Effects of Dietary Enterococcus faecalis Supplementation on Production Performance, Egg Quality, Lipid Metabolism, and Intestinal Microbial Count in Laying Hens (Postprint)

Authors: Liu Song, Dong Xiaofang, Tong Jianming, Yan'e Bao, Wang Zhihong

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Abstract

This study aimed to investigate the effects of dietary Enterococcus faecalis supplementation on production performance, egg quality, lipid metabolism, and intestinal microbiota in laying hens. A total of 450 Hy-Line Brown laying hens at 137 days of age were randomly allocated to 5 groups with 6 replicates per group and 15 hens per replicate, and fed basal diets supplemented with 0, $1.0\$\times10^4, 1.0\times10^6, 1.0\times10^8, and 1.0\times10^{10} CFU/g Enterococcus faecalis (CGMCC 1.2135T) for 168 days. Theres$
$1) During days 113–140 and 141–168, egg production in the 1.0\times10^6 CFU/g Enterococcus faecalis supplemented g$
$0.01). During days 141–168, the feed conversion ratio of the 1.0\times10^4 CFU/g Enterococcus faecalis supplemented$
$0.05). 2) On day 56, eggshell thickness in all Enterococcus faecalis supplemented groups was significantly higher th$
$0.05), and albumen height in the control group and the 1.0\times10^6 CFU/g Enterococcus faecalis supplemented groupw$
$0.05); on days 84 and 140, albumen height in the 1.0\times10^8 CFU/g Enterococcus faecalis supplemented group was assig$
$0.05). On day 56, Haugh units in the 1.0\times10^6 CFU/g Enterococcus faecalis supplemented group were highly signi fi$
$0.01); on day 84, Haugh units in the 1.0\times10^6 and 1.0\times10^8 CFU/g Enterococcus faecalis supplemented groups were s$
$0.05). On day 28, yolk color in the 1.0\times10^{10} CFU/g Enterococcus faecalis supplemented group was highly signi fican$
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$0.01), while yolk color in the 1.0\times10^{10} CFU/g Enterococcus faecalis supplemented group was highly significantly$
$0.01). 3) On days 56 and 112, egg yolk total cholesterol in the 1.0\times10^8 CFU/g Enterococcus faecalis supplemented gro$
$0.01). Compared with the control group, dietary Enterococcus faecalis supplementation significantly or highly sig$
$0.01), low-density lipoprotein cholesterol content (P < 0.01) on day 84, and serum triglyceride content on day 168 (P$
$0.05). 4) Ileal Escherichia coli counts in the 1.0\times10^6, 1.0\times10^8, and 1.0\times10^{10} CFU/g Enterococcus faecalis supplem$
$0.05); jejunal Escherichia coli counts decreased linearly with increasing Enterococcus faecalis supplementation$
$0.05). Ileal Enterococcus faecalis counts in the 1.0\times10^{10} CFU/g Enterococcus faecalis supplemented group were h$
$0.01); cecal Enterococcus faecalis counts in the 1.0\times10^8 and 1.0\times10^{10} CFU/g Enterococcus faecalis supplemented$

0.01).*Theresultsindicatedthatdietary Enterococcus faecalissupplementationcouldimproveeggproduction*, *alb*
CFU/g.

Full Text

Effects of Dietary Enterococcus faecalis on Performance, Egg Quality, Lipid Metabolism and Intestinal Microflora Numbers of Laying Hens

LIU Song, DONG Xiaofang*, TONG Jianming, BAO Yan' e, WANG Zhihong

Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing 100193, China

Abstract

This experiment was conducted to investigate the effects of dietary Enterococcus faecalis on performance, egg quality, lipid metabolism and intestinal microflora numbers of laying hens. Four hundred and fifty 137-day-old Hy-Line brown laying hens were randomly allocated to 5 groups with 6 replicates per group and 15 hens per replicate. Hens were fed the basal diet supplemented with 0, 1.0$×10^{4}, 1.0×10^{6}, 1.0×10^{8}$ and 1.0$×10^{10}$ CFU/g Enterococcus faecalis (CGMCC1.2135T), respectively. The experiment lasted for 168 days. The results showed as follows: 1) Egg mass of laying hens in the 1.0$×10^{6}$ CFU/g Enterococcus faecalis supplemental group was significantly higher than that in the control group and the other Enterococcus faecalis supplemental groups during days 113-140 and days 141-168 (P<0.01). During days 141-168, the feed-to-egg ratio in the 1.0$×10^{4}$ CFU/g Enterococcus faecalis supplemental group was significantly lower than that in the 1.0$×10^{8}$ CFU/g Enterococcus faecalis supplemental group (P<0.05). 2) On day 56, eggshell thickness in all Enterococcus faecalis supplemental groups was significantly higher than that in the control group (P<0.05), and albumen height in the control group and the 1.0$×10^{6}$ CFU/g Enterococcus faecalis supplemental group was significantly higher than that in the 1.0$×10^{4}$ and 1.0$×10^{10}$ CFU/g Enterococcus faecalis supplemental groups (P<0.05). On days 84 and 140, albumen height in the 1.0$×10^{8}$ CFU/g Enterococcus faecalis supplemental group was significantly higher than that in the 1.0$×10^{4}$ CFU/g Enterococcus faecalis supplemental group (P<0.05). On day 56, Haugh unit in the 1.0$×10^{6}$ CFU/g Enterococcus faecalis supplemental group was significantly higher than that in the 1.0$×10^{4}$ and 1.0$×10^{10}$ CFU/g Enterococcus faecalis supplemental groups (P<0.01). On day 84, Haugh unit in the 1.0$×10^{6}$ and 1.0$×10^{8}$ CFU/g Enterococcus faecalis supplemental groups was significantly higher than that in the 1.0$×10^{4}$ CFU/g Enterococcus faecalis supplemental group (P<0.05). On day 28, yolk color in the 1.0$×10^{10}$ CFU/g Enterococcus faecalis supplemental group was significantly higher than that in the control group and the 1.0$×10^{4}$ CFU/g

Enterococcus faecalis supplemental group (P<0.01). On day 56, yolk color in the 1.0$×10^{4}, 1.0×10{6}$ and 1.0$×10^{10}$ CFU/g Enterococcus faecalis supplemental groups was significantly higher than that in the control group (P<0.01). On day 112, yolk color in all Enterococcus faecalis supplemental groups was significantly higher than that in the control group (P<0.01), and yolk color in the 1.0$×10^{8}$ CFU/g Enterococcus faecalis supplemental group was significantly higher than that in the 1.0$×10^{6}$ CFU/g Enterococcus faecalis supplemental group (P<0.01). On day 140, yolk color in the 1.0$×10^{8}$ CFU/g Enterococcus faecalis supplemental group was significantly higher than that in the control group (P<0.01), while yolk color in the 1.0$×10^{10}$ CFU/g Enterococcus faecalis supplemental group was significantly lower than that in the control group and the other Enterococcus faecalis supplemental groups (P<0.01). 3) The content of total cholesterol in egg yolk of laying hens in the 1.0$×10^{8}$ CFU/g Enterococcus faecalis supplemental group was significantly lower than that in the control group and the 1.0$×10^{4}$ and 1.0$×10^{6}$ CFU/g Enterococcus faecalis supplemental groups on days 56 and 112 (P<0.01). Compared with the control group, dietary Enterococcus faecalis significantly decreased the contents of total cholesterol and low-density lipoprotein cholesterol in serum on day 84 (P<0.01) and the content of triglyceride in serum on day 168 (P<0.05). 4) The number of Escherichia coli in ileum in the 1.0$×10^{6}, 1.0×10{8}$ and 1.0$×10^{10}$ CFU/g Enterococcus faecalis supplemental groups was significantly lower than that in the control group (P<0.05), and the number of Escherichia coli in jejunum had a linear decrease with the increasing Enterococcus faecalis supplemental level (P<0.05). The number of Enterococcus faecalis in ileum in the 1.0$×10^{10}$ CFU/g Enterococcus faecalis supplemental group was significantly higher than that in the control group and the 1.0$×10^{4}$ and 1.0$×10^{8}$ CFU/g Enterococcus faecalis supplemental groups (P<0.01), and the number of Enterococcus faecalis in caecum in the 1.0$×10^{8}$ and 1.0$×10^{10}$ CFU/g Enterococcus faecalis supplemental groups was significantly higher than that in the control group (P<0.01). This study indicates that dietary Enterococcus faecalis can increase egg mass, albumen height and yolk color, decrease the content of cholesterol in serum and egg yolk and regulate intestinal microflora numbers of laying hens, and the appropriate supplemental amount of Enterococcus faecalis in diets of laying hens is 1.0$×10^{6}$ or 1.0$×10^{8}$ CFU/g.

Key words: Enterococcus faecalis; laying hen; performance; egg quality; lipid metabolism; intestinal microflora numbers

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Introduction

Enterococcus faecalis is a lactic acid-producing Gram-positive aerobic or facultative anaerobic bacterium commonly found in the intestines of humans and animals [1-3]. It is one of the feed-grade microbial additive strains listed in the "Feed Additive Catalogue (2013)" published by the Ministry of Agriculture of

China. As early as 1998, the U.S. Food and Drug Administration (FDA) and the Association of American Feed Control Officials (AAFCO) recognized Enterococcus faecalis as a safe microbial strain that can be fed directly to animals. Studies have found that dietary supplementation with Enterococcus faecalis can improve animal performance, enhance nutrient metabolism and improve immune function [4-8]. Shi et al. [4] reported that dietary Enterococcus faecalis could reduce diarrhea rate in piglets, increase blood total protein and globulin contents, decrease albumin-to-globulin ratio and alanine aminotransferase activity, improve protein metabolism and immune function, increase average daily feed intake and average daily gain, and improve growth performance. Liu et al. [5] found that adding Enterococcus faecalis to weaned piglet diets increased average daily gain by 8.79% and decreased feed-to-gain ratio by 8.43%. Hou [6] reported that Enterococcus faecalis supplementation in weaned piglet diets increased average daily gain by 8.51% and decreased feed-to-gain ratio by 7.57%. Ross et al. [7] found that Enterococcus faecalis supplementation in weaned piglet diets significantly reduced feed intake while significantly improving feed utilization efficiency. Gong et al. [8] reported that adding 1$\times10^{8}$ CFU/kg Enterococcus faecalis to growing blue fox diets resulted in ideal nutrient digestibility, nitrogen retention, net protein utilization and protein biological value, along with better growth performance. However, there are currently no reports on the research and application of Enterococcus faecalis as a probiotic in laying hens. Therefore, this experiment aimed to investigate the effects of Enterococcus faecalis on performance, egg quality, lipid metabolism and intestinal microflora numbers of laying hens through feeding trials, providing experimental basis for the application of Enterococcus faecalis in laying hens.

Materials and Methods

1.1 Preparation of Enterococcus faecalis Suspension Enterococcus faecalis (CGMCC1.2135T) freeze-dried powder was inoculated into tryptic soy broth (TSB) medium and cultured at 37°C with shaking at 160 r/min for activation and propagation. The fermentation broth was diluted with physiological saline, and 0.2 mL of the diluted solution was evenly spread on tryptic soy agar (TSA) medium plates, cultured at 37°C for 24 h for viable cell counting to calculate total bacterial count. The broth was then centrifuged at 4,500 r/min for 15 min, the supernatant was discarded, and the bacterial cells were washed with sterile physiological saline, followed by centrifugation at 4,500 r/min for 15 min and discarding the supernatant. This washing process was repeated twice to obtain bacterial cell pellets. The pellets were diluted with physiological saline to a determined concentration (CFU/mL) to obtain Enterococcus faecalis suspension with known concentration. The suspension was evenly sprayed onto diets and mixed thoroughly. The supplemental levels of Enterococcus faecalis in diets, suspension concentration and suspension supplemental levels are shown in Table 1 .

1.2 Experimental Design and Diets Four hundred and fifty 137-day-old Hy-Line brown laying hens were randomly divided into 5 groups with 6 replicates per group and 15 hens per replicate. There were no significant differences in body weight and laying rate among groups (P>0.05). The control group was fed the basal diet without Enterococcus faecalis, while groups I, II, III and IV were fed the basal diet supplemented with 1.0$×10^{{4}, 1.0×10}{6}, 1.0×10^{8}$ and 1.0$×10^{10}$ CFU/g Enterococcus faecalis, respectively. The basal diet was formulated according to the nutrient requirements of laying hens from NRC (1994) [9]. The composition and nutrient levels of the basal diet are shown in Table 2 .

1.3 Management Experimental laying hens were raised in a semi-open chicken house with 3-tier step cages, 3 hens per cage. Feed was provided at fixed times three times daily (06:00, 12:00, 18:00) with free access to feed and water. Natural light was supplemented with artificial light to maintain a constant photoperiod of 16 h/d (06:00–22:00, controlled by automatic lighting system) at an intensity of 14 lx. Daily temperature and humidity were measured with a dry-wet bulb thermometer, and management measures were taken promptly to ensure house temperature at (20$±$3)°C and relative humidity at 60%–80%. The experimental period lasted 168 days.

1.4 Measurement Indicators and Methods

1.4.1 Performance During the experiment, body weight, egg number, egg weight and mortality were recorded daily per replicate to calculate laying rate, average egg weight and egg mass. Feed was weighed weekly to calculate average daily feed intake and feed-to-egg ratio.

1.4.2 Egg Quality On days 28, 56, 84, 112, 140 and 168 of the experiment, all eggs laid on that day were collected and egg quality was measured within 12 h. Eggshell strength was measured using an eggshell strength tester (Model-III, Robotmation, Japan). Eggshell thickness was measured using an eggshell thickness gauge (Model P-1, Ozaki MFG, Japan). Albumen height, Haugh unit and yolk color were measured using an egg quality tester (EMT-2500, Robotmation, Japan).

1.4.3 Serum Total Cholesterol (TCHO), Triglyceride (TG) and Low-Density Lipoprotein Cholesterol (LDL-C) Contents On days 1, 28, 56, 84, 112, 140 and 168 of the experiment, 3 hens were randomly selected from each replicate, and blood was collected from wing veins and centrifuged at 3,000 r/min for 10 min to separate serum, which was stored at -20°C. Serum total cholesterol content was measured using a total cholesterol assay kit (COD-PAP method), triglyceride content using a triglyceride assay kit (GPO-PAP method), and low-density lipoprotein cholesterol content using a low-density lipoprotein

cholesterol assay kit (polyvinyl sulfate precipitation method). All kits were provided by Beijing Zhongsheng Beikong Biotechnology Co., Ltd. Absorbance was measured using a double-beam UV-Vis spectrophotometer (TU-1901, Beijing Puxi General Instrument Co., Ltd.).

1.4.4 Yolk Total Cholesterol Content On days 28, 56, 84, 112, 140 and 168 of the experiment, 8 eggs were randomly collected from each replicate, yolks were separated, mixed evenly and stored at -20°C. Yolk total cholesterol content was measured using a total cholesterol assay kit (COD-PAP method) provided by Beijing Zhongsheng Beikong Biotechnology Co., Ltd.

1.4.5 Intestinal Microflora Numbers On day 168 of the experiment, 1 hen was randomly selected from each replicate, sacrificed by bloodletting, and jejunum, ileum and caecum were collected aseptically and ligated, then quickly transferred to a microbiology laboratory for selective culture and plate counting of Escherichia coli and Enterococcus faecalis in intestinal contents. The selective medium for Escherichia coli was eosin methylene blue agar (Beijing Luqiao Technology Co., Ltd.), and for Enterococcus faecalis was KF streptococcus agar (CM0701) (Beijing Luqiao Technology Co., Ltd.).

Approximately 0.5 g of intestinal content was weighed into a 10 mL centrifuge tube, and physiological saline was added for 10-fold gradient dilution. The mixture was vortexed to appropriate gradient. Then 0.1 mL of diluted bacterial suspension was spread on each medium with a spreader, with 3 replicates per gradient, and cultured at 37°C for 24 h before counting.

Number of colonies per gram sample = lg[(colony count × dilution factor × 10 mL/0.1 mL)/0.5 g].

1.5 Data Analysis Experimental data were analyzed by one-way ANOVA using the GLM procedure of SAS 9.1 statistical software. Significant differences were further analyzed by Duncan' s multiple comparison test, with $P<0.05$ and $P<0.01$ as the criteria for significant and highly significant differences, respectively. Meanwhile, orthogonal polynomial analysis was used to analyze the linear and quadratic effects of dietary Enterococcus faecalis on performance, egg quality, yolk cholesterol content, serum cholesterol, low-density lipoprotein cholesterol and triglyceride contents, and intestinal microflora numbers. Laying rate data were analyzed after arcsine transformation, and intestinal microflora numbers data were analyzed after logarithmic transformation.

Results

2.1 Effects of Dietary Enterococcus faecalis on Performance of Laying Hens Dietary Enterococcus faecalis had no significant effects on body weight, laying rate, average egg weight or average daily feed intake of laying hens (data not shown) ($P>0.05$). As shown in Table 3 , compared with the control

group and other Enterococcus faecalis supplemental groups, dietary supplementation with 1.0$×10^{6}$ CFU/g Enterococcus faecalis significantly increased egg mass during days 113-140 and days 141-168 (P<0.01). During days 141-168, the feed-to-egg ratio in all Enterococcus faecalis supplemental groups was not significantly different from that in the control group (P>0.05), but the feed-to-egg ratio in the 1.0$×10^{4}$ CFU/g Enterococcus faecalis supplemental group was significantly lower than that in the 1.0$×10^{8}$ CFU/g Enterococcus faecalis supplemental group (P<0.05). There were no significant differences in feed-to-egg ratio among groups in other stages (P>0.05).

2.2 Effects of Dietary Enterococcus faecalis on Egg Quality of Laying Hens Dietary Enterococcus faecalis had no significant effect on eggshell strength (data not shown) (P>0.05). As shown in Table 4 , on day 56, eggshell thickness in all Enterococcus faecalis supplemental groups was significantly higher than that in the control group (P<0.05), with no significant differences among Enterococcus faecalis supplemental groups (P>0.05). There were no significant differences in eggshell thickness among groups in other stages (P>0.05). On day 56, albumen height in the control group and the 1.0$×10^{6}$ CFU/g Enterococcus faecalis supplemental group was significantly higher than that in the 1.0$×10^{4}$ and 1.0$×10^{10}$ CFU/g Enterococcus faecalis supplemental groups (P<0.05). On days 84 and 140, albumen height in all Enterococcus faecalis supplemental groups was not significantly different from that in the control group (P>0.05), but albumen height in the 1.0$×10^{8}$ CFU/g Enterococcus faecalis supplemental group was significantly higher than that in the 1.0$×10^{4}$ CFU/g Enterococcus faecalis supplemental group (P<0.05). Throughout the experiment, there were no significant differences in Haugh unit between all Enterococcus faecalis supplemental groups and the control group (P>0.05). On day 56, Haugh unit in the 1.0$×10^{6}$ CFU/g Enterococcus faecalis supplemental group was the highest and significantly higher than that in the 1.0$×10^{4}$ and 1.0$×10^{10}$ CFU/g Enterococcus faecalis supplemental groups (P<0.01). On day 84, Haugh unit in the 1.0$×10^{6}$ and 1.0$×10^{8}$ CFU/g Enterococcus faecalis supplemental groups was significantly higher than that in the 1.0$×10^{4}$ CFU/g Enterococcus faecalis supplemental group (P<0.05). On day 28, yolk color in the 1.0$×10^{10}$ CFU/g Enterococcus faecalis supplemental group was significantly higher than that in the control group and the 1.0$×10^{4}$ CFU/g Enterococcus faecalis supplemental group (P<0.01). On day 56, yolk color in the 1.0$×10^{{4}, 1.0×10}{6}$ and 1.0$×10^{10}$ CFU/g Enterococcus faecalis supplemental groups was significantly higher than that in the control group (P<0.01), with no significant differences among Enterococcus faecalis supplemental groups (P>0.05). On day 112, yolk color in all Enterococcus faecalis supplemental groups was significantly higher than that in the control group (P<0.01), and yolk color in the 1.0$×10^{8}$ CFU/g Enterococcus faecalis supplemental group was significantly higher than that in the 1.0$×10^{6}$ CFU/g Enterococcus faecalis supplemental group (P<0.01). On day 140, yolk color in the 1.0$×10^{8}$ CFU/g

Enterococcus faecalis supplemental group was significantly higher than that in the control group (P<0.01), while yolk color in the 1.0$×10^{10}$ CFU/g Enterococcus faecalis supplemental group was significantly lower than that in the control group and the other Enterococcus faecalis supplemental groups (P<0.01).

2.3 Effects of Dietary Enterococcus faecalis on Yolk Total Cholesterol Content As shown in Table 5 , on day 56, yolk total cholesterol content in the 1.0$×10^{8}$ CFU/g Enterococcus faecalis supplemental group was significantly lower than that in the control group and the 1.0$×10^{4}$ and 1.0$×10^{6}$ CFU/g Enterococcus faecalis supplemental groups (P<0.01). On day 112, yolk total cholesterol content in the 1.0$×10^{8}$ and 1.0$×10^{10}$ CFU/g Enterococcus faecalis supplemental groups was significantly lower than that in the control group and the 1.0$×10^{4}$ and 1.0$×10^{6}$ CFU/g Enterococcus faecalis supplemental groups (P<0.01). There was no significant difference in yolk total cholesterol content between the 1.0$×10^{8}$ and 1.0$×10^{10}$ CFU/g Enterococcus faecalis supplemental groups (P>0.05).

2.4 Effects of Dietary Enterococcus faecalis on Serum Total Cholesterol, Triglyceride and Low-Density Lipoprotein Cholesterol Contents As shown in Table 6 , on day 84, serum total cholesterol content in all Enterococcus faecalis supplemental groups was significantly lower than that in the control group (P<0.01), with no significant differences among Enterococcus faecalis supplemental groups (P>0.05). On day 168, serum total cholesterol content showed a quadratic decrease with increasing Enterococcus faecalis supplemental level (P<0.05). On day 168, serum triglyceride content in all Enterococcus faecalis supplemental groups was significantly lower than that in the control group (P<0.05), with no significant differences among Enterococcus faecalis supplemental groups (P>0.05). On day 84, serum low-density lipoprotein cholesterol content in all Enterococcus faecalis supplemental groups was significantly lower than that in the control group (P<0.01), with no significant differences among Enterococcus faecalis supplemental groups (P>0.05).

2.5 Effects of Dietary Enterococcus faecalis on Intestinal Microflora Numbers As shown in Table 7 , the number of Escherichia coli in ileum in the 1.0$×10^{\{6\},1.0×10}${8}$ and 1.0$×10^{10}$ CFU/g Enterococcus faecalis supplemental groups was significantly lower than that in the control group (P<0.05), with no significant differences among Enterococcus faecalis supplemental groups (P>0.05). The number of Escherichia coli in jejunum showed a linear decrease with increasing dietary Enterococcus faecalis supplemental level (P<0.05). The number of Enterococcus faecalis in ileum in the 1.0$×10^{10}$ CFU/g Enterococcus faecalis supplemental group was significantly higher than that in the control group and the 1.0$×10^{4}$ and 1.0$×10^{8}$ CFU/g Enterococcus faecalis supplemental groups (P<0.01). The number of Enterococcus faecalis in caecum in the 1.0$×10^{8}$ and 1.0$×10^{10}$ CFU/g Enterococcus faecalis supplemental groups was significantly higher than that in the control group

(P<0.01), with no significant differences among Enterococcus faecalis supplemental groups (P>0.05).

Discussion

Enterococcus faecalis is a lactic acid-producing Gram-positive aerobic or facultative anaerobic bacterium commonly found in the intestines of humans and animals [1-3]. A key factor for probiotics to exert their function is acid and bile salt tolerance, which ensures their arrival at the intestinal region where they exert probiotic effects. Previous studies have shown that the Enterococcus faecalis CGMCC1.2135T used in this experiment has good acid and bile salt tolerance [10]. Hou [6] also reported that Enterococcus faecalis has characteristics of acid tolerance, bile salt tolerance and high temperature resistance, enabling it to successfully reach and survive in the intestinal tract of livestock and poultry. Studies have also found that Enterococcus faecalis CGMCC1.2135T has the ability to inhibit Salmonella and Escherichia coli in vitro [10]. Hugas et al. [11] also found that Enterococcus faecalis can produce organic acids and bacteriocins that inhibit the growth of pathogenic and spoilage microorganisms in the intestine, reduce pH in caecum and ileum, and thus optimize the intestinal environment. Furthermore, Pereira et al. [12] found that Enterococcus faecalis has high bile salt hydrolase activity, which can enzymatically hydrolyze bile salts in the enterohepatic circulation, thereby reducing intestinal reabsorption of bile salts. This leads to increased cholesterol in the blood for compensatory synthesis of bile salts, providing a theoretical possibility for Enterococcus faecalis as a cholesterol-lowering probiotic.

In this experiment, dietary Enterococcus faecalis supplementation for 24 weeks had no significant effects on body weight, laying rate, average egg weight or average daily feed intake of laying hens. Currently, there are no reports on the effects of Enterococcus faecalis on body weight, laying rate, average egg weight and average daily feed intake of laying hens. Studies have shown that dietary supplementation with lactic acid bacteria [13-15], Bacillus licheniformis and Bacillus subtilis [16] and Saccharomyces cerevisiae [17] had no significant effects on body weight of laying hens. Dietary supplementation with lactic acid bacteria [14], Bacillus licheniformis and Bacillus subtilis [16,18] could improve laying rate of laying hens. However, Nahashon et al. [15,19], Balevi et al. [20], Salma et al. [21] and Mikulski et al. [22] reported that probiotics such as lactic acid bacteria, Rhodobacter capsulatus and Pediococcus acidilactici had no significant effects on laying rate of laying hens. Nahashon et al. [13], Kurtoglu et al. [16], Yousefi et al. [17], Goodling et al. [23] and Mohan et al. [24] found that probiotics including lactic acid bacteria, Bacillus licheniformis, Bacillus subtilis, Saccharomyces cerevisiae, Lactobacillus acidophilus, Lactobacillus casei and Bifidobacterium had no significant effects on egg weight of laying hens. The results of this study showed that dietary supplementation with 1.0$×10^{6}$ CFU/g Enterococcus faecalis significantly increased egg mass during days 113–168. Nahashon et al. [19], Tortuero et al. [25] and Xu et al. [26] also found that lactic acid

bacteria, Streptococcus faecalis and Bacillus subtilis could increase egg mass of laying hens. During days 141–168, dietary supplementation with 1.0$×10^{4}$ CFU/g Enterococcus faecalis significantly reduced feed-to-egg ratio compared with 1.0$×10^{8}$ CFU/g Enterococcus faecalis, improving feed conversion efficiency. This may be related to the ability of Enterococcus faecalis to inhibit harmful intestinal microorganisms, regulate intestinal pH, and thus optimize intestinal environment and improve intestinal enzyme activity and nutrient digestibility [11,27].

Eggshell thickness and eggshell strength are commonly used indicators to evaluate eggshell quality. In this experiment, on day 56, dietary Enterococcus faecalis supplementation significantly increased eggshell thickness, presumably related to its ability to improve intestinal environment and function and enhance intestinal calcium absorption. Dietary Enterococcus faecalis had no significant effect on eggshell strength, which is consistent with reports by Nahashon et al. [15] and Mahdavi et al. [18]. Haugh unit and albumen height are indicators for evaluating egg freshness. Studies have reported that Haugh unit and albumen height decrease significantly with storage time [28-30]. In this experiment, on day 56, dietary supplementation with 1.0$×10^{6}$ CFU/g Enterococcus faecalis significantly increased albumen height and Haugh unit compared with 1.0$×10^{4}$ and 1.0$×10^{10}$ CFU/g Enterococcus faecalis. On day 84, dietary supplementation with 1.0$×10^{8}$ CFU/g Enterococcus faecalis significantly increased albumen height and Haugh unit compared with 1.0$×10^{4}$ CFU/g Enterococcus faecalis, thereby improving egg freshness and extending shelf life of commercial eggs. Regarding yolk color, although there was no clear dose-effect or consistent pattern, the experiment observed that on days 28, 56, 84 and 112, yolk color in all Enterococcus faecalis supplemental groups was improved compared with the control group. The mechanism of dietary Enterococcus faecalis affecting yolk color needs further investigation.

The mechanism by which Enterococcus faecalis reduces yolk and serum cholesterol content may be related to its ability to produce bile salt hydrolase. Bile salts are water-soluble substances synthesized from cholesterol as a precursor in the liver. Bile salt hydrolase is an N-terminal nucleophilic hydrolase that can specifically hydrolyze the amide bond of conjugated bile salts, releasing free bile salts and amino acid residues of glycine or taurine [31]. Enterococcus faecalis can produce bile salt hydrolase [32-34]. Free bile salts hydrolyzed by bile salt hydrolase have lower solubility than conjugated bile salts and are less easily reabsorbed by the intestine [35], thus being excreted with feces [36-37]. Therefore, to maintain normal enterohepatic circulation and compensate for bile salt loss, the liver utilizes cholesterol from blood to synthesize new bile salts, thereby reducing serum cholesterol content [12,37]. In addition to reducing cholesterol through the dissociation action of bile salt hydrolase activity, researchers have also speculated that probiotics may reduce cholesterol through cytoplasmic assimilation of extracellular cholesterol, cell membrane integration of extracellular cholesterol, and cell wall adsorption of extracellular cholesterol [38-39].

Some pathogenic Escherichia coli can cause local or systemic infections in chronic asymptomatic carrier states, making them a worldwide public health problem. Additionally, Escherichia coli residues on eggs are also a food safety concern. In this experiment, the number of Escherichia coli in ileum in the 1.0$×10^{6}, 1.0×10{8}$ and 1.0$×10^{10}$ CFU/g Enterococcus faecalis supplemental groups was significantly lower than that in the control group, and the number of Escherichia coli in jejunum showed a linear decrease with increasing Enterococcus faecalis supplemental level. Bao et al. [10] reported that Enterococcus faecalis CGMCC1.2135T could inhibit the growth of Escherichia coli O1 and O78. Studies have confirmed that bacteriocins produced by Enterococcus faecalis have broad-spectrum antimicrobial activity, including not only Gram-positive bacteria but also Gram-negative bacteria such as Listeria spp. [40-43], Salmonella spp. [44], Escherichia coli [44] and Staphylococcus aureus [45]. Therefore, dietary Enterococcus faecalis supplementation helps reduce the risk of Escherichia coli infection in laying hens and may also help reduce Escherichia coli contamination of eggs.

Conclusions

1. Dietary supplementation with 1.0$×10^{6}$ CFU/g Enterococcus faecalis significantly increased egg mass of laying hens during days 113–168.
2. On day 56, dietary supplementation with 1.0$×10^{6}$ CFU/g Enterococcus faecalis significantly increased albumen height and Haugh unit compared with 1.0$×10^{4}$ and 1.0$×10^{10}$ CFU/g Enterococcus faecalis. On day 84, dietary supplementation with 1.0$×10^{8}$ CFU/g Enterococcus faecalis significantly increased albumen height and Haugh unit compared with 1.0$×10^{4}$ CFU/g Enterococcus faecalis. On days 28, 56, 84 and 112, all Enterococcus faecalis supplemental groups improved yolk color, but dietary supplementation with 1.0$×10^{10}$ CFU/g Enterococcus faecalis reduced yolk color on day 140.
3. On days 56 and 112, yolk total cholesterol content in the 1.0$×10^{8}$ CFU/g Enterococcus faecalis supplemental group was significantly lower than that in the control group and the 1.0$×10^{4}$ and 1.0$×10^{6}$ CFU/g Enterococcus faecalis supplemental groups. Dietary Enterococcus faecalis significantly reduced serum total cholesterol (day 84), triglyceride (day 168) and low-density lipoprotein cholesterol (day 84) contents.
4. Dietary supplementation with 1.0$×10^{6}, 1.0×10{8}$ and 1.0$×10^{10}$ CFU/g Enterococcus faecalis significantly reduced ileal Escherichia coli numbers. The number of Escherichia coli in jejunum showed a linear decrease with increasing Enterococcus faecalis supplemental level. Compared with the control group and the 1.0$×10^{4}$ and 1.0$×10^{8}$ CFU/g Enterococcus faecalis supplemental groups, dietary supplementation with 1.0$×10^{10}$ CFU/g Enterococcus faecalis significantly increased ileal Enterococcus faecalis numbers. Dietary supplementation

with 1.0$×10^{8}$ and 1.0$×10^{10}$ CFU/g Enterococcus faecalis significantly increased caecal Enterococcus faecalis numbers.

5. The appropriate supplemental amount of Enterococcus faecalis in laying hen diets is 1.0$×10^{6}$ or 1.0$×10^{8}$ CFU/g.

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