

Dietary lactosucrose suppresses influenza A (H1N1) virus infection in mice

Eriko KISHINO^{1*}, Naho TAKEMURA¹, Hisaharu MASAKI¹, Tetsuya ITO¹ and Masatoshi NAKAZAWA²

¹Carbohydrate Research Laboratory, Ensuiko Sugar Refining Co., Ltd., 1-1-1 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan

²Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan

Received April 16, 2015; Accepted June 23, 2015; Published online in J-STAGE July 11, 2015

This study examined the effects of lactosucrose (4^G-β-D-galactosylsucrose) on influenza A virus infections in mice. First, the effects of lactosucrose on fermentation in the cecum and on immune function were investigated. In female BALB/c mice, lactosucrose supplementation for 6 weeks promoted cecal fermentation and increased both secretory IgA (SIgA) levels in feces and total IgA and IgG2a concentrations in serum. Both the percentage of CD4⁺ T cells in Peyer's patches and the cytotoxic activity of splenic natural killer (NK) cells increased significantly in response to lactosucrose. Next, we examined the effects of lactosucrose on low-dose influenza A virus infection in mice. After 2 weeks of dietary supplementation with lactosucrose, the mice were infected with low-dose influenza A virus. At 7 days post infection, a comparison with control mice showed that weight loss was suppressed, as were viral titers in the lungs. In the spleens of lactosucrose-fed mice, there was an increase in the percentage of NK cells. Lastly, mice fed lactosucrose were challenged with a lethal dose of influenza A virus. The survival rate of these mice was significantly higher than that of mice fed a control diet. These results suggested that lactosucrose supplementation suppresses influenza A virus infection by augmenting innate immune responses and enhancing cellular and mucosal immunity.

Key words: cytotoxic activity, splenic NK cells, lactosucrose, mucosal immunity, secretory IgA, serum IgA

INTRODUCTION

The intestinal microbiota plays an important role in the development of host immune responses [1–5]. Probiotics are live microorganisms that alter the intestinal microbial flora. For example, lactobacilli and bifidobacteria are probiotic bacteria present in the small and large intestines. Probiotics influence human health via positive effects on the metabolic fermentation of prebiotics such as oligosaccharides and indigestible dietary fiber. Probiotics can influence the immune response of the host by enhancing humoral and cellular immunity [6]. A fermented milk drink containing *Lactobacillus casei* Shirota increased the cytotoxic activity of natural killer (NK) cells in healthy humans [7], while supplementation with *Lactobacillus rhamnosus* HN001 or *Bifidobacterium lactis* HN019 probiotics during pregnancy increased the

total IgA concentration in breast milk [8]. The ability of probiotics to modulate and regulate immune responses in the gastrointestinal tract probably reflects their interactions with various receptors on intestinal epithelial cells, M cells, and dendritic cells [2–4]. Probiotics metabolize prebiotics into short-chain fatty acids (SCFA) such as butyrate or acetate, which strengthen epithelial barrier integrity [3, 9].

Probiotics may be helpful in preventing upper respiratory tract infection [10, 11]. Reviews on the use of probiotics in humans to prevent respiratory infections have shown a beneficial effect. A meta-analysis was published on probiotics for preventing acute upper respiratory tract infections and strain specificity of probiotics [12]. The respiratory tract itself is colonized by commensal bacteria, and the intestinal microbiota influences immunity in the respiratory tract and systemic immunity in general [13, 14]; thus, probiotics also determine local respiratory immunity. Some probiotic strains have been shown to have protective effects against influenza virus infection. In a murine model [15–20], oral administration of *Lactobacillus casei* Shirota augmented cytotoxic activity in splenic and pulmonary cells and resulted in defensive effects against influenza virus infection [16, 17]. Oral administration of heat-killed *Lactobacillus plantarum* L-137 to mice conferred

*Corresponding author. Mailing address: Eriko Kishino, Carbohydrate Research Laboratory, Ensuiko Sugar Refining Co., Ltd., 1-1-1 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan. Phone: +81-45-780-1922, Fax: +81-45-780-1920. E-mail: eriko_kishino@pearlance.co.jp

©2015 BMFH Press

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <<http://creativecommons.org/licenses/by-nc-nd/3.0/>>.

enhanced protection against influenza virus infection by increasing the production of type I interferon (IFN) [18]. In mice orally administered *Lactobacillus pontosus* strain b240, the enhanced defenses against influenza virus infection included increased secretory IgA (SIgA) production [19] and the regulation of antiviral gene expression in mouse lungs [20]. Thus, the strengthened immune responses induced by probiotics can protect against influenza virus infection.

At the 2008 Annual Meeting of the International Scientific Association for Probiotics and Prebiotics, prebiotics were defined as follows: “a dietary prebiotic is a selectively fermented ingredient that results in specific changes, in the consumption and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health” [21]. Recently, prebiotics were shown to be similar to probiotics in their effects on immune function and in their ability to protect against viral infection of the respiratory tract [22–25]. A supplement for infants containing a mixture of neutral short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides administered during the first 6 months of life provided protection against respiratory infections through modification of the intestinal microbiota [25]. However, most studies of prebiotics have focused on microbial infections of the digestive tract, with few reports on their effects on viral infections of the respiratory tract [23].

Lactosucrose (4^G-β-D-galactosylsucrose) is a prebiotic oligosaccharide that selectively enhances the proliferation of *Bifidobacterium* species in the large intestine [26, 27]. It has been approved by the Japanese Ministry of Health, Labour and Welfare for special dietary use due to its beneficial effects, including the improvement of intestinal disorders and the enhancement of intestinal calcium absorption [28]. In rodents, lactosucrose supplementation was shown to modulate immune functions [29–31]. The amounts of SIgA in the feces and cecum were increased in mice fed a 5% lactosucrose-supplemented diet for 4 weeks. In addition, IgA, transforming growth factor (TGF)-β1, and interleukin (IL)-6 secretion by Peyer’s patch (PP) cells were increased in lactosucrose-fed mice [29]. Lactosucrose supplementation also decreased the production of antigen-specific serum IgE in mice immunized with ovalbumin/alum [30] and reduced the ratios of CD80/CD86 and IFN-γ/IL-4 in a rat model of colitis induced by 2,4,6-trinitrobenzenesulfonic acid [31]. However, the effects of lactosucrose on the cytotoxic activity of NK cells and on lymphocyte populations have yet to be reported. Based on the ability of prebiotic supplementation to increase the numbers of beneficial

intestinal bacteria (probiotics), modulate host immunity, and improve host immune defenses, we hypothesized that lactosucrose would protect against viral infections in the respiratory tract. Thus, the aim of this study was to investigate the effect of lactosucrose on immune function and whether it would suppress influenza virus infections in mice.

MATERIALS AND METHODS

Mice

Five-week-old female BALB/c mice were obtained from Japan SLC (Hamamatsu, Japan). The mice were given free access to water and laboratory food (MF, Oriental Yeast Co., Ltd, Tokyo, Japan) during an adaptation period, that is, until they were assigned to individual groups. This study was approved by the Laboratory Animal Care Committee of Yokohama City University. The mice were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals of Yokohama City University.

Lactosucrose administration

Lactosucrose was mixed with the control diet and fed to the mice. The control diet was based on the AIN-93G formulation [32]. Lactosucrose (LS-98, Ensuiko Sugar Refining Co., Ltd., Tokyo, Japan) was included in the test diet at 50 g/kg, replacing an equal amount of cornstarch. The lactosucrose product consisted of 99.2% lactosucrose, 0.3% sucrose, and 0.5% lactose.

Experimental design

1. Effect of lactosucrose on intestinal fermentation and the immune response in mice

After an adaptation period, the mice were divided into two groups: those fed a control diet ($n = 9$) and those fed a lactosucrose-supplemented diet for 6 weeks ($n = 8$). Feces were collected every week. The mice were euthanized by administration of an overdose of anesthesia. Blood drained from the ventricles was centrifuged at $2,000 \times g$ at 4°C for 10 min to collect the serum, which was stored at -80°C until further use. The lungs, spleens, PPs, mesenteric lymph nodes (MLNs), and cecum were collected as well.

2. Effects of lactosucrose on low-dose influenza A virus infection

After the adaptation period, the mice were divided into control and lactosucrose groups as described above and then infected intranasally with $10 \mu\text{L}$ of influenza A

virus (H1N1, minimum dose lethal to 50% of animals [MLD50] = 150 plaque-forming units [PFU]) (75 PFU) diluted in PBS. To monitor the progression of the disease, the daily body weight and food intake of the mice were assessed for 7 days, after which the mice were euthanized as described above. The lungs, spleens, PPs, and MLNs were collected at 7 days post infection. Blood was collected, centrifuged, and stored as described above.

3. *Effects of lactosucrose on lethal-dose influenza A virus infection*

After an adaptation period, the mice were divided into two groups: those fed a control diet and those fed a lactosucrose-supplemented diet for 4 weeks ($n = 39$ each). They were then infected intranasally with 10 μ L (300 PFU) of influenza A (H1N1) virus diluted in PBS. The mice were selected randomly and euthanized 1, 2, and 3 days after infection ($n = 6$ in each group and at each time point), as described above, and their spleens and lungs were collected. Blood was collected, centrifuged, and stored also as described above. Disease progression was investigated in another 21 mice, the body weights and food intakes of which were recorded daily for 16 days.

Assays

1. *Cell isolation*

The lungs were removed and weighed. The right lung, spleen, PPs, and MLNs were diced and digested with 0.1% collagenase (Wako Pure Chemical Industries, Osaka, Japan). After digestion, the lung tissue was ground and sandwiched between two glass slides. All samples were filtered through cotton gauze and then centrifuged for 5 min at 1,500 rpm. Red blood cells from the lung and spleen were lysed with cell lysis buffer. Mononuclear cells in a single-cell suspension from the lungs were collected by Percoll gradient centrifugation (GE Healthcare Bio Sciences Corp., Piscataway, NJ, USA). All samples were washed before the cell populations were analyzed by flow cytometry.

2. *Flow cytometry*

Isolated cells ($1-2 \times 10^6$) were stained for flow cytometry analysis using the following antibodies: FITC-conjugated anti-mouse CD19 (clone 1D3) (BD Biosciences, San Diego, CA, USA), FITC-conjugated anti-mouse CD4 (clone GK1.5) and IgM (clone RMM-1) (BioLegend, San Diego, CA, USA), FITC-conjugated anti-mouse CD11c (clone N418), (eBioscience, San Diego, CA, USA), PE-conjugated anti-mouse CD8a

(clone 53-6.7) and CD49b (clone DX5) (eBiosciences), PE-conjugated anti-mouse IgA (clone 11-44-2) and MHC class II (clone NIMR-4) (eBioscience), and PE-Cy5-conjugated anti-mouse CD3e (clone 145-2C11) (eBioscience). Stained samples were analyzed using a BD FACSCanto II instrument and the BD FACSDiva version 6.1.3 software (BD Biosciences).

3. *NK cell isolation and cytotoxicity assay*

NK cells from the spleens of mice fed the experimental diets for 4 weeks were enriched by negative selection using a NK cell isolation kit (Miltenyi Biotec Inc., San Diego, CA, USA) and MACS technology according to the manufacturer's protocols. Briefly, spleen cells were treated with a biotin-antibody cocktail and then mixed with anti-biotin microbeads. After a wash with buffer, the cell suspension was applied to a column and placed in the magnetic field of a MidiMACS Separator (Miltenyi Biotec). Unlabeled cells that passed through the column were collected. Spleen cells and purified NK cells were cocultured with the target cells (YAC-1; American Type Culture Collection) at different effector: target (E:T) ratios in 96-well plates for 24 or 20 hr at 37°C in a 5% CO₂ atmosphere. Cytotoxicity was determined colorimetrically using an LDH (lactate dehydrogenase) cytotoxicity Detection Kit (Takara Bio Inc., Otsu, Japan) to measure LDH released from the cytosol. The assay was performed according to the manufacturer's instructions. LDH activity in the supernatants was measured using an ELISA reader (at 490 nm).

4. *Pathological examination*

The lungs were removed and weighed 16 days after infection with a lethal dose of influenza virus. The left lung were embedded in paraffin and cut into 4- μ m-thick sections; one section from each tissue sample was stained using a standard hematoxylin and eosin procedure. Lung sections were examined to determine the degree of inflammation and extent of pneumonia in a blinded fashion: 0 = no pneumonia; 1 = slightly mild interstitial pneumonia (< 10% of the lung exhibiting inflammation); 2 = mild interstitial pneumonia (10–30% of the lung); 3 = moderate interstitial pneumonia (30–60% of the lung); 4 = moderately severe interstitial pneumonia (60–80% of the lung) and 5 = severe interstitial pneumonia (\geq 80% of the lung). The scores of the individual samples were summed to yield a composite score.

5. *Preparation of lung homogenates and viral titration in Madin-Darby canine kidney (MDCK) cells*

The right lung was weighed and then homogenized

in 1 mL of ice-cold PBS using a tissue homogenizer. Following centrifugation ($20,000 \times g$ at 4°C for 20 min), the supernatant was collected. Plaque assays were performed on monolayers of MDCK cells in 12-well tissue culture plates. Quadruplicate serial dilutions were prepared from the lung homogenates and 50 μL of each dilution was incubated in monolayers for 1 hr at 37°C in a 5% CO_2 atmosphere, with occasional rocking. The inoculum was aspirated, and the monolayers were overlaid with 1 mL of minimum essential medium supplemented with 0.8% agarose and 1 μg of acetylated trypsin. After incubation of the cultures for 3 days at 35°C , the viral plaques were identified by fixing the cultures with 2% paraformaldehyde followed by staining with 0.1% crystal violet. The results are presented as PFU per lung.

6. ELISA for cytokines, IgA, IgG1, IgG2a, and surfactant protein-D (SP-D)

The levels of cytokines, IgA, IgG1, IgG2a, and SP-D were measured using commercial ELISA kits according to the instructions of the respective manufacturer. The IL-6, IL-10, IL-12, tumor necrosis factor (TNF)- α , TGF- β 1, and IFN- γ levels in lung homogenates were measured using an OptEIA ELISA kit (BD Biosciences). IFN- α and IFN- β levels were measured using the respective kits (Invitrogen, Merelbeke, Belgium). The levels of IgA in the feces, lung homogenates, and serum and of IgG1 and IgG2a in serum were measured using IgA, IgG1, and IgG2a kits (Bethyl Laboratories, Montgomery, TX, USA). SP-D levels were measured using an ELISA kit for mouse pulmonary SP-D (CUSABIO Biotech, Wuhan, P.R. China). The levels of IgA, IgG1, and IgG2a in the lung homogenates were converted and expressed per lung.

7. Cecum analysis

The cecal contents were mixed, and the pH was measured using a compact pH electrode (B-112; Horiba, Kyoto, Japan). A portion of the cecal contents was dried at 105°C for 24 hr and weighed to determine the water content. Organic acids (acetate, propionate, butyrate, lactate, and succinate) in the cecum were measured by high-performance liquid chromatography (HPLC) with an internal standard [33]. The cecal contents were prepared by mixing 300 mg with 0.3 mL of 10 mM crotonic acid (Wako Pure Chemical Industries) as an internal standard followed by centrifugation at $10,000 \times g$ for 10 min; the supernatant was used in the HPLC analysis. The organic acids were separated using an ion-exclusion column and detected using the post-column pH-buffered electroconductivity detection method. The H-type cation exchanger column (Shim-pack SCR-102H,

8-mm 8 mm internal diameter \times 30 cm length; Shimadzu, Kyoto, Japan) had a temperature of 45°C , a mobile phase of 5 mM p-toluene sulfonic acid (flow rate, 0.6 mL/min at 45°C), an electroconductivity detector of positive polarity at 45°C , and a detection reagent consisting of 20 mM bis-Tris aqueous solution containing 5 mM p-toluene sulfonic acid and 100 μM EDTA (flow rate of 0.6 mL/min, 45°C).

Statistical analyses

Each experiment was performed at least twice. The data are presented as means \pm SDs. All statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Changes in fecal SIgA, body weight, the cytotoxic activity of NK cells, lung virus titer and SIgA on lethal-dose influenza A virus infection, and cytokine and SP-D levels were analyzed by a two-way ANOVA. Significant differences between two groups were identified using Tukey post hoc tests. The survival rate was estimated by the Kaplan-Meier method and analyzed by the log-rank test. All other data were analyzed using the Student's t-test. A p-value < 0.05 was considered to indicate statistical significance.

RESULTS

Effect of lactosucrose on intestinal fermentation and the immune response in mice

Lactosucrose supplementation did not affect body weight gain, food intake, or organ weights. The exception was the cecum, as the cecal mass and the cecal acetate, propionate, butyrate, lactate, succinate, and SCFA levels were significantly increased and the pH of the cecal contents was significantly decreased in mice fed the lactosucrose supplement (Table 1). Significant increases in fecal (Fig. 1A) and lung SIgA concentrations and in the concentrations of total IgA and IgG2a in the serum (Fig. 1B) were also recorded in these mice. In the spleen and PPs, lactosucrose supplementation altered the proportions of lymphocyte subsets. The percentages of CD8^+ T cells in the spleen and of CD4^+ T cells in PPs were significantly increased compared with the control group (Fig. 1C). However, in the lungs and MLNs, there were no significant differences in the lymphocyte subsets of lactosucrose-fed and control mice (data not shown).

Effect of dietary lactosucrose on cytotoxic activity of NK cells

Lactosucrose supplementation in mice for 4 weeks significantly increased NK cell cytotoxic activity in splenocytes at E:T ratios of 80:1 and 40:1 (Fig. 2A) and

Table 1. Organ weights, cecal pH, water content, organic acid content, and total amounts of short-chain fatty acids in mice fed the control or lactosucrose-supplemented diet for 6 weeks

	Control	Lactosucrose
<i>n</i>	9	8
Organ weights (g)		
Spleen	0.10 ± 0.01	0.11 ± 0.02
MLNs	0.03 ± 0.01	0.03 ± 0.01
Lung	0.13 ± 0.01	0.15 ± 0.02
Cecum	0.19 ± 0.04	0.32 ± 0.05 *
Cecum (pH)	8.2 ± 0.2	7.5 ± 0.4 *
Water (%)	69.8 ± 2.1	71.6 ± 2.6
Organic acid concentrations (μmol/g cecal contents) ^a		
Acetate	25.2 ± 5.4	56.9 ± 33.9 *
Propionate	5.2 ± 0.7	8.6 ± 3.0 *
Butyrate	3.3 ± 0.7	10.0 ± 8.0 *
Short-chain fatty acid ^b	33.7 ± 6.5	75.5 ± 42.6 *
Lactate	6.8 ± 1.4	17.3 ± 13.0 *
Succinate	1.3 ± 0.5	2.8 ± 0.9 *

The values are means ± SDs. The data were analyzed by Student's *t*-test. An asterisk indicates a significant difference between the control and lactosucrose groups ($p < 0.05$). ^aThe data were calculated from the amounts of the organic acids contained in the cecal contents. ^bShort-chain fatty acid is the sum of acetate, propionate, and butyrate.

significantly increased the cytotoxic activity of purified NK cells in the spleen at an E:T ratio of 50:1 (Fig. 2B).

Effects of lactosucrose on low-dose influenza A virus infection

Lactosucrose supplementation did not affect body weight gain or food intake before influenza A virus infection. The average daily food intake of the lactosucrose group (9.72 g/mouse, 7 days) after influenza A virus infection was greater than that of the control group (7.52 g/mouse, 7 days). One of the control mice died 5 days after infection, whereas all mice in the lactosucrose group survived until 7 days post infection. Body weight loss (Fig. 3A) and lung viral titers (Fig. 3B) were significantly lower in the lactosucrose group than in the control group at 7 days post infection. Furthermore, the percentages of NK cells in the spleen and of CD4⁺ and CD8⁺ T cells in PPs were significantly higher in the lactosucrose group than in the control group (Fig. 3C). The percentage of IgM⁺ cells in PPs was significantly lower in the lactosucrose group than in the control group. There was no significant difference in lung weight. The average lung weight in mice fed the normal diet was 0.32 ± 0.08 g, while that of mice fed lactosucrose was 0.28 ± 0.09 g.

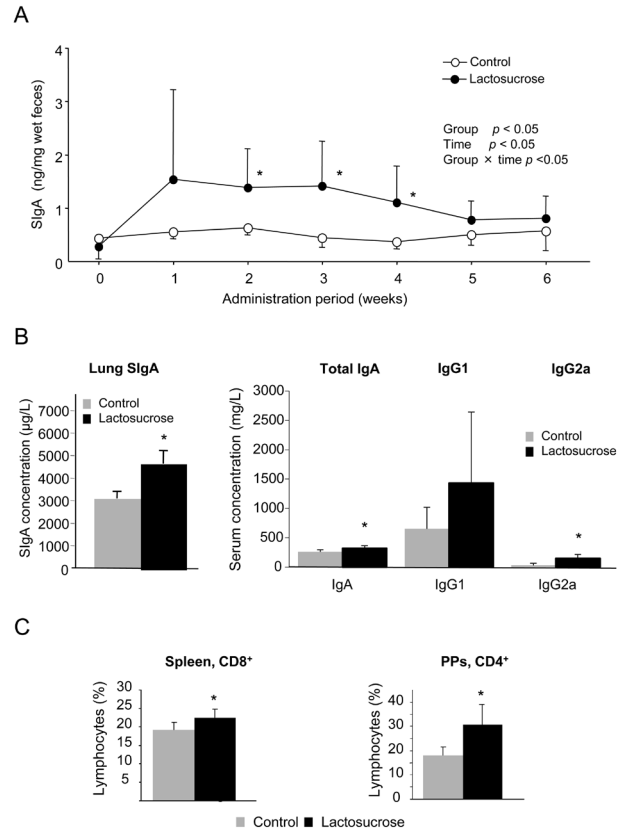


Fig. 1. Effects of lactosucrose supplementation on immune modulation in mice. All data are representative of two independent experiments. Fecal SIgA was determined each week by ELISA. Lactosucrose supplementation increased SIgA production (A). *, Significant increases in fecal SIgA levels determined using a two-way ANOVA and Tukey post hoc tests in comparison with mice fed the control diet ($p < 0.05$). Each point represent the mean ± SD. ○, Mice fed the control diet ($n = 9$). ●, Mice fed the lactosucrose diet ($n = 8$). The concentration of SIgA in lung homogenates and the serum concentrations of total IgA, IgG1, and IgG2a in mice fed the experimental diet for 6 weeks (B). Lactosucrose supplementation increased the concentration of SIgA in lung homogenates (B) and the concentrations of total IgA and IgG2a in serum. It also altered lymphocyte subset ratios in the spleen and PPs (C). Significant increases in the percentages of CD8⁺ T lymphocytes in the spleen and CD4⁺ T lymphocytes in PPs. *, $p < 0.05$ as determined by the Student's *t*-test in comparison with mice fed the control diet.

Effects of lactosucrose on lethal-dose influenza A virus infection

Lactosucrose supplementation protected mice against a lethal influenza virus A infection as evidenced by their higher survival rates (Fig. 4A) and lower weight loss (Fig. 4B) compared with the control mice. In addition, in the lactosucrose group, viral titers in the lungs were significantly lower at 1 day post infection (Fig. 4C),

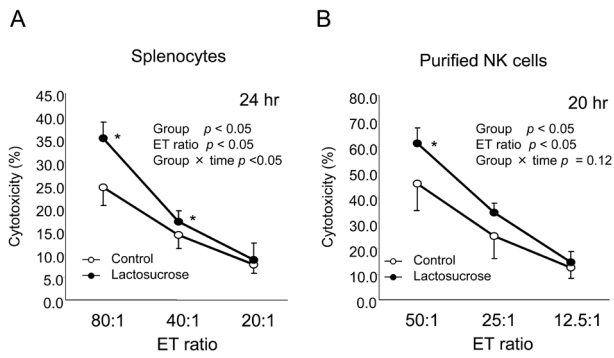


Fig. 2. Effects of lactosucrose supplementation on the cytotoxic activity of NK cells. Values are means \pm SDs ($n = 5$). *, $p < 0.05$ as determined by two-way ANOVA and Tukey post hoc tests in a comparison with mice fed a control diet. Lactosucrose supplementation in mice significantly increased cytotoxic activity in splenocytes at ratios of 80:1 and 40:1 (A) and significantly increased cytotoxic activity in purified NK cells at a ratio of 50:1 (B).

and lung SIgA levels were significantly higher at 1 and 2 days post infection (Fig. 4D). Three mice survived in the control group compared to 12 in the lactosucrose group. There were no significant differences in lung weight or pathology score. The average lung weight in the mice fed the normal diet was 0.29 ± 0.08 g, while that in the mice fed the lactosucrose-supplemented diet was 0.26 ± 0.05 g. The average pathology score in the mice fed the normal diet was 4.3 ± 1.2 , while that in the mice fed the lactosucrose-supplemented diet was 3.7 ± 1.1 . Differences between the two groups with respect to lymphocyte subset populations in the spleen and lungs were also noted. The percentages of splenic CD11c⁺ MHC class II⁺ and class II⁻ dendritic cells were significantly lower at 1 day post infection (Fig. 5A), and the percentage of CD4⁺ T cells was significantly higher at 1 and 3 days post infection in the lactosucrose group than in the control group. The percentage of CD8⁺ T cells in the spleen tended to be higher at 1 day post infection ($p = 0.06$). At 2 days post infection, the difference in CD8⁺ T cells in the lungs was significant (Fig. 5B). There were no significant differences in the concentrations of cytokines (IFN- α , IFN- β , IFN- γ , IL-6, IL-10, IL-12, TNF- α , and TGF- β 1) and SP-D in the lungs of the control versus the lactosucrose-fed mice at 3 days post infection.

DISCUSSION

This study evaluated the effects of lactosucrose on cecal fermentation, immune responses, and influenza A virus infection in mice. In the absence of influenza A

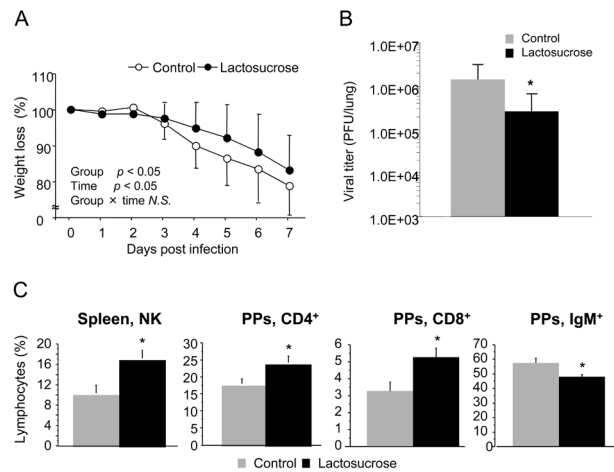


Fig. 3. Effects of lactosucrose in mice infected with a low dose of influenza A virus. All data are representative of two independent experiments. Values are means \pm SDs. Control group, $n = 8$; lactosucrose group, $n = 9$. The mice were intranasally infected with one-quarter of the lethal dose (low dose) of influenza A (H1N1) virus after being fed the control diet or the lactosucrose diet for 2 weeks. One mouse in the control group died 5 days after infection, whereas all mice in the lactosucrose group survived until 7 days after infection. Body weight loss (A) and lung viral titers were significantly lower in the lactosucrose group than in the control group at 7 days postinfection (B). The percentages of NK cells in the spleen and of CD4⁺ and CD8⁺ T cells in PPs were significantly higher and that of IgM⁺ cells in PPs was significantly lower in the lactosucrose group than in the control group (C). *, $p < 0.05$ as determined by the Student's *t*-test in a comparison with mice fed the control diet.

virus infection, lactosucrose supplementation increased fecal and lung SIgA concentrations and total serum IgA and IgG2a concentrations. It also altered lymphocyte subset populations in the spleen and PPs. In a mouse model of low-dose influenza virus infection, lactosucrose supplementation significantly reduced weight loss and viral titers in the lungs. In mice infected with a lethal dose of influenza A virus, it significantly increased their survival.

The cytotoxic activity of splenic NK cells was also augmented by lactosucrose supplementation, consistent with the significantly decreased viral titers in the mouse lungs 1 day post infection with a lethal dose of influenza A virus. Enhancement of local cellular immunity in the respiratory tract is important for protection against influenza virus infection [34, 35]. NK cells display a potent ability to control viral infections through the production of cytokines and the elimination of virus-infected cells [17, 36, 37]. In a previous study, oral administration of *Lactobacillus casei* Shirota [17] and *Bifidobacterium longum* MM [37] augmented the

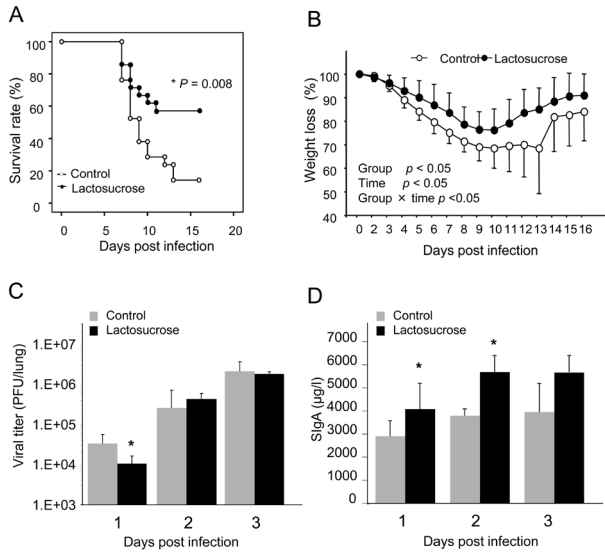


Fig. 4. Effects of lactosucrose on mice infected with a lethal dose of influenza A virus. Mice were intranasally infected with a lethal dose of influenza A virus after being fed the control diet or the lactosucrose diet for 4 weeks. All data are representative of two independent experiments. The survival rates of the lactosucrose-fed mice were significantly higher than those of the control mice (A). Significant decreases in weight loss were observed compared with mice fed the control diet at each time point as determined by two-way ANOVA and Tukey post hoc tests (B, $p < 0.05$). All values are means \pm SDs ($n = 21$). A significant decrease in the viral titer (C) and a significant increase in the SIgA concentration (D) were observed in lung homogenates. All values are means \pm SDs ($n = 6$). *, $p < 0.05$ as determined by two-way ANOVA and Tukey post hoc tests in comparison with mice fed the control diet.

cytotoxic activity of splenic and pulmonary NK cells in mice, thus demonstrating the anti-viral effects of probiotics. Prebiotic supplementation enhanced the number of probiotic bacteria in the small and large intestines [21]. Lactosucrose was fermented by probiotic bacteria, including *Bifidobacterium* species in the large intestine [26, 27]. We did not investigate the change in intestinal microflora, but our results indicate increases in the weight of the cecum and the SCFA levels in mice fed a lactosucrose-supplemented diet, suggesting an increase in probiotic bacteria in their large intestines. These changes might enhance the cytotoxicity of NK cells targeting influenza virus-infected cells during the early phase of virus infection.

Both cellular and humoral immunity are important defense responses to influenza virus infection. IgG antibodies are responsible for the majority of serum antiviral activity, while SIgA is directed toward viruses that infect mucosal surfaces, and SIgA protects against

viral infections of the upper respiratory tract [35, 38, 39]. In this study, lactosucrose supplementation significantly increased the concentration of SIgA in fecal and lung homogenates and the concentration of total IgA in serum. In a previous study, suppression of influenza virus infection by *Lactobacillus* pontosus strain b240 was suggested to be mediated by enhanced SIgA production [19]. Accordingly, in lactosucrose-fed mice, the increased SIgA production may have enhanced the mucosal immunity of the upper respiratory tract, thereby enabling a defensive response to influenza A virus infection.

Similarly, 5% yacon-derived fructo-oligosaccharide supplementation in mice increased the concentration of SIgA in feces, but did not increase serum IgA [40]. Fructo-oligosaccharide and manno-oligosaccharide supplementation in dogs increased the concentration of SIgA in the gastrointestinal tract, but did not increase serum IgA [41]. Supplementation with probiotics and/or prebiotics has been reported to increase except for serum IgA level [23]; however, a few studies have reported that probiotic and/or prebiotic supplementation increased total serum IgA [42–45]. We found higher concentrations of SIgA in the lungs and total IgA in the serum and feces of lactosucrose-fed mice. However, it is not clear how lactosucrose supplementation influenced antibody production; in-depth consideration of this is necessary. Our study invites the following questions: 1) Does IgA in the serum and SIgA in lungs also increase at the time of infection with other types of influenza virus? 2) Can this phenomenon be observed in other animals and humans?

Influenza infection cannot be prevented by increased SIgA production alone. Defense mechanisms against influenza virus infection comprise several effector cells and molecules [35–37]. The prevention of an influenza virus infection or recovery following an infection requires the induction of innate and adaptive immune responses in the respiratory mucosa. Most influenza viruses are detected and destroyed within a few hours by innate immune mechanisms such as mucus; macrophages; IFN- α , IFN- β , and other cytokines; fever; NK cells; and the complement system [35]. NK cells can be activated by cytokines during the initial stages of viral infection. Cytokines can also enhance activating receptor-mediated NK cell activation. The four principal cytokines involved in NK cell activation are type I IFNs, IL-12, IL-15, and IL-18. These cytokines can be produced directly by infected cells or by activated dendritic cells or macrophages [37]. In this study, to identify the factors that contributed to protection against influenza virus infection in lactosucrose-fed mice, cytokine, SP-D, and SIgA levels in lung homogenates and the lymphocyte subset populations

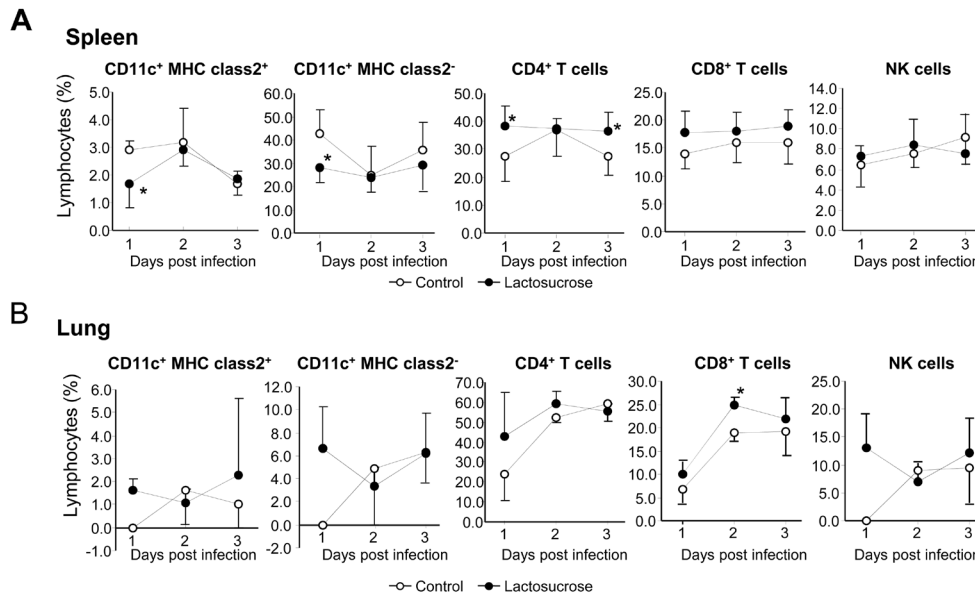


Fig. 5. Lymphocyte subsets, cytokine levels, and SP-D levels in mice fed the control or lactosucrose diet for 4 weeks and then infected intranasally with a lethal dose of influenza A virus. Lymphocyte subsets and lung homogenate cytokine levels were determined at 1, 2, and 3 days post infection. Lactosucrose supplementation altered the lymphocyte subset populations in the spleen (A) and lungs (B). The percentages of CD11c⁺ MHC class II⁺ double-positive and CD11c⁺ MHC class II⁻ dendritic cells in the spleen were significantly lower in the lactosucrose group than in the control group at 1 day post infection whereas the percentage of splenic CD4⁺ T cells was significantly higher in the lactosucrose group than in the control group at 1 and 3 days post infection. The percentage of CD8⁺ T cells in the spleen showed a non-significant increase 1 day postinfection ($p=0.06$). The percentage of CD8⁺ T cells in the lung was significantly higher in the lactosucrose group than in the control group at 2 days post infection. All values are means \pm SDs ($n = 6$). *, $p < 0.05$ as determined by two-way ANOVA and Tukey post hoc tests in comparison with mice fed the control diet.

in the spleen and lungs were assessed at the early stages of influenza virus infection (1, 2, and 3 days post infection). Our results show that lactosucrose supplementation augmented the cytotoxic activity of splenic NK cells in the absence of influenza virus infection; however, there was no significant difference between the lactosucrose and control groups in the concentrations of cytokines and SP-D in lung homogenates 3 days after infection of the mice with a lethal dose of influenza A virus. Lee et al. [46] reported that in mice orally administered red ginseng, IFN- γ levels in lung homogenates increased significantly 5 days post influenza virus infection. In the lung homogenates of mice orally administered *Lactobacillus plantarum* DK119, IFN- γ concentrations were significantly higher 4 days post influenza virus infection [47]. Thus, a difference between the lactosucrose and control groups might have developed after 3 days. Moreover, we did not evaluate the differences in upper respiratory tract and nasal immunity between the control and lactosucrose groups. Measurements of cytokines and antibodies in bronchoalveolar lavage fluid and nasal washes in lactosucrose-fed mice infected or not infected with influenza virus may provide information on the

effects of supplementation on mucosal immunity and protection against influenza virus infection of the upper respiratory tract.

In summary, we demonstrated the defensive effects of lactosucrose supplementation on influenza virus infection in this study. It increased splenic NK cell cytotoxicity, SIgA concentrations in the feces and lungs, and total IgA and IgG2a concentrations in serum. Higher survival rates, decreased body weight loss, and lower viral titers in the lungs also characterized mice fed a lactosucrose-supplemented diet prior to influenza virus infection. These findings suggest that lactosucrose supplementation enhances innate and mucosal immunity in the respiratory tract. However, there were no significant differences in the levels of type I IFN or cytokines, possibly because the virus was removed at an early stage by the increases in antibody levels in the respiratory tract and by greater NK cell cytotoxicity. The protective effects of lactosucrose on mice infected with influenza virus were attributed to the augmentation of innate immune responses, enhanced cellular and mucosal immunity, and possibly the more potent adaptive immunity following a boost observed in intestinal fermentation. Thus, in humans, lactosucrose

supplementation may also aid in improving resistance to influenza viral infection, ameliorating intestinal disorders, and increasing intestinal calcium absorption through mechanisms related to *Bifidobacterium* proliferation.

REFERENCES

1. Cahenzli J, Balmer ML, McCoy KD. 2013. Microbial-immune cross-talk and regulation of the immune system. *Immunology* 138: 12–22. [Medline] [CrossRef]
2. Kayama H, Takeda K. 2012. Regulation of intestinal homeostasis by innate and adaptive immunity. *Int Immunol* 24: 673–680. [Medline] [CrossRef]
3. McDermott AJ, Huffnagle GB. 2014. The microbiome and regulation of mucosal immunity. *Immunology* 142: 24–31. [Medline] [CrossRef]
4. Maynard CL, Elson CO, Hatton RD, Weaver CT. 2012. Reciprocal interactions of the intestinal microbiota and immune system. *Nature* 489: 231–241. [Medline] [CrossRef]
5. Hooper LV, Littman DR, Macpherson AJ. 2012. Interactions between the microbiota and the immune system. *Science* 336: 1268–1273. [Medline] [CrossRef]
6. Vieira AT, Teixeira MM, Martins FS. 2013. The role of probiotics and prebiotics in inducing gut immunity. *Front Immunol* 4: 445. [Medline] [CrossRef]
7. Nagao F, Nakayama M, Muto T, Okumura K. 2000. Effects of a fermented milk drink containing *Lactobacillus casei* strain Shirota on the immune system in healthy human subjects. *Biosci Biotechnol Biochem* 64: 2706–2708. [Medline] [CrossRef]
8. Prescott SL, Wickens K, Westcott L, Jung W, Currie H, Black PN, Stanley TV, Mitchell EA, Fitzharris P, Siebers R, Wu L, Crane J, Probiotic Study Group 2008. Supplementation with *Lactobacillus rhamnosus* or *Bifidobacterium lactis* probiotics in pregnancy increases cord blood interferon-gamma and breast milk transforming growth factor-beta and immunoglobulin A detection. *Clin Exp Allergy* 38: 1606–1614. [Medline] [CrossRef]
9. Hardy H, Harris J, Lyon E, Beal J, Foey AD. 2013. Probiotics, prebiotics and immunomodulation of gut mucosal defences: homeostasis and immunopathology. *Nutrients* 5: 1869–1912. [Medline] [CrossRef]
10. Allan GM, Arroll B. 2014. Prevention and treatment of the common cold: making sense of the evidence. *CMAJ* 186: 190–199. [Medline] [CrossRef]
11. Homayouni Rad A, Torab R, Mortazavian AM, Mehrabany EV, Mehrabany LV. 2013. Can probiotics prevent or improve common cold and influenza? *Nutrition* 29: 805–806. [Medline] [CrossRef]
12. Hao Q, Dong BR, Wu T. 2015. Probiotics for preventing acute upper respiratory tract infections. *Cochrane Database Syst Rev* 2: CD006895. [Medline]
13. Vissers M, de Groot R, Ferwerda G. 2014. Severe viral respiratory infections: are bugs bugging? *Mucosal Immunol* 7: 227–238. [Medline] [CrossRef]
14. Nagalingam NA, Cope EK, Lynch SV. 2013. Probiotic strategies for treatment of respiratory diseases. *Trends Microbiol* 21: 485–492. [Medline] [CrossRef]
15. Yasui H, Kiyoshima J, Hori T, Shida K. 1999. Protection against influenza virus infection of mice fed *Bifidobacterium breve* YIT4064. *Clin Diagn Lab Immunol* 6: 186–192. [Medline]
16. Hori T, Kiyoshima J, Shida K, Yasui H. 2002. Augmentation of cellular immunity and reduction of influenza virus titer in aged mice fed *Lactobacillus casei* strain Shirota. *Clin Diagn Lab Immunol* 9: 105–108. [Medline]
17. Yasui H, Kiyoshima J, Hori T. 2004. Reduction of influenza virus titer and protection against influenza virus infection in infant mice fed *Lactobacillus casei* Shirota. *Clin Diagn Lab Immunol* 11: 675–679. [Medline]
18. Maeda N, Nakamura R, Hirose Y, Murosaki S, Yamamoto Y, Kase T, Yoshikai Y. 2009. Oral administration of heat-killed *Lactobacillus plantarum* L-137 enhances protection against influenza virus infection by stimulation of type I interferon production in mice. *Int Immunopharmacol* 9: 1122–1125. [Medline] [CrossRef]
19. Kobayashi N, Saito T, Uematsu T, Kishi K, Toba M, Kohda N, Suzuki T. 2011. Oral administration of heat-killed *Lactobacillus pentosus* strain b240 augments protection against influenza virus infection in mice. *Int Immunopharmacol* 11: 199–203. [Medline] [CrossRef]
20. Kiso M, Takano R, Sakabe S, Katsura H, Shinya K, Uraki R, Watanabe S, Saito H, Toba M, Kohda N, Kawaoka Y. 2013. Protective efficacy of orally administered, heat-killed *Lactobacillus pentosus* b240 against influenza A virus. *Sci Rep* 3: 1563. [Medline] [CrossRef]
21. Roberfroid M. 2007. Probiotics: the concept revisited. *J Nutr* 137 Suppl 2: 830S–837S. [Medline]
22. Schley PD, Field CJ. 2002. The immune-enhancing effects of dietary fibres and prebiotics. *Br J Nutr* 87 Suppl 2: S221–S230. [Medline] [CrossRef]
23. Lomax AR, Calder PC. 2009. Probiotics, immune function, infection and inflammation: a review of the evidence. *Br J Nutr* 101: 633–658. [Medline] [CrossRef]
24. Weichert S, Schrotten H, Adam R. 2012. The role of prebiotics and probiotics in prevention and treatment of childhood infectious diseases. *Pediatr Infect Dis J* 31: 859–862. [Medline] [CrossRef]
25. Arslanoglu S, Moro GE, Boehm G. 2007. Early supplementation of prebiotic oligosaccharides protects formula-fed infants against infections during the first 6 months of life. *J Nutr* 137: 2420–2424. [Medline]
26. Hara H, Li S, Sakaki M, Maruyama T, Terada A, Ogata Y, Fujita K, Ishigami H, Hara K, Fujimori I, Mitsuoka T. 1994. Effective dose of lactosucrose on fecal flora and fecal metabolites of humans. *Bifidobact Microflora* 13: 51–63. [CrossRef]
27. Ohkusa T, Ozaki Y, Sato C, Mikuni K, Ikeda H. 1995. Long-term ingestion of lactosucrose increases *Bifidobacterium* sp. in human fecal flora. *Digestion* 56:

- 415–420. [Medline] [CrossRef]
28. Teramoto F, Rokutan K, Sugano Y, Oku K, Kishino E, Fujita K, Hara K, Kishi K, Fukunaga M, Morita T. 2006. Long-term administration of 4^G-beta-D-galactosylsucrose (lactosucrose) enhances intestinal calcium absorption in young women: a randomized, placebo-controlled 96-wk study. *J Nutr Sci Vitaminol (Tokyo)* 52: 337–346. [Medline] [CrossRef]
 29. Hino K, Kurose M, Sakurai T, Inoue S, Oku K, Chaen H, Fukuda S. 2007. Effect of dietary lactosucrose (4^G-beta-D-galactosylsucrose) on the intestinal immune functions in mice. *J Appl Glycosci* 54: 169–172. [CrossRef]
 30. Taniguchi Y, Mizote A, Kohno K, Iwaki K, Oku K, Chaen H, Fukuda S. 2007. Effects of dietary lactosucrose (4^G-beta-D-galactosylsucrose) on the IgE response in mice. *Biosci Biotechnol Biochem* 71: 2766–2773. [Medline] [CrossRef]
 31. Zhou Y, Ruan Z, Zhou X, Huang X, Li H, Wang L, Zhang C, Deng Z, Wu G, Yin Y. 2015. Lactosucrose attenuates intestinal inflammation by promoting Th2 cytokine production and enhancing CD86 expression in colitic rats. *Biosci Biotechnol Biochem* 79: 643–651. [Medline] [CrossRef]
 32. Reeves PG, Nielsen FH, Fahey Jr GC, American Institute of Nutrition 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* 123: 1939–1951. [Medline]
 33. Kishino E, Ito T, Fujita K, Kiuchi Y. 2009. A mixture of *Salacia reticulata* (*Kotala himbutu*) aqueous extract and cyclodextrin reduces body weight gain, visceral fat accumulation, and total cholesterol and insulin increases in male Wistar fatty rats. *Nutr Res* 29: 55–63. [Medline] [CrossRef]
 34. Lee SH, Miyagi T, Biron CA. 2007. Keeping NK cells in highly regulated antiviral warfare. *Trends Immunol* 28: 252–259. [Medline] [CrossRef]
 35. Tamura S, Kurata T. 2004. Defense mechanisms against influenza virus infection in the respiratory tract mucosa. *Jpn J Infect Dis* 57: 236–247. [Medline]
 36. Brandstadter JD, Yang Y. 2011. Natural killer cell responses to viral infection. *J Innate Immun* 3: 274–279. [Medline] [CrossRef]
 37. Kawahara T, Takahashi T, Oishi K, Tanaka H, Masuda M, Takahashi S, Takano M, Kawakami T, Fukushima K, Kanazawa H, Suzuki T. 2015. Consecutive oral administration of *Bifidobacterium longum* MM-2 improves the defense system against influenza virus infection by enhancing natural killer cell activity in a murine model. *Microbiol Immunol* 59: 1–12. [Medline] [CrossRef]
 38. McGill J, Heusel JW, Legge KL. 2009. Innate immune control and regulation of influenza virus infections. *J Leukoc Biol* 86: 803–812. [Medline] [CrossRef]
 39. Mbawuike IN, Pacheco S, Acuna CL, Switzer KC, Zhang Y, Harriman GR. 1999. Mucosal immunity to influenza without IgA: an IgA knockout mouse model. *J Immunol* 162: 2530–2537. [Medline]
 40. Asahi Y, Yoshikawa T, Watanabe I, Iwasaki T, Hasegawa H, Sato Y, Shimada S, Nanno M, Matsuoka Y, Ohwaki M, Iwakura Y, Suzuki Y, Aizawa C, Sata T, Kurata T, Tamura S. 2002. Protection against influenza virus infection in polymeric Ig receptor knockout mice immunized intranasally with adjuvant-combined vaccines. *J Immunol* 168: 2930–2938. [Medline] [CrossRef]
 41. Delgado GT, Thomé R, Gabriel DL, Tamashiro WM, Pastore GM. 2012. Yacon (*Smallanthus sonchifolius*)-derived fructooligosaccharides improves the immune parameters in the mouse. *Nutr Res* 32: 884–892. [Medline] [CrossRef]
 42. Swanson KS, Grieshop CM, Flickinger EA, Bauer LL, Healy HP, Dawson KA, Merchen NR, Fahey GC Jr. 2002. Supplemental fructooligosaccharides and mannanoligosaccharides influence immune function, ileal and total tract nutrient digestibilities, microbial populations and concentrations of protein catabolites in the large bowel of dogs. *J Nutr* 132: 980–989. [Medline]
 43. Marteau P, Vaerman JP, Dehennin JP, Bord S, Brassart D, Pochart P, Desjeux JF, Rambaud JC. 1997. Effects of intrajejunal perfusion and chronic ingestion of *Lactobacillus johnsonii* strain La1 on serum concentrations and jejunal secretions of immunoglobulins and serum proteins in healthy humans. *Gastroenterol Clin Biol* 21: 293–298. [Medline]
 44. Yin QQ, Chang J, Zuo RY, Chen LY, Chen QX, Wei XY, Guan QF, Sun JW, Zheng QH, Yang X, Ren GZ. 2010. Effect of the Transformed *Lactobacillus* with phytase gene on pig production performance, nutrient digestibility, gut microbes and serum biochemical indexes. *Asian-australas J Anim Sci* 23: 246–252. [CrossRef]
 45. Link-Amster H, Rochat F, Saudan KY, Mignot O, Aeschlimann JM. 1994. Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. *FEMS Immunol Med Microbiol* 10: 55–63. [Medline] [CrossRef]
 46. Lee JS, Hwang HS, Ko EJ, Lee YN, Kwon YM, Kim MC, Kang SM. 2014. Immunomodulatory activity of red ginseng against influenza A virus infection. *Nutrients* 6: 517–529. [Medline] [CrossRef]
 47. Park MK, Ngo V, Kwon YM, Lee YT, Yoo S, Cho YH, Hong SM, Hwang HS, Ko EJ, Jung YJ, Moon DW, Jeong EJ, Kim MC, Lee YN, Jang JH, Oh JS, Kim CH, Kang SM. 2013. *Lactobacillus plantarum* DK119 as a probiotic confers protection against influenza virus by modulating innate immunity. *PLoS ONE* 8: e75368. [Medline] [CrossRef]