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Effect of Probiotic Supplementation in Diet on Egg Production, Egg Quality, Intestinal Morphology and Bacterial Population in Caecum of Laying Hens

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Abstract

The objective of this study was to study the effect of probiotic supplementation in diet on egg production, egg quality, intestinal morphology, and bacterial population in caecum of laying hens. A total of 768 Lohmann Brown-Classic laying hens, aged 42 - 54 weeks, were used. The laying hens were divided into 3 groups, each with 8 subgroups of 32 birds each. The experiment was planned to use a completely randomized design method. The 3 experimental groups were classified based on their dietary treatment as follows: 1) Basal diet (without probiotic supplementation), 2) Basal diet with probiotic 0.01 and 0.02 % in 3, respectively. Data was collected for 12 weeks. Adding probiotic in the diet was no significant difference on egg production, egg quality, and bacterial population in caecum. However, probiotic supplementation in the diet increased villi height of the duodenum significantly improved ($p < 0.02$), villi height per depth crypt of the duodenum significantly improved ($p < 0.05$), villi height per depth crypt of the jejunum significantly improved ($p < 0.01$), and villi height per depth crypt of the ileum significantly improved ($p < 0.02$). Therefore, studies have shown that dietary probiotic supplementation can improved small intestinal morphology are the most important indexes for measuring the digestive and absorption function of the small intestine are more efficient.

Keywords: Egg production, Laying hens, Egg quality, Probiotic, Intestinal morphology

Introduction

Probiotics contain a variety of beneficial microorganisms. It has several mechanisms of action together, including increasing barrier function through the process of producing mucus in the digestive tract. To block and reduce the amount of pathogenic microorganisms [1]. Microbial inhibition (antimicrobial effect) by the process of creating substances that are factors that inhibit the growth of pathogenic microorganisms, such as the production of lactic acid and short chain fatty acids (SCFAs), causing the digestive system to become more acidic [2]. Competition in adhesion increases the number of probiotics, which are microorganisms in the *Lactobacillus*, *L. plantarum* and *B. subtilis* compete to inhibit *E. coli* from adhering to the intestinal wall [3]. They can also block pathogen entry sites. When animals receive probiotics into their bodies, they pass through the stomach to grow and compete with pathogenic microorganisms in adhering to the gastrointestinal mucosa and increase in number in the small intestinal mucosa. By inserting itself between the villi, it reduces clumping and causes the expulsion of pathogens from the gastrointestinal tract [4] decomposed by white blood cells (macrophages), which are part of the immune system, improves the animal's ability to digest and absorb. Probiotic supplements currently being used to feed chickens are specific to growth in the digestive tract. It may be in the form of powder containing capsules, granules, in the form of wet cells, live cultures, or spores, which can be fed to animals. The properties of good probiotics must be able to survive in the digestive tract of animals. It is resistant to stomach acid, bile, and must have properties that are beneficial to animals.

Microorganisms in the *B. subtilis* group are rod-shaped (bacilli, rod) and usually live alone or has different shape, such as creating spores inside the cell (spore forming rod), etc. There is an endospore, a structure that makes bacteria resistant to inappropriate environmental conditions. Endospores are resistant to drought, dyes, chemical disinfectants, radiation, and heat at various temperatures, but most can tolerate temperatures as high as 80 °C for at least 10 min [5]. The ability to create spores has been applied for use in the animal feed industry [6]. It could produce lactic acid and various types of enzymes such as protease and amino acids, amylase and lipase help in the digestion of carbohydrates and proteins [7]. They are microorganisms that can grow in both aerobic and anaerobic conditions. In the presence of oxygen, the process of breathing is used. In conditions where there is no oxygen, fermentation is used. Using hydrogen as an energy source, *B. subtilis* has begun to be used as an alternative to antibiotics used in poultry production [8] as it improves the egg production percentage, egg weight, egg mass, and feed conversion ratio [9,10]. The productivity of laying hens improves both in eggshell quality and in reducing the amount of egg damage [11,12], improving the efficiency of the gut microbiota and controlling morphology of the small intestine affects the absorption of nutrients in the small intestine more efficiently.

From the above, probiotics (*B. subtilis*) have many benefits in industry, thus researchers are interested in studying the impact of probiotics on egg production, eggs quality, intestinal morphology, and bacterial content in the caecum of laying hens.

Materials and methods

Experimental animals and management

This experiment used 768 laying hens of the Lohmann Brown-Classic breed. The experiment began when the laying hens were 42 weeks old. The laying hens were divided into 3 experimental groups, 8 replicates per group, 32 birds per replicate, and raised in a closed, temperature-controlled house. The house has an evaporative cooling system (EVAP) and tunnel ventilation system. They are raised in cages measuring 41×46×40 cm^3, using a density of 2 birds per cage. The house is controlled with an automatic system for the hens to lay eggs. Receive photoperiod for 16 h per day. Automatic watering system with nipple and full feeding (*ad libitum*). Total experimental period was 12 weeks.

Experimental diets

Experimental diet is a powder based basic diet (mash feed) that meets the needs of Lohmann Brown Classic laying hens and contains complete nutritional components. **Tables 1** and **2** are divided into the following formulas: Experimental group 1 receives the basic formula control (T1), and experimental group 2 receives the formula supplemented with probiotic (*B. subtilis* 1×10^9 cfu/g) was 0.01 % (T2) and experimental group 3 received a formula supplemented with probiotic (*B. subtilis* 1×10^9 cfu/g) at 0.02 % (T3).

Table 1 Diet composition and calculated nutrient content.

Ingredient name	Amount (kg)
Corn	32.482
Cassava pulp	20.624
Soybean oil	2.000
Soybean meal 44 %	24.392
Rice solvent bran	5.000
Pork meal 50 %	3.000
DL-Methionine	0.100
$CaCO_3$ (Limestone)	5.000
$CaCO_3$ (Limestone Granular)	6.450
Mono calcium phosphate 21 %	0.250
Salt	0.300
Choline chloride 60 %	0.067
Vit + Min PX[1]	0.250
Mold inhibitor	0.050
Antioxidant	0.010
Phytase	0.010
Non-starch polysaccharides Enzyme	0.015
Total	100.00

Note: [1]Vit + Min (Premix), Vit + Min PX consist of vitamin A 4 MIU, D 30.8 MIU, E 4.0 g, K 30.8 g, B 22 g, B 120.006 g, nicotinic acid 8 g, calcium pantothenate 4.8 g, choline chloride 40.0 g, folic acid 0.2 g, Fe 12 g, Co 0.2 g, Mn 40.0 g, Cu 4.0 g, Zn 32 g, I 0.8 g, Se 0.04 g, feed preservative 20.0 g, flavor 4.0 g and carrier added to 1.00 kg premix.

Table 2 Calculated nutrient content.

Nutrient	Unit
ME. For Poultry (Kcal/kg)	2,700.00
Crude Protein (%)	17.00
Fat (%)	3.98
Crude Fiber (%)	4.06
Calcium (%)	5.00
Total Phosphorus (%)	0.70
Avail. P for Poultry (%)	0.43
Salt (%)	0.35
Lysine (%)	0.93
Methionine (%)	0.36
Methionine + Cystine (%)	0.66
Threonine (%)	0.68
Tryptophan (%)	0.23
Choline (mg/kg)	400.00

Egg production and egg quality

Bird weight before and after trial, feed intake, daily egg production and egg weigh, survival rate, feed conversion ratio, and feed cost were record.

When chickens are at 45, 49, or 53-week-old, a random experiment is conducted on 4 eggs in each group to record the weight of eggs, yolk weight, albumin weight, and eggshell weight are used to calculate the percentage of egg yolk, egg white, and eggshell. Measure eggshell thickness, measure egg white height, calculate Haugh unit (HU) = $100\times\log(H + 7.57 - 1.7\times W^{0.37})$. When H is the height of the egg white (mm) and W is the weight of the egg (g), measure the color of the egg yolk according to the method [12] by Roche colorimetric fan scale or DSM® yolk color fan.

Intestinal morphology

At the end of the experiment randomly select 1 laying hen per experimental unit. Selected from chickens with average production performance of the experimental group to collect small intestinal samples. The laying hens were asphyxiated by gassing them with carbon dioxide and then killed. It was dissected and sent for small intestine collection. Each section of the small intestine was sampled approximately 3 cm in length. The duodenum region was sampled from the loop of duodenum. The jejunum region was sampled in the middle of the bile duct and Meckel's diverticulum and the ileum region were sampled from the area next to the ileocecal junction. The small intestine was cut open lengthwise and the small intestine was cleaned with phosphate buffered saline until the small intestine was free of feed particles that is not digested or other tissues the small intestinal specimen was then fixed to paper and immersed in 10 % buffered neutral formalin to preserve its integrity. Before taking the sample through the cutting and dyeing process small intestinal samples were processed through an automatic tissue processer, starting with tissue samples soaked in 10 % buffered neutral formalin with varying concentrations of ethanol and dried. The biopsy sample is then embedded in paraffin (embedding) using a paraffin dropper and left for the paraffin to harden. Before being cut, the samples were cut to a thickness of 6 µm using a microtome (thermo scientific, Waltham, USA). The cut sections were placed on hematoxylin-stained slides, (hematoxylin) and eosin. In the final step, a drop of mounting median was placed on the sample slide and covered with a coverslip and left for approximately 2 days. A total of 3 photographs were taken of the biopsy sample using a digital camera. A micropublisher 5.0 (Qimage, Surrey, BC, Canada) connected to a bright light microscope (Olympus, Tokyo, Japan) analyzed the images for villi height and crypt depth, using a computer program Image-PRO®PLUS 6.0 (Media Cybernatics, Inc, Bethesda, MD, USA) in which 5 points were measured in each image, then the obtained values were averaged for the height of the villi. Crypt depth and proportions of villi height and crypt depth according to the method of Tsirtsikos [13].

Bacterial population in caecum

At the end of the experiment randomly select 1 laying hen per experimental unit. Detect the total amount of bacteria (total viable count) by standard plate count or aerobic plate count. Collect 1 g of sample from both appendices and dilute the sample to tenfold increments. After that, use spread plate technique for culturing bacteria. Use a 0.1 mL sample volume to grow on nutrient agar or plate count agar. Incubate the culture medium at 35 - 37 °C for 24 - 48 h. Count the bacterial colonies and the results are reported as colony forming unit/gram (cfu/g). Detection of *E. coli* bacteria using the most probable number (MPN) method begins with the sample being diluted to 3 levels. Then, 1 mm sample was placed in 10 mL of lauryl sulphate broth medium at 3 tubes per dilution and incubated at 35 °C for 48 h. The cultures that were positive in the initial test were grown in EC broth agar by incubating at a temperature of 45 ± 0.2 °C within 48 h. The tubes that were positive in EC broth medium were inoculated on to eosin methylene blue agar (EMB) medium and incubated at 35 °C for 24 h, then take the culture for gram staining including the shape and study the IMVIC test (Indole test, Methyl red - Vogesproskauer: MR-VP test, Citrate utilixation) will give the test result as + + – or – + – take the value from the table according to the method of [14] comes to report the results (**Figure 1**).

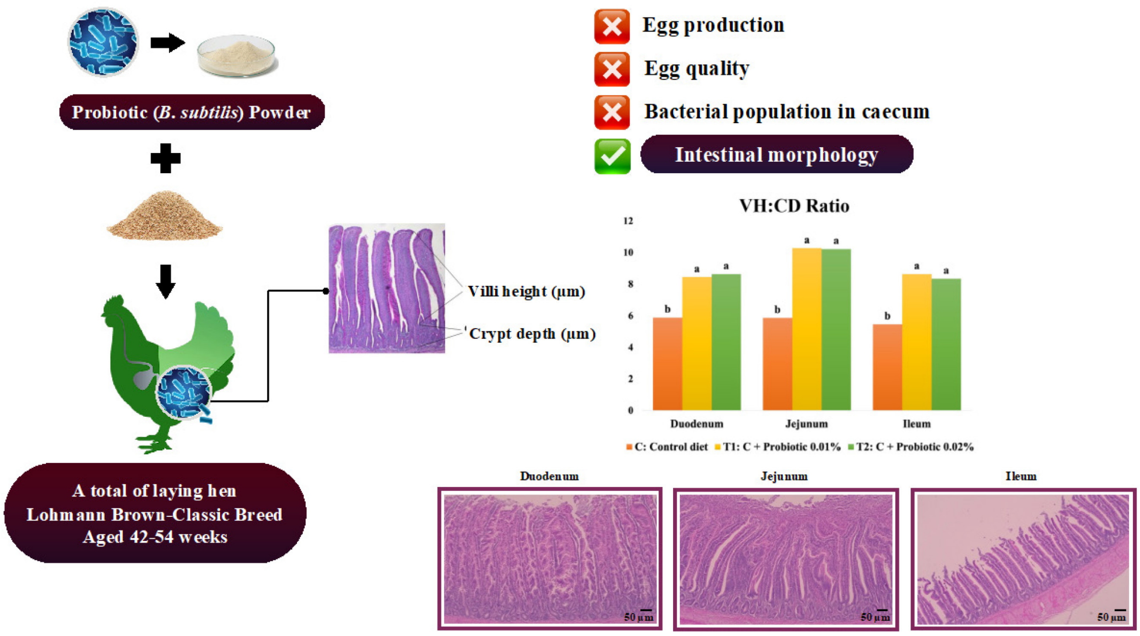

Figure 1 Experimental group 1 received a basic formula diet control (T1), experimental group 2 received a formula supplemented with probiotic probiotic (*B. subtilis* 1×10^9 cfu/g) at a level of 0.01 % (T2) and experimental group 3 received the formula supplemented with probiotic probiotic (*B. subtilis* 1×10^9 cfu/g) at a level of 0.02 % (T3).

Statistical data analysis

The experiment was conducted as a completely randomized design. The results are presented as mean ± SD. Means were analyzed with ANOVA followed by Duncan's multiple range test to determine significant differences between groups. All percentage data were arcsine transformed before statistical analysis. A probability level of $p \leq 0.05$ was considered as significant.

Results and discussion

Egg production

From the supplementation of probiotics (*B. subtilis*) at 0 % (control), 0.01 and 0.02 % in the diets of Lohmann Brown-Classic laying hens, aged 42 - 54 weeks, duration of the experiment 12 week, found that egg production, mount of feed intake, average egg weight, egg mass, feed conversion efficiency, egg size, feed cost and mortality rate throughout the experimental period, were not statistically different ($p > 0.05$) (**Table 3**). It is reported that the reduction of feed intake substantially contributes to impaired egg production during hyperthermia [15]. Moreover, the feed kept at room temperature (22 °C) began to show a decline in the microbial cell counts of the organisms and their viability after 5 days of storage [16]. It is also reported that using 1 and 2 g probiotic per kg feed has caused serious damage to the absorptive area of digestive system [17]. There was also non-significant reduction in egg production, egg mass, and feed intake due to probiotic addition (0.4, 1 and 2 g per kg) in laying hens diet [18]. Likewise, the egg production was not affected by adding a product containing *B. subtilis* a mixture of bacteria and fungi in laying hens diets [18]. These controversial results might be related to the dosage of probiotics, bacterial concentration and viability, species of probiotic, types of prebiotics, breed of birds, age of birds, the stage of production, climatic conditions, the degree of pollution and bacteria's tolerance of climatic conditions.

Table 3 Effect of probiotic supplementation in diet on egg performance of Lohmann Brown-Classic hens at 42 - 54 weeks (1st - 12th weeks trial).

Item	Control diet	Control diet		*p*-value
		Probiotic 0.01 %	Probiotic 0.02 %	
Hen-day egg production (%)	95.62 ± 1.88	95.46 ± 1.78	94.75 ± 2.91	0.59
Average feed intake (g/hen/day)	125.77 ± 3.81	128.93 ± 2.40	126.47 ± 3.86	0.23
Average egg weight (g)	62.35 ± 1.45	62.55 ± 0.87	61.91 ± 0.95	0.63
Egg mass (g/hen/day)	59.61 ± 1.29	59.78 ± 1.77	58.78 ± 2.34	0.56
Feed conversion ratio (FCR)	2.11 ± 0.01	2.13 ± 0.01	2.10 ± 0.02	0.48
Feed cost (baht/egg)	1.71 ± 0.04	1.73 ± 0.02	1.72 ± 0.02	0.37
Feed cost (baht/egg)	27.49 ± 0.00	27.59 ± 0.00	1.72 ± 0.03	0.41
Mortality (%)	0.00 ± 0.00	0.00 ± 0.00	0.57 ± 1.61	0.83

Egg quality

The effect of supplementing probiotics (*B. subtilis*) in the diet of laying hens on egg quality shown in **Table 4**. We found that the eggshell color, eggshell hardness, eggshell thickness, height of egg white, Haugh unit, yolk color, yolk weight, yolk height, and the ratio of yolk to egg white there is no significant difference ($p > 0.05$). Similar results were reported earlier [19] in respect of shell weight, while egg shape index was not significantly affected in case of hens treated with probiotic inclusion did not significantly affect the egg quality [20]. Haugh unit is a measure of protein quality and freshness of egg [21]. The eggs are graded in descending order based on its desirability as AA (72 or more), A (71 - 60), and B (59 - 31) by the United States Department of Agriculture (USDA) based on Haugh unit [22]. Scores of 90 and above are considered excellent, 70 is acceptable, and buyers generally reject eggs that score below 60. Further, Haugh unit and egg shape were not affected by dietary groups fed on probiotic (*B. subtilis*) [23]. Also, the different levels of probiotic (*saccharomyces cerevisiae*) did not have a significant effect on Haugh unit, albumen (%) and egg shape index, but yolk (%) was improved [24]. On the contrary, 1 g *B. subtilis* per kg diet exhibited the maximum increase in shell weight and shell thickness compared to the other treatments [25]. Likewise, dietary supplementation at different levels of probiotics (*Lactobacillus fermentum*, *Bacillus* spp. and *Saccharomyces cerevisiae*) significantly increased shell thickness compared to non-treated laying hens [26].

Table 4 Effect of probiotic supplementation in diet on egg quality of Lohmann Brown-Classic hens at 42 - 54 weeks (1st - 12th weeks trial).

Item	Control diet	Control diet		p-value
		Probiotic 0.01 %	Probiotic 0.02 %	
Shell color	5.79 ± 0.03	5.79 ± 0.29	5.92 ± 0.22	0.63
Shell breaking strength (N)	41.99 ± 2.62	42.07 ± 1.78	42.59 ± 2.85	0.87
Yolk color	8.46 ± 0.47	8.53 ± 0.48	8.56 ± 0.34	0.89
Albumen height (mm)	6.36 ± 0.38	6.59 ± 0.21	6.40 ± 0.33	0.31
Haugh unit (HU)	77.21 ± 4.93	79.29 ± 1.53	78.64 ± 2.30	0.44
Yolk weight ratio (%)	25.28 ± 0.84	25.31 ± 0.49	25.43 ± 0.75	0.91
Albumen weight ratio (%)	64.61 ± 0.83	64.46 ± 0.55	64.29 ± 0.59	0.64
Shell weight ratio (%)	10.11 ± 0.25	10.27 ± 0.25	10.32 ± 0.29	0.26
Yolk: Albumen ratio	39.15 ± 1.78	39.26 ± 1.08	39.57 ± 1.51	0.84
Shell thickness (mm)	0.36 ± 0.01	0.36 ± 0.01	0.37 ± 0.01	0.71

Intestinal morphology

The effect of supplementing probiotics (*B. subtilis*) in the diet of laying hens on intestinal morphology (**Table 5**). Probiotic supplementation in the diet increased villi height of the duodenum significantly improved ($p < 0.02$). Villi height per crypt depth of duodenum significantly improved ($p < 0.05$), villi height per crypt depth of jejunum significantly improved ($p < 0.01$) and villi height per crypt depth of ileum significantly improved ($p < 0.01$). This is consistent with the experiment conducted by [27] the intestinal morphology such as villi height, and width, and total thickness of the muscularis externa of birds were significantly ($p < 0.05$) affected by probiotic supplementation and control diets. Dunham *et al.* [28] found larger villi and lower crypts in the ileum of domestic fowls fed diets supplemented with probiotic compared with the control, which is in agree with our results. Similarly, Marković *et al.* [29] reported that the use of probiotic in the chickens increased length and width of intestinal villi and decreased depth of crypts. It is assumed that the enlargement of the length and width of the intestinal villi increases the absorptive surface of the intestine that enhances the absorption of nutrients to maintain optimum growth and production of chickens [30]. At the same time, the observed thicker ileal muscularis externa of chickens fed probiotic indicates a stronger gut integrity that prevents various infections caused by the microorganisms [31].

Table 5 Effect of probiotic supplementation in diet on intestinal morphology of Lohmann Brown-Classic hens at 54 weeks.

Item	Control diet	Control diet		*p*-value
		Probiotic 0.01 %	Probiotic 0.02 %	
Duodenum				
Villi height (µm)	1956.02 ± 231.51^b	2701.48 ± 353.34^a	2786.84 ± 241.90^a	< 0.02
Crypt depth (µm)	339.21 ± 78.25	329.13 ± 82.08	323.20 ± 9.22	0.96
VH: CD Ratio	5.89 ± 0.79^b	8.45 ± 1.64^a	8.64 ± 0.98^a	< 0.05
Jejunum				
Villi height (µm)	1416.13 ± 187.02	1983.02 ± 832.79	1688 ± 832.79	0.58
Crypt depth (µm)	257.61 ± 94.11	189.94 ± 64.09	166.10 ± 70.57	0.38
VH: CD Ratio	5.86 ± 1.51^b	10.28 ± 0.94^a	10.23 ± 0.86^a	< 0.01
Ileum				
Villi height (µm)	996.55 ± 305.31	1201.99 ± 512.95	1121.18 ± 295.87	0.81
Crypt depth (µm)	197.28 ± 109.11	136.35 ± 39.99	132.89 ± 25.59	0.48
VH: CD Ratio	5.46 ± 1.12^b	8.64 ± 1.37^a	8.35 ± 0.73^a	< 0.02

Note: [a, b] Means within the same row with different superscripts differ significantly ($p < 0.05$).

Bacterial population in caecum

The effect of supplementing probiotics (*B. subtilis*) in diets on bacterial population in caecum of laying hens (**Table 6**) found that no difference in the total bacterial count and *E. coli* ($p > 0.05$). Similarly, Li *et al.* [32] reported that the effects of probiotic supplementation with *B. subtilis* in concentrate diet at 5×10^8 and 2.5×10^9 cfu/kg^2 in 28-week-old laying hens. No differences in the composition and balance of microorganisms were found caecum of laying hens ($p > 0.05$). Previously, an experiment by [33] studied the effectiveness of supplementation with *B. subtilis* and *B. licheniformis* at 8×10^5 cfu/g in the diet during the egg production period of the Hy-line Brown laying hens, it was found that the chickens in the group supplemented with *B. subtilis* and *B. licheniformis* in the diet had a reduction in the amount of *E. coli* bacteria in the fecal but had no effect on the amount of lactic acid bacteria, *C. perfringens*, and *Salmonella* compared with the control group [34]. This is consistent with the experimental work of [35]. It was found that supplementing *B. subtilis* in laying hens' diet resulted in a decrease in the number of *E. coli* bacteria and the number of *Lactobacillus* in the intestines increased and the experimental work of [36] found that the number of *E. coli* and *Salmonella* in chickens in the group supplemented with *B. licheniformis* and *B. subtilis* had a significant decrease in the number of pathogenic bacteria ($p > 0.05$).

Table 6 Effect of probiotic supplementation in diet on bacteria in caecum of Lohmann Brown-Classic hens at 54 weeks.

Item	Control diet	Control diet		*p*-value
		Probiotic 0.01 %	Probiotic 0.02 %	
Total bacterial count (cfu/g)	7.40 ± 0.47	7.21 ± 0.35	7.26 ± 0.39	0.64
E. coli (MPN/g)	5.48 ± 0.80	5.15 ± 1.38	5.08 ± 0.52	0.71

Conclusions

In a study of the effect of supplementing probiotics (*B. subtilis*) in the diet of laying hens at levels of 0.01 and 0.02 % in the diet of laying hens of the Lohmann Brown-Classic breed, aged 42 - 54 weeks, the experimental period was 12 weeks, there was no effect on egg production, egg quality and bacterial population in caecum. Therefore, studies have shown that dietary probiotic supplementation can improved small intestinal morphology are the most important indexes for measuring the digestive and absorption function of the small intestine are more efficient.

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