

Full Paper

Anti-Influenza virus effects of *Enterococcus faecalis* KH2 and *Lactobacillus plantarum* SNK12 RNA

Takumi WATANABE^{1,*}, Kyoko HAYASHI¹, Tatsuhiko KAN², Makoto OHWAKI³ and Toshio KAWAHARA⁴

¹Graduate School of Engineering, Chubu University, 1200 Matsumoto, Kasugai, Aichi 487-8501, Japan

²Bio-Lab Co., Ltd., 2-1-3 Komagawa, Hidaka, Saitama 350-1249, Japan

³Non-Profit Organisation, The Japanese Association of Clinical Research on Supplements, 1-9-24 Shihogi, Hidaka, Saitama 350-1248, Japan

⁴College of Life and Health Sciences, Chubu University, 1200 Matsumoto, Kasugai, Aichi 487-8501, Japan

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Bacterial RNA has recently emerged as an immune-stimulating factor during viral infection. The immune response in an organism is directly related to the progression of virus infections. Lactic acid bacteria in particular have anticancer, bioprotective, and anti-allergic effects by modulating immunity. Here, we aimed to demonstrate the effect of bacterial RNA on *in vitro* production of IL-12, a proinflammatory cytokine, and on *in vivo* activity against influenza A virus (IFV) infection. Oral administration of heat-killed *Enterococcus faecalis* KH2 (KH2) or *Lactobacillus plantarum* SNK12 (SNK) in IFV-infected mice suppressed viral replication and stimulated production of virus-specific antibodies. However, ribonuclease-treated KH2 or SNK abrogated the effect, reducing IL-12 production *in vitro* and anti-IFV effects *in vivo*. Taken together, KH2 or SNK showed antiviral effects *in vivo* when administered orally, and the RNAs of KH2 and SNK play a part in these effects, despite the phylogenetic differences between the bacteria.

Key words: viral infection, influenza virus, lactic acid bacteria, *E. faecalis*, *L. plantarum*, bacterial RNA

INTRODUCTION

Lactic acid bacteria (LAB) intake has several major health benefits, such as improvement of fecal microbiota [1–3] and antibacterial [4, 5], anti-allergy [6, 7], antitumor [8, 9], and antiviral effects [10–12]. Bacterial cell walls [13] and extracellular polysaccharides produced by bacteria [14] have been reported as factors affecting the immune stimulation and biological defense provided by LAB, but no active component has yet been clearly elucidated. In this study, we aimed to identify immunomodulators of LAB and evaluate their effects on influenza virus infection in a mouse model. First, we focused on the RNA of LAB because *Staphylococcus aureus* DSM20231 23S rRNA has been reported to stimulate toll-like receptor (TLR) 13 and produce various cytokines [15]. Furthermore, a sequence containing 13 nucleotides near the active site of 23S rRNA ribozyme, which catalyzes peptide bond synthesis, was necessary and sufficient to trigger TLR13-dependent interleukin (IL)-1 β production [16]. It has also been reported that the RNAs of other bacteria, including *Enterococcus faecalis* EC-12, *Lactobacillus gasseri* JCM5344, *Bifidobacterium breve* JCM1192 [17], *Pediococcus acidilactici* strain K15, *Lactobacillus plantarum* ATCC14197T, *Lactobacillus*

pentosus ATCC8041T, and *Lactococcus lactis* subsp. *lactis* ATCC19435 [18], influence IL-12 production. That is, RNase A treatment of heat-killed bacteria significantly decreased the IL-12 production of human peripheral blood mononuclear cells. IL-12 production induced by bacterial RNA was reduced by a treatment with siRNA against TLR8, suggesting that the recognition of bacterial RNA was mediated by TLR8. In addition, IL-12 is a proinflammatory cytokine produced by dendritic cells, macrophages, and B cells [19, 20] that have immunomodulatory effects, such as antitumor and antiviral effects. These reports also suggest that bacterial RNA influences immune stimulation and that the health benefits of LAB via the immune system are affected by bacterial RNA. We selected the same bacterial species as reported in previous studies [17, 18] from the species we use in our studies and tested whether RNase treatment would affect IL-12 production in the species we have been studying. Although it is a classical method, we evaluated the production of IL-12 in bacteria using mouse splenocytes and examined the influence of RNase treatment [21, 22].

Influenza continues to be a serious infectious disease worldwide. On average, influenza viruses infect 5–15% of the global population annually, resulting in approximately 500,000

*Corresponding author. Takumi Watanabe (E-mail: t.watanabe@bio-ken.jp)

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deaths each year [23]. Influenza viruses belong to the family *Orthomyxoviridae* and are classified into four different types: A, B, C, and D [24, 25]. Among these viruses, influenza A viruses exhibit a broad host spectrum, including mammals and birds, and they can quickly mutate into highly pathogenic strains [26, 27]. The two principal clinical approaches for combating influenza are antiviral drugs and vaccines. Among the antivirals, neuraminidase inhibitors are commonly used for treatment of influenza. However, drug-resistant viruses have been reported clinically [28, 29]. Therefore, the importance of prophylaxis by vaccination has been increasing. However, vaccination has several limitations and problems that need to be resolved, such as egg adaptation [30], antigen mismatching [31], and insufficient antibody responses by intradermal or intramuscular injection. Therefore, we believe that the countermeasures for influenza should involve not only vaccines and drugs but also enhancement of host defense functions. LAB have been reported to have an anti-influenza effect [32], and we hoped that this study would reveal the active components of LAB in host defense against this virus. In the present study, we investigated two strains of heat-killed LAB, *E. faecalis* KH2 (KH2) and *L. plantarum* SNK12 (SNK), to confirm the effects of KH2 and SNK on viral load and antiviral antibody production in mice infected with influenza virus. By comparing the effects of ribonuclease-treated and untreated LAB, we also tested whether LAB RNA is necessary for the antiviral effects.

MATERIALS AND METHODS

Preparation of heat-killed bacteria and ribonuclease treatment

E. faecalis KH2 (International Patent Organism Depository in Japan number, NITE P-14444; GenBank Accession number, AB534553) and *L. plantarum* SNK12 (International Patent Organism Depository in Japan number, NITE P-1445; GenBank Accession number, AB715330) were stored at Bio-Lab Co., Ltd. All 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 bacterial suspension in distilled water (20–30 mg [wet bacteria weight]/mL) was heated at 105°C for 30 min using an autoclave (HV-25IILB, Hirayama Manufacturing Corp., Saitama, Japan).

Ribonuclease treatment was performed with RNase A (Invitrogen, Tokyo, Japan). RNase A was added to heat-killed KH2 and SNK suspended in distilled water at a final concentration of 10 µg/mL. After 120 min of incubation at 37°C, the ribonuclease-treated bacteria were washed with distilled water and resuspended in distilled water. The ribonuclease-treated KH2 and SNK were designated as R-KH2 and R-SNK, respectively.

To determine the RNA content of the bacteria, the solutions of the bacteria (n=3) with and without ribonuclease treatment were centrifuged at 10,000 × g for 3 min, and the bacteria pellets were collected. Next, 100 µL of distilled water was added, 100 mg of 0.1-mm zirconia beads (BioSpec Products, Bartlesville, OK, USA) were added, the bacteria were crushed in a Micro Smash™ MS-100 (TOMY, Tokyo, Japan). RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA), and the RNA concentration was measured using a NanoDrop One (Thermo Fisher Scientific, Waltham, MA, USA).

IL-12 production by mouse splenocytes

The bacterial suspension was added at a final concentration of 1 µg/mL (culture medium, RPMI1640, Wako, Osaka, Japan) to 6 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% CO₂ incubator at 37°C. After incubation for 24 hr, the culture supernatants of the mixtures were collected to measure the concentration of IL-12 by enzyme-linked immunosorbent assay (ELISA). The reagents used in the ELISA were the 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 standard (Recombinant Mouse IL-12 [p70] [ELISA Std.], BioLegend), and the IL-12 levels were measured by sandwich ELISA method [33].

Animal experiments

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 due to drug administration were detected throughout the experiments. Mice were intranasally infected with influenza A virus (A/NWS/33, H1N1 subtype) [34] at 2 × 10⁴ plaque-forming units (PFU)/50 µL per mouse (n=10) on day 0. KH2 and SNK (5 mg/mouse/day; contents of RNAs in 5 mg of KH2, R-KH2, SNK, and R-SNK: 543.7 ± 17.5 ng, 54.9 ± 5.8 ng, 397.8 ± 5.8 ng, and 40.7 ± 2.3 ng, respectively) with or without ribonuclease treatment were suspended in distilled water. Oseltamivir phosphate (OSL; 0.2 mg/mouse/day) was used as a positive control for antiviral effect and was also dissolved in distilled water. Each of KH2, R-KH2, SNK, R-SNK, and OSL was given by oral administration twice per day from 7 days before virus inoculation until 14 days after inoculation. Control mice were administered orally with vehicle (distilled water) alone. As IFV infection causes a reduction in body weight [35, 36], mice in each treatment group were weighed daily for 14 days 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 sec after the addition of 10 µL phosphate-buffered saline (PBS) per 1 mg of lung tissue and centrifuged at 10,000 × g for 30 min to separate the supernatants, which were stored at –80°C. BALFs were collected by four washes with 0.8 mL of ice-cold PBS via a tracheal cannula and centrifuged at 1,500 rpm for 10 min; 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 were quantified by plaque assays on Madin–Darby canine kidney (MDCK) cell monolayers. Sera and BALFs were subjected to neutralizing antibody titer assays using a 50% plaque reduction method, as

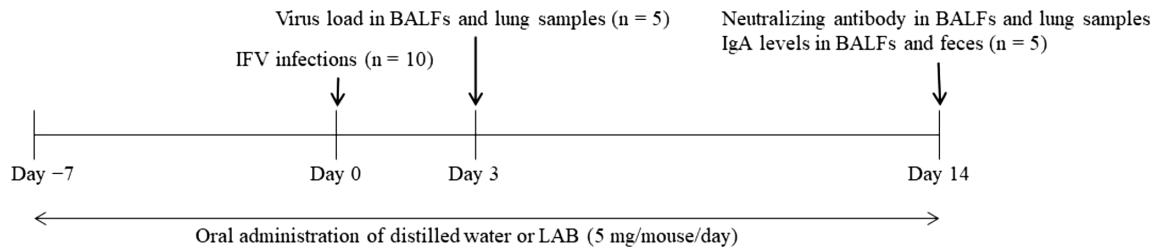


Fig. 1. Experimental procedure of influenza virus infection.

Mice in the control or LAB groups were administered distilled water or LAB (5 mg/day in two doses per day) during the study period (day -7 to 14). Mice were intranasally infected with IFV on day 0. On day 3 after IFV infection, five mice from each group were sacrificed to quantify virus loads in BALFs and lungs. The remaining five mice were sacrificed for measurement of neutralizing antibody and IgA levels on day 14. BALF: bronchoalveolar lavage fluid; IFV: influenza A virus; LAB: lactic acid bacteria.

described previously [37, 38], and BALFs and fecal extracts were assessed for mucosal IgA levels by ELISA.

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.

RESULTS

Comparison of RNA concentration with and without ribonuclease treatment

The RNA contents were 108.74 ± 3.50 ng/mg for KH2, 10.98 ± 1.16 ng/mg for R-KH2, 79.55 ± 1.16 ng/mg for SNK, and 8.13 ± 0.47 ng/mg for R-SNK. Ribonuclease treatment reduced the quantity of RNA to 1/10.

Effects of ribonuclease-treated KH2 and SNK on IL-12 production in mouse splenocytes

Ribonuclease treatment of both KH2 and SNK markedly reduced the levels of IL-12 produced by mouse splenocytes ($p < 0.01$) (Fig. 2). The reduction in IL-12 production was more pronounced in the SNK strain than in the KH2 strain.

Effects of ribonuclease-treated KH2 and SNK on IFV infection in mice

The effects of ribonuclease-treated (R) or untreated KH2 and SNK on the change in body weight of mice infected with IFV were examined (Fig. 3). The control group without KH2 or SNK showed approximately 16% loss of body weight on day 7 following IFV infection. The KH2, R-KH2, SNK, and R-SNK groups showed approximately 14%, 22%, 17%, and 21% losses, respectively, on day 7 post-infection. Although no significant difference was observed between the ribonuclease-treated and untreated groups, KH2 and SNK slightly suppressed weight loss more so than R-KH2 and R-SNK. Thereafter, mice of these groups gradually gained body weight, and the mice in the KH2 group returned to their pre-infection body weight levels on day 14 post-infection.

The virus yields in the lungs and BALFs of IFV-infected mice on day 3 post-infection are shown in Fig. 4A and Fig. 4B, respectively. Oral administration of the ribonuclease-treated or untreated forms of KH2 and SNK significantly reduced the virus

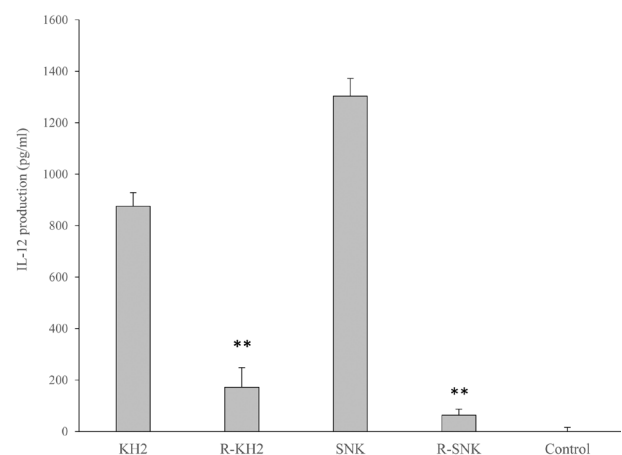


Fig. 2. Effect of ribonuclease treatment of LAB on IL-12 production in mouse splenocytes.

Heat-killed *E. faecalis* KH2 and *L. plantarum* SNK12 were treated with or without ribonuclease and co-cultured with mouse splenocytes for 24 hr. IL-12 protein concentration in the culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA). KH2: non-treated *E. faecalis* KH2; R-KH2: ribonuclease-treated *E. faecalis* KH2; LAB: lactic acid bacteria; SNK: non-treated *L. plantarum* SNK12; R-SNK: ribonuclease-treated *L. plantarum* SNK 12; control: culture medium only. Each value is presented as the mean \pm SD. $n = 6$. ** $p < 0.01$ vs. untreated for each LAB.

load in the lungs compared with the control group ($p < 0.05$), except for the lung samples of R-KH2, which showed no significant difference. A similar tendency was observed in BALF samples. Virus loads in the OSL group were markedly low, as shown in Fig. 4.

Figure 5 shows the effects of the ribonuclease-treated and untreated forms of KH2 and SNK on the neutralizing antibody response to IFV in BALFs (Fig. 5A) and sera (Fig. 5B) at day 14 post-infection. The antibody titers of BALFs and sera in the mice administered with untreated KH2 or SNK were significantly high as compared with those obtained in the control group ($p < 0.01$). By contrast, antibody titers in the ribonuclease-treated KH2 and SNK groups were almost equivalent to those of the control group, but those in BALF samples were decreased significantly by ribonuclease treatment ($p < 0.05$) (Fig. 5A). OSL group titers were significantly lower than those of the control group ($p < 0.01$)

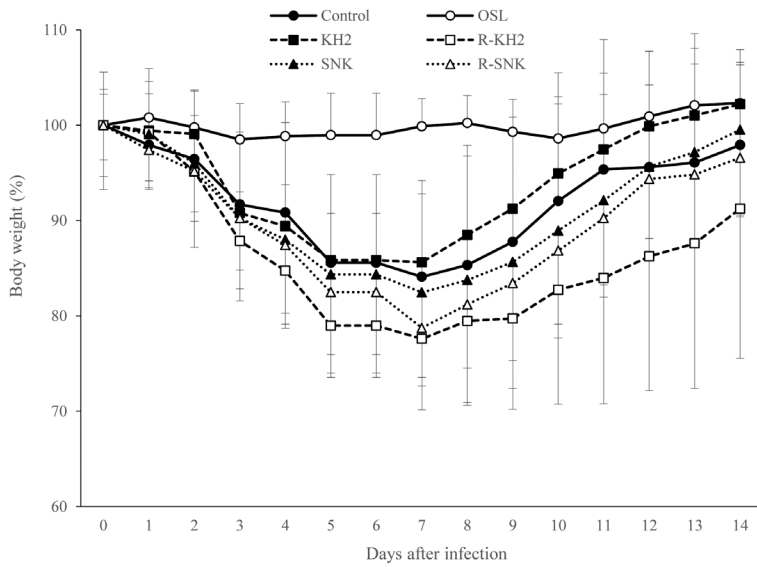


Fig. 3. Body weight changes of mice infected with the IFV. IFV-infected mice were orally administered distilled water (control, filled circle), 0.2 mg/day of oseltamivir (OSL, white circle), 5 mg/day of bacteria (KH2, untreated *E. faecalis* KH2, filled square; R-KH2, ribonuclease-treated *E. faecalis* KH2, white square; SNK, untreated *L. plantarum* SNK12, filled triangle; R-SNK, ribonuclease-treated *L. plantarum* SNK12, white triangle) from 7 days prior to virus infection to 14 days 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 \pm SD. n=5. IFV: influenza A virus.

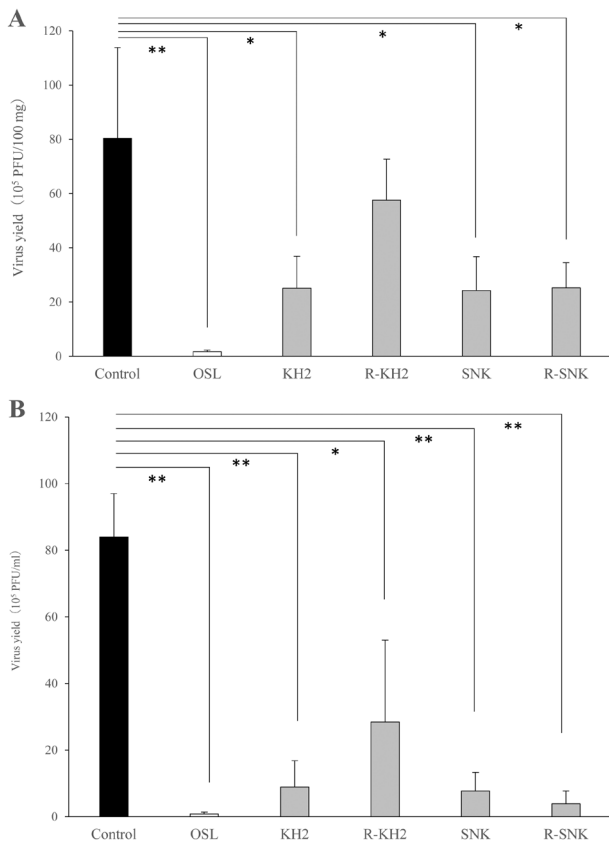


Fig. 4. Effect of LAB administration on virus load in the mice. Virus yield in BALFs (A) and lung samples (B) were measured by a plaque assay on day 3 post-infection. Each value is presented as the mean \pm SD. n=5. **p<0.01; *p<0.05. BALF: bronchoalveolar lavage fluid; KH2: non-treated *E. faecalis* KH2; R-KH2: ribonuclease-treated *E. faecalis* KH2; LAB: lactic acid bacteria; OSL: oseltamivir; PFU: plaque-forming units; SNK: non-treated *L. plantarum* SNK12; R-SNK: ribonuclease-treated *L. plantarum* SNK12.

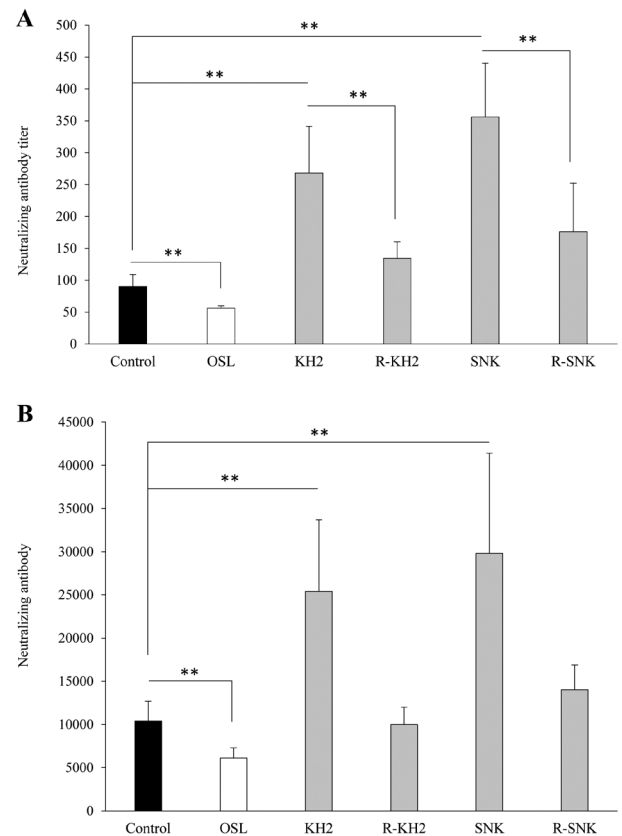


Fig. 5. Effect of LAB administration on the neutralizing antibody titer against IFV in the mice. The titer of the virus-neutralizing antibody is presented as the reciprocal of the dilution of BALFs (A) and sera (B) that reduced the plaque number to a level below 50% of that seen in the virus control. Each value is presented as the mean \pm SD. n=5. **p<0.01; *p<0.05. BALF: bronchoalveolar lavage fluid; IFV: influenza A virus; KH2: non-treated *E. faecalis* KH2; R-KH2: ribonuclease-treated *E. faecalis* KH2; LAB: lactic acid bacteria; OSL: oseltamivir; SNK: non-treated *L. plantarum* SNK12; R-SNK: ribonuclease-treated *L. plantarum* SNK12.

in both BALF and serum samples.

To elucidate whether ribonuclease treatment of KH2 and SNK stimulates the local immune response in mice, the levels of IFV-specific IgA in BALFs and feces were determined at day 14 post-infection (Fig. 6A and Fig. 6B). IgA production in the KH2 group was significantly increased ($p < 0.01$). The IgA levels of the ribonuclease-treated KH2 group were almost equivalent to those of the control group and significantly lower than those of the KH2 group in the BALFs ($p < 0.05$) and feces ($p < 0.01$). For the SNK and R-SNK groups, no significant difference in IgA levels was observed in the BALF samples, whereas R-SNK showed significantly reduced IgA production in the feces ($p < 0.05$). By contrast, marked suppression of IgA production was observed in the OSL group as compared with the control group ($p < 0.01$) in BALF samples.

DISCUSSION

To validate the immune-related active components of KH2 and SNK, IL-12 production by splenocytes and a mouse IFV infection model were used. The results showed that oral administration of KH2 or SNK produced an anti-IFV effect. In addition, the RNAs of KH2 and SNK were degraded by ribonuclease, which markedly reduced IL-12 production in splenocytes and had an impact on the anti-IFV effects. The RNAs of KH2 and SNK were suggested to be important factors for the anti-IFV effects based on the relationship between IL-12 production and anti-IFV effects. Although the cell wall has been reported to be an important factor in IL-12 production [13, 39], the present study found that RNA in bacteria is also a major factor affecting IL-12 production, though it should be noted that the validation method differed between this study and previous studies. The cell wall of *L. plantarum* and *Streptococcus mutans* strongly induces IL-12 production via TLR2 and TLR4 signaling in DCs and macrophages [13, 39], whereas KH2 and SNK may be signaling differently due to reduced IL-12 production by RNA degradation. Since bacteria have been reported to produce IL-12 via TLR3, 7, and 8 [17, 18], KH2 and SNK may also be signaling via TLR3, 7, and 8.

TLR3, which recognizes double-stranded RNA, and TLR7, which recognizes single-stranded RNA (TLR8 in humans), are also receptors that recognize viruses [40, 41]. This suggests that the anti-IFV effects of KH2 and SNK are influenced by RNA. The degradation of the RNAs of KH2 and SNK reduced the antiviral effect, suggesting that the RNAs of KH2 and SNK acted similarly to viral RNAs. Therefore, we would like to analyze the entire genome of KH2 or SNK to determine whether there are any sequences similar to those possessed by influenza viruses. In contrast, our observations that LAB reduced virus loads and increased antiviral antibodies were similar to those reported from previous studies on the anti-influenza virus effects of other LAB strains [42–46]. Because many reports on the anti-influenza effects of bacteria are conducted with probiotic strains and the heat-killed bacteria used in this study exerted similar effects to those of live bacteria, the viability of bacteria is not related to their immune-mediated antiviral effects. The mechanism of action is the uptake of bacteria from M cells in the intestinal tract and phagocytosis of them by DCs and macrophages, stimulating immunity [47]. Bacteria endocytosed by M cells have been reported to be transported to immunocompetent cells and to induce an immune response systemically or in the immune

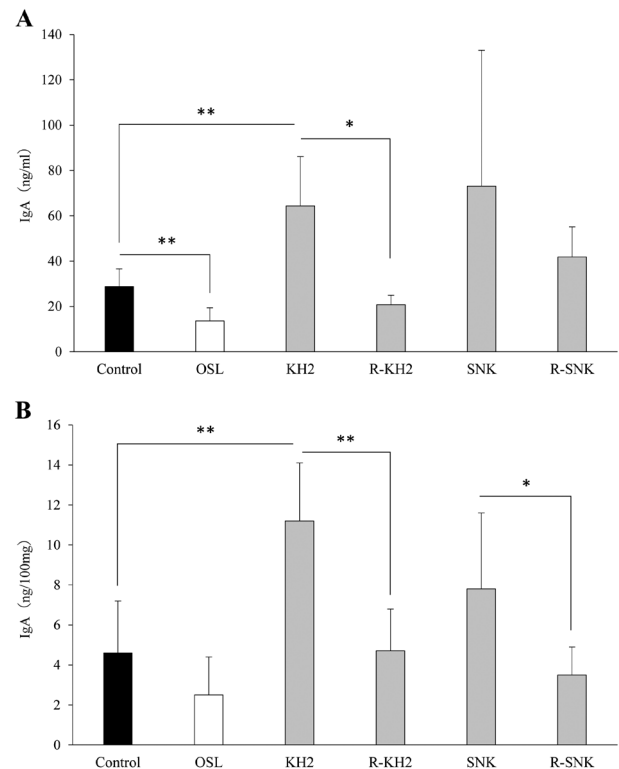


Fig. 6. Effect of LAB administration on the production of IFV-specific IgA in mice.

The IFV-specific IgA levels in BALFs (A) and feces (B) were determined by ELISA. Each value is presented as the mean \pm SD. $n = 5$. ** $p < 0.01$; * $p < 0.05$. BALF: bronchoalveolar lavage fluid; IFV: influenza A virus; KH2: non-treated *E. faecalis* KH2; R-KH2: ribonuclease-treated *E. faecalis* KH2; LAB: lactic acid bacteria; OSL: oseltamivir; SNK: non-treated *L. plantarum* SNK12; R-SNK: ribonuclease-treated *L. plantarum* SNK12.

system [47, 48]. Since we confirmed the uptake of KH2 from the intestinal Peyer's patch, we believe that this is a similar mechanism. However, it is unclear whether the mechanism is the same as that of other LAB, so we will use KH2 and SNK in future research to investigate their movement after transport from the Peyer's patch and to analyze the influence in each tissue. In addition, it was interesting that the serum and BALF neutralizing antibody titers, BALF and fecal IgA, were significantly lower in the ribonuclease-treated KH2 and SNK intake groups after 14 days of IFV infection (Figs. 5 and 6). The R-KH2 group showed a trend in viral load than the KH2 group, but there was no difference between the SNK and R-SNK groups after 3 days of IFV infection (Fig. 4). Ribonuclease treatment also had a slight influence on weight change, with the R-KH2 group losing weight compared with the KH2 group, although not significantly; the R-SNK group also showed a slight loss of weight compared with the SNK group, although not as much as the R-KH2 group (Fig. 3). This difference may be related to the immune response to the viral infection. During virus infection, inflammatory cytokines such as antiviral type I interferon are produced, and the innate immune system plays an important role in viral control [49]. The major receptors involved in the recognition of a virus during innate immunity are TLRs, retinoic acid-inducible gene-1 like receptor, and nucleotide-binding oligomerization domain-

containing protein 2 [50–52], and it is possible that the effects of the RNA of KH2 at the initial stages of infection (Fig. 4) affected the abovementioned receptors. In the future, we would like to use KH2 RNA to analyze the receptors involved in innate immunity. By contrast, SNK had little influence on the initial viral suppression effect on nuclease treatment (Fig. 4), as it has a different immune activation pathway from KH2. The titers of neutralizing antibody and IgA production at day 14 post-infection were significantly reduced by ribonuclease treatment (Figs. 5 and 6), suggesting that the RNAs of KH2 and SNK affect acquired rather than innate immunity.

In conclusion, herein we showed that both orally administered KH2 and SNK have potent profiles as influenza therapeutic agents involved in protection against IFV infection, inhibition of viral replication, and increased immune response. Furthermore, the RNAs of KH2 and SNK were shown to be active components and were suggested to affect the acquired immunity. We would also like to compare the quality and quantity of RNAs in the future by analyzing the RNAs of KH2 and SNK by RNA-seq. Based on the results obtained herein, we elucidated at least one mechanism of the protective effects of KH2 and SNK against virus infection. Future studies will search for more effective LAB species and explore not only the effect but also the mechanism through comparisons with live bacteria. Because the threat from viral infections still exists, the need for safer and more effective immunomodulators remains pressing. Among them, LAB are generally accepted as safe functional foods; we therefore hope that the widespread use of LAB species such as the KH2 and SNK used in this study will reduce the risk of viral infections.

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