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Characterization of Structure and Cellular Immunity Bioactivity of Milk-Derived Galactooligosacchrides Prepared by *Lactobacillus delbrueckii subsp. bulgaricus* Fermentation

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ABSTRACT

In this study, the milk-derived Galactooligosaccharides (GOS) were produced by *Lactobacillus delbrueckii subsp. bulgaricus* and refined by an ultrafiltration-nanofiltration continuous membrane. By further investigation, we found that the GOS product purified by gel permeation chromatography mainly contained low molecular weight disaccharide and trisaccharide, that is, 4-*β*-galactobiose and tri-galacto-oligosaccharides. The cellular immune activity of the purified GOS was evaluated by using Intestinal Epithelial Cells (IECs). Results showed that GOS could significantly ($P< 0.05$) promote IECs proliferation in a dose and time dependent manner, and the relative proliferation rate after 24 hours culture was high up to 158% at the concentration of 100 μg mL^{-1}, which was three time the value after 4 hours culture without GOS. Moreover, the production of IL-6 was observably increased and up to 133.54 ng L^{-1} with addition of 100 μg mL^{-1} GOS. These data implied that the purified GOS might have a role in promoting the immune adjustment, which could be utilized as a novel and natural immunoregulatory agent in the field of medicine and functional food. This work also revealed that the employment of transgalactosylation activity of *β*-galactosidase derived from the fermentation of probiotics such as *Lactobacillus delbrueckii subsp. bulgaricus* would enhance the value of the milk product due to the form of GOS.

Keywords: Fermentation, Galacto-oligasacchrides, Immunoregulatory activity, Probiotics.

INTRODUCTION

GalactoOligoSaccharides (GOS), non-digestible functional oligosaccharides, generally, are a mixed-length galactosylated product with a Degree of Polymerization (DP) ranging from 2 to 6. GOS are typically produced from lactose by the enzymatic activity of *β*-galactosidase, therefore, production of GOS in milk product may lower lactose concentration, which is beneficial to lactose intolerant consumers (Gosling *et al*., 2010). GOS are regarded as prebiotics since they can stimulate the proliferation of lactobacilli and bifidobacteria, which play a beneficial role in improving gastrointestinal health (Pan *et al*., 2009). Moreover, GOS have other multiple physiological effects on human health, such as reducing travelers' diarrhea (Drakoularakou *et al*., 2010), preventing colon cancer (Wijnands *et al*., 2001), enhancing calcium absorption (Chonan *et al*., 1995), and other physiological effects (Boehm and Stahl, 2007). Since consumers are more and more aware of the importance of diet for health, the demand for functional foods, or foods that promote health beyond providing basic nutrition, increases constantly, and a lot of attention has been paid to GOS production (Gosling *et al*., 2010).

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The enzymatic transgalactosylation is a typical method to synthesize GOS and has been applied to commercialized production. The process is that lactose acts as the substrate, both hydrolysis reaction releasing galactose and transgalactosylation are catalyzed by the commercial *β*-galactosidase (EC 3.2.1.23), namely, a bidirectional reaction (Cho *et al*., 2003; Jokar and Karbassi, 2011). The ratio of transferase and hydrolase activities of the enzyme affects the amount and nature of the formed oligosaccharides, which are also influenced by lactose concentration, temperature, pH and time (Park *et al*., 2007). Currently, the industrial *β*-galactosidases are mainly derived from microorganisms (bacteria, fungi, yeasts), such as *Aspergillus oryzae*, and Kluyveromyces lactis (Husain, 2010). In our previous work, milk-derived GOS derived from the fermentation of *Lactobacillus delbrueckii subsp. bulgaricus* strain, were produced under the optimum fermentation conditions, and then were refined by application of ultrafiltration-nanofiltration membrane combination technology (An *et al*., 2013). *Lactobacillus delbrueckii subsp. bulgaricus* is a probiotics, which could produce GOS. Fermented milk product by this strain could be directly edible and do not need external GOS, and this could reduce the cost of production, and enhance the nutritional and functional value of fermented product.

Although many documents have reported the production and physiological functions of GOS produced by commercial enzymes, there are few literatures on the study of structural and functional characterization of milk-derived GOS by *Lactobacillus delbrueckii subsp. bulgaricus*. Therefore, the objective of this study was to investigate the isolation, characterization, and immunological effects of natural product of milk-derived GOS, which would propose a new insight on the natural GOS produced by way of the fermentation of probiotics in milk manufacture and reveal its potential applications in the future.

MATERIALS AND METHODS

Materials

New Zealand (NZMP) skim milk power was obtained from Fonterra Commercial Trading Co., Ltd (Shanghai, China). The *Lactobacillus delbrueckii subsp. bulgaricus* (LB340 LYO) was offered by Danisco Co., Ltd (Kunshan, China). Beta-galactosidases were provided by Amar, Amano Enzymes Trading Co., Ltd (Shanghai, China). Sephadex G-10 was bought from Shanghai Bioroot Biological Technology Co., Ltd (Shanghai, China). The Folin phenol reagent was purchased from Sigma-Aldrich Co. Ltd (Missouri, USA). Acetonitrile was obtained from Tedia Company Inc. (Shanghai, China). Low-glucose Dulbecco's Modified Eagle's Medium (DMEM), penicillin streptomycin, 0.25% Trypsin-EDTA in Phosphate-Buffered Saline (PBS) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific Inc (Rockford, USA). 3-(4, 5-dimethlthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and Concanavalin A (ConA) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Enzyme-Einked Immunosorbent Assay (ELISA) kits were purchased from Yan Jing Biotech (Shanghai, China). Other chemical reagents were acquired from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). ICR rats were provided by Comparative Experiment Center of Yangzhou University (Yangzhou, China).

Fermentation and Refining of Milk-gerived GOS

Fermentation and refining of GOS from skim milk by *Lactobacillus delbrueckii subsp. bulgaricus* were prepared as described in our previous paper (An *et al*., 2013). Briefly, *Lactobacillus delbrueckii subsp. bulgaricus* [1‰ (w/v)] were inoculated into sterile skim milk solution

[12% (w/v)] and cultured at 37°C for 8 hours. After inactivating the enzymes by boiling the fermentation broth for 10 minutes, trichloroacetic acid (1%) and lead acetate solution (2%) were added into the fermentation broth to precipitate proteins. Protein precipitate was then sonicated, centrifuged, and filtered to get GOS. Next, the crossflow membrane separation equipment (MSM-2008, Shanghai MoSu science equipment Co., LTD, China) with the ultrafiltration (HPS-3)-nanofiltration (NF-270) continuous membranes was used to refine GOS. The optimal operation condition for ultrafiltration was 0.05 Mpa at the feed concentration of 2.5 g L^{-1} to get rid of proteins and for nanofiltration was 0.4 Mpa at 45°C to remove reducing sugars. The content of protein was determined by the Lowry method (Lowry *et al*., 1951) and the content of reducing sugars was analyzed by 3, 5-dinitrosalicylic acid (DNS) assay (Ghose, 1987; Arce-Cervantes *et al*., 2013).

Compositions Analysis of Milk-derived GOS

A Dionex ICS-5000 multifunctional ion chromatography with high performance liquid chromatography gradient pump and pulsed amperometric detection module (HPAEC-PAD) was used to analyze the compositions of GOS according to method described by de Slegte (2002) with minor modifications. The chromatographic analysis conditions for the monosaccharide compositions of GOS were as follows: CarboPac PA20 chromatographic column, 0.5 mL min^{-1} of flow rate, 30°C of detection temperature, and 20 μL of injection volume. The gradient elution was performed with 5 psi nitrogen and the mobile phase contained ultrapure water, 250 mM NaOH and 1.0M NaAc solution. The elution steps were as follows: firstly, the chromatography column was eluted by 98.2% ultrapure water and 1.8% NaOH solution for 21 minutes; secondly, pure water percentage was decreased to 93.2%, the proportion of NaAc solution was increased to 5.0% while NaOH was kept the constant percentage from 21 to 21.1 minutes; thirdly, ultrapure water percentage continued fell to 78.2% and the proportion of NaAc was raised to 20% and the proportion of NaOH was also invariant from 21.1min to 30 min; finally, the proportion of ultrapure water and NaOH was held at 20 and 80%, respectively, from 30.1 to 50 minutes. The detection method by using integrated pulsed amperometric detector with four potential values was applied and the detection voltages were *E1= +0.10V, E2= -2.00V, E3= +0.60V, E4= -0.10V*, respectively. The data were analyzed by Chromeleon chromatographic data system (An *et al*., 2013).

Purification of GOS by Gel Permeation Chromatography

Gel Permeation Chromatography (GPC) was widely used to separate carbohydrates (Martínez-Villaluenga *et al*., 2008). The milk-derived GOS refined by the ultrafiltration-nanofiltration continuous membranes were applied to GPC on Sephadex G-10 column (80×1.0 cm, the ratio of diameter to height 1:30) equilibrated previously with ultrapure water at a flow rate of 0.2 mL min^{-1}. The separated fractions containing sugars were collected by a distribution collector with a collection rate at 10 min per tube, and then were lyophilized for further analysis. The content of sugar in fraction was detected by measuring the absorbance of the eluent at 490 nm using the classical phenol-sulfuric acid method (DuBois *et al*., 1956).

Characterization of Purified GOS

Chemical structure of the purified GOS was analyzed by electrospray ionization mass spectrometry (ESI-MS) (Hernández *et al*., 2009), Fourier transforms infrared (FT-IR) spectroscopy (Moussa and Ali, 2008; Coimbra *et al*., 1999) and ion chromatography (Bashari *et al*., 2013).

ESI-MS analysis was performed using an Aglient 1100 LC MS system. The parameters for the analysis were as follows: photomultiplier tube voltage, 1,500V;

desolvation temperature, 250°C; ESI source temperature, 200°C, reflector, positive; mass range, 50-1,000 m z^{-1}.

The dried GOS (3.0 mg) were ground with spectroscopic grade potassium bromide (KBr, 200 mg) powder and then pressed into 1 mm pellets for FT-IR measurement (Nicolet 6700, Thermo Fisher, America) in the wave number range from 4,000 to 400 cm^{-1}, spectral resolution 4 cm^{-1}, and scan times 32.

Monosaccharide composition analysis was conducted using the method described by Bashari *et al*. (2013), with some modifications. Sample was hydrolyzed in 2M trifluoroacetic acid (TFA) at 120°C for 0.5 hour, and then TFA was removed by rotary evaporation at 45°C. Hydrolyzed sample was washed three times by adding anhydrous methanol and evaporated methanol completely at 45°C, further diluted with ultrapure water. The diluted sample was filtered by using a 0.45 μm membrane and injected into HPAEC-PAD (ICS-5000, Dionex Corporation, USA), equipped with a CarboPac™ PA20column (250-4 mm ID) and a guard column (25-3 mm ID). Separation was achieved with isocratic eluent (250 mM NaOH) at a flow rate of 0.5 mL min^{-1}. A post-column delivery solvent system of 5 and 20% 1M NaAc was added to the HPAEC-PAD system at a flow rate of 0.5 mL min^{-1} after 21.1 and 30 minutes, respectively.

Evaluation of Cellular Immunity Function of Purified GOS

Cell Culture

Newborn ICR rats provided by Comparative Experiment Center of Yangzhou University without feeding were killed, and then the small intestine of rats were taken out in a sterile environment. All animals were treated in accordance with the Guidelines of the Principle of Laboratory Animal Care (NIH Publication, revised 1985). The small intestinal was split and put into the PBS. Then, the turbid PBS was centrifuged and the liquid decanted. The precipitation was repeatedly washed by 0.01M PBS buffer (pH= 7.4) and centrifuged until the precipitation had been washed clean when the supernate was clear. Further, Intestinal Epithelial Cells (IECs) were cultured in the low-glucose DMEM medium supplemented with 10% FBS and antibiotics (100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin) (Barnard *et al*., 1989; Li *et al*., 2007). Cells were cultured at 37°C in a 5% CO_2 humidified atmosphere.

MTT Assay for Cell Viability

Cells were divided into eight groups: the control group, the ConA (10 mg L^{-1}) group, the coculture groups of the ConA (10 mg L^{-1}) and GOS groups (adding with 40, 70, 100, 130, 160, and 190 μg mL^{-1} GOS, respectively). Each group was set three time points (4, 8, 24 hours) with three replications. Cell viability was determined using the MTT cell viability assay (Marks *et al*., 1992). The logarithmic phase cells were re-suspended in the medium described above, and plated into culture flasks at a density of 1,000-10,000 cells/hole and a total volume of 200 μL per well. Cells were incubated until 96-well plates paved with monolayer cell at 37°C with 5% CO_2. Then, cells were incubated with a range of drug concentrations, incubated for 4, 8, and 24 hours, respectively and observed using the inverted microscope (Leica DMI3000B). Afterwards, 20 μL MTT (5 mg mL^{-1}) was added to each well. After 4 hours, the medium was removed and 150 uL dimethyl sulfoxide (DMSO) was added to dissolve the blue crystals of formazan with gentle shaking (Bandyopadhyay *et al*., 2013; Li *et al*., 2007). The Optical Density (OD) was detected in the enzyme-linked immunometric meter at 490 nm, and the relative cell proliferation rates (%) was calculated as the following equation:

$$\text{Relative proliferation rate of IECs}(\%) = \frac{\text{ODsample} - \text{ODcontrol}}{\text{ODcontrol}} \times 100$$

Where, ODsample was the optical density of each test sample at 490 nm, while ODcontrol was the optical density of untreated control sample (without GOS) at 490 nm and at the starting culture time (zero h).

Flow Cytometry Analysis

Cell counts were performed using a hemocytometer, as described literature (Li *et al*., 2001). Approximately 1×10^6 IECs were suspended in PBS and fixed by 70% ethanol. Cells were incubated at 37°C for 10 minutes after being washed with PBS and re-suspended in sodium citrate buffer containing RNAase. Then, the cells were centrifuged and the cellular DNA was stained by Propidium Iodide (PI) (50 μg mL^{-1}). The stained cells were analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) at excitation wavelength of 488 nm. Data were acquired with CellQuest acquisition software (BD Biosciences).

IL-6 Determination

The intracellular cytokine production (IL-6) of IECs was determined according to the instruction of ELISA kits (BD Biosciences, R and D Systems, and Minneapolis, MN). Briefly, IECs were stimulated by 10 μg mL^{-1} LipoPolySaccharide (LPS) with or without GOS at a series of concentrations (50, 100, 150 μg mL^{-1}) for 24 hours at 37°C. After incubation, cells were harvested and washed with PBS buffer, and then the supernatants were collected. The level of IL-6 was determined. The absorbance of each well at 490 nm was read using the microplate reader (Multiskan MK3) (Hu *et al*., 2013; Li *et al*., 2007).

Statistical Analysis

All data were given as the mean±SD. The survival curve was constructed by the Kaplan-Meier method. The data were analyzed by one way ANOVA. All statistical analyses were performed with IBM SPSS Statistics. $P< 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Purification and Structural Characteristic of GOS

The contents of GOS for fermented and refined GOS were about 1.24±0.02 and 26.80±0.01%, respectively. Thus, the refinement fold of ultrafiltration-nanofiltration membrane combination technology for GOS was about 22 times. Furthermore, the removal rate of proteins was high up to 96.70%. The refined GOS were then separated by GPC. Figure 1 shows the gel filtration chromatography chromatogram, which displays the peak appeared from the 11th tube based on the determined results of sugar content. Generally speaking, bigger Molecular Weight (MW) sugars were obtained firstly based on the molecular sieve principle.

In order to get sugars with different MW, the eluent of different tubes was merged and analyzed by ESI-MS. The results are shown in Figure 2. It can be seen that the fraction pooled from the 11th to the 14th tubes (Figure 1) mainly contained disaccharide and trisaccharide with the two intense ions at 365 and 527 m/z, respectively (Figure 2-a). However, for the fraction pooled from the 15th to the 18th tubes (Figure 1), the monosaccharide peak corresponding to the ion at 203 m z^{-1} began to come out and the main sugar component was disaccharide, whereas the relative content of trisaccharide corresponding to the ion at 527 m z^{-1} decreased obviously (Figure 2-b). Moreover, for the fraction pooled from the 22nd to 24th,

the predominant peak was monosaccharide as shown in the Figure 2-c. Thus, the possible transgalactosylation products

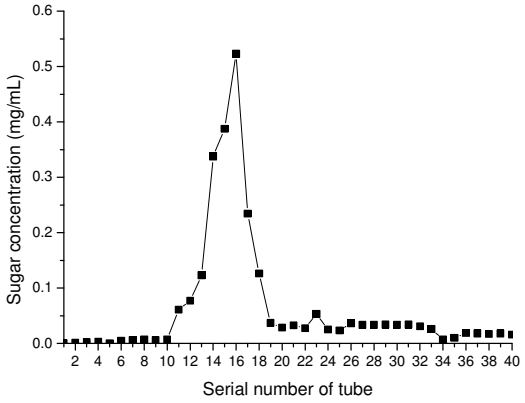


Figure 1. Gel filtration chromatography chromatogram of the refined GOS.

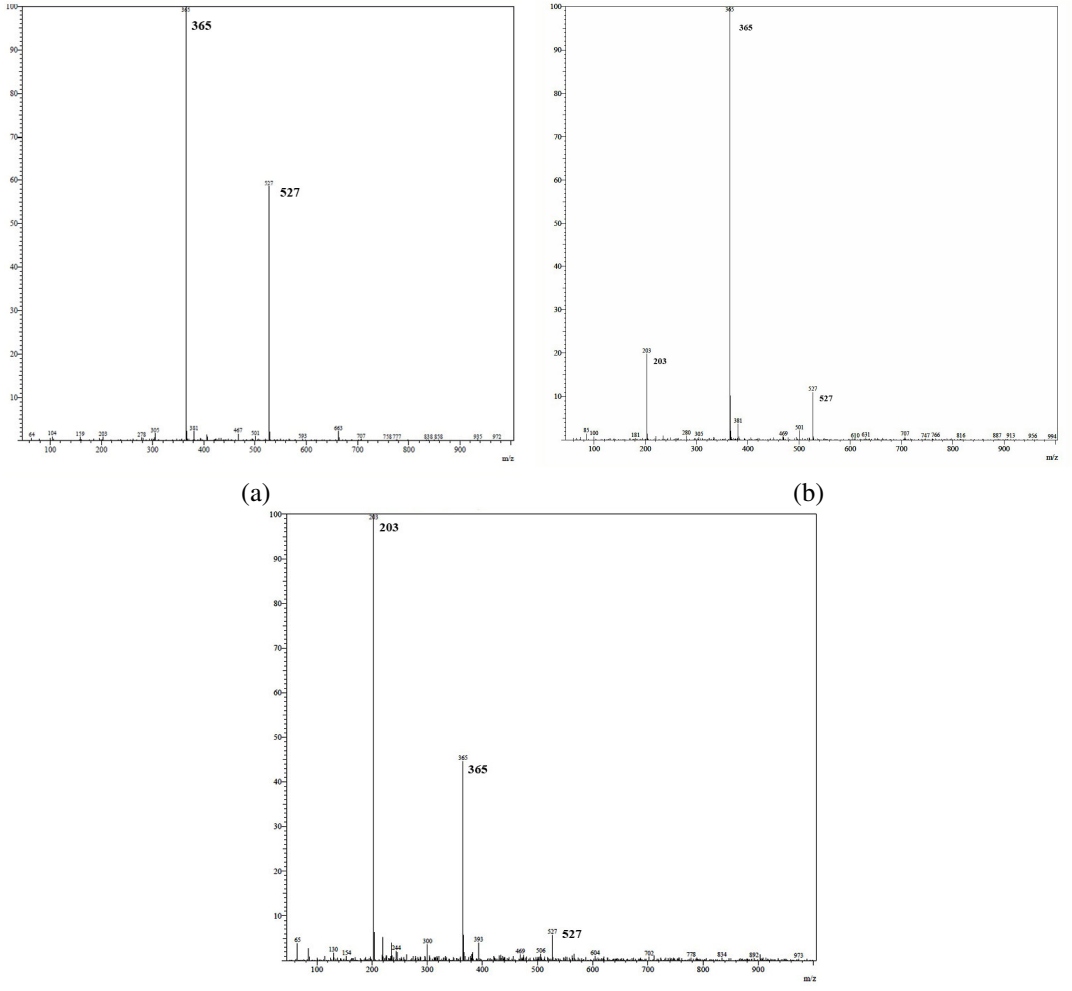


Figure 2. Mass spectrogram of the fractions pooled from gel chromatography as shown in Figure 1. (a) The fraction of the 11th to the 14th tubes; (b) The fraction of the 15th to 17th tubes, and (c) The fraction of the 18th to the 19th tubes.

mainly existed in the fraction pooled from the 11th to the 14th tubes, and the fraction sample was considered as a purified GOS (abbreviated as GOS-md).

The compositions of the purified GOS-md were then analyzed by HPAEC-PAD. Figure 3 illustrates the monosaccharide, including galactose (peak 1) and glucose (peak 2), had almost been removed by comparison of relative peak area with the standard sugars. Peak 3 was identified to the mixture of lactose and 3-*α*-galactobiose or 4-*β*-galactobiose. Peak 4 was assigned to the tri-galacto-oligosaccharides, and this result agreed with the reported findings that the major constituent of the synthesized GOS mixture was a trisaccharide by using active *β*-galactosidase Inclusion Bodies (IBs) (Lee *et al*., 2011) and thermotolerant yeasts isolated from different dairy products possessed a high yield of the tri-galacto-oligosaccharides (Petrova and Kujumdzieva, 2010). Peak 5 was suspected to the tetra-galacto-oligosaccharides. As shown in Figure 3, the relative ration of peak area of peak 3 to peak 4 was consistent with the relative ratio of abundance of disaccharide to trisaccharides as shown in Figure 2, namely, the relative amount of disaccharide was a little more than that of trisaccharides. Moreover, the purified GOS-md was acidic hydrolyzed and its monosaccharide composition by HPAEC-PAD was analyzed. Results showed that the purified GOS-md was consistent with two monosaccharides, i.e., galactose and glucose (Figure 4). These data suggested that the possible mechanism of GOS production during the fermentation of *Lactobacillus delbrueckii subsp. bulgaricus* strain was that *β*-Galactosydases produced by *Lactobacillus delbrueckii subsp. bulgaricus* strain could hydrolyze the lactose to produce galactose monosaccharide and further catalyze the transgalactosyl reaction with the lactose and galactose as the substrates to form the polymer of galacto-oligosaccharides.

The FT-IR spectrum of the purified GOS-md is represented in Figure 5. It can be seen that the purified GOS-md had peaks in the wavenumbers of 3,353, 2,892.63, 1,635.94, 1,428.57, 1,071.89, and 889 cm^{-1}, which indicated that there were hydroxyl groups (-OH), the vibration of methanetriyl groups (-CH), stretching vibration of the carbonyl group (C=O), non-symmetric vibration of glucosidic bond (C-O-C) and the presence of *β*-gylcosidic linkages in the purified GOS, respectively (Moussa *et al*., 2008; Coimbra *et al*., 1999). Therefore, it could be inferred that the GOS-md was a β-galactose polymer, which might contain 4-*β*-galactobiose, since disaccharide was the highest amount of ingredient in the compositions of the purified GOS compound.

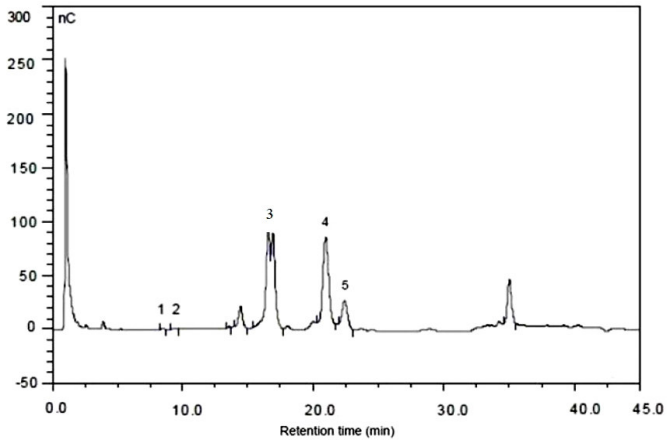
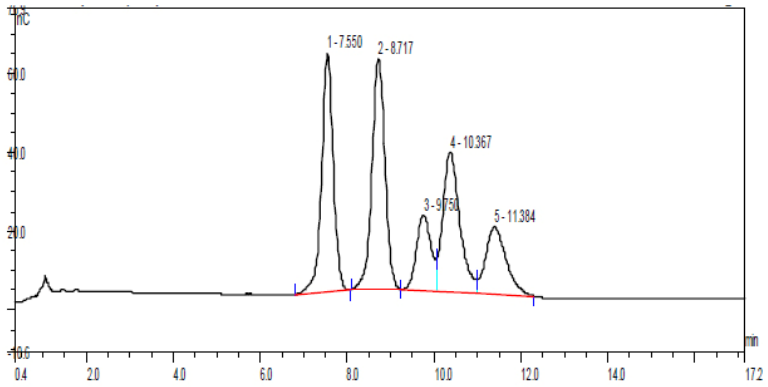
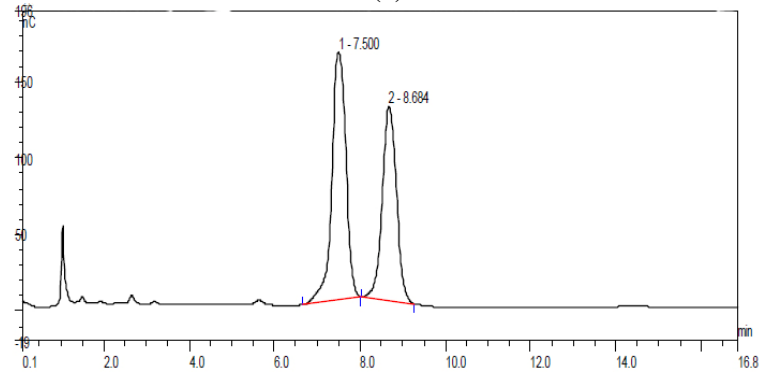


Figure 3. The chromatogram of the GOS-md was analyzed by HPAEC-PAD. Each sugar peak was identified by the following standard sugars: (1) Galactose; (2) Glucose, (3) 3-*α*-Galactobiose, lactose; (4) 4-*β*-Galactobiose or tri-galacto-oligosaccharides, and (5) Tetra-galacto-oligosaccharides.



(a)



(b)

Figure 4. HPAEC-PAD chromatograms of standard monosaccharides (a) and the purified GOS-md (b). The identified standard monosaccharides in (a) are indicated as (1) Galactose; (2) Glucose; (3) Sucrose; (4) Lactose, and (5) Mannose.

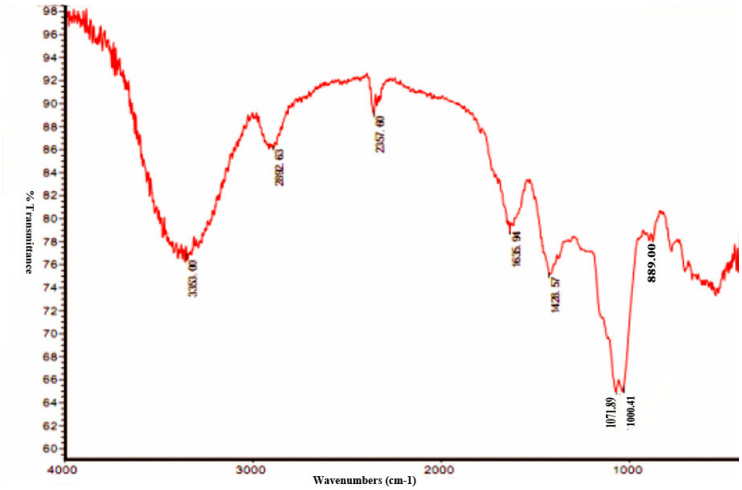


Figure 5. The FT-IR spectra of the purified GOS-md.

Therefore, based on these findings, it could be concluded that the main kinds of GOS-md derived from lactose transformation by the fermentation of *Lactobacillus delbrueckii subsp. bulgaricus* strain in the lactose containing milk media were 4-*β*-galactobiose and tri-galacto-oligosaccharides.

Cellular Immunity Function of the Purified GOS

GOS as a kind of prebiotics, can promote *Bifidobacterium* and *Lactobacillus* proliferation in the mucosal surfaces of gut, and thus are able to produce one or more antibacterial substances to influence intestinal physiology and have additional immunomodulatory roles (Gopalakrishnan *et al*., 2012). IECs are known to play an important role in the immune response at the intestinal mucosa, not only in maintaining a physical barrier to the external environment but also in adjusting alongside cells of the immune system to prevent infection and epithelial injury or promoting the immunologic defense by secretion of proinflammatory cytokines (Pitman and Blumberg, 2000). Therefore, the proliferation of IECs was used for the evaluation of the cellular immunity function of the purified GOS-md.

The effect of GOS-md promoting the proliferation of IECs was determined by culturing IECs with different GOS-md dosages (0, 40, 70, 100, 130, 160, and 190 μg mL^{-1}, respectively) for 4, 8, and 24 h and results are shown in Figure 6. Results showed that the values of relative proliferation rate were significantly ($P<0.05$) elevated when culture times increased gradually, and after being cultured 24 hours, the relative proliferation rate value of samples with GOS-md supplement were obviously higher than the control sample without the addition of GOS-md. Moreover, the various dosages of GOS-md exhibited different effects on the proliferation of IECs in a dose- and a time-dependent manner, and the optimal adding amount was determined as 100 μg mL^{-1}. Figure 6 shows that, after being cultured for 24 hours, the relative proliferation rate of samples with 100 μg mL^{-1} GOS-md supplement was high up to 158%, which was about three times the value after the 4 hours culture without GOS-md. However, the effect of GOS on the IECs proliferation decreased when the concentration of GOS was more than 100 μg mL^{-1}. The reason of the reduced effect might be due to the impact of osmotic pressure. GOS is a saccharide, which are usually osmotic agent (Tomo *et al*., 2000), therefore, it was conjectured that when the concentration of GOS was greater than 100 μg mL^{-1}, the osmotic pressure might be obvious, which might ultimately restrict the cell proliferation (Havard *et al*., 2011).

The MTT experiments showed that 40-190 μg mL^{-1} GOS-md were able to promote IECs proliferation. Therefore, the concentrations

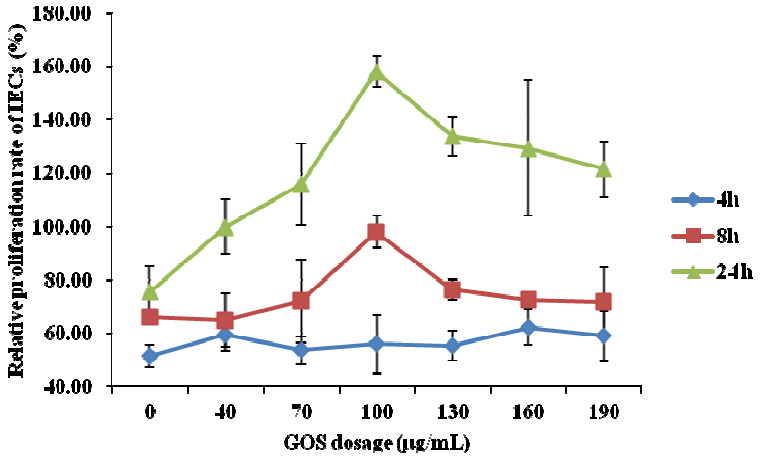


Figure 6. Relative proliferation rate of IECs with different concentrations of the purified GOS-md compound determined by MTT assay.

of 50, 100, and 150 μg mL^{-1} and 24 hours were chosen for the following experiment. In order to accurately quantify the degree of cell proliferation, cell cycle distribution was analyzed by flow cytometry. Cell proliferation can be efficiently analyzed by multiparameter flow cytometry, and viable cell production may be assessed by measurement of *S*-phase influx or mitotic rate. According to the results shown in Figure 7 and Table 1, after 24 hours culture, the area of *S* phase in the IECs group treated with 100 μg mL^{-1} GOS-md displayed the biggest value among the three tested IECs

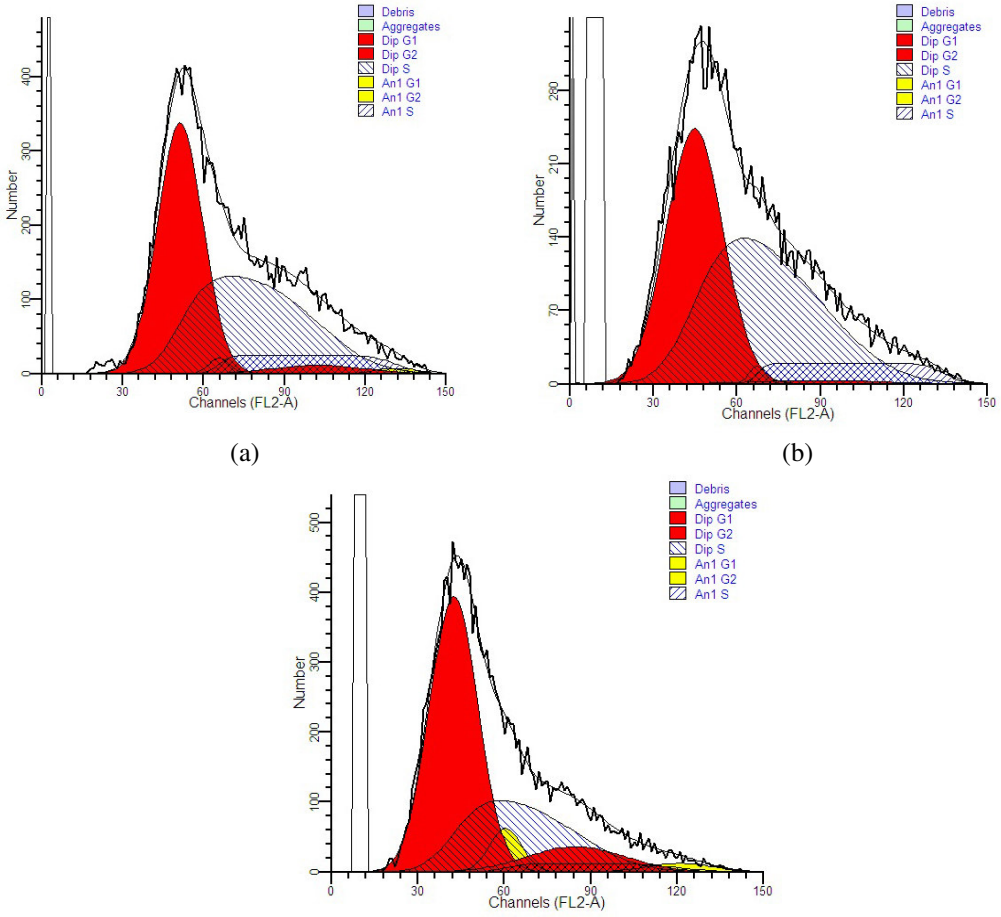


Figure 7. The induced cell cycle extension in IECs with the different concentration of GOS-md at: (a) 50; (b) 100, and (c)150 μg mL^{-1}.

Table 1. Cell cycle extension induced by different concentration of GOS-md in IECs at 24 hours.

Phase of cell cycle[a]		GOS-md		
		50 (μg mL^{-1})	100 (μg mL^{-1})	150 (μg mL^{-1})
Diploid	G1 % of cells	48.85 ±0.9	45.89 ±1.4	56.98 ±2.3
	S % of cells	47.99 ±2.1	52.80 ±3.2	32.52 ±2.8
	G2 % of cells	3.17 ±1.0	1.31 ±0.6	10.50 ±1.2
Aneuploid	G1 % of cells	9.99 ±0.8	8.47 ±0.7	44.78 ±2.6
	S % of cells	83.05 ±2.4	91.27 ±1.8	37.03 ±0.6
	G2 % of cells	6.96 ±1.1	0.26 ±0.01	18.19 ±1.7

[a] Cell cycle distributions of GOS-md-treated cells were determined by PI staining and flow cytometric analysis.

groups, and its area percentage was high up to 52.80%. This finding suggested that milk-derived GOS obtained in this study had the immunity function by promoting the proliferation of IECs, which were vital active component relative to the defense mechanism of the gut to endotoxin.

IECs have been shown to produce IL-6 in response to bacterial infection. IL-6 as a multifunctional cytokine is involved in diverse biological processes, such as host response to enteric pathogens, hematopoiesis, acute-phase reactions and growth factor (Vinderola *et al*., 2005). Thus, levels of IL-6 of IECs treated by 10 μg mL^{-1} LPS and a series of concentrations of GOS (0, 50, 100, and 150 μg mL^{-1}, respectively, treated for 24 hours) were detected by ELISA. As shown in Figure 8 and Table 2, LPS stimulation in IECs increased the levels of IL-6 in comparison with the control group ($P > 0.05$). What is more, the IL-6 production by cultured (24 h) IECs with adding GOS-md (100 and 150 μg mL^{-1}, respectively) showed a significant difference with the LPS versus control group ($P < 0.05$). Moreover, a remarkable finding was that GOS-md in the concentration 100 μg mL^{-1} could induce the highest IL-6 production, and the amount of IL-6 was up to 133 ng L^{-1}. IL-6 is involved in both inflammatory and normal immune responses. IL-6 has traditionally been considered as the product of proinflammatory cells (Bromberg and Wang, 2009), however, IL-6 is also known to possess several anti-inflammatory characteristics, such as its ability to down-regulate LPS-induced monocyte IL-1 and TNF-α mRNA expression (Miller and McGee, 2002). Thus, it is necessary to make an intensive study of the cellular immunity mechanism of milk-derived GOS in the future.

CONCLUSIONS

The contents of milk-derived GOS by being fermented and refined were disaccharide and trisaccharide, and further confirmed as lactose, 4-β-galactobiose and tri-galacto-oligosaccharides by MS, HPAEC-PAD and RT-IR. Furthermore, the cellular immune function of GOS-md was observed and it could be found that GOS-md at the concentration of 100 μg mL^{-1} could significantly ($P < 0.05$) promote IECs proliferation and elevate IL-6 production.

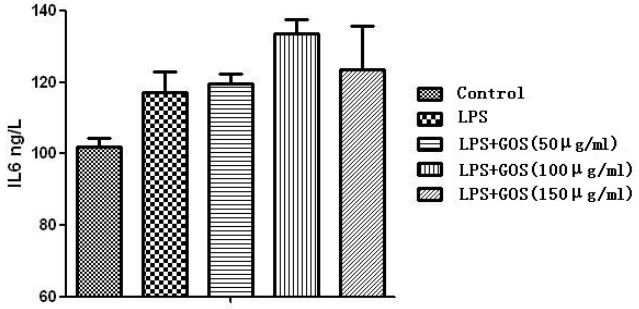


Figure 8. The effects of GOS-md on the promotion of IL-6 production in LPS-induced IECs.

Table 2. The effects of GOS-md on the production of IL-6 in LPS-induced IECs.[a]

	Control	LPS	LPS+GOS (50 μg mL^{-1})	LPS+GOS (100 μg mL^{-1})	LPS+GOS (150 μg mL^{-1})
IL-6 (ng/L)	102.05±0.49	117.16±0.19	119.50±0.56	133.54±0.81*	123.60±2.46*

[a] Results presented were representative of three independent experiments. Values are presented as means ±SD. * $P < 0.05$ vs. control.

Therefore, milk-derived purified GOS obtained in this study had cellular immunity bioactivity, which indicated that it could also be utilized as a novel and natural immunoregulatory agent. Thus, this work can contribute to further development of an effective technology for production of valuable galacto-oligosaccharides by lactose hydrolysis and enzyme catalysis with *Lactobacillus delbrueckii subsp. bulgaricus* strain cells.

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شناسایی ساختمان و فعالیت زیستی ایمنی یاخته ای گالاکتواولیگوساکارید مشتق از شیر و تهیه شده با تخمیر *Lactobacillus delbrueckii subsp. Bulgaricus*

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چکیده

در این پژوهش، گالاکتواولیگوساکارید (GOS) به دست آمده از شیر با تخمیر *Lactobacillus delbrueckii subsp. Bulgaricus* تولید شد و با غشای فراپالایش-نانوصافی تصفیه شد. در ادامه بررسی ها، آشکار شد که محصولات GOS خالص شده با کروماتوگرافی غربالی، بیشتر حاوی دی ساکارید و تری ساکاریدها با وزن ملکولی کم، مانند ۴-β-galactobiose و tri-galacto-oligosaccharides، بودند. فعالیت ایمنی یاخته ای GOS خالص شده با استفاده از یاخته های epithelial معده (IECs) ارزیابی شد. یافته ها نشان داد که GOSنفوذ IECs ها را به صورتی که وابسته به دُز و زمان بود به طور معنی داری ($P<0.05$) افزایش داد و نرخ نفوذ نسبی ۲۴ ساعت پس از کشت در غلظت ۱۰۰ میکرو گرم در میلی لیتر تا ۱۵۸٪ افزایش یافت که سه برابر مقدار مشابه در ۴ ساعت بعد از کشت بدون GOS بود .همچنین، با افزودن ۱۰۰ میکرو گرم در میلی لیتر GOS، تولید IL-6 به طور قابل مشاهده ای تا حد ۱۳۳/۵۴ نانوگرم در لیتر افزایش یافت. این داده هاچنین اشاره دارد که GOS خالص شده ممکن است نقشی در بهبود ایمنی داشته باشد و بنا بر این می توان آن را به عنوان ماده ای بدیع و طبیعی که تنظیم کننده ایمنی است در رشته های پزشکی و غذایی به کار بست. این پژوهش این موضوع را هم آشکار کرد که کار برد فعالیت transgalactosylation ماده-β galactosidase به دست آمده از تخمیرریزاندامگان فرا سودمند(probiotics) مانند *Lactobacillus delbrueckii subsp. Bulgaricus* می تواند ارزش محصولات شیری را به لحاظ شکل GOS بالا ببرد.