Anti-influenza virus effects of both live and non-live Lactobacillus acidophilus L-92 accompanied by the activation of innate immunity

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Abstract

The antiviral effects of both a live and non-live Lactobacillus acidophilus strain L-92 (L-92) were investigated by oral administration (10 mg/mouse per d) daily for 21 d in a mouse model infected intranasally with influenza virus (H1N1). Virus titres in the lung of mice administered either live or non-live L-92 cells daily for 15 d were repressed 6 d after virus infection compared with the control group. Natural killer (NK) activity in the orally administered non-live L-92 group was higher compared with that of the control group before virus infection and on day 6. In contrast, NK activity in the live L-92 group compared with the control group was not significantly changed on both days, but was significantly higher on day 1. In contrast, live L-92 showed a greater repression of virus proliferation compared with non-live L-92, 6 d after the infection. Live L-92 decreased the number of neutrophils in the lung and suppressed lung weight, leading to the consequent deterioration of consolidation scores of the lung. These results indicated that pretreatment of live or non-live L-92 cells had protective effects against influenza virus infection. Among the measured cytokines and chemokines, eotaxin, macrophage colony-stimulating factor, IL-1β, RANTES (regulated on activation, normal T cell expressed and secreted) and interferon-α were significantly increased in the lung; IL-17 was significantly increased in Peyer’s patch of the live L-92 group compared with the control group. A mechanistic study suggested that the enhancement of NK activity in the lung caused by stimulating various antiviral cytokines and chemokines after the oral administration of L-92 cells might be important in protecting against virus infection.

Key words: Lactobacillus acidophilus L-92: Innate immunity: Antiviral effects: Natural killer activity: Cytokine profiles

Many probiotic lactic acid bacteria and Bifidobacterium with health benefits to humans, such as improvement of the intestinal environment and allergy symptoms, preventive effects on cancer and lowering of serum cholesterol levels, have been reviewed¹⁻³. Recent studies have revealed that intranasal administration of Lactobacillus species might be effective in protecting against virus infection and decreasing the relevant inflammation⁴⁻⁵. Activation of host innate immunity and/or adaptive immunity has been suggested to be important for the protective effect against virus infection⁴⁻⁵. An orally administered Lactobacillus acidophilus strain L-92 (L-92) has an anti-allergy effect in a mouse model: it has been shown to modulate a T helper (Th) 2-skewed Th1/Th2 immunobalance by increasing the production of IL-12, known as a Th1-type cytokine, and reducing the production of IL-4, known as a Th2-type cytokine, in a mouse model⁶⁰. The anti-allergy effects of the oral administration of L-92 have been proven in several previous human trials, including pollen⁵⁻⁷ and perennial allergies⁵⁻⁸, and atopic dermatitis⁵⁻⁹. However, immunostimulating effects, such as the antiviral and anti-pathogenic bacterial effects of L-92, have not yet been elucidated.

The recent outbreak of pandemic H1N1 influenza has been a big social problem. Influenza virus infection to the host respiratory tract mucosa leads to high mortality, and there have been many influenza pandemics, such as the 1918
influenza pandemic\(^{10–15}\). Excessive production of inflammatory cytokines in the lung (cytokine storm) has often been observed in influenza infection, and this causes a severe clinical condition due to an inflammation in the lung\(^{16,17}\). At present, the use of drugs is the best approach for treatment against virus infection, but there is a risk of the emergence of drug-resistant bacteria due to repeated drug use\(^{18}\) or that a drug may not be sufficiently effective if they are not taken within about 48 h of infection\(^{19}\). Humans have encountered many pandemics caused by new types of influenza virus and tens of thousands of people have died, even though there are many antiviral drugs\(^{15,14,20}\). We are investigating alternative treatments against virus infection. Many challenging studies have been reported in mice using various probiotic strains to investigate the protective effects against influenza virus infections\(^{21–23}\). However, these probiotic effects are thought to be species and strain dependent\(^5\). The anti-allergy effect of L-92 is thought to be linked to certain characteristic features in the cell-wall components\(^{24,25}\). In the present study, we report for the first time the potency of the probiotic L-92 against influenza virus infections. Changes in various cytokines and chemokines were analysed to understand specific L-92 defence mechanisms against virus infection using a mouse model. In addition, the antiviral effects of both live and non-live L-92 cells were compared.

**Materials and methods**

**Mice**

Female BALB/c mice (4 weeks old) were purchased from Japan SLC and allowed to acclimatise for 1 week. Mice were housed in an air-conditioned animal room at 21–27°C and 40–80% humidity under a 12 h light–12 h dark cycle. The experiments were performed in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006). All experimental procedures were approved by the Calpis Animal Ethical Committee.

**Preparation of a live and non-live Lactobacillus acidophilus strain L-92**

L-92 isolated from a healthy Japanese volunteer in our stock culture collection was cultured in a medium consisting of yeast extract (Organotechnie) and cascin hydrolysate for 21 h at 37°C. The cell culture was centrifuged at 7000 rpm for 15 min and the pellet cells were washed with sterile PBS. Then, the cells washed with PBS were resuspended in PBS at a concentration of 100 mg wet cells/ml (approximately 4 x 10^10 cells/ml: colony-forming units/ml). The number of viable cells was monitored by counting the colonies on the de Man, Rogosa and Sharpe agar plate after the fermentation of the diluted suspension cells for 72 h at 37°C. For the preparation of non-live L-92 cells, the washed cells, prepared by the aforementioned method, were heat-killed until the temperature reached 85°C, then completed freeze-dry treatment and resuspended in PBS at a concentration of 33 mg dried cells/ml.

**Experimental design**

Mice were fed FR-2 (Funabashi Farms) and water was available at all times during the experimental period. As shown in Fig. 1, two sets of experiments were carried out: non-live L-92 was used in Expt I and live L-92 was used in Expt II. In both experiments, L-92 suspension, as prepared above, was administered (Expt I: doses of 300μl of non-live L-92 suspension/d, corresponding to 10 mg dry cells/mouse per d for both experiments; Expt II: doses of 300μl of live L-92 suspension/d) to mice by an oral zonde daily for 21 d. Mice in the control groups received doses of 300μl of saline instead of L-92 suspension. The 1st day of sample administration (L-92 or saline) was defined as day −15, and mice were infected with the influenza virus on day 0. In Expt I, fifty mice were divided into two groups: group I in which NK assays were performed on day 0 before the virus infection; group III in which virus titre,NK assays and Ig assays were performed on day 6, as illustrated in Fig. 1. In Expt II, fifty-four mice were divided into three groups: group I in which NK assays were performed on day 0 before the virus infection; group II in which virus titre and NK assays were performed on day 1; group III in which virus titre, NK assays, cytokine/chemokine assays, Ig assays and macroscopic inspections were performed on day 6. On day 0, mice in groups II and III were inoculated with the influenza virus. Virus titre analysis of part of the left lung lobe and NK activity measurements from part of the right lung lobe were performed on day 6 in Expt I, and on days 1 and 6 in Expt II (Fig. 1). The remaining left and right lobes were mixed and used for Ig assays on day 6 in Expt I and Expt II, and also cytokine/chemokine analysis on day 6 in Expt II.

**Influenza virus and infection**

Influenza A/PR/8/34 (H1N1) was prepared by Japan Biological Science, Inc., as described previously\(^{21}\). Mice were inoculated intranasally in the nasal cavity with a 50μl drop of influenza virus at 5 x 10^5 plaque-forming units (PFU)/mouse using a micropipette (Eppendorf Company, Limited).

**General symptom score**

General symptom scores were calculated by averaging four different health condition scores: eyelid, fur appearance, behaviour and others such as breath and body temperature, as listed in Table 1. Mice were visually inspected for these health conditions, every morning for 21 d, before sample administration, according to the standard operating procedures of Japan Biological Science, Inc. Points were given for each health condition, ranging from 1 (good) to 5 (bad).

**Virus titre**

Virus titres in the lung were counted according to the method described previously\(^{21}\). In short, on day 1 or 6, a sliced left lung was homogenised with protease inhibitor cocktail (Sigma-Aldrich), and inoculated into MDCK (Madin–Darby...
canine kidney) cells (10^6 cells/ml). Agar medium (1.5 ml) was
then added and MDCK cells were incubated for 2 d at 37°C.
The number of virus plaques was then counted as PFU.

Lung weight and macroscopic evaluation
Mice were killed and the thorax opened. Lungs were weighed
and scored macroscopically, ranging from 1 (no consolidation)
to 5 (consolidation through the left or right pulmonary
lobe). Scores were averaged if two scores were applied. The
consolidation score was calculated by adding the left and
right lobe scores, ranging from 2 to 10.

Number of neutrophils in lung slides
The left lung was stained using the Giemsa method to count
the number of neutrophils. Thereafter, five sites were counted
for each lung and the number of cells at each site was
averaged.

Natural killer activity in the lung
NK activity in the lung was evaluated according to a method
described previously. In short, NK activity was determined
using a 51Cr release assay that employed 51Cr-labelled YAC-1
cells as target cells. On day 0 before the virus infection, days

Table 1. General symptom scores for the evaluation of health condition of mice

<table>
<thead>
<tr>
<th>Score</th>
<th>Eyelid</th>
<th>Fur appearance</th>
<th>Behaviour</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Fatal</td>
<td>Fatal</td>
<td>Fatal</td>
<td>Fatal/Respiratory failure/skinny/obvious</td>
</tr>
<tr>
<td>4</td>
<td>Blepharosynechia</td>
<td>Dull fur</td>
<td>No reaction when touched</td>
<td>Decrease of body temperature</td>
</tr>
<tr>
<td>3</td>
<td>Loss of eyelid reflex</td>
<td>Piloerection/lose lustre</td>
<td>Loss of reaction when touched</td>
<td>Obvious respiratory irregularity/skinny</td>
</tr>
<tr>
<td>2</td>
<td>Eyelid closure</td>
<td>Slight piloerection</td>
<td>React when touched</td>
<td>Increased or decreased breathing rate</td>
</tr>
<tr>
<td>1</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>
1 and 6, right lung cells were incubated with radiolabelled \( ^{31}\text{Cr} \) YAC-1 cells. A total of 2 \( \times 10^5 \) lung cells were incubated with 1 \( \times 10^4 \) target cells at an effector:target ratio of 20:1. NK activity was calculated by measuring the radioactivity released from YAC-1 cells according to the following formula:

\[
\text{Cytotoxicity} \; (\%) = 100 \times \frac{\text{experimental count} - \text{spontaneous count}}{\text{total count} - \text{spontaneous count}}
\]

Cytokines and chemokines in the lung and Peyer’s patch

The following thirty-four different cytokines and chemokines in the lung and Peyer’s patch (PP) extracts prepared from mice on day 6 in Expt I were measured using Multiplex based on an immunological method, according to the manufacturer’s instructions (Millipore or Affymetrix): eotaxin; granulocyte colony-stimulating factor; granulocyte macrophage colony-stimulating factor; interferon (IFN)-α; IFN-β; IFN-γ; IL-10; IL-12 (p40); IL-12 (p70); IL-13; IL-17; IL-1α; IL-1β; IL-2; IL-3; IL-4; IL-5; IL-6; IL-7; IL-9; inducible protein-10; keratinocyte-derived chemokine (KC); leukaemia inhibitory factor; lipopolysaccharide-induced CXC chemokine (LIX); macrophage colony-stimulating factor (M-CSF); monocyte chemotactic protein-1; monokine induced by IFN-γ; macrophage inflammatory protein (MIP)-1α; MIP-1β; MIP-2; RANTES (regulated on activation, normal T cell expressed and secreted); TNF-α; vascular endothelial growth factor.

Virus-specific IgA and IgG

MDCK cells seeded on a ninety-six-well plate were infected by PR8 influenza and cultured for 2 d at 37°C, according to the method described by Liu et al.\(^{26}\) with some modification. Then, the influenza-infected cells were immobilised with methanol. The plate prepared above was washed twice with PBS before 100 μl of lung extracts from mice were added to each well and incubated for 30 min at room temperature. Then, a secondary antibody (goat anti-mouse IgA or goat anti-mouse IgG (both peroxidase labelled); Bethyl Laboratories) was added to each well of the plate after being washed with PBS. After the incubation of the plate for 3 h at room temperature, each 100 μl of PBS containing 1% (w/v) o-phenylenediamine dihydrochloride, 4·5% (w/v) and 3% (v/v) H₂O₂ was added to each well of the washed plate, and incubated for 30 min at room temperature. After the addition of 100 μl of 10% (v/v) H₂SO₄ to each well to stop the reaction, optical density at 492 nm was measured to evaluate optical density. The total amount of Ig (IgA and IgG) was quantified, according to the method described previously.\(^{26}\)

Statistical analyses

Data are presented as means with their standard errors. Statistical analyses were calculated using Student’s \( t \) test, two-way ANOVA or the Mann–Whitney test. Differences were considered significant at \( P < 0·05 \) or less.

Results

The antiviral effects of live and non-live L-92 were investigated in two sets of mouse model studies (Expt I and II), as illustrated in Fig. 1. There was no significant difference in virus titres on day 6 after the infection between the control groups in Expt I (38 688 (SE 3877) PFU) and in Expt II (19 330 (SE 10 250) PFU). Throughout the test period, there was no significant difference in body weight or severe fatal damage (data not shown). General symptom scores were high in the control groups of both experiments throughout the test period, while the non-live group tended to have lower symptom scores compared with the control group (\( P < 0·1 \)), but not the live group (\( P = 0·14 \)) (Fig. 2). In Expt I using non-live L-92 cells, virus titres in the lung were significantly (\( P < 0·05 \)) lowered in the L-92 group (1 85 \( \times 10^4 \) (SE 0 44 \( \times 10^3 \) PFU, \( n \) 15) compared with the control group (3 87 \( \times 10^4 \) (SE 0 39 \( \times 10^3 \) PFU, \( n \) 15) on day 6 (Fig. 3(a)). NK activity in the lung was significantly (\( P < 0·001 \)) higher in the non-live L-92 group (4 70 (SE 0·55) %, \( n \) 15) compared with the control group (0 90 (SE 0·16) %, \( n \) 15) on day 6 (Fig. 3(b)). Then, in Expt II using live L-92 cells (viability over 70%), virus titre and NK activity in the lung were monitored on day 1 to understand the quick host response after the virus infection. As a result, virus titre of the control group on day 1 (300 (SE 300) PFU, \( n \) 6) increased dramatically on day 6 in the control group (1 9 \( \times 10^4 \) (SE 1 0 \( \times 10^3 \) PFU, \( n \) 6), but not in the L-92 group (Fig. 4(a)). NK activity in the lung on day 1

\[\text{Symptom score} = \frac{n}{m} \times 2\]

Fig. 2. General symptom scores of mice administered (a) live (●) or (b) non-live (●) \textit{Lactobacillus acidophilus} strain L-92 (L-92) cells and the saline-administered control (●) group from days 0 to 6. L-92 or saline was administered daily for the whole study period (days 1 to 6). Values are means (a) \( n \) 11 and (b) \( n \) 15, with their standard errors represented by vertical bars. * Mean values between groups were marginally significantly different (\( P < 0·1 \); two-way ANOVA).
Thus, live L-92 cells were selected and used in the subsequent study for a more detailed understanding of the host response after the infection of the influenza virus. After the infection of influenza virus, inflammatory host damage in the lung is usually observed as the main host event, so the consolidation score of the lung for the live L-92-treated group was compared with that for the saline-treated control group. The live L-92 group showed a significant decrease in consolidation scores (3.00 (SE 0.35), n 5, P < 0.001) compared with the control group (6.00 (SE 0.55), n 5) (Fig. 6(a)). In addition, the number of neutrophils in the lung was significantly (P < 0.05) lowered in the L-92 group (46.7 (SE 8.0), n 5) compared with the control group (80.3 (SE 11.1), n 5) (Fig. 6(b)). Lung weight is known to be increased by inflammation; here it was significantly (P < 0.05) lowered in the live L-92 group (171.3 (SE 13.7) mg, n 5) compared with the control group (232.7 (SE 26.4) mg, n 5).

To understand the host response, the antiviral effect mechanism and anti-inflammatory events most probably linked to the virus infection: the release of various cytokines and chemokines in the lung and PP was measured in the live L-92 group on day 6. The thirty-four different cytokines and chemokines were measured, which showed that eotaxin, M-CSF, IL-1β, RANTES and IFN-α were significantly increased in the lung of the live L-92 group compared with the control group (Fig. 7(a)). Furthermore, there were significant increases in the production of IL-17, while IFN-α tended to be increased and IL-4 and IL-6 tended to be decreased in PP, 6 d after the virus infection (Fig. 7(b)). As expected from the protective anti-influenza virus effect shown in Figs. 2–4, notable increases in IFN-α, which is known to be activated in response to virus infection, were observed in the lung and PP (Fig. 7(a) and (b)). In addition, IFN-β was increased in the lung of mice from the L-92 group (310 (SE 280) pg/ml, P = 0.33), although this was not significant when compared with the control group (174 (SE 5.5) pg/ml). IL-6 was decreased in the lung in the L-92 group (4.4 (SE 2.3) ng/ml, P = 0.31), although this was not significant when compared with the control group (8.9 (SE 3.5) ng/ml).

IgA and IgG were measured in the lung on day 6 to understand the effect of live and non-live L-92 on adaptive immunity. A small, but significant (P < 0.05) decrease in the amount of IgG was observed in the live L-92 group (0.12 (SE 0.04), n 6) when compared with the control group (0.14 (SE 0.005), n 6), but not in the non-live L-92 group (0.15 (SE 0.005), n 15) when compared with the control group (0.14 (SE 0.005), n 15). There was no significant change in IgA in the live and non-live L-92 groups compared with the control group (data not shown).

Discussion

There have been many reports on the protective effects of the probiotic Lactobacillus and Bifidobacterium strains against influenza virus infection in mice5,21,25,27. These effects seem to be species and strain dependent. Youn et al.5 reported strain-specific clinical efficacy and differences in the effects among strains used in their clinical study. So far,
there has been only one report of an antiviral effect of *L. acidophilus* in a human trial(28), and no detailed mechanism studies or discussions about the mode of action have been published. This is the first report of the protective effects of a *L. acidophilus* strain against influenza virus infection in a mouse model and the first mechanism study considering the effect. This is also the first comparative study using live and non-live *L. acidophilus* cells analysing the antiviral effects.

L-92 is known for the immunomodulatory effect in some allergy symptoms, such as pollen(7) and perennial allergies(8), and atopic dermatitis (9) in humans. The present study also reveals a potential immunomodulatory effect of L-92, not only for anti-allergy effects, but also for an immunostimulation effect, such as protective effects against pathogenic bacteria and virus infection *in vivo*.

In the present study, both live and non-live L-92 cells showed antiviral effects in separate mouse model experiments. However, host responses after the oral administration of live and non-live *L. acidophilus* cells differed. NK activity before the virus infection was higher in the non-live group compared with the live group; however, virus titres in the lung 6 d after the infection were more repressed in the live group (more than 99 %) than in the non-live group (55 %) when compared with the control groups. Furthermore, NK activity in the non-live group was stimulated more than 5-fold 6 d after the virus infection when compared with the control group, but not in the live group. These results suggest that elimination of the virus from the lung, based on NK activity-dependent innate immunity, was higher in the non-live group than in the live group. However, elimination of the virus from the lung in the live group was higher than that in the non-live group. One of the possible explanations would be quick and temporary activation of NK activity by the virus infection on day 1 (Fig. 4(b)) that reaches to following thorough elimination of the virus in the live L-92 group observed on day 6 (Fig. 4(a)), and disappeared NK activation on day 6 (Fig. 4(b)). In contrast, the increase of NK activity in the non-live L-92 group appearing on day 6 may be due to a delayed host response compared with the live group, which may affect on the following elimination of the virus in the lung after day 6 (not tested). Higher preventive effects against influenza virus infection in a live bacteria-administered group compared with a heat-killed bacterial group have been reported in a previous study(5). With respect to the general symptom score, there seemed to be a greater improvement in the non-live group (*P* < 0.01 v. control group, *n* 15) than in the live group (*P* = 0.14 v. control group, *n* 11) throughout the test period (Fig. 2). The possible reason for this difference must be...
because of the smaller number of mice used in the experiments for the live group. The statistical calculation suggested that the general symptom score in the live group would be significantly lower than that of the control group \((P < 0.05)\) if the number of mice was increased to 15. However, a detailed comparative study on the mechanism of host responses against virus infection and immune response including NK activity and some cytokines between live and non-live, and also different administration routes, should be addressed in the future.

In the present study, among the measured cytokines and chemokines, eotaxin, M-CSF, IL-1\(\beta\), RANTES and IFN-\(\alpha\) were significantly increased in the lung of the live L-92 group compared with those in the control group. In a previous study, influenza infection of C57BL/6j mice induced chemokine gene expression for monocyte chemotactic proteins, monocyte chemotactic protein-1, MIP-1\(\alpha\), MIP-1\(\beta\), RANTES and IFN-inducible protein 10\(^{(29)}\). RANTES was considered to be important for virus infection because knockout \((-/-)\) mice did not demonstrate increased susceptibility to influenza infection\(^{(29)}\). M-CSF enhancement has been suggested to be important for early host resistance to influenza virus\(^{(30)}\). A potent eosinophil chemoattractant, eotaxin, well known to be induced after influenza virus infection in nasal epithelial cells\(^{(31)}\), was significantly induced in the present study. At 6 d after the virus infection, there was a significant increase in the production of IL-17, IFN-\(\alpha\) tended to be increased and IL-4 and IL-6 were decreased in PP. Influenza virus infection was accompanied by IL-1\(\beta\) and IFN-\(\alpha\) production in the bronchoalveolar lavage of mice\(^{(32)}\). The production of antiviral cytokines, such as IFN-\(\alpha\) and -\(\beta\) which have been reported to induce NK activity\(^{(33–35)}\), was measured 6 d after the virus infection in the present experiment. The antiviral cytokine IFN-\(\alpha\) was significantly increased in both lung and PP compared with the control group. IFN-\(\beta\) in the L-92 group was present at a higher value than that in the control group \((P = 0.33)\). In addition, other cytokines (IL-2 and IL-15), which are also known to induce NK activity, were not significantly different, but their average values were both higher in the L-92 group compared with the control group. The enhancement of NK activity in the lung may be caused by stimulating various antiviral cytokines after the oral administration of L-92 cells. An evaluation of various cytokines and chemokines before the virus infection would be of interest in considering the host response against probiotic challenge with or without virus infection.

Mohamadzadeh et al.\(^{(36)}\) reported that surface components of a Lactobacillus strain were the most important factor in the intake of active cells via M cells in the gastrointestinal tract and the activation of dendritic cells to induce a host immunomodulation effect. Perdigón et al.\(^{(37)}\) also reported that several kinds of surface components in lactic acid...
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bacteria, such as surface layer protein (Slp), lipoteichoic acid and peptidoglycan, are involved in the interaction with host cells. In addition, the importance of SlpA in *L. acidophilus* NCFC in association of the cell with immature dendritic cells, which is essential for immunomodulatory action, has been suggested (38). A recent study has reported on the potential of *L. acidophilus* ATCC 4356 to inhibit Junin virus entry using Slp on *L. acidophilus* to bind to dendritic cell-specific intracellular adhesion molecule (ICAM)-3-grabbing nonintegrin that inhibited the entry of the virus (39). An inhibitory effect of Slp on the adhesion and entry of several virus families has also been suggested. In our previous study, probiotic L-92-enriched GroEL and GroES have been considered to have the potential to affect immunomodulation in mouse splenocytes (24). Moreover, a comparison of *L. acidophilus* strains showed that L-92 expressed higher amounts of cell-wall Slp and produced greater amounts of the Th1-type cytokine IL-12 after incubation with splenocytes (25). These reports have suggested that L-92 has the potential to protect against virus infection via Slp binding to DC-specific ICAM-3-grabbing nonintegrin on epithelial cells. There may also be structural changes in the cell wall and membrane components of live and non-live cells after heat treatment, leading to changes in host responses in the gastrointestinal immune system. To further understand the antiviral effect of L-92 cells, clarification of the key active components and the differences between live and non-live cells should be addressed.

In the present study, adaptive immunity was not thought to be involved because there were no significant increases in IgA and IgG in the live L-92 group. IgA in the lung 6 d after the infection in the L-92 group was not significantly changed compared with the control group. IgG in the live L-92 group was slightly, but significantly, lower compared with the control group. The lower adaptive immune responses are thought to be due to the delayed production of IgG and IgA after the virus infection (about 1 week after the infection). Generally, lactobacilli have been reported to induce adaptive immunity by increasing IgG (40); however, in the present study, they were not increased in the live L-92 group. We suggest that pretreatment of L-92 cells activates innate immunity so effectively that it causes immediate elimination of virus infection, or the mode of L-92 action differs from other lactobacilli.

A number of studies in animals have demonstrated the potential of using probiotics as immune adjuvants. *Lactobacillus* species and other probiotics stimulate both the cellular and innate immune systems (41). Clinical trials have also supported the potential of *Lactobacillus* in the prevention of influenza infection (22, 27). In a clinical trial on thirty-nine subjects, the authors thank A. Morikawa and H. Tsunoda (Nihon Bioresearch, Inc.) for technical assistance throughout the two animal studies. H. G. and N. Y. analysed the data, and revised the paper; A. S., N. A., T. H. and T. S. conceived the design, performed the experiments and acquired the data; S. K. performed the experiments. There was no funding for the present study. The authors have no conflicts of interest to declare.

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