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), 10^6, 10^8, 10^{10} 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 $\log_{10}$ cfu, and the pathogen linearly reduced ($P \leq 0.015$) 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 ($P \leq 0.019$) 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

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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 in the feed additive industry by 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), 10^6, 10^8, or 10^{10} 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. 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-Acriflavin-Lithium chloride-Ceftazidime-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 administrated with 1 mL of 10^4 cfu/kg of *L. monocytogenes*.

On 7 and 14 D after administration, 5 birds were randomly selected and euthanized by CO_2 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 ($\log_{10}$) 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 ($2^{-\Delta\Delta Ct}$, 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'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

Table 1. Ingredient and nutrition levels in the basal diet[1] (as fed basis).

Items	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[2]	1.0
Calculated composition	
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; niacin, 40 mg; pyridoxine, 2 mg; biotin, 0.1 mg; folic acid, 0.55 mg; vitamin B_{12}, 0.01 mg; manganese, 120 mg; iodine, 1.2 mg; iron, 40 mg; copper, 16 mg; zinc, 100 mg; and selenium, 0.3 mg.

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 *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* deceased 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 2. Information of genes for quantitative real-time PCR.

Items	GenBank	Primers (5'→3') Forward	Reverse	Length (bp)
Genes from *Listeria monocytogenes*				
Ami	AF035424.1	tggggagcaggacaatatgc	cagtatgggttgttccgcct	223
FlaA	FJ234183.1	gtgcacttctaggtgctggt	tcggacatttcagcagccat	127
HlyA	DQ988349.1	acaccaggagttcccattgc	gcaacgtatccctccagagt	150
InlA	EF445938.1	gaaaaatgtgacgggcgctt	tgcgtcacggttccactaaa	174
InlB	EU408886.1	attgtgccacttgcaggttt	ggagtcactaacgacccatca	206
16sRNA	M58822.1	gattgtaggctgcaactcgc	atctgtcccaccttcggcg	178
Genes from broilers				
IL-10	AJ621614.1	tggcagcttaacgttcggtc	attcaggggtggaaactcgc	268
HIF1A	NM_204297.1	ccagcagttcctcatgcaat	aaatgctgctagcccttccc	215
PTGER2	NM_001083365.1	tgatggtcatgatggcgagg	ttgcacgtcaccttctcgtt	235
PTGS2	NM_001167718.1	acgtacctcgtgactccgaa	aacgagttccacttgcacga	158
ACTB	NM_205518.1	Ttactcgcctctgtgaaggc	tcctagactgtgggggactg	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, prostaglandin E receptor 2; PTGS2, prostaglandin-endoperoxide synthase 2.

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

	L. monocytogenes infection						
		Lactobacilli ($\log_{10}$ cfu/kg of diet)[1]				*P*-value	
Items	Control	10^6	10^8	10^{10}	SEM	Linear	Quadratic
7 D after administration ($\log_{10}$ cfu/g)							
Cecum	3.365[a]	2.713[b]	2.432[b]	2.472[b]	0.080	0.068	0.153
Skin	0.159[a]	0.120[b]	0.105[b,c]	0.089[c]	0.008	0.015	0.974
Liver	0.337[a]	0.261[b]	0.237[b,c]	0.220[c]	0.009	0.011	0.789
Spleen	0.307[a]	0.256[b]	0.233[b]	0.244[b]	0.011	0.427	0.192
14 D after administration ($\log_{10}$ cfu/g)							
Cecum	3.799[a]	3.109[b]	2.902[b]	3.148[b]	0.069	0.672	0.011
Skin	0.266[a]	0.202[b]	0.179[b,c]	0.134[c]	0.011	0.002	0.467
Liver	0.441[a]	0.282[b]	0.257[b]	0.265[b]	0.010	0.149	0.096
Spleen	0.493[a]	0.363[b]	0.330[b]	0.281[b]	0.013	0.004	0.709

[a-c]Means within a row with no common superscripts are significantly different ($P < 0.05$).

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

whereas serum IL-6 and TNF-α were quadratically responded ($P \leq 0.031$) 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 ($P \leq 0.023$) 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,

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

	L. monocytogenes infection						
		Lactobacilli (cfu/kg of diet)[1]				*P*-value	
Items	Control	10^6	10^8	10^{10}	SEM	Linear	Quadratic
7 D after administration (mRNA, $2^{-\Delta\Delta Ct}$)							
Ami	0.645[a]	0.531[b]	0.465[b]	0.435[b]	0.029	0.037	0.629
FlaA	3.332[a]	2.642[a,b]	2.164[b]	2.045[b]	0.119	0.006	0.282
HlyA	0.714[a]	0.527[b]	0.468[b]	0.444[b]	0.035	0.097	0.668
InlA	0.257[a]	0.166[b]	0.133[b]	0.140[b]	0.009	0.012	0.027
InlB	0.129[a]	0.088[b]	0.076[b]	0.075[b]	0.004	0.058	0.327
14 D after administration (mRNA, $2^{-\Delta\Delta Ct}$)							
Ami	0.758[a]	0.421[b]	0.416[b]	0.415[b]	0.025	0.894	0.960
FlaA	3.342[a]	3.008[b]	2.819[b,c]	2.356[c]	0.110	0.005	0.437
HlyA	0.922[a]	0.694[b]	0.616[c]	0.480[d]	0.017	0.000	0.293
InlA	0.574[a]	0.363[b]	0.318[b]	0.300[b]	0.028	0.171	0.733
InlB	0.885[a]	0.602[b]	0.462[c]	0.431[c]	0.018	0.000	0.023

[a-c]Means within a row with no common superscripts are significantly different ($P < 0.05$).

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

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

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

	Listeria monocytogenes infection						
		Lactobacilli (cfu/kg of diet)[1]				*P*-value	
Items	Control	10^6	10^8	10^{10}	SEM	Linear	Quadratic
7 D after administration (pg/mL)							
IL-1β	155.9[a]	107.2[b]	96.0[b]	76.8[c]	4.456	<0.001	0.455
IL-6	181.3[a]	108.9[b]	113.2[b]	90.8[c]	3.841	0.005	0.013
TNF-α	150.6[a]	96.5[b]	77.7[b]	84.5[b]	4.933	0.075	0.031
IFN-γ	92.6[a]	61.0[b,c]	64.8[b]	49.8[c]	3.585	0.045	0.051
14 D after administration (pg/mL)							
IL-1β	169.6[a]	109.0[b]	87.5[c]	82.9[c]	2.903	<0.001	0.021
IL-6	189.7[a]	124.1[b]	121.7[b]	108.9[b]	4.265	0.023	0.326
TNF-α	159.2[a]	91.0[b]	88.3[b]	79.8[b]	2.891	0.011	0.415
IFN-γ	96.2[a]	71.8[b]	70.9[b]	63.7[b]	3.745	0.081	0.403

[a-c]Means within a row with no common superscripts are significantly different ($P < 0.05$).

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

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

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

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

	Listeria monocytogenes infection						
		Lactobacilli (cfu/kg of diet)[1]				*P*-value	
Items	Control	10^6	10^8	10^{10}	SEM	Linear	Quadratic
7 D after administration (mRNA, $2^{-\Delta\Delta Ct}$)							
IL-10	0.126[d]	0.390[c]	0.485[b]	0.592[a]	0.017	0.000	0.785
HIF1A	0.041[c]	0.162[b]	0.236[a]	0.210[a]	0.010	0.010	0.003
PTGER2	0.034[c]	0.185[b]	0.201[b]	0.304[a]	0.009	0.000	0.005
PTGS2	0.028[d]	0.123[c]	0.153[b]	0.220[a]	0.008	0.000	0.152
14 D after administration (mRNA, $2^{-\Delta\Delta Ct}$)							
IL-10	0.455[c]	0.647[b]	0.620[a,b]	0.690[a]	0.013	0.042	0.012
HIF1A	0.106[c]	0.281[b]	0.386[a]	0.360[a]	0.019	0.015	0.019
PTGER2	0.050[b]	0.277[a]	0.290[a]	0.295[a]	0.016	0.492	0.851
PTGS2	0.045[b]	0.169[a]	0.235[a]	0.216[a]	0.012	0.027	0.025

[a-d]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). 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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