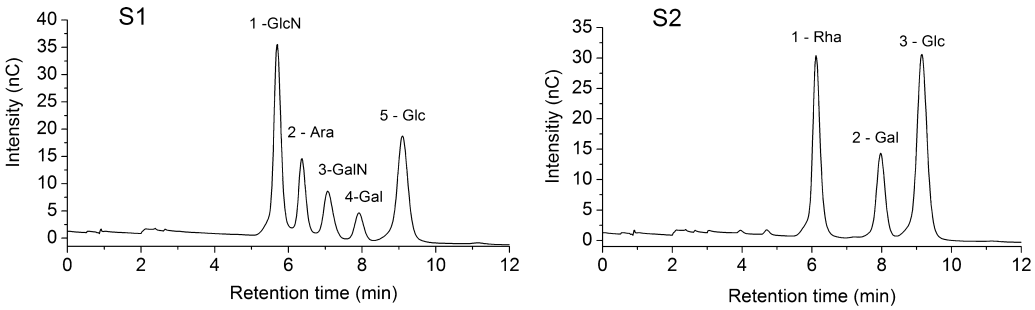

Fig. 3. HPAEC profile of monosaccharide composition of EPS fractions (S1 and S2). (GlcN: glucosamine; Glc: glucose; Gal: galactose; Ara: arabinose; GalN: galactosamine; Rha: rhamnose).

GlcN in an approximate ratio of 6:5:4:1, whilst the polysaccharides produced by *S. thermophilus* NCFB 2393 was composed of *N*-acetylgalactosamine (GalNAc), Gal, Rha and Glc in a ratio of 1:1:2:3 (Almirón-Roig, Mulholland, Gasson & Griffin, 2000). Doco, Carcano, Ramos, Loones & Fournet, 1991 studied ropy strains *Streptococcus salivarius* subsp. *thermophilus* CNCMI 733, 734 and 735 grown in skim milk were capable of producing EPS containing different amounts of four sugars: Glc, Gal, Rha and GlaNAc. Other heteropolysaccharides contained acetylated amino sugars GalNAc or GlcNAc (e.g. *L. acidophilus* LMG 9433 and *Streptococcus macedonicus* Sc136) (Robijn et al., 1996; Vincent, Faber, Neeser, Stingele & Kamerling, 2001). The LAB EPS containing arabinose scarcely appeared before (Badel, Bernardi & Michaud, 2011).

S2, a fraction with high molecular weight, contained Rha, Glc and Gal in a molar ratio of approximately 1.73:1.47:1.00 (Fig. 3). The constituting monosaccharides of S2 were almost present in other LAB EPS, but with different ratios (De Vuyst et al., 2001), such as the heteropolysaccharides produced by *L. rhamnosus* RW-9595M and R (Calsteren, Pau-Roblot, Bégin & Roy, 2002), *L. casei* LC2W (Ai et al., 2008) and *S. thermophilus* EU20 (Marshall et al., 2001). In general, the monosaccharide composition of EPS by lactic acid bacteria can be influenced by various factors, such as the types of strain, mediums and culture conditions.

3.4. FT-IR spectral analysis of the EPS

The FT-IR spectra of S1 and S2 exhibited a variety of typical absorption peaks of polysaccharides, as shown in Fig. 4. The broader bands at 3419 and 3418 cm^{-1} were due to stretching of the hydroxyl groups. The weak bands at 2922, 2856 cm^{-1} (S1) and 2931 cm^{-1} (S2) were attributed to C—H stretching, whilst the bands at 1372 (S1) and 1373 cm^{-1} (S2) were ascribed to the C—H bending vibrations. Each particular polysaccharide has a specific band in the 1200–1000 cm^{-1} region, and the position and intensity of the bands in this region were specific for each polysaccharide (Filippov, 1992; Wu, Cui, Tang, Wang & Gu, 2007). The broad bands at 1068 and 1030 cm^{-1} (S1) dominated by the C—OH stretching vibrations and the (C—O—C) glycosidic band vibration and also indicated the pyranose configuration of the sugar residues. Meanwhile, 1131, 1078 and 1044 cm^{-1} in the FT-IR spectra of S2 were also attributed to C—OH stretching and the (C—O—C) glycosidic band vibration. The band at 914 cm^{-1} was attributed to pyranose ring asymmetrical

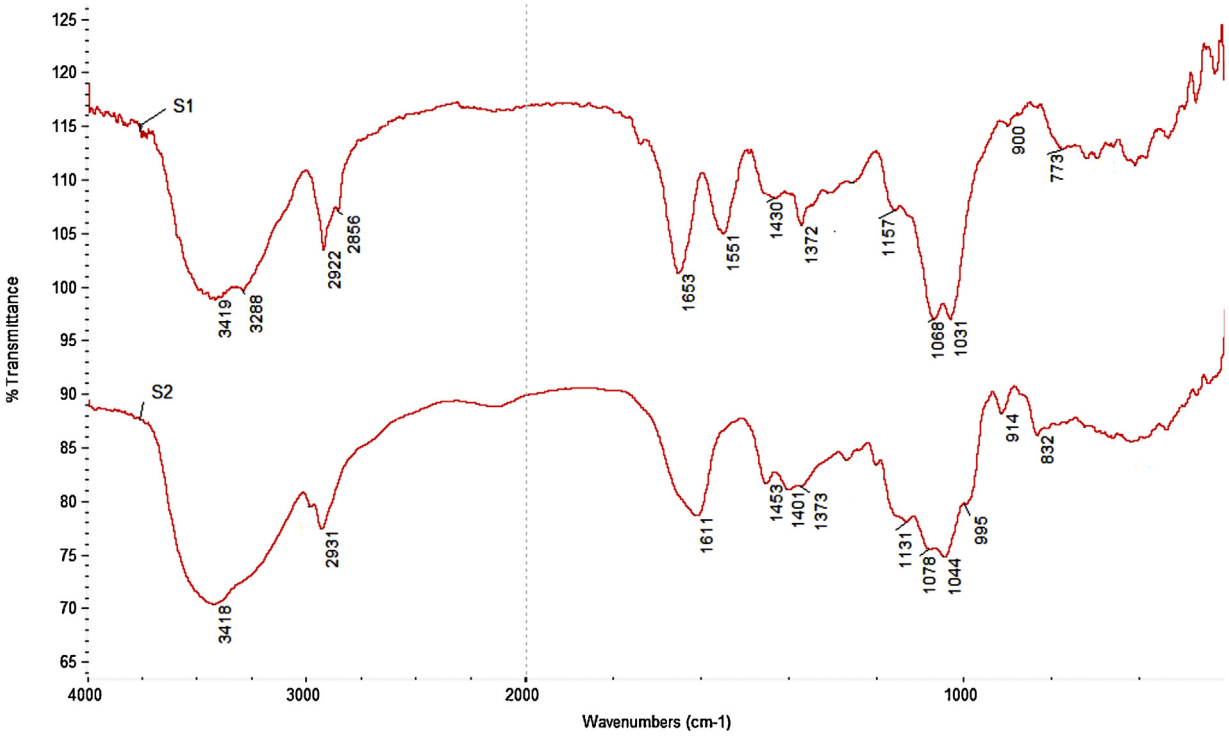

Fig. 4. FT-IR spectrum of exopolysaccharides produced by *L. rhamnosus* KF5.

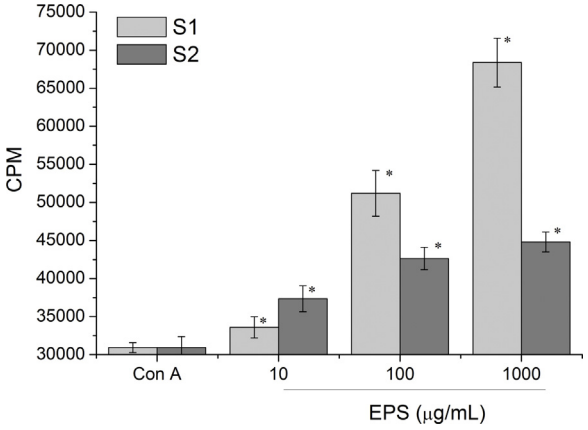

Fig. 5. Effect of EPS fractions on splenocyte proliferation in vitro. Splenocytes were stimulated with EPS (from 10 to 1000 μg/mL) or ConA (5 μg/mL) for 72 h. Proliferation was measured by ^{3}H- TdR method, and results were expressed in counts per minute (CPM). Data were analyzed by one-way analysis of variance (ANOVA) and the Student's *t*-test. Differences were considered statistically significant for $^{*}p < 0.05$ vs ConA.

stretching vibration, while the band at 832 cm^{-1} was associated with stretching of the α-isomeric carbon. Therefore, it could be speculated that S2 contained some amount of α-type isomeric carbons. The broad band at 1611 and 1401 cm^{-1} in the FT-IR spectra of S2 could be ascribe to the asymmetric and symmetric stretching of the carboxylate anions groups, —COO^{-}. The stronger absorption at 1653 and 1551 cm^{-1} in the FT-IR spectra of S1 was respectively dominated by C=O stretching and N—H bending vibration of amide groups. Meanwhile, a weak band at 3288 cm^{-1} was also observed due to the N—H stretching vibration, which was obviously affected by the strong absorption of —OH (Helm & Naumann, 1995). As S1 was rich in GlcN (37%) and GalN (15.6%), it could be speculated that the amino sugar residue in S1 was present in the form of *N*-acetylgalactosamine or *N*-acetylglucosamine.

3.5. Immunostimulatory activity of EPS

Several LAB strains and their extracellular polysaccharides were reported to stimulate the immune system through promoting T/B lymphocyte proliferation, natural killer (NK) cell tumoricidal activity, mononuclear cell phagocytic capacity, mitogenic activity, eliciting cytokines and thus might increase the host immune defense against pathogen (Cross, Stevenson & Gill, 2001; Kitazawa, Yamaguchi, Miura, Saito & Itoh, 1993; Makino et al., 2006).

The effects of EPS fraction S1 and S2 on lymphocyte proliferation were assayed by the ^{3}H- TdR incorporation, a classic method as described by Bounous et al. (1992). ConA, a typical T-lymphocyte mitogen, was used as the positive control. As shown in Fig. 5, both S1 and S2 were able to significantly stimulate the proliferation of splenocytes compared with 5 μg/mL ConA ($P < 0.05$), indicating both of EPS fractions exhibited mitogenic activity. The stimulating effect of S1 and S2 on the proliferation of spleen cells was in a dose-dependent manner at the concentrations from 10 to 1000 μg/mL. Compared with the positive control ConA, at a concentration of 100 μg/mL, the proliferation activity was 65% for S1 and 38% for S2, while at a higher concentration of 1000 μg/mL, the proliferation activity of S1 reached 121%, much higher than that of S2 (45%). As a mitogen, S1 was much stronger than S2.

It was observed that immunomodulatory activities of EPS synthesized by lactic acid bacteria were related to the physicochemical properties of the polymers, such as average molecular weight (López et al., 2012; Yasuda et al., 2008), electric charges (Sato et al., 2004), monosaccharide composition, stereochemistry and water-solubility (Jouault et al., 1995; Zjawiony, 2004). Based on the correlation between the potential immune-modulating activities of LAB EPS and their physical–chemical properties (i.e. molecular weight and charge), Hidalgo-Cantabrana et al. (2012) hypothesized that EPS having negative charges and/or small molecular weight were able to act as strong/mild stimulators of immune cells, whereas those neutral polymers with a large size exhibited a weak stimulation and even suppressive profile. This was in agreement with López et al. (2012), who proposed that acidic and smaller molecular weight EPS were able to present stronger immune response stimulation than neutral polymers with high molecular weight. The result of this study which S1, the low-molecular weight EPS was a stronger mitogen in stimulating the proliferation of splenocytes than S2 in vitro was in accordance with the reported results listed above.

4. Conclusions

The exopolysaccharides produced by *L. rhamnosus* KF5 in skim milk were composed of two EPS fractions (S1 and S2), and both S1 and S2 were homogeneous, but different in average molecular weight and monosaccharide composition. S1, with a low molecular weight, was composed of five sugars, i.e. glucose, glucosamine, arabinose, galactosamine and small amounts of galactose, which was likely to be an unusual type of LAB exopolysaccharides. S2, the high molecular weight EPS fraction, consisted of rhamnose, galactose and glucose. Both S1 and S2 could stimulate splenocytes proliferation in vitro, moreover, S1 demonstrated stronger mitogenic activity than that of S2. The primary task of further understanding about the relationship between the physicochemical properties of EPS fractions produced by *L. rhamnosus* KF5 and their immunomodulatory activities is the elucidation of the EPS structures.

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