Structural analysis and mucosal immune regulation of exopolysaccharide fraction from \textit{Bifidobacterium animalis} RH

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ABSTRACT
An exopolysaccharide fraction (EPSa) produced by \textit{Bifidobacterium animalis} RH was purified to dissect its chemical structure and to investigate its ability to modulate the mucosal immunity. Our results showed that EPSa had a backbone of (1→6)-linked glucose, (1→3)-linked galactose, (1→4, 6)-linked mannose, (1→3, 4)-linked mannose and (1→4)-linked glucose, branched with glucose and arabinose, and terminated with glucose residues. Scanning electron microscopy identified spherical particle to be the basic structural component of EPSa. The Congo red test indicated that EPSa was not in triple-helical conformation. Our \textit{in vitro} results showed that EPSa was able to stimulate the proliferation of peripheral blood mononuclear cells and intestinal Peyer's patch lymphocytes in the presence of concanavalin A. Our \textit{in vivo} results demonstrated that EPSa can enhance the mucosal immunity by maintaining the Peyer's patch number in small intestine and the IFN-\textgamma level in serum, and balancing the Th1/Th2 responses.

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Introduction
Lactic acid bacteria (LAB) are generally recognized as safe and widely used as starter cultures in the food industry. These bacteria produce lactic acid as the metabolic end product. The production of lactic acid can lower the pH of the food substrates, which can assist to develop unique flavors in the final products and simultaneously inhibit the growth of some spoilage and pathogenic microorganisms (Leroy & De Vuyst, 2004). In addition, many studies reported that LAB produce antimicrobial peptides bacteriocins, which can enhance the safety and extend the shelf life of the food products by killing or inhibiting the growth of un-desirable microorganisms (Garneau, Martin, & Vederas, 2002; Kaletta & Entian, 1989; Zhang et al., 2009). Besides their applications in the food industry, LAB are gaining considerable scientific attention due to their health promoting attributes. Amongst the LAB, \textit{Bifidobacterium} is the genus largely present in the human gut. The health benefits of bifidobacteria have been well documented in the literature.
Bifidobacteria are potent anti-oxidants which can efficiently eliminate reactive oxygen species \textit{in vitro} and \textit{in vivo} (Lin & Chang, 2000; Lin & Yen, 1999; Shen et al., 2010; Shen, Shang, & Li, 2011). In addition, bifidobacteria produce bacteriocins, which may help us to combat gastrointestinal infections (Cheikhyoussef, Pogori, Chen, & Zhang, 2008; Collado et al., 2005; Yildirim & Johnson, 1998). More importantly, the interaction between bifidobacteria and the host can modulate the host immune system and subsequently contribute to the elimination of the invading pathogens or inhibit the proliferation of tumor cells (Ashraf & Shah, 2014; Fanning et al., 2012; Sekine et al., 1985).

Exopolysaccharides (EPSs) is a type of polysaccharide that is either bound to the cells’ surface or being secreted into the environment. EPSs are composed of repeating units of monosaccharides, some of which are associated with proteins and lipids. EPSs such as xanthan, dextran and alginate are widely used in various industries. More attractively, EPSs are demonstrated to be potentially beneficial to the mammalian host. Microbial EPSs showed a great anti-oxidant activity both \textit{in vitro} and \textit{in vivo} (Lehman & Long, 2013; Xiang, Xu, & Li, 2012; Xu, Shang, & Li, 2011). Also, several studies revealed that in mammalian hosts, EPSs can reduce inflammation caused by either chemicals or bacterial infections (Jones, Paynich, Kearns, & Knight, 2014; Mello et al., 2017; Totte et al., 2015). In addition, EPSs are reported to be excellent anti-tumor agents as the tumor growth was reduced or inhibited in tumor bearing mice after the administration of EPSs (Cao et al., 2016; Zhao, Wang, Lv, Bie, & Lu, 2015). \textit{Bifidobacterium} is one of the LAB that can produce EPSs. Many studies have been conducted to isolate EPSs-producing \textit{Bifidobacterium} strains from human intestinal tract (Cunlei, Li, Wang, Guo, & Xu, 2015; Liu et al., 2007; Ruas-Madiedo et al., 2007; Salazar et al., 2009). EPSs produced by \textit{Bifidobacterium} showed great anti-oxidant and anti-tumor activities (Li et al., 2014; Prosekova et al., 2015; Xu, Shen, Ding, Gao, & Li, 2011). Recently, there an increasing number of studies focusing on the immunomodulatory activity of EPSs produced by \textit{Bifidobacterium}. However, studies focusing on the impact of \textit{Bifidobacterium} EPSs on the mucosal immunity are limited.

In our previous studies, we isolated an EPS fraction (EPSa) from \textit{Bifidobacterium animalis} RH and its preliminary structure was characterized by Fourier-transform infrared spectra and monosaccharide analysis in our previous study (Xu, Shen, et al., 2011). The identification of EPS structure is a critical step towards understanding the mechanisms by which it exerts bioactive effects in the host. Therefore, in the present study, our first objective was to further elucidate the structure of EPSa by Smith degradation, methylation analysis, Congo red test and scanning electron microscopy (SEM) to gain a more detailed picture of the EPSa structure. Secondly, due to the scarcity of knowledge on whether \textit{Bifidobacterium} EPS can influence the mucosal immunity, we investigated the impact of EPSa on the mice mucosal immunity by assessing its ability to stimulate the proliferation of immune cells \textit{in vitro}, maintain the number of Peyer’s patch in small intestine and the production of cytokines and antibodies \textit{in vivo}.

\textbf{Materials and methods}

\textbf{Structural analysis}

The strain \textit{B. animalis} RH was cultured at 37\(^\circ\)C under anaerobic conditions in PTYG medium for 48 h. Then EPS crude extracts were collected from the supernatant of
B. animalis RH using ethanol precipitation followed by protein hydrolysis and dialysis. Subsequently, one of the EPS fractions, namely EPSa, was separated from the crude extracts using anion exchange chromatography on DEAE-Sepharose fast flow column. After being purified by Sepharose CL-6B column, the glycosyl configuration, glycosidic bond and linkage order of EPSa was determined by periodate oxidation, Smith degradation and methylation analysis coupled with GC-M. The conformational characterization was performed using SEM and Cong red test. The procedure of all structural characterization except SEM followed the methods described in our previous study (Shang, Xu, & Li, 2013). Dry EPSa was examined under SEM (S-4800 Scanning Electron Microscope, Hitachi) after being coated with electric golden film using ion sputter (E-1010 Ion Sputter, Hitachi). SEM was performed at 500 x, 2000 x, 5000 x and 10,000 x with 10 kV acceleration voltage. Representative fields were selected to record.

**Animals**

Specific pathogen free Kunming mice (19.0–21.0 g, 6–8 weeks old, male-to-female ratio of 1:1) were purchased from the Animal Research Center, Beijing. They were randomized and housed 10 per cage in polycarbonate cages containing sawdust bedding. They received standard mouse chow and water ad libitum. The room conditions were maintained at 22 ± 2°C with relative humidity of 50 ± 5% and 12/12 h light/dark cycle. All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

**Effect of EPSa on stimulating the proliferation of peripheral blood mononuclear cell (PBMC) and Peyer’s patch lymphocyte (PPL) in vitro**

The PBMCs and PPLs were isolated from mice using density gradient centrifugation. Ten ml of heparinized blood was mixed with 10 ml of RPMI media. Mixed blood solution was overlaid upon a 5 ml of Ficoll–Hypaque in a 50 ml centrifuge tube. Thereafter, centrifugation was performed at 2000 r/min for 20 min at 18–20°C. The PBMCs were collected from the Ficoll–Hypaque interface. The PBMCs were washed with RPMI media twice and resuspended in RPMI supplemented with 5% Newborn Bovine Serum (NBS). Peyer’s patches were extracted from the intestine by mechanical extrusion and filtering through a 300 μm nylon net. The flow through was collected and centrifuged at 1500 r/min for 10 min and cells were resuspended in 5 ml of 40% (v/v) Percoll. Thereafter, 4 ml of 70% (v/v) Percoll was added at the bottom of centrifuge tube and centrifugation was performed at 2000 r/min for 20 min at 25°C. The fraction between 40% and 70% (v/v) Percoll was collected. The collected cells were washed in RPMI media for three times and resuspended in RPMI supplemented with 5% NBS.

The cells were counted using hemocytometer and 100 μl of PBMC or PPL were seeded into 96-well plate at the density of 1 x 10^6/ml in RPMI-1640 supplemented with 5% NBS. Thereafter, 50 μl of concanavalin A (ConA, 16 μg/ml as a final concentration) and 50 μl of EPSa at different final concentrations (400, 200, 100, 50 and 25 μg/ml dissolved in phosphate buffered saline (PBS)) were added into each well. In the control well, the cells received 50 μl of PBS instead of EPSa solution. In the background control well, neither ConA nor EPSa was added. The cells were incubated in 5% (v/v) CO₂ for 64 h at 37°C. In the end of incubation, the culture supernatant was replaced with fresh media and 10 μl of MTT
(5 mg/ml) was added and incubated at 37°C in the dark for 4 h. Finally, 100 μl of 0.04 M acidic isopropanol was added and incubated for 10 min. The absorbance value \((A_{570\text{nm}})\) was measured. The proliferation rate was calculated using the following formula:

\[
Proliferation\ rate = \left( \frac{A_{570\text{nm}} \text{ of EPSa treated well} - A_{570\text{nm}} \text{ of control well}}{A_{570\text{nm}} \text{ of control well}} \right) \times 100\%.
\]

**Effect of EPSa on protecting mucosal immunity in vivo**

Mice were randomly classified into six groups with eight mice in each group, according to their body weights. The six groups included a blank control group which neither received cyclophosphamide (CTX) nor EPSa, a model control group which only received PBS prior to CTX challenge, a high EPSa dosage group (400 mg/kg), a medium EPSa dosage group (200 mg/kg), a low EPSa dosage group (100 mg/kg) and a positive control group which received lentinan (170 mg/kg) instead of EPSa. Intragastric administration of EPSa or PBS was performed every 24 h for 15 days. On the 14th day, except the ones in the blank control group, all the other mice received intraperitoneal injection of 100 mg/kg CTX to induce lymphoid tissue lesions in the intestinal mucosa (Matsunaga et al., 1987; Nakasaki, Tajima, Mitomi, Fujii, & Kamijoh, 1996).

On the 15th day, the blood samples were collected from the mice by retro-orbital bleeding after 1 h ether anesthesia. The blood samples were incubated at 37°C to coagulate followed by 30 min incubation at 4°C to separate the serum. The serum samples were collected by 20 min centrifugation at 3000 r/min and kept frozen at −70°C for further use. The concentrations of IFN-γ, IL-2, IL-10 and IgG in the serum were measured using the ELISA kit (Shanghai Westang Bio-Tech Co., Ltd). After collecting the serum, the mice were sacrificed and the number of Peyer's patch on the entire small intestine was counted. Thereafter, 10 cm of small intestine which was 1 cm away from cecum was collected and homogenized in 2 ml of PBS. The homogenates were centrifuged at 4°C and the supernatant was collected. The concentrations of IFN-γ, IL-2 and IL-10 in small intestine were measured by the ELISA kit as mentioned above and the concentration of SIgA was measured by the method of double antibody sandwich ABC-ELISA (Vinderola, Perdigon, Duarte, Farnworth, & Matar, 2006).

**Statistical analysis**

Data were expressed as mean ± SD by at least three biological replicates for each sample. Statistical significance was evaluated by one-way analysis of variance (ANOVA) to determine the difference among different groups (SPSS 19.0, IBM). Values of \(P < .05\) were considered as statistically significant.

**Results**

**Analysis of periodate oxidation and Smith degradation**

The results of periodate oxidation showed that 0.995 mmol of periodate was consumed and 0.050 mmol of formic acid was produced per sugar residue, indicating the existence of small amount of 1→ linked or 1→6 linked monosaccharides. The complete acid hydrolysis of the periodate-oxidized products and three fractions after partial acid
hydrolysis (precipitation in the dialysis sack, supernatant in the dialysis sack and fraction out of dialysis sack) were obtained and subjected to GC analysis (Table 1).

The existence of mannose prior to acid hydrolysis and large amount of glycerol and erythritol outside of the dialysis sack revealed that some mannose residues could be oxidized by sodium periodate (HIO₄) and some mannose could not, but all mannose residues were located on the backbone. No glucose and arabinose were observed and large amount of glycerol and erythritol were obtained, demonstrating that glucose and arabinose were in linkages which can be oxidized, namely 1→3, (1→6), (1→2), (1→2, 6), (1→4) or (1→4, 6) linkage. The existence of glucose and arabinose outside of the dialysis sack also indicated that these residues can be oxidized by HIO₄ and some of them could be the branched structure of EPSa. There were no precipitations in the sack and no substance was detected in the supernatant of sack, suggesting that the linkages of backbone of EPSa should be all oxidized by HIO₄ or linkages cannot be oxidized and should not be arranged continuously.

### Analysis of methylation coupled with GC–MS analysis

The fully methylated EPSa was hydrolyzed by acid, converted into alditol acetates and analyzed by GC–MS (Figure 1 and Table 2). The results showed that the presence of seven components, namely 2, 3, 5-Me₂-L-Ara; 2, 5-Me₂-Man; 2, 3-Me₂-D-Man; 2, 3, 4-Me₃-D-Glc; 2, 4, 6-Me₃-D-Gal; 2, 3, 4, 6-Me₄-D-Glc and 2, 3, 6-Me₃-D-Glc in the molar ratio of 1.0: 1.4: 1.1: 1.5: 0.9: 1.2: 1.3. Therefore, the mannose had two types of linkage: (1→3, 4)-linkage and (1→4, 6)-linkage. In line with the analysis of periodate oxidation and Smith degradation, (1→3, 4)-linked mannose could not be oxidized by HIO₄ but (1→4, 6)-linked mannose could be oxidized. Hence, 2, 5-Me₂-Man (1→3, 4-linked mannose), 2, 4, 6-Me₃-Gal (1→3 linked galactose) and 2, 3-Me₂-D-Man (1→4, 6-linked mannose) were present discontinuously on the backbone, and residues of branches terminated with Ara and Glc. Among them, Glc showed 1→3, 1→4 and 1→6 linkages, which indicated that Glc was not only present in the backbone but also in the branches. The predicted structure of EPSa monomer was summarized in Figure 2.

### Conformational characterization

To determine whether the EPSa exists in an ordered conformation (triplex) which can form a complex with Congo red in diluted aqueous NaOH solution, causing a shift in
the maximum absorption wavelength (λ<sub>max</sub>), the transition of the triple-helical conformation to the single-stranded conformation in EPSa was studied by measuring the shift of λ<sub>max</sub> in the presence of Congo red solutions with different NaOH concentrations ranging from 0.05 to 0.4 M. As a result, the same pattern was observed for both Congo red-EPSa solution and Congo red alone, suggesting that triple-helical conformation was not present in EPSa (Figure 3).

Images of EPSa obtained by SEM were shown in Figure 4. Seen From the exterior structure, EPSa was composed of freely distributed small spherical particles. A structural overview showed that EPSa was present in pieces or columns (Figure 4(a)). However, under

![Figure 1](image1.png)

**Figure 1.** Gas chromatography profile of methylated EPSa on GC–MS.

| Retention time (min) | Methylated sugars<sup>a</sup> | Linkage types | Molar ratio |
|----------------------|-------------------------------|---------------|-------------|
| 11.084               | 2,3,5-Me<sub>3</sub>-L-Ara    | L-Araf (1→)   | 1.0         |
| 13.553               | 2,5-Me<sub>2</sub>-Man        | →3,4-Manf (1→)| 1.4         |
| 13.844               | 2,3-Me<sub>2</sub>-D-Man      | →4,6-D-Man (1→)| 1.1        |
| 18.731               | 2,3,4-Me<sub>3</sub>D-Glc    | →6)-α-D-GlcP (1→)| 1.5        |
| 20.484               | 2,4,6-Me<sub>3</sub>D-Gal    | →3)-α-D-GalP (1→)| 0.9        |
| 21.209               | 2,3,4,6-Me<sub>4</sub>D-Glc | α-D-GlcP (1→) | 1.2         |
| 22.866               | 2,3,6-Me<sub>3</sub>D-Glc    | →4)-α-D-GlcP (1→)| 1.3        |

<sup>a</sup>2,3,6-Me<sub>3</sub>Gal = 1,4-di-O-acetyl-2,3,6-tri-O-methyl-galactose, etc.

the maximum absorption wavelength (λ<sub>max</sub>), the transition of the triple-helical conformation to the single-stranded conformation in EPSa was studied by measuring the shift of λ<sub>max</sub> in the presence of Congo red solutions with different NaOH concentrations ranging from 0.05 to 0.4 M. As a result, the same pattern was observed for both Congo red-EPSa solution and Congo red alone, suggesting that triple-helical conformation was not present in EPSa (Figure 3).

Images of EPSa obtained by SEM were shown in Figure 4. Seen From the exterior structure, EPSa was composed of freely distributed small spherical particles. A structural overview showed that EPSa was present in pieces or columns (Figure 4(a)). However, under

![Figure 2](image2.png)

**Figure 2.** The predicted structure of EPSa monomer.
higher magnifications, the basic structural component of EPSa was shown to be spherical particle (Figure 4(b–d)). Spherical particles were tightly arrayed and some of them were connected into a piece in which the boundaries were not distinct. The surface of spherical particles was smooth. The diameter of those spherical particles varied from 2.10 to 5.78 µm (Figure 4(c,d)).

Figure 3. Change in the maximum absorption of the Congo red-EPSa complex at various concentrations of sodium hydroxide solution.

Figure 4. SEM images of EPSa. Magnifications were 500x(a), 2000 x(b), 5000 x(c) and 10,000 x(d).
**Effects of EPSa on stimulating the proliferation of PBMCs and PPLs**

The results of MTT assay showed that EPSa was able to synergy with ConA to stimulate the proliferation of PBMCs and PPLs, but the stimulatory effect was not in a dose-dependent manner. When the EPSa concentration was at 50 μg/ml, the highest proliferation rate 57.18% and 10.49% was observed for PBMCs and PPLs, respectively (Figure 5). Moreover, our results showed that EPSa did not exhibit any toxicity to the lymphocytes as the proliferation rate was still positive at the highest concentration of 200 μg/ml.

**Effects of EPSa on maintaining the number of mice Peyer’s patch in small intestine**

Peyer’s patches are lymphoid follicle in the small intestinal mucosa and the number of Peyer’s patch reflects the immune status of individuals. As shown in Figure 6(a), compared to the blank control group, intraperitoneal injection of 100 mg/kg CTX significantly \( P < .05 \) reduced the number of Peyer’s patch by 16.9%, indicating the successful establishment of animal model with impaired mucosal immunity in the intestine. Compared to the model control group, all EPSa-treated groups showed 20–30% \( P < .05 \) higher amount of Peyer’s patch. Compared to the blank control group, medium dosage showed 15.7% \( P < .05 \) higher number of Peyer’s patch, whereas the low and high dosage groups did not show any difference. These results demonstrated that EPSa can maintain the number of Peyer’s patch at a normal level after CTX challenge, suggesting its protective role on the mucosal immunity in the intestine.

**Effects of EPSa on cytokines’ production in small intestine and serum**

Compared to the blank control group, injection of CTX resulted in reduced amount of IFN-γ in serum (Figure 7(a)). Low dosage group failed to maintain the IFN-γ level after CTX challenge as its IFN-γ level was not significantly \( P > .05 \) higher than that of model control group. However, compared to the model control group, both medium and high dosage groups showed 2.7–4.4 fold \( P < .05 \) greater level of IFN-γ in the

![Figure 5. Effect of EPSa on the proliferation of PBMC and PPL in vitro.](image)
serum, which was similar to that of blank control group (Figure 7(a)). On the other hand, compared to the blank control group, CTX challenge did not significantly ($P > .05$) lower the amount of IFN-$\gamma$ in the small intestine. Except the high dosage group, the IFN-$\gamma$ levels in both low and medium dosage groups were not significantly ($P > .05$) higher than that of the model control group in the intestine (Figure 6(b)).

As shown in Figure 7(b), the IL-2/IL-10 ratio in serum of the model control group were significantly ($P < .05$) lower than that of the blank control group by 80.3%, indicating that CTX resulted in the imbalanced production of T helper cells 1 (Th1) and T helper cells 2 (Th2). Among the EPSa administration groups, only the high dosage group can maintain the normal ratio of IL-2/IL-10 in the serum after CTX challenge (Figure 7(b)). On the other hand, although the IL-2/IL-10 ratio of the model control group in small intestine was lower than that of the blank control group, the difference was not significant ($P > .05$) (Figure 6(c)). Similarly, both medium and high dosage EPSa administration was able to bring the IL-2/IL-10 ratio in small intestine back to normal level (Figure 6(c)), but no significant ($P > .05$) difference was observed between the model control group and the EPSa groups. These results indicated that EPSa administration may protect the mucosal immunity by balancing the Th1/Th2 responses in vivo.

Figure 6. Effect of EPSa on the number of Peyer’s patch (a), intestinal level of IFN-$\gamma$ (b), IL-2/IL10 ratio (c) and SIgA (d). Data were presented as mean ± SD ($n = 8$). Blank control only received PBS without CTX challenge. Positive control and model group received lentinan (170 mg/kg) or PBS after CTX challenge, respectively. Three EPS groups received 100, 200 or 400 mg/kg EPSa, respectively, after CTX challenge. Columns marked with different lowercase letters indicate mean significant differences based on one-way ANOVA test ($P < .05$).
Figure 7. Effect of EPSa on the serumal level of IFN-γ (a), IL-2/IL10 ratio (b) and IgG (c). Data were presented as mean ± SD (n = 8). Blank control only received PBS without CTX challenge. Positive control and model group received lentinan (170 mg/kg) or PBS after CTX challenge, respectively. EPS groups received 100, 200 or 400 mg/kg EPSa, respectively, after CTX challenge. Columns marked with different lowercase letters indicate mean significant differences based on one-way ANOVA test (P < .05).
Effects of EPSa on antibodies’ production in small intestine and serum

As shown in Figures 6(d) and 7(c), CTX challenge did not alter the SIgA production in small intestine or IgG production in serum as compared to the blank control group, no significant ($P > .05$) reduction of antibody production was observed. Also, compared to the model control group, no significant ($P > .05$) increase of IgG in the serum was observed in the EPSa groups. Though high dosage group showed 21.2% ($P < .05$) greater production of SIgA in the small intestine, the difference did not appear to be biologically significant. These results suggest that CTX challenge did not exhibit adversary effect on the overall antibody production in vivo and EPSa had little impact on modulating the antibody production in either serum or small intestine.

Discussion

The immunomodulatory activity of EPSs produced by LAB has been reported in a wealth of literature (Fanning et al., 2012; Sato et al., 2004; Vinderola et al., 2006; Wu et al., 2010). However, the mechanisms by which the EPSs exhibit immunomodulatory activity remain unclear. In order to fully understand the mechanisms, the interactions between the EPSs and the host immune cells first need to be illustrated. Unfortunately, only little information is available on how LAB EPSs interact with host immune cells. The structural analysis of LAB EPSs can provide some clues that may lead to understanding the interactions between EPSs and host immune cells. The structures of some Bifidobacterium EPSs that exhibited immunomodulatory activities have been identified (Hidalgo-Cantabrana et al., 2012, 2014). Hopefully, a certain correlation can be found between the structure (e.g. monosaccharides composition, monomer structure and conformation) of EPS and its impact on immunity, thereby stimulating novel hypothesis to test to elucidate the EPS–host cell interactions. Our structural data of EPSa from B. animalis RH provide additional information that can facilitate to achieve this goal.

It was noticed that in the in vitro PBMCs and PPLs proliferation assay, the optimal concentration to induce cell proliferation with ConA was 50 μg/ml instead of the highest concentration 200 μg/ml (Figure 5). ConA is a lectin that can bind to sugars (Brewer, Sternlicht, Marcus, & Grollman, 1973). Hence, high concentrations of EPSa may tend to sequester the ConA to interact with the PBMCs and PPLs, which subsequently reduce the synergy stimulatory effect of ConA and EPSa. In addition, compared to PBMCs, the PPLs were much less responsive to the stimulation by ConA and EPSa (Figure 5). PBMCs and PPLs were isolated from different locations in the mice, which suggests that the composition of these cells may be quite different. It is possible that EPSa can only stimulate certain types of immune cells, resulting in the different proliferation rates between PBMCs and PPLs. Therefore, the stimulatory effect of EPSa on the proliferation of individual type of immune cells will be the focus of our future work.

The majority of previous work focuses the immunomodulatory activity of LAB EPS in vitro (Hidalgo-Cantabrana et al., 2012). However, the in vitro work rarely reflects what is occurring in vivo. Therefore, we investigated the impact of EPSa on the mucosal immunity using a mouse model. Our results demonstrated that EPSa was able to maintain the number of Peyer’s patch in the mice small intestine, which suggests that the number of
immune cells may not be altered after CTX challenge (Figure 6(a)). Most likely, this protection effect can be conferred by the stimulatory effect of EPSa on the proliferation of immune cells. However, it is not clear whether EPSa can directly stimulate the proliferation of local immune cells in the small intestine or EPSa stimulates the proliferation of immune cells in other organs, which then migrate to the small intestine.

It is noticed that treatment of 200 mg/kg of EPSa or 170 mg/kg of lentinan resulted in similar immune responses except the IL-2/IL-10 (Figures 6 and 7), which suggests that EPSa from B. animalis and lentinan have similar immune stimulatory activity. Compared to lentinan, which is a β-(1,3)-glucan with β-(1,6) branching, our EPSa has much more heterogeneous monosaccharide composition and types of glycosidic linkage. However, these structural differences did not have an impact on the immune stimulatory activity between EPSa and lentinan suggesting that monosaccharide composition and types of glycosidic linkage do not dictate the immune stimulatory activity of our EPSa. Increasing evidence indicates that EPSs with low molecular weight or/and negative charge most likely have stimulatory activity (Hidalgo-Cantabrana et al., 2012; Kitazawa et al., 1998). On the other hand, high-molecular-weight EPS with neutral charge EPSs tend to have immunosuppressive effect (Ryan, Ross, Fitzgerald, Caplice, & Stanton, 2015). Our previous study shows that EPSa is a neutral EPS with a molecular weight of $2.31 \times 10^4$ Da, which is relatively small compared to that of most other LAB EPSs (Ryan et al., 2015). Therefore, the low-molecular-weight might be one important factor that contributes to the immune stimulatory activity of our EPSa.

CTX is known to be an immunosuppressant used to decrease the immune response and it is also demonstrated to have anti-inflammatory property (Hurd, 1973; Winkelstein, 1973). Our results showed that CTX challenge significantly ($P < .05$) reduced the production of the pro-inflammatory cytokine IFN-γ in serum (Figure 7(a)). This is detrimental to the host since IFN-γ is the most important cytokine that activates macrophages to exhibit pathogen-killing capacity (Schroder, Hertzog, Ravasi, & Hume, 2003). Insufficient amount of IFN-γ can render the host vulnerable to microbial infections. A similar pattern was observed in the production of IL-2/IL-10. The ratio of IL-2/IL-10 represents the balance between Th1 and Th2 cells in the host. The CTX injection significantly ($P < .05$) reduced the IL-2/IL-10 ration in serum (Figure 7(b)), which is consistent with its Th2 skewing property (Karni et al., 2004). It is noteworthy that the EPSa administration can maintain the cytokines level similar to those of the blank control group without triggering any unbalanced Th1/Th2 responses (Figures 6 and 7), which makes EPSa a good candidate to be developed into an immunomodulatory agent.

In conclusion, our data revealed additional structural information of EPSa from B. animalis RH, which may contribute to elucidating its function mechanisms. In addition, we demonstrated that EPSa can enhance the mucosal immunity by stimulating the proliferation of immune cells in vitro, maintaining the number of Peyer’s patch in small intestine, stabilizing the pro-inflammatory cytokine production in serum and balancing the Th1/Th2 responses in vivo.

**Disclosure statement**

No potential conflict of interest was reported by the authors.
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