IgA-enhancing effects of membrane vesicles derived from Lactobacillus sakei subsp. sakei NBRC15893

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IgA-enhancing effects of membrane vesicles derived from *Lactobacillus sakei* subsp. *sakei* NBRC15893

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Immunoglobulin (Ig) A in the mucus of the intestinal tract plays an important role in preventing the invasion of pathogenic microorganisms and regulating the composition of the gut microbiota. Several strains of probiotic lactic acid bacteria (LAB) are known to promote intestinal IgA production. Bacteria are also known to naturally release spherical membrane vesicles (MVs) that are involved in various biological functions such as quorum sensing, pathogenesis, and host immunomodulation. However, the production of MVs by LAB and their effects on host immunity remain poorly understood. In this study, we investigated the MV production by *Lactobacillus sakei* subsp. *sakei* NBRC15893 isolated from *kimoto*, the traditional seed mash used for brewing sake. MVs were separated from the culture broth of *L. sakei* NBRC15893 through filtration and density gradient ultracentrifugation and were observed by transmission electron microscopy. The MVs showed a spherical morphology, with a diameter of 30–400 nm, and contained proteins and nucleic acids. In addition, both the LAB cells and purified MVs promoted IgA production by murine Peyer's patch cells. This MV- and cell-induced IgA production was suppressed by neutralization of Toll-like receptor (TLR) 2, which recognizes cell wall components of gram-positive bacteria, using an anti-TLR2 antibody. Collectively, our results indicate that MVs released from *L. sakei* NBRC15893 enhance IgA production by activating host TLR2 signaling through its cell wall components. Thus, it is important to consider novel interactions between gut microbiota and hosts via MVs, and MVs derived from probiotic bacteria could have promising applications as safe adjuvants.

Key words: lactic acid bacteria, membrane vesicle, gut immunity, IgA

INTRODUCTION

The intestinal tract is equipped with multilevel barriers to prevent the invasion of pathogenic microorganisms, including chemical barriers such as the mucus and antimicrobial peptides, a physical barrier created by epithelial cells, and a biological barrier established by intestinal microflora and immune cells [1]. The gut mucosal immune system is characterized by the production of immunoglobulin (Ig) A, which not only plays an important role in preventing the invasion of pathogenic microorganisms into epithelial cells [2] but also regulates the composition of the gut microbiota [3, 4]. Therefore, enhancement of IgA production in the gut through dietary components and probiotics such as lactic acid bacteria (LAB) and bifidobacteria can be an effective strategy for enhancing the immune system, thereby preventing infectious diseases and maintaining the optimal composition of the gut microbiota.

IgA production in the gut involves the induction of IgA class-switch recombination (CSR) in which IgM^+ B cells change to IgA^+ B cells in gut-associated lymphatic tissues, including Peyer's patches (PPs). This step is followed by the differentiation of the IgA^+ B cells into IgA-producing plasma cells in the intestinal mucosal lamina propria. During this process, $CD4^+$ T cells, which are activated by antigen-specific dendritic cells (DCs), induce the IgA CSR of IgM^+ B cells in the presence of stimuli such as transforming growth factor (TGF)-β, interleukin (IL)-4, and IL-6 [5]. IgA CSR is also induced by retinoic acid, a proliferation-inducing ligand, or B-cell activating factor, which are produced by DCs in the PPs through a route independent of T cells [6]. Indeed, several reports have demonstrated the IgA-enhancing effects of LAB strains in the gut [7], including *Lactobacillus pentosus* strain b240 [8], *L. plantarum* AYA [9], and *L. gasseri* SBT 2055 [10]. These strains activate DCs in the PPs via Toll-like receptors (TLRs) and induce the production of retinoic acid, IL-6, and TGF-β, which in turn enhance IgA production *in vitro*.

Bacteria also naturally release membrane vesicles (MVs) with diameters of 20–500 nm under various growth conditions. Although the majority of studies on MVs have focused on gram-negative bacteria, recent studies have also

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reported MV production by gram-positive bacteria with thick peptidoglycan (PG). Moreover, the mechanism of production and functions of MVs have been predominantly examined in pathogenic strains. MVs contains bacterial components such as nucleic acids, proteins, and cell wall components and are involved in processes such as quorum sensing, relief of environmental stresses, delivery of pathogenic factors, and host immunomodulation [11–13]. For example, MVs produced by gram-negative bacteria *Neisseria meningitidis* [14] and *Helicobacter pylori* [15] act on macrophages to induce the inflammatory cytokine IL-6 and exert immunomodulatory effects. Similar mechanisms have been demonstrated for gram-positive bacteria. For instance, MVs of *Mycobacteria tuberculosis* inhibit the antigen presentation of DCs and macrophages [16], and the MVs of *Clostridium perfringens* induce IL-6 production from macrophage-like cells [17]. However, there are few reports on the production of MVs by nonpathogenic and probiotic bacteria, including LAB [18, 19]. The MVs of *L. rhamnosus* JB-1 were found to modulate regulatory T cells [18], and those of *L. plantarum* WCFS1 were reported to enhance the immune response against vancomycin-resistant enterococci [19]. Besides these examples, it remains unclear whether other LAB strains produce MVs or show immunomodulatory effects, including IgA-enhancing effects.

We found that the cells of *Lactobacillus sakei* subsp. *sakei* NBRC15893, which was isolated from *kimoto*, the traditional seed mash used for brewing sake, and the microcomponents obtained from the culture supernatant by ultracentrifugation promote IgA production from PP cells *in vitro*. In the present study, we aimed to elucidate whether *L. sakei* NBRC15893 produces MVs and to identify specific microcomponents that mediate the IgA-enhancing effect.

MATERIALS AND METHODS

Bacterial strain and culture conditions

L. sakei subsp. *sakei* NBRC15893 was purchased from Biological Resource Center, NITE (Tokyo, Japan). The strain was precultured anaerobically in deMan, Rogosa and Sharpe (MRS) medium (Becton Dickinson and Company [BD], Tokyo, Japan) using an AnaeroPack system (Mitsubishi Gas Chemical Company, Tokyo, Japan) and then cultured statically in MRS medium at 30°C. The optical density at 660 nm (OD_{660}) of the culture broth was measured using a UV-1850 spectrophotometer (Shimadzu, Kyoto, Japan).

Preparation of MVs

The microcomponents in the culture broth were purified according to standard purification procedures for MVs [17]. The culture broth was centrifuged (8,500 × g, 5 min, 4°C), and the precipitated LAB cells were autoclaved at 121°C for 15 min and then dried under a reduced pressure at 22–25°C. The supernatant was filtered (0.45 µm, Thermo Fisher Scientific, Waltham, MA, USA) and then ultracentrifuged (100,000 × g, 1 hr, 4°C). The precipitate was washed with 10 mM HEPES containing 0.85% NaCl, pH 6.8 (hereafter HEPES-NaCl), and ultracentrifuged again (100,000 × g, 1 hr, 4°C). For investigation of MV, the precipitate obtained from 400 ml of culture broth was resuspended in 50 µl of HEPES-NaCl (the suspension is hereafter referred to as the crude MV fraction). For purification of MVs, the precipitate obtained from 2.2 l of culture broth at 24 hr was suspended in 1 ml of 45% (v/v) OptiPrep (iodixanol; Axis-Shield, Dundee, Scotland) in HEPES-NaCl and then overlaid by 1-ml aliquots of 40%, 35%, 30%, 25%, 20%, 15%, and 10% (v/v) OptiPrep in HEPES-NaCl. After ultracentrifugation (100,000 × g, 6 hr, 4°C), 1.5-ml fractions were collected from the tops of the ultracentrifuge tubes (Fr. 1–5). Each fraction was washed with HEPES-NaCl by ultracentrifugation (100,000 × g, 1 hr, 4°C), and each precipitate was resuspended in 50 µl of the buffer.

Absorbance at 260 and 280 nm was measured using a NanoDrop ND-1000 system (Thermo Fisher Scientific). Protein concentrations were determined with a bicinchoninic acid protein assay reagent kit (Thermo Fisher Scientific).

Transmission electron microscopy (TEM)

Fractionated samples were stained with 2% sodium phosphotungstate on a collodion-coated grid (150-mesh, Okenshoji, Tokyo, Japan) and observed by TEM (JEM-1400, JOEL, Tokyo, Japan) at 80 kV.

Preparation and culture of PP cells

The experimental protocols used in this study followed the Guide for the Care and Use of Experimental Animals issued by the Prime Minister's Office of Japan and were reviewed and approved by the Animal Ethics Committee of Kansai University (Approval No. 1706). The breeding room was maintained under the following conditions: temperature, 21–23°C; humidity, 55–65%; light period, 08:00 AM to 08:00 PM. Female BALB/c mice (7–14 weeks old, Japan SLC, Shizuoka, Japan) were used to obtain murine PP cells. PPs were harvested from the small intestine of mice and then incubated in RPMI 1640 medium (Sigma-Aldrich, Tokyo, Japan) containing 0.5 mg/ml collagenase, 2% (v/v) fetal bovine serum (FBS; Thermo Fisher Scientific), 100 U/ml penicillin, and 100 µg/ml streptomycin for 30 min. The PP cells were filtered through a cell strainer (100 µm; BD) and washed with the medium without collagenase. The cells were suspended in RPMI 1640 medium containing 10% (v/v) FBS, 55 µM 2-mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml streptomycin and then cultured at 1.0×10^5 cells/well in a U-bottom 96-well plate with or without LAB cells or fractionated samples for 4 days at 37°C under 5% CO_2 in air.

Neutralization of TLR2 in PP cells

Murine PP cells were preincubated with 0.5 µg/ml anti-TLR2 antibody (purified anti-mouse/human CD282, clone T2.5, Biolegend, San Diego, CA, USA) or isotype antibody (purified mouse IgG_1, clone MOPC-21, Biolegend) at 37°C for 30 min and then cultured with or without the purified MVs (Fr. 3 obtained by the density gradient ultracentrifugation) or

L. sakei NBRC15893 (50 µg/well) cells for 4 days at 37°C under 5% CO_2 in air.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IgA were determined by ELISA. In brief, 96-well plates (MaxiSorp; Thermo Fisher Scientific) were coated with 50 µl of 10 µg/ml goat anti-mouse IgA (Bethyl Laboratories, Montgomery, TX, USA) for 1 hr at 22–25°C. After washing with phosphate-buffered saline (PBS) containing 0.05% (w/v) Tween 20, the wells were blocked with 300 µl PBS containing 1% bovine serum albumin (Sigma-Aldrich) at room temperature for 1 hr, and 50 µl of the diluted samples was added to each well and incubated at room temperature for 1 hr. After washing, 50 µl of 25 ng/ml goat anti-mouse horseradish-peroxidase-conjugated IgA (Bethyl Laboratories) was added, and the wells were further incubated at room temperature for 1 hr. After washing, 50 µl of the TMB microwell peroxidase substrate system (SeraCare, Milford, MA, USA) was added and incubated at room temperature. After the addition of 25 µl of 1 M sulfuric acid, the absorbance at 450 nm was measured using an infinite F200 microplate reader (Tecan, Zürich, Switzerland). Purified mouse IgA (κ isotype control; BD) was used as a standard.

The concentrations of IL-6 were determined by ELISA using a Mouse IL-6 DuoSet ELISA (R&D Systems, Minneapolis, MN, USA).

RESULTS

Production of MVs by L. sakei *NBRC15893*

To demonstrate whether *L. sakei* subsp. *sakei* NBRC15893 produces MVs, the microcomponents in the culture supernatant (crude MV fraction) were first recovered by ultracentrifugation. The time courses of the OD_{660} and pH of the culture and the amount of protein recovered as the crude fraction are shown in Fig. 1. The amount of recovered protein increased with the increase in bacterial concentration. A typical TEM image of the crude MV fraction at 24 hr is shown in Fig. 2A. MV-like spherical structures with a diameter of 30–400 nm, which were similar to the MVs produced by other bacteria, were observed. This indicated that *L. sakei* NBRC15893 releases MVs. The MVs were purified using a routine purification method. The protein concentrations and absorbance at 260 and 280 nm of each fraction (Fr. 1–5) obtained by density gradient ultracentrifugation are shown in Table 1. Brown and white bands were observed in Fr. 2 and 3, respectively, and the protein concentration of Fr. 3 was higher than that of the other fractions. TEM observation of each fraction further revealed that the MVs were much more heavily concentrated in Fr. 3 than in the other fractions and that Fr. 2 and Fr. 4 contained only a small number of MVs (Fig. 2B–F). These results indicated that the MVs derived from *L. sakei* NBRC15893 contained proteins and nucleic acids, similar to other bacterial MVs.

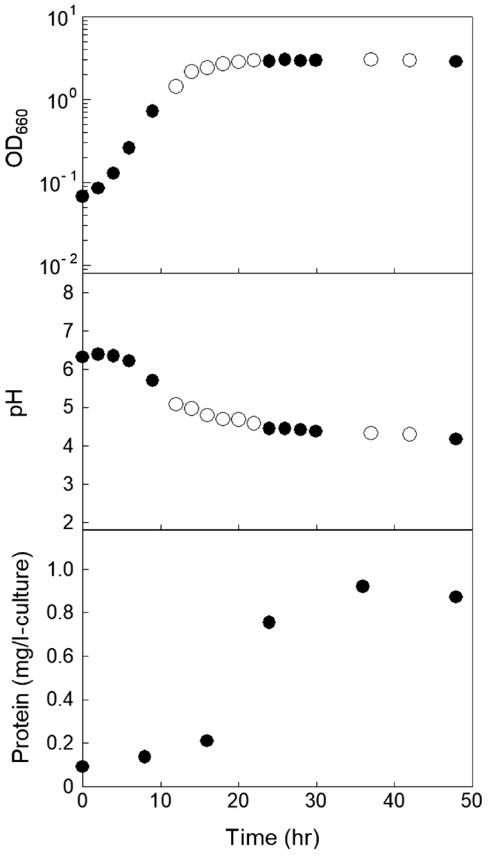

Fig. 1. Time course of the growth of *L. sakei* NBRC15893, pH of the culture broth, and MV production.

Protein concentration is expressed as the amount of protein obtained from 1 l of the culture supernatant.

Open circles and closed circles indicate the values of OD_{660} and pH of the culture broth in different runs, respectively. The values are the average of three measurements, and the measurement error was within approximately 5%. Repeatability was confirmed by repeating the experiments at least three times, and typical results are shown.

IgA-enhancing effects of L. sakei *NBRC15893 MVs*

IgA production from the PP cells was evaluated by ELISA with the crude MV fraction at 24 hr and the fractions obtained by the density gradient ultracentrifugation. The crude MV fraction enhanced IgA production by the PP cells, showing that the microcomponents derived from *L. sakei* NBRC15983 were involved in the IgA-enhancing effects. As shown in Fig. 3, only the IgA concentration of the cells cultured with Fr. 3 was significantly increased compared with that of the negative control group ($p=0.021$). These results indicated that MVs derived from *L. sakei* NBRC15893 in the culture broth enhanced IgA production by the PP cells.

Involvement of TLR2 in the IgA-enhancing effect of MVs

The IgA-enhancing effects of several LAB have been shown to be mediated by TLR2 expressed on DCs, which

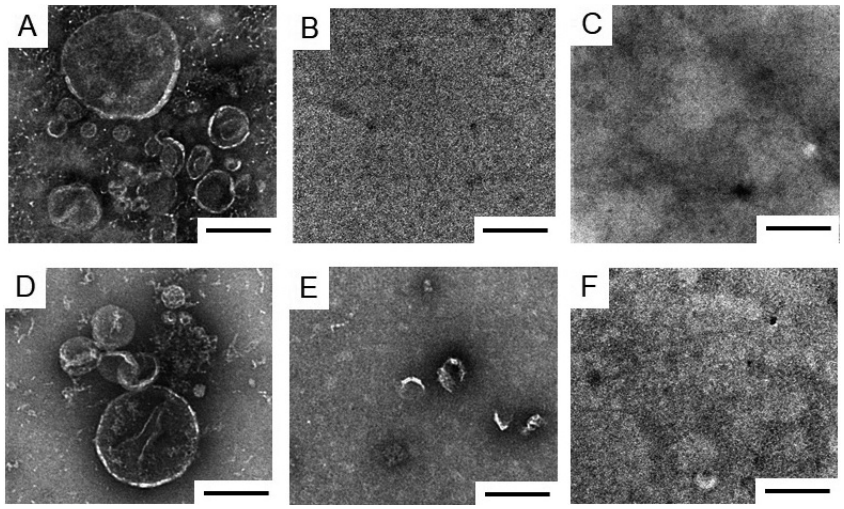

Fig. 2. Typical transmission electron microscope images of MVs.

(A) Crude membrane vesicle fraction precipitated by the ultracentrifugation and the fractions obtained by the density-gradient ultracentrifugation of the precipitate: (B) Fr. 1, (C) Fr. 2, (D) Fr. 3, (E) Fr. 4, (F) Fr. 5. Scale bars, 200 nm. Repeatability was confirmed by repeating the similar experiment at least three times, and typical results are shown.

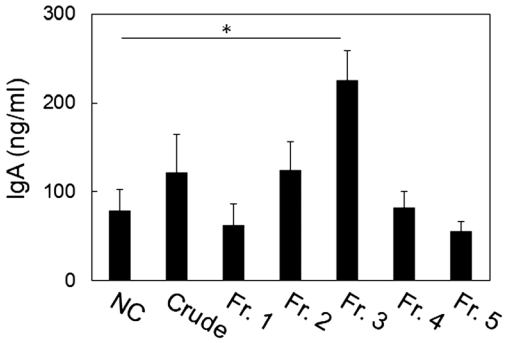

Fig. 3. IgA-enhancing effects of fractions obtained by density gradient ultracentrifugation in Peyer's patch (PP) cells.

The PP cells (1.0×10^5 cells/well) were cultured with the crude membrane vesicle (MV) fraction (protein concentration, 37 μg/ml) or equal volumes of each fraction obtained by the density-gradient ultracentrifugation of 10–45% (v/v) OptiPrep (one-hundredth the protein concentration of each fraction shown in Table 1) for 4 days. NC, negative control. Crude, crude MV fractions. The data are presented as means ± standard deviations of triplicate samples. *$p<0.05$, Student's t-test.

recognize the cell wall components of gram-positive bacteria [8, 10]. Thus, the involvement of TLR2 in the IgA-enhancing effects of the MVs derived from *L. sakei* NBRC15893 and the LAB cells was investigated using an anti-TLR2 antibody for neutralization of TLR2. The increases in the IgA production induced by the addition of purified MVs (Fr. 3) and the LAB cells were completely inhibited by neutralization of TLR2 with anti-TLR2 (Fig. 4A), indicating that both *L. sakei* NBRC15893 cells and the MVs they release enhance IgA production from PP cells via TLR2. In addition, since it was reported that several LAB strains induce IL-6 production from DCs [8–10], which activates B cells to produce IgA, the IL-6 concentration in the culture supernatant was measured. MVs and LAB cells induced IL-6 production from PP cells, and the induction by MVs and LAB cells was suppressed by addition of anti-TLR2 antibody ($p=0.071$ and 0.045, respectively; Fig. 4B), similar to that in IgA production. It was revealed that MVs of the strain induced IL-6 production from PP cells via TLR2. These results suggested that both MVs and LAB cells enhance IL-6 production via TLR2, resulting in promotion of IgA production.

DISCUSSION

We demonstrated that the nonpathogenic gram-positive bacterium *L. sakei* NBRC15893 produced MVs, which, along with the cells themselves, exhibit IgA-enhancing effects in PP cells via TLR2.

Envelope structures of gram-negative bacteria are stabilized via protein cross-links that reach from the inner membrane (IM) through the PG to the outer membrane (OM). MVs are produced through liberation of the OM from the covalent and noncovalent OM-PG-IM cross-links without concomitant damage and loss of membrane integrity either due to the loss of OM proteins, periplasmic lipoproteins, and PG or through the accumulation of misfolded proteins in the periplasm [20, 21]. In addition, it has been reported that explosive cell lysis produces membrane fragments that rapidly form MVs through the activity of a cryptic prophage endolysin [22]. Although little is known about the mechanism of MV production of

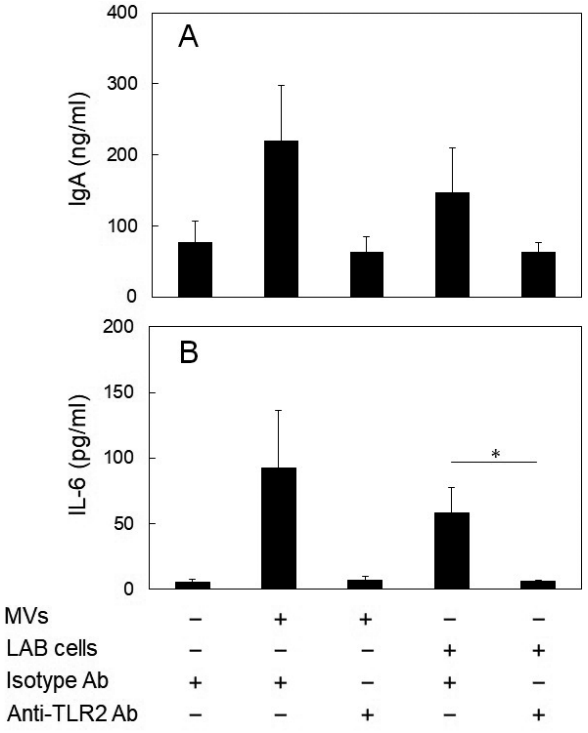

Fig. 4. Involvement of TLR2 in the IgA-enhancing effects of the purified MVs and the cells of *L. sakei* NBRC15893.

Peyer's patch (PP) cells (1.0×10^5 cells/well) were cultured in the presence or absence of the anti-TLR2 antibody for 4 days with or without the MVs (protein concentration; 34 μg/ml) or *L. sakei* NBRC15893 cells (50 μg/ml). (A) IgA concentration and (B) IL-6 concentration in the culture supernatant was measured by ELISA. The data are expressed as means ± standard deviations of triplicate samples. *$p<0.05$, Student's t-test.

Table 1. Concentrations of cytoplasmic constituents of fractions obtained by density gradient ultracentrifugation

Fraction No.	Protein (mg/ml)	A_{260}	A_{280}
1	0.02	0.07	0.02
2	0.11	0.29	0.14
3	3.42	1.59	0.92
4	0.30	1.26	0.76
5	0.01	0.53	0.11

The values are the average of three measurements, and the measurement error was within approximately 5%. Repeatability was confirmed by repeating the experiments at least three times, and typical results are shown.

gram-positive bacteria, it is predicted that an increase in the turgor pressure of the cytoplasm, followed by distortion of the cell wall, and presence of a channel that penetrates the cell wall result in MV release [23]. Recently, Toyofuku *et al.* reported that the cell membrane was extruded from the pores of PG degraded by endolysin to release MVs in *Bacillus subtilis* [24].

According to the Kyoto Encyclopedia of Genes and Genomes database (KEGG; http://www.genome.jp/kegg/), several lactobacilli such as *L. plantarum* WCFS1, *L. casei* BL23, *L. salivarius* JCM1046, and *L. rhamnosus* GG possess genes encoding endolysin. Although we did not confirm whether *L. sakei* NBRC15893 also possesses an endolysin-encoding gene, it is possible that endolysin is also involved in the MV production by the LAB and that MV production is not limited to certain strains but occurs widely in most LAB strains.

Cell wall components such as lipoteichoic acid of *L. casei* YIT 9029 and *L. fermentum* YIT 0159 [25], teichoic acid of *L. plantarum* ATCC 14917^T [26], PG of *L. pentosus* strain b240 [8] and *L. casei* YIT 9029 [27], and lipoprotein of *L. acidophilus* X37 [28] have been reported to exert immunomodulatory effects via TLR2. MVs generally contain these cell wall components in addition to nucleic acids and proteins. For example, the lipoproteins contained in the MVs of *M. tuberculosis* were shown to inhibit the antigen presentation of DCs and macrophages via TLR2 [16], and components considered to be lipoproteins contained in the MVs of *C. perfringens* also induced the IL-6 production of macrophage-like cells [17]. Here, we found that the whole cells of *L. sakei* NBRC15893 also enhanced IgA production via TLR2 signaling, suggesting that IgA production by the MVs may be stimulated by the TLR2 ligands that are derived from the cells and also contained in the MVs.

Regarding the induction of IgA production from PP cells, it has been reported that several LAB cells produce IL-6 and other cytokines via TLR2 of DCs [8–10], resulting in activation of B cells promoting IgA production. In this study, it was suggested that IL-6 induced by MVs of *L. sakei* NBRC15893 promote IgA production as well as the LAB cells. It is necessary to clarify the detailed mechanism of the IgA-enhancing effect of MVs derived from the strain.

Furthermore, the MVs of *L. sakei* NBRC15893 may be taken up by PP cells *in vivo*, resulting in enhancement of IgA production in the gut, as Al-Nedawi *et al.* [18] demonstrated that MVs were taken into the DCs of the PP after oral administration of the MVs derived from *L. rhamnosus* JB-1. With increasing recognition of the influence of gut microbiota on health and diseases of the host, the finding that LAB in the host microbiota produce MVs with immunomodulatory effects, including an IgA-enhancing effect, indicates an important role of MVs in mediating interactions between the host and bacteria. Moreover, MVs are frequently isolated from biofilms and have been related to the aggregation of bacteria and biofilm formation [29, 30]. When LAB are used as probiotics, they are expected to become established in the intestinal tract of the host. Since the aggregation of bacterial cells is an important step leading to the successful establishment [31], gaining a detailed understanding of the mechanism of MV production and its functions is very important to ensure a beneficial effect.

In summary, we found that the MVs of *L. sakei* NBRC15893 could activate the mucosal immune system

via enhanced IgA production. Moreover, antigen-specific secretion of IgA to the mucosal surface can be induced by the nasal immunization of MVs derived from pathogenic bacteria such as *Porphyromonas gingivalis*, indicating that MVs are effective agents for the control of the mucosal immune system [32]. Our findings highlight the potential of the application of MVs derived from probiotics with established safety based on food experience for overall health benefits via control of the mucosal immune system and their potential as a vaccine adjuvant via the mucosa.

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