

Full Paper

Oral administration of the probiotic bacterium *Lactobacillus acidophilus* strain L-55 modulates the immunological parameters of the laying hen inoculated with a Newcastle disease virus-based live attenuated vaccine

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Probiotic supplements containing living bacteria have attracted interest as a potential source of health benefits for humans and livestock. The aim of this study was to determine whether administration of *Lactobacillus acidophilus* strain L-55 (LaL-55) enhances the immune response among chicks exposed to a Newcastle disease virus (NDV)-based live attenuated vaccine. Oral administration of LaL-55 augmented the elevation in the total numbers of leukocytes and lymphocytes following inoculation with the NDV-based live attenuated vaccine. Monocyte counts increased after LaL-55 administration independent of inoculation with the NDV vaccine. Among chicks that were administered LaL-55, there was a dose-dependent increase in the NK cell activity measured by a ⁵¹Cr release assay at 2 weeks after the secondary NDV vaccine inoculation. Two weeks after the secondary inoculation with the NDV vaccine, interferon (IFN)- γ -mRNA expression was significantly elevated in mononuclear splenocytes from chicks that were administered LaL-55. Meanwhile, LaL-55 administration did not change the mRNA levels of IFN- α , IFN- β , and interleukin-1 β . These results may suggest that coadministration of LaL-55 with an NDV vaccine augments the immune response against the virus. Therefore, LaL-55 may help protect against viral diseases in poultry.

Key words: *Lactobacillus acidophilus* L-55, chicken, Newcastle disease vaccine, immunomodulatory effect

INTRODUCTION

Various pathogens, such as highly pathogenic strains of influenza and porcine fever, have become major threats to public health and livestock suppliers around the world. Furthermore, excessive use of antibiotics among humans and livestock has promoted the emergence of antibiotic-resistant organisms, which are rapidly spreading and causing problems for public health. Along with the production of drugs and vaccines, probiotics are being considered as additional tools for the prevention and treatment of diseases. Live bacteria supplements have recently become widely used, and probiotics have become an important target for providing health benefits in humans and animals.

Lactic acid bacteria (LAB), including members of the genera *Lactobacillus* and *Bifidobacterium*, are normal inhabitants of the gastrointestinal tract that are classified as probiotic bacteria. Several studies in mammals have reported the benefits of intestinal bacteria, especially LAB. Some of the benefits provided

by LAB include the production of various nutrients for the host, prevention of infections caused by intestinal pathogens, and modulation of the normal immune response [1]. However, there is only a small number of published reports demonstrating the benefits of probiotic bacteria on the avian immune system. The effects of probiotics against viral infection in avian species have not yet been investigated. *Lactobacillus* spp. are the main type of LAB used in probiotic formulations because they possess potentially advantageous properties [2]. In addition, *Lactobacillus acidophilus* has been proven to elevate immunological activity by stimulating both the innate and adaptive immune responses [3–5]. *In vitro* studies have indicated that *L. acidophilus* is a strong inducer of T helper type 1 (Th1) cytokines, including interleukin (IL)-12 and interferon (IFN)- γ [6, 7]. Also, *L. acidophilus* is involved in enhancement of the activity of NK cells [8]. Clinical trials involving oral administration of *L. acidophilus* strain L-55 (LaL-55) show that this strain effectively suppresses experimental allergic rhinitis [8] and experimental atopic dermatitis in mice

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[9]. Therefore, *L. acidophilus*, specifically LaL-55, was selected for this study to determine whether it may help protect chickens against infectious diseases by boosting immune function. The aim of this study was to evaluate the immunomodulatory and beneficial effects of LaL-55 administration in chicks inoculated with a live attenuated Newcastle disease virus (NDV) vaccine.

The attenuated NDV vaccine used in this study was a lyophilized version of an attenuated NDV clone 30 injected into a growing chicken egg. Live and inactivated NDV vaccines have been widely used. Live vaccines based on lentogenic strains are widely used because of their high efficacy and availability [10]. Inactivated oily vaccines are used for enhancing and sustaining immunity [10, 11]. Vaccines are commonly administered by the eye- or nose-drop method. Generally, cellular immunity is crucial in viral infection because the viral pathogenesis includes an intracellular phase. It is important to understand this response, and therefore estimation of the cellular immunity against a viral infection such as NDV should be aligned with estimation of the humoral antibody response. Cell-mediated immune response is essential for virus clearance and may be a key player in vaccinal immunity to NDV [12]. Lambrecht *et al.* have reported the presence of antigen-specific chicken IFN- γ production as an indicator of actively acquired immunity to NDV [13]. Furthermore, cell-mediated immune response was detected in the spleens of chickens vaccinated twice or vaccinated and challenged with wild-type virus [14]. The data obtained from this study are also expected to provide useful information for future probiotic formulations, which may help control the spread of infectious diseases in chickens.

MATERIALS AND METHODS

Animals

Male chicks (White Leghorn Julia strain, newborn chicks) were purchased from Akebono Noen (Ehime, Japan). Chicks ingested feed and water *ad libitum*. Animal care and experiments were carried out in accordance with the guidelines for animal experiments at Okayama University. The animals were housed at a constant temperature ($27 \pm 1^\circ\text{C}$) with a 12 hr dark/light cycle. All procedures were conducted in accordance with the Policy on the Care and Use of the Laboratory Animals at Okayama University. The body weights of chicks were measured daily during this experiment.

Administration of *L. acidophilus* L-55 and NDV vaccine inoculation

Freeze-dried powder of LaL-55 was prepared as previously reported [8]. Chicks ($n=24$) were randomly divided into four groups and were maintained on different concentrations of freeze-dried LaL-55 (high-dose group, 0.5 mg/100 g body weight; middle-dose group, 0.15 mg/100 g body weight; low-dose group, 0.05 mg/100 g body weight; and control group, 0 mg/100 g body weight). Each volume of freeze-dried LaL-55 powder was suspended in 1 mL of distilled water and compulsively administered orally to chicks daily from 1 to 6 weeks of age. Identical volumes of distilled water were administered to chicks in the control group.

The live attenuated NDV vaccine (10 μL ; ND clone 30, Intervet, Osaka, Japan) was dropped into the eye and nose at 2 weeks of age (primary inoculation). The same dose of NDV

vaccine was administered at the same site in 4-week-old chicks in all treatment groups (the secondary inoculation).

Peripheral blood cell counts

Heparinized blood samples were collected from a wing vein on a weekly basis from 2 weeks of age (before primary inoculation with the NDV vaccine) to 6 weeks of age (2 weeks after secondary inoculation with the NDV vaccine). To determine the total number of leukocytes, lymphocytes, monocytes, and granulocytes, peripheral blood samples from each treatment group were stained with Natt and Herrick staining solution. Cells were counted using a hemocytometer [15].

Cytolytic activity of mononuclear cells

Mononuclear cells were harvested from the small intestine using the following protocol: briefly, chicks were euthanized at the end of the experiment (6 weeks of age, 2 weeks after secondary inoculation), and a portion of the small intestine (5 cm upper from the base of the cecum, approximately 10 cm long) was collected. A portion of the mucosa from the small intestine was collected in RPMI 1640 medium (Nissui Pharmaceutical, Osaka, Japan). After stirring sufficiently to disperse and wash cells, mononuclear cells in the mucosa were isolated using mononuclear cell separation medium (Ficoll-Paque, Amersham, Uppsala, Sweden). The collected cells were washed three times and resuspended in RPMI 1640 medium at a density of 5×10^7 cells/mL. A sample of the liver was collected from a Wistar rat that was anesthetized with ether. Liver cells were dispersed by cutting the liver with scissors and homogenizing the tissue fragment using a 25G needle and syringe. The cells were washed three times and resuspended in RPMI 1640 medium at a density of to 1×10^6 cells/0.7 mL. Chromium-51 (^{51}Cr ; PerkinElmer Inc., Waltham, MA, USA) solution (0.3 mL) prepared to 3.7×10^4 Bq/mL was added to 0.7 mL of the target cell suspension. The mixture was incubated for 1 hr at room temperature under mild shaking conditions. The cells were washed and resuspended in RPMI 1640 medium at a density of 1×10^6 cells/mL. The effector cell suspension (100 μL) and the same volume of target cell suspension were added to a 96-well microplate. The cell suspensions were incubated in a CO_2 incubator (5% CO_2) at 37°C for 2 hr. After incubation and centrifugation, the γ -ray count of the supernatant was measured using a γ -counter (count A). The cytolytic activity of effector cells was calculated using count A, the count obtained under the condition of spontaneous ^{51}Cr release from target cells, and the count obtained under the condition of 100% ^{51}Cr release from target cells treated with 3% sodium lauryl sulfate (Nacalai Tesque, Inc., Kyoto, Japan). The cytolytic activity was expressed as a percentage.

Semiquantitative RT-PCR

The levels of mRNA for pro-inflammatory cytokines (IFN- α , IFN- β , IFN- γ , IL-1 β) were measured using semiquantitative RT-PCR. The sequences for the primer pairs used in this experiment are listed in Table 1 [16–19]. The chicks were euthanized at the end of the experiment (6 weeks of age, 2 weeks after secondary inoculation), and spleen tissue was collected. Mononuclear splenocytes were isolated using mononuclear cell separation medium (Ficoll-Paque; Amersham Bioscience, Uppsala, Sweden). Collected cells were washed three times and resuspended in RPMI-1640 medium (Nissui Pharmaceutical, Tokyo, Japan) to

a density of 5×10^6 cells/mL. The cells were preserved in an RNA stabilizing agent (Ambion, Austin, TX, USA) at -20°C until ready for use. RNA extraction was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The extracted RNA was treated with DNase I (Takara Bio, Otsu, Shiga, Japan), and cDNA synthesis was carried out using oligo dT₁₅. The PCR cycling conditions were as follows: denaturation at 95°C for 1 min, annealing for 1 min at the annealing temperature for each primer set, elongation at 72°C for 30 sec, and a single extension step for 5 min at 72°C . β -actin amplification was carried out over 22 cycles and used as a positive control. The amplification products were visualized using an agarose gel and ethidium bromide staining. The intensity of the amplified target fragments was quantified using the ImageJ 1.42q software (NIH, Bethesda, MD, USA). The relative expression of each cytokine was calculated by dividing the band intensity for each cytokine by the corresponding β -actin intensity.

Statistical analysis

Data represent the mean \pm SE of the samples (n=6 in each

experimental group). Statistical analyses were carried out using Student's t-test. Statistical significance was determined by $p < 0.05$.

RESULTS

Leukocyte counts

Total leukocyte counts in the peripheral blood of chicks increased 1 week after the primary and secondary inoculations with the NDV vaccine (Fig. 1a). The leukocyte count in the high-dose group at 1 week after the primary inoculation (3 weeks old in Fig. 1a) was significantly higher than the average count in the control group ($p < 0.05$). The counts in the three groups that received LaL-55 were significantly higher than that in the control group at 1 week after the secondary NDV inoculation (5 weeks old in Fig. 1a, $p < 0.05$). Lymphocyte counts in the peripheral blood of both the low-dose and high-dose chick groups were significantly higher than that of the control group ($p < 0.05$) at 1 week after the second inoculation with the NDV vaccine (5 weeks old in Fig. 1b). The lymphocyte count of the middle-dose group was significantly higher than that of the control at 2 weeks

Table 1. Primer sets for semiquantitative RT-PCR

Gene name	Primer sequence (5'-3')	Annealing temperature ($^\circ\text{C}$)	References
IFN- γ	Forward: AATGACTTGCCAGACTTACAACTTG	69.0	[16]
	Reverse: TTAGCAATTGCATCTCCTCTGAGACTGG		
IFN- α	Forward: GGGTACGACATCCTGTTGCTC	68.8	[17]
	Reverse: CGGCTGATCCGGTTGAGGAG		
IFN- β	Forward: GCCACAGCCTCCTCAACCAGAT	69.5	[17]
	Reverse: CAACGTCCCAGGTACAAGCACT		
IL-1 β	Forward: TGAGGCTCAATTGCGCTGTA	62.1	[18]
	Reverse: TGTCCAGGCGGTAGAAGATAGGA		
β -actin	Forward: TCTGGCACCACACTTTCTACAA	58.0	[19]
	Reverse: CACAGGACTCCATACCCAAGA		

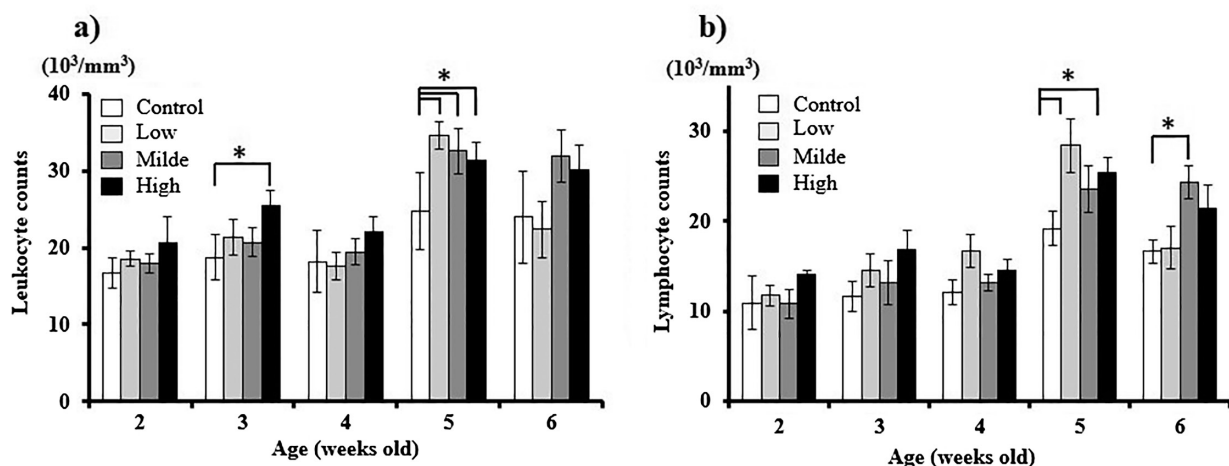


Fig. 1. The course of leukocyte (a) or lymphocyte (b) counts in the peripheral blood of chicks that received the Newcastle disease virus (NDV) vaccine and oral LaL-55. Chickens were divided into four groups (n=6) and were maintained on different concentrations of freeze-dried LaL-55 (high-dose group, 0.5 mg/100 g body weight; middle-dose group, 0.15 mg/100 g body weight; low-dose group, 0.05 mg/100 g body weight; and control group, 0 mg/100 g body weight). Heparinized blood samples were collected every week from 2 weeks of age (before the primary NDV vaccine inoculation) to 6 weeks of age (2 weeks after the secondary inoculation). Representative data from three independent experiments are shown. * $p < 0.05$ vs. control. Error bars represent the SE.

after the second inoculation (6 weeks old in Fig. 1b, $p < 0.05$). Monocyte counts tended to be higher in groups that received LaL-55, and the monocyte count of the middle-dose group was significantly higher than that of the control at 5 weeks of age. Monocyte counts did not change following inoculation with the NDV vaccine. Granulocyte counts were not significantly different among the treatment groups (data not shown).

Cytolytic activity

LaL-55 administration increased the cytolytic activity of mononuclear cells from the intestinal mucosa in a dose-dependent manner (Fig. 2). The cytolytic activity of mononuclear cells in the high-dose group ($25.0 \pm 2.3\%$) was significantly higher ($p < 0.01$) than the cytolytic activities of cells in the control ($10.3 \pm 2.9\%$) and low-dose groups ($12.8 \pm 1.7\%$; Fig. 2).

Cytokine expression

Levels of IFN- γ mRNA were increased in a dose-dependent manner after oral administration of LaL-55. IFN- γ expression was significantly higher in the middle- and high-dose groups ($p < 0.01$) than that in the control group (Fig. 3a). The mRNA levels of IL-1 β , IFN- α , and IFN- β did not change after administration of LaL-55 (Fig. 3b–3d).

DISCUSSION

Various strategies, including vaccination, have been used to combat pathogenic organisms. However, due to the excessive use of antibiotics among humans and livestock and changes in the environment, antibiotic-resistant organisms and some pathogens have posed a huge problem for public health. In parallel with the production of drugs and vaccines, probiotics are being considered as an additional effective method for disease prevention and treatment. Recently, live or dead bacteria have been used to enhance the efficacy of vaccines. Probiotic bacteria have become a target of interest for promoting health benefits in humans and animals. LAB enhance vaccine efficacy by stimulating both the innate and adaptive immune responses [3–5]. In this study, we focused on LaL-55 and determined its beneficial effects on vaccine-administered animals. Some researchers have also reported that oral administration of LaL-55 induced immunosuppressive effects against experimental allergic rhinitis and experimental atopic dermatitis [8, 9]. Hence, LaL-55 has been expected to promote beneficial effects on the immune system of the chicken.

Differential white blood cell counts are important for detecting active infection by viruses and bacteria. The results of this study indicated that total and individual lymphocyte counts in the peripheral blood were increased by all doses of LaL-55 after inoculation with the live attenuated NDV vaccine. It has been reported that the number of lymphocytes was increased by *L. acidophilus* administration in mice and chicken [1, 2, 6, 9]. Few studies have shown the effects of a probiotic on either differential or total leukocyte counts [20–22]. A previous report also showed that the administration of lactobacilli augmented the proliferation of lymphocytes depending upon the dose and strain of the bacteria [23]. Administration of *L. acidophilus* has been shown to affect T cell populations in the blood and lymphoid organs of chickens [2, 9]. In this study, we did not clarify the composition of lymphocytes after LaL-55 administration in detail.

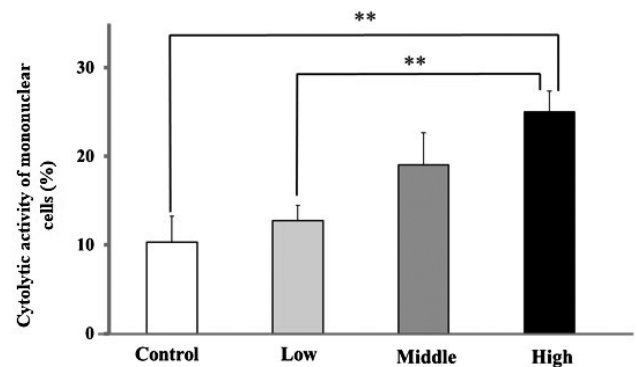


Fig. 2. Effect of LaL-55 administration on cytolytic activity of mononuclear cells. Mononuclear cells were collected from the intestinal mucosa. Birds ($n = 6$) were inoculated with the live attenuated Newcastle disease virus (NDV) vaccine at 2 weeks of age. Two weeks later (4 weeks of age), chicks were inoculated a second time with the NDV vaccine. Representative data from three independent experiments are shown. ** $p < 0.01$ vs. control. Error bars represent the SE.

However, the more prominent effects of LaL-55 administration on lymphocyte counts that were observed during the secondary response compared with the primary response indicate that LaL-55 may affect the memory mechanisms of the immune system. The elevation of clonal expansion of lymphocytes responsible for eliminating NDV after exposure to attenuated NDV indicates that oral administration of LaL-55 may reinforce the resistance to other viruses, such as avian influenza, through the expansion of corresponding lymphocyte clones. Elevated numbers of monocytes in the peripheral blood of LaL-55-administered chicks show that the elimination of antigens by phagocytosis and antigen-presenting activity are consequentially elevated following LaL-55 administration. However, it is necessary to conduct more experiments to determine how LaL-55 affects the total numbers and composition of lymphocytes.

In the present study, the cytolytic activity of intestinal mucosal mononuclear cells was measured at 2 hr after the addition of target cells. NK cells are part of the innate immune system and are known to secrete cytokines and kill tumor cells or cells infected with viruses. Therefore, NK cells protect the host from pathogens through targeted elimination. The ^{51}Cr release assay (CRA) is a standard method for measuring the cytotoxic effects of NK cells and the activity of cytolytic T lymphocytes [24–28]. Antigen-specific cell lysis exerted by CTLs begins after 2–3 days of exposure to the antigen. Therefore, the cytolytic activity measured in this study is due to NK cells. The results of the present study indicate that oral administration of LaL-55 elevates the activity of NK cells in the intestinal mucosa. Previous studies have also reported that *L. casei* Shirota and a heat-killed strain of *L. acidophilus* La205 enhance the activity of NK cells in the peripheral blood [29, 30]. The enhanced activity of NK cells was previously explained to be due to the interaction between dendritic cells (DCs) and LAB [31]. *In vitro* studies have also indicated that *L. acidophilus* enhances NK cell activity [30].

LAB stimulate the production of IL-12 by DCs, which increases the IFN- γ production, activation, and cytotoxic activity of NK cells [32]. In our study, we also observed the upregulation of

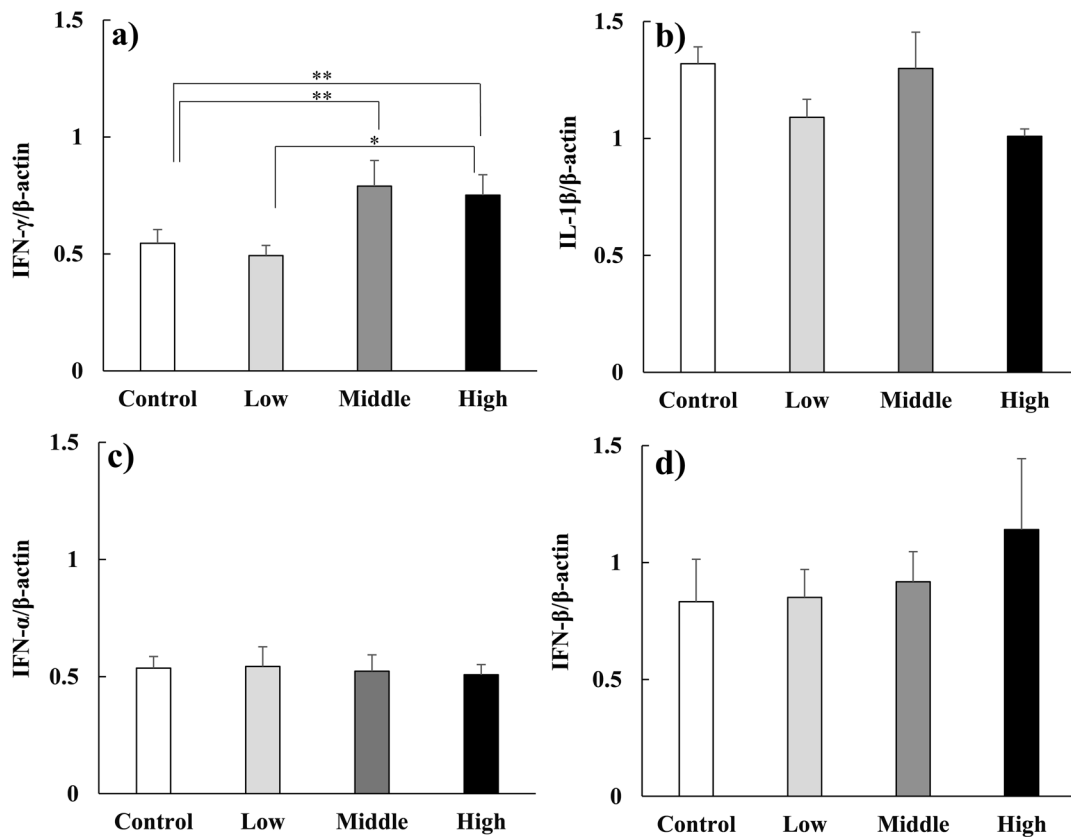


Fig. 3. Effect of LaL-55 administration on mRNA levels of cytokines in splenic mononuclear cells. (a) Relative gene expression of IFN- γ . (b) Relative gene expression of IL-1 β . (c) Relative gene expression of IFN- α . (d) Relative gene expression of IFN- β . * $p < 0.05$ vs. β -actin; ** $p < 0.01$ vs. β -actin. Error bars represent the SE.

IFN- γ mRNA in the mononuclear cells from the middle- and high-dose groups; this upregulation of IFN- γ may explain the enhanced activity of the NK cells. In addition, our results showed that the total number of monocytes in the peripheral blood increased after LaL-55 administration, but the expression of IL-1 β mRNA did not change. This may suggest that LaL-55 administration affects DCs, but not macrophage function. However, it is necessary to confirm this by studying the effects of LaL-55 administration on DC activity and DC-mediated production of IL-12 in chicks. *In vitro* studies have indicated that *L. acidophilus* is a strong inducer of Th1 cytokines, including IL-12 and IFN- γ [6, 7]. The data in this study show that IFN- γ mRNA expression in the splenic mononuclear cells was upregulated following oral administration of the middle or high dose of LaL-55 at 2 weeks after secondary immunization with the NDV vaccine. By contrast, the mRNA levels of type I interferons (IFN- α and IFN- β) and IL-1 β did not change. IFN- γ , which is produced by Th1, CTL, and NK cells, is involved in macrophage activation, Th1 differentiation, B cell differentiation, and NK cell activation. In the present study, it is not clear which type of cells upregulated IFN- γ mRNA, since the spleen contains all of the previously mentioned types of cells. However, it is possible to assume that IFN- γ production is related to the enhanced activity of NK cells and Th1 cells. The elevation of IFN- γ mRNA levels has been shown in studies related to the effect of LAB on the Th1/Th2 balance. Administration of other strains of LAB (*L. brevis*, *L. acidophilus*) increases the production of Th1 cell-specific cytokines (IFN- γ and IL-12) [23,

33]. Our data support these previous findings, although we used a different strain of *Lactobacillus*. Takeda *et al.* reported that oral administration of killed LAB augments the production of IFN- α in the bronchoalveolar lavage fluid of mice [29]. In the present study, LaL-55 administration did not alter type I interferon gene expression in splenocytes. This discrepancy may be caused by differences in the cell types and LAB species used in these studies.

Overall, LaL-55 enhances the immune response following administration of a live attenuated NDV vaccine by increasing the number of leukocytes in the peripheral blood, augmenting NK cell activity, and increasing IFN- γ expression. Therefore, LaL-55 could be used as a tool to enhance the immune response against viral infection in avian species.

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