Oral intake of *Lactobacillus rhamnosus* M21 enhances the survival rate of mice lethally infected with influenza virus

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**Background:** Influenza viruses cause acute respiratory disease. Because of the high genetic variability of viruses, effective vaccines and antiviral agents are limited. Considering the fact that the site of influenza virus entry is the mucosa of the upper respiratory tract, probiotics that can enhance mucosal immunity as well as systemic immunity could be an important source of treatment against influenza infection.

**Methods:** Mice were fed with *Lactobacillus rhamnosus* M21 or skim milk and were challenged with influenza virus. The resulting survival rate, lung inflammation, and changes in the cytokine and secretory immunoglobulin A (sIgA) levels were examined.

**Results:** Because of infection (influenza virus), all the mice in the control group and 60% of the mice in the *L. rhamnosus* M21 group died; however, the remaining 40% of the mice fed with *L. rhamnosus* M21 survived the infection. Pneumonia was severe in the control group but moderate in the group treated with *L. rhamnosus* M21. Although there were no significant changes in the proinflammatory cytokines in the lung lysates of mice collected from both groups, levels of interferon-γ and interleukin-2, which are representative cytokines of type I helper T cells, were significantly increased in the *L. rhamnosus* M21-treated group. An increase in sIgA as well...
**Introduction**

Influenza is an acute viral respiratory infection that results in high morbidity and significant mortality. There have been 10 influenza A pandemics in the past 300 years. Many experts are concerned that even a mild pandemic could kill many millions of people\(^1,2\), as such, influenza has become one of the most threatening infectious diseases for humans. Influenza virus consists of eight single-stranded RNA segments, and is classified into subtypes based on antigenic differences in two major surface glycoproteins, namely, hemagglutinin (HA) and neuraminidase. It is recognized that only three subtypes (H1, H2, and H3) of 15 HAs have established stable lineage in humans.\(^2,3\) However, the H5, H7, and H9 subtypes, which were initially thought to infect only birds, were also recently isolated from humans,\(^1,2\) indicating the potential for influenza viruses to be transferred from bird populations to humans. The fact that other subtypes could cross species barriers to infect humans has provoked much concern that such infections could lead to another influenza pandemic.\(^4\) Thus, the need for effective control against influenza virus infection is critical.

In general, prevention is more effective than therapeutic treatment in protecting hosts from viral infections. However, the development of effective preventive vaccines against influenza virus has been hampered due to its high genetic variability.\(^2,3\) Moreover, the effectiveness of antiviral drugs therapy is also hindered by the development of drug-resistant viruses; in addition, the use of such drugs is limited owing to their severe side effects. Thus, there has been a continued search for alternative ways to control influenza infection, and probiotics have emerged as a strong candidate.

Probiotics are live microorganisms that confer health benefits to the host when administered in appropriate doses.\(^5\) Because the site of influenza virus entry is the mucosa of the upper respiratory tract and one of the beneficial properties of probiotics is enhancing mucosal immunity, probiotics have long been recognized as an important protective candidate against influenza infection. For example, some strains of Lactobacillus or Bifidobacterium have been shown to have protective potential against influenza infection in animals.\(^9-11\)

We have previously reported that intranasal\(^1,2\) as well as sublingual\(^3\) administration of Lactobacillus rhamnosus M21 ameliorated influenza infection in mice by enhancing mucosal immunity. Although we demonstrated the anti-influenza activity of L. rhamnosus M21 through these studies, nasal as well as sublingual administration of probiotics has limitations in terms of practical application to the host. Probiotics are usually consumed by eating or drinking. In this regard, it is necessary to prove that L. rhamnosus M21 can maintain its anti-influenza activity when exposed to strong acids in the stomach upon oral administration. The aims of the present study were therefore to investigate whether orally administered L. rhamnosus M21 could provide the host beneficial effects against influenza virus infection as well as to reveal responsible immunological mechanisms.

**Materials and methods**

**Mice and L. rhamnosus M21**

Specific pathogen-free, female BALB/c mice (age, 4 weeks; weight, 16–18 g) were purchased from Orient Bio (Gapyeong, Gyeonggi, Korea). The mice were divided into the following two groups: the experimental group, which received oral administration of 0.3 mL of \(1 \times 10^9\) colony forming units/mL of L. rhamnosus M21 (KCTC 10965BP) daily for 2 weeks; and the control group that received the same volume of 5% skim milk, which was treated for 30 minutes at 63°C to destroy any possible bacteria. Administration of L. rhamnosus M21 or skim milk was continued until termination of the experiment. All procedures conformed to the Guidelines for the Care and Use of Laboratory Animals of the Institutional Animal Care and Use Committee of Konkuk University, Seoul, Korea. L. rhamnosus M21 was propagated in de Man, Rogosa, and Sharpe (MRS; Difco, Sparks, MD, USA) broth at 37°C for 24 hours. The bacterial cells were then harvested by centrifugation at \(5,000 \times g\) for 10 minutes at 4°C, and suspended in 5% nonfat milk (Sigma-Aldrich, St. Louis, MO, USA). The cell counts were determined on an MRS agar plate.

**Influenza virus infection**

Influenza virus A/NWS/3 3 (H1N1) was grown in the chorioallantoic fluid of 10-day-old embryonic hen eggs. After harvesting, the allantoic fluid was separated into aliquots and stored at \(-70\)°C until use. On the day after oral administration of L. rhamnosus M21 or skim milk for 2 weeks, lightly anesthetized mice were inoculated intranasally with a 5.5 log10 50% tissue culture infectious dose of influenza virus in 90 μL of phosphate-buffered saline (PBS).

For survival rate analysis, 10 mice/group were challenged and observed daily after the infection for up to 20 days. For as the diminution of inflammatory cells in bronchoalveolar lavage fluid was also observed in the L. rhamnosus M21-treated group.

**Conclusion:** These results demonstrate that orally administered L. rhamnosus M21 activates humoral as well as cellular immune responses, conferring increased resistance to the host against influenza virus infection.

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Cytokine analysis

To determine cytokine levels in the lung, lung tissues were removed from noninfected and infected mice on Day 0, Day 1, Day 3, and Day 6 after the infection. Lung tissues were homogenized in 1 mL of cold PBS, and centrifuged at 9,000 × g at 4°C for 30 minutes. The clarified cell lysates were collected and their cytokine levels were determined [interleukin-1β (IL-1β), IL-6, IL-4, IL-12, and interferon-γ (IFN-γ)] using enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA).

Analysis of bronchoalveolar lavage cells

Anesthetized mice were exsanguinated, and the chest cavity was dissected to expose the trachea and lungs. A 24-gauge catheter was inserted and secured in the trachea. Lavage was performed twice by injecting 0.75 mL of prewarmed PBS into the lungs through the tracheal cannula. Bronchoalveolar lavage (BAL) cells were concentrated by centrifuging the lung fluid at 600 rpm for 5 minutes at 4°C. BAL cells were resuspended in 1 mL of PBS, cytospun, stained with Diff-Quick (American Scientific Products, McGaw Park, IL, USA), and counted using an ADAM automatic cell counter (Digital Bio Technology, Seoul, Korea).

Determination of immunoglobulin A level in BAL fluid

Total protein content in the BAL fluid was quantified by Bradford reagent (Sigma-Aldrich). Plates were coated with 100 µL of 1 µg/mL purified immunoglobulin A (IgA) monoclonal antibody (Ab; BD Pharmingen, San Jose, CA, USA) by incubating overnight at 4°C. Blocking buffer, 300 µL [3% bovine serum albumin (BSA) in PBS], was added and the plates were incubated at room temperature for 2 hours. BAL fluid and standard IgA (BD Pharmingen) were diluted (1.5% BSA in PBS) and added to the plates, which were incubated at room temperature for 2 hours. After washing three times with 0.1% PBS–Tween-20, the plates were incubated with horseradish peroxidase-conjugated anti-IgA Ab (dilution range, 1:25,000; Serotec, Raleigh, NC, USA) at room temperature for 1 hour. The plates were developed with 3,3′,5,5′-tetramethylbenzidine (Chemicon International, Temecula, CA, USA). Reactions were terminated by adding 100 µL of 2N H2SO4 and the optical density was read at 450 nm.

Statistical analyses

Statistical significance of cytokine and IgA levels in the lungs between groups were determined by one-way analysis of variance and Duncan’s post hoc test. All data were presented as the statistical mean ± standard error of the mean and p < 0.05 was considered significant. Survival analysis was performed using Kaplan–Meier curves and log-rank test with Bonferroni adjustment.

Results

Survival rate of L. rhamnosus M21-fed mice

In the search for probiotics that can confer beneficial effects to the host against influenza infection, we evaluated the efficiency of L. rhamnosus M21. When mice were intranasally challenged with a lethal dose of influenza virus, those fed with skim milk (control) began to die at Day 5 or Day 6, and by Day 17 all the mice had died. Although L. rhamnosus M21-treated mice showed sickness on similar days, approximately 40% survived the infection (Fig. 1). As the surviving mice began to eat and drink thereafter and completely recovered, we choose Day 20 as the terminal point to demonstrate the significant difference between groups. These experiments were repeated three times, and all results were consistent. These results suggest that L. rhamnosus M21 may be useful to confer the increased resistance to the host against influenza virus infection, which prompted us to investigate the underlying mechanisms associated with the beneficial effects of this bacterial strain.

Pathological analysis

To determine the relevance of pathological events occurring in the lungs on survival rate, five mice in each group were subjected to lung pathological analysis following influenza virus infection. Up to Day 3 after the infection, lungs from mice in the control and experiment groups showed no changes compared with noninfected mice with regard to gross lesions (data not shown). However, on Day 6, the lungs of all mice in the control group showed severe pneumonia with massive hemorrhage (Fig. 2A), whereas the lungs of the experimental group mice exhibited moderate pneumonia with mild hemorrhage (Fig. 2B). H&E-stained pathological analysis, on Day 3 and Day 6 after the infection, lung tissues were fixed in 10% buffered formalin for routine hematoxylin and eosin (H&E) staining.

Figure 1. Effect of Lactobacillus rhamnosus M21 treatment on the survival rate of influenza virus-infected mice. Ten mice/group were orally administered with skim milk (broken line) or L. rhamnosus M21 (unbroken line) for 2 weeks and were intranasally challenged with influenza virus. The survival rate of each group was observed for 20 days.
tissue sections revealed that treatment with \textit{L. rhamnosus} M21 led to a significant reduction in influenza-induced lung pathology. In the control group mice (i.e., mice infected with influenza A virus and received skim milk treatment), severe lung damages including extensive cellular infiltrates, reduced alveolar spaces, hemorrhage, progressed fibrosis, and alveolar edema were observed (Fig. 2C and E). By contrast, the \textit{L. rhamnosus} M21-treated mice showed moderate pneumonia (Fig. 2D and F).

Infiltration of inflammatory cells in BAL

Although BAL cells do not represent all the infiltrated cells in the lungs, they are commonly examined to reveal the pathological states of the lungs. The total cell numbers in the lungs started to increase at Day 3 and peaked on Day 6 after the infection (Fig. 3). The experimental group also showed an increase in infiltrated cell numbers in the lungs, but at a 19.7% lower level on average compared with the control group. Although statistical significance between the groups was not obtained due to high individual differences, these data showed that the infiltration of inflammatory cells into the lungs was reduced by administration of \textit{L. rhamnosus} M21. On Day 0, BAL cells mainly consisted of macrophages and a few neutrophils. After Day 1, neutrophils started to increase, and became predominant on Day 3 when only a few lymphocytes were observed. On Day 6, remarkable lymphocyte infiltration was seen (data not shown).

Cytokine production in the lung

Because there is plenty of evidence supporting the fact that cytokines influence the inflammatory response in influenza...
virus-infected lungs, we investigated whether administration of *L. rhamnosus* M21 could modulate the production of cytokines. As shown in Fig. 4, 1 day after the infection, IL-1β (Fig. 4A) and IL-6 (Fig. 4B) reached peak levels and declined gradually thereafter, showing that an inflammatory response was immediately induced by influenza virus infection. However, there was no apparent difference between the experimental and control groups. Some probiotics can affect the T-helper type 1 (Th1) and T-helper type 2 (Th2) balance, thereby modulating disease outcomes.14 In order to determine whether *L. rhamnosus* M21 shifts the Th1/Th2 paradigm, we investigated the levels of IL-4 and IFN-γ, representative cytokines of Th1 and Th2 cells, respectively. Whereas the level of IL-4 in the lungs from both the control and experiment groups was too low to detect (data not shown), the level of IFN-γ started to increase from Day 1 and was highest on Day 6 after the infection. Even though the significant difference in the level of IFN-γ between the control group and the experiment group on Day 6 vanished, the level of IFN-γ in the experimental group on Day 3 was three times higher than that of the control group (Fig. 5A). We also examined IL-12 levels, another main cytokine of Th1 cells, which increased greatly on Day 1 in the experimental group but not in the control group, and found that it declined thereafter in both groups (Fig. 5B).

**IgA levels in BAL fluid**

Early studies have shown that some probiotics administered orally exhibit beneficial effects by production of secretory IgA (sIgA).15 Therefore, the production of sIgA was examined in BAL fluid on Day 0, Day 1, Day 3, and Day 6 after the infection. The sIgA levels started to increase in both groups 3 days after the infection, but the experimental group showed a significant increase compared with the control group on Day 6 after the infection (Fig. 6).

**Discussion**

In this study, we found that orally administered *L. rhamnosus* M21 increased survival rate of mice that were lethally challenged with influenza virus. Histopathological analysis of the lungs confirmed the correlation between the lethality and severity of the acute pneumonia. The infiltration of mononuclear cells in the lungs also decreased in the experimental group. These results suggested that oral administration of *L. rhamnosus* M21 inhibited the inflammatory response in the lungs after the influenza virus infection. The significant increase in the IFN-γ and IL-12 levels in the lungs of the mice in the experimental group demonstrated that *L. rhamnosus* M21 shifted the type of helper T cells to Th1, thereby enhancing the antiviral activities. The levels of sIgA, a well-known frontline protector against viral invasion, were also increased in the experimental group. These results demonstrate that *L. rhamnosus*
M21 may be a useful probiotic for controlling influenza virus infection. It has been reported that one of the causes of death due to influenza virus infection is the burst of inflammatory cytokines. Although cytokines facilitate elimination of pathogens, their excessive production can be harmful, in part by causing excessive infiltration of inflammatory cells into tissues, resulting in tissue destruction. Previous studies have shown that the therapeutic effects of some agents against influenza virus infection in mice were attributed to a reduction in proinflammatory cytokines. Therefore, we evaluated whether administration of L. rhamnosus M21 could modulate inflammatory cytokine production by measuring periodic changes in the levels of IL-1 and IL-6 in the lungs. Our results showed that cytokines levels peaked on Day 1 after the infection and declined thereafter, which indicates that these cytokines are primarily induced by influenza virus infection in the lung. However, there was no significant difference between the cytokines levels in the control and experiment groups, indicating that the beneficial effect of L. rhamnosus M21 was not related to changes in the IL-1 and IL-6 levels.

M21 may be a useful probiotic for controlling influenza virus infection.

Figure 5. Effect of Lactobacillus rhamnosus M21 on Th1-derived cytokines in lung lysates after the influenza virus challenge. Mice were orally administered with skim milk (open squares) or L. rhamnosus MK21 (closed squares) for 2 weeks and were intranasally infected with influenza virus. (A) Interferon-γ (IFN-γ) and (B) interleukin-12 (IL-12) levels in the lung lysate at the indicated days were determined with enzyme-linked immunosorbent assay. Data are shown as mean ± standard error of the mean for each group (n = 5). Significant differences from the control group are denoted for the L. rhamnosus-treated group (*p < 0.05).

Figure 6. Effect of Lactobacillus rhamnosus M21 on secretory IgA (sIgA) levels in bronchoalveolar lavage (BAL) fluid after the influenza virus challenge. Mice were orally administered with skim milk (open squares) or L. rhamnosus MK21 (closed squares) for 2 weeks and were intranasally infected with influenza virus. The sIgA levels in BAL fluid at the indicated days were determined with enzyme-linked immunosorbent assay. Data are shown as mean ± standard error of the mean for each group (n = 5). Significant differences from the control group are denoted for the L. rhamnosus-treated group (*p < 0.05).
infection. Effect contributes to the host defense against viral effect subsided later. However, it is presumed that this increased the early production of these cytokines, but this was not addressed. Renegar and Small27 showed that passive immune response. Treatment with probiotics in controlling virus replication prior to the onset of adaptive immune response.M21 could be explained by the kinetic behavior of these cytokines. Both IL-12 and IFN-γ are antiviral cytokines that are secreted within a few hours after viral invasion, and their early production is important in controlling virus replication prior to the onset of adaptive immune response. Treatment with L. rhamnosus M21 increased the early production of these cytokines, but this effect subsided later. However, it is presumed that this effect contributes to the host defense against viral infection.

The importance of IgA in protective immunity against influenza viral infections has also been previously addressed. Renegar and Small27 showed that passive transfer of IgA Ab could protect mice from intranasal influenza virus infection, suggesting a pivotal role of IgA in influenza immunity. An interesting feature in this context is that probiotics that were administered orally could modulate the levels of sIgA in the lungs, and this phenomenon can be explained as follows: orally administered probiotics enter microfold (M) cells in the small intestine, where they stimulate immune cells such as dendritic cells, macrophages, and lymphocytes. Activated B cells migrate to the lungs through mucosal-associated tissues that secrete IgA.28 Influenza virus-specific IgA has been involved in neutralizing the virus, whereas a nonspecific IgA prevents attachment of the virus to the mucosal surface.29 Therefore, for a probiotic, with the aim of controlling influenza virus infection, the increase of IgA level following administration is a critical feature. Indeed, L. rhamnosus M21-treated mice showed significantly much higher levels of IgA in the lungs 6 days after the infection. These results support that of Pérignon et al15, who showed that oral administration of lactic acid bacteria increased the number of IgA + cells in bronchi. Although what we presented here were total IgA levels, the influenza-specific IgA should play an important role in improving the beneficial effects of orally administered L. rhamnosus M21. Regrettably, we tried but failed to detect the specific IgA in this study, and therefore, the involvement of specific IgA remains in speculation.

In summary, our results show that L. rhamnosus M21 exhibits anti-influenza activity by shifting the host response to Th1 type, enhancing IgA production as well as diminishing the recruitment of inflammatory cells into the lungs, all of which could contribute to lessening of lungopathies. In addition, considering the fact that L. rhamnosus M21 was administered orally, other factors such as changes in the gut environment should be considered for identifying the underlying mechanisms. Interesting to note is that all probiotics with anti-influenza activities do not exhibit the same mechanisms as L. rhamnosus M21. Because a mixture of several probiotics rather than a single probiotic produces beneficial effects for various diseases,30 we can expect that a combination of probiotics with similar anti-influenza activities but with different mechanisms can compensate each other’s drawbacks, thereby maximizing their activities against influenza infection. Additional studies to address this concept are in progress.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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References

Effectiveness of *L. rhamnosus* M21 against influenza


