

## Effect of lactic acid bacteria on *Listeria monocytogenes* infection and innate immunity in rabbits

HEPING ZHAO<sup>1</sup>, FEIKE ZHANG<sup>2</sup>, JUN CHAI<sup>3</sup>, JIANPING WANG<sup>1\*</sup>

<sup>1</sup>College of Animal Science, Henan University of Science and Technology, Luoyang, P.R. China

<sup>2</sup>Luoyang Xintai Agro-pastoral Technology Co., Ltd, Luoyang, P.R. China

<sup>3</sup>Luoyang Polytechnic, Luoyang, P.R. China

\*Corresponding author: [jp\\_wang68@163.com](mailto:jp_wang68@163.com)

**Citation:** Zhao H., Zhang F., Chai J., Wang J. (2020): Effect of lactic acid bacteria on *Listeria monocytogenes* infection and innate immunity in rabbits. Czech J. Anim. Sci., 65: 23–30.

**Abstract:** The present study aimed to investigate the effect of probiotic lactic acid bacteria (LAB) addition on *Listeria monocytogenes* translocation and its toxin listeriolysin O (LLO), proinflammatory factors, immune organ indexes and serum immunoglobulins in farmed rabbits. Five treatments included negative control (NC), positive control (PC) with *L. monocytogenes* infection and supplemental LAB at  $3.0 \times 10^6$  (low-LAB, L-LAB),  $3.0 \times 10^8$  (medium-LAB, M-LAB) and  $3.0 \times 10^{10}$  (high-LAB, H-LAB) CFU/kg of diet, respectively. The LAB was a mixture of equal amounts of *Lactobacillus acidophilus* (ACCC11073), *Lactobacillus plantarum* (CICC21863) and *Enterococcus faecium* (CICC20430). A total of 180 weaned rabbits (negative for *L. monocytogenes*) were randomly assigned to 5 groups with 6 replicates of 6 rabbits each in response to the 5 treatments. *L. monocytogenes* infection occurred on the first day of feeding trial and dietary LAB supplementation lasted for 14 days. The results showed that on days 7 and 14 post administration, *L. monocytogenes* in caecum, liver, spleen and lymph nodes was reduced in M-LAB and H-LAB compared to PC ( $P < 0.05$ ), and linear and quadratic reducing trends were found in liver on day 7 ( $P \leq 0.002$ ). On day 14, mucosa LLO mRNA expression and serum TNF $\alpha$ , IL1 $\beta$  and IFN $\gamma$  were reduced in the three LAB treatments ( $P < 0.05$ ), and linear and quadratic trends were found on TNF $\alpha$  and IL1 $\beta$  ( $P \leq 0.025$ ); indexes of thymus and spleen, serum IgA and IgG were increased in the LAB treatments ( $P < 0.05$ ). It is concluded that LAB can be used to alleviate *L. monocytogenes* infection and to improve the immune function of farmed animals.

**Keywords:** immune organ; immunoglobulins; listeriolysin O; pathogen translocation; proinflammatory factor

Listeriosis is a serious infection usually caused by eating food contaminated with the bacterium *Listeria monocytogenes*, a lower incidence rate but higher mortality (Radoshevich and Cossart 2018). *L. monocytogenes* can flourish with or without oxygen, thrive and reproduce at 0° C, and infect many animal species including rabbits. The hardiness of *L. monocytogenes*, combined with its relative ubiquity, makes it a serious ongoing concern both in food safety and in animal production (Johansson and Freitag 2019). *L. monocytogenes* typically enters the host through the intestines and infects the liver, spleen and immune cells where the bacteria multiply until tackled by the host's immune system (Regan et al. 2014). Animals infected with

*L. monocytogenes* can show the clinical signs of restlessness, loss of appetite, fever and nervous system disorders (Papic et al. 2019).

Lactic acid bacteria (LAB) including *Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Enterococcus faecium* are commonly used as probiotic strains, i.e. live microorganisms as food supplement in order to benefit health. The health claims range from rather vague like regulation of bowel activity and exerting an antagonistic effect on the gastroenteric pathogens, neutralising food mutagens produced in the colon, to shifting the immune response towards a Th2 response (Riaz Rajoka et al. 2017; Liu et al. 2018; Wang et al. 2019a, b). Besides secreting antimicrobial substances with

activity against the homologous strain, LAB strains also produce microbicidal substances with the effect on gastric and intestinal pathogens and other microbes, or compete for cell surface and mucin binding sites. This could be the mechanism behind reports that some probiotic strains inhibit or decrease translocation of bacteria from the gut to the other organs (Scaffaro et al. 2018).

Recent *in vitro* studies showed that *L. acidophilus*, *L. plantarum*, *E. faecium* or their secondary metabolites inhibited biofilm-forming ability or activity of *L. monocytogenes* (Ehsani et al. 2019; Rocha et al. 2019; Wei et al. 2019). In farm animals, literature about LAB effect on the prevention or control of *L. monocytogenes* infection is unavailable. Additionally, with the increasing trend of health awareness, rabbit meat is popular due to its low levels of fat, cholesterol and sodium, but high levels of protein (Dalle Zotte and Szendro 2011; Ding et al. 2019a; Wang et al. 2019d). The present study aimed to investigate the effect of LAB on immune organ index, lymphocyte activity and immune globulins in farmed rabbits.

## MATERIAL AND METHODS

**Lactic acid bacteria and basal diet.** The LAB strains included *L. acidophilus* (ACCC11073), *L. plantarum* (CICC21863) and *E. faecium* (CICC20430) from the Animal Biological Laboratory at Henan University of Science and Technology (Luoyang, China) at a ratio of 1 : 1 : 1. Nutrition levels of basal diet were recommended by China Agricultural Standard for Farm Rabbits (NY/T2765-2015). Lyophilised LAB strains were separately recovered and aerobically enriched in

De Man, Rogosa and Sharpe (MRS) broth (HB0384 1; Qingdao Hopebio Co., Ltd., China) at 37 °C for 48 h, then they were transferred onto corn powder using the step-by-step method and added at the expense of corn to the basal diet. All diets were considered as isonitrogenous and isocaloric and were prepared in the form of pellets (cold formed; diameter × length, 3.5 × 8.0 mm). No antibiotics were offered to rabbits via either feed or water throughout the trial. The formulation and nutrition levels of basal diet are listed in Table 1.

**Treatments and animals.** The trial protocol was approved by the Institutional Committee for Animal Use and Ethics of the College of Animal Science of the Henan University of Science and Technology. Treatments included negative control (NC), positive control (PC) with *L. monocytogenes* infection and based on PC, LAB were added at  $3.0 \times 10^6$  (low-LAB, L-LAB),  $3.0 \times 10^8$  (medium-LAB, M-LAB) and  $3.0 \times 10^{10}$  (high-LAB, H-LAB) colony forming units (CFU)/kg of diet, respectively.

A total of 180 weaned male Rex rabbits at approximately 35 days of age with similar initial body weight were randomly assigned to 5 groups with 6 replicates of 6 rabbits each in response to the 5 treatments. Before a feeding trial, all rabbits were negative for *L. monocytogenes* by rectal swab detection, and they were individually raised in stainless steel cages (length × width × height, 35 × 45 × 40 cm) in a house and had free access to diets and water (Wang et al. 2019c). The feeding trial lasted for 14 days. All rabbits were monitored for general health at least twice a day.

***L. monocytogenes* infection.** The strain of *L. monocytogenes* (CMCC54002) used in the present study was obtained from the China Microbiological

Table 1. Ingredient and nutrition levels in the basal diet<sup>1</sup> (as fed basis)

Ingredient	Content (%)	Calculated composition	Content (%)
Corn	24.2	crude protein	17.02
Soybean meal	14.3	digestible energy (MJ/kg)	10.91
Corn germ meal	8.0	crude fibre	14.35
Alfalfa meal	33.0	lysine	0.79
Wheat bran	10.0	methionine + cysteine	0.53
Dicalcium phosphate	1.5	Ca	1.02
Premix <sup>2</sup>	2.0	P	0.53

<sup>1</sup>calculated by Chinese Feed Database, Version 25, 2014

<sup>2</sup>premix provided the following per kg of diets: vitamin A 12 000 IU; vitamin D 2 000 IU; vitamin E 30 IU; Cu 12 mg; Fe 64 mg; Mn 56 mg; Zn 60 mg; I 1.2 mg; Se 0.4 mg; Co 0.4 mg; NaCl 6.4 g

<https://doi.org/10.17221/247/2019-CJAS>

Culture Collection Centre (Beijing, China). The strain was activated from a stock culture stored at  $-80^{\circ}\text{C}$  and was grown overnight at  $37^{\circ}\text{C}$  in polymyxin acriflavine lithium chloride ceftazidime aesculin mannitol (PALCAM) broth (HB8497; Qingdao Hopebio Co., Ltd.) under microaerophilic conditions. On the first day of feeding trial, each rabbit in treatments PC and LAB was orally administered 1 mL of  $10^7$  CFU/kg of *L. monocytogenes* and rabbits in treatment NC received the same liquid without the strain.

**Sample collection.** On days 7 and 14 post administration, two rabbits close to the mean value of body weight per replicate were weighed. Blood was collected from the right marginal ear vein and sera were prepared by centrifuging at 1000 g for 10 min (Liu et al. 2010) and stored at  $-20^{\circ}\text{C}$  till their further use. Then, the rabbits were euthanised with  $\text{CO}_2$  and dissected. Approximately 5 g of caecal content was collected and stored at  $-40^{\circ}\text{C}$  for the enumeration of spores and live bacteria of *L. monocytogenes*. The caecal mucosa was collected and stored in RNeasy lysis buffer for an mRNA assay. Liver (approximately 5 g), spleen, thymus and mesenteric lymph node (approximately 5 g) were collected and stored at  $-40^{\circ}\text{C}$  for bacterial enumeration (Ding et al. 2019b). The spleen and thymus were weighed and the immune organ index was expressed as a ratio of organ weight to live body weight.

**Enumeration of bacteria.** Each sample including the broth with *L. monocytogenes*, caecal content or tissue was homogenised, weighed, and diluted at 1 : 10 (wt/vol) with PBS and mixed thoroughly (Liu et al. 2018). The suspension of each sample was serially diluted between  $10^{-1}$  to  $10^{-7}$  dilutions, and 100  $\mu\text{L}$  of each diluted sample was spread onto duplicate PALCAM agar,  $37^{\circ}\text{C}$  for 24 h. Similarly, each LAB strain in cultured medium was counted using MRS agar at  $37^{\circ}\text{C}$  for 48 h. The amount of bacteria was expressed as a logarithmic ( $\log_{10}$ ) transformation per gram of sample. For the enumeration of spores, 1-mL sample was heated at  $75^{\circ}\text{C}$  for 20 min to kill vegetative cells and induce the germination of mature spores. The heat-treated culture was then serially diluted in sterile peptone, plated on PALCAM agar (HB4188; Qingdao Hopebio Co., Ltd.), and incubated anaerobically at  $37^{\circ}\text{C}$ . The colonies represented germinated spores which were included in the counts of *L. monocytogenes*.

**Quantification of mRNA.** mRNA was isolated from the tissues using the guanidine thiocyanate-acid phenol procedure. The procedure used for mRNA isolation, as well as reverse transcription and real-time qPCR were 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 *L. monocytogenes* listeriolysin O (LLO) were TAAGACGCCAATCGAAAAGAA and GTCACATGCATCTCCGTGGTAT, respectively, according to the report by Lukic et al. (2017). SYBR Green Master Mix for qPCR Kit was used and reactions were performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, USA). The qPCR reactions were set at 10  $\mu\text{L}$  with 5  $\mu\text{L}$  of SYBR Green Master Mix, 1  $\mu\text{L}$  of primer, 4  $\mu\text{L}$  of  $10\times$  diluted cDNA. The conditions of the two-step qPCR were set as follows: activation for 3 min at  $95^{\circ}\text{C}$  followed by 40 cycles of 15 s at  $95^{\circ}\text{C}$  and 1 min at  $60^{\circ}\text{C}$ . Reagents and primers for real-time qPCR were manufactured by TaKaRa Co., Ltd. (China). In the relative quantification analysis, a 1 : 10 dilution of cDNA template was used with the sample from PC treatment group set as a calibrator. Controls without reverse transcriptase were amplified to ensure a lack of DNA contamination.

**Immunity and inflammation assay.** Rabbit enzyme-linked immunosorbent assay kits from Cusabio Technology LLC (USA) were used for the detection of cytokines according to manuals, including tumour necrosis factor  $\alpha$  (TNF $\alpha$ , detection range 0.27 to 200 pg/mL), interleukin 1 $\beta$  (IL1 $\beta$ , detection range 0.27 to 200 pg/mL) and interferon  $\gamma$  (IFN $\gamma$ , detection range 3.13 to 800 pg/mL). Serum immunoglobulins were detected using commercial kits for IgA (H108) and IgG (E026) from Nanjing Jiancheng Biological Institute (China) as described by Wang et al. (2019c).

**Statistical analysis.** Data are presented as the means and SEM using SPSS software (IBM SPSS, Version 23). Differences between mean values of normally distributed data were assessed by 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 days 7 and 14 post administration, the average mean of 2 rabbits per replicate was a statistical unit. The trend of LAB doses was analysed using contrasts of linear and quadratic polynomial.

## RESULTS AND DISCUSSION

**Effect of LAB on caecal and tissue carriage of *L. monocytogenes*.** In the present study, after the administration of *L. monocytogenes* infection and probiotic LAB additive for 7 days, rabbits in PC treatment carried greater *L. monocytogenes* counts whereas they showed a lower number in LAB treatment, indicating the effective inhibition of the additive ( $P < 0.05$ ), except for L-LAB in liver (Table 2). With the increase of LAB doses, linear responses were found in the caecum, liver, spleen and lymph node ( $P \leq 0.043$ ) and a quadratic trend in the liver ( $P = 0.002$ ) post administration for 7 days. Similar results were found post administration for 14 days, LAB at the three doses decreased the *L. monocytogenes* carriage of L-LAB treatment ( $P < 0.05$ ), except for the pathogen number of L-LAB treatment, and linear responses of LAB doses were found in the caecum and spleen ( $P \leq 0.012$ ). Additionally, the *L. monocytogenes* in rabbits of NC treatment were also detectable post administration due to all rabbits raised in one house.

Recently, *in vitro* studies have well documented the antilisterial effect of probiotics. Supplemental *L. acidophilus* (PTCC1643) at  $10^9$  or  $10^{12}$  CFU/mL to the milk showed a significant protective effect against *L. monocytogenes* in cheese (Ehsani et al. 2019). The underlying molecular mechanism may be due to the upregulated *luxS* gene of *L. acidophilus* (NCFM) in response to *L. monocytogenes* by co-cultivation (Moslehi-Jenabian et al. 2011).

*L. plantarum* strains or their cell-free supernatants significantly inhibited pathogenic bacteria *L. monocytogenes*, *Salmonella enteritidis*, *Escherichia coli* and *Staphylococcus aureus* (Arena et al. 2016). Incorporation of *L. plantarum* (TN8) at  $10^8$  CFU/g into raw minced beef meat inhibited the proliferation of spoilage microorganisms, such as *L. monocytogenes* and *Salmonella spp.*, delayed the lipid oxidation, improved texture parameters, and extended the shelf life (Trabelsi et al. 2019). Growth of *L. monocytogenes* was significantly inactivated by *E. faecium* (KE82) and its enterocins A and B *in situ* in milk (Vandera et al. 2017).

In contrast, *in vivo* research on the effect of probiotics on *L. monocytogenes* is limited, especially in farm animals. A study found that *L. plantarum* (423) competitively excluded *L. monocytogenes* from the gastrointestinal tract of mice (Van Zyl et al. 2016). Probiotic *Lactobacillus brevis* supplementation resulted in a significant reduction in dispersion and propagation of *L. monocytogenes* in liver, spleen and intestine, and it also led to a significant elevation of the LAB number and reduction of total plate count, anaerobic count and coliform population in the intestine of mice (Riaz et al. 2019). *Lactobacillus lactis* and *Lactobacillus salivarius* lowered the listeria count in the spleen of mice (Lukic et al. 2017). Apparently, the *in vitro* and *in vivo* studies demonstrated the effectiveness of probiotic LAB in inhibiting *L. monocytogenes*. Similarly, in the present study, the supplementation of LAB including *L. acidophilus*, *L. plantarum*

Table 2. Carriage of *L. monocytogenes* in caecum, liver, spleen and lymph node of rabbits

Items	NC	<i>L. monocytogenes</i> infection				SEM	<i>P</i> -value	
		PC	L-LAB	M-LAB	H-LAB		linear	quadratic
7 days post administration (Log <sub>10</sub> CFU/g)								
Caecum	0.23 <sup>d</sup>	3.93 <sup>a</sup>	3.34 <sup>b</sup>	2.92 <sup>c</sup>	2.77 <sup>c</sup>	0.072	0.001	0.281
Liver	0.02 <sup>c</sup>	0.18 <sup>a</sup>	0.14 <sup>a</sup>	0.09 <sup>b</sup>	0.09 <sup>b</sup>	0.006	<0.001	0.002
Spleen	0.03 <sup>d</sup>	0.39 <sup>a</sup>	0.30 <sup>b</sup>	0.26 <sup>bc</sup>	0.24 <sup>c</sup>	0.009	0.002	0.743
Lymph node	0.03 <sup>c</sup>	0.38 <sup>a</sup>	0.30 <sup>b</sup>	0.27 <sup>b</sup>	0.27 <sup>b</sup>	0.007	0.043	0.395
14 days post administration (Log <sub>10</sub> CFU/g)								
Caecum	0.24 <sup>c</sup>	4.17 <sup>a</sup>	3.55 <sup>ab</sup>	3.12 <sup>b</sup>	3.22 <sup>b</sup>	0.095	0.065	0.084
Liver	0.08 <sup>d</sup>	0.27 <sup>a</sup>	0.22 <sup>b</sup>	0.18 <sup>c</sup>	0.17 <sup>c</sup>	0.010	0.012	0.516
Spleen	0.18 <sup>c</sup>	0.43 <sup>a</sup>	0.30 <sup>b</sup>	0.29 <sup>b</sup>	0.29 <sup>b</sup>	0.007	0.395	0.319
Lymph node	0.07 <sup>d</sup>	0.51 <sup>a</sup>	0.40 <sup>b</sup>	0.33 <sup>bc</sup>	0.26 <sup>c</sup>	0.013	<0.001	0.932

LAB = lactic acid bacteria; NC = negative control; PC = positive control; L/M/H-LAB = low/middle/high-LAB at  $3.0 \times 10^6/10^8/10^{10}$  CFU/kg of diet

<sup>a-d</sup> means in a row without the same superscript were significantly different ( $P < 0.05$ )

<https://doi.org/10.17221/247/2019-CJAS>

and *E. faecium* significantly reduced the number of *L. monocytogenes* in the caecum, liver, spleen and lymph nodes of rabbits, indicating LAB can be an alternative for preventing listeriosis. However, in the present study, the interactions between LAB and *L. monocytogenes*, *luxS* gene profile and enterocin secretion, as mentioned above, deserve further study.

**Mucosal LLO and serum proinflammatory cytokines.** On day 7 post treatment, the mRNA level of LLO in the PC treatment was the highest, while it was decreased by LAB addition by 35.4 to 48.2% ( $P < 0.05$ ) (Table 3). There was no difference between the three doses of LAB, but the trend analysis showed that with the increased doses of LAB, LLO decreased linearly ( $P = 0.012$ ) and quadratically ( $P = 0.007$ ). On day 14 post treatment, LLO was decreased by 25.3 to 56.4% ( $P < 0.05$ ) compared to the PC treatment, and there were significant decreases in LLO levels between the three doses of LAB ( $P < 0.05$ ), which exhibited a linear trend ( $P < 0.001$ ).

On day 7 post treatment, proinflammatory factors including TNF $\alpha$ , IL1 $\beta$  and IFN $\gamma$  were also decreased by the addition of LAB ( $P < 0.05$ ) (Table 3). Similar results were found for these parameters on day 14 post treatment ( $P < 0.05$ ), except for IL1 $\beta$  and IFN $\gamma$  in M-LAB treatment. Furthermore, linear responses were found for TNF $\alpha$  and IL1 $\beta$  ( $P \leq 0.006$ ) and a quadratic effect for IFN $\gamma$  ( $P = 0.005$ ).

Among pathogenic bacteria, a group of species causes infections relying on their ability to enter host cells at an early stage of inflammation, in-

cluding *L. monocytogenes*. LLO is a haemolysin produced by *L. monocytogenes* and is crucial for its invasion (Osborne and Brumell 2017; Nguyen et al. 2019). Host macrophages produce the cytokines interleukins, which initiate proinflammatory responses that are important for recruiting neutrophils to the liver to kill *L. monocytogenes* (Meixenberger et al. 2010). In the present study, LAB supplementation reduced the mucosal mRNA levels of LLO, indicating that the virulence of *L. monocytogenes* can be alleviated by the additive.

To the authors' knowledge, the effect of probiotic species used in the present study on the toxin LLO is very limited. *L. plantarum* (CICC6257) inhibited the growth of *L. monocytogenes* in cabbage and decreased the survival rate of *L. monocytogenes* during passage through the simulated gastrointestinal tract (Dong et al. 2020). *Lactobacillus salivarius* caused a decrease of LLO mRNA expression in intestinal villi and Peyer's patches, but an increase in mesenteric lymph nodes of rats (Lukic et al. 2017). High concentrations of bacteriocin produced by *L. plantarum* (ST8SH) were more effective in the biofilm inhibition of *L. monocytogenes* (Todorov et al. 2018). For 17 days after inoculation in sludge, the concentration of *L. monocytogenes* decreased rapidly but the concentrations of culturable *E. faecium* (T) were stable (Wery et al. 2006). *Enterococcus hirae* and *Pediococcus pentosaceus* aggregated with *L. monocytogenes* facilitated the elimination of this pathogen (Cavicchioli et al. 2019). Addi-

Table 3. Listeriolysin O and proinflammatory cytokines in the serum of rabbits

Items	NC	<i>L. monocytogenes</i> infection				SEM	<i>P</i> -value	
		PC	L-LAB	M-LAB	H-LAB		linear	quadratic
<b>7 days post administration</b>								
LLO <sup>1</sup>	0.241 <sup>c</sup>	2.597 <sup>a</sup>	1.678 <sup>b</sup>	1.346 <sup>b</sup>	1.411 <sup>b</sup>	0.076	0.012	0.007
TNFα (ng/L)	34.38 <sup>d</sup>	306.6 <sup>a</sup>	191.4 <sup>b</sup>	148.0 <sup>c</sup>	138.5 <sup>c</sup>	7.072	< 0.001	0.067
IL1β (ng/L)	64.59 <sup>c</sup>	387.4 <sup>a</sup>	266.1 <sup>b</sup>	229.3 <sup>b</sup>	226.4 <sup>b</sup>	12.19	0.058	0.327
IFNγ (ng/L)	22.86 <sup>c</sup>	218.4 <sup>a</sup>	161.1 <sup>b</sup>	143.1 <sup>b</sup>	130.8 <sup>b</sup>	9.252	0.051	0.821
<b>14 days post administration</b>								
LLO <sup>1</sup>	0.532 <sup>e</sup>	4.766 <sup>a</sup>	3.562 <sup>b</sup>	2.832 <sup>c</sup>	2.078 <sup>d</sup>	0.138	< 0.001	0.954
TNFα (ng/L)	31.36 <sup>d</sup>	334.3 <sup>a</sup>	287.4 <sup>b</sup>	197.0 <sup>c</sup>	167.6 <sup>c</sup>	9.319	< 0.001	0.025
IL1β (ng/L)	70.09 <sup>d</sup>	392.7 <sup>a</sup>	321.8 <sup>b</sup>	349.7 <sup>ab</sup>	256.9 <sup>c</sup>	12.20	0.006	0.004
IFNγ (ng/L)	89.88 <sup>c</sup>	246.0 <sup>a</sup>	180.6 <sup>b</sup>	226.5 <sup>ab</sup>	163.2 <sup>b</sup>	16.12	0.378	0.005

LLO = listeriolysin O; LAB = lactic acid bacteria; NC = negative control; PC = positive control; L/M/H-LAB = low/middle/high-LAB at  $3.0 \times 10^6/10^8/10^{10}$  CFU/kg of diet

<sup>a-e</sup> means in a row without the same superscript were significantly different ( $P < 0.05$ )

<sup>1</sup>LLO, mRNA,  $2^{-\Delta\Delta Ct}$

tionally, in the future study it is noteworthy how the bacteriocin of LAB function, toxin LLO and its genes, as well as whether LAB and *L. monocytogenes* can aggregate in the gastrointestinal tract.

In the present study, the serum levels of inflammatory factors including TNF $\alpha$ , IL1 $\beta$  and IFN $\gamma$  increased in PC treatment and decreased in LAB treatments, further implying the inflammatory alleviating potential of LAB. Similarly, orally administered *L. plantarum* reduced proinflammatory interleukin secretion in sera from *L. monocytogenes* infected mice (Puertollano et al. 2008). Paradoxically, Choi et al. (2012) found that heat-killed *E. faecium* (JWS833) stimulated mouse peritoneal macrophages to produce NO, IL1 $\beta$  and TNF $\alpha$  and that the oral administration of viable *E. faecium* enhanced NO, IL1 $\beta$  and TNF $\alpha$  synthesis upon *L. monocytogenes* challenge. A low concentration of the aloe fermentation supernatant fermented by *L. plantarum* (HM218749.1) reduced the production of IL1 $\beta$ , TNF $\alpha$  and IL6 in both mRNA and protein levels *in vitro* (Jiang et al. 2016). There is no literature reporting the effect of probiotic species used in the present study on the inflammatory responses in farm animals with *L. monocytogenes* infection. The present study is the first report, however, more studies are needed to confirm the effect of LAB on controlling listeriosis in farm animals.

**Immune organ index and serum immunoglobulins.** On day 7 post treatment, the spleen index was not influenced by *L. monocytogenes* infection and LAB addition, but there were linearly and quadrati-

cally increased trends with LAB doses ( $P \leq 0.014$ ) (Table 4). The thymus index was decreased in PC treatment and was compensated in M-LAB treatment ( $P < 0.05$ ), and linearly responded to LAB doses ( $P = 0.001$ ). As for the immunoglobulins in the serum, IgA was increased by the addition of LAB ( $P < 0.05$ ), but there was no difference between LAB doses; IgG was increased only by M- and H-LAB ( $P < 0.05$ ).

On day 14 post treatment, the thymus and spleen indexes were decreased in PC treatment, while they were increased by LAB addition ( $P < 0.05$ ), but only the thymus index reached the level of NC treatment (Table 4). Serum IgA and IgG were also decreased in PC treatment and increased in LAB treatments. For the parameters of thymus, spleen, IgA and IgG, there were no differences between LAB doses and no significant linear or quadratic trends.

It is well documented that probiotics can promote the innate immunity of farm animals, however, information is unavailable when it is concurrent with *L. monocytogenes* infection. In the present study, immune organ indexes and humoral immunity of rabbits were reversely influenced by *L. monocytogenes* and LAB addition. This is consistent with the finding that *E. faecium* (JWS833) isolated from the duck intestinal tract showed immunomodulatory properties by producing NO and cytokines in mice (Choi et al. 2012). The molecule with the antilisterial effect from *E. faecium* (BGPAS1-3) was a bacterial cell-wall protein and prevented tight junction disruption in differentiated Caco-2 monolayer, stimulated the production of protective transforming growth

Table 4. Immune organ index and serum immunoglobulins of rabbits

Items	NC	<i>L. monocytogenes</i> infection				SEM	<i>P</i> -value	
		PC	L-LAB	M-LAB	H-LAB		linear	quadratic
<b>7 days post administration</b>								
Spleen (g/kg)	0.68	0.63	0.63	0.65	0.65	0.008	0.020	0.014
Thymus (g/kg)	2.91 <sup>a</sup>	2.66 <sup>b</sup>	2.74 <sup>ab</sup>	2.84 <sup>a</sup>	2.73 <sup>ab</sup>	0.029	0.640	0.001
IgA (mg/dL)	4.41 <sup>a</sup>	3.94 <sup>b</sup>	4.29 <sup>a</sup>	4.31 <sup>a</sup>	4.35 <sup>a</sup>	0.036	0.418	0.875
IgG (mg/dL)	4.41 <sup>a</sup>	3.78 <sup>c</sup>	4.03 <sup>bc</sup>	4.12 <sup>b</sup>	4.17 <sup>ab</sup>	0.067	0.264	0.843
<b>14 days post administration</b>								
Spleen (g/kg)	0.94 <sup>a</sup>	0.76 <sup>c</sup>	0.86 <sup>b</sup>	0.86 <sup>b</sup>	0.88 <sup>b</sup>	0.015	0.277	0.459
Thymus (g/kg)	2.89 <sup>a</sup>	2.68 <sup>b</sup>	2.84 <sup>a</sup>	2.80 <sup>a</sup>	2.84 <sup>a</sup>	0.034	0.948	0.375
IgA (mg/dL)	5.31 <sup>a</sup>	3.76 <sup>b</sup>	5.28 <sup>a</sup>	5.26 <sup>a</sup>	5.46 <sup>a</sup>	0.132	0.370	0.504
IgG (mg/dL)	3.52 <sup>a</sup>	2.30 <sup>b</sup>	3.20 <sup>a</sup>	3.20 <sup>a</sup>	3.06 <sup>a</sup>	0.084	0.285	0.512

LAB = lactic acid bacteria; NC = negative control; PC = positive control; L/M/H-LAB = low/middle/high-LAB at  $3.0 \times 10^6/10^8/10^{10}$  CFU/kg of diet

<sup>a-c</sup> means in a row without the same superscript were significantly different ( $P < 0.05$ )

<https://doi.org/10.17221/247/2019-CJAS>

factor  $\beta$  in intestinal epithelial cells, and modulated myeloid differentiation primary response 88 protein dependent toll-like receptor 2 and 4 pathways (Popovic et al. 2019). Additionally, host resistance to an intragastric infection with *L. monocytogenes* in mice is dependent on cellular immunity and intestinal bacterial flora of mice (Okamoto et al. 1994).

In the present study, the changed indexes of thymus and spleen as well as serum IgA and IgG by dietary LAB demonstrated the modulation of cellular and humoral immunity induced by *L. monocytogenes* infection. Traditionally, mechanisms of probiotics inhibiting enteropathogens in the gastrointestinal tract include the competition for nutrients, production of antimicrobial compounds, competitive exclusion of pathogens for adhesion to binding sites on the epithelial cells, stimulation of immune responses, and inhibition of virulence gene or protein expression in pathogens (Corr et al. 2009). Most recently, research that has looked more deeply at each of these mechanisms has found that many are actively mediated by various probiotic effector molecules, such as surface-located molecules, metabolites related to tryptophan and histamine, 5'-C-phosphate-G-3'-rich DNA and various enzymes in *Lactobacillus* and *Bifidobacterium* strains (Lebeer et al. 2018). Anyway, in the present study, the probiotic effectors and their interaction with rabbit gut flora deserve further study in animals suffering from listeriosis.

## CONCLUSION

Probiotic LAB in the rabbit diet attenuated *L. monocytogenes* infection by reducing its counts in caecum, liver, spleen and lymph nodes, mucosal LLO, serum levels of TNF $\alpha$ , IL1 $\beta$  and IFN $\gamma$ , and in parallel, it improved the immune status by increasing immune organ indexes and serum IgA and IgG. The results suggest that the LAB species can be used as an additive against *L. monocytogenes* infection in farm animals.

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Received: November 30, 2019

Accepted: January 20, 2020