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Original Article

Dietary Lactic Acid Bacteria Modulate Yolk Components and Cholesterol Metabolism by Hmgr Pathway in Laying Hens

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■Keywords

Cholesterol metabolism; HMGR pathway; lactic acid bacteria; laying hens; yolk components.

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ABSTRACT

This study aimed to investigate the effect of dietary lactic acid bacteria (LAB) on egg production, yolk components, cholesterol metabolism, and enterohepatic circulation of bile acids in hens. Four treatment diets included a control and LAB added at 3×10^5 (low), 3×10^7 (medium), or 3×10^9 (high) cfu/kg. The treatment LAB contained equal amounts of *Lactobacillus acidophilus*, *Lactobacillus plantarum*, and *Enterococcus faecium*. Results showed that high LAB increased ($p<0.05$) laying rate, egg mass, and yolk phospholipid, but decreased ($p<0.05$) yolk triglyceride and phosvitin. Diets with LAB decreased ($p<0.05$) yolk and serum cholesterol content, and serum bile acid by 9.3 to 39.9%. In liver, high LAB downregulated ($p<0.05$) mRNA expression of serine/threonine kinase 11 (STK11), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), AMP-activated protein kinase catalytic subunit (PRKAA1, 2), and protein phosphatase catalytic subunits (PPP2CA, PPP2CB and PPP3CA) by 49.5 to 175.4%. In mucosa, high LAB downregulated ($p<0.05$) PRKAA1 and HMGR by 68.2 and 69.6%, respectively; but upregulated ($p<0.05$) PPP2CA and PPP2CB by 51.2 and 45%, respectively. Linear decreasing ($p \leq 0.035$) responses to LAB doses were found on cholesterol, phosvitin, bile acid, and hepatic gene expressions, and quadratic ($p \leq 0.006$) effects on yolk cholesterol and hepatic STK11. It is concluded that probiotic LAB can improve yolk components and decrease hepatic cholesterol synthesis by regulating HMGR pathway in hens.

INTRODUCTION

Lactic acid bacteria (LAB) such as *Lactobacillus acidophilus* (*L. acidophilus*), *Lactobacillus plantarum* (*L. plantarum*), and *Enterococcus faecium* (*E. faecium*) are among the most important groups of microorganisms used in food fermentations and health promotion. Clinical trials showed that LAB ingested in sufficient numbers produced nutrition and health benefits with regards to nutrient digestibility, gut microbiota, immunity, and metabolism (Saez-Lara *et al*., 2015; Wang *et al*., 2018; Wang *et al*., 2019a,b; Zhao *et al*., 2020). Cholesterol is an essential structural component of lipid membranes and a precursor to steroid hormones and bile acids; biosynthesized by all animal cells, but primarily by hepatocytes (Howles, 2016). Eggs and meat are the main dietary sources for cholesterol (Ahn *et al*., 1999). Excess dietary cholesterol coupled with its hepatic biosynthesis can lead to hypercholesterolemia and so threaten cardiovascular health (Cha and Park, 2019). It is therefore considered desirable to reduce the cholesterol content in animal derived foods.

Recent dietary intervention with LAB found that it contributed to improving lipid metabolism and reducing cholesterol. *L. plantarum* reduced serum triglyceride, cholesterol, low-density lipoprotein

and very-low-density lipoprotein cholesterol, and atherogenic index in rats (Aminlari *et al*., 2018). The combination of *L. acidophilus* and β-cyclodextrin lowered 27.9% serum low-density lipoprotein cholesterol in pigs (Alonso *et al*., 2016). *Pediococcus acidilactici* supplements fed to hens reduced yolk cholesterol by more than 10% (Mikulski *et al*., 2012). One of the potential mechanism for cholesterol reduction by probiotics is the deconjugation or excretion of bile salts. *L. plantarum* induced bile acid synthesis, enhanced bile flow and biliary glutathione output, and fecal bile acid excretion in mice (Zhai *et al*., 2019). *L. acidophilus* affected intestinal bile acid composition and vitamin E acetate metabolism of mice (Roager *et al*., 2014), but *L. plantarum* had no effect on stool bile acids, serum lipids, glucose, and insulin in healthy obese adults (Culpepper *et al*., 2019). Notably, besides into bile acid, cholesterol is also metabolized into steroids and vitamin D, whether probiotics influence their enterohepatic circulation or circulating concentrations is unknown.

The reaction catalyzed by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) is the rate limiting step in de *novo* cholesterol biosynthesis (Howles, 2016). This enzyme is further modified by AMP-activated protein kinase (AMPK) and protein phosphatases of the 2A family (PP2A), and serine/threonine kinase 11 (STK11). Because probiotics are capable of improving lipid metabolism and reducing cholesterol, we hypothesized that they may act in part through changes in HMGR activity and its downstream products in farm animals. The present study aimed to investigate the effect of LAB on the egg production, yolk components, cholesterol and its metabolites, and gene expression related to cholesterol synthesis in laying hens.

MATERIALS AND METHODS

LAB strains and diets

Lactic acid bacteria strains including *L. acidophilus* (ACCC11073), *L. plantarum* (CICC21863), and *E. faecium* (CICC20430) were obtained from the Animal Biotechnology Lab in Henan University of Science and Technology (Luoyang, China). The strains were combined at an equal amount and added into a basal diet at final dose rates of 0 (control), 3.0×10^5 (low), 3.0×10^7 (medium), or 3.0×10^9 (high) colony forming unit (cfu)/kg of diet. A total of 1.00 g of the strain powder was diluted with 9 mL of 1% Tryptic Soy Broth medium (TSB, Hopebio-Technology Co., Ltd, Qingdao, China) and then homogenized. Viable counts of bacteria were conducted by plating serial 10-fold dilutions onto TSB agar plates after cultured at anaerobically 37°C, pH 6.2, for 24 h. Compositions and nutrient levels of the basal diet are listed in Table 1.

Table 1 – Composition and nutrient level of basal diet (air-dry basis).

Ingredient	Content, g/kg	Nutrient **	Content, g/kg
Corn	604	Metabolizable energy, MJ/kg	11.28
Soybean meal	235	Crude protein	175.3
Corn gluten	45	Ca	35.1
Met	2	Non-phytate P	4.5
Salt	4	Met	5.0
Di-calcium phosphate	15	Lys	8.2
Limestone	85	Met+Cys	7.9
Premix *	10		

*Provides (per kg of diet): vitamin A (retinyl acetate), 8,000 IU; cholecalciferol, 1,600 IU; vitamin E (DL-α-tocopheryl acetate), 5 IU; vitamin K, 0.5 mg; riboflavin, 2.5 mg; D-pantothenic acid, 2.2 mg; niacin, 20 mg; pyridoxine, 3.0 mg; biotin, 0.10 mg; folic acid, 0.25 mg; vitamin B12, 0.004 mg; choline, 500 mg; manganese, 60 mg; iodine, 0.35 mg; iron, 60 mg; copper, 8 mg; zinc, 80 mg; and selenium, 0.30 mg.

**Calculated by Chinese Feed Database (2014, 25th ed).

Animals and samples

The Institutional Committee for Animal Use and Ethics of Henan University of Science and Technology approved the experimental protocol of this study.

One hundred and ninety two, 23 wk old Hyline brown laying with similar body weights (mean ± SEM, 1796 g ± 4.10) were allocated randomly into 4 treatment groups, with 6 replicates per treatment and 8 laying hens per replicate. All hens were housed in 3-tiered cages at the Gonghua Poultry Company (Luoyang, China) with free access to feed and water at all times. During the period, hens received 16 h light/d within ventilated houses (airflow rate here). Hens were observed during a 1-wk adjustment period to monitor health status and egg production prior to the start of the 6 wk experiment. Feed intake, laying rate, and egg weight were recorded daily for each replicate.

All eggs laid during the last 3 d of the trial were collected from each replicate. A subsample of 15 eggs

from each replicate were selected randomly for analysis. At the end of the feeding trial, 6 hens were randomly selected from each replicate and venous blood collected from the right wing for serum preparation (Liu *et al*., 2018 a,b). The birds were euthanized by CO_2 inhalation prior to harvest of the entire liver. Intestinal mucosa was collected from a 10 cm (approximate) section of proximal jejunum following removal of digesta by deionized water (0 to 4°C) flushes. The liver and mucosa samples were halved. One portion of each tissue type was stored in RNA later for gene expression analysis, while the other portion was stored at -80°C for chemical quantification.

Biochemical analysis

Phospholipid (Catalog No. MET-5085), total cholesterol (Catalog No. STA-390), and triglyceride (STA-397) contents of yolk and serum were measured using commercial kits (Cell Biolabs, Inc., San Diego, CA, USA) and Fluoroskan™ Microplate Fluorometer (Thermo Scientific, Waltham, MA, USA). Yolk phosvitin was quantified by ELISA kit (Abbkine Scientific Co., Ltd., Wuhan, China). IgY Chicken SimpleStep ELISA™ Kit, Total Bile Acid Assay Kit (Fluorometric, MET-5005) from Abcam Trading Co. Ltd. (Shanghai, China), Pregnenolone ELISA Kit (Catalog No. E4633) from Biovision Incorporated (Milpitas, CA, USA), and Calcitriol ELISA Kit (Catalog No. KC0962) from Aviva Systems Biology, Corp. (San Diego, CA, USA) were also used for biochemical parameter analysis. The sample preparation and analytical procedures were carried out according to manuals and literature (Liu *et al*., 2010; Ding *et al*., 2019 a,b). All samples were assayed in triplicate. Biochemical components in jejunal mucosa were expressed as concentration relative to per gram mucosal protein.

Reagents, primer synthesis and cDNA sequencing for RT-qPCR analysis were provided by TaKaRa Co. Ltd. (Dalian, China). Gene expression data was analyzed by the comparative C_T method using beta-actin gene as an internal control in the treated and untreated samples according to the method by Schmittgen and Livak (2008). The qPCR reactions were set at 10 µL with 5 µL of SYBR Green Master Mix, 1 µL of primer (Table 2), 4 µL of 10 × diluted cDNA. All qPCRs were run in triplicate using 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) on the ABI Prism 7900HT Fast Real-Time PCR System. No amplification signal was detected in water or no-RT RNA control samples.

Statistical analysis

Data are reported as grand means and standard error of the mean (SEM) for the six replicate means in each treatment group. All calculations were carried out using SPSS software (IBM SPSS, version 23, Armonk, NY, USA). Differences between mean values of normally distributed data were assessed with one-way ANOVA and Tukey'b-test at $p<0.05$ level of significance, and Tamhane's T2 test for parameters with heterogeneity variance. The mean values from each replicate pen was the statistical unit for growth performance and parameters from the serum, liver, and jejunal mucosa, and the mean of the 15 eggs subsamples from each replicate pen was the statistical unit for yolk components. The response trend of experimental parameter to the three LAB doses was analyzed using contrasts of linear and quadratic polynomial.

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

		Primers (5'→3')		Length
Name	GenBank	Forward	Reverse	(bp)
STK11	NM_001045833.1	tggtgcccatacctcctagc	tggtgcccatacctcctagc	244
PRKAA1	NM_001039603.1	caagtagtgtctcgcacggt	gactgatagctggtcccacg	133
PRKAA2	NM_001039605.1	ttcggcaaagtcaaggttgg	gaagaagtctgttggcgtgc	192
HMGR	NM_204485.2	ttctcggccgggcgattt	tacagcgtgtgattgtcagga	256
PPP2CA	nm_001006152.1	tgttcaccaaggagctcgac	ctgtgacaggacatcgcact	147
PPP2CB	NM_205124.1	gctcaccaactcgtcatgga	gtacgacgggtaacgtgagg	194
PPP3CA	XM_025149952.1	cgtgtaaacacagcccgttt	ggccatggatgtctccacaa	271
Beta-actin	NM_205518	gccgagagagaaattgtgcg	cacaggactccatacccaaga	208

HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; PPP2CA, protein phosphatase 2 catalytic subunit alpha; PPP2CB, protein phosphatase 2 catalytic subunit beta; PPP3CA, protein phosphatase 3 catalytic subunit alpha; PRKAA1, AMP-activated protein kinase catalytic subunit alpha-1; PRKAA2, protein kinase AMP-activated catalytic subunit alpha-2; and STK11, serine/threonine kinase 11.

RESULTS

Effect of dietary LAB on egg production and yolk components

The addition of LAB at 3 × 10^9 cfu/kg improved ($p<0.05$) laying rate and egg mass, compared to the control diet, and linear responses ($p\leq0.043$) to LAB doses

were found for feed intake, laying rate, egg mass, and egg weight (Table 1). For yolk components, LAB at 3 × 10^9 cfu/kg decreased (p<0.05) triglyceride and phosvitin, but increased (p<0.05) phospholipid; the three doses of LAB decreased (p<0.05) cholesterol by 19.9 to 43.7%. Linear responses (p≤0.002) were found for phospholipid, cholesterol, and phosvitin, and quadratic responses (p≤0.008) for phospholipid and cholesterol.

Table 3 – Effect of dietary lactic acid bacteria on egg production and yolk components of laying. hens.

		Lactic acid bacteria, cfu/kg				*p* value	
Item	Control	3×10^5	3×10^7	3×10^9	SEM	Linear	Quadratic
Egg production							
Feed intake, g/day	97.00	96.77	96.89	97.50	1.417	0.019	0.307
Laying rate, %	80.76^b	81.00^b	82.59^{ab}	83.63^a	0.512	0.013	0.532
Egg mass, g/day	41.87^b	41.84^b	42.67^{ab}	43.13^a	0.274	0.012	0.377
Egg weight, g/egg	51.86	51.65	51.68	51.58	0.303	0.043	0.714
Feed/mass	2.317	2.313	2.271	2.260	0.030	0.086	0.235
Yolk components, mg/g							
Triglyceride	206.9^a	196.3^{ab}	194.9^{ab}	189.2^b	3.668	0.134	0.579
Phospholipid	91.69^b	93.10^b	91.92^b	97.61^a	0.896	0.002	0.004
Cholesterol	15.50^a	12.93^b	12.35^c	10.79^d	0.115	<0.001	0.008
Phosvitin	27.63^a	28.15^a	26.41^{ab}	24.86^b	0.526	<0.001	0.878
IgY	3.73	3.95	4.13	4.13	0.155	0.436	0.628

$^{a-d}$ Means within a row without a common superscript are significantly different, p<0.05.

Effect of dietary LAB on tissue cholesterol and its metabolites

Compared to the control, the three doses of dietary LAB also decreased (p<0.05) cholesterol by 14.3 to 43.1% in the serum and jejunal mucosa, and serum bile acid by 17.4 to 41.5% (Table 4). In the jejunal mucosa, bile acid content was reduced (p<0.05) with LAB at 3 × 10^5 and 3 × 10^9 cfu/kg. Linear effects (p≤0.034) were found on cholesterol, bile acid, and calcitriol in the serum, and cholesterol in the jejunal mucosa.

Effect of dietary LAB on the expression of genes related to cholesterol synthesis

In the liver, the three doses of dietary LAB downregulated (p<0.05) the mRNA expression of STK11, AMP-activated protein kinase catalytic subunit alpha-1 (PRKAA1), HMGR, protein phosphatase 2 catalytic subunit alpha (PPP2CA), and protein phosphatase 2 catalytic subunit beta (PPP2CB), compared to the control (Table 5). LAB doses at 3 × 10^7 and 3 × 10^9 cfu/kg downregulated (p<0.05) protein kinase AMP-activated catalytic subunit alpha 2 (PRKAA2) and protein phosphatase 3 catalytic subunit alpha (PPP3CA). Linear responses (p≤0.035) were found for all the target genes, and quadratic responses (p≤0.006) for STK11 and PPP2CB.

In the jejunal mucosa, compared to the control, LAB at 3 × 10^9 cfu/kg downregulated (p<0.05) the gene expression of PRKAA1, but upregulated (p<0.05) PPP2CA. LAB at any of the three doses downregulated (p<0.05) HMGR by 30.7 to 93.2%, but upregulated (p<0.05) PPP2CB by 43.0 to 57.0%. Expression of PRKAA1, PRKAA2, and HMGR decreased linearly in response to LAB doses (p≤0.035).

Table 4 – Effect of dietary lactic acid bacteria on tissue cholesterol and its metabolites in laying hens.

		Lactic acid bacteria, cfu/kg				*p* value	
Item	Control	3×10^5	3×10^7	3×10^9	SEM	Linear	Quadratic
Serum							
Cholesterol, mg/dL	169.9^a	145.6^b	129.5^b	124.1^b	5.780	0.009	0.392
Pregnenolone, ng/dL	337.1	346.5	336.6	349.2	9.368	0.860	0.409
Bile acid, µg/dL	159.9^a	132.1^b	110.2^c	93.61^d	3.264	<0.001	0.540
Calcitriol, µg/dL	2.83^{ab}	2.65^b	2.82^{ab}	2.95^a	0.048	0.002	0.779
Jejunal mucosa, per g mucosal protein							
Cholesterol, mg	64.94^a	42.62^b	37.55^b	36.90^b	1.757	0.034	0.314
Pregnenolone, ng	197.6	200.9	198.3	197.6	6.087	0.703	0.902
Bile acid, µg	76.85^a	70.48^b	72.99^{ab}	68.91^b	1.550	0.476	0.098
Calcitriol, µg	1.06	1.04	1.02	0.94	0.062	0.385	0.728

$^{a-d}$ Means within a row without a common superscript are significantly different, p<0.05.

Table 5 – Effect of dietary lactic acid bacteria on the expression of genes related to cholesterol synthesis in laying hens.

		Lactic acid bacteria, cfu/kg				*p* value	
Item	Control	3×10^5	3×10^7	3×10^9	SEM	Linear	Quadratic
Liver (mRNA, $2^{-\Delta\Delta Ct}$)							
STK11	7.75^a	6.02^b	6.47^b	4.70^c	0.241	0.005	0.006
PRKAA1	5.28^a	3.73^b	3.53^{bc}	2.79^c	0.148	0.001	0.212
PRKAA2	2.67^a	2.76^a	2.11^b	1.72^c	0.077	<0.001	0.204
HMGR	4.23^a	2.86^b	2.14^c	1.53^d	0.135	<0.001	0.764
PPP2CA	4.88^a	3.85^b	4.04^b	3.20^c	0.118	0.003	0.004
PPP2CB	2.32^a	1.89^b	1.66^{bc}	1.50^c	0.079	0.005	0.771
PPP3CA	3.25^a	2.80^{ab}	2.17^{bc}	2.30^c	0.131	0.035	0.061
Jejunal mucosa (mRNA, $2^{-\Delta\Delta Ct}$)							
STK11	1.79	1.63	1.49	1.76	0.165	0.562	0.309
PRKAA1	3.15^a	2.79^{ab}	2.40^{bc}	1.87^c	0.155	0.001	0.709
PRKAA2	3.40	3.35	3.10	2.86	0.143	0.035	0.968
HMGR	5.25^a	4.01^b	2.71^c	3.09^c	0.185	0.002	0.001
PPP2CA	3.33^b	4.29^{ab}	3.75^{ab}	5.04^a	0.345	0.155	0.052
PPP2CB	3.63^b	5.19^a	5.70^a	5.26^a	0.231	0.811	0.076
PPP3CA	3.50	3.48	3.19	3.73	0.334	0.607	0.341

$^{a-d}$ Means within a row without a common superscript are significantly different, $p<0.05$.

HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; PPP2CA, protein phosphatase 2 catalytic subunit alpha; PPP2CB, protein phosphatase 2 catalytic subunit beta; PPP3CA, protein phosphatase 3 catalytic subunit alpha; PRKAA1, AMP-activated protein kinase catalytic subunit alpha-1; PRKAA2, protein kinase AMP-activated catalytic subunit alpha-2; STK11, serine/threonine kinase 11.

Figure 1 exhibits the relationship of HMGR pathway activity and mRNA expression influenced by dietary LAB at 3 × 10^9 cfu/kg of feed compared to the control diet. In the liver, all target genes were downregulated marked as (-), indicating that the LAB may slow cholesterol biosynthesis. In the jejunal mucosa, similar results were found for PRKAA1 and HMGR, but STK11 and PRKAA1 were not affected as (o), and PP2A subunits PPP2CA and PPP2CB were upregulated as (+).

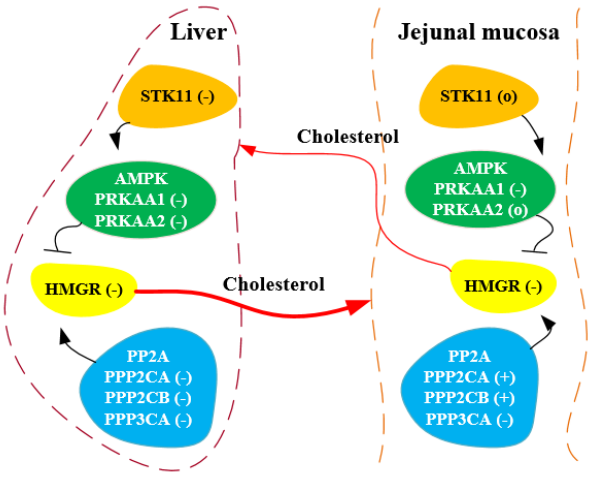

Figure 1 – Effect of lactic acid bacteria on HMGR pathway of cholesterol synthesis.

(-), (o), and (+) denote that gene mRNA expression is downregulated, unaffected, and upregulated, respectively, compared to the control diet, for hens fed a diet containing lactic acid bacteria at 3 × 109 cfu/kg. AMPK, AMP-activated protein kinase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; PP2A, protein phosphatases of the 2A family; PPP2CA, protein phosphatase 2 catalytic subunit alpha; PPP2CB, protein phosphatase 2 catalytic subunit beta; PPP3CA, protein phosphatase 3 catalytic subunit alpha; PRKAA1, AMP-activated protein kinase catalytic subunit alpha-1; PRKAA2, protein kinase AMP--activated catalytic subunit alpha-2; STK11, serine/threonine kinase 11.

DISCUSSION

In the present study, dietary LAB including *L. acidophilus*, *L. plantarum*, and *E. faecium* increased laying rate and egg mass, indicating the potential benefits of the probiotics in the host health and nutrition. This was supported by the studies that LAB improved growth performance and nutrient digestibility of broiler chickens (Liu *et al*., 2018a,b). Furthermore, the per gram yolk concentrations of triglyceride, cholesterol, phospholipid, and phosvitin were also influenced by the dietary LAB in the present study. This was similar to the report that LAB increased hemoglobin, albumin and total protein, but decreased cholesterol, total bilirubin, creatinine of broilers (Liu *et al*., 2018c; Wang *et al*., 2018). Others reported that *L. acidophilus* (strain D2/CSL CECT 4529) decreased egg lipid content of laying hens (Forte *et al*., 2016); *L. plantarum* (strain MDL1118) and *E. faecium* strain (MDF1104) removed yolk cholesterol by 58 and 38%, respectively, when incubated in medium containing 4% egg yolk (Liu *et al*., 2013). However, commercial probiotics Bioplus and Primalac increased the concentration of total cholesterol in broilers (Kivi *et al*., 2015).

Some probiotic LAB strains have been reported promising treatments for high cholesterol and bile acid content (Singhal *et al*., 2019), but information about LAB effects on cholesterol metabolism is unavailable. The present study is the first to report the effect of dietary probiotics on the gut mucosal

cholesterol content and its downstream metabolites in animals. The observed decreased concentration of cholesterol and bile acid in serum and jejunal mucosa reported here suggest that LAB may slow the rates of cholesterol synthesis or promote its catabolism during the enterohepatic circulation. Bile acids, the major end-product of cholesterol metabolism, are synthesized in the liver and secreted via bile into the intestine to aid the absorption of fat-soluble vitamins and dietary fat (Di Ciaula *et al*., 2017).

Simultaneous measurements of biliary and intestinal cholesterol secretion in mice showed that plasma cholesteryl ester transfer protein (CETP) altered the route of cholesterol elimination from the body in mice fed a high fat, high cholesterol diet (Li *et al*., 2019). Overexpression of bile salt hydrolase by a *Lactobacillus* strain reduced cholesterol absorption and increased cholesterol catabolism in mice fed a high cholesterol diet (Wang *et al*., 2019c). In the present study, diets did not contain cholesterol, and only biosynthesis *de novo* provided cholesterol to the laying hens. Chickens have CETP, but typically consume low fat, lower (in case of diets containing animal derived components) or no cholesterol diets, and so whether the LAB strains affect the route of cholesterol elimination by influencing CETP needs further study. Factors that influence cholesterol absorption, *de novo* synthesis and metabolism are subjects of extensive research in humans (Alphonse and Jones, 2016). Similar studies are not available for hens, and details of these same topics are still throughout egg production are poorly described.

Notably, in addition to processes related to changes in bile metabolism and excretion, Reis *et al*. (2017) suggested that incorporation, and assimilation of cholesterol into cell membranes or gametes are possible potential mechanisms responsible for the cholesterol lowering effect of probiotics. Outcomes reported here showed that LAB treatments lowered cholesterol concentrations in yolk, serum and mucosa, coincident with reduced tissue HMGR expression, which indicates reduced cholesterol biosynthesis in these hens fed a cholesterol free diet. Thus, the inhibition of cholesterol biosynthesis in the liver or other tissues can be another potential mechanism by which probiotics could produce hypocholesterolaemic effects. Furthermore, it is known that hypocholesterolaemic effects vary among probiotics strains, as well as the physiological state and diet type of the animal ingesting them.

Cholesterol is also a precursor molecule in the biosynthesis of steroid hormones and vitamin D. Pregnenolone is a cholesterol metabolite and precursor/metabolic intermediate in the biosynthesis of progestogens, androgens, estrogens, glucocorticoids, and mineralocorticoids (Weng & Chung, 2016). Thus, there could be concern whether probiotic mediated cholesterol lowering has a negative effect on the critical hormone and vitamin D production by precursor limitation. While literature is unavailable on this point, results from the present study found that dietary LAB did not affect tissue concentrations of pregnenolone, indicating that LAB has no negative influences on the synthesis of steroid hormones. In addition, hens continued to lay eggs and in fact increased egg production. Cholesterol is a precursor for bile acids and there may be preferential use of cholesterol for critical precursor functions. Calcitriol, the active form of vitamin D, decreased in the serum of hens fed the low LAB diet, but not in hens fed the medium and high LAB dose diets. Likely, LAB have negligible negative effects on vitamin D metabolism and its related functions, however, confirmation studies are of interest.

Cholesterol biosynthesis *de novo*, is largely controlled by a crucial rate limiting enzyme HMGR. This enzyme is controlled by four distinct mechanisms: feed-back inhibition, control of gene expression, rate of enzyme degradation and phosphorylation-dephosphorylation (DeBose-Boyd, 2008; Liu *et al*., 2009). The present study measured the mRNA expression of HMGR and related genes including PPP2CA, PPP2CB, PPP3CA, PRKAA1, PRKAA2, and STK11, and found that dietary LAB dose above 3×10^7 cfu/kg significantly downregulated the expression of these genes in the liver. The findings indicate that cholesterol biosynthesis is reduced by high-dose LAB and suggests that ingested LAB may also act by regulating liver and gut gene expression.

In the liver, the effect of LAB on mRNA profiles was a general dose-dependent reduction in gene expression of varying levels of significance. However, in the jejunal mucosa, LAB did not influence STK11, PRKAA2, and PPP3CA, whereas only high dose of LAB upregulated PPP2CA and PPP2CB. Expression of PPKAA1 and HMGR genes were reduced by LAB in both the liver and mucosa, with mucosa less affected than liver. A study reported that STK11 downregulation decreased intracellular cholesterol levels in macrophages treated with the isoflavone puerarin (Li *et al*., 2017). AMPK subunits, PRKAA1 and PRKAA2, harbored largely non-overlapping genetic determinants for body fat mass, glucose and cholesterol metabolism (Randrianarisoa *et al*., 2019), and were activated by aerobic exercise

training in mice (Ferreira *et al*., 2017). There are no data in the nutrition on possible roles played by dietary factors in the activities of AMPK subunits. It is known that PPP2CA and PPP2CB, the coding genes of protein phosphatase catalytic subunits, can be inhibited by a cholesterol rich diet in mice (Pichavaram *et al*., 2019). Besides HMGR gene expression for cholesterol regulation, other mechanisms such as enzyme degradation rates as well as rates or regulators of phosphorylation-dephosphorylation need further study.

In summary, the three doses of LAB decreased tissue cholesterol and bile acid concentrations. Diets containg 3×10^9 cfu/kg of the LAB mixture increased laying rate, egg mass, and yolk phospholipid concentrations, but decreased yolk concentrations of triglyceride and phosvitin. These changes accompanied increased expression of the PPP2CA and PPP2CB genes in jejunal mucosa, as well as reduced expression of all hepatic genes related to cholesterol synthesis and PRKAA1 and HMGR expression in the jejunal mucosa. Dose-dependent decreases in cholesterol, phosvitin, serum bile acid concentrations and associated changes in hepatic gene expression in conjunction with improved egg performance suggest that LAB can be an effective additive to decrease cholesterol in yolk. The probiotic mixture acts in part by regulating hepatic cholesterol synthesis at the point of HMGR.

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N. L., Q. D., H. S. and J. W. conceived the project, and developed the overall research plan and had study oversight. Q. D. and Y. L. conducted the research and oversaw the analysis of the samples. N. L. and H. S. performed the statistical analysis. Prof. R. Walzem at Texas A&M University reviewed the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

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