Long-term effects of oral tea polyphenols and *Lactobacillus brevis* M8 on biochemical parameters, digestive enzymes, and cytokines expression in broilers

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Abstract: This study investigates the long-term effects of oral tea polyphenols (TPs) and *Lactobacillus brevis* M8 (LB) on biochemical parameters, digestive enzymes, and cytokines expression in broilers. In experiment 1, 240 broiler chickens were selected to investigate the effects of 0.06 g/kg body weight (BW) TP and 1.0 ml/kg BW LB on broilers; in experiment 2, 180 broiler chickens were assigned randomly to three groups to investigate the effects of different dosages of TP (0.03, 0.06, and 0.09 g/kg BW) combined with 1.0 ml/kg BW LB on broilers; in experiment 3, 180 broiler chickens were assigned randomly to three groups to investigate the effects of different dosages of LB (0.5, 1.0, and 1.5 ml/kg BW) combined with 0.06 g/kg BW TP on broilers. The results showed that TP and LB affected serum biochemical parameters, and TP reduced serum cholesterol (CHO) and low-density lipoprotein cholesterol (LDL-C) abundances in a dosage-dependent manner ($P < 0.05$) on Day 84. Meanwhile, broilers fed a diet supplemented with TP or LB had a lower intestinal lipase activity on Day 84 compared with the control group ($P < 0.05$). Middle and high dosages of TP increased pancreatic lipase and proventriculus pepsin activities ($P < 0.05$). Also middle and high dosages of LB significantly enhanced pancreatic lipase activity ($P < 0.05$), while high LB supplementation inhibited intestinal trypsase ($P < 0.05$) on Day 84. Furthermore, both TP and LB reduced intestinal cytokine expression and nuclear factor-κ B (NF-κB) mRNA level on Days 56 and 84. In conclusion, long-term treatment of TP and LB improved lipid metabolism and digestive enzymes activities, and affected intestinal inflammatory status, which may be associated with the NF-κB signal.

Key words: Tea polyphenols, *Lactobacillus brevis*, Cytokines, Broilers
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1 Introduction

Previous studies have examined the bioavailability of tea polyphenols (TPs) and indicated that the possible mechanism for the beneficial effects of TP include vasculoprotective, anti-oxidative, anti-thrombogenic, anti-inflammatory, and lipid-lowering properties (Kanwar *et al*., 2012; Murakami, 2014; Chen and Lin, 2015). Shen *et al*. (2011a) reported that dietary TP attenuates lipopolysaccharide-induced myocardial fibrosis through a suppression of chronic inflammation and innate immune responses in rats. In chickens, TP supplementation has been demonstrated to alleviate obesity through the regulation of lipid-metabolism-related genes and transcription factor expression (Huang *et al*., 2013) and reduce glucocorticoid-

induced growth inhibition and oxidative stress in broiler chickens (Eid *et al*., 2003).

Lactic acid bacteria (LAB) are comprised of an ecologically diverse group of microorganisms united by formation of lactic acid as the primary metabolite of sugar metabolism. Currently, LAB have been considered to be the most common type of microbes used as probiotics and have been successfully used in improving intestinal microflora, prevention of infectious diseases and food allergies, reduction of serum cholesterol (CHO), anticarcinogenic activity, stabilization of the gut mucosal barrier, immune adjuvant properties, alleviation of intestinal bowel disease symptoms, and improvement in the digestion of lactose in intolerant hosts (Kumari *et al*., 2011; Berlec *et al*., 2012; Zhong *et al*., 2014; Rashti *et al*., 2015). Meanwhile, LAB have been suggested as improving growth performance and production in swine (Yang *et al*., 2015) and broilers (Chen *et al*., 2012). Our previous studies suggested that co-dietary TP and LAB supplementation improve growth performance and intestinal microflora in chickens, while little is known about the long-term effects of co-dietary TP and LAB supplementation in birds. Thus, the current study was to investigate the effects of TP and LAB on lipid-metabolic parameters, digestive enzymes, and cytokines expression in chickens.

2 Materials and methods

2.1 Chicks, diets, and experimental design

One-day-old yellow-feathered broiler chickens were obtained from the Hunan Animal Husbandry and Veterinary Research Institute (Changsha, China). The animal care protocol was approved by the Hunan Agricultural University Animal Care and Use Committee (Changsha, China). Birds were housed in an electrically heated thermostatically controlled room with feeders, nipple drinkers, and steel cages. Feed and tap water were available *ad libitum*. All birds were offered the same basal diet, which was formulated to meet or slightly exceed the NRC (1994) broilers requirements for all nutrients.

Experiment 1: 240 broiler chickens were assigned randomly to four groups: one control group (Control), one TP group (TP) in which birds were administered 0.06 g/kg body weight (BW) TP by oral gavage, one *Lactobacillus brevis* M8 (LB) group (LB) in which birds were administered 1.0 ml/kg BW LB by oral gavage, and a combined group (TPLB) in which birds orally received 0.06 g/kg BW TP and 1.0 ml/kg BW LB. Each group comprised 6 replicate cages with 10 birds per cage in a completely randomized design. LB, after being grown in de Man-Rogosa-Sharpe (MRS) liquid medium (Difco, USA), was adjusted to 2.15×10^8 CFU/ml and stored at $-80 \,^\circ\mathrm{C}$ prior to feeding. TP was purchased from Xi'an Xuhuang Bio-Tech Co., Ltd. (Xi'an, China), with a purity higher than 98%. Every 1000 mg TP contained 69.8 mg caffeine, 495 mg (−)-epigallocatechin gallate, 112 mg (−)-epicatechin gallate, 100 mg (−)-epicatechin, 78 mg (−)-epigallocatechin, and 96 mg (−)-gallocatechin gallate according to high-performance liquid chromatography-ultraviolet (HPLC-UV) analysis.

Experiment 2: 180 broiler chickens were assigned randomly to three groups, with 6 replicate cages with 10 birds per cage in a completely randomized design. All birds received 1.0 ml/kg BW LB combined with 0.03 (LTP), 0.06 (MTP), and 0.09 (HTP) g/kg BW TP.

Experiment 3: 180 broiler chickens were assigned randomly to three groups, with 6 replicate cages with 10 birds per cage in a completely randomized design. All birds received 0.06 g/kg BW TP combined with 0.5 (LLB), 1.0 (MLB), and 1.5 (HLB) ml/kg BW LB.

On Days 56 and 84, five birds from each replicate cage were slaughtered. Blood, glandular stomach, pancreas, and middle section of ileum were collected for further analyses (Guan *et al*., 2013).

2.2 Determination of serum biochemical parameters

Blood was centrifuged at 3000 r/min for 10 min. Serum was separated into Eppendorf tubes, and stored at $-80 \,^\circ\mathrm{C}$ for further analyses. Assay kits for the analysis of serum biochemical parameters were obtained from Nanjing Jiancheng Biotechnology Institute, China. Serum fasting blood glucose (GLU), low-density lipoprotein cholesterol (LDL-C), total glycerin trilaurate (TG), CHO, and high-density lipoprotein cholesterol (HDL-C) were determined using an Automatic Biochemistry Radiometer (Au640, Olympus).

2.3 Determination of digestive enzymes

Glandular stomach, pancreas, and middle intestine samples were collected for analyzing intestinal amylase, pancreatic amylase, intestinal lipase, pancreatic lipase, proventriculus pepsin, intestinal trypsase, and pancreatic trypsase using spectrophotometric kits in accordance with the manufacturer's instructions (Nanjing Jiancheng Biotechnology Institute, China).

2.4 Complementary DNA (cDNA) synthesis and mRNA quantification by real-time polymerase chain reaction (PCR) analysis

Extraction of total RNA and its reverse transcription were performed according to previous reports (Yin *et al.*, 2013a; 2014a). Primers were designed with Primer 5.0 according to the gene sequence of chicken (http://www.ncbi.nlm.nih.gov/pubmed) to produce an amplification product: IL-1β (forward: 5'-CGA GGA GCA GGG ACT TTG C-3'; reserve: 5'-GAA GGT GAC GGG CTC AAA AA-3'), IL-4 (forward: 5'-GCT CTC AGT GCC GCT GAT G-3'; reserve: 5'-GAA ACC TCT CCC TGG ATG TCA T-3'), IL-6 (forward: 5'-GCT TCG ACG AGG AGA AAT GC-3'; reserve: 5'-GGT AGGTC TGA AAG GCG AAC AG-3'), IL-10 (forward: 5'-CGC TGT CAC CGC TTC TTC A-3'; reserve: 5'-CGT CTC CTT GAT CTG CTT GAT G-3'), TNF-α (forward: 5'-TGT GTA TGT GCA GCA ACC CGT AGT-3'; reserve: 5'-GGC ATT GCA ATT TGG ACA GAA GT-3'), IFN-γ (forward: 5'-GTC TTG CTC CTT CAA CGA CA-3'; reserve: 5'-GCG CTG TAA TCG TTG TCT TG-3'), NF-κB (forward: 5'-TTG CTG CTG GAG TTG ATG TC-3'; reserve: 5'-TGC TAT GTG AAG AGG CGT TG-3'), Myd88 (forward: 5'-AGC GTG GAG GAG GAC TGC AAG AAG-3'; reserve: 5'-CCG ATC AAA CAC ACA CAG CTT CAG-3'), and TLR4 (forward: 5'-AGT CTG AAA TTG CTG AGC TCA AAT-3'; reserve: 5'-GCG ACG TTA AGC CAT GGA AG-3'). β-Actin was used as a house keeping gene to normalize target gene transcript levels (forward: 5'-CAT CCT GCG TCT GGA CCT GG-3'; reserve: 5'-TAA TGT CAC GCA CGA TTT CC-3'). Real-time PCR was performed according to previous studies (Yin *et al.*, 2014b; 2015a). Relative expression was normalized and expressed as a ratio to the expression in the control group.

2.5 Statistical analysis

All statistical analyses were performed using SPSS 17.0 software. Data were obtained using the one-way analysis of variance (ANOVA) to test homogeneity of variances via Levene's test followed with Tukey's multiple comparison tests. The results are expressed as the mean±standard error of the mean. Values in the same row with different superscripts are significant ($P<0.05$), while values with the same superscripts are not significantly different ($P>0.05$).

3 Results

3.1 Effects of TP and LB on serum biochemical parameters in chickens

The serum biochemical parameters on Day 84 are shown in Table 1. Supplementation with TP or LB significantly decreased serum LDL-C and TG concentrations ($P<0.05$) compared with the control group. LDL-C in the TPLB group was markedly lower than that in the LB group, and TG in the TPLB group was much lower compared with other groups ($P<0.05$). Supplementation with TP decreased serum HDL-C concentrations ($P<0.05$).

In experiment 2, supplementation with middle dosage of TP markedly decreased the serum LDL-C level compared with other groups ($P<0.05$). TP reduced serum CHO and HDL-C abundances in a dosage-dependent manner ($P<0.05$). In experiment 3, high dosage of LB enhanced serum GLU and reduced serum TG compared with the LLB and MLB groups ($P<0.05$).

3.2 Effects of TP and LB on digestive enzymes in chickens

Broilers fed a diet with TP or LB had a lower intestinal lipase activity on Day 84 of age compared with the control group ($P<0.05$). Pancreatic lipase activities in TP and TPLB groups were significantly higher than those in the control and LB groups ($P<0.05$; Table 2).

Compared with the LTP group, middle and high dosages of TP increased pancreatic lipase and proventriculus pepsin activities ($P<0.05$). Meanwhile, middle and high dosages of LB significantly enhanced pancreatic lipase activity ($P<0.05$), while high LB supplementation inhibited intestinal trypsase compared with the LLB and MLB groups ($P<0.05$; Table 2).

Table 1 Effects of TP and LB on serum biochemical parameters in broilers

Group	LDL-C (mmol/L)	GLU (mmol/L)	TG (mmol/L)	CHO (mmol/L)	HDL-C (mmol/L)
Experiment 1					
Control	1.36±0.11[a]	10.89±0.61	0.44±0.04[a]	3.43±0.45	2.13±0.09[a]
TP	0.92±0.02[bc]	8.62±0.24	0.34±0.05[b]	3.18±0.18	1.86±0.38[b]
LB	1.03±0.13[b]	11.32±0.74	0.26±0.01[b]	2.65±0.68	2.09±0.07[ab]
TPLB	0.88±0.03[c]	12.86±0.63	0.15±0.01[c]	3.14±0.23	2.05±0.05[ab]
Experiment 2					
LTP	0.51±0.05[b]	11.02±0.50	0.19±0.05	3.37±0.06[a]	2.20±0.04[a]
MTP	0.88±0.03[a]	12.86±0.33	0.15±0.03	3.14±0.04[ab]	2.05±0.05[b]
HTP	0.56±0.03[b]	12.89±0.24	0.14±0.02	2.82±0.10[b]	2.04±0.04[b]
Experiment 3					
LLB	0.84±0.09	11.33±0.43[b]	0.40±0.02[a]	3.23±0.08	2.23±0.06
MLB	0.88±0.03	12.86±0.33[ab]	0.15±0.01[b]	3.14±0.04	2.05±0.05
HLB	0.80±0.07	13.45±0.78[a]	0.12±0.01[b]	3.06±0.08	2.27±0.08

Data are expressed as means±standard error of the mean (SEM) (*n*=60). Values in a row with different superscripts differ significantly (*P*<0.05)

Table 2 Effects of TP and LB on digestive enzymes in broilers

Group	Intestinal amylase (U/mg prot)	Pancreatic amylase (U/mg prot)	Intestinal lipase (U/mg prot)	Pancreatic lipase (U/mg prot)	Proventriculus pepsin (U/mg prot)	Intestinal trypsase (U/mg prot)	Pancreatic trypsase (U/mg prot)
Experiment 1							
Control	0.53±0.04	0.76±0.07	177.67±7.59[a]	162.82±8.76[b]	5.82±0.87[ab]	0.20±0.02	0.20±0.06[ab]
TP	0.45±0.07	0.71±0.04	137.39±5.04[b]	215.70±10.75[a]	3.05±0.08[b]	0.22±0.05	0.27±0.03[a]
LB	0.59±0.08	0.77±0.02	142.00±10.13[b]	194.53±3.48[ab]	5.28±0.46[ab]	0.21±0.02	0.16±0.02[b]
TPLB	0.64±0.04	0.78±0.01	144.40±6.74[b]	217.58±4.15[a]	6.39±0.43[a]	0.22±0.01	0.29±0.03[a]
Experiment 2							
LTP	0.60±0.05	0.77±0.02	132.24±6.64	141.70±4.09[b]	5.85±0.69[b]	0.22±0.01	0.28±0.06
MTP	0.64±0.04	0.78±0.01	144.40±6.74	217.58±4.15[a]	6.39±0.43[ab]	0.22±0.01	0.29±0.03
HTP	0.58±0.00	0.79±0.01	149.78±8.33	230.13±4.78[a]	8.69±0.45[a]	0.22±0.02	0.22±0.02
Experiment 3							
LLB	0.59±0.05	0.77±0.02	157.59±5.39	163.13±6.98[b]	5.63±0.64	0.22±0.02[a]	0.22±0.04
MLB	0.64±0.04	0.78±0.01	144.40±6.74	217.58±4.15[a]	6.39±0.43	0.22±0.01[a]	0.29±0.03
HLB	0.59±0.05	0.77±0.01	125.81±4.07	218.47±5.15[a]	5.94±0.26	0.16±0.02[b]	0.24±0.05

Data are expressed as means±SEM (*n*=60). Values in a row with different superscripts differ significantly (*P*<0.05)

3.3 Effects of TP and LB on intestinal gene expression

On Day 56, supplementation with TP or LB significantly down-regulated IL-1β expression compared with the control group (*P*<0.05). Co-administration of TP and LB markedly reduced IL-6, IL-10, and TNF-α mRNA abundances (*P*<0.05; Fig. 1a). Co-administration of TP and LB changed intestinal TLR4 and NF-κB expression (*P*<0.05; Fig. 1b). On Day 84, broilers in the TPLB group exhibited lower abundances of IL-1β, IL-6, TNF-α, and NF-κB mRNA (*P*<0.05; Fig. 1) compared with the control group.

In experiment 2, different dosages of TP affected intestinal IL-1β, IL-4, IL-10, and NF-κB expression (*P*<0.05) on Day 56 (Fig. 2a) and influenced IL-1β, IFN-γ, and NF-κB expression (*P*<0.05) on Day 84 (Fig. 2b) in chickens. In experiment 3, administration of a high dosage of LB markedly inhibited IL-6 and IL-10 expression and enhanced TLR4 mRNA abundance (*P*<0.05) on Day 56 (Fig. 2c). High dosage of LB down-regulated IL-1β and TNF-α expression (*P*<0.05) on Day 84 (Fig. 2d). Compared with the LLB and HLB groups, NF-κB mRNA abundance was significantly lower in the MLB group (*P*<0.05; Fig. 2d).
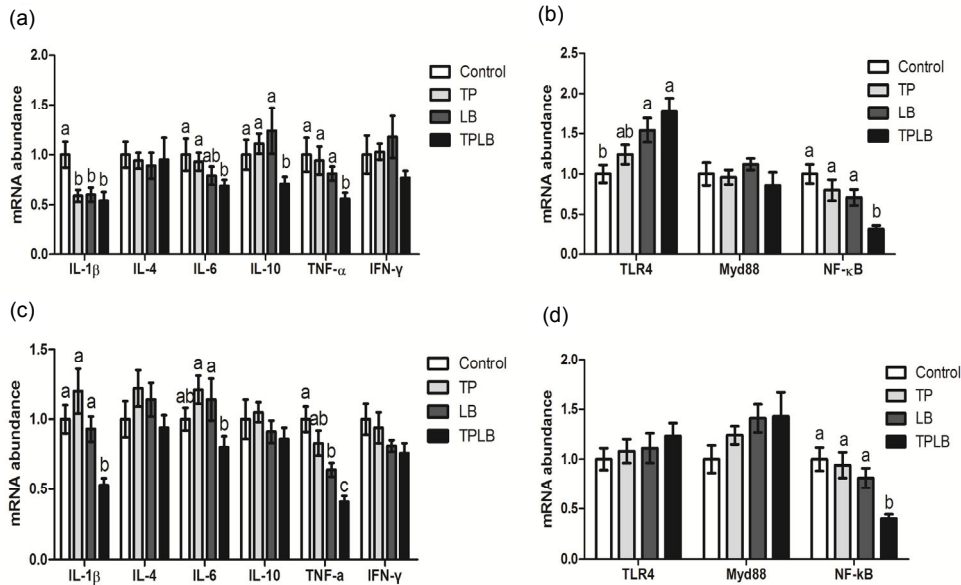
Fig. 1 Effects of TP and LB on expression of genes related to cytokines and NF-κB in broilers in experiment 1
(a) and (b): on Day 56 of age; (c) and (d): on Day 84 of age. The mRNA levels of target genes were normalized using β-actin. Data are expressed as mean±SEM (*n*=60). Values with different superscripts differ significantly (*P*<0.05)
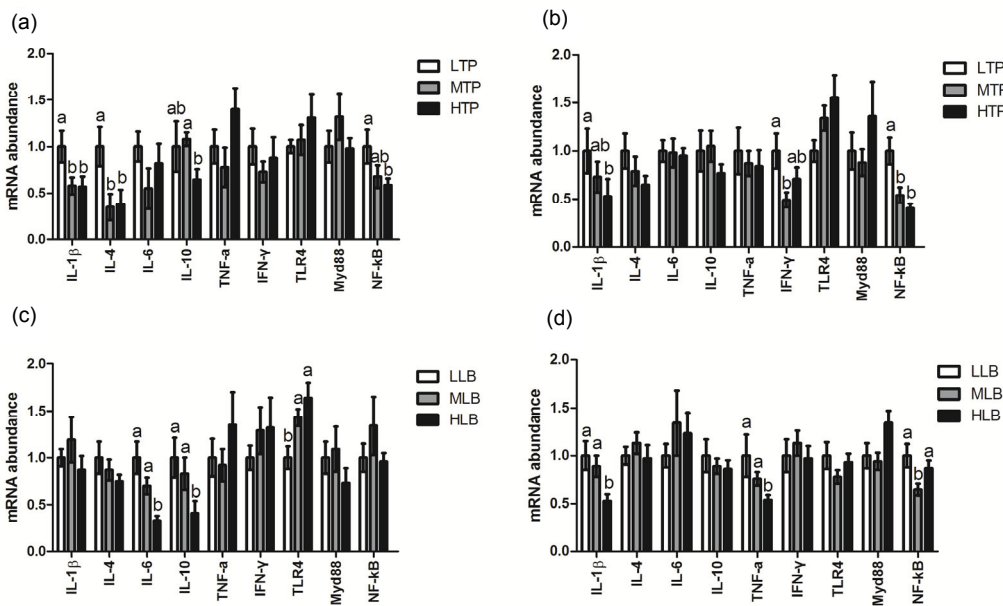

Fig. 2 Effects of TP and LB on expression of genes related to cytokines and NF-κB in broilers in experiments 2 and 3
(a) and (b): experiment 2; (c) and (d): experiment 3. (a) and (c): on Day 56 of age; (b) and (d): on Day 84 of age. The mRNA levels of target genes were normalized using β-actin. Data are expressed as mean±SEM (*n*=60). Values with different superscripts differ significantly (*P*<0.05)

4 Discussion

Serum levels of GLU, LDL-C, TG, CHO, and HDL-C were tested as a reflection of the metabolism and inner organ status of the animal (Wu *et al.*, 2013). The present study revealed that supplementations with TP and LB influence serum LDL-C, TG, and HDL-C concentrations in chickens. TP reduced serum CHO and HDL-C abundances in a dosage-dependent manner in the presence of LB, while LB affected serum GLU and TG levels. Bornhoeft *et al.* (2012) reported that dietary 0.2% TP improved lipid

metabolism in rats fed an atherogenic diet via mediating serum LDL-C and HDL-C concentrations. The mechanism may be that TP can regulate fatty acid synthesis and lipolysis-related genes, including carnitine palmitoyl transferase I, acyl-CoA oxidase 1, peroxisome proliferator-activated receptor-α (PPARα), adipose triglyceride lipase, and lipoprotein lipase (LPL) (Huang *et al.*, 2013; Choi *et al.*, 2015).

LAB also have been demonstrated to affect the health of the host by modifying fatty acid composition (Kishino *et al.*, 2013). Ito *et al.* (2015) further investigated the role of LAB on lipid metabolism and found that LAB exhibited an anti-oxidative activity against LDL oxidation *in vitro*. Thus, long-term TP and LB treatment may improve lipid metabolism in chickens.

Digestive enzymes are considered to be reliable indicators of the nutritional condition of the individuals due to their species and age specificity, sensitivity, and short latency (Lazo *et al.*, 2007; Eastep *et al.*, 2015; Hirai *et al.*, 2015). Various factors can influence digestive enzyme activity, including different dietary supplements (Charron *et al.*, 2014). Kusano *et al.* (2008) reported that TP exhibited lipase and amylase inhibitory activities. The present study also found that oral administration of TP with or without LB inhibited intestinal lipase activity. However, dietary TP in experiment 1 and middle or high dosage of TP in experiment 2 significantly enhanced pancreatic lipase activity. The reason may be our long-term treatment and the *in vivo* study. LAB have been widely reported to interact with food matrix components and to be involved in nutrient metabolism and absorption (Burgain *et al.*, 2014; Michlmayr and Kneifel, 2014), while little is known about the effect of LAB on digestive enzymes. Thus, this is the first study to report the inhibitory effect of LAB on intestinal lipase activity. Combined with TP, high dosage of LAB increased pancreatic lipase activity and reduced intestinal trypsase in this study.

Our previous data revealed that TP and LAB improve immune function in chickens (Liu *et al.*, 2014), so we further investigated the effects of TP and LAB on cytokines expression. We found that administration of TP and LAB mediated cytokines expression (IL-1β, IL-6, IL-10, TNF-α, IFN-γ) on Days 56 and 84. Cytokines play an important role in immune system and inflammatory response (Postal and Appenzeller, 2015). Shen *et al.* (2011b) reported that

green TP reversed LPS-induced chronic inflammation via suppression of TNF-α generation; Qin *et al.* (2010) also found that supplementation with TP (200 mg/kg BW daily) for 6 weeks decreased TNF-α, IL-1β, and IL-6 mRNA abundances and enhanced the anti-inflammatory protein expression in insulin-resistant rats. LAB have also been reported to regulate pro-inflammatory cytokines expression (i.e. IL-1β, IL-6, IL-10, TNF-α, and IFN-γ) and reduce inflammation in animals, including broiler chicks (Chen *et al.*, 2012; Park *et al.*, 2014).

The nuclear factor-κ B (NF-κB) is involved in cellular response to stimuli such as cytokines, ultra-violet irradiation, oxidized LDL, and microbes or viral antigens. After activation, NF-κB mediates cell growth, differentiation, development, apoptosis, and oxidative stress (Yin *et al.*, 2013b). Previous reports indicated that LAB (i.e. *Lactobacillus plantarum* 10hk2 and *L. johnsonii* HY7042) suppressed pro-inflammatory cytokine production via inhibiting NF-κB activation (Chon *et al.*, 2010; Joo *et al.*, 2011), which is consistent with the present result that LB down-regulated NF-κB expression. We also found that TP decreased intestinal NF-κB mRNA abundance on Days 56 and 84, which might mediate cytokines expression. Li *et al.* (2014) reported that TP pretreatment could suppress the TLR4/NF-κB signaling pathway, protecting renal tubular epithelial cells against ischemia/reperfusion-induced apoptosis and inflammation. The mechanism may be that TP blocked phosphorylation and subsequent degradation of inhibitor of κB-α and κB-β subunits (Singh *et al.*, 2011), which contributes to NF-κB activation (Yin *et al.*, 2015b).

In conclusion, results from the current study indicated that long-term treatments of TP and LB improved lipid metabolism, activity of digestive enzymes, and intestinal inflammatory status, which might be associated with the NF-κB signal.

Compliance with ethics guidelines

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中文摘要

题 目：茶多酚和乳酸菌影响肉鸡血液生化、消化酶以及肠道细胞因子表达的研究

目 的：茶多酚和乳酸菌在动物营养上的作用已经得到广泛的验证，但是关于二者联合作用的研究鲜有报道。本文采用肉鸡作为试验模型，研究了茶多酚和乳酸菌联合灌喂对肉鸡血液生化、消化酶以及肠道细胞因子表达的影响。

创新点：本研究首次采用联合灌喂茶多酚和乳酸菌，探讨了二者联合作用对肉鸡的影响，并通过模拟生产，长期观察了茶多酚和乳酸菌对肉鸡的影响。

方 法：对肉仔鸡灌喂不同浓度的茶多酚和乳酸菌，在第56和84天随机屠宰取样。收集血液检查血液生化指标，并测定消化酶活性。取肠道样品提取RNA，采用反转录聚合酶链反应（RT-PCR）检测细胞因子的表达以及相关信号通路的激活。

结 论：长期灌喂茶多酚和乳酸菌改善了肉鸡脂质代谢、消化酶活性以及炎症反应，其机制可能是通过影响了NF-κB信号通路。

关键词：茶多酚；乳酸菌；NF-κB；肉鸡