Effects of *Lactobacillus plantarum* and *Leuconostoc mesenteroides* Probiotics on Human Seasonal and Avian Influenza Viruses

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Received: April 4, 2018
Revised: April 10, 2018
Accepted: April 11, 2018

First published online
April 20, 2018

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pISSN 1017-7825, eISSN 1738-8872

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Influenza viruses that cause recurrent seasonal epidemics to humans can be controlled with vaccine and antiviral therapy. However, the medical treatments often exhibit limited efficacy in the elderly or immunosuppressed individuals. In these cases, daily uptake of probiotics may be an option to bring in health benefits against influenza. Here, we demonstrate the effects of probiotics *Lactobacillus plantarum* (Lp) and *Leuconostoc mesenteroides* (Lm) against seasonal and avian influenza viruses. As assessed by the plaque size reduction of human H1N1 and avian influenza H7N9 viruses, including green fluorescent protein-tagged H1N1 strain in cells, the selected Lp and Lm strains restrained viral replication in mouse lungs with statistical significance. Against lethal viral challenge, the Lp and Lm strains exhibited their beneficial effects by increasing the mean days and rates of survival of the infected mice. These results suggest that, despite rather narrow ranges of protective efficacy, the dietary supplement of *Lactobacillus* and *Leuconostoc* probiotics may promote health benefits against influenza.

Keywords: Dietary supplement, influenza, *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, probiotic

Introduction

Recurrent seasonal epidemic and sporadic pandemic influenzas have caused severe public health burdens [1, 2]. Vaccination is considered as the best preventive measure. However, there are often mismatches between the vaccine virus and circulating strains of the season. Furthermore, owing to the constant presence of the virus among aquatic birds as its reservoir [3], zoonotic transmission from animals to humans, as shown in recent outbreaks of H7N9 [4], cannot be prevented effectively. Vaccine mismatch is due to the constantly changing antigenicity of the virus, termed “antigenic drift.” Zoonotic transmission of the virus introduces to humans completely different influenza virus strains, which is termed “antigenic shift” [5]. Antigenic drifts and shifts can be prepared against theoretically by a

“universal” vaccine [6], which is yet to come. In the meantime, the empirical “word of mouth” preventive measures to reduce susceptibility to the virus or to have reduced illnesses from influenza have come under scientific scrutiny.

Many lactic acid bacteria (LAB) have been recognized as probiotics. LAB are a group of bacteria belonging to many genera, including *Lactobacillus* and *Leuconostoc*. Reported health benefits of LAB from the uptake of fermented food are numerous, ranging from the reduction of gut symptoms and diabetes to antitumor effects and enhanced defenses against infectious diseases through immune modulation. The versatility of LAB might be attributed to the diversity of the metabolic products of different probiotic LAB strains [7, 8].

The effects of LAB on respiratory tract infections have also been studied actively. In a double-blind, randomized

controlled trial in the elderly, dietary consumption of LAB was reported to reduce the duration and severity but not the incidence of common cold episodes [9]. Similarly, reduction of the duration of respiratory infections in the elderly by consumption of fermented dairy products containing the probiotic *Lactobacillus* was observed in a randomized controlled trial [10]. Immune enhancement effects of LAB could have been one of the potential mechanisms explaining such results. However, human trials have not shown definitively the immune enhancement effects of LAB. Boge *et al.* [11] reported that daily consumption of fermented dairy drink containing *Lactobacillus casei* DN-114 001 improved antibody responses to influenza vaccination in the elderly in two randomized controlled trials. On the other hand, in a different double-blind, randomized placebo-controlled trial with healthy elderly nursing home residents, daily consumption of fermented dairy drink containing *Lactobacillus casei* Shirota showed no statistically or clinically significant effects on the protection against respiratory symptoms and no significant differences regarding the influenza-vaccination immune response [12]. Interestingly, the authors of the latter report cautiously pointed out that there was a nonsignificant trend with relative risk reduction whereas most studies often demonstrated beneficial effects that are often difficult to reproduce [8]. Different cocktails of bioactive molecules and metabolites that were released from and by the activity of different strains of LAB could have produced non-uniform outcomes [13].

Kimchi is a traditional Korean food of fermented vegetables. Kimchi is known for its rich source of LAB [14, 15], and LAB isolated from kimchi have manifested many beneficial effects in experiments using animal models [16, 17]. It was also recognized that different LAB strains isolated from different types of kimchi show different immune modulatory functions [18].

The protective effects of LAB isolated from kimchi have been studied against influenza [19]. We also reported anti-influenza effects of an oral uptake of heat-killed kimchi-isolated LAB, *Lactobacillus plantarum* (nF1), in H1N1 and H3N2 influenza virus-infected mice [17]. As with many other studies looking for anti-influenza effects of LAB using different heat-killed LAB strains and feeding regimens in mice [20, 21], we could see a “trend” of protective effects of LAB against influenza. It appears that the efforts to identify a specific strain of LAB that is definitely effective against the virus need to be continued with diverse approaches. One might also take note of the

differences of responses between using live and heat-killed LAB [22]. In this study, we determined the effects of a panel of *Lactobacillus plantarum* and *Leuconostoc mesenteroides* strains, originally isolated from kimchi, against H1N1 and H7N9 influenza viruses in vitro and in vivo.

Materials and Methods

Ethics

The animal experiments were conducted in strict accordance with the recommendations delineated in the Guide for the Care and Use of Laboratory Animals of the Animal, Plant and Fisheries Quarantine and Inspection Agency of Korea. The experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Korea University College of Medicine (Permit No. KOREA-2016-0113).

Viruses and Cells

A/Korea/01/2009 (2009 pandemic influenza A H1N1 virus, rK09; provided by Korean Centers for Disease Control and Prevention, Korea) [23, 24], K09 harboring serine residues at neuraminidase (NA) residues 58-59 (rK09/NA:NN58SS) [25], A/Puerto Rico/8/1934 expressing green fluorescent protein (rPR8-GFP; provided by Dr. Adolfo Garcia-Sastre, Icahn School of Medicine at Mount Sinai, USA) [26, 27], and A/Anhui/01/2013 (avian influenza A H7N9 subtype 6:2 vaccine virus, rAH01; previously generated by Lee *et al.* [28]) were propagated in 10-day-old embryonated chicken eggs. Virus titers were determined by a standard plaque assay in Madin-Darby canine kidney (MDCK) cells, which were obtained from the American Type Culture Collection (ATCC, USA). The MDCK cells were maintained in Eagle's Minimum Essential Medium (Lonza, Switzerland) supplemented with 10% fetal bovine serum (BioTechnics Research Inc., USA) and 1% penicillin and streptomycin (Gibco, USA) at 37°C in a 5% CO₂ incubator. Genomic sequences of all the viruses were confirmed using reverse-transcription PCR before use.

Probiotics Preparation

Lactobacillus plantarum (Lp; strains J1-8, 330, CK10, and 920; Patent Application Publication No. Korea/10-1249824, 10-1485182, 10-10-1287120, and 10-1600940, respectively) and *Leuconostoc mesenteroides* (Lm; strains DRC1506 and 218; Patent Application Publication No. Korea/10-1809447 and 10-1055949, respectively) were provided by Daesang Corporation (Korea). Bacterial culture of Lp and Lm strains was performed using De Man-Rogosa-Sharpe (MRS) broth media (MB Cell, USA) at 37°C. The cultured bacteria were washed with phosphate-buffered saline (PBS; Lonza, USA) by repeating centrifugation (3,500 ×g, 37°C, 20 min) and finally resuspended with PBS again. OD values of the bacteria were measured, and their colony-forming unit (CFU) titers were determined with serial 10-fold dilutions (down to 10⁻⁸) by

counting the number of colonies grown in MRS agar plates (MB Cell, USA).

Cell-Based Assays

A virucidal assay was performed by modifying a protocol described previously [29]. Briefly, 100 plaque-forming units (PFU) of a virus (in 100 μ l PBS; rK09 and rAH01) were mixed with the same volume of Lp and Lm strains (2-fold dilutions from 10^8 CFU) and incubated at 37°C for 1 h. A standard plaque assay was then performed to determine viral plaque formation in MDCK cells [28]. For the plaque-reduction assay, MDCK cells were infected with 100 PFU of a virus (in 100 μ l PBS; rK09, rAH01, and rPR8-GFP) for 1 h. After being washed three times with cell culture media, the cells were overlaid with Oxoid agar (Thermo Fisher Scientific, USA) containing 2-fold dilutions of 10^8 CFU Lp or Lm strain. The virus-infected, agar-overlaid cells were then stained with crystal violet at 48–72 h post-infection (hpi). For the detection of GFP expression from the cells infected with rPR8-GFP, each viral plaque was assessed using the EVOS FL Imaging System (Thermo Fisher Scientific, USA) from low to high magnification at 48–72 hpi. PBS was used for the mock treatment.

Mouse Experiment

Twelve BALB/c mice (5-week-old females; NaraBiotech, Korea) per group were acclimated for 1 week before the start of the experiment. The mice were fed once daily with Lp or Lm strain (10^9 CFU in 200 μ l PBS) using a feeding needle (model JD-S-124; Jeung Do B&P, Korea) for 14 days prior to and 14 days after lethal viral challenge with rK09/NA:NN58SS ($2 \times 50\%$ mouse lethal dose; 2 MLD₅₀). At 3 and 6 days post-infection (dpi), three mice per group were sacrificed for lung collection, which was used for the titration of viral replication by the plaque assay. Body weight changes and survival rates of the rest of the six mice per group were monitored daily for 14 dpi. Mice that lost greater than 25% of their initial body weight were considered dead and euthanized humanely. PBS was used for the mock infection and treatment as controls.

Statistical Analysis

Differences in viral lung replication were tested for significance using Student's *t*-test ($*p < 0.05$). Differences in mouse survival rates were tested for significance using the Kaplan-Meier test ($p < 0.05$).

Fig. 1. Virucidal efficacy of *Lactobacillus plantarum* (Lp) and *Leuconostoc mesenteroides* (Lm) probiotics against 2009 pandemic influenza H1N1 and avian influenza H7N9 viruses.

Four Lp (J1-8, 330, CK10, and 920) and two Lm (DRC1506 and 218) strains were evaluated for their virucidal efficacy against rK09 (A, 2009 pandemic H1N1 virus) and rAH01 (B, human-infecting avian influenza H7N9 vaccine virus) in MDCK cells. Serial 2-fold dilutions of 10^8 CFU of each probiotic were used in the assay, and PBS was used as a negative control.

Results

Lactobacillus plantarum and *Leuconostoc mesenteroides* Probiotics Restrain Plaque Formation of Human Seasonal H1N1 and Avian Influenza H7N9 Viruses in MDCK Cells

Four strains (J1-8, 330, CK10, and 920) of Lp and two strains (DRC1506 and 218) of Lm were evaluated for their anti-influenza activity in cell-based assays. In the virucidal assay, which might examine the direct efficacy of the Lp and Lm strains, the probiotics exhibited no effectiveness against both rK09 and rAH01. Neither plaque number nor diameter were affected, compared with the PBS-treated wells (Fig. 1). As seen in the virucidal assay, Lp J1-8 and Lm DRC1506 did not demonstrate their effectiveness in the plaque-reduction assay, either. However, treatment with Lp 330, CK10, and 920 and Lm 218 reduced the sizes of viral plaques of rK09 and rAH01 in a dose-dependent manner, even though the plaque numbers appeared to be unaffected (Fig. 2).

To further validate the effects of Lp and Lm probiotics,

we used rPR8-GFP in the same plaque-reduction assay. In fluorescent microscopy, we observed that viral plaques of PBS-treated controls expressed green fluorescence. However, treatment with Lp 330, CK10, and 920 and Lm 218 reduced fluorescence expression in a dose-dependent manner, whereas Lp J1-8 and Lm DRC1506 exhibited no effects, either (Fig. 3), as demonstrated against both rK09 and rAH01 (Fig. 2). In particular, at 10^8 CFU treatment with Lp 330, CK10, and 920 and Lm 218, it was apparent that viral plaques were extremely reduced in diameter (Fig. 3). These results demonstrate the protective effects of Lp 330, CK10, and 920 and Lm 218 against both human and avian influenza viruses.

Treatment with *Lactobacillus plantarum* and *Leuconostoc mesenteroides* Probiotics Promotes Survival of Mice Infected with Influenza Virus

We then investigated the protective effects of the Lp 330, CK10, and 920 and Lm 218 strains against lethal viral challenge in mice (Fig. 4A). Given the viral titers determined

Fig. 2. Plaque-reduction efficacy of *Lactobacillus plantarum* (Lp) and *Leuconostoc mesenteroides* (Lm) probiotics against 2009 pandemic influenza H1N1 and avian influenza H7N9 viruses.

The Lp (J1-8, 330, CK10, and 920) and Lm (DRC1506 and 218) strains were evaluated for their plaque-reduction efficacy against rK09 (A) and rAH01 (B) in MDCK cells. Serial 2-fold dilutions of 10^8 CFU of each probiotic were used in the assay, and PBS was used as a negative control.

Fig. 3. Inhibition of GFP expression by *Lactobacillus plantarum* (Lp) and *Leuconostoc mesenteroides* (Lm) probiotics in GFP-expressing influenza virus-infected cells.

The plaque-reduction efficacy of the Lp (J1-8, 330, CK10, and 920) and Lm (DRC1506 and 218) strains were validated using rPR8-GFP. Under a fluorescence microscope, GFP expression of each viral plaque was examined from low to high magnification at 48–72 hpi. The images above were observed with $\times 100$ magnification.

at 3 dpi, mice treated with Lp 330, CK10, and 920 and Lm 218 appeared to be never protected from viral infection because higher viral titers were observed in the lungs of these mice than those of PBS-treated mice. However, at 6 dpi, treatment with Lp 330, CK10, and 920 greatly reduced viral titers with statistical significance ($p < 0.05$) (Fig. 4B). Even though no statistical significance was found, Lm 218 also reduced the viral titer at 6 dpi.

In terms of viral titers in mouse lungs, Lp 330, CK10, and 920 and Lm 218 appeared to be comparable to oseltamivir, which has been prescribed for influenza patients under the name of Tamiflu, because similar ranges of viral titers were determined in the lungs of these mice at 6 dpi. In this viral

challenge experiment, oseltamivir treatment saved 83.33% of the mice from death. However, as Lp 330, CK10, and 920 and Lm 218 might not have direct effects against influenza viruses (Fig. 1), treatment with Lp 330, CK10, and 920 and Lm 218 did not protect all the mice from lethal viral challenge. Even so, both Lp CK10 and Lm 218 saved half of the infected mice from death (Lm 218, $p < 0.05$), and Lp 920 and 330 also resulted in 33.33% and 16.67% survival rates, respectively (Lp 920, $p < 0.05$) (Fig. 4C). Given that all the PBS-treated mice had succumbed to death at 8 dpi, it is noteworthy that treatment with Lp 330, CK10, and 920 and Lm 218 extended mouse survival from 2.4 to 4.4 days on average (Table 1). These results suggest that despite the

Fig. 4. Effects of *Lactobacillus plantarum* and *Leuconostoc mesenteroides* probiotics against lethal viral challenge in mice.

(A) Overall experimental scheme indicating the duration of probiotics administration, infection titer, and time points for infection and lung collection. (B) Viral replication in the lungs of mice was determined by the plaque assay in MDCK cells. PBS was used for mock treatment. The error bar denotes standard deviation (SD). * $p < 0.05$. (C) Body weight changes and survival rates of infected mice were monitored daily for 14 dpi. PBS was used for the mock infection and treatment. The error bar denotes SD. * $p < 0.05$.

rather narrow ranges of protective efficacy, the dietary supplement of *Lactobacillus* and *Leuconostoc* probiotics may promote health benefits against influenza

Discussion

Given the continuous threats of influenza that have been caused mainly by recurrent human seasonal viruses [30] and avian influenza viruses, such as human-infecting H5

and H7 subtypes [31, 32], effective medical arsenals should be prepared for protection not only of human beings but also of animals. Currently, vaccines and antivirals are the two main intervention methods that can be used for prophylactic and therapeutic purposes against human influenza [33, 34]. However, owing to ceaselessly changing patterns of viral genotypes and phenotypes, which may result in the problems of low vaccine effectiveness and antiviral resistance, the quest for reliable new medical

Table 1. Efficacy of probiotics administration in infected mice.

Group	% Survival	Body weight loss (% mean $\pm$ SD (dpi)) ^a	MDD $\pm$ SD ^b	Increase in survival days ^c
Non-infection	100	-	14.0 $\pm$ 0	14
Infection	0	23.73 $\pm$ 0.98 (6)	5.8 $\pm$ 0.8	0
Oseltamivir	83.33	12.25 $\pm$ 4.58 (8)	12.7 $\pm$ 3.3	6.9
330	16.67	23.56 $\pm$ 1.33 (8)	8.2 $\pm$ 3.0	2.4
CK10	50	19.47 $\pm$ 4.88 (8)	9.8 $\pm$ 4.6	4.0
920	33.33	21.55 $\pm$ 5.69 (8)	9.2 $\pm$ 3.8	3.4
218	50	22.44 $\pm$ 1.59 (7)	10.2 $\pm$ 4.3	4.4

^aThe mean maximum body weight losses $\pm$ standard deviations (SD).^bMDD, mean number of days to death for the mice that died prior to 14 dpi.^cMean increase in days of mouse survival, compared with mean survival days of the mice of infection groups.

countermeasures have intensified [35–38].

Even though an effective spectrum of probiotics appeared to be substantially variable based on the experimental conditions, such as the kind of virus/probiotic/strain and administration dosage/duration, it was suggested that probiotics supplements may improve vaccine efficacy [39, 40]. Viral pathogenicity might also be reduced with probiotics treatment [41], or certain microbial protein(s) might enhance the efficacy of probiotics treatment [42]. In this regard, dietary supplements of beneficial intestinal microbes as probiotics can be a good option for influenza and influenza-related respiratory patients, especially of the elderly and immunocompromised individuals who might experience the problem of low vaccine effectiveness [43, 44]. Moreover, given the immunomodulatory effects of probiotics [45–47], the administration of probiotics may promote the overall health status of patients. Hence, even though no direct effects of probiotics have been reported on viral pathogenic mechanisms, as demonstrated in our results of virucidal and plaque-reduction assays (Figs. 1–3), daily or regular uptake of probiotics can be suggested as a dietary supplement for targeted population groups. To investigate this, we treated mice with the three Lp and one Lm strains, which were selected on the basis of the results of the plaque-reduction assay (Figs. 2 and 3), 14 days prior to and 14 days after lethal viral challenge. Given the maximal body weight changes of mice, the administration of probiotics appeared to exhibit limited efficacy. However, the Lp- or Lm-treated mice showed maximal body weight loss 1 or 2 days later, compared with the mice of the infection group (Fig. 1C). In addition, by the supplementation with probiotics, the mean days of mouse survival was extended at least 2.4–4.4 days (Table 1). As expected, oseltamivir treatment helped the infected mice to regain

their body weights from 6–8 dpi and increased the mean days of survival by approximately 6.9 days (Table 1). Regarding the mechanism of action of oseltamivir, targeting influenza neuraminidase and blocking the release of progeny virions budding out of infected cells [48], it is apparent that oseltamivir might exhibit superior efficacy to the Lp and Lm probiotics. Despite this, the Lp- or Lm-treated mice that experienced approximately 19.47% to 23.56% body weight loss started to recover from 8 dpi, and 16.67% (Lp 330), 33.33% (Lp 920), and 50% (Lp CK10 and Lm 218) of them survived until 14 dpi (Fig. 4C and Table 1). Consistent with these results, viral replication titers in the lungs of mice were reduced in the Lp- or Lm-treated mice (Fig. 4B).

Here, we do not provide exact evidence of the mechanism(s) of action of the Lp and Lm probiotics against influenza. However, given our results obtained from in vitro and in vivo efficacy analyses, we believe that the tested Lp and Lm probiotics retain beneficial effects against influenza, and our results may underline the usefulness of probiotics as an anti-influenza dietary supplement for overall age groups, including the elderly and immunocompromised patients.

Acknowledgments

This study was supported by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through High Value-added Food Technology Development Program, funded by the Ministry of Agriculture, Food and Rural Affairs (MAFRA) (Grant No. 314054-3). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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