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Full Paper

Differential effects of orally administered *Lactobacillus acidophilus* L-55 on the gene expression of cytokines and master immune switches in the ileum and spleen of laying hen with an attenuated Newcastle disease virus vaccine

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This study aimed to evaluate the benefits of oral administration of *Lactobacillus acidophilus* strain L-55 (LaL-55) to chickens inoculated with a Newcastle disease virus (NDV)-based live-attenuated vaccine by examining the mRNA expression of several genes related to viral infection in the spleen and ileum by quantitative reverse transcription polymerase chain reaction. In the spleen, interferon (IFN)-α was significantly higher in the low- and middle-dose LaL-55 groups at 6 weeks than at 4 weeks. IFN regulatory factor (IRF)-3 and IRF-7 expression was significantly higher in the low-dose LaL-55 group than in the middle- and high-dose LaL-55 groups. In the ileum, melanoma differentiation-associated gene 5 showed a dose-dependent increase at 4 weeks. IFN-γ and IRF-7 showed dose-dependent increases at 6 weeks. These results suggested that LaL-55 boosts the immune response to the NDV vaccine, albeit by different mechanisms in the spleen and ileum.

Key words: *Lactobacillus acidophilus*, Newcastle disease virus, MDA5, IRF, ileum

INTRODUCTION

Lactic acid bacteria (LAB) are normal inhabitants of the gastrointestinal tract and classified as probiotic bacteria. 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]. Probiotics such as LAB can regulate inflammatory response by stimulating immune cells to produce cytokines. However, only a few published reports have demonstrated the benefits of probiotic bacteria with respect to the avian immune system. Furthermore, 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]. *Lactobacillus acidophilus* is considered a probiotic strain with anticancer and cholesterol-lowering properties and an antagonist against enteric pathogens. *L. acidophilus* has also been proven to elevate immunological activity by stimulating the innate and adaptive immune responses [3–5]. Clinical trials involving the oral administration of *L. acidophilus* strain L-55 (LaL-55) showed that this strain effectively suppresses experimental allergic rhinitis [6] and experimental atopic dermatitis in mice [7]. *In vitro* studies have indicated that some specific strains of LAB are strong inducers of T helper type 1 (Th1) cytokines, such as interleukin (IL)-12 and interferon (IFN)-γ [8]. One study has indicated that LaL-55 modulates the immune reaction of chickens inoculated with a Newcastle disease virus (NDV)-attenuated vaccine [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 in the ileum, one of the gates for NDV. The data obtained from this study are also expected to provide useful information for future probiotic formulations that may help control the spread of infectious diseases in chickens.

Newcastle disease is one of the most important poultry viral diseases worldwide. NDV is classified into lentogenic (low virulence), mesogenic (moderate virulence), and velogenic (high

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virulence) strains according to the pathogenicity in chickens [10]. 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 [11]. Inactivated oily vaccines are used for enhancing and sustaining immunity [11, 12]. The attenuated NDV vaccine used in this study was a lyophilized version of an attenuated NDV clone 30 injected into a growing chicken egg to evaluate the immune responses. Vaccines are commonly administered by the eye- or nose-drop method. Generally, cellular immunity is crucial in viral infection because viral pathogenesis includes an intracellular phase. It is important to understand this response; therefore, the estimation of the cellular immunity against viral infection, such as NDV, should be aligned with the estimation of the humoral antibody response. A cell-mediated immune response is essential for virus clearance and may be a key player in vaccinal immunity to NDV [12]. Lambrecht *et al.* reported the presence of antigen-specific chicken interferon-gamma (IFN-γ) production as an indicator of actively acquired immunity to NDV [13]. Furthermore, a cell-mediated immune response has been detected in the spleen of chickens vaccinated twice or vaccinated and challenged with wild-type virus [14].

IFNs play an essential role in innate immunity against viruses [15]. The crucial role of IFNs is the recognition of viral pathogen-associated molecular patterns by cellular pattern recognition receptors (PRRs) [16]. There are three major classes of PRRs associated with the activation of IFN pathways. The first category of PRRs is the family of retinoic acid-inducible gene-I-like receptors, including melanoma differentiation-associated gene 5 (MDA5). The second class of PRRs is the family of Toll-like receptors (TLRs), such as TLR3 and TLR7 [17]. The third category of PRRs is the family of DNA sensors [18]. When these PRRs are activated, they can recruit specific adaptor proteins, such as myeloid differentiation primary response gene 88 (MyD88) or Toll/IL-1 receptor domain-containing adaptor-inducing IFN-β. These signaling pathways ultimately converge on the activation of IFN regulatory factors (IRFs). Activated IRFs translocate into the nucleus to regulate IFN expression. However, the immune status in the ileum of chickens after NDV vaccination has been unclear. The differences in immune status between the ileum and spleen in chickens inoculated with an attenuated NDV vaccine have also been unclear.

This study aimed to evaluate the beneficial effects of LaL-55 oral administration on the gene expression of cytokines, MDA5, and transcriptional factors (Blimp-1, IRF-3, IRF-7, MyD88, and STING) associated with antiviral immunity in the ileum and spleen of chicks inoculated with a live-attenuated NDV vaccine.

MATERIALS AND METHODS

Animals

Eggs (White Leghorn) were purchased from Kui Poultry Co., Ltd. (Mihara, Hiroshima, Japan). Eggs were incubated at 37.7°C ± 1°C until hatching. After hatching, chicks were maintained in a coccidian-free room and provided feed and water *ad libitum*. The animals were housed at a constant temperature (27°C ± 1°C) with a 12 hr dark/light cycle. All procedures were approved by the Animal Care and Use Committee of Okayama University (OKU-2020201) and conducted in compliance with the Policy on the Care and Use of the Laboratory Animals of Okayama University. Animal care and experiments were also carried out in accordance with the guidelines for animal experiments at Okayama University. The body weights of the chicks were measured daily during this experiment.

LaL-55 administration and NDV vaccine inoculation

Freeze-dried LaL-55 powder was prepared as reported previously [19]. Chicks (n=24) were randomly divided into four groups and provided different concentrations of the 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 the freeze-dried LaL-55 powder was suspended in 1 mL 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 NDV vaccine dose was also administered to the same sites at 4 weeks of age in all treatment groups (secondary inoculation).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The mRNA levels for cytokines (IFN-α, IFN-β, IFN-γ, IFN-λ, and IL-1β), MDA5, and transcriptional factors (Blimp-1, IRF-3, IRF-7, MyD88, and STING) were determined using qRT-PCR. The sequences for the primer pairs used in this experiment are listed in Table 1. The chicks were euthanized at the end of the experiment (4 and 6 weeks of age, 2 weeks after primary and secondary inoculation), and ileum and spleen tissues were collected. The tissues were preserved at −20°C until they were used for analyses 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 (dT_{18}). Real-time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was conducted in a MiniOpticon Real-Time PCR System (Bio-Rad Laboratories Inc.) using a Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix Kit (Agilent Technologies, West Cedar Creek, TX, USA). The amplification of β-actin was used as an internal control. The samples were heated at 95°C for 1 min and then subjected to 40 cycles of denaturation at 95°C for 15 sec and annealing/elongation for 1 min at 60°C. Amplification was performed for three independent samples per group, with triplicate reactions for each sample on the same plate. The relative mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method [20].

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Data were statistically evaluated by two-way analysis of variance (ANOVA) with Tukey's test using IBM SPSS Statistics 20.0. Differences were considered significant at $p<0.05$ and $p<0.01$.

RESULTS

The results for the mRNA expression levels in the spleen are indicated in Fig. 1. The expression levels of the cytokines examined other than IFN-β and IFN-λ showed dose-dependent

Table 1. Primer sets for quantitative RT-PCR

	Primer sequence (5′-3′)	Annealing temperature (°C)	Accession No.
IFN-α	Forward: GGGTACGACATCCTGTTGCTC	68.8	AB_021153
	Reverse: CGGCTGATCCGGTTGAGGAG		
IFN-β	Forward: GCCACAGCCTCCTCAACCAGAT	60.0	AY_831397
	Reverse: CAACGTCCCAGGTACAAGCACT		
IFN-γ	Forward: AAGTCAAAGCCGCTACATCAAAC	60.0	NM_205149.1
	Reverse: CTGGATTCTCAAGTCGTTCATCG		
IFN-λ	Forward: GGAGGATGAAGGAGCAGTTTG	60.0	NM_001128496.1
	Reverse: ACGGTGATGGTGAGGTCC		
IL-1β	Forward: GTACCGAGTACAACCCCTGC	60.0	XM_015297469.1
	Reverse: AGCAACGGGACGGTAATGAA		
Blimp-1	Forward: GGCAGCCTGTCAGAATGGAAT	60.0	XM_004940353.3
	Reverse: GCTCCTTCTTTGGGACGCTCT		
MDA5	Forward: GCAAAAACCAGCACTGAATGGG	60.0	GU570144.1
	Reverse: CGTAAATGCTGTTCCACTAACGG		
IRF-3	Forward: ACCACATGCAGACAGACTGACACT	60.0	AF268079.1
	Reverse: GGAGTGGATGCAAATGCTGCTCTT		
IRF-7	Forward: GCCTGAAGAAGTGCAAGGTC	60.0	NM_205372.1
	Reverse: CTCTGTGCAAAACACCCTGA		
MyD88	Forward: AAGGTGTCGAGGATGGTGGTC	60.0	NM_001030962.4
	Reverse: GGAATCAGCCGCTTGAGACGAG		
STING	Forward: GGTCCTACTACATCGGCTACCTGA	60.0	XM_015293528.2
	Reverse: GGCCTGAGCTTGTTGTCCTTATCT		
β-actin	Forward: GAGAAATTGTGCGTGACATCA	60.0	NM_205518.1
	Reverse: CCTGAACCTCTCATTGCCA		

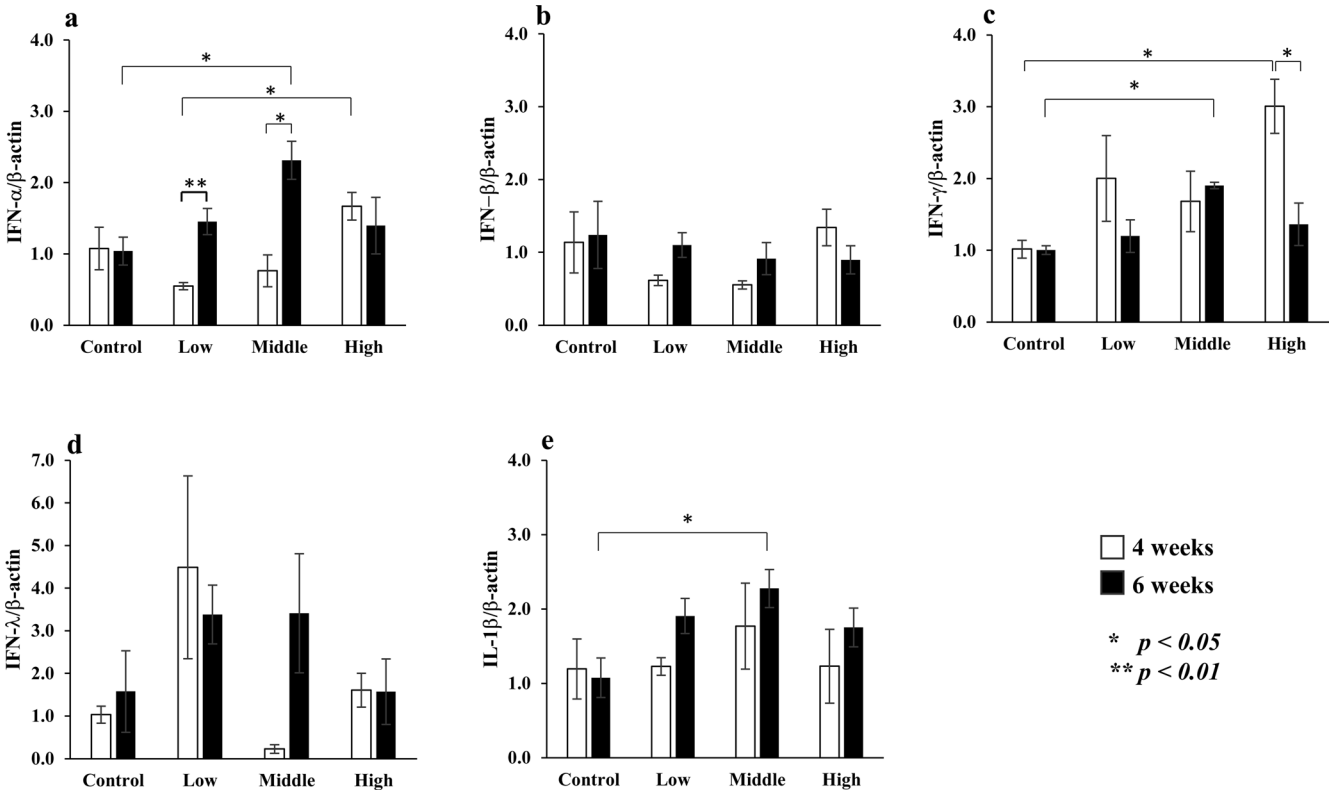

Fig. 1. mRNA expression levels of cytokines in the spleen of chickens inoculated with an NDV vaccine: (a) IFN-α, (b) IFN-β, (c) IFN-γ, (d) IFN-λ, and (e) IL-1β. Open columns represent 4-week-old chicks; filled columns represent 6-week-old chicks. Amplification was performed for three independent samples with triplicate reactions carried out for each sample. The relative mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method. Data are presented as the mean ± SEM and were analyzed by two-way ANOVA with Tukey's test using IBM SPSS Statistics 20.0. *p<0.05; **p<0.01.

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increases at 6 weeks, reaching their peaks in the middle-dose LaL-55 group. The IFN-α and IFN-γ mRNA levels increased in a dose-dependent manner after LaL-55 administration (Fig. 1a and 1c). The IL-1β expression level also increased in a dose-dependent manner after oral administration of LaL-55 (Fig. 1e). The IFN-β and IFN-λ mRNA levels did not change after LaL-55 administration (Fig. 1b and 1d). IFN-α mRNA expression was significantly higher in the low- and middle-dose groups at 6 weeks than in the spleen samples at 4 weeks ($p<0.01$ and 0.05).

The results for the cytokine mRNA expression levels in the ileum are indicated in Fig. 2. The expression levels of all the cytokines examined in this study did not differ among the groups at 4 weeks. IFN-α and IFN-β mRNA expression decreased in a dose-dependent manner after oral administration of LaL-55 (Fig. 2a and 2b). In contrast, IFN-γ showed a dose-dependent increase after 6 weeks of oral administration of LaL-55, but increase was not statistically significant. However, the IFN-γ mRNA expression level in the high-dose group was significantly higher at 6 weeks than at 4 weeks (Fig. 2c). The IFN-λ expression level at 6 weeks showed a dose-dependent increase after the oral administration of LaL-55, the increase was not statistically significant (Fig. 2d). The IL-1β mRNA level did not change after LaL-55 administration (Fig. 2e).

The mRNA expression levels of transcriptional factors in the spleen were also determined. The MDA5 expression level in the spleen decreased in a dose-dependent manner after the oral administration of La-L55 (Fig. 3a). The IRF-3 and IRF-7 mRNA expression levels were significantly higher in the low-dose group than in the middle- and high-dose groups (Fig. 3b and 3c). The Blimp-1, STING, and MyD88 mRNA levels did not change in the spleen after LaL-55 administration (Fig. 3d–3f).

Finally, the mRNA expression levels of transcriptional factors in the ileum were determined. The MDA5 expression level showed a dose-dependent increase at 4 weeks. However, the expression levels of MDA5 at 6 weeks were not different (Fig. 4a). The IRF-3 and MyD88 mRNA expression levels did not change in the ileum after LaL-55 administration (Fig. 4b and 4f). The IRF-7 expression levels after 6 weeks of oral administration of LaL-55 showed dose-dependent increases and were higher than that of the high-dose group at 4 weeks (Fig. 4c; $p<0.05$). The Blimp-1 and STING levels showed dose-dependent decreases at 4 weeks (Fig. 4d and 4e).

DISCUSSION

Infectious diseases caused by various pathogens, such as highly pathogenic strains of influenza and porcine fever, not only cause stunting of the livestock and poultry industries but can also be fatal at times, causing enormous economic losses to these industries. Various strategies, including vaccination, have been used to combat pathogenic organisms. However, due to the excessive use of antibiotics among humans and livestock and environmental changes, antibiotic-resistant organisms and some pathogens pose a huge public health problem. In parallel with the

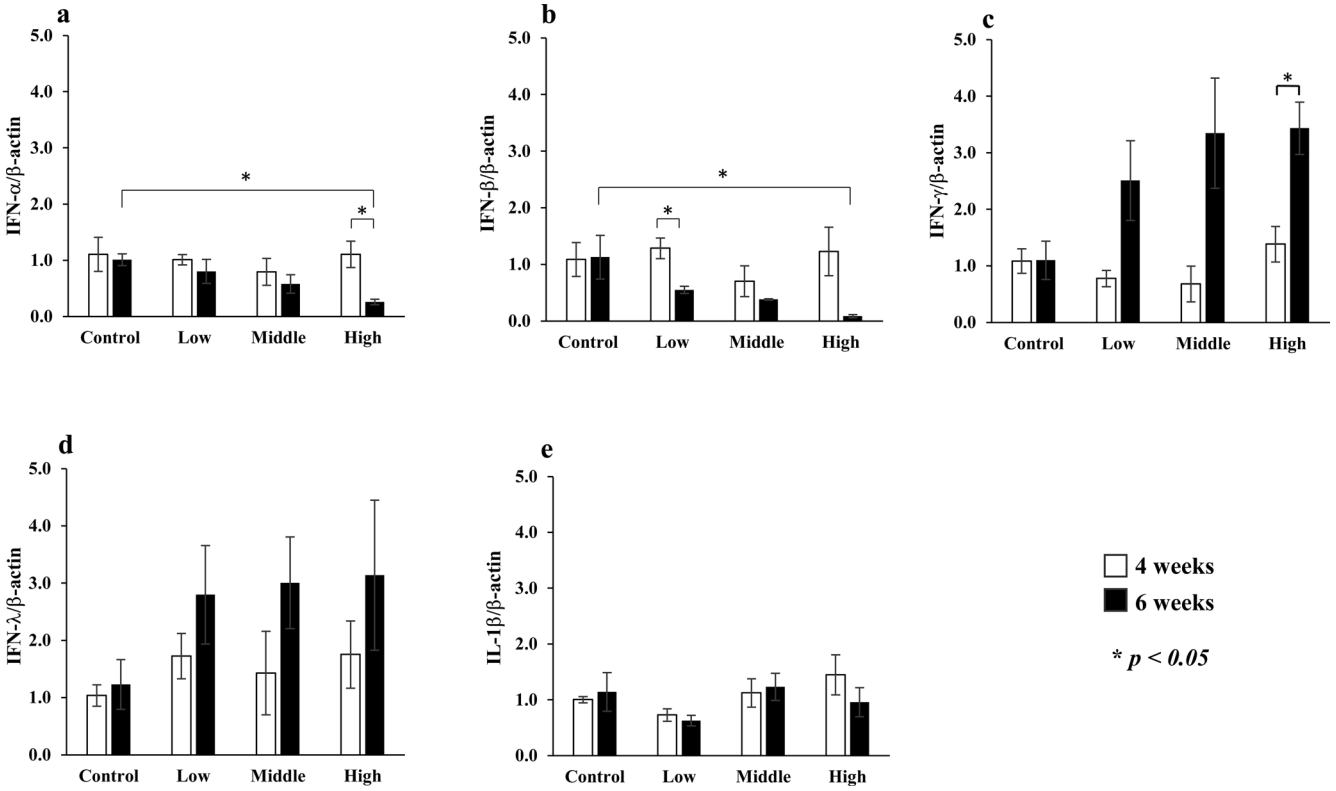

Fig. 2. mRNA expression levels of cytokines in the ileum of chickens inoculated with an NDV vaccine: (a) IFN-α, (b) IFN-β, (c) IFN-γ, (d) IFN-λ, and (e) IL-1β. Open columns represent 4-week-old chicks; filled columns represent 6-week-old chicks. Amplification was performed for three independent samples with triplicate reactions carried out for each sample. The relative mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method. Data are presented as the mean ± SEM and were analyzed by two-way ANOVA with Tukey's test using IBM SPSS Statistics 20.0. *$p<0.05$.

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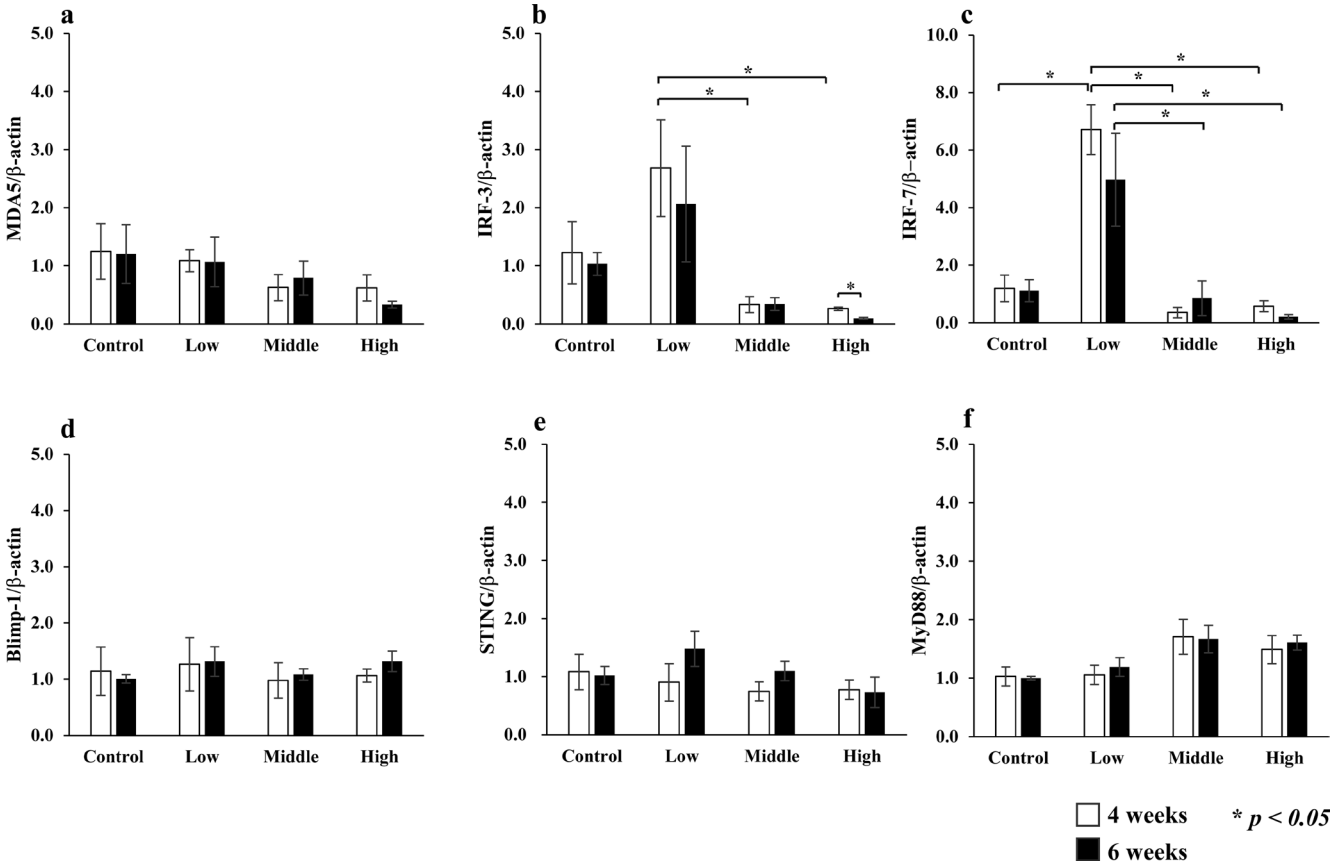

Fig. 3. mRNA expression levels of transcriptional factors in the spleen of chickens inoculated with an NDV vaccine: (a) MDA5, (b) IRF-3, (c) IRF-7, (d) Blimp-1, (e) STING, and (f) MyD88. Open columns represent 4-week-old chicks; filled columns represent 6-week-old chicks. Amplification was performed for three independent samples with triplicate reactions carried out for each sample. The relative mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method. Data are presented as the mean ± SEM and were analyzed by two-way ANOVA with Tukey's test using IBM SPSS Statistics 20.0. *p<0.05; **p<0.01.

production of drugs and vaccines, probiotics are considered an additional effective method for disease prevention and treatment. Live bacteria supplements, such as LAB, have recently become widely used, and probiotics have become an important target for providing health benefits in humans and animals. Recently, live and 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 enhances vaccine efficacy by stimulating the innate and adaptive immune responses [2–5]. This study focused on LaL-55 and determined its beneficial effects on chicks administered a vaccine. In a previous study, chickens supplemented with various lactobacilli showed enhanced IgM and IgG responses to soluble antigens [8]. In another study, the administration of probiotics containing *L. acidophilus* enhanced the serum IgA response to keyhole limpet hemocyanin (a highly immunogenic T-cell dependent antigen) [19]. Hence, LaL-55 is expected to promote beneficial effects on the immune system of the chicken. This study evaluated the effects of LaL-55 oral administration on the gene expression of cytokines and master immune switches in the ileum and spleen of chicks inoculated with an attenuated NDV vaccine.

IFNs are known as essential factors of innate immune responses against virus infection [14, 15]. This study showed that IFN-γ mRNA expression in the spleen was upregulated after oral administration of the middle dose of LaL-55 at 2 weeks after secondary immunization with the NDV vaccine (Fig. 1c). In contrast, the IFN-β and IFN-λ mRNA levels did not change (Fig. 1b and 1d). The results also showed that dose-dependent upregulation of IFN-γ mRNA was observed in the ileum at 6 weeks (Fig. 2c). LAB stimulates IL-12 production by dendritic cells, increasing IFN-γ production and activation [6, 7, 21]. IFN-γ produced by Th1 cells, cytotoxic T lymphocytes, and natural killer (NK) cells is involved in macrophage activation, Th1 differentiation, B-cell differentiation, and NK cell activation. In vitro studies have indicated that some specific strains of LAB are strong inducers of Th1 cytokines, such as IL-12 and IFN-γ [8]. Administration of other LAB strains (*Lactobacillus brevis* and *L. acidophilus*) increases the production of Th1 cell-specific cytokines (IFN-γ and IL-12) [22, 23]. Data in a previous study also showed that IFN-γ mRNA expression in splenic mononuclear cells was upregulated after the oral administration of middle or high doses of LaL-55 at 2 weeks after secondary immunization with the NDV vaccine [9]. Cytotoxic CD8 T cells are one of the major sources of IFN-γ production. However, the CD8 mRNA levels did not change in this study (data not shown). In this study, it is not clear which type of cells upregulated IFN-γ mRNA, as the spleen contains all previously mentioned types of cells. However, it is possible to assume that IFN-γ production is related

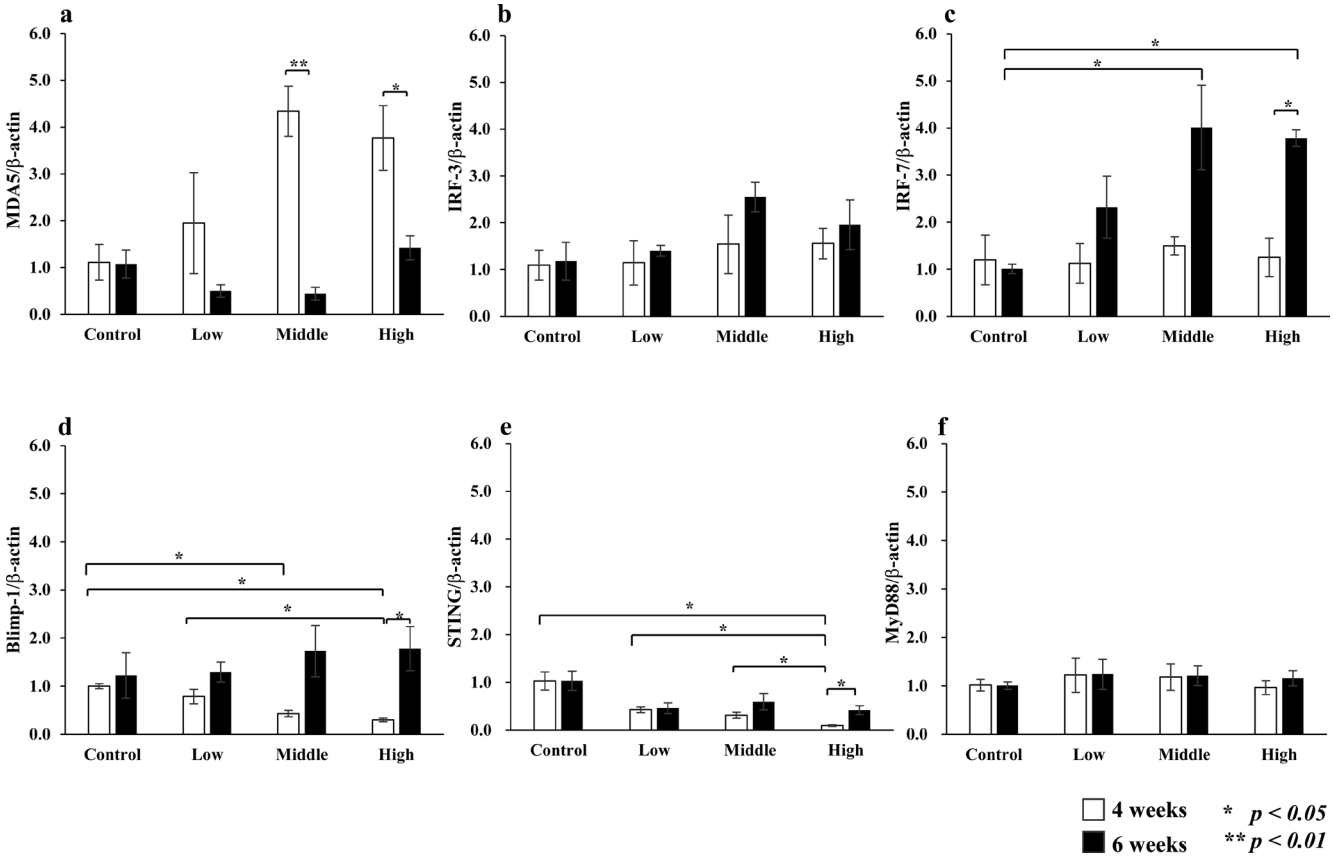

Fig. 4. mRNA expression levels of transcriptional factors in the ileum of chickens inoculated with an NDV vaccine: (a) MDA5, (b) IRF-3, (c) IRF-7, (d) Blimp-1, (e) STING, and (f) MyD88. Open columns represent 4-week-old chicks; filled columns represent 6-week-old chicks. Amplification was performed for three independent samples with triplicate reactions carried out for each sample. The relative mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method. Data are presented as the mean ± SEM and were analyzed by two-way ANOVA with Tukey's test using IBM SPSS Statistics 20.0. *p<0.05; **p<0.01.

to the enhanced activity of NK and Th1 cells. Elevation of IFN-γ mRNA levels has been observed in studies related to the effect of LAB on the Th1/Th2 balance.

The main function of the IFN-α is to alert the organism in the case of viral infection [24]. IFN-α expression in spleen was enhanced at 6 weeks by the middle dose of LaL-55 in this study. However, our previous study showed that LaL-55 administration did not alter the expression levels of IFN-α in splenocytes at 6 weeks [9]. The difference between this and the previous study is that the entire spleen was used in this study rather than just spleen monocytes. Since the entire spleen was used in this study, it is possible that there was a wide variety of IFN-α-producing cells, including monocytes, present. IFN-α-producing cells include a variety of cells, such as macrophages, dendritic cells, lymphocytes, neutrophils, and fibroblasts. In the previous study and this study, we did not examine granulocytes such as neutrophils. Our previous study indicated that total number of lymphocytes increased in the peripheral blood [9]. It has also been reported that *L. acidophilus* promotes heterophil activation [25]. Therefore, regarding the usefulness of LaL-55 administration, it may be necessary to investigate IFN-α-producing cells that may contain granulocytes, such as heterophils, other than monocytes in the future.

The mRNA levels of IFN-β were similar in all groups at the two time points, demonstrating different effects of LaL-55 on different types of type I IFN. Comparing the IFN-α mRNA levels at the two time points, the expression of IFN-α was found to be low after the first vaccination with the low and medium doses of LaL-55. Initially, low levels developed in the 2 weeks after the first vaccination, but the level became even more pronounced at 2 weeks after the second vaccination. In the high-dose group, the expression level of IFN-α was higher than those in the other groups at 4 weeks. The difference compared with the low-dose group was significant. It is unknown why this changed at 6 weeks. Further research may be needed on the dose of LaL-55. On the other hand, IFN-α expression at 6 weeks was correlated with IL-1β (Fig. 1a and 1e). IL-1β is an inflammatory cytokine and is widely known as a potent modulator of IFN-α-induced antiviral gene expression [26]. IL-1β is also known to play an important role in both inflammation and viral pathogenesis [27, 28]. Guo et al. reported that IL-1β may enhance the antiviral response of IFN-α by regulating the JAK-STAT signaling pathway [29]. These facts may suggest that the expression of IFN-α in the spleen in the chicks administered both the low and medium doses of LaL-55 was controlled by IL-1β.

In the ileum at 6 weeks, IFN-α and IFN-β gene expression after NDV vaccination was inversely correlated with the amount of LaL-55 administered (Fig. 2a and 2b). The reduced expression of

type I IFN observed as a result of the administration of high doses of LaL-55 may have occurred to avoid excessive downmodulation of the immune response in the negative feedback loop [30]. The results of this study may require further investigation of the appropriate doses and methods of administration for LaL-55.

Our results show that there are differences in the chicken immune response in the spleen and ileum to NDV vaccination in conjunction with frequent intake of an LaL-55 supplement. The most striking difference was the difference in the expression patterns of type I IFN in the spleen and ileum of the chicks administered LaL-55 compared with the chicks in the control group. In the case of type II IFN, significant upregulation was observed in the spleen (Fig. 1c), and an upward trend was observed in the ileum of chicks administered LaL-55, but it was not significant (Fig. 2c). These reactions evoked by LaL-55 administration primarily involved the spleen, which is a secondary immune organ. In our previous research, we showed that the cytotoxic activity of NK cells was enhanced by LaL-55 administration [9]. Since activated NK cells are also considered to be type II IFN-producing cells, it is possible that IFN was locally induced because of NK cell activation in the ileum. On the other hand, the expression of IFN-α and IFN-γ in the spleen may reflect the immune response as a secondary lymphoid organ. In other words, it may indicate an antiviral response as a result of antigen-presenting cells migrating to the spleen due to NDV infection of the ileum. As the spleen is one of the secondary lymphoid organs, boosting the immune defense in this organ is of great benefit for the animal to combat harmful agents. In fact, the enhancement of cytokine expression in the chicken spleen by administration of a probiotic supplement has been reported previously [31, 32].

In this study, oral administration of LaL-55 increased the levels of IRF-7 and IFN-γ mRNA in the ileum after the second NDV vaccination in a dose-dependent manner (Figs. 2c and 4c). IRF-7 expression in the spleen was significantly higher in the low-dose LaL-55 group than in the middle- and high-dose groups at both 4 and 6 weeks. Similarly, IRF-3 expression was more prominent in the low-dose group compared with the middle- and high-dose groups, but the differences were only significant at 4 weeks. The expression patterns of IRF-3 and IRF-7 in the spleen were not consistent with the expression patterns of IFN-α and IFN-γ; however, the expressions of these IFNs at 6 weeks were significantly increased in the middle-dose group compared with the control group (Fig. 1a and 1c). The expression of IFN-γ in the spleen at 4 weeks was also significantly higher in the high-dose group compared with the control group (Fig. 1c). The IRFs comprise a growing family of related transcriptional proteins that were first identified as regulators of the IFN-α/β gene promoter and interferon-stimulated response components of several IFN-stimulating genes [33]. IRF-3 and IRF-7 have additional self-suppressing motifs that are relaxed in response to double-stranded RNA induced by serine phosphorylation of TANK-binding kinase 1, but IRF-3 is limited in function without stimulation. IRF-7 is known to have additional elements that increase both basal and virus-inducing activity and is thought to be more indiscriminate with its binding motifs [34]. Further studies may be needed on the mechanism of regulation of gene expressions of antiviral factors after LaL-55 administration, including post-transcriptional modification of these factors.

Recently, Carlin *et al.* reported that mice deficient in key transcriptional factors, including IRF-3 and IRF-7, remain resistant to severe dengue virus infection [35]. Splenocytes from their gene knockout mice predominantly activated a transcriptional program compatible with the IL-12/IFN-γ/IRF-1 signaling pathway after dengue virus infection. These results may suggest that the oral administration of LaL-55 induces IFN-γ expression by an alternative pathway of IFN-γ production rather than the IRF-3, IRF-7, MDA5, and MyD88 signaling pathways and induces the local antiviral activity against NDV in the ileum of the chicken. Future studies are needed to investigate whether an alternative pathway of IFN-γ production is activated by LaL-55 administration.

Overall, LaL-55 enhances the immune response after the administration of a live-attenuated NDV vaccine by increasing the expression levels of IFN-γ but not type I IFN production in the ileum. Therefore, LaL-55 could be used as a tool to enhance the local immune response against viral infection in avian species. Additionally, LaL-55 enhances IFN-α and IFN-γ gene expression in the spleen after the administration of a live-attenuated NDV vaccine. However, it is unclear from the results of this study which signaling pathway is responsible for stimulation of the IFN-α and IFN-γ gene expression induced by LaL-55 administration. Further research is needed to make more effective use of LaL-55 in livestock production and human health.

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