Effect of dietary Lactobacilli mixture on Listeria monocytogenes infection and virulence property in broilers

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ABSTRACT The present study aimed to investigate the effect of probiotic Lactobacilli addition on Listeria monocytogenes load, inflammatory reaction, and virulence properties in broilers from 1 to 14 D of age. A total of 480 broiler chicks were randomly allocated to 4 treatments of 6 replicates each. All birds were infected with L. monocytogenes on the first day and supplemented an equal amount mixture of Lactobacillus acidophilus and Lactobacillus plantarum at doses of 0 (control), 10⁶, 10⁷, 10¹⁰ cfu/kg of diet. The results showed that on 7 and 14 D after administration, Lactobacilli addition at the 3 doses decreased (P < 0.05) L. monocytogenes loads in the cecum, skin, liver, and spleen by 0.065 to 0.933 log₁₀ cfu, and the pathogen linearly reduced (P ≤ 0.015) with the increasing doses of probiotics in the skin. Serum cytokines including IL-1β, IL-6, tumor necrosis factor-α, and interferon-γ in probiotics treatments were decreased (P < 0.05) by 25.4 to 51.1%. Transcriptional levels of genes related to anti-inflammatory reactions including IL-10, hypoxia inducible factor 1 alpha (HIF1A), prostaglandin E receptor 2, and prostaglandin-endoperoxide synthase 2 in the intestinal mucosa were upregulated (P < 0.05) in Lactobacilli treatments, and linear and quadratic responses (P ≤ 0.019) were found on HIF1A. Furthermore, the probiotics attenuated (P < 0.05) listerial adhesion, pore-forming, and invasion properties by downregulating autolysin Ami, listeriolysin O, internalin A and B, and a linear (P = 0.006) dose response of probiotics was exhibited on flagellin. The findings indicate that dietary coadministration of L. acidophilus and L. plantarum can attenuate L. monocytogenes infection by depressing its intestinal inoculation, translocation, inflammatory reaction, and virulence property in broilers and suggest that the probiotics can be an alternative against listerial infection in broilers.

Key words: broiler, inflammatory reaction, Lactobacilli, Listeria monocytogenes, virulence property

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INTRODUCTION

Listeria monocytogenes is a facultative intracellular foodborne pathogen that infects a wide variety of species especially farm animals and immunocompromised humans, causing the life-threatening disease listeriosis. Virulence of L. monocytogenes stems from its capacity of adhesion, invasion, and translocation across intestinal barrier during the gastrointestinal phase of infection (Radoshevich and Cossart, 2018). The virulence factors are involved in several surface proteins, of which autolysin Ami and flagellin are responsible for adhesion, internalins are required for intracellular invasion, and listeriolysin O is contributed to the disruption of cell membrane (Portman et al., 2017). Studies have shown that the virulence activity of these proteins could be influenced directly or indirectly by ambient factors such as oxygen, oligopeptides, and betulin (Portman et al., 2017; Rinehart et al., 2018; Lu et al., 2019).

Probiotic bacteria have long been used as a health-promoting agent in the aspects of prevention or alleviation of enteric infection, allergic diseases, and chronic inflammatory diseases (Wilkins and Sequoia, 2017). Furthermore, these beneficial effects of probiotics are gaining increasingly attention as a substitute for antibiotic or anti-inflammatory drugs due to the threat from antibiotic resistance and foodborne pathogens to public health (Ayala et al., 2019). Possible action mechanisms of probiotics are involved in the modulation of intestinal barrier, defensin, inflammation, short-chain fatty acids, as well as cell interactions with hosts and...
pathogens (Markowiak and Śliżewska, 2017; Wang et al., 2019a,b). In recent studies, probiotic *Lactobacillus plantarum* and *Lactobacillus acidophilus* have been shown a significant anti-listerial activity (Ehsani et al., 2019; Reuben et al., 2019; Van Zyl et al., 2019; Zhao et al., 2020). However, whether probiotics influence the activities of virulence factors of pathogens including *L. monocytogenes* remains mostly unclear.

It is hypothesized that probiotics can deactivate the activities of virulence factors of pathogens. This study aimed to investigate the effect of *L. plantarum* and *L. acidophilus* mixture on the inoculation and translocation of *L. monocytogenes*, mRNA expression of anti-inflammatory reactions, and virulence factors in broilers.

**MATERIALS AND METHODS**

The experimental protocol (no. 2018016) was approved by the Institutional Committee for Animal Use and Ethics of Henan University of Science and Technology (HAUST).

**Bacterial Strains and Diets**

*L. acidophilus* ACCC11073 and *L. plantarum* CICC21863 were obtained from Animal Biological Lab at Henan University of Science and Technology (Luoyang, China). The two probiotics are permitted to be used as a feed additive by the Ministry of Agriculture and Rural Affairs of the People’s Republic of China (no. 2045-2013). *L. monocytogenes* CMCC54002 was from China Microbiological Culture Collection Center (Beijing, China). A basal diet (Table 1) was formulated referring to Manuals of Arbor Acres Broilers (Liu et al., 2018). The two probiotic strains were mixed at a ratio of 1:1 and added into 4 experimental treatments at doses of 0 (control), 10⁶, 10⁸, or 10¹⁰ cfu/kg of diet.

**Animal Model and Samples**

A total of 480 one-day-old male Arbor Acres broilers were randomly allocated into 4 groups with 6 cages (replicates) of 20 chicks each. The broilers in 4 treatments were given ad libitum access to feed (containing respective dose of *Lactobacilli*) and water, continuous light, auto-ventilation, and health monitor twice a day throughout the trial. The room temperature was maintained at 32°C for first 3 D and then gradually decreased to 25°C on 14 D.

The *L. monocytogenes* strain was activated from a stock culture stored at −80°C and was grown overnight at 37°C in Polymyxin-Acridin-Lithium chloride-Cef-tazidime-Aesculin-Mannitol (PALCAM) broth (HB8497; Qingdao Hopebio Co., Ltd., Shandong, China) under microaerophilic conditions. At night of the first day of feeding trial, each chick was orally administered with 1 mL of 10³ cfu/kg of *L. monocytogenes*.

On 7 and 14 D after administration, 5 birds were randomly selected and euthanized by CO₂ and dissected, respectively. Approximately 1 g of cecal content, liver, spleen, and cloacal skin from each bird was collected, pooled per replicate, and stored at −40°C for the enumeration of bacteria of *L. monocytogenes*. Approximately 1 g of cecal mucosa, liver, spleen, and cloacal skin was collected (Liu et al., 2010; Ding et al., 2019a), pooled per replicate, and stored in RNAlater for mRNA assay. Experimental animal feeding and sampling is strictly carried out in isolated houses to ensure biosafety, and animal waste and corpses were collected in sealed packages for harmless disposal.

**Bacterial Enumeration**

Each sample including broth with *L. monocytogenes*, cecal content, or tissue was homogenized, weighed, and diluted at 1:10 (wt/vol) with phosphate buffer saline and mixed thoroughly. The suspension of each sample was serially diluted between 10⁻¹ to 10⁻⁷ dilutions, and 100 μL of each diluted sample was spread onto duplicate PALCAM condition, 37°C for 24 h. The amount of bacteria was expressed as a logarithmic (log₁₀) transformation per gram of sample.

**Cytokine Assay**

The serum concentrations of cytokines were measured using chicken speciation enzyme-linked immunosorbent assays kits from Nanjing Jiancheng Biological Institute (Nanjing, China) for IL-6 (assay range, 15.0–1000 pg/mL; product no. H007), IL-1β (assay range, 20–600 pg/mL; product no. H002), tumor necrosis factor-α (TNF-α; assay range, 0.30–200 pg/mL; product no. H052), and interferon-γ (IFN-γ; assay range, 7.5–120 pg/mL; product no. H025). Three parallel tests with aliquots of the same sample were performed for all samples and all chemical and biochemical analyses.

**Gene Quantification**

Messenger RNA from the tissues was isolated using guanidine thiocyanate acid phenol procedure. Procedure used for mRNA isolation, as well as reverse transcription and real-time qPCR, was performed as previously described by Ding et al. (2019b). Random hexamers and RNase inhibitor were used in the reaction. Controls without reverse transcriptase were included for the genomic DNA contamination check. Forward and reverse primers of genes are listed in Table 2. The transcriptional profiles of target genes were expressed as the relative expression to a housekeeping gene (2⁻ΔΔCt, Livak and Schmittgen, 2001).

SYBR Green Master Mix for qPCR Kit was used and reactions were performed using ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The qPCR reactions were set at 10 μL with 5 μL of SYBR, 1 μL of primer, 4 μL of 10 × diluted cDNA. The conditions of the two-step qPCR were set as follows: activation for 3 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Reagents and
primers for real-time qPCR were purchased from TaKaRa Co. Ltd. (Dalian, China). All tests were performed in triplicate. No amplification signal was detected in water or no-RT RNA samples.

**Statistical Analysis**

Data are represented as mean and SEM using SPSS software (IBM SPSS, version 23, Armonk, NY). Differences between mean values of normally distributed data were assessed with one-way ANOVA (Tukey’s b-test) at $P < 0.05$ level of significance, and Tamhane’s T2 test for parameters with heterogeneity variance. For samples collected on 7 and 14 D after administration, the detected value of pooled sample of 5 birds per replicate was a statistical unit. The trend of probiotics doses at $10^6$, $10^8$, and $10^{10}$ cfu/kg was analyzed using contrasts of linear and quadratic polynomial.

**RESULTS AND DISCUSSION**

**Effect of Dietary Lactobacilli on L. Monocytogenes Load**

As shown in Table 3, on 7 D after administration, Lactobacilli addition at the 3 doses decreased ($P < 0.05$) the amounts of _L. monocytogenes_ in cecum, skin, liver, and spleen by 0.065 to 0.933 log_{10} cfu, compared to the control treatment, and the pathogen linearly decreased with the increasing doses of Lactobacilli in the liver ($P = 0.015$) and skin ($P = 0.011$). Similar effects of Lactobacilli were obtained on 14 D after administration, and the pathogen amounts linearly responded to the Lactobacilli doses in the skin ($P = 0.002$) and spleen ($P = 0.004$), and quadratically responded in the cecum ($P = 0.011$). The results indicate that dietary Lactobacilli mixture can effectively inhibit _L. monocytogenes_ proliferation and translocation in broilers.

In the present study, the mortalities between treatments were not statistically different (data not shown), and the growth performance was not measured due to the short duration of feeding trial, but the symptoms of birds in the control treatment were consistent with the known knowledge that animals suffered from listeriosis showed some clinical signs such as restlessness, loss of appetite, fever, and nervous system disorders (Papić et al., 2019; Zhao et al., 2020). Notably, dietary Lactobacilli significantly inhibited _L. monocytogenes_ proliferation in the gastrointestinal tract and its invasion into other critical organs in broilers.

It has been well documented that probiotic Lactobacilli confer a health benefit on the host by optimizing gut microflora, inhibiting some pathogens. _L. acidophilus_ was reported the antibacterial activity against _L. monocytogenes_ in cheese (Ehsani et al., 2019). _Enterococcus faecium_ isolated from poultry gastrointestinal tract could be used as a potential probiotic for preventing _L. monocytogenes_ infection (Reuben et al., 2019), and _E. faecium_ selected from rabbit faeces showed an inhibitory activity against _E. avium_, _L. innocua_, and _L. monocytogenes_ (Simonová and Lauková, 2007). The anti-listerial activity of _L. plantarum_ was via bacteriocin production and adhesion properties in vitro and in mice (Van Zyl et al., 2019). In the present study, whether the bacteriocins of Lactobacilli mixture influence listerial activity needs deserves further study.

In farm animals, the information about the antimicrobial activity of Lactobacilli as additives against _L. monocytogenes_ is very limited. In the present study, the inhibition of Lactobacilli against _L. monocytogenes_ in broilers indicates that Lactobacilli can be an alternative for growth-promoting antibiotics in farm animal production, and the inhibition capacity of high-dose Lactobacilli was more pronounced on _L. monocytogenes_ carriage in the liver and skin. In addition, _L. acidophilus_ in response to _L. monocytogenes_ induced quorum sensing luxS gene (Moslehi-Jenabian et al., 2011), and the quorum sensing for the probiotics and pathogen in such a scenario of the present study needs further investigation.

**Effect of Lactobacilli on Virulence Factors From L. Monocytogenes**

On 7 D after administration, dietary Lactobacilli decreased ($P < 0.05$) mRNA profiles of _L. monocytogenes_ virulence factors including autolysin amidase (Ami), flagellin (FlaA), listeriolysin O (HlyA), internalin A (InlA), and internalin B (InlB) in the intestinal mucosa of broilers (Table 4), except FlaA in the low Lactobacilli dose treatment. There were no differences for 3 doses of Lactobacilli on Ami, HlyA, InlA, and InlB, but linear ($P \leq 0.037$) decreasing effects were found on Ami, FlaA, and InlA, a decreasing quadratic ($P = 0.027$) effect on InlA.

On 14 D after administration, all doses of Lactobacilli showed decreasing ($P < 0.05$) effects on the detected genes of virulence factors. By contrast, Lactobacilli at a high dose was more pronounced than the low-dose treatment on FlaA gene and greater effects of middle and high dose Lactobacilli on InlB, but the 3 doses of Lactobacilli were significant ($P < 0.05$) on HlyA. Furthermore, these effects of Lactobacilli converged linear ($P \leq 0.005$) effects on FlaA, HlyA, and InlB and a quadratic effect ($P = 0.023$) on InlB.

Adherence to the cell surface is a key event during infection of _L. monocytogenes_. Ami in pathogenesis is to promote an efficient listerial adherence and internalization into host cells, and the production of TNF-α, IL-6, and IFN-γ (Asano et al., 2011; Drolia et al., 2018). FlaA is one of _L. monocytogenes_ flagellar motility genes and encodes adhesion protein flagellin, a natural ligand of inflammasome and a potent proinflammatory molecule in inducing IL-1β secretion in porcine cells (Reis et al., 2019). The transcriptional profiles of the 2 genes can be regulated by temperature or media nutrients (Way et al., 2004; Skovager et al., 2013). As known, probiotics can be used to eliminate some pathogens in the gastrointestinal tract; however, literature about
the effect of probiotics on adhesion proteins is unavailable. In the present study, treatments with Lactobacilli addition showed lower mRNA levels of Ami and FlaA, indicating that Lactobacilli can attenuate listerial adhesion, localization, and proinflammatory status in broilers.

Listeriolysin O encoded by HlyA gene is a pore-forming toxin produced by the bacterium to disrupt vacuolar membranes and promote bacterial entry into the cytosol (Kunishige et al., 2020). Lactobacillus salivarius decreased listeriolysin O mRNA expression in intestinal villi and Peyer’s patches but increased the gene level in mesenteric lymph nodes, and both intestinal villi and Peyer’s patches but increased the gene level in mesenteric lymph nodes, and both Lactococcus lactis and L. salivarius lowered Listeria count in spleens of infected rats (Lukic et al., 2017). High concentrations of bacteriocin produced by L. plantarum ST8SH were effective in biofilm inhibition of L. monocytogenes (Todorov et al., 2018). Similar results were found in the present study that Lactobacilli decreased transcriptional level of HlyA gene. Paradoxically, L. plantarum CICC6257 had no effect on Hly gene (Dong et al., 2020). Therefore, more studies are needed to confirm the effect of Lactobacilli strains used in the present study on listerial elimination.

Internalins including InlA and InlB are used by L. monocytogenes to invade host cells by binding to cadherin and inducing phagocytosis and may cooperate at different steps of infection, in particular, the targeting to specific organs (Phelps et al., 2018). The gastrointestinal tract is the primary route of infection for L. monocytogenes, and crossing the intestinal barrier is the first step. In the present study, the gene profiles of InlA and InlB in broiler intestinal mucosa were lowered in the Lactobacilli treatments, implying that the probiotics can attenuate the invasion capacity of the pathogen. This is consistent with the report that L. plantarum CICC6257 decreased the survival ratio of L. monocytogenes during passage through the simulated gastrointestinal tract and downregulated the relative expressions of InlA, InlB, and prfA genes (Dong et al., 2020). In addition, these virulence genes associated with listerial adhesion, invasion, and pore-forming were repressed by some antimicrobial substances (Vazquez-Armenta et al., 2020); therefore, whether they can be influenced by Lactobacilli metabolites deserves further study.

### Effect of Lactobacilli on Serum Cytokines

On 7 D after administration, the addition of Lactobacilli mixture decreased ($P < 0.05$) serum levels of cytokines including IL-1β, IL-6, TNF-α, and IFN-γ by 30.0 to 50.7%, compared to the control treatment (Table 5), and the high dose of probiotics was more pronounced ($P < 0.05$) on IL-1β, IL-6, and IFN-γ. The serum levels of IL-1β, IL-6, and IFN-γ were linearly responded ($P \leq 0.045$) to the doses of probiotics.

### Table 1. Ingredient and nutrition levels in the basal diet

| Item          | Content (%) |
|---------------|-------------|
| Ingredient    |             |
| Corn          | 56.7        |
| Soybean meal  | 30.0        |
| Corn gluten meal | 5.5   |
| Soybean oil   | 2.5         |
| Met           | 0.2         |
| Lys           | 0.4         |
| Salt          | 0.4         |
| Limestone     | 1.8         |
| Dicalcium phosphate | 1.5 |
| Premix        | 1.0         |

### Calculated composition

| Ingredient | Content (%) |
|------------|-------------|
| Crude protein | 21.7       |
| ME (MJ/kg)   | 12.41       |
| Crude fiber  | 2.73        |
| Lysine       | 1.39        |
| Met          | 0.55        |
| Met + Cys    | 0.88        |
| Ca           | 1.02        |
| Non-phytate P | 0.49       |

1 calculated by Chinese Feed Database, version 25, 2014.
2 The premix provided the following per kg of diets: vitamin A (retinyl acetate), 9,000 IU; cholecalciferol, 4,000 IU; vitamin E (DL-tocopheryl acetate), 50 IU; vitamin K, 2 mg; thiamin, 2 mg; riboflavin, 5 mg; d-pantothenic acid, 15 mg; nicin, 40 mg; pyridoxine, 2 mg; biotin, 0.1 mg; folic acid, 0.55 mg; vitamin B12, 0.01 mg; manganese, 120 mg; iodine, 1.2 mg; iron, 40 mg; copper, 16 mg; zinc, 100 mg; and selenium, 0.3 mg.

### Table 2. Information of genes for quantitative real-time PCR.

| Items          | GenBank   | Forward | Reverse | Length (bp) |
|----------------|-----------|---------|---------|-------------|
| Genes from Listeria monocytogenes |           |         |         |             |
| Ami            | AF035424.1| tggggagcagcgcagatagc | cagctatgggtgttcgcct | 223         |
| FlaA           | FJ234183.1| tggccgtcttcaggtggttt | tggccatcttcagcgcct | 127         |
| HlyA           | DQ988349.1| acacccgagttccctcagtc | gcaagcttcccctcgctt | 150         |
| InlA           | EF445938.1| gaaaaagtgacgggccttt | tgcgcctctccctcaaaa | 174         |
| InlB           | EU408886.1| atttgcctctctgctctgc | gggctcttcctcctcctc | 206         |
| 16sRNA         | M58822.1  | gattgtagctgtaacctgct | atctcctctccctgtggt | 178         |
| Genes from broilers |         |         |         |             |
| IL-10          | AJ621614.1| tggccggttaacctcctgc | atcgacggtggaactctgc | 268         |
| HIF1A          | NM_204297.1| cagcagccttcctcttcagc | aatagctgtctccccttc | 215         |
| PTGER2         | NM_001083365.1| tggctgtctctctctctc | tggctgtctctctctc | 235         |
| PTGDS2         | NM_001167718.1| agctactcctgactcga | aagctgtcctctcctc | 158         |
| ACTB           | NM_205518.1| Tacctctgcctgcaaga | tctcagcctgtggggcagc | 228         |

Abbreviations: ACTB, beta-actin; Ami, autolysin amidase gene; HlyA, listeriolysin O gene; InlA, internalin A; InlB, internalin B; HIF1A, hypoxia inducible factor 1 alpha; PTGER2, prostanoid E receptor 2; PTGDS2, prostaglandin-endoperoxide synthase 2.
whereas serum IL-6 and TNF-α were quadratically responded \((P < 0.031)\) to the additive doses. On 14 D after administration, *Lactobacilli* decreased \((P, 0.05)\) serum cytokines by 25.4 to 51.1%, and middle and high doses were more pronounced \((P < 0.05)\) than the low dose, which contributed to linear \((P ≤ 0.023)\) reduction on IL-1β, IL-6, and TNF-α, and quadratic \((P = 0.021)\) reduction on IL-1β.

The decreased effects on the detected cytokines in the present study further demonstrated the anti-inflammatory property of *Lactobacilli* mixture. Importantly, these findings also confirmed the attenuating effect of *Lactobacilli* on virulence property of *L. monocytogenes*. To authors’ knowledge, the linkage among virulence factors, proinflammatory cytokines, and probiotics is a first report, which could be a novel action mechanism for probiotics. *L. plantarum* DR7 reduced proinflammatory cytokines, such as interferon-γ and transforming growth factor-α, but increased plasma anti-inflammatory cytokine IL-10 in stressed adults (Chong et al., 2019). Serum levels of proinflammatory cytokines IL-1β, IL-2, IL-6, IFN-γ, and anti-inflammatory IL-4, IL-10 changed linearly or quadratically both at the initial and final phases of broilers fed with *E. faecium* NCIMB 11181 (Wu et al., 2019). Mucosa listeriolysin O mRNA expression and serum TNFα, IL1β, and IFNγ were reduced by a cocktail of *L. acidophilus*, *L. plantarum*, and *E. faecium*, but indexes of thymus and spleen, serum IgA, and IgG were increased in farm rabbits (Zhao et al., 2020). Anyway, the interrelation among virulence property of pathogens, inflammatory response of hosts, and the probiotics deserves more studies.

### Effect of *Lactobacilli* on the Anti-inflammatory Reaction

In Table 6, on 7 D after administration, dietary *Lactobacilli* upregulated \((P < 0.05)\) mRNA profiles of anti-inflammatory reactions related genes including IL-10, IL-1β, IL-6, IFN-γ, and anti-inflammatory IL-4, IL-10 changed linearly or quadratically both at the initial and final phases of broilers fed with *E. faecium* NCIMB 11181 (Wu et al., 2019). Mucosa listeriolysin O mRNA expression and serum TNFα, IL1β, and IFNγ were reduced by a cocktail of *L. acidophilus*, *L. plantarum*, and *E. faecium*, but indexes of thymus and spleen, serum IgA, and IgG were increased in farm rabbits (Zhao et al., 2020). Anyway, the interrelation among virulence property of pathogens, inflammatory response of hosts, and the probiotics deserves more studies.

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**Table 3. Effect of *Lactobacilli* on *Listeria monocytogenes* loads in broilers.**

| Items         | L. monocytogenes infection | SEM | Linear | Quadratic |
|---------------|----------------------------|-----|--------|-----------|
| Control       | 10⁶                        | 10⁸ | 10¹⁰   |           |
| Cecum         | 3.365⁰                     | 2.713⁰ | 2.432⁰ | 2.472⁰    | 0.080 | 0.068 | 0.153 |
| Skin          | 0.159⁰                     | 0.120⁰ | 0.103⁰  | 0.089⁰    | 0.008 | 0.015 | 0.974 |
| Liver         | 0.337⁰                     | 0.261⁰ | 0.237⁰  | 0.220⁰    | 0.009 | 0.011 | 0.789 |
| Spleen        | 0.30⁰                      | 0.25⁰  | 0.23⁰   | 0.24⁰     | 0.011 | 0.427 | 0.192 |

**Table 4. Effect of lactic acid bacteria on virulence factors from *Listeria monocytogenes* in the cecal mucosa of broilers.**

| Items         | L. monocytogenes infection | SEM | Linear | Quadratic |
|---------------|----------------------------|-----|--------|-----------|
| Control       | 10⁶                        | 10⁸ | 10¹⁰   |           |
| Cecum         | 3.79⁰                      | 3.10⁰  | 2.90⁰  | 3.14⁰     | 0.069 | 0.672 | 0.011 |
| Skin          | 0.26⁰                      | 0.20⁰  | 0.17⁰  | 0.13⁰     | 0.011 | 0.002 | 0.467 |
| Liver         | 0.44¹                      | 0.28²  | 0.25⁰  | 0.26⁰     | 0.010 | 0.14⁰ | 0.09⁰ |
| Spleen        | 0.49³                      | 0.36³  | 0.33⁰  | 0.28¹     | 0.013 | 0.004 | 0.70⁰ |

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**Abbreviations:** Ami, autolysin amidase gene; HlyA, listeriolysin O gene; InlA, intercalin A; InlB, intercalin B; HIF1A, hypoxia inducible factor 1 alpha.

1. An equal amount mixture of *Lactobacillus acidophilus* and *Lactobacillus plantarum*. 

2. Means within a row with no common superscripts are significantly different \((P < 0.05)\).
hypoxia inducible factor 1 alpha (HIF1A), prostaglandin E receptor 2 (PTGER2), and prostaglandin-endoperoxide synthase 2 (PTGS2) in the cecal mucosa of broilers, and linear ($P \leq 0.010$) responses were found on the 4 genes, quadratic ($P \leq 0.005$) responses on HIF1A and PTGER2. On day 14 after administration, greater ($P < 0.05$) effects were found for the high dose of Lactobacilli on IL-10, middle and high doses on HIF1A ($P < 0.05$), but there were no differences between the 3 doses of Lactobacilli on PTGER2 and PTGS2. Linear and quadratic ($P \leq 0.042$) responses to the Lactobacilli doses were found on IL-10, HIF1A, and PTGS2. These data imply that Lactobacilli can improve the secretion of anti-inflammatory factors and attenuate inflammatory reactions.

Cytokine synthesis inhibitory factor IL-10 has a complex and predominantly opposing roles in inflammation and plays a major role in suppressing immune and inflammatory responses (Wei et al., 2019). HIF1A is a key transcriptional factor to dampen hypoxia-induced inflammation through the enhanced production and signaling effects of anti-inflammatory signaling molecules (Novak et al., 2016; Fujii et al., 2020). In the gastrointestinal tract, pathogens can develop hypoxic microenvironments and subsequent inflammatory damage of epithelial cells. Recent literature has shown that HIF1A can protect B cells in autoimmunity by driving IL-10 expression (Meng et al., 2018; Qian et al., 2019).

In the present study, IL-10 and HIF1A in the control treatment with L. monocytogenes infection exhibited the lowest mRNA profiles, whereas they were upregulated with Lactobacilli addition at 3 doses, indicating the 2 genes are collaboratively integrated by either L. monocytogenes or Lactobacilli. Unfortunately, information about the 2 genes expressions is unavailable in the presence of L. monocytogenes and probiotics. Furthermore, a study reported an inverse correlation between IL-10 and HIF1A in macrophages infected with pathogenic fungus Histoplasma capsulatum (Fecher et al., 2016). Therefore, the new mechanism of probiotics dampening inflammation by anti-inflammatory factors, HIF1A and IL-10, deserves further study.

The PTGER2, one of various oxygenated metabolites of arachidonic acid, produces a broad range of biologic

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### Table 5. Effect of Lactobacilli on the contents of cytokines in the serum of broilers.

| Listeria monocytogenes infection | Lactobacilli (cfu/kg of diet) | $P$-value |
|----------------------------------|-------------------------------|-----------|
|                                 | Control                       | Linear    | Quadratic |
| 7 D after administration (pg/mL) |                               |           |           |
| IL-1β                           | 155.9a                        | 107.2b    | 96.0b     | 76.8b    | 4.456     | <0.001    | 0.455     |
| IL-6                            | 181.3a                        | 108.9c    | 113.2c    | 90.8c    | 3.841     | 0.005     | 0.013     |
| TNF-α                           | 150.6a                        | 96.5b     | 77.7b     | 84.5b    | 4.933     | 0.075     | 0.031     |
| IFN-γ                           | 92.6a                         | 61.0ab    | 64.8b     | 49.8b    | 3.585     | 0.045     | 0.051     |
| 14 D after administration (pg/mL) |                               |           |           |
| IL-1β                           | 169.6a                        | 109.0b    | 87.5b     | 82.9b    | 2.903     | <0.001    | 0.021     |
| IL-6                            | 189.7a                        | 124.1a    | 121.7b    | 108.9b   | 4.265     | 0.023     | 0.326     |
| TNF-α                           | 159.2a                        | 91.0b     | 88.3b     | 79.8b    | 2.891     | 0.011     | 0.415     |
| IFN-γ                           | 96.2a                         | 71.8b     | 70.9b     | 63.7b    | 3.745     | 0.081     | 0.403     |

**Means within a row with no common superscripts are significantly different ($P < 0.05$).**

Abbreviations: IL-10, interferon γ; TNF-α, tumor necrosis factor α.

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### Table 6. Effect of Lactobacilli on anti-inflammatory reaction in the cecal mucosa of broilers.

| Listeria monocytogenes infection | Lactobacilli (cfu/kg of diet) | $P$-value |
|----------------------------------|-------------------------------|-----------|
|                                 | Control                       | Linear    | Quadratic |
| 7 D after administration (mRNA, $2^{-ΔΔCt}$) | |           |           |
| IL-10                           | 0.126a                        | 0.390b    | 0.485b    | 0.592a   | 0.017     | 0.000     | 0.785     |
| HIF1A                           | 0.041a                        | 0.162b    | 0.230b    | 0.210b   | 0.010     | 0.010     | 0.003     |
| PTGER2                          | 0.034a                        | 0.183b    | 0.201b    | 0.304a   | 0.009     | 0.000     | 0.005     |
| PTGS2                           | 0.028a                        | 0.122b    | 0.153b    | 0.220a   | 0.008     | 0.000     | 0.152     |
| 14 D after administration (mRNA, $2^{-ΔΔCt}$) | |           |           |
| IL-10                           | 0.455a                        | 0.647b    | 0.620b    | 0.690a   | 0.013     | 0.042     | 0.012     |
| HIF1A                           | 0.106a                        | 0.281b    | 0.386b    | 0.360a   | 0.019     | 0.015     | 0.019     |
| PTGER2                          | 0.050b                        | 0.277a    | 0.290a    | 0.295a   | 0.016     | 0.492     | 0.851     |
| PTGS2                           | 0.045b                        | 0.169b    | 0.235b    | 0.216a   | 0.012     | 0.027     | 0.025     |

**Means within a row with no common superscripts are significantly different ($P < 0.05$).**

Abbreviations: HIF1A, hypoxia inducible factor 1 alpha; PTGER2, prostaglandin E receptor 2; PTGS2, prostaglandin-endoperoxide synthase 2.

1 An equal amount mixture of Lactobacillus acidophilus and Lactobacillus plantarum.
actions in diverse tissues of humans and animals. PTGS2, also known Cox2, is an enzyme responsible for the synthesis of prostanoids. Studies have shown that PTGER2 has potential in producing anti-inflammatory cytokines including IL-10 in vivo (Okla et al., 2019; Tomić et al., 2019). IL-10/Cox2/PTGER2 could be influenced by Mycobacterium avium infection in dendritic cells and by Antrodia cinnamomea fermented product in chicken cells (Kim et al., 2019; Lee et al., 2019). Lactobacilli in product in chicken cells (Kim et al., 2019; Lee et al., 2019). IL-10/Cox2/PTGER2 could be influenced by Mycobacterium avium infection in dendritic cells and by Antrodia cinnamomea fermented product in chicken cells (Kim et al., 2019; Lee et al., 2019). In addition, interaction of mesenchymal stem cells, S. typhimurium, and L. acidophilus increased Cox2, IL-6, IL-8, and PTGER2 (Kol et al., 2014). Lactobacillus fermentum attenuated TNF-α expression and liver injury via an IL-10- and PTGER2-EP4-dependent mechanism (Jin et al., 2015). In the present study, broilers fed with Lactobacilli exhibited greater levels of IL-10/PTGS2/PTGER2 in the cecal mucosa than the control infected with L. monocytogenes, implying that Lactobacilli may increase the activity of these genes, but more studies are needed.

CONCLUSIONS

Administration of probiotic Lactobacilli for broilers infected with L. monocytogenes repressed the colonization or translocation of the pathogen by decreasing its loads in the cecum, liver, spleen, and skin of broilers. The Lactobacilli also attenuated inflammatory status by deregulating proinflammatory cytokines IL-1β, IL-6, TNF-α, and IFN-γ and mRNA levels of anti-inflammatory factors IL-10, HIF1A, PTGER2, and PTGS2 in the intestinal mucosa of broilers. The lower profiles of virulence genes Ami, FlaA, HlyA, InlA, and InlB further demonstrated that Lactobacilli effectively decreased the adhesion, invasion, and pore-forming of the pathogen. The results suggest that Lactobacilli could be applied as a supplement for L. monocytogenes infection in broilers.

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