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Lactobacillus acidophilus reduces *Listeria monocytogenes* infection by inhibiting mitogen-activated protein kinase genes in growing rabbits

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ABSTRACT - We aimed to investigate the effect of *Lactobacillus acidophilus* ACCC11073 on the growth performance, oxidation, inflammation, and mitogen-activated protein kinase (MAPK) family genes of rabbits infected with *Listeria monocytogenes* (*L. monocytogenes*) using antibiotic enrofloxacin hydrochloride (EH) as a reference. There were four treatments including negative control, positive control with *L. monocytogenes* infection on the first day of feeding trial (PC), PC + EH at 40 mg kg^{-1}, and PC + *L. acidophilus* at 10^8 CFU kg^{-1} of diet using 240 weaned growing rabbits. The results showed that *L. monocytogenes* infection worsened growth performance of rabbits, whereas EH or *L. acidophilus* supplementation partially recovered body weight gain, but did not reach the levels of the negative control. *Listeria acidophilus* and EH decreased *L. monocytogenes* loads in caecum, liver, spleen, and lymph node, serum oxidative markers including diamine oxidase, malondialdehyde, and protein carbonyl, serum IL-1β, IL-6, and TNF-α. The decreased effects of EH on IL-1β and TNF-α were more pronounced than that of the probiotic. Treatments EH and probiotic also de-regulated the mRNA levels of MAPK1, 3, 6, and 14. *Listeria acidophilus* exhibits a similar effect to EH against *L. monocytogenes* in rabbits, and the regulation on inflammatory process is via MAPK family genes. The results suggest that *L. acidophilus* can be used as a feed additive against *L. monocytogenes* infection.

Keywords: growth performance, inflammation, *L. monocytogenes* load, oxidation

1. Introduction

Listeria monocytogenes is an opportunistic pathogen responsible for listeriosis that is especially hard to control and can result in intermittent contamination of food (Johansson and Freitag, 2019). Infected animals most commonly show neurologic signs, fever, loss of appetite, and decreased activity level. Sometimes, animals can carry *L. monocytogenes* without appearing sick, and shed the bacteria in their feces (Papić et al., 2019). Rabbit meat with low fat and high proteins is becoming popular (Dalle Zotte and Szendrő, 2011; Ding et al., 2019a; Wang et al., 2019b,c); however, investigations show that farm rabbits are susceptible to *L. monocytogenes* and their meat is also a source of listerial foodborne pathogen (Rodríguez-Calleja et al., 2006; De Cesare et al., 2017; Zhao et al., 2020).

In recent studies, some probiotics including *Lactobacilli* spp. have shown a preventive and curative effect on listerial infection in foods (Fernandes et al., 2013) or mice (Dos Santos et al., 2011). In farm animals, information about the effect of probiotics on listeriosis is very limited. Two studies reported

that dietary lactic acid bacteria attenuated listerial infection and virulence but improved innate immunity in rabbits (Zhao et al., 2020) and broilers (Deng et al., 2020). Host mitogen-activated protein kinase (MAPK) links multiple signaling pathways in response to a myriad of stimuli including bacterial infections. *In vitro* or rodent studies have shown that *L. monocytogenes* activates p38 MAPK and induces IL-8 secretion, while *Lactobacillus casei* triggers innate immunity via p38 MAPK pathway (Kim et al., 2006; Opitz et al., 2006). It is unclear whether the modulation of *L. monocytogenes* by probiotics is associated with MAPK family members in farm animals.

It is hypothesized that dietary *Lactobacillus acidophilus* protects against *L. monocytogenes* infection by influencing MAPK genes. This study was developed to investigate the effect of dietary *L. acidophilus* on the growth performance, oxidative status, and transcriptional levels of MAPK family genes using Rex rabbits infected with *L. monocytogenes*.

2. Material and Methods

The trial protocol was approved by the institutional Ethics Committee on Animal Use (no. 2018016), and the experiment was carried out in Luoyang, China (112°45' N, 34°+62' E).

Lactobacillus acidophilus ACCC11073 was obtained from the Animal Biological Laboratory at Henan University of Science and Technology (Luoyang, China). Lyophilized *L. acidophilus* was recovered and aerobically enriched in De Man, Rogosa and Sharpe (MRS) broth (HB0384-1; Qingdao Hopebio Co. Ltd., Shandong, China) at 37 °C for 48 h. After bacterial enumeration, the broth loaded *L. acidophilus* was sprayed onto corn powder (40 meshes) using a step-by-step method and was added into the basal diet at 10^8 colony forming units (cfu) kg^{-1} in the expense of corn referring to the pilot study of authors and literature by Liu et al. (2018a).

Nutrition levels of basal diet were recommended by China Agricultural Standard for Farm rabbits (NY/T2765-2015; Yu et al., 2015). 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). Based on a basal diet (Table 1), there were four treatments including a negative control without *L. monocytogenes* infection, a positive control (PC) with *L. monocytogenes* infection, a PC + commercial enrofloxacin hydrochloride (EH) at 40 mg kg^{-1}, and a PC + *L. acidophilus* at 10^8 cfu kg^{-1} of basal diet. The doses of EH was recommended by the producer (Beijing Ding Niu Biotechnology Co., Ltd, Beijing, China) and literature by Huff et al. (2004); and *L. acidophilus* dose was referred to Deng et al. (2020).

A total of 240 weaned male Rex rabbits at approximately 35 days old with initial body weight (744±7.13 g, mean±SD) from a big group were randomly assigned to four groups with six replicates of 10 rabbits each in response to the four treatments. Before feeding trial, all rabbits were confirmed to be *L. monocytogenes*-free by rectal swabs detection, and then were individually raised in stainless steel cages (35 × 45 × 40 cm; length × width × height) in an automatic house in temperature, ventilation, and lighting and had free access to diets and water according to Technical Specification for Feeding and Management of Rex Rabbit (Yu et al., 2015). The feeding trial lasted for 35 days. Rabbits and feed in

Table 1 - Ingredient and nutrition levels in the basal diet (as fed basis)

Ingredient	Content (g kg^{-1})	Calculated composition[1]	Content (g kg^{-1})
Corn	275	Crude protein	171.4
Soybean meal	150	Digestible energy (MJ kg^{-1})	10.91
Brewers dried grain	50	Crude fiber	146.0
Alfalfa meal	400	Lysine	8.0
Wheat bran	100	Methionine + cysteine	5.0
Dicalcium phosphate	15	Ca	10.4
Premix[2]	10	P	5.3

[1] Calculated by Chinese Feed Database, version 25, 2014.
[2] The premix provided the following per kg of diets: vitamin A, 12,000 IU; vitamin D, 2000 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.

each replicate were weighed at 35 and 70 days old. Feed intake (FI), body weight gain (BWG), and feed conversion ratio (FCR, BWG/FI) were immediately adjusted when mortality occurred.

Listeria monocytogenes CMCC54002 was from China Microbiological Culture Collection Center (Beijing, China), of a stock culture stored at −80 °C. The strain was grown overnight at 37 °C in Polymyxin-Acriflavin-Lithium Chloride-Ceftazidime-Aesculin-Mannitol (PALCAM) broth (HB8497; Qingdao Hopebio) under microaerophilic conditions. On the first day of feeding trial, each rabbit in treatments PC and probiotic was orally administrated with 1 mL of 10^7 CFU kg^{-1} of *L. monocytogenes*, and rabbits in the negative control treatment received the same liquid without the strain (Zhao et al., 2020).

On day 35 post administration, five rabbits close to the mean value of body weight per replicate were weighed. Blood was collected from right marginal ear vein, and sera were prepared by centrifuging at 1000 × *g* for 10 min and stored at −20 °C for quantification of oxidative status parameters. Then, the rabbits were euthanized by CO_2. The caeca were vertically dissected and washed with phosphate-buffered saline (0 to 4 °C). Liver (approximately 5 g), spleen, thymus, and mesenteric lymph node (approximately 5 g) were collected and stored at −40 °C for bacterial enumeration (Ding et al., 2019b). Caecal content (approximately 5 g) was collected and stored at −40 °C for *L. monocytogenes* enumeration. Caecal mucosa (approximately 2 g) was collected and stored in RNAlater (Dalian TaKaRa Co. Ltd., Liaoning, China) for mRNA assay.

For enumeration of *L. acidophilus* and *L. monocytogenes*, each sample (1 g) was diluted with sterile buffered peptone water (0.1%, 9 mL, 0 to 4 °C) and mixed. The suspension of each sample was serially diluted between 10^{-1} to 10^{-7} dilutions, and each diluted sample (100 µL) was subsequently spread onto triplicate selective agar plates for bacterial count. The number of cfu was expressed as a logarithmic (log_{10}) transformation per gram of sample. The MRS agar (HB0384-2) and PALCAM agar (HB4188) from Qingdao Hopebio were used for counting of *L. acidophilus* and *L. monocytogenes*, respectively.

Serum concentrations of oxidative products and inflammatory factors were measured using kits from Nanjing Jiancheng Biological Institute (Nanjing, China) for diamine oxidase (DAO; EC 1.4.3.6; A088), malondialdehyde (MDA; A003), protein carbonyl (PCO; A087), interleukin-6 (IL-6; assay range, 15.0 to 1000 ng L^{-1}), interleukin -1β (IL-1β; assay range, 20 to 600 ng L^{-1}), and tumor necrosis factor- α (TNF-α; assay range, 0.30 to 200 ng L^{-1}). Three parallel tests with aliquots of the same sample were performed for all samples and all chemical and biochemical analyses.

All reagents for mRNA isolation, cDNA synthesis, qPCR amplification, and primers synthesis (Table 2) were provided by Dalian TaKaRa Co. Ltd. (Liaoning, China). Total mRNA from cecal samples was isolated using guanidine thiocyanate-acid phenol procedure according to the product manual. The mRNA concentration was determined by the optical density reading at 260 nm, and the purity was checked using A260:A280 ratio (1.8 to 2.0) and A260:A230 ratio (>1.5) on a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The cDNA synthesis was carried out according to the instructions of the kit. Random hexamers and RNase inhibitor were utilized in the reaction. Controls without reverse transcriptase were included for the genomic DNA contamination check.

Table 2 - Information of primers for quantitative real-time PCR

Name	GenBank	Exon	Primer (5'→3')		Length (bp)
			Forward	Reverse	
MAPK1	XM_008272180.2	6-7	attacgacccgagtgacgag	agcacgtccagtcctctgaa	175
MAPK3	XM_008257945.1	1-2	cgaacaccagacctactgcc	gtcgttgcttagttgctggc	196
MAPK4	XM_017344177.1	-	ccactagcctcaaccgacag	ggagccagagaggaatacgc	258
MAPK6	XM_002717751.3	5-6	tgacgtttagccccatggac	aggccaatcatgctccgaaa	215
MAPK8	XM_002722670.3	-	taaagccagtcaggcaaggg	ggctgccctcgtataactcc	261
MAPK14	XM_002714646.3	11-12	tttgcccagtaccacgatcc	tctttgcaacgagaggcact	261
ACTB	NM_001101683.1	-	tgtggccgaggactttgatt	ttacacaaatgcgatgctgcc	172

- unavailable; ACTB - beta-actin; MAPK - mitogen-activated protein kinase.

Transcript levels of target genes were expressed as the relative expression of target genes to a reference gene such as actin-beta ($2^{-\Delta\Delta Ct}$, Livak and Schmittgen, 2001).

Quantitative PCR reaction was set at 10 μL with 5 μL of SYBR Green Master Mix, 1 μL of primer, 4 μL of 10 × diluted cDNA or DNA. Plates were run on the ABI Prism 7900HT Fast Real-Time PCR System. All qPCR were run in triplicates on the same thermal cycles (50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min). No amplification signal was detected in water or no-RT RNA samples. Important details and precautions for the RT-qPCR were in accordance with the descriptions by Bustin et al. (2009).

Data were subjected to ANOVA, and means were separated by Tukey's b test at P<0.05 using IBM SPSS (version 23). The average BW of all rabbits per replicate was the statistical unit for growth performance, but the average mean of five rabbits dissected was used for gut bacteria (Log_{10} cfu) and the mRNA expression of genes.

Variables were analyzed according to the following mathematical model:

$$Y_{ij} = \mu + \beta_i + \varepsilon_{ij},$$

in which Y_{ij} = observation j of experimental unit subjected to treatments i, μ = general constant, β_i = effects of probiotic or antibiotic, and ε_{ij} = random error associated to each observation.

3. Results

Listeria monocytogenes infection in positive treatment worsened (P<0.05) BWG, FI, and FCR of rabbits compared with the negative control (Table 3). Supplementation of EH or *L. acidophilus* partially recovered BWG and FI, but did not reach (P<0.05) the levels of negative control. There were no differences between antibiotic and probiotic for the growth performance. The percentage of mortality in the positive control was greater than in other treatments, but there were no statistical differences among treatments due to a bigger difference within a group than among groups.

In the caecal content of rabbits, treatments antibiotic and probiotic decreased (P<0.05) *L. monocytogenes* loads compared with the positive control, and the pathogen load was greater (P<0.05) in treatments probiotic than in antibiotic (Table 4). The *L. monocytogenes* loads in the liver, spleen, and lymph node were decreased (P<0.05), but there were no differences between antibiotic and probiotic. Similarly, serum levels of DAO, MDA, and PCO were also decreased (P<0.05) in treatments antibiotic and probiotic, and the decreased effect of antibiotic on PCO was more pronounced (P<0.05).

Serum inflammatory factors IL-1β, IL-6, and TNF-α in positive control were greatest (P<0.05) among treatments, and were lowered (P<0.05) in treatments antibiotic and probiotic compared with positive control (Table 5). The decreased effects of antibiotic on IL-1β and TNF-α were pronounced (P<0.05). For MAPK family numbers, antibiotic and probiotic also de-regulated (P<0.05) the mRNA levels of MAPK (1,3,6, 14) compared with positive control.

Table 3 - Effects of *Lactobacillus acidophilus* and enrofloxacin hydrochloride (EH) on growth performance and mortality of growing rabbits

Item		*Listeria monocytogenes* infection			SEM	P-value
	NC	PC	EH	*L. acidophilus*		
Initial BW (g/rabbit)	744.3a	743.3a	745.3a	743.8a	1.506	0.830
Final BW (g/rabbit)	1426a	1311c	1342b	1331b	4.861	<0.001
BWG (g rabbit^{-1})	682a	567c	596b	587b	4.955	<0.001
FI (g rabbit^{-1})	2180a	1905b	1958b	1949b	14.25	<0.001
FCR (g:g)	3.20b	3.36a	3.28ab	3.32ab	0.034	0.024
Mortality (%)	1.67a	5.00a	1.67a	3.33a	1.919	0.575

NC - negative control, without *Listeria monocytogenes* infection; PC - positive control; BW - body weight; BWG - body weight gain; FI - feed intake; FCR - feed conversion ratio, BWG/FI; SEM - standard error of the mean.
a-c - Means within a row with a different letter differ significantly (P<0.05).

Table 4 - Effects of *Lactobacillus acidophilus* and enrofloxacin hydrochloride (EH) on *Listeria monocytogenes* load and oxidative status of growing rabbits

Item	NC	*Listeria monocytogenes* infection			SEM	P-value
		PC	EH	*L. acidophilus*		
L. monocytogenes load (log_{10} cfu g^{-1})						
Caecum	-	4.89a	1.92c	2.97b	0.156	<0.001
Liver	-	0.56a	0.27b	0.30b	0.068	0.019
Spleen	-	0.48a	0.27a	0.33a	0.060	0.066
Lymph node	-	0.31a	0.08b	0.15b	0.033	0.004
Serum level (nmol L^{-1})						
DAO	0.39c	2.20a	0.98b	1.06b	0.028	<0.001
MDA	0.73c	4.89a	3.56b	3.89b	0.127	<0.001
PCO	9.06d	25.4a	15.7c	18.3b	0.577	<0.001

NC - negative control, without *Listeria monocytogenes* infection; PC - positive control; - undetectable; DAO - diamine oxidase; MDA - malondialdehyde; PCO - protein carbonyl; SEM - standard error of the mean.
a-c - Means within a row with a different letter differ significantly (P<0.05).

Table 5 - Effects of *Lactobacillus acidophilus* and enrofloxacin hydrochloride (EH) on inflammatory factors and MAPK pathway of growing rabbits

Item	NC	*Listeria monocytogenes* infection			SEM	P-value
		PC	EH	*L. acidophilus*		
Serum inflammatory factors (ng L^{-1})						
IL-1β	29.1d	154.2a	65.7c	98.7b	3.909	<0.001
IL-6	42.3c	167.9a	93.4b	104.3b	3.910	0.008
TNF-α	18.6d	148.6a	80.8c	94.4b	3.472	<0.001
Caecal mucosal (mRNA, $2^{-\Delta\Delta Ct}$)						
MAPK1	0.093c	0.455a	0.223b	0.248b	0.009	0.006
MAPK3	0.109c	0.299a	0.211b	0.200b	0.007	0.009
MAPK4	0.041b	0.076a	0.060ab	0.043b	0.007	0.022
MAPK6	0.073b	0.096a	0.075b	0.080b	0.002	0.031
MAPK8	0.369c	0.482a	0.436b	0.456ab	0.010	0.028
MAPK14	0.096c	0.439a	0.271b	0.256b	0.009	0.010

NC - negative control, without *Listeria monocytogenes* infection; PC - positive control; IL - interleukin; MAPK - mitogen-activated protein kinase; TNF - tumor necrosis factor; SEM - standard error of the mean.
a-c - Means within a row with a different letter differ significantly (P<0.05).

4. Discussion

In the present study, the worsened growth performance in positive control treatment indicates the subclinical or clinical listeriosis of rabbits. Listerial contamination occurring in slaughter plants worldwide were frequently reported (Rodríguez-Calleja, et al., 2006; De Cesare et al., 2017); however, information is very limited about the negative effect of listeriosis on the growth of animals, especially rabbits. Actually, for farmers, animal growth depression and consequent economical loss from listeriosis must not be ignored because there were 5 to 20% decrease in body weight and feed efficiency based on the present study model. Importantly, the addition of EH or *L. acidophilus* partially compensated the growth parameters, and similar effects were found on the growth resulted from the two supplements, implicating that the probiotic can attenuate the listeriosis of rabbits.

The greater loads of *L. monocytogenes* in caecal content and tissues in the present study further demonstrated the successful establishment of the pathogenic infection. The lowered loads in the liver, spleen, and lymph node in treatments EH and probiotic indicate that the two supplements can decrease the pathogenic translocation, and the probiotic has a similar effect to the antibiotic. The decreased effect of the probiotic on *L. monocytogenes* load was supported by findings in rabbits (Zhao et al., 2020)

and mice (Dos Santos et al., 2011; Riaz et al., 2019). Additionally, some antilisterial bacterial species or strains were co-residents in the gut of farm animals (Lo Verso et al., 2018; Riaz et al., 2019). It is speculated that selection probiotics from rabbit gut as *Listeria* antagonists are more efficient, which needs further study.

Blood DAO is involved in the metabolism and oxidation in the intestinal epithelium (Fukudome et al., 2014). Malondialdehyde is a metabolite of lipid peroxidation of polyunsaturated fatty acids (Tsikas, 2017), and PCO groups are biomarkers of protein oxidation (Dalle-Donne et al., 2003; Liu et al., 2018c,d). In the present study, increased serum levels of DAO, MDA, and PCO in treatment PC indicate oxidative stress resulted from *L. monocytogenes*, whereas this status was alleviated by the addition of either EH or probiotic. It is well known the antioxidant activity of probiotics, but literature is very limited on listerial infection. *Lactobacilus rhamnosus* or *sakei* controlled *L. monocytogenes* growth, excessive proteolysis, and the production of spoilage odor, but increased antioxidant activity by radical scavenging activity assays in cheese or ham (Gao et al., 2015; Kariyawasam et al., 2019).

The elevation of peripheral IL-1β, IL-6, and TNF-α is a typical pro-inflammatory state associated with *L. monocytogenes* infection in rabbits. The beneficial effect of probiotics on an inflammatory process has been documented (Liu et al., 2018a,b; Wang et al., 2019a). In the present study, it was noted that the attenuation of *L. acidophilus* on IL-1β and TNF-α did not reach the levels of antibiotic treatment, and whether an amplified effect exists for the probiotic by elevating its inclusion dose deserves further study. The regulation on oxidation and inflammation is mainly directed by MAPK family (Kaminska, 2005), which is considered as one of molecular mechanisms of probiotics alleviating inflammatory responses (Marco et al., 2006).

As known, MAPK functions as integration point for multiple biochemical signals in response to a diverse array of stimuli, such as hyperosmosis, oxidative stress, DNA damage, low osmolarity, infection, and proinflammatory cytokines (Kaminska, 2005). In this study, both antibiotic and probiotic comparably down-regulated the mRNA profiles of MAPK (1, 3, 6, and 14), implying that inhibition of *L. monocytogenes*-induced infection is related to deactivation of MAPK. Based on the present infectious model, literature about this is unavailable, but there are some studies using other models. *Lactobacillus acidophilus*, *plantarum*, *Lactis*, or their metabolites attenuated the production of TNF-α, IL-1β, IL-8, and reactive oxygen species by inhibiting the MAPK-related pathways in oxidative-induced cells of rodents (Kim et al., 2017; Wang et al., 2018).

5. Conclusions

Inclusion of EH or *L. acidophilus* partially recovers BW gain, but does not reach the levels of the negative. Also, *L. acidophilus* and enrofloxacin hydrochloride decreases *L. monocytogenes* colonization and translocation, serum oxidative markers, and inflammatory factors and downregulates the mRNA levels of MAPK (1, 3, 6, and 14) in the intestinal mucosa. Similar results are found for most parameters between the two supplements, except that decreased effects of enrofloxacin hydrochloride on IL-1β and TNF-α are more pronounced than that of the probiotic. The findings suggest that *L. acidophilus* can be an alternative for antibiotic in rabbits infected with *L. monocytogenes*, and the regulation on growth performance and pathology is via decreased MAPK family genes.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: H. Zhao and J. Wang. Formal analysis: H. Zhao. Investigation: F. Zhang and J. Chai. Methodology: H. Zhao and J. Wang. Project administration: H. Zhao, F. Zhang, J. Chai and J. Wang. Resources: J. Chai. Writing-review & editing: J. Wang.

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