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Article

Enhanced Protection against Influenza Virus Infection with Dispersion Controlled Lactic Acid Bacteria Powder

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Abstract: We evaluated the change in water dispersibility of lactic acid bacteria (LAB, *Enterococcus faecalis* KH2) upon powderization and its influence on their efficacy. When cultured LAB are washed, heat-killed, and powdered, adhesion between LAB occurs and they form aggregation (non-treated LAB, n-LAB). However, a dispersed LAB (d-LAB) powder with less aggregates can be prepared by treating them with a high-pressure homogenizer and adding an excipient during powdering. n-LAB or d-LAB was administered to mice and the Peyer's patches in the small intestine were observed. n-LAB administration showed a high amount of aggregated LAB drifting in the intestinal mucosa, whereas d-LAB reached the Peyer's patches and was taken up into the Peyer's patches. Evaluation in a mouse influenza virus infection model showed that d-LAB was more effective than n-LAB in influenza yield of bronchoalveolar lavage fluids (BALFs) on day 3 post-infection, neutralizing antibody titers of sera and influenza virus-specific IgA in the feces on day 14 post-infection. Thus, the physical properties of LAB affect their efficacy; controlling their water dispersibility can improve their effectiveness.

Keywords: lactic acid bacteria; enterococcus faecalis; dispersion; viral infection; particle size; Peyer's patch

1. Introduction

Lactic acid bacteria (LAB) play an important role in various fermentation processes. In his book "Essais Optimistes [1]," the Russian microbiologist Metchnikov advocated the consumption of yogurt to increase life expectancy. This led to a flurry of research on the health benefits of LAB. In recent years, many health benefits have been reported, including improved gut microbiota [2,3] and biological defense [4,5] and anti-allergenic [6,7] and anti-tumorigenic [8,9] effects. The isolation and cultivation of LAB has made LAB consumption convenient. The active ingredients present in LAB, including lipoteichoic acid [10] and nucleic acid [11], are involved in modulating the immune response. LAB are used in food supplements, beverages, confectionery, cereals, and others. They are also consumed as powders. As the market for LAB has grown, research, and development has increased and various kinds of LAB, such as anti-obesity [12] and antiviral [13], have been developed. However, reports evaluating and verifying LAB powder raw materials produced in a factory are scarce. We consider that there is a difference in physical properties, especially dispersion, between factory-produced LAB powder and lab-prepared LAB powder, based on processes such as thermal history and powderization. Studies have reported that the mechanism by which LAB improve the immune response includes LAB being taken up through the Peyer's patches into the

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intestinal tract, phagocytosed by antigen-presenting cells, such as dendritic cells and macrophages [14,15], and recognized by PRRs, such as toll-like receptors, nucleotide binding oligomerization domain-like receptors (NLRs) and retinoic acid inducible gene-like receptors (RLRs) to produce immune-related substances, such as cytokines [16]. LAB are taken up by the M cells that are scattered on the Peyer's patches, but if LAB aggregate and become larger than M cells, they are difficult to take up physically. When we checked some LAB materials, we found that LAB were agglomerated, which is common in powdered materials. Therefore, we resuspended the LAB before and after powderization in distilled water and compared their physical properties with the distribution of particle size to investigate whether the effect of the difference in the LAB species or powderization was due to the difference in the species. The mean particle size of LAB before powderization was smaller than that after powderization and bacteria aggregated after powderization. This may lead to a loss of the beneficial effects of LAB on health. We prepared a non-agglomerating LAB powder by dispersing bacteria in a high-pressure homogenizer and adding dextrin as a vehicle. Then, LAB powders with higher and lower number of aggregates were compared. Water dispersibility was analyzed by a laser diffraction particle size analyzer, uptake from mouse Peyer's patches was observed under a microscope, and the protective effect of LAB on viral infection [17–21], which is also the main health effect of LAB, was compared in a mouse influenza infection model.

2. Materials and Methods

2.1. Sample Preparation and Particle Size Measurement

Enterococcus faecalis KH2 (International Patent Organism Depositary, Japan; number NITE P-14444; GenBank Accession number, AB534553) was stored at Bio-Lab Co., Ltd. LAB were grown aerobically overnight at 37 °C in MRS broth (Difco, Detroit, MI, USA) and washed with distilled water, followed by centrifugation at 10,000 × *g* for 3 min. The suspension of bacteria in distilled water (20–30 mg (wet bacteria weight)/mL) was heated at 105 °C for 30 min using an autoclave (HV-25 II LB; Hirayama Manufacturing Corp., Saitama, Japan). The untreated LAB powder was designated "non-treated LAB: n-LAB." To increase the water dispersibility of the prepared LAB, the sample was treated with a high-pressure homogenizer (ECONIZER LABO-01; Sanmaru Machinery Co., Ltd. Shizuoka, Japan) at 15 MPa and an equal amount of dextrin (NSD300; San-ei Sucrochemical Co., Ltd. Aichi, Japan) was added. The powdered sample was designated "dispersed LAB: d-LAB." A spray dryer (ADL311S-A; Yamato Scientific Co., Ltd. Tokyo, Japan) was used to powderize the samples. Each sample was diluted with distilled water to a concentration of 10 mg/mL, and particle size distribution was measured using a laser diffraction particle size analyzer (SALD-2300; Shimadzu Corporation, Kyoto, Japan) to calculate average and median particle sizes.

2.2. The State of LAB in Mouse Peyer's Patches

LAB was diluted with distilled water to 25 mg/mL and Cy3 (Amersham Cy3 Mono-Reactive Dye Pack, GE Healthcare, Chicago, USA) was added to reach a final concentration of 0.6 mg/mL and incubated for 2 h in the dark. Then, the samples were centrifuged at 3,000 rpm for 15 min and washed with phosphate-buffered saline (PBS)(-). Subsequently, PBS(-) was added to the sample for administration. Male specific pathogen-free Slc:ddY mice (6 weeks old, 16–18 g) were obtained from Tokyo Laboratory Animals Science (Tokyo, Japan). A 100-fold diluted sample was injected into the intestines of mice fasted overnight and incubated for 1 h for the loop assay. After the intestines were collected, actin was stained with phalloidin (Alexa Fluor™ 488 Phalloidin, Thermo Fisher Scientific, Waltham, MA, USA) and nuclei were stained with DAPI (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h. The stained small intestine was observed under a fluorescence microscope (OPTIPHOTO, NIKON, Tokyo, Japan) or confocal laser scanning microscope (LSM 5EXCITER, ZEISS, Jena, Germany).

2.3. IL-12 Production by Mouse Splenocytes

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The LAB suspension was added at a final concentration of 1 μg/mL (culture medium, RPMI1640, Wako, Osaka, Japan) to six wells of a 96-well cell culture plate, seeded with mouse splenocytes collected from BALB/c mice (8 to 9 weeks old) obtained from CLEA Japan (Tokyo, Japan). The mixtures of mouse cells and bacteria were cultured in a humidified 5% CO_2 incubator at 37 °C. After incubation for 24 h, the culture supernatants of the mixtures were collected to measure the concentration of IL-12 by enzyme-linked immunosorbent assay (ELISA). The reagents used for ELISA included a primary antibody (purified anti-mouse IL-12 [p70] antibody, BioLegend Inc., San Diego, CA, USA), secondary antibody (Biotin anti-mouse IL-12/IL-23 p40 antibody; BioLegend), blocking reagent (Block Ace Powder, KAC Co., Ltd., Kyoto, Japan), capture antibody (HRP Avidin, BioLegend), substrate (tetramethylbenzidine, Sigma-Aldrich, St. Louis, MO, USA), and standards (Recombinant Mouse IL-12 [p70] [ELISA Std.], BioLegend), and IL-12 levels were measured using sandwich ELISA [24].

2.4. Model of Mouse Influenza Infection

Female, specific pathogen-free BALB/c mice (5–6 weeks old, 16–18 g) were obtained from Japan SLC (Shizuoka, Japan). All experiments were conducted in accordance with the animal experimentation guidelines of Chubu University and permitted by the Animal Care Committee of Chubu University (Permission number: 3010057). No side effects of drugs were detected throughout the experiments. Mice were intranasally infected with influenza A virus (A/NWS/33, H1N1 subtype) [25] at 2×10^4 plaque-forming units (PFU)/50 μL per mouse (n = 10) on day 0. n-LAB (5 mg/mouse/d) and d-LAB (as dextrin was mixed with LAB in equal amounts, we doubled the dose to 10 mg/mouse/d) were suspended in distilled water. Oseltamivir phosphate (OSL; 0.2 mg/mouse/d) was used as a positive control for antiviral effects and dissolved in distilled water. LAB, d-LAB, or OSL was given by oral administration two times per day, from 7 d before viral inoculation to 14 d after inoculation. The control mice were administered orally with vehicle (distilled water) alone. Because influenza virus (IFV) infection causes a reduction in body weight [26,27], mice of each treatment group were weighed daily for 14 d, beginning on the day of IFV inoculation (designated day 0). Lung samples and bronchoalveolar lavage fluid (BALF) were collected from each group on days 3 and 14, and blood and fecal samples were collected on day 14 (Fig. 1). Lung samples were sonicated for 10 s after the addition of 10 μL PBS per mg of lung tissue and centrifuged at 1,500 rpm for 30 min to separate the supernatants, which were stored at −80 °C. BALFs were collected after four washes with 0.8 mL ice-cold PBS via a tracheal cannula and centrifuged at 1,500 rpm for 10 min; the supernatants were stored at −80 °C. Blood samples were centrifuged at 3,000 rpm for 10 min, and the sera were stored at −20 °C. Fecal extracts were prepared by adding PBS at 10 μL per mg of feces. The amount of virus in the lung and BALF samples collected on day 3 post-infection was quantified using the plaque assays on Madin–Darby canine kidney (MDCK) cell monolayers. Sera and BALFs were subjected to neutralizing antibody titer assays using the 50% plaque reduction method, as described [28,29]. BALFs and fecal extracts were assessed for mucosal virus-specific IgA levels by ELISA, as described [30].

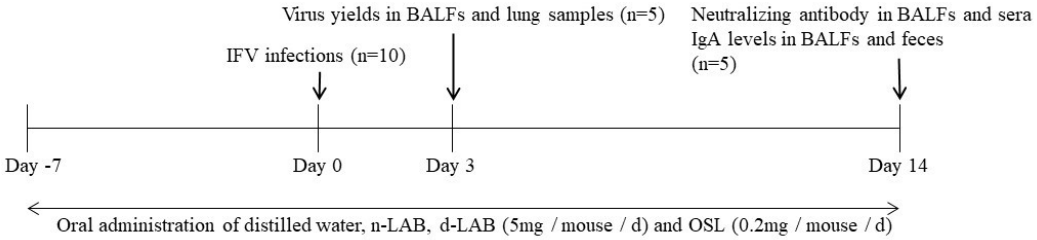

Figure 1. Experimental procedure of influenza virus infection. Mice in the control, LAB, d-LAB, and OSL groups were administered distilled water, LAB, and d-LAB (5 mg/d, two doses per day) and OSL (0.2 mg/d, two doses per day), respectively, during the study period (days −7 to 14). Mice were intranasally infected with IFV on day 0. On day 3 after IFV infection, five mice from each group were euthanized to quantify virus loads in BALFs and lungs. Five mice were euthanized for measuring neutralizing antibody and IgA levels on day 14. BALF, bronchoalveolar lavage fluid; d-LAB,

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dispersed lactic acid bacteria; IFV, influenza A virus; n-LAB, non-treated lactic acid bacteria; OSL, oseltamivir phosphate.

2.5. Statistical Analysis

The effects of the drugs were analyzed by one-way analysis of variance, and correction for multiple comparisons was done by Tukey's multiple comparison test. A p value of <0.05 was considered to be significant.

3. Results

3.1. Particle Size of n-LAB and d-LAB

Figure 2A shows the particle size distribution of LAB that was washed with distilled water and powdered using a spray dryer. Figure 2B shows the particle size distribution of d-LAB that was washed with distilled water, treated with a high-pressure homogenizer, and powdered with an equal amount of dextrin. The mean and median particle sizes of n-LAB and d-LAB are shown in Table 1. n-LAB and d-LAB were found to be more abundant around 55 and 0.7 μm in particle size. The mean particle size of d-LAB was smaller, 0.679 μm compared to 35.454 μm for n-LAB; the median was 0.633 μm for d-LAB compared to 40.761 μm for n-LAB (Table 1).

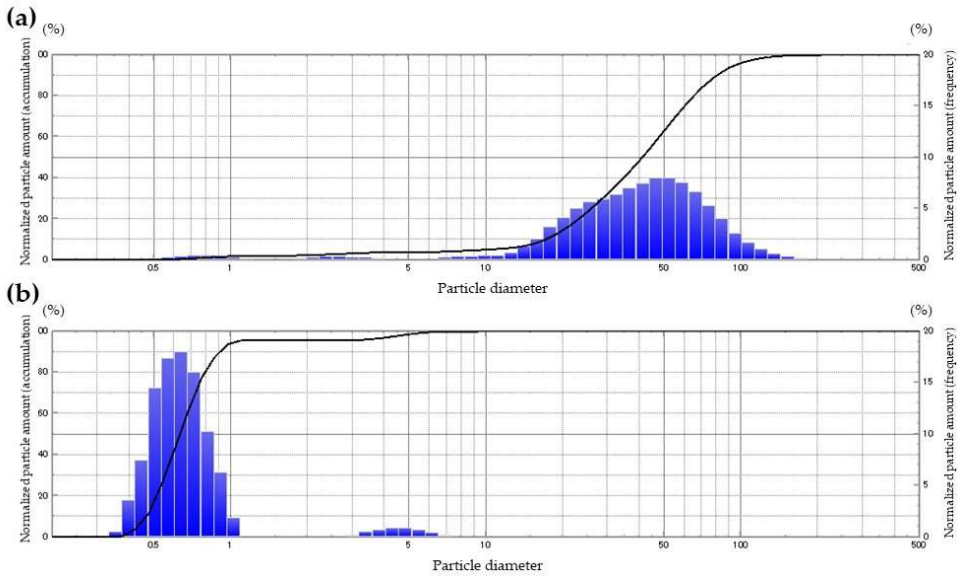

Figure 2. Measurement of n-LAB and d-LAB using a laser diffraction particle size analyzer (SALD-2300). n-LAB (**a**) and d-LAB (**b**) were suspended in distilled water and the relative particle mass (frequency and integration) was measured using a laser diffraction particle size analyzer. d-LAB, dispersed lactic acid bacteria; n-LAB, non-treated lactic acid bacteria; n = 3.

Table 1. Measurement of n-LAB and d-LAB using a laser diffraction particle size analyzer

	particle sizes (μm)

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	Mean	±	SD	Median
n-LAB	35.454	±	0.378	40.761
d-LAB	0.679	±	0.203	0.633**

The n-LAB (**a**) or d-LAB (**b**) were suspended in distilled water and particle sizes (mean and median) were measured using a laser diffraction particle size analyzer (SALD-2300). d-LAB, dispersed lactic acid bacteria; n-LAB, non-treated lactic acid bacteria; n = 3; **p < 0.01 vs. n-LAB.

3.2. Observation of Mice Peyer's Patches After n-LAB and d-LAB Administration

n-LAB was not visible on Peyer's patches but was observed on the mucosa around the Peyer's patches (Fig. 3A). n-LAB was in an agglomerated state. In contrast, d-LAB was observed on the surface of the Peyer's patches (Fig. 3B). Furthermore, cLSM was used to confirm the underlying layer of the Peyer's patches upon d-LAB administration and bacterial uptake into the body was present (Fig. 3C).

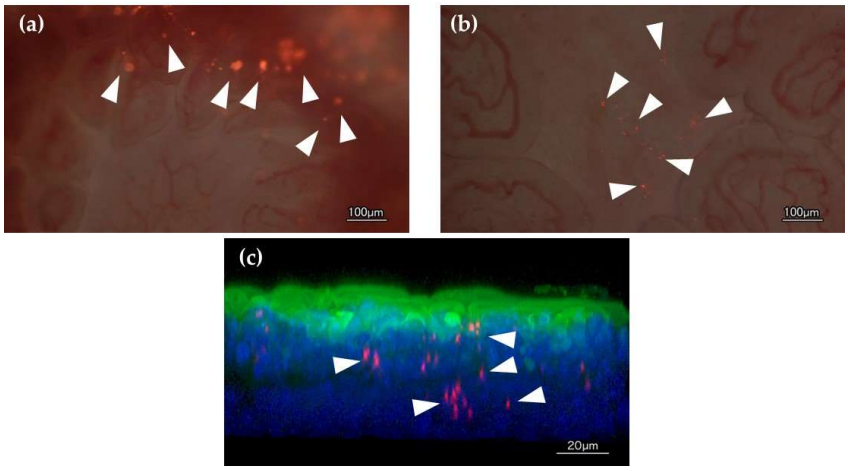

Figure 3. Microscopy images of the Peyer's patch in mice after LAB and d-LAB administration and Peyer's plate images after d-LAB administration with cLSM. n-LAB (**a**) or d-LAB (**b**) stained with Cy3 and Peyer's patches were imaged with a fluorescence microscope. The uptake of d-LAB by Peyer's patches after d-LAB administration was imaged with cLSM (**c**).The white triangular arrow shows the bacteria. cLSM, confocal laser scanning microscopy; d-LAB, dispersed lactic acid bacteria; n-LAB, non-treated lactic acid bacteria.

3.3. Effects of n-LAB and d-LAB on IL-12 Production in Mouse Splenocytes

We compared IL-12 production after n-LAB and d-LAB administration using mouse splenocytes and found that d-LAB was significantly higher than n-LAB (Fig. 4).

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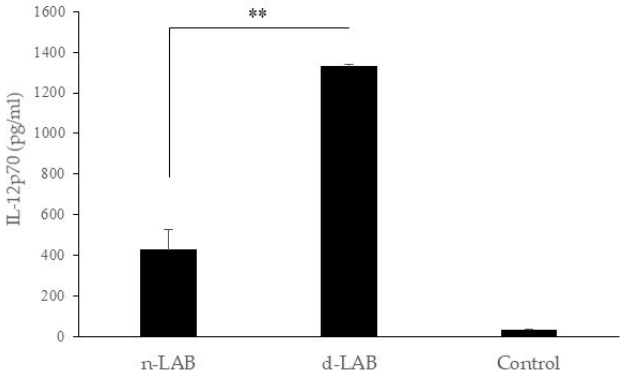

Figure 4. Effect of ribonuclease treatment after n-LAB and d-LAB administration on IL-12 production in mouse splenocytes. n-LAB and d-LAB co-cultured with mouse splenocytes for 24 h. IL-12 concentration in the culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA). Control, culture medium only; d-LAB, dispersed lactic acid bacteria; n-LAB, non-treated lactic acid bacteria. Each value is presented as the mean ± SD; n = 6; **p < 0.01 vs. n-LAB.

3.4. Effects of n-LAB and d-LAB on IVF Infection in Mice

The effects of n-LAB and d-LAB on the change in body weight of mice infected with IFV were examined (Fig. 5). The control, n-LAB, and d-LAB groups showed approximately 15.9%, 14.9%, and 14.4% loss, respectively, on day 7 post-infection. Although no significant difference was observed between the n-LAB and d-LAB groups, d-LAB suppressed weight loss slightly more than n-LAB. Thereafter, mice gradually gained body weight, and the mice in the d-LAB group returned to their pre-infection body weights on day 12 post-infection.

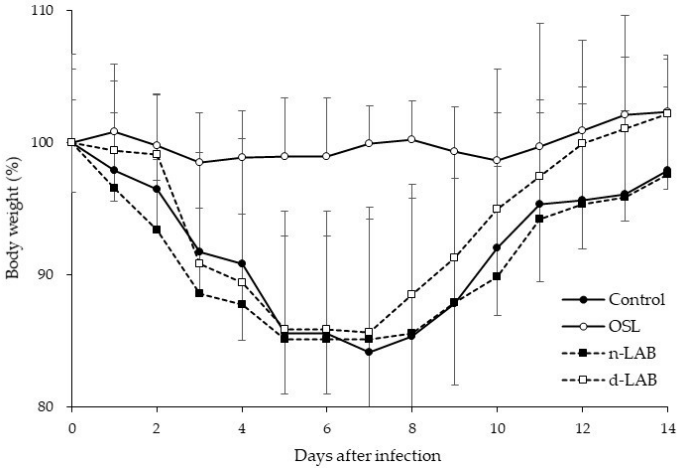

Figure 5. Body weight change in mice infected with IFV. IFV-infected mice were orally administered distilled water (control, filled circle), 0.2 mg/d of oseltamivir (OSL, white circle), 5 mg/d of non-treated lactic acid bacteria (n-LAB, filled square), and 5 mg/d of dispersed lactic acid bacteria (d-LAB, white square) from 7 d before viral infection to 14 d post-infection. Body weights are relative to those on the day of viral infection (day 0), which was set as 100%. Each value is presented as the mean ± SD; n = 5; IFV, influenza A virus.

Virus yields in the BALF and lung of IFV-infected mice on day 3 post-infection are shown in Figures 6A and 6B, respectively. Oral administration of n-LAB and d-LAB significantly reduced the virus load in BALFs ($p < 0.01$) and lungs ($p < 0.05$) compared with the control group. The d-LAB group had a significantly lower viral load than the n-LAB group in the BALFs ($p < 0.05$). Virus loads in the OSL group were markedly low, as shown in Figure 6A, B.

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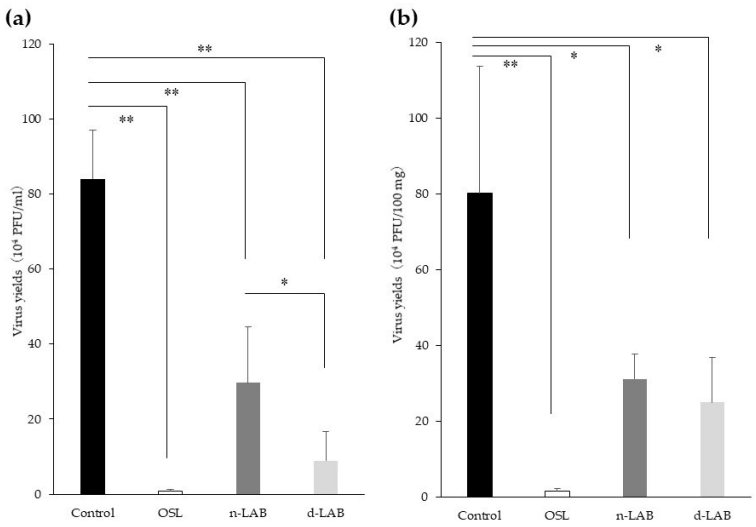

Figure 6. Effect of LAB or d-LAB administration on virus load in mice. Virus yield in BALFs (**a**) and lung samples (**b**) were measured using a plaque assay on day 3 post-infection. Each value is presented as the mean ± SD; n = 5; **p < 0.01; *p < 0.05. BALF, bronchoalveolar lavage fluid; d-LAB, dispersed lactic acid bacteria; n-LAB, non-treated lactic acid bacteria; OSL, oseltamivir; PFU, plaque-forming units.

Figures 7A, B show the effects of n-LAB and d-LAB on the neutralizing antibody response to IFV in BALFs (Fig. 7A) and sera (Fig. 7B) at day 14 post-infection. The antibody titers of BALFs in the mice administered with n-LAB or d-LAB were significantly higher than those in the control group. Moreover, it was significantly higher in the d-LAB than the n-LAB group ($p < 0.05$). By contrast, the antibody titer in the d-LAB group was significantly higher ($p < 0.01$) than that in the control group.

To elucidate whether n-LAB and d-LAB stimulate the local immune response in mice, the levels of IFV-specific IgA in BALFs and feces were determined at day 14 post-infection (Fig. 7C, D). IgA production in the d-LAB group was significantly higher than the control group ($p < 0.01$). The IgA levels in the feces were significantly higher in the d-LAB group than the n-LAB group to ($p < 0.05$).

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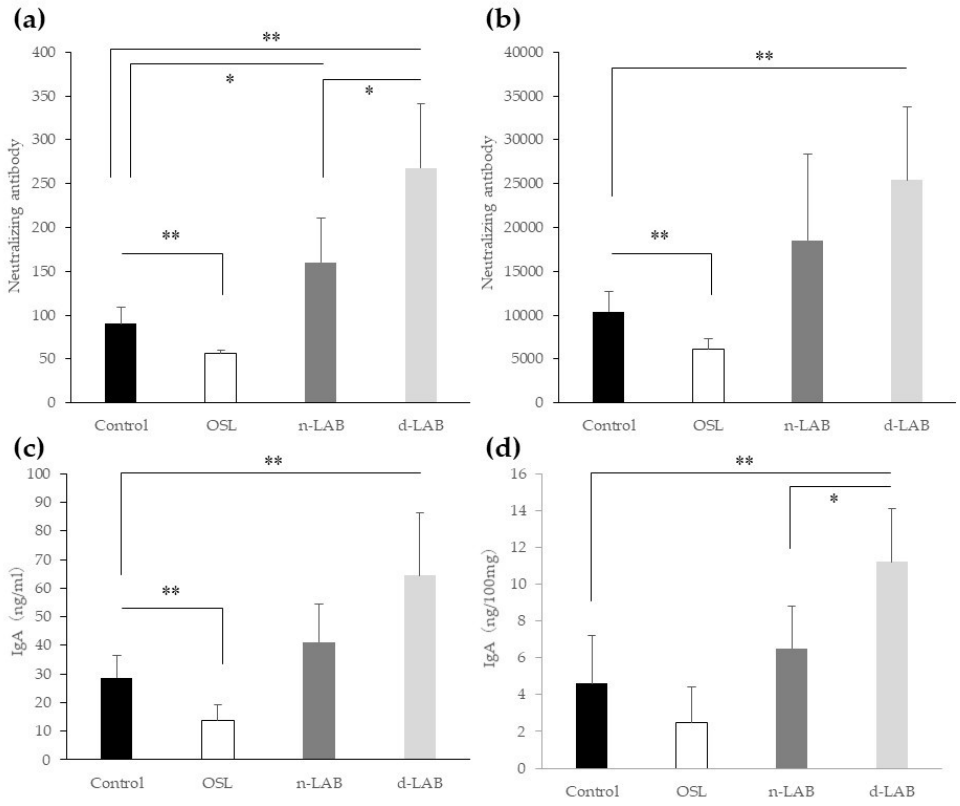

Figure 7. Effects of n-LAB or d-LAB administration on the neutralizing antibody titer and production of IFV-specific IgA in mice. The titer of the virus-neutralizing antibody is presented as the reciprocal of the dilution of BALFs (**a**) and sera (**b**) that reduced the plaque number to a level below 50% of that seen in the virus control. The IFV-specific IgA levels in BALFs (**c**) and feces (**d**) were determined by ELISA. Each value is presented as the mean ± SD; n = 5; **p < 0.01; *p < 0.05. BALF, bronchoalveolar lavage fluid; IFV, influenza A virus; d-LAB, dispersed lactic acid bacteria; n-LAB, non-treated lactic acid bacteria; OSL, oseltamivir.

4. Discussion

When cultured LAB are washed and dried into powder, the bacteria adhere to each other and form aggregates (Table 1, Fig. 2). One possible cause is that the polysaccharides produced by LAB and the capsules present on the surface of the bacteria may help in bonding during drying. Furthermore, it has been reported that bacterial surface proteins [31] are involved in charge stability and that non-specific electrostatic effects may be a factor in adhesion. LAB were reported to adhere to polysaccharides, such as xylan and mucin, by surface layer protein action [32], and it is natural for bacteria to adhere to a variety of substrates to survive. Thus, polysaccharide-producing LAB may stick to each other. Further, powderization may strengthen the binding. However, in consideration of quality and cost, it is desirable to powderize LAB for distribution, but if LAB agglomerate with each other by powderization (Fig. 2A), the product may be affected. Therefore, we developed a LAB powder (d-LAB) with less agglomerates, by homogenizing the culture and adding dextrin to the powder to prevent the agglomerates from re-agglomerating (Fig. 2B). d-LAB was measured using a laser diffraction particle size analyzer and average particle size was 0.679 μm, indicating few bacterial aggregates. Then, n-LAB, with several bacteria aggregates, and d-LAB, with excellent water dispersibility and fewer aggregates, were administered to mice and observed in the vicinity of the Peyer's patches in the small intestine. n-LAB failed to reach the Peyer's patches and drifted on the mucus covering the Peyer's patches, whereas d-LAB reached the Peyer's patches. This difference may be due to the presence of membrane-tethered mucin in the intestinal cells. [33,34]. We think that n-LAB, which has many bacteria aggregates of about 50 μm in size (Fig. 3A), is physically prevented

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from contacting the intestinal tract by mucin, whereas d-LAB, which has fewer aggregates, can pass through mucin and reach the Peyer's patches (Fig. 3B, C). Furthermore, larger aggregates of LAB were found to reduce the immune response to phagocytosis by macrophages and dendritic cells (Fig. 4). These results suggest that LAB aggregation may not only reduce their uptake from M cells in Peyer's patches, but also affect their phagocytosis and decrease the immune response of LAB; therefore, we compared n-LAB and d-LAB in a mouse influenza infection model. n-LAB differed significantly from d-LAB in IFV yield of BALFs at 3 d after infection (Fig. 6) and in neutralizing antibody titers of BALFs and IFV-specific IgA in feces at 14 d after infection (Fig. 7). Although the difference was not significant, n-LAB showed almost the same transition in body weight as control, whereas d-LAB showed rapid weight recovery after 7 d of infection and returned to pre-infection weight by day 12 (Fig. 5). The results of this study show that the effectiveness of LAB decreases when there are many aggregates of LAB. The effects of *Lactobacillus* species, strains [35], culture methods [36], and use [37] have been studied extensively. However, there are few reports of changes in the physical properties of LAB during powderization that affect their health benefits. To make LAB use effective, we recommend studying and verifying the properties of LAB at the consumer stage. Therefore, not only will we evaluate the LAB prepared in our laboratory, but we will also study LAB in a form close to the final product to confirm whether the effectiveness of the LAB has been lost. We will continue to develop better LAB and improve their quality.

5. Conclusions

In this study, it was found that LAB could not reach the Peyer's patches through the intestinal mucosa because of the formation of aggregates when LAB were powdered, although this was true for all LAB (Fig. 8A). n-LAB were found to have an effect on the efficacy (protection against viral infection). Therefore, we developed a LAB powder (d-LAB) with less agglomerates by treating it with a high-pressure homogenizer and adding an excipient to prevent agglomeration. When the d-LAB were administered to mice, they were able to pass through the intestinal mucosa and reach the Peyer's patches because of the improved water dispersibility of the bacteria (Fig. 8B). Thus, the effectiveness of the LAB with improved dispersibility was higher. These results indicate that differences in water dispersibility of LAB has an effect on protection against IFV infection. Therefore, we can increase the health benefits of LAB by improving the dispersibility of LAB that have low water dispersibility.

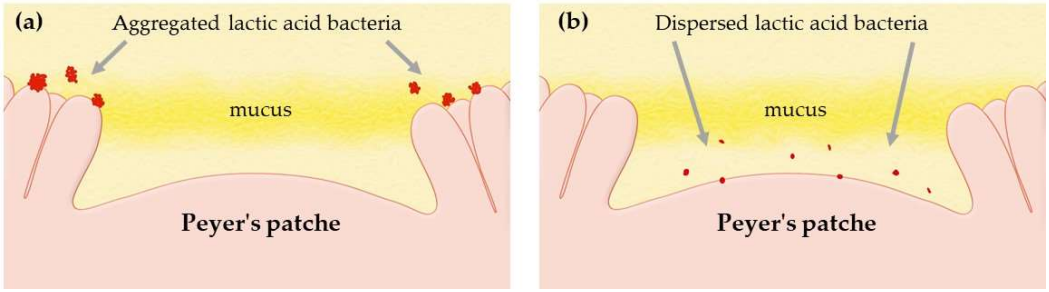

Figure 8. Image of aggregated lactic acid bacteria or dispersed lactic acid bacteria in contact with the small intestinal Peyer's patches. Aggregated lactic acid bacteria could not reach the Peyer's patches because of large particle size, which is blocked by mucus (a). Dispersed lactic acid bacteria were small particle size and can pass through mucus to reach the Peyer's patches (b).

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