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Isolation, Characterization, and Immunological Effects of α-Galacto-oligosaccharides from a New Source, the Herb *Lycopus lucidus* Turcz.

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This study was designed to isolate and characterize a mixture of α-galacto-oligosaccharides (GOS) from a new source, the roots of *Lycopus lucidus* Turcz. (RL), a traditional dietary treatment. In this study, the chemical components and immunological function of RL-GOS were investigated. HPLC analysis showed that the purified RL-GOS was a typical raffinose family oligosaccharide (RFO) with a high stachyose content of 51.8% (w/w), followed by 26.5% raffinose and 10.1% verbascose. Further functional evaluation showed that RL-GOS could elicit a significant increase ($p < 0.05$ vs control) in humoral immunity, as measured by plaque-forming cell (PFC) generation and serum hemolysin level in response to sheep red blood cells (SRBC) at all three tested doses of RL-GOS (0.75, 1.5, and 3.0 g/kg of BW) in mice. In addition, the cellular immune activity of RL-GOS was also demonstrated by enhancing in vivo delayed-type hypersensitivity (DTH) reaction to SRBC and spleenocyte proliferation response to concanavalin A ($p < 0.05$, compared with control group). Nevertheless, there were no significant differences in weight gain, lymphoid organ indices, and phagocytosis capacity following RL-GOS treatment. This study provides evidence for the discovery of a new GOS source (20% w/w GOS in fresh roots of *L. lucidus* Turcz.) and its potential application as an immune stimulant in functional foods.

KEYWORDS: *Lycopus lucidus* Turcz.; galacto-oligosaccharides; HPLC-RI; immunological activity

INTRODUCTION

Nondigestible oligosaccharides of various origins (plants, viruses, bacteria, and fungi) present important physiological functions and have been used extensively as both food ingredients and pharmacological supplements to aid weight control, regulate glucose, reduce serum lipid levels, and control some acute or chronic diseases (*1*−*5*). Raffinose, which has an α-D-galactosyl residue linked (1→6) to the D-glucosyl residue of sucrose, is the first member of a family of α-galacto-oligosaccharides (α-GOS), which also includes stachyose (two galactosyl residues) and verbascose (three galactosyl residues) (**Figure 1**). It is an excellent dietary fiber and prebiotic and also is one of the best known and most commonly applied oligosaccharides. Therefore, α-GOS application as food ingredient has increased rapidly (*6*−*9*).

α-GOS are widely distributed in legume seeds, such as soybean, lupin, lentil, and pea, chickpea, etc. (*10*−*13*). Although the intake of α-GOS is indigestible in the upper intestinal tract and is associated with the production of flatulence, it may be regarded as a potential human nutriment because it can be selectively consumed by intestinal commensally beneficial microflora to exert a crucial contribution to human nutrition and welfare (*2*, *3*). Therefore, it is claimed that the ingestion of α-GOS dietary sources could regularly promote health and lessen the severity of many disorders (*13*), and this function possibly depends on their detailed structures.

The aerial parts (leaves and stems) of *Lycopus lucidus* Turcz., which belongs to the family Lamiaceae (Labiatae), have been used for centuries as an oriental traditional medicine and dietary herb for its anti-inflammatory, cardiac, sedative, wound-healing, pain relief, and tonic effects (*14*, *15*). In recent years, the roots of *L. lucidus* Turcz. (RL), also called "small ginseng" in China, have been popularly consumed as agricultural vegetable product and botanical food supplements among peasants in China. In the present study, we report for the first time, in detail, the chemical characterization of the isolated GOS from dietary herb RL with the aim of discovering and selecting a new GOS source with high amounts of α-galactoside for use as a functional food ingredient. It is well-known that functional foods possess not only nutritional value but also physiological functions. For nondigestible oligosaccharides, the immune-modulatory activity is very important from the food point of view. Therefore, we also further investigated the systemic immunomodulatory activity of RL-GOS. Such information would facilitate the sustainable use of *L. lucidus* Turcz. as a new source of GOS functional food ingredients.

MATERIALS AND METHODS

Materials and Chemicals. The roots of *L. lucidus* Turcz. were harvested from the countryside region of Jingbian County (northeastern

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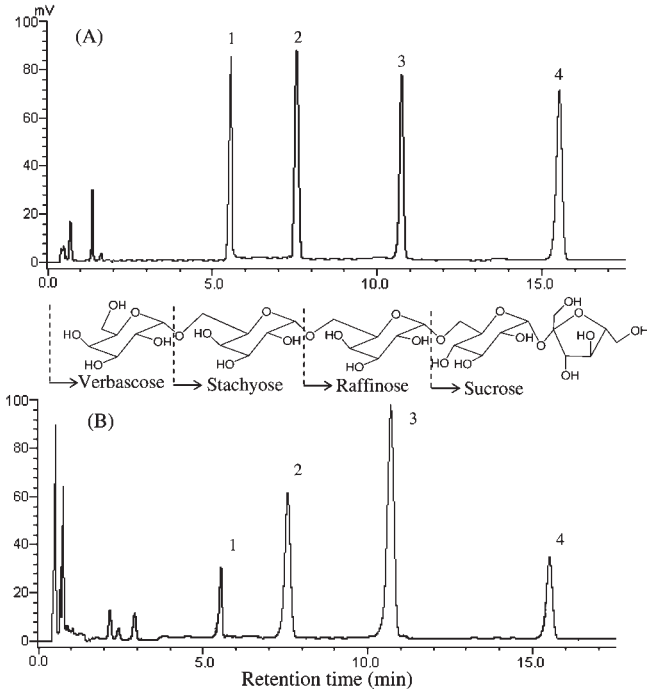

Figure 1. HPLC-RI chromatographic profile of the standard mixture (**A**) and compositional oligosaccharides released from RL-GOS (**B**). HPLC-RI conditions: column, TSKgel Amide-80 (4.6 i.d. × 250 mm, 5 μm); mobile phase, acetonitrile/water (75:25, v/v); flow rate, 1.5 mL/min. The analytical procedures were performed as described under Materials and Methods. Peaks: 1, sucrose; 2, raffinose; 3, stachyose; 4, verbascose.

China) of Shaanxi province, China. The samples were thoroughly washed with water, air-dried, and finely pulverized into a powder. Verbascose, stachyose, raffinose, sucrose (all >98%), concanavalin A (Con A), sodium dodecyl sulfate (SDS), and activated charcoal (Darco G-60, 100 mesh) were obtained from Sigma-Aldrich (St. Louis, MO). Thiazolyl blue tetrazolium bromide (MTT), RPMI 1640, dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), and fetal bovine serum were from Gibco BRL (Gaithersburg, MD). All other chemicals were of analytical grade.

Isolation and Purification of RL-GOS. RL-GOS were extracted and purified as previously described (*8*, *16*) with some modifications. Briefly, the powder (300 g) of the dried rhizomes was soaked for 24 h with 100% ethanol at ambient temperature. Solvent was removed by filtration, and then the defatted residue was extracted twice with 3.0 L of stirred 10% aqueous ethanol at 60 °C for 2 h. The combined extract was centrifuged for 15 min at 3000 rpm, and the supernatants were concentrated under reduced pressure below 80 °C and further deproteinized by freeze−thaw process, repeated three times, followed by a centrifugation for 15 min at 3000 rpm. The resulting supernatant fluid was stirred with 3 g of activated charcoal Darco G-60 (100 mesh) for 30 min to remove mono- and disaccharides. This mixture was filtered under vacuum, and the activated charcoal was further washed with 10% aqueous ethanol. The oligosaccharides adsorbed onto the activated charcoal were desorbed by stirring in 50% aqueous ethanol for 30 min. Activated charcoal was eliminated by filtering, and the solution was evaporated under vacuum. The remaining sample was filtered through a 0.45 μm filter and freeze-dried to obtain the oligosaccharides (RL-GOS) as a fine white powder.

Analytical Identification of RL-GOS. The quantification of the component oligosaccharides in RL-GOS was carried out using an Agilent 1100 series HPLC system (Agilent, USA) with a refraction index detector (RI, G1362A). Chromatography was performed on a TSKgel amide-80 column (4.6 i.d. × 250 mm, 5 μm), using acetonitrile/water (75:25, v/v) as the mobile phase at a flow rate of 1.5 mL/min. Stock standard solutions of each carbohydrate at a concentration of 2.0 mg/mL were prepared in deionized water. Working standard solutions were prepared as needed by appropriate dilution of the stock solutions in deionized water and stored at 4 °C. Quantification of each carbohydrate was performed by comparing the peak areas with those of the standard solutions. Before injection, all samples were filtered through a 0.45 μm Millipore membrane.

Experimental Animals and Treatments. A total of 200 female Kunming mice weighing 18−22 g (6−8 weeks old) were used in the present study. Animals were acclimatized for at least 7 days prior to use and maintained in a temperature-controlled environment (22 ± 2 °C) with a 12 h light−dark cycle and with free access to water and standard rodent chow. All animals were treated in accordance with the Guidelines of the Principle of Laboratory Animal Care (NIH Publication, revised 1985) and with the approval of the Medical Research Committee, Disease Control Centre of Shaanxi Province, China.

Animals used in each experiment were randomly assigned to four groups of 10 animals each, that is, vehicle control group and 0.75, 1.50, and 3.0 g/kg of body weight (g/kg of BW) RL-GOS groups. RL-GOS was intragastrically administered to mice for 30 consecutive days, and the control mice were given vehicle (normal saline) only. The dose volume was 0.2 mL/10 g of BW. Male guinea pigs were used for complement preparation, where blood was collected from guinea pig hearts, and serum was prepared by centrifugation and diluted 1:10 with physiological saline.

Lymphoid Organ and Body Weights. The body weights of the mice in each group were measured every week throughout the experimental period, and the mice were sacrificed by cervical dislocation. Animals were weighed and sacrificed after the last dose of RL-GOS treatment, and the weights of vital organs such as spleen and thymus were recorded and indexed as relative organ weights (w%).

Plaque-Forming Cell (PFC) Assay. On the 25th day of RL-GOS treatment, the mice were immunized with 0.2 mL of 10% (v/v) sheep red blood cells (SRBC) by intraperitoneal (ip) injection, and RL-GOS supplement was continued for 5 days. The following parameters of humoral immunity were studied in those animals. The PFC assay was performed according to the method reported by Cunningham and Szenberg (*17*). Briefly, the animals were sacrificed after the immunization with SRBC for 5 days, spleen cells were separated in RPMI 1640 medium, washed twice, and suspended in the same medium (10^6 cells/mL). One hundred microliters of 5×10^6 splenocyte suspension and 100 μL of 20% SRBC were mixed with 100 μL of 1% agarose and then added on a slide covered with a 1.2% agarose layer in PBS (0.05 M, pH 7.4, 0.15 M NaCl). The Petri dishes were followed by an incubation period of 90 min at 37 °C; then 2 mL of 1:9 diluted fresh guinea pig serum was added to the Petri dishes, and the plate was re-incubated for another 90 min to allow plaque formation. The number of plaques was counted immediately, and values are expressed as counts per 10^6 spleen cells.

Assay for Serum Hemolysis to SRBC. After animals were treated as described for the PFC assay, serum was collected through the eye orbit of mice and then diluted with PBS. Serial 2-fold dilution of serum (100 μL) was transferred to hemagglutination microplate, and 100 μL of SRBC (0.5%) was added, followed by 100 μL of guinea pig serum in sequence. For negative controls, the complement was substituted with PBS. The degree of agglutination was observed after 3 h of incubation at 37 °C for complement-mediated SRBC hemolysis and terminated on ice. Antibody product was calculated as follows: antibody product $= S_1 + 2S_2 + 3S_3 + \ldots + nS_n$, where S is defined as the degree of agglutination and n is defined as the dilution rate (*18*).

Delayed-Type of Hypersensitivity (DTH) Response. The DTH response was determined using the method of Langrange et al. (*19*). In brief, on the 26th day of RL-GOS administration, mice were immunized ip with 200 μL of SRBC (2%, about 5×10^8 SRBC). The RL-GOS administration was continued for 4 days, and then all of the animals were again subcutaneously (sc) challenged with SRBC (2%) in the left hind footpad on the fourth day after SRBC ip immunization. The right footpad injected sc with the corresponding volume of PBS served as trauma control for nonspecific swelling. The increase in footpad thickness was measured 24 h after the challenge using a dial caliper (pitch, 0.01 mm, Guanglu, China). The results were expressed as difference in the reading between the footpads injected with SRBC and that with PBS.

Splenic Lymphocyte Proliferation Assay. The effect of RL-GOS on Con A-induced lymphocyte proliferation was measured by MTT as described by Zhang et al. (*20*). The splenocytes of the mice treated with vehicle or RL-GOS were isolated by gently pressing spleen tissue through nylon mesh filters. Cells were washed twice with RPMI 1640, counted, and diluted to the appropriate concentrations. Samples of 2×10^6 cells/100 μL

were distributed into 96-well plates in 100 μL of RPMI 1640 medium with 1.5 μg/mL Con A (T cell and B cell mitogen). Cells were incubated at 37 °C under 5% CO_2 for 48 h, 20 μL of 5 mg/mL MTT was then added to each well, and the plates were incubated at 37 °C for 4 h. Cell dissociation solution (20% SDS, 50% DMSO, pH 4.5) was then added to each well to dissolve the purple crystal. Spleen cell proliferation was measured by determining the absorbance at 570 nm using an automated microplate reader (Bio-Rad model 680). The proliferation ratio of control cells induced by Con A was taken as 100%.

Phagocytosis Assay by Carbon Clearance Method. The phagocytosis function of monocytes was assessed through the test of carbon particle clearance (*21*). After 30 days of oral administration with RL-GOS, Indian ink at a dose of 100 μL/10 g of BW was injected by tail vein. Blood (20 μL) were drawn from the retro-orbital venous plexus with a capillary at intervals of 2 min (t_1) and 10 min (t_2) after the carbon particle injection. Each blood sample was immediately added into 2 mL of 0.1% Na_2CO_3 solution. The optical density of each sample at 600 nm was read and used to calculate the phagocytic index K as $K = (\ln OD_1 - \ln OD_2)/(t_2 - t_1)$, where OD_1 and OD_2 are the optical densities at time t_1 and t_2, respectively.

Macrophage Phagocytosis for Chicken Red Blood Cells (CRBC). The phagocytic capacity of peritoneal cavity phagocytes was assessed following the methods previously described (*22*). After the last pretreatment with RL-GOS, mice were immunized with 1.0 mL of 20% (v/v) CRBC by ip injection. Thirty minutes later, they were sacrificed and peritoneal macrophages were aseptically harvested by peritoneal lavage with 2 mL of PBS. One milliliter of cell-rich lavage fluid was smeared on a slide. After incubation at 37 °C for 30 min, nonadherent cells were washed off with PBS, and then slides were fixed and stained using a 9-fold-diluted Giemsa solution (Sigma, St. Louis, MO) with PBS (pH 7.4). The percentage of phagocytosis was determined by counting the number of phagocytes that swallowed the CRBC per 100 macrophages on each slide.

Statistical Analysis. The data were expressed as means ± SEM. Significance was tested by Student's t test by variance analysis. The data were considered to be statistically different from control at $p < 0.05$.

RESULTS AND DISCUSSION

HPLC-RI Analysis for Compositional Characterization of RL-GOS. A routine HPLC-RI chromatographic procedure was performed to measure the component carbohydrates in the RL-GOS preparation. As shown in **Figure 1A**, the tested standard oligosaccharides can be completely baseline separated within 16 min, and the peaks in the chromatogram were identified in the order sucrose, raffinose, stachyose, and verbascose. Method precision was excellent, with CV values of < 5% for all variables and excellent linearity from 10 to 800 μg/mL. The limit of detection (LOD), calculated as 3 times the baseline noise, was below 65 ng (10 μL injection). These results indicate that this method was accurate and reliable.

A typical HPLC-RI chromatogram for oligosaccharide composition in RL-GOS samples is shown in **Figure 1B**, and the quantified constituents are listed in **Table 1**. The component oligosaccharides could be identified by comparison with the standard oligosaccharide mixture (**Figure 1A**). The results indicate that RL-GOS is a typical raffinose family oligosaccharide mixture (RFO) and is composed of sucrose and α-galactosides raffinose (trisaccharide), stachyose (tetrasaccharide), and verbascose (pentasaccharide) in the amounts of 2.7, 26.5, 51.8, and 10.1% (grams per 100 g of RL-GOS product), respectively. Moreover, recovery experiments were performed to investigate the accuracy of the method. As shown in **Table 1**, the recoveries of all four tested short-chain carbohydrates ranged between 90.1 and 104.0%, and the CV values fell within 2.8−4.7%. Such results further demonstrated that this method is precise and practical for the analysis of the RL-GOS samples.

The HPLC-RI identification results clearly showed that the major oligosaccharides present in RL-GOS were stachyose and raffinose, accounting for up to 78.3% of the RL-GOS preparation, and only a trace amount of sucrose (2.7%) was detected.

Table 1. Average Contents and Recovery Analysis ($n = 3$) of the Digestible Sucrose and Nondigestible RFO in the RL-GOS Preparation

component	initial amount (mg/100 mg of extract)	added amount (mg)	found amount (mg)	accuracy (%)	precision (CV%)
sucrose	2.7	5.0	7.9	104.0	4.7
raffinose	26.5	20	47.0	102.4	3.9
stachyose	51.8	50	101.7	99.8	2.8
verbascose	10.1	20	28.1	90.1	5.3

This is in agreement with previous reports that the methodology with activated charcoal to remove most of the monosaccharides and part of the disaccharides was specific for the isolation of relatively high-purity oligosaccharides (*8*, *16*). With this method, 91.1% of the RL-GOS are short-chain carbohydrates, indicating that RL-GOS is a high-purity oligosaccharide preparation. This compositional feature of RL-GOS is similar to that of legume lupin RFO preparations, where the main oligosaccharides were identified as stachyose and raffinose and a low level of verbascose (*10*−*12*, *23*). However, our work demonstrates that the extraction yield of the purified RL-GOS could reach approximately 20% of the fresh RL. In previous research on GOS distribution in a wide species of lupin seeds, which are regarded as the best GOS source, *L. luteus* was characterized by a remarkably high amount of total α-galactosides (9.5−12.3%), which was about twice that of other lupin cultivars (*10*−*12*). This finding indicates that traditional dietary herb RL is a novel plant-based oligosaccharide source, which was richer in functional GOS than legume lupin for this purpose.

Humoral Immune Properties of RL-GOS. The effect of RL-GOS on PFC generation of spleen in response to SRBC (T-dependent particulate antigen) was investigated in mice, and the results are presented in **Figure 2A**. The number of PFC in spleen cells was significantly enhanced by 40.6, 29.1, and 56.2% at all three tested doses of RL-GOS (0.75, 1.5, and 3.0 g/kg of BW), respectively, compared with that in the control ($p < 0.05$). The increasing effect of RL-GOS was very significant at a high dose of 3.0 g/kg ($p < 0.01$). To further confirm the humoral immunity of RL-GOS, we also evaluated the levels of serum hemolysin antibody in response to SRBC in mice. As shown in **Figure 2B**, the daily administration to mice of a 0.75 g/kg RL-GOS dose for 30 days resulted in an 18.8% enhancement of serum hemolysin level in comparison with the control value ($p < 0.05$). Animals receiving the mid (1.5 g/kg) and high doses (3.0 g/kg) displayed corresponding elevations by 32.2 and 32.4% in hemolysin production to SRBC ($p < 0.01$), respectively, both of which were significantly higher than the low-dose group ($p < 0.05$). From the results it was concluded that RL-GOS possessed the potential of humoral immune-enhancing activity.

In Vivo Cellular Immune Function of RL-GOS. To understand whether the specific immune response of RL-GOS was also involved in the cellular immune mechanism, we evaluated the DTH reaction to SRBC and proliferation response of splenic lymphocyte to Con A in mice. As expected, mice receiving RL-GOS at doses of 1.5 and 3.0 g/kg had significant DTH responses in vivo by 12.3 and 21.6% in a dose-dependent manner, respectively ($p < 0.05$, versus control; **Figure 3A**). However, no significant immunological effect was observed at the low dose of 0.75 g/kg when compared to control group (**Figure 3A**, $p > 0.05$). From the results shown in **Figure 3B**, it was further confirmed that in the mice treated with RL-GOS at the doses of 1.5 and 3.0 g/kg, the Con A-stimulated spleenocyte proliferation was markedly enhanced by 32.6 and 79.1%, respectively, in comparison with the control group ($p < 0.05$ and 0.01, respectively). However, the

Table 2. Effect of RL-GOS on Body Weight Gain, Relative Weights of Lymphoid Organs of Mice, and Nonspecific Phagocytic Activities of Macrophages after 30 Days of Treatment

			percentage of body wt		phagocytic index	
treatment	dose (g/kg)	body wt gain (%)	thymus	spleen	carbon particles	CRBC
control		12.1 ± 2.4	0.36 ± 0.10	0.54 ± 0.08	4.38 ± 0.33	0.82 ± 0.15
RL-GOS	0.75	12.4 ± 2.1	0.33 ± 0.06	0.55 ± 0.12	4.44 ± 0.93	0.88 ± 0.17
RL-GOS	1.5	11.6 ± 3.0	0.32 ± 0.08	0.64 ± 0.35	4.46 ± 0.39	0.93 ± 0.11
RL-GOS	3.0	12.0 ± 3.2	0.37 ± 0.07	0.57 ± 0.10	4.78 ± 0.70	0.81 ± 0.15

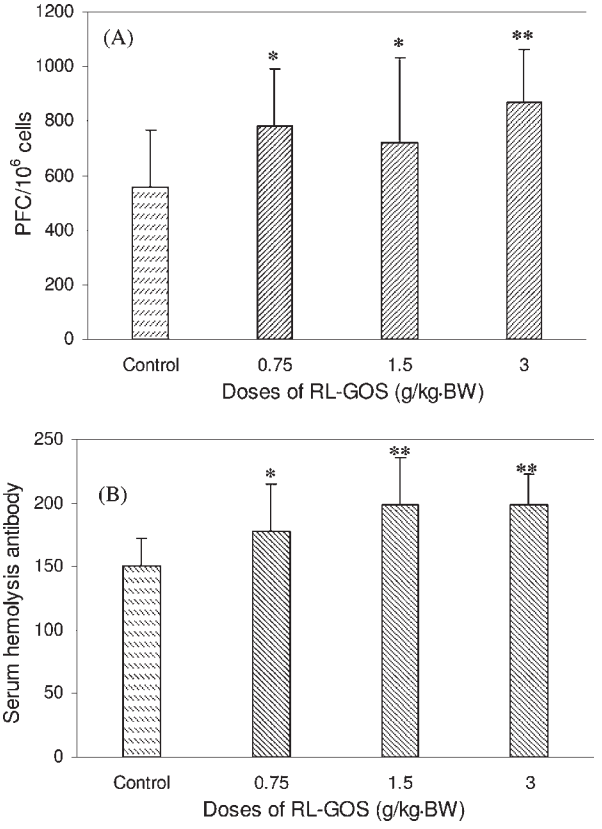

Figure 2. Effect of RL-GOS on humoral immunity as assessed by the PFC assay (**A**) and the measurement of serum hemolysis antibody to SRBC (**B**). Data are shown as mean ± SEM of 10 mice. ∗, $p < 0.05$, and ∗∗, $p < 0.01$, indicate a significant difference versus control group.

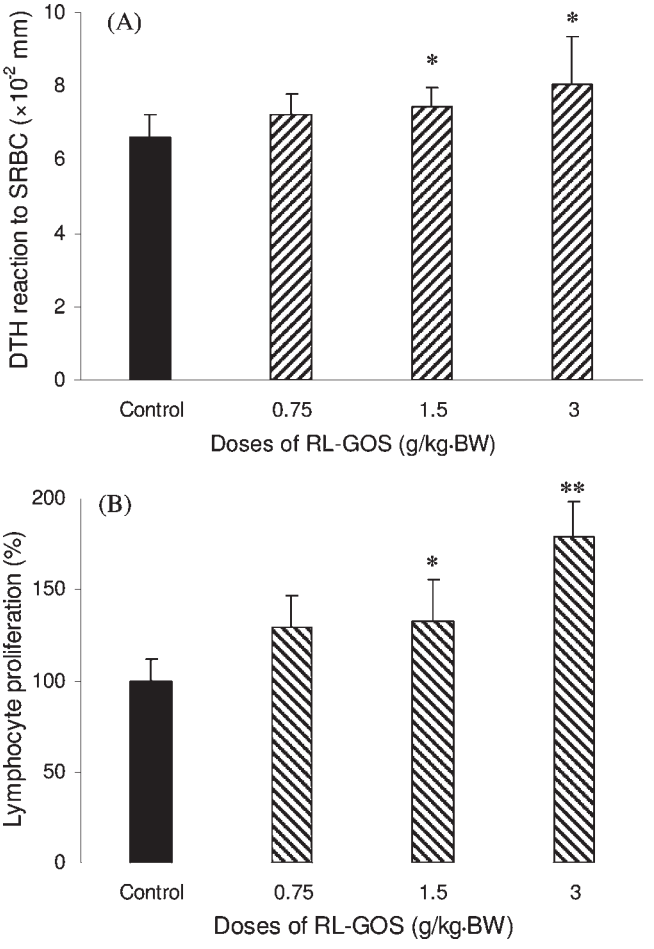

Figure 3. Effect of RL-GOS on cellular immune function as assessed by the DTH response (**A**) and the proliferation response of Con A-induced splenic lymphocytes (**B**). Footpad swelling for DTH response was tested as the difference between the thickness of footpads challenged with SRBC and saline, respectively. The proliferation ratio of Con A-induced lymphocytes separated from control mice was regarded as 100%. Each bar represents the mean ± SEM of results obtained from 10 mice. Asterisks denote a significant difference compared with the control groups (∗, $p < 0.05$; ∗∗, $p < 0.01$).

lymphocyte proliferation ratio at the low dose of RL-GOS (0.75 g/kg) did not reach statistical significance ($p > 0.05$). In agreement with the results of humoral immunity assay, the tested oligosaccharides could effectively exert cellular immune functions via enhancing DTH response and spleenocyte proliferation in vivo.

Effects of RL-GOS on Nonspecific Immune Functions. Phagocytic activity of RL-GOS-activated monocytes in vivo was examined using a carbon clearance test. The changes in the phagocytic index observed in mice were not statistically significant at all three doses (**Table 2**). Furthermore, macrophage phagocytosis of CRBC was also measured in mice. As shown in **Table 2**, phagocytosis rates in the three groups receiving RL-GOS were not significantly greater than those in the control group ($p > 0.05$). The results indicate that the systemic immunological effects of RL-GOS were not associated with nonspecific immune modulation in vivo, probably due to the indigestibility feature of the tested oligosaccharides, which cannot be absorbed by the gut to directly stimulate in vivo macrophages (*24*, *25*).

Moreover, the effects of RL-GOS on body and lymphoid organ weights in mice are also shown in **Table 2**. There were no significant differences in mice treated with RL-GOS alone compared with control mice during the 30 day study ($p > 0.05$).

There is good evidence that the functional oligosaccharides have positive influences in reducing the risk of development of nutrition-related chronic diseases (*26*). This fact consequently has stimulated the search for new oligosaccharide sources. Among these, legume α-GOS including those from pea and lupin have been extensively investigated (*27*, *28*). However, little is known about α-GOS from traditional herbs and their possible application as modern functional food ingredients. The findings outlined above demonstrated first that the dietary herb RL is a potential source rich in α-GOS (raffinose, stachyose, and verbascose) with

the content of up to 20% of fresh weight. However, for most oligosaccharide sources the concentration range is between 0.3 and 10% (*10*−*12*,*23*). Therefore, these findings are of significance in the discovery of new functional food sources and development of agricultural products.

It is well-known that GOS prebiotic nutriment can support the growth of beneficial bacteria in the gut. There is increasing evidence that prebiotic oligosaccharides are also associated with immunostimulatory effects including both innate and specific immunity, although most data originate from mucosal immune system of the intestine and in vitro experiments (*24*−*29*). However, living bifidobacteria and lactobacilli as probiotic agents have immune-modulatory effects on innate and mucosal immune parameters, as well as on systemic immune responses (*27*−*29*). For these reasons, studies on the systemic immunological potential of oligosaccharides are especially interesting and necessary. It is assumed that prebiotics have effects on the systemic immune responses similar to probiotics, because both enhance the number of beneficial bacteria in the gut. Therefore, in our study we further investigated the immunological activity of novel herb-based RL-GOS in mice. This study showed that RL-GOS intervention significantly enhanced cellular and humoral immune responses, although without any changes of the nonspecific immune parameters. This suggests that RL-GOS is a potential plant-based systemic immunomodulator. Although the mechanism behind the effects has not been established, there is strong evidence indicating that the changes in the intestinal microflora that occur with the consumption of prebiotic oligosaccharides may potentially mediate immune responses via the direct contact of lactic acid bacteria or bacterial products (cell wall or cytoplasmic components) with immune cells in the intestine, the production of short-chain fatty acids from oligosaccharide fermentation, or changes in mucin production (*24*−*29*). Therefore, oligosaccharide-induced immune modulation has often been attributed to microbiota-dependent effects (*24*−*29*). As a result, this research opens the possibility of exploitation of RL-GOS as a new source of bioactive oligosaccharides with immunostimulatory activity. Together with the effects of other potential functional oligosaccharides on gut bacteria and health, this finding further supports the challenging new concept that functional food ingredients should exert a beneficial effect on host health beyond nutritive value.

ABBREVIATIONS USED

RL, roots of *Lycopus lucidus* Turcz.; GOS, galacto-oligosaccharides; HPLC-RI, high-performance liquid chromatography with refraction index detector; RFO, raffinose family oligosaccharide; PFC, plaque-forming cell; DTH, delayed-type hypersensitivity; Con A, concanavalin A; SRBC, sheep red blood cells; CRBC, chicken red blood cells.

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LITERATURE CITED

(1) Lindsay, J. O.; Whelan, K.; Stagg, A. J.; Gobin, P.; Al-Hassi, H. O.; Rayment, N.; Kamm, M. A.; Knight, S. C.; Forbes, A. Clinical, microbiological, and immunological effects of fructooligosaccharide in patients with Crohn's disease. *Gut* **2006**, *55*, 348–355.

(2) Steed, H.; Macfarlane, G. T.; Macfarlane, S. Prebiotics, synbiotics and inflammatory bowel disease. *Mol. Nutr. Food Res.* **2008**, *52*, 898–905.

(3) Ishizuka, S.; Iwama, A.; Dinoto, A.; Suksomcheep, A.; Maeta, K.; Kasai, T.; Hara, H.; Yokota, A. Synbiotic promotion of epithelial proliferation by orally ingested encapsulated bifidobacterium breve and raffinose in the small intestine of rats. *Mol. Nutr. Food Res.* **2009**, *53*, S62–S67.

(4) Matsukawa, N.; Matsumoto, M.; Chiji, H.; Hara, H. Oligosaccharide promotes bioavailability of a water-soluble flavonoid glycoside, αG-rutin, in rats. *J. Agric. Food Chem.* **2009**, *57*, 1498–1505.

(5) Ariefdjohan, M. W.; Martin, B. R.; Lachcik, P. J.; Weaver, C. M. Acute and chronic effects of honey and its carbohydrate constituents on calcium absorption in rats. *J. Agric. Food Chem.* **2008**, *56*, 2649–2654.

(6) Vulevic, J.; Drakoularakou, A.; Yaqoob, P.; Tzortzis, G.; Gibson, G. R. Modulation of the fecal microflora profile and immune function by a novel *trans*-galactooligosaccharide mixture (B-GOS) in healthy elderly volunteers. *Am. J. Clin. Nutr.* **2008**, *88*, 1438–1446.

(7) Depeint, F.; Tzortzis, G.; Vulevic, J.; I'Anson, K.; Gibson, G. R. Prebiotic evaluation of a novel galactooligosaccharide mixture produced by the enzymatic activity of *Bifidobacterium bifidum* NCIMB 41171, in healthy humans: a randomized, double-blind, crossover, placebo-controlled intervention study. *Am. J. Clin. Nutr.* **2008**, *87*, 785–791.

(8) Sanz, M. L.; Polemis, N.; Morales, V.; Corzo, N.; Drakoularakou, A.; Gibson, G. R.; Rastall, R. A. In vitro investigation into the potential prebiotic activity of honey oligosaccharides. *J. Agric. Food Chem.* **2005**, *53*, 2914–2921.

(9) Montilla, A.; Corzo, N.; Olano, A.; Jimeno, M. L. Identification of oligosaccharides formed during stachyose hydrolysis by pectinex ultra SP-L. *J. Agric. Food Chem.* **2009**, *57*, 5007–5013.

(10) Martínez-Villaluenga, C.; Frias, J.; Gulewicz, K.; Vidal-Valverde, C. Improved method to obtain pure α-galactosides from lupin seeds. *J. Agric. Food Chem.* **2004**, *52*, 6920–6922.

(11) Giannoccaro, E.; Wang, Y. J.; Chen, P. Y. Comparison of two HPLC systems and an enzymatic method for quantification of soybean sugars. *Food Chem.* **2008**, *106*, 324–330.

(12) Xiang, X. L.; Yang, L. Y.; Hua, S.; Li, W.; Sun, Y.; Ma, H.; Zhang, J. S.; Zeng, X. X. Determination of oligosaccharide contents in 19 cultivars of chickpea (*Cicer arietinum* L) seeds by high performance liquid chromatography. *Food Chem.* **2008**, *111*, 215–219.

(13) Crittenden, R. G.; Playne, M. J. Production, properties and applications of food grade oligosaccharides. *Trends Food Sci. Technol.* **1996**, *7*, 353–361.

(14) Ślusarczyk, S.; Hajnos, M.; Skalicka-Woźniak, K.; Matkowski, A. Antioxidant activity of polyphenols from *Lycopus lucidus* Turcz. *Food Chem.* **2009**, *113*, 134–138.

(15) Lee, Y. J.; Kang, D. G.; Kim, J. S.; Lee, H. S. *Lycopus lucidus* inhibits high glucose-induced vascular inflammation in human umbilical vein endothelial cells. *Vasc. Pharmacol.* **2008**, *48*, 38–46.

(16) Morales, V.; Sanz, M. L.; Olano, A.; Corzo, N. Rapid separation on activated charcoal of high oligosaccharides in honey. *Chromatographia* **2006**, *64*, 233–238.

(17) Cunningham, A. J.; Szenberg, A. Further improvements in the plaque technique for detecting single antibody forming cells. *Immunology* **1968**, *14*, 599–600.

(18) Yang, X. P.; Guo, D. Y.; Zhang, J. M.; Wu, M. C. Characterization and anti-tumor activity of pollen polysaccharide. *Int. Immunopharmacol.* **2007**, *7*, 401–408.

(19) Langrange, P. H.; Mackaness, G. B.; Miller, T. E. Influence of dose and route of antigen injection on the immunological induction of T-cell. *J. Exp. Med.* **1974**, *39*, 528–530.

(20) Zhang, Q.; Zeng, X. K.; Guo, J. X.; Wang, X. Oxidant stress mechanism of homocysteine potentiating Con A-induced proliferation in murine splenic T lymphocytes. *Cardiovasc. Res.* **2002**, *53*, 1035–1042.

(21) Biozzi, G.; Bernacrraf, B.; Halpern, B. N. Quantitative study of the granulopectic endothelial system: II. A study of the kinetics of the granulopectic activity of the RES in relation to the dose of carbon injected. *Br. J. Exp. Pathol.* **1953**, *34*, 441–456.

(22) Davis, L.; Kuttan, G. Immunomodulatory activity of *Withania somnifera*. *J. Ethnopharmacol.* **2000**, *71*, 193–200.

(23) Martínez-Villaluenga, C.; Frías, J.; Vidal-Valverde, C. Raffinose family oligosaccharides and sucrose contents in 13 Spanish lupin cultivars. *Food Chem.* **2005**, *91*, 645–649.

(24) Simões, J.; Madureira, P.; Nunes, F. M.; Domingues Mdo, R.; Vilanova, M.; Coimbra, M. A. Immunostimulatory properties of coffee mannans. *Mol. Nutr. Food Res.* **2009**, *53*, 1036–1043.

(25) Kelly-Quagliana, K. A.; Nelson, P. D.; Buddington, R. K. Dietary oligofructose and inulin modulate immune function in mice. *Nutr. Res.* (*N.Y.*) **2003**, *23*, 257–267.
(26) Mussatto, S. I.; Mancilha, I. M. Non-digestible oligosaccharides: a review. *Carbohydr. Polym.* **2007**, *68*, 587–597.
(27) Vos, A. P.; M'Rabet, L.; Stahl, B.; Boehm, G.; Garssen, J. Immune-modulatory effects and potential working mechanisms of orally applied nondigestible carbohydrates. *Crit. Rev. Immunol.* **2007**, *27*, 97–140.
(28) Swennen, K.; Courtin, C. M.; Delcour, J. A. Non-digestible oligosaccharides with prebiotic properties. *Crit. Rev. Food Sci. Nutr.* **2006**, *46*, 459–471.
(29) Amit-Romach, E.; Uni, Z.; Reifen, R. Multistep mechanism of probiotic bacterium, the effect on innate immune system. *Mol. Nutr. Food Res.* **2010**, *54*, 1–8.

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