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Intranasal administration of live *Lactobacillus* species facilitates protection against influenza virus infection in mice

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ABSTRACT

Influenza virus infections continue to be a significant public health problem. For improved therapies and preventive measures against influenza, there has been an increased tendency in modern medicine involving the use of probiotics. In this study, we compared the protective efficacy of various live and dead *Lactobacillus* species against challenge with influenza virus in mice according to the administration route and dose. In addition, to understand the underlying mechanism behind this clinical protective effect, we performed immunologic assays including examination of IgA levels and cytokine profiles in the lung. The survival rate of mice receiving intranasal administration of *Lactobacillus* was higher than after oral administration, and administration of live bacteria was more protective than of dead bacteria. The lung levels of interleukin (IL)-12 and IgA were significantly increased ($P < 0.05$). Conversely, the levels of the pro-inflammatory cytokines tumor necrosis factor-alpha and IL-6 were decreased. Interestingly, there were huge differences in protective effects of various *Lactobacillus* strains on influenza virus infection. Therefore, for clinical applications, selection of effective strains could be critical and individually optimized application regimens of the selected strains are required.

1. Introduction

Influenza virus infection is an acute respiratory infectious disease that causes significant morbidity and mortality in annual epidemics and pandemic outbreaks worldwide (Tang et al., 2010). In 2009, swine-origin H1N1 virus caused a pandemic with serious public health issues (Dawood et al., 2009). For prevention and treatment of influenza infection, during the initial phases of the 2009 H1N1 pandemic, the use of neuraminidase inhibitors was very effective when vaccines were not available (Boltz et al., 2010). However, seasonal and 2009 H1N1 pandemic influenza viruses resistant to these drugs have emerged and spread worldwide (Sheu et al., 2011; Webster et al., 2011). There is an urgent need for novel antiviral therapeutic approaches.

In previous studies, various probiotics showed significant antimicrobial effects via immunomodulatory activities (Botic et al., 2007; Dalloul et al., 2003; Harikrishnan et al., 2010; Son et al., 2009; Urdaci et al., 2004). Particularly, *Lactobaillus* spp. were found to be effective in the prevention or treatment of influenza virus infection (Harata et al., 2010; Hori et al., 2001, 2002; Izumo et al., 2010; Kobayashi et al., 2011; Maeda et al., 2009; Yasui et al., 2004). Although oral administration of *Lactobacillus* spp. have proven to be effective in preventing influenza virus infection in mice, there have been concerns about the passage of intact bacteria through the acidic conditions of the stomach, since this might deplete or even eliminate the successful routing of the bacteria to (Hori et al., 2002; Kobayashi et al., 2011; Maeda et al., 2009; Yasui et al., 2004). It has been suggested that intranasal administration of inactivated *Lactobacillus* spp. could be effective in protection against respiratory infection due to direct augment of the respiratory immune system (Harata et al., 2010; Hori et al., 2001; Izumo et al., 2010).

In the present study, we evaluated the intranasal administration of *Lactobacillus* spp. for reducing the numbers of bacteria required for induction of antiviral efficacy compared to oral administration. Further, we compared the protective efficacy of live (LLr) and dead (DLr) *Lactobacillus rhamnosus* against challenge with influenza virus in mice, and determined the minimum effective dose for clinical application. Moreover, to understand the underlying mechanism behind this clinical protective effect, we

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performed immunological assays including examination of immunoglobulin A (IgA) levels and cytokine profiles in the lung. In addition, we compared protective efficacy of various *Lactobacillus* strains against challenge with influenza virus in mice according to the administration route.

2. Materials and methods

2.1. Animals

Female specific pathogen-free (SPF) BALB/c mice (Orient Bio Laboratories, Seoul, Korea) weighing 18–20 g were used. All experiments were carried out in compliance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Konkuk University.

2.2. Bacteria

Lactobacillus spp. were cultured for 24 h at 37 °C in MRS broth (Difco Laboratories, Detroit, MI), harvested by centrifugation at 1400*g* at 4 °C for 10 min, and resuspended in 0.5% skim milk. For inactivation of bacteria, 3% formalin was added and centrifuged at 1400*g* at 4 °C for 10 min. The inactivated preparation was washed three times with saline and resuspended in 0.5% skim milk. Finally, the absence of live *Lactobacillus* was tested by culture in MRS agar.

2.3. Virus

Influenza A/NWS/33 (H1N1) virus was grown in allantoic sacs of 11-day-old chicken embryos at 37 °C for 2 days. The allantoic fluid was harvested and stored at −70 °C until used. The titer of virus in the allantoic fluid was determined as the 50% egg infective dose (EID_{50}). Briefly, serial 10-fold dilutions of the allantoic fluid were injected into embryonated eggs, and the presence of virus in the allantoic fluid of each egg was determined based on the hemagglutinating capacity of virus. In challenge studies, mice were challenged intranasally with 100 μl of $10^{4.0}$ EID_{50} influenza virus after anesthetizing mice by intraperitoneal injection of Avertin (375 mg/kg).

2.4. Experimental design

2.4.1. Protective effect of L. rhamnosus according to the administration route

Animals were assigned to three experimental groups (n = 10 per group); LLr, DLr, and positive control. The experimental procedure for the influenza virus infection model is summarized in Fig. 1. Volumes of LLr and DLr solutions containing 10^8 plaque forming units (pfu) were administered orally or intranasally to the treatment groups of mice for 21 days before viral challenge. Skim milk was also given in the same manner and for the same time to the control mice. After challenge with influenza virus at day 0 post-infection (p.i.), survival rate and clinical signs were observed daily for 14 days p.i.

2.4.2. Dose-dependent effect of intranasal administration of L. rhamnosus

Mice were treated with various concentrations (1×10^8, 1×10^7, 1×10^6 and 1×10^5 colony forming units (cfu) of LLr or DLr for 21 days. In addition, skim milk was also given in the same manner to control groups for 21 days before challenge. After challenge with influenza virus, survival rate and clinical signs were observed daily for 14 days p.i. For evaluation of safety, four mice in each group were sacrificed on day 0 p.i. and lung samples were collected for histopathological examination.

2.4.3. Effects of intranasal administration of L. rhamnosus on lung virus titer and immune response in lung

Mice were treated with 10^8 and 10^6 cfu per mouse of LLr or DLr for 21 days before challenge as described above. Four mice in each group were sacrificed on days 0, 2, 5 and 7 p.i. for IgA determinations and cytokine analyses. Furthermore, four mice in each group were also sacrificed on days 3 and 7 p.i. and their lungs were removed, weighed, and assigned a consolidation score ranging from 0 (normal) to 4 (maximal consolidation), depending on the percentage of the lung exhibiting typical plum coloration. Each lung was assayed for infectious virus titer.

2.4.4. Protective effect of various Lactobacillus species on influenza virus infection

Live strains of *Lactobacillus plantarum* (n = 4), 2 strains of *Lactobacillus fermentum*, 2 strains of *Lactobacillus brevis* and a strain of *L. rhamnosus* (10^8 pfu per mouse) were administrated orally or intranasally to mice (n = 10) for 21 days. Skim milk was also given in the same manner to control mice. After challenge with influenza virus, the survival rate and clinical signs were observed daily for 14 days p.i.

2.5. Determination of IgA concentration

The level of total IgA in lung homogenates was determined using a mouse IgA enzyme-linked immunosorbent assay (ELISA) kit (Bethyl Laboratories, Montgomery, TX) according to the manufacturer's instructions in duplicate against a standard curve.

2.6. Quantitation of cytokine mRNA

Total cellular RNA was extracted from lung tissue using a RNeasy mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instruction. The mRNA expression levels of gamma interferon (IFN-r), tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and IL-12 were determined by quantitative real-time RT-PCR. Primer and probes for murine IFN-γ, TNF-α, IL-6, IL-12 and

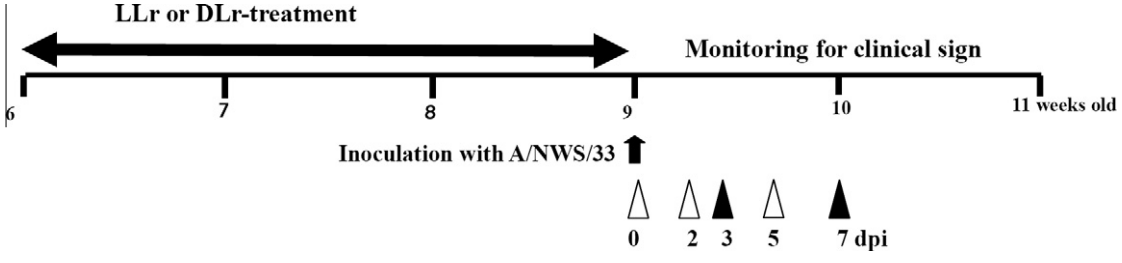

Fig. 1. Schedule for the animal experiments. BALB/c mice were treated intranasally with LLr and DLr for 3 weeks before inoculation with A/NWS/33 virus. Mice were monitored daily for clinical signs for 14 days. ▲, Determination of virus titers in lungs; △, preparation of lung homogenates and measurement of IgA and cytokine mRNA level.

β-actin (a housekeeping gene) were designed based on the sequences from identical conditions as previously reported (Giulietti et al., 2001). The assay was standardized using the One-step SYBR® PrimeScript™ RT-PCR kit II (TaKaRa Bio, Shiga, Japan) and master mix recipes were prepared according to the manufacturer's instructions. Each sample was tested and a cycle threshold (Ct) value was determined for each using the log-linear phase of each reaction.

2.7. Histopathological examination

For histopathological examination, all lungs collected from each group were fixed in 10% phosphate-buffered formalin. Lungs were excised and embedded in paraffin wax, and stained with hematoxylin and eosin (H&E) for microscopic examination.

2.8. Determination of lung virus titers

Each mouse lung was homogenized and centrifuged at 1400*g* for 20 min at 4 °C. The supernatants were 10-fold serially diluted with phosphate-buffered saline. The infectivity of virus in the supernatant was determined from the median cell culture infective dose ($CCID_{50}$) by using Madin–Darby canine kidney (MDCK) cells.

2.9. Statistical analyses

Statistical significance of lung parameters was determined by nonparametric ANOVA with Kruskal–Wallis test. For differences in IgA levels and cytokine profiles, we first determined overall means' equality using ANOVA and then compare values with control group using Dunnett's test. The protective effects of *Lactobacillus* spp. in mice infected with virus were evaluated using chi-square analysis and Fisher's exact test. A value of $P < 0.05$ was considered significant in these tests. Kinetics of mortality was analyzed by Kaplan–Meier curves and log-rank test with Bonferroni adjustment.

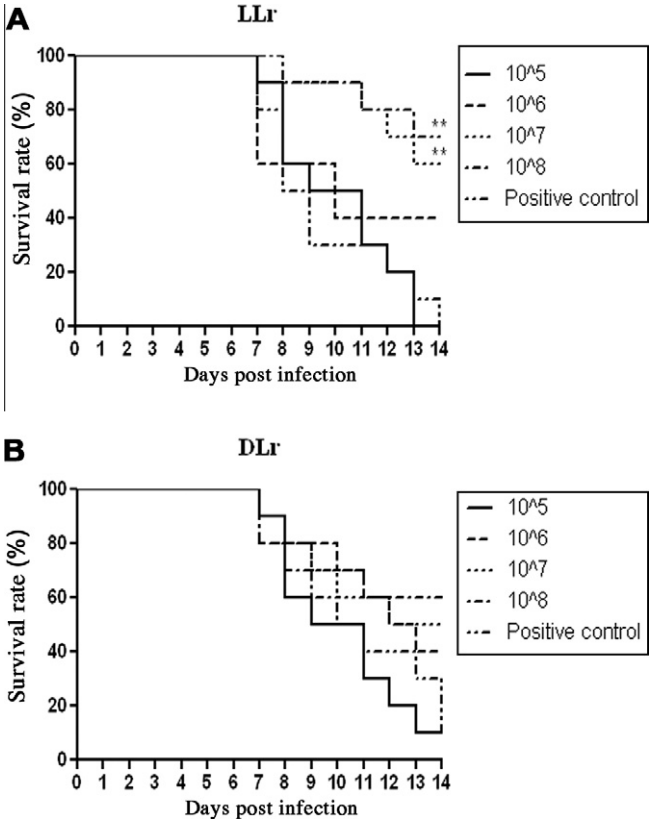

Fig. 2. The effect of intranasal administration of *L. rhamnosus* on survival rate of mice challenged with influenza virus. BALB/c mice were treated intranasally with LLr and DLr (10^5–10^8 cfu per mouse) for 3 weeks before inoculation with A/NWS/33 virus. Mice were monitored daily for clinical signs for 14 days. Treatments groups, $n = 10$; positive control group, $n = 10$. Statistical significance was determined by Fisher's exact test. Asterisks indicate significant differences ($^*P < 0.05$; $^{**}P < 0.01$) compared with results in positive control.

3. Results

3.1. Anti-influenza effect of LLr and DLr according to administration route

We compared oral and intranasal routes of administration to evaluate the protective efficacy of LLr and DLr. In a mouse challenge study, intranasal administration bestowed higher protective efficacy than the oral route (Table 1). LLr showed higher protective efficacy than DLr by both administration routes. The survival rates of mice orally administered LLr and DLr were 40% and 0%, respectively. However, the survival rates of mice intranasally administered with LLr and DLr were 70% and 40%, respectively (Table 1). None of the challenged mice in the control group survived.

3.2. Anti-influenza effect of LLr and DLr according to administration dose

We compared the protective efficacy of LLr and DLr according to the administration dose and investigated the safety profiles by histopathological examination to establish the minimum effective dose. In a mouse challenge study, administration of LLr and DLr reduced mortality in a dose-dependent manner. The survival rates of mice administered LLr at 10^8, 10^7, and 10^6 cfu/mouse were 70%,

Table 1
Protective effects of oral or intranasal administration of *L. rhamnosus* on survival rate of mice challenged with influenza virus.

Group	Dose (cfu/mice)	Administration route	Mortality		PI_{14}[d]
			Survival/total	MDT[c]	
LLr-treated[a]	$10^{8.0}$	Oral	4/10	8.2	1.6
DLr-reated[b]	$10^{8.0}$	Oral	0/10	8.5	2.0
LLr-treated[a]	$10^{8.0}$	Intranasal	7/10**	8.0	1.3
DLr-reated[b]	$10^{8.0}$	Intranasal	4/10	8.0	1.6
Positive control[c]	–	–	0/10	8.0	2.0

[a] Live *L. rhamnosus* solution ($10^{8.0}$ cfu/mouse) was administered orally or intranasally for 3 weeks before virus challenge ($10^{4.0}$ EID_{50}/0.1 ml).
[b] Dead *L. rhamnosus* solution ($10^{8.0}$ cfu/mouse) was administered orally or intranasally for 3 weeks before virus challenge ($10^{4.0}$ EID_{50}/0.1 ml).
[c] Mean death time.
[d] Pathogenicity index 14 (PI_{14}): the mean score per mouse per observation over a 14 day period when each day, mouse are scored 0 if normal, 1 if sick, 2 if dead.
** $P < 0.01$ compared to positive control group. Statistical significance was determined using Kaplan–Meier survival curve and log-rank test with Bonferroni adjustment.

Table 2
Effect of intranasal administration of *L. rhamnosus* on replication of influenza virus in lung.

Group	Dose (cfu)	Mean lung parameters					
		Day 3			Day 7		
		Lesion score[d] ± S.D.	Lung weight (mg ± S.D.)	Virus titer (log_{10}/g ± S.D.)	Lesion score[d] ± S.D.	Lung weight (mg ± S.D.)	Virus titer (log_{10}/g ± S.D.)
LLr-treated [a]	$10^{8.0}$	1.8 ± 0.3	200.0 ± 31.6	7.2 ± 0.1**	3.1 ± 0.3	385.0 ± 28.9	3.0 ± 0.1**
	$10^{6.0}$	2.1 ± 0.3	207.5 ± 15.0	7.6 ± 0.1	3.3 ± 0.3	410.0 ± 64.8	5.6 ± 0.1
DLr-treated [b]	$10^{8.0}$	2.3 ± 0.3	210.0 ± 18.3	7.6 ± 0.3	3.4 ± 0.5	367.5 ± 45.0	5.8 ± 0.2
	$10^{6.0}$	2.6 ± 0.5	192.5 ± 15.0	7.7 ± 0.1	3.6 ± 0.5	426.7 ± 15.3	5.8 ± 0.2
Positive control[c]	–	2.4 ± 0.3	202.5 ± 9.6	8.5 ± 0.1	4.0 ± 0.3	460.0 ± 73.5	6.5 ± 0.2

[a] Live *L. rhamnosus* solution ($10^{8.0}$ cfu/mouse) was administered intranasally by twice a week for 3 weeks before virus challenge ($10^{4.0}$ EID_{50}/0.1 ml).
[b] Dead *L. rhamnosus* solution ($10^{8.0}$ cfu/mouse) was administered intranasally by twice a week for 3 weeks before virus challenge ($10^{4.0}$ EID_{50}/0.1 ml).
[c] Positive control group was not treated after virus challenge.
[d] Lung was assigned a consolidation score ranging from 1 (normal) to 4 (maximal coloration).
** $P < 0.01$; compared to positive control group. Statistical significance was determined by nonparametric ANOVA with Kruskal–Wallis test.

60%, and 40%, respectively (Fig. 2A). The survival rate of mice treated with DLr at 10^8, 10^7, and 10^6 pfu/mouse was 60%, 50%, and 40%, respectively (Fig. 2B). On day 3 and 7 p.i., the viral titer in LLr-treated mice was significantly lower than that in the positive control group (Table 2). Histopathological examination revealed that, although intranasal administration with LLr or DLr (10^8 and 10^7 cfu per mouse, respectively) conferred significantly high protection against influenza in mice without gross lesions as described above, acute exposure to high concentration of LLr or DLr distinctly caused histological lesions of pneumonia. However, 10^6 cfu of LLr and DLr did not cause any histological lesions in the lung and still conferred 40% protection.

3.3. Effect of intranasal administration of LLr and DLr on IgA levels and cytokine profile

IgA concentrations in lung homogenates from mice treated with 10^8 cfu of LLr were significantly higher than that from the positive control group on days 0, 2, 5, and 7 p.i. (Fig. 3A). Furthermore, IgA concentrations in lung homogenates from mice treated with 10^8 cfu of DLr and 10^6 cfu of LLr were significantly higher than those in the positive control group on days 0 and 2 p.i. (Fig. 3A and B). TNF-α levels in lung homogenates from mice treated with 10^8 cfu of either LLr or DLr were significantly lower than those in the positive control group on day 2 p.i. (Fig. 4A). The levels of IL-6 in lung homogenates from these mice and from mice treated with 10^6 cfu of LLr or DLr 10^8 were significantly lower than those in the positive control group on day 2 p.i. (Fig. 4B). The IL-12 level in lung homogenates from the LLr 10^8 cfu group was significantly higher than that from the positive control group on day 7 p.i. (Fig. 4C). IFN-γ levels were not significantly different between the positive control and administration groups (data not shown).

3.4. Protective effect of the various live Lactobacillus species on influenza virus infection

The protective efficacy of various live *Lactobacillus* species against challenge with influenza virus in mice according to the administration route was compared. The survival rates of mice receiving nasal administration were higher than orally administered groups (Table 3). Interestingly, live *Lactobacillus* species showed a diverse protective efficacy (20–100%) with intranasal administration. Among the 9 strains of *Lactobacillus* spp., intranasal administration of *L. fermentum*-1 and *L. brevis*-2 conferred a significantly high protection rate (100% and 70%, respectively) against influenza virus.

4. Discussion

In previous studies, *Lactobacillus* spp. were administrated via the oral route (Hori et al., 2002; Kobayashi et al., 2011; Maeda et al., 2009; Yasui et al., 2004) and the intranasal route in mouse models in an effort to bestow protection against subsequent influenza virus infection (Harata et al., 2010; Hori et al., 2001; Izumo et al., 2010). Both routes produced marked antiviral activity against influenza virus and activated the host immune system, but a direct comparison of both routes using various strains of *Lactobacillus* has not hitherto been made. In the present study, we compared the oral and intranasal routes to specifically evaluate the antiviral effects and immunomodulating activities. The present results

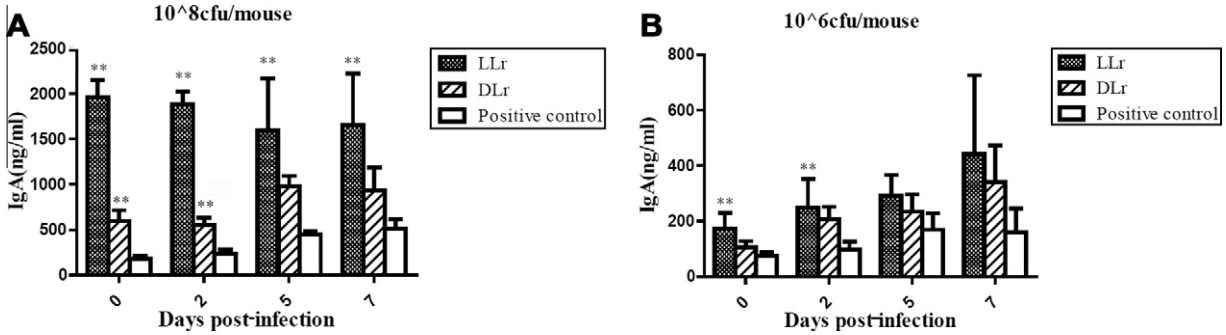

Fig. 3. Level of total IgA in lungs of LLr- or DLr-treated mice influenza virus challenge. BALB/c mice were treated intranasally with 10^8 cfu per mouse (A) and 10^6 cfu per mouse (B). Four mice per of all group each were also sacrificed on days 0, 2, 5, and 7 p.i. for examination of IgA levels by ELISA. Statistical significance was determined by ANOVA with Dunnett's test. Asterisks indicate significant differences (**$P < 0.05$) compared with results in positive control.

establish that these outcomes were more potent following intranasal administration. This might be correlated to the direct modulation of the immune system in the respiratory tract.

Most previous studies used heat-killed *Lactobacillus* spp. to protect mice from influenza virus challenge (Harata et al., 2010; Hori et al., 2001; Izumo et al., 2010; Kobayashi et al., 2011; Maeda et al., 2009; Yasui et al., 2004). To our knowledge, the present study is the first to use intranasal delivery of live *Lactobacillus* spp. Live *L. rhamnosus*, were more effective than dead bacteria. However, concerns over safety of administering live *Lactobacillus* spp. necessitated an investigation of the safety profiles of LLr and DLr. Interestingly, adverse reaction including aspiration pneumonia was caused by not only the intranasal administration of 10^8 cfu LLr, but also by the same dose of DLr. Further investigation of intranasal administration of serially-diluted LLr and DLr (10^5–10^8 cfu/mouse) did not reveal significant differences. The use of 10^6 cfu/mouse of either LLr or DLr struck a balance between safety and efficacy. In addition, bacteria taken up by an abnormal portal of entry could induce an abnormal immune response such as a type I hypersensitivity response.

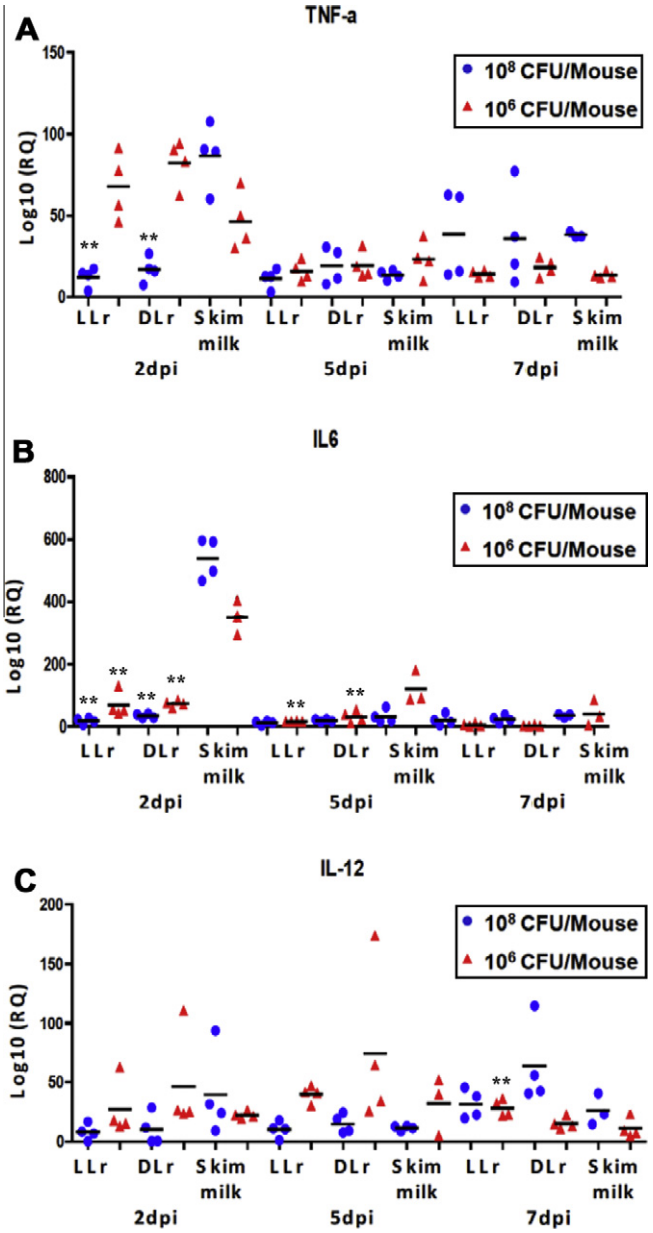

Fig. 4. Levels of TNF-α (A), IL-6 (B) and IL-12 (C) mRNA expression in lungs isolated from test mice. Mice were treated with 10^8 and 10^6 cfu per mouse of LLr or DLr for 21 days before challenge. Skim milk was also given in the same manner and for the same time to the control mice. The blue circle identifies the 10^8 cfu/mouse group and the red triangle identifies the 10^6 cfu/mouse. The blue circle of skim milk group identifies the control group of 10^8 cfu/mouse and the red triangle of skim milk group identifies the control group of 10^6 cfu/mouse. After challenge, mRNA expression was measured by quantitative RT-PCR using the total RNA extracted from lungs. Values are normalized to β-actin and are expressed as fold change compared to positive control. Relative quantities (RQ) of mRNAs were calculated by $2^{-\Delta\Delta Ct}$ method. Statistical significance was determined by ANOVA with Dunnett's test. Asterisks indicate significant differences ($^{**}P < 0.05$) compared with results for positive control.

Mucosal secretory IgA antibodies in the respiratory tract provide cross-protection against variant respiratory virus infections, which may confer higher antiviral effects than systemic IgG antibodies (Liew et al., 1984; Nishino et al., 2009). Particularly, mucosal secretory IgA antibody is essential and crucial for immune protection against influenza virus infection (Takahashi et al., 2010). Previous studies demonstrated that *Lactobacillus* spp. characteristically stimulate the production of IgA antibodies and prevent invasive infection of pathogens (Galdeano and Perdigón, 2006; Kobayashi et al., 2011; Kotani et al., 2010; Olivares et al., 2006; Tsai et al., 2010). In the present study, intranasal administration of LLr and DLr also increased mucosal IgA level in the lung. Elevated levels of IgA in the respiratory tract seem to play a role in the clearance of influenza virus.

Over-reaction of the immune response (i.e., cytokine storm) induces a hyperinflammatory process, and is involved in the pathogenicity of influenza virus (de Jong et al., 2006; Wang et al., 2010). Especially, the level of IL-6 and TNF-α was demonstrated to positively correlate with lung inflammation and vascular dysfunction (Wang et al., 2010). In this study, high levels of the pro-inflammatory cytokines IL-6 and TNF-α were also detected in lungs of influenza-infected mice. However, the IL-6 and TNF-α levels were significantly reduced in the LLr- and DLr-treated groups. The decreased level of pro-inflammatory cytokine on day 2 p.i. might positively correlate with survival of infected mice after day 6 p.i. via reduction of lung inflammation.

In addition, it was found that LLr administered intranasally significantly induced the production of IL-12 in lungs, which is known to stimulate cytotoxic T cells and natural killer cells, and to enhance the proliferation of Th1 cells (Harata et al., 2010). Although we could not demonstrate evidence of IFN-r elevation, increased

Table 3
Protective efficacy of various live *Lactobacillus* species against influenza A virus in mice.

Lactobacillus species[a]	Survival/total[b] (mean death time)	
	Oral route	Nasal route
L. plantarum-1	3/10 (8.9)	6/10** (9.5)
L. plantarum-2	0/10 (8.0)	5/10* (10.4)
L. plantarum-3	3/10 (9.4)	6/10** (9.8)
L. plantarum-4	1/10 (8.1)	2/10 (9.0)
L. fermentum-1	2/10 (8.0)	10/10*** (0.0)
L. fermentum-2	2/10 (8.4)	5/10* (10.2)
L. rhamnosus-1	0/10 (8.6)	5/10* (10.2)
L. brevis-1	1/10 (7.8)	6/10** (8.8)
L. brevis-2	0/10 (8.0)	7/10** (8.3)
Positive control [c]	0/10 (7.8)	0/10 (8.0)

*$P < 0.05$; **$P < 0.01$, ***$P < 0.001$ compared to positive control group. Statistical significance was determined by chi-square analysis.

[a] *Lactobacillus* species solution ($10^{8.0}$ cfu/mouse) was administered by once a day for 3 weeks.

[b] Mice were challenged by intranasal route with $10^{4.0}$ EID_{50}/0.1 ml of A/NWS/33 (H1N1) virus.

[c] Virus challenge without *Lactobacillus* administration.

IL-12 might enhance innate immune cells and play a role in early viral clearance (Fensterl and Sen, 2009).

In previous studies of the prevention and treatment of influenza virus, various *Lactobacillus* strains were used (Harata et al., 2010; Hori et al., 2001, 2002; Izumo et al., 2010; Kawase et al., 2010; Kobayashi et al., 2011; Maeda et al., 2009; Yasui et al., 2004). In the present study, we compared the protective efficacy of various live *Lactobacillus* spp. against challenge with influenza virus in mice according to the administration route. Interestingly, there were huge differences in the protective effects of various *Lactobacillus* strains of the same species on influenza virus infection. Therefore, for clinical applications, differences among strains could be critical for the selection of effective strains. Furthermore, after selection, an optimized application regimen for each selected strain would be required.

In conclusion, intranasal administration of live *Lactobacillus* provided higher protection against influenza virus infection by enhancement of secretory IgA production and down-regulation of pro-inflammatory cytokines in the respiratory immune system. Further studies should consider effective strain selection in certain species because each *Lactobacillus* strains could provide a protective efficacy in a strain-dependent manner.

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