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Original Research Article

Effect of Probiotic Supplementation to the Diet on Performance and Egg Quality of Laying Hens

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ABSTRACT

Probiotics are live microorganisms that provide many health benefits to the host when administered in sufficient amounts. The use of probiotics in poultry diets has steadily increased over the years as an alternative to antibiotics to avoid the use of antibiotics in the feed as a result of increasing demand. The objective of this study, determine the effect of different probiotic types on laying hen performance and egg quality. A total of 140 laying hens, aged 33 weeks of age, were involved in a 56-day long experiment. The study was conducted in 5 treatment groups, each with 7 replications, and were fed with five diets. The diets contained *Bacillus megaterium* and *Bacillus amyloliquefaciens* at one colony forming unit (0.5 g/kg with $1x10^8$ cfu/g), in addition to a control diet. Results indicated that initial body weight, final body weight, body weight gain, feed intake, feed conversion ratio, and damaged egg ratio were not significantly different among the groups ($p>0.05$). The addition of *Bacillus megaterium* and *Bacillus amyloliquefaciens* to the laying hen diets had no significant effects on albumen and yolk index, shell breaking strength, shell weight, shell thickness, shell ratio, and egg yolk color characteristics (L* and b* values). However, the a* value and Haugh unit of yolk color characteristics significantly decreased compared to the control group ($p<0.05$; $p<0.01$). According to the results, it was concluded that the inclusion of *Bacillus megaterium* and *Bacillus amyloliquefaciens* to the diets of laying hens did not result a significant change growth performance and egg quality parameters.

Keywords: ***Bacillus amyloliquefaciens*, *Bacillus megaterium*, egg quality, laying hen, performance**

INTRODUCTION

Eggs have great economic value and excellent source of animal protein, and due to their low cost, they have become an important consumer product worldwide (Applegate 2000). The main goal of the commercial laying hen industry is to meet public demand by producing eggs in high quantity and quality. In recent years, the use of organic acids, herbal extracts, enzymes, probiotics, antimicrobial peptides, and their combinations as feed additives in poultry nutrition have become widespread in order to improve animal health, performance characteristics, and feed efficiency (Macit *et al.* 2021, Chen *et al.* 2020). Probiotics have emerged as an effective alternative in the poultry feeding industry, preferred over antibiotics and pathogen inhibitors (Patterson and Burkholder 2003). In recent years, probiotic preparations containing *Lactobacillus*, *Bacillus* species, *Bifidobacterium*, and yeast have been used as probiotics primarily in broilers and laying hens (Khan 2013). *Bacillus* species are technologically suitable feed additives because they are stable in the presence of various stress factors and can produce various enzymes such as proteases, amylases and lipases. It has been reported that Bacillus probiotic supplementation in the diet of laying hens improves performance and eggshell properties, and that these positive effects are proportional to the dose of probiotic given to the hens, namely the colony forming unit (Lei *et al.* 2013). However, the formulation technology for probiotics is typically industry specific, but the key requirement is that the probiotic product should be in powder form and generally stable at $1x10^9$ cfu/g (Teo and Tan 2007, Kim *et al.* 2017 , Jeong and Kim 2014). In order for probiotics to show beneficial effects on the host, they must be at a minimum level of 10^6-10^7 cfu/g and reach the intestinal tract safely (Bosnea *et al.* 2009). In addition, a probiotic must remain active in the digestive system and maintain its effect in the intestine (Masco *et al.* 2007).

Bacillus species have been accepted as the most promising probiotic type due to their ability to survive through the process, colonize the system and be excreted through the faeces (Cartman *et al.* 2008, Shivaramaiah *et al.* 2011). *Bacillus amyloliquefaciens* is a soil-isolated probiotic, one of the most effective bacteria among *Bacillus* species, and it has been reported in various studies that it can be used as an alternative to antibiotics to improve intestinal epithelial barrier and immune function of broiler chickens by modulating intestinal flora (Du *et al.* 2018, Wang *et al.* 2021). Adding *Bacillus amyloliquefaciens* to the diets of laying hens reduced stress, improved their performance and egg quality by regulating their immune systems (Zhou *et al.* 2020). It has been reported that

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Bacillus amyloliquefaciens improves egg production, sperm quality, egg quality and hatchability, and slows reproductive aging in chickens (Prazdnova *et al.* 2019). Among the *Bacillus* species, *Bacillus megaterium* has become a research material as a probiotic due to its unique properties such as resistance to stress conditions and high temperatures, and easy storage (Vary *et al.* 2007, De Vos 2009). Ding and Wang (2015) found that adding 100 mg/kg *Bacillus megaterium* to the diet of laying hens significantly increased egg weight and egg production compared to the control group. However, it is thought that more studies are needed to clarify the uncertainties of the effects of *Bacillus amyloliquefaciens* and *Bacillus megaterium* in layer hens compared to *Bacillus subtilis*, which is more commonly used in poultry. This study aimed to evaluate the effects of *Bacillus amyloliquefaciens* and *Bacillus megaterium* on performance and egg quality traits in laying hens and to provide information to researchers based on the comparison of data between these two probiotics.

MATERIALS AND METHODS

Animal material and diets

This research was conducted at the Animal Husbandry Research and Application Facility of the Department of Animal Science at Selcuk University, Faculty of Agriculture. The hens were housed in an environmentally controlled room equipped with 90 metal battery cages. Hens were kept in cages (50 cm length, 50 cm width, 45 cm height) with 4 hens per cage. The experimental group consisted of 140 white strain laying hens (Tinted, H&N International) at 33 weeks of age. The study was carried out in 35 subgroups, with 7 replications in 5 treatment groups. The study lasted for a total of 56 days as 2 periods of 28 days. During the experiment, feed and water were given ad libitum and lighting was applied daily for 16 hours. In the study, a total of 5 different diets were used: control diet based on corn-soybean meal, and *Bacillus megaterium* and *Bacillus amyloliquefaciens* with one colony forming unit at 0.5 g/kg level, $1x10^8$ cfu/g. The probiotic strains used in the study were obtained from a commercial company. Experimental diets were prepared in accordance with the management guideline of the commercial layer chicken line used in the study and the needs reported by the NRC (1994) (Table 1).

Table 1. The compositions of experimental basal diet.

Ingredients	**%**
Corn	56.00
Soybean meal (% 45 CP)	29.00
Vegetable oil (8800 kcal ME/kg)	3.33
Limestone	9.30
Dicalcium phosphate	1.67
Salt	0.30
Vitamin-Mineral Premix[1]	0.25
DL-Methionine	0.15
Total	**100**
Calculated nutrient content	
Metabolizable energy (Kcal/kg)	2750
Crude Protein (%)	16.50
Calcium (%)	3.90
Available phosphorus (%)	0.40
Lysine (%)	0.76
Methionine (%)	0.38
Methionine+cystine (%)	0.70

[1]Vitamin-Mineral premix provides per kilogram of the diet: manganese: 80 mg; iron: 60 mg; copper: 5 mg; iodine: 1 mg; selenium: 0.15 mg; vitamin A: 8.800 IU; vitamin D3: 2.200 IU; vitamin E: 11 mg; niacin: 44 mg; Cal-D-Pan: 8.8 mg; riboflavin: 4.4 mg; thiamine: 2.5 mg; vitamin B12: 6.6 mg; folic acid: 1 mg; biotin: 0.11 mg; choline: 220 mg

Performance parameters

The body weights of the hens were determined as a group by weighing them with a scale with a sensitivity of 1 g at the beginning and end of the experiment, and the body weight gains were calculated from these data. The egg production was calculated by this formula: Egg production (%) = (Total number of eggs in the period (count) / Number of animals in the group (count)) × 100. The egg weights were determined by weighing all the eggs collected from each subgroup during the last two days of each 28 days using a digital scale with a precision of 0.01 grams. The egg mass was calculated by multiplying the percentage of egg production for each period with the average egg weight and then dividing by 100. The hens were weighed as groups and their daily feed intake was calculated. The feed conversion ratio was calculated by dividing the average daily feed intake per hen for each period by the corresponding egg mass (grams of feed/grams of egg mass).

Egg quality parameters

Eggshell quality parameters such as shell breaking strength, shell weight, shell thickness, white index, yolk index, Haugh unit and yolk color were determined in a total of 6 eggs from each subgroup collected on the last two days of each 28-day period. Eggshell breaking strength was determined using an eggshell strength measurement device (Egg Force Reader, Orka Food Technology, Israel). The eggshell weight (with membrane) was determined by cracking the eggs and separating the contents, then thoroughly washing them and drying them at room temperature for 3 days. Afterward, the eggshells were weighted using a precision digital scale to determine their weight. The percentage of eggshell ratio was calculated by dividing the eggshell weight by the egg weight. Eggshell thickness was determined by averaging the measurements made from the blunt, pointed, and equatorial regions of the broken eggshells with a digital micrometer. The internal quality characteristics of the eggs were also determined in the eggs used for determining eggshell quality parameters. For this purpose, eggs were broken on a glass table and egg internal quality parameters were measured. The height of the egg yolk and albumen was determined with a digital height gauge, and the yolk diameter and egg albumen length and width were determined with a digital caliper (Mutitoyo, Japan). The calculations were used to determine the yolk index, albumen index, and Haugh unit using this formula: Yolk index = (yolk height/yolk diameter) × 100; Albumen index = (albumen height/ (albumen length + albumen diameter)) × 100; Haugh unit = 100 × log (albumen height + 7.57-1.7 × egg weight$^{0.37}$). Egg yolk color was measured using a colorimeter device (Konica Minolta CR-200) and recorded as CIELab (L*, a*, b*) values. Egg internal quality analyses were completed within 12 hours after the eggs were collected.

Statistical analysis

To determine whether there were any significant effects of treatments on the examined parameters, one-way analysis of variance (ANOVA) was performed using the Minitab 17 statistical software package, and also Duncan's Multiple Comparison Test was applied to determine the differences between treatment groups (Düzgüneş *et al.* 1987).

RESULTS AND DISCUSSION

Performance parameters

The effects of adding *Bacillus amyloliquefaciens* (at levels of 1x10^8 cfu/g) and *Bacillus megaterium* (at levels of 1x10^8 cfu) to the diets of laying hens on initial body weight, final body weight, body weight gain, egg production, egg weight, and feed conversion ratio are presented in Table 2. According to Table 2, the examined performance parameters were not significantly affected by the addition of the probiotic strains to the diet compared to the control group ($p>0.05$). According to previous studies, probiotics affect numerous performance parameters in laying hens. These parameters include dynamics of body weight (weight gain), feed conversion ratio, egg laying performance, and egg quality (improved shell thickness, egg weight) (Lei *et al.* 2013, Chaucheyras-Durand and Durand 2010, Smith 2014, Bai *et al.* 2016). *Bacillus* probiotics are widely used in poultry nutrition, and it has been revealed that these probiotics offer a promising approach to improving poultry health (Jia *et al*. 2016). These probiotics exhibit resilience to different climatic conditions and have a long shelf life. *Bacillus* species including *Bacillus amyloliquefaciens* are found in the normal intestinal microbiota and have the ability to grow and produce spores in the gastrointestinal system (Cartman *et al.* 2008, Cutting 2011, Barbosa *et al.* 2005). In addition, their ability to form biofilms is important from a medical perspective (Ushakova *et al.* 2009). Our findings showed that the addition of different types of probiotics to the diets of laying hens had no significant effect on the performance parameters examined. In the studies, Tsai *et al.* (2023) compared the addition of 0.3% *Bacillus subtilis* and 0.1% *Bacillus amyloliquefaciens* to the diets of laying hens and reported that *Bacillus subtilis* increased body weight and body weight gain more than *Bacillus amyloliquefaciens* and the control group. They attributed this to *Bacillus subtilis'* better promotion of nutrient absorption. The same researchers found that *Bacillus amyloliquefaciens* did not affect egg production, feed consumption, egg mass, or the rate of damaged eggs. In contrast to these findings, Mazanko *et al.* (2018) reported that *Bacillus amyloliquefaciens* increased egg production in their study where they added various *Bacillus* species probiotics to the diets of laying hens. However, Weili *et al.* (2014) claimed that *Bacillus megaterium* improved performance in laying hens and reduced ammonia release in feces. Although the results of the studies reported and the results of the current study are partially compatible, it is thought that the reasons why the performance parameters reported in our study were not affected by the treatments may be due to the lack of any stress (heat stress, etc.) in hens. (Mazanko *et. al.* 2018). May be the fact that the animals are not exposed to any stress factors and optimum conditions (temperature, humidity, ventilation, etc.) are provided in the coop.

Table 2. The effect of adding different types of probiotics to the diets of laying hens on performance.

Performance Parameters	**Control**	***Bacillus megaterium* 10^8 cfu/g**	***Bacilllus megaterium* 10^8 cfu/g**	***Bacillus amyloliquefaciens* 10^8 cfu/g**	***Bacillus amyloliquefaciens* 10^8 cfu/g**	**P-value**
IBW, g	1728.43±42.95	1751.10±36.58	1785.80±25.14	1739.80±39.48	1774.50±26.15	0.76
FBW, g	1687.29±50.72	1713.43±47.81	1744.29±27.67	1703.57±39.96	1717.14±25.35	0.89
BWG, g	-41.43±11.66	-37.86±18.82	-41.71±17.74	-36.43±8.47	-57.86±12.66	0.84
FI, g/hen/day	102.99±1.88	101.03±2.43	101.08±1.06	101.55±2.45	101.81±1.21	0.95
Egg production, %	96.02±1.03	95.51±0.92	94.69±0.84	95.51±0.69	95.87±0.76	0.84
Egg weight, g	62.95±1.16	62.23±0.47	61.66±0.42	62.67±1.00	62.56±0.73	0.83
Egg mass, g/d	60.39±0.84	59.45±0.90	58.39±0.64	59.84±0.99	59.99±1.00	0.57
FCR, FI/EM	1.71±0.03	1.70±0.02	1.73±0.02	1.70±0.03	1.70±0.03	0.81
Damaged egg ratio, %	1.69±0.55	2.57±0.46	2.28±0.54	1.60±0.64	1.85±0.41	0.65

IBW: Initial body weight, FBW: Final body weight, BWG: Body weight gain, FI: Feed intake, EM: Egg mass, FCR: Feed conversion ratio

Egg quality parameters

The effect of adding 0.5 g/kg *Bacillus amyloliquefaciens* (1x10^8 cfu/g) and *Bacillus megaterium* (1x10^8 cfu/g) to the diets of laying hens on egg quality characteristics is given in Table 3. According to this, the values of albumen index, yolk index, shell breaking strength, eggshell weight, eggshell thickness, eggshell ratio, L* and b* values of the yolk color measurements were not significantly affected by the addition of *Bacillus megaterium*, and *Bacillus amyloliquefaciens* compared to the control group (p>0.05). The addition of different types of probiotics to the diet in laying hens significantly affected the Haugh unit (p<0.05) and a* value (p<0.01) as an egg yolk color measurement, and both values were significantly lower than the control group. In the study, the highest Haugh unit value was observed in the control group, and *Bacillus megaterium* and *Bacillus amyloliquefaciens* decreased this value. There was no statistically significant difference among the treatments, but *Bacillus amyloliquefaciens* at a level of 1x10^8 cfu/g reduced this value the most (p<0.05). The same findings were also observed in the* values of egg yolk color measurements. In this parameter examined, the control group was also the highest, and *Bacillus amyloliquefaciens* at a level of 1x10^8 cfu/g reduced it the most compared to the control group (p<0.01). Egg quality generally covers various parameters such as shell weight, albumen, and yolk quality. Egg quality has a genetic basis and varies among breeds of laying hens. However, egg quality is also affected by the housing conditions, age, and diet of the hens used (Jha *et al.* 2020). Abd El-Hack *et al.* (2017) reported that *Bacillus subtilis* increased yolk index, yolk color, and shell thickness compared to the control group in their study examining egg quality characteristics of laying hens. They reported that *Bacillus subtilis* did not affect Haugh unit values. On the other hand, Tsai *et al.* (2023) reported that *Bacillus subtilis* and *Bacillus amyloliquefaciens* did not affect eggshell thickness, shell weight, and egg yolk color. In another study, *Bacillus amyloliquefaciens* significantly increased eggshell quality. Researchers attributed this to an increase in calcium absorption due to increased feed utilization, which also passed into the eggshell. This is possible because probiotics create an acidic environment suitable for the ionization of minerals and lower the intestinal pH, which is especially necessary for the dissolution and optimal absorption of both calcium and phosphorus (Resta-Lenert and Barrett 2003). Lei *et al.* (2013) reported that the addition of *Bacillus amyloliquefaciens* at different doses led to varying increases in the Haugh unit, indicating that probiotics have the potential to improve egg quality by enhancing egg protein metabolism. By reducing the stress response, Bacillus amyloliquefaciens can enhance the egg production and quality of hens in a way that depends on the amount of bacteria given (Zhou et al. 2020). While our study findings do not align with those of various researchers reported because it is worth noting that the lack of any stress factors on the animals in our study may contribute to this discrepancy. This notion is supported by Jia *et al.* (2016), who propose that probiotic supplementation may be more effective under conditions of animal stress, thereby reducing the adverse effects of mycotoxins on laying performance, effectively improving egg quality, and reducing the accumulation of aflatoxin residues in eggs.

Table 3. The effect of adding different types of probiotics to the diets of laying hens on egg quality traits.

Egg quality parameters	Control	*Bacillus megaterium* 10^8 cfu/g	*Bacilllus megaterium* 10^8 cfu/g	*Bacillus amyloliquefaciens* 10^8 cfu/g	*Bacillus amyloliquefaciens* 10^8 cfu/g	P-value
Albumen index, %	9.38±0.27	8.45±0.32	8.56±0.15	8.66±0.19	8.24±0.38	0.06
Yolk index. %	43.74±0.61	43.14±0.34	42.42±0.46	43.18±0.26	43.70±0.53	0.32
Haugh unit	84.18±0.75^a	80.41±1.29^b	80.30±0.79^b	80.28±0.78^b	78.70±1.55^b	0.02
Shell breaking strength, kg	4.57±0.15	4.60±0.13	4.41±0.09	4.51±0.11	4.51±0.09	0.84
Eggshell weight, g	6.22±0.12	6.10±0.08	6.05±0.10	6.04±0.10	6.05±0.07	0.66
Eggshell thickness, mm	0.380±0.003	0.378±0.005	0.378±0.004	0.372±0.005	0.372±0.004	0.50
Eggshell ratio, %	9.88±0.05	9.81±0.09	9.81±0.13	9.65±0.12	9.67±0.10	0.43
Egg yolk color measurements						
L*	47.98±0.43	47.86±0.67	48.76±0.44	47.80±0.53	48.30±0.29	0.63
a*	2.70±0.29^A	1.99±0.15^B	1.93±0.11^B	2.08±0.09^B	1.82±0.11^B	0.001
b*	24.41±0.55	25.30±0.79	25.74±0.69	25.79±0.46	25.33±0.47	0.52

$^{a, b}$: Differences shown with different letters in the same row are statistically significant (P<0.05),
$^{A, B}$: Differences shown with different letters in the same row are statistically significant (P<0.01)

CONCLUSIONS

In conclusion, it was observed that the addition of *Bacillus megaterium* and *Bacillus amyloliquefaciens* at a level of 0.5 g/kg to the diets of laying hens had no significant effect on performance. It has been found that the addition of two different probiotics to the laying hen diets significantly reduces the Haugh unit and a* value of the yolk color measurement compared to the control group. However, the other quality parameters were not affected by the probiotic supplementation. In the study, compared to the control group, *Bacillus amyloliquefaciens* at a level of $1x10^8$ cfu/g reduced these parameters the most. According to the results obtained from the study, the lack of any effect of probiotic supplementation on animal performance was attributed to the optimal provision of animal welfare. Probiotic supplementation may have greater effects under stress than under normal conditions so, it would be more beneficial to use these probiotics individually or in combination at different levels, and to use them after a stress factor has been created in hens.

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