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Effect of the Probiotic *Bifidobacterium animalis* as a Substitute to Growth-Promoting Antibiotics on Performance and Egg Quality in Laying Hens

Research Article

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ABSTRACT

The present study was conducted to determine the effects of a dietary probiotic (*Bifidobacterium animalis* ssp.) as a substitute to a mixture of growth-promoting antibiotics (zinc bacitracin and colistin sulfate on the laying performance, egg quality characteristics, blood parameters, and organ morphological characteristics of early-phase laying hens. Seventy-two (72) 20-week-old Lohmann White hens were randomized into three treatment groups. The dietary treatments are as follows: Growth-promoting antibiotics (GPA; 0.5 % of COLI-ZIN), probiotic (PRO; 0.1% *Bifidobacterium animalis* ssp. lactis) or without any additive (control; CON) for 90-days feeding trial. Significant differences were observed in feed intake and feed conversion ratio of the bird's fed PRO compared to GPA and CON at 90 days of the experimental period. The air cell height, yolk volume, and yellowness of yolk color were lower, and thick and thin albumen diameters were the greatest in the PRO group than in CON and GPA groups ($P<0.05$). Dietary PRO decreased the number of heterophils (H) and increased the number of lymphocytes (L), improving the H:L index ($P<0.05$). Probiotic treatment increased crop and duodenum relative weight compared to GPA ($P<0.05$). No significant changes ($P>0.05$) were observed in the relative weight of reproductive organs. This study shows that the dietary supplementation of 0.1% *Bifidobacterium animalis* improves laying performance and egg traits and can be a substitute for antibiotics in hen diets.

KEY WORDS dietary additives, egg quality, laying hens, probiotics.

INTRODUCTION

Livestock production, in general, and domestic chicken production play a vital socioeconomic role for people living in low-income countries in Africa and Asia (Mohammadifar *et al*. 2014; Moazeni *et al*. 2016a).

Domestic chickens are widely distributed avian species worldwide due to their short generation interval and adaptability in various agroecologies (Moazeni *et al*. 2016b; Mohammadifar and Mohammadabadi, 2018). Domestic chickens provide high-quality protein and income for poor rural households and are the world's most widely

kept livestock species (Mohammadabadi *et al*. 2010; Mohammadifar and Mohammadabadi, 2017). This is due to the presence of the valuable traits of chickens, like disease resistance, adaptation to harsh environments, and the ability to utilize poor-quality feeds (Shahdadnejad *et al*. 2016). Due to the high demand in poultry production, growth-promoting antibiotics (GPAs) are used to improve feed conversion to reduce mortality and the weight gain and production of poultry birds (Suresh *et al*. 2018; Muhammad *et al*. 2020).

Since the prohibition of GPAs in 2006 by the European Union due to the emergence of bacterial resistance and due to the possible presence of antibiotic residues in tissues and products such as milk, eggs, and meat. Other countries such as Japan, the United States, and Canada have joined the initiative of establishing guidelines for prohibiting GPAs in poultry production. Moreover, South Korea, New Zealand, Mexico, and others, have reduced in-feed antibiotic use (McNamee *et al*. 2013; Mund *et al*. 2017; Vieco-Saiz *et al*. 2019).

In this context, probiotics are the most recommended substitute in-feed antibiotics. Probiotics are known as living microorganisms that provide substantial benefits to the health of the host. Though there are a wide variety of strains, the most used include *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Bacillus*, or *Bifidobacterium* alone or in combination. Probiotics can be administered in powder form dissolved in water or incorporated into food (Forte *et al*. 2016; Alagawany *et al*. 2018; Yan *et al*. 2019).

Therefore, the aim of this study was to evaluate the productive performance, egg quality, and health status of laying hens treated with *Bifidobacterium animalis* ssp. lactis as compared to those treated with a mixture of GPAs.

MATERIALS AND METHODS

Birds, husbandry, and experimental diets

The protocol of bird experiments was approved by the Institutional Committee of Bioethics in Research of the University of Guanajuato (CIBIUG-P36-2020). A total of 72 hens from the 18-week-old Lohmann White strain (1.4±0.7 kg) were purchased from a local commercial layer farm (Jalisco, Mexico). All birds were previously given a complete vaccination schedule by intramuscular or drinking water injection against the Newcastle disease virus, infectious bronchitis virus, and infectious bursitis virus. All birds were introduced into wire cages (25 cm×35 cm×40 cm) with a floor slope of 12˚, and individual feeders and waterers were provided. They were housed under controlled environmental conditions with room temperature at 25-27 ˚C.

The birds were exposed to 16 hours of continuous artificial lighting and 8 hours of darkness. The birds were fed a balanced diet for two weeks prior (the adaptation period) to and during the 90-days experimental period. The diet was formulated based on the nutritional requirements of laying hens (LOHMANN LSL CLASSIC Management guide, 2020). Table 1 presents the ingredients and the composition of the diets. The 20-week-old laying hens were randomly assigned into three dietary treatment groups, with 24 birds in each group with three replicates. The diets were supplemented with 0.5% of COLI-ZIN (colistin sulfate and zinc bacitracin) as a growth-promoting antibiotic (GPA), 0.1% of *Bifidobacterium animalis* ssp. lactis as probiotic (PRO), and without supplement (control; CON). The *Bifidobacterium animalis* ssp. lactis strain showed the viability of 3×10^{11}/g (Bi-07 300B, FloraFIT®, Danisco, USA) (Figure 1).

Sample collection and laying performance

At the start, all birds were weighed once a week throughout the experimental phase. The amount of feed intake was recorded daily (ADFI), and cumulative egg production was recorded three times (30, 60, and 90 days) of the experimental period at 32 weeks of age. Eggs produced were collected twice daily at 8:00 a.m. and 3:00 p.m. and were weighed individually with an electronic balance (Velab VE-5000).

Egg production percent was calculated by the number of eggs produced daily divided by the number of hens. The feed conversion ratio (FCR) was calculated by dividing kilograms of intake to kilograms of egg produced (Tapingkae *et al*. 2018).

Egg quality traits

Egg quality was evaluated at the end of the experiment at 32 weeks of age. A total of 36 eggs were collected (12 eggs for each treatment) to determine egg quality parameters. Egg width, egg length, shell thickness, air cell height, thick albumen diameter, thin albumen diameter, albumen height, yolk height, yolk length, and total diameter were measured using a digital caliper (model HER-411, Steren, Mexico), and expressed as mm, and shape index (%) was calculated (Abanikannda *et al*. 2007; Hanusová *et al*. 2015). Egg surface (ES) was calculated using the formula: ES (cm^2)= 4.68 $W^{2/3}$; were: W= egg weight (Sauveur, 1988). Yolk volume, improved albumen index (AIi) used as an alternative to Haugh units, and egg quality Index (EQI) was determined according to Narushin *et al*. (2021). Egg yolk color was determined by the color profile L* (lightness value), a* (redness value), and b* (yellowness value) using Hunterlab Colour Flex (Hunterlab, USA).

Table 1 Ingredients and nutritional composition of the experimental diets

Ingredients (%)	Treatments		
	Control	Growth-promoting antibiotic	Probiotic
Sorghum grain	36.9	37.3	37.2
Soya bean meal (CP %)	20.4	20.4	20.4
Corn	28.5	28.5	28.7
Fish meal (CP %)	10.0	10.3	10.0
Soya bean oil	1.1	1.1	1.1
Calcium carbonate	1.5	1.5	1.5
Orthophosphate	1	1	1
Vitamins and minerals premix[1]	1	1	1
L-threonine	0.05	0.05	0.05
L-tryptophan	0.05	0.05	0.05
Colizin	0	0.2	0
Bifidobacterium animalis ssp. lactis	0	0	0.1
Total	100	100	100
Calculated analysis			
ME (kcal/kg)	2900	2895	2898
Crude protein (%)	19.4	19.3	19.3
Crude fiber (%)	6.0	6.0	6.0
Calcium (%)	2.5	2.5	2.5
Phosphorus (%)	0.6	0.6	0.6

[1] Supplied per kilogram of diet: vitamin A: 39378 IU; vitamin D_3: 5358 IU; vitamin B_1: 8.17 mg; B_2: 21.60 mg; B_6: 16.66 mg; B_{12}: 108 mcg; Pantothenic acid: 82.5 mg; Folic acid: 5.3 mg; Copper: 36 mg; Biotin: 7 mg; Selenium: 0.75 mg and Zinc: 360 mg.

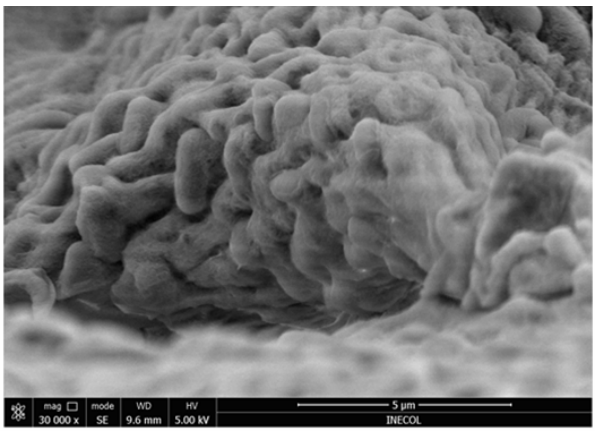

Figure 1 Scanning electron microscopy micrograph showing freeze-dried *Bifidobacterium animalis* sub. lactis Bi-07 300B powder (scale bar: 5 µm; magnification: 30000 X)

Sampling and estimation of heterophile and lymphocyte (H:L) index

Ten birds per treatment were selected randomly for blood collection based on the technical bulletin for the appropriate one for the strain used. Briefly, blood was collected from the alar vein with a 22-gauge needle in heparinized tubes (BD Vacutainer®) after 12 h fasting. The total count of leukocytes was used in a Neubauer chamber diluting the sample 1:20 in Natt and Herrick solution (Natt and Herrick, 1952).

Then, the nine quadrants of the entire chamber were counted (40X), multiplied by 20, and divided by 1000, reporting the number of leukocytes × 10^3/µL (Acevedo *et al.* 2012). To perform leukocyte differential counts, the wright stain was used. To calculate the Heterophile: Lymphocyte (H:L) index, which is a direct indicator of animal stress, we divide the number of heterophils (H) by the number of lymphocytes (L) (Gross and Siegel, 1983).

Determination of weight and pH of various organs

At the end of the experiment, ten hens from each treatment were sacrificed by cervical dislocation according to the guidelines of NOM-033-ZOO (1995). Crop, proventriculus, gizzard, duodenum, jejunum, ileum, large intestine, ceca, heart, liver, spleen, kidney, lung, brain, ovary, infundibulum, magnum, isthmus, uterus, and vagina were immediately removed, and weighed. The data are expressed in % of body mass. Finally, the determination of the pH (model Hi98103, Hanna, USA) of gastrointestinal and reproductive organs was performed (Özek *et al.* 2011).

Statistical Analysis

Data were analyzed by one-way ANOVA using Statistica software (version 8.0, Statsoft, USA). The statistical model used was as follows:

$$Y_{ijk} = \mu + T_i + e_{ijk}$$

Where:
Y_{ij}: observation.
μ: general mean.
T_i: effect of each treatment.
e_{ijk}: random error.

A post hoc test to measure specific differences between pairs Duncan's test considering a $P \leq 0.05$ as significant.

RESULTS AND DISCUSSION

Table 2 shows the effect of CON, GPA, and PRO treatments on the performance of laying hens three times (30, 60, and 90 days) from 18 to 32 weeks of age. No significant differences were observed in the amount of daily feed intake between treatments for the two first times in response to the different functional ingredients. However, in the last period, PRO treatment significantly decreased the amount of daily feed intake compared to GPA and CON groups (P<0.001). On the other hand, no significant differences were observed between treatments during three experimental periods in hen-day egg production and egg weight. Interestingly, FCR was reduced significantly in PRO treatment compared to those fed GPA and CON at the end of the third phase (P<0.001).

Table 3 presents the effects of probiotics on the quality parameters of eggs collected at the end of the experimental period (32 weeks of age). No significant differences were observed between treatments for the width, length, surface, egg quality index (EQI), shape index, or shell thickness. However, the air cell height was lower in the PRO treatment compared to the GPA treatment and CON groups. Moreover, thick and thin albumen diameters were greater in PRO than in CON and GPA treatment (P<0.05). Furthermore, PRO treatment significantly reduced yolk volume with respect to CON and GPA groups (P<0.05). The yellowness (b*) of the yolk color was lower in the PRO compared to the CON groups (P<0.05).

Figure 2 shows the number of total leucocytes, percent heterophils and lymphocytes, and H:L index of laying hens at 32 weeks of age. No statistically significant difference (P>0.05) was observed in the total leucocyte count. However, the number of heterophils was significantly lower in PRO as compared to GPA and CON (P<0.05). Conversely, the number of lymphocytes was significantly increased in PRO compared to GPA and CON groups (P<0.05). Therefore, the H:L ratio decreased significantly with respect to GPA and CON groups (P<0.05).

Table 4 presents the weight and pH of the gastrointestinal tract and visceral and reproductive organs in hens at 32 weeks of age.

Regarding the gastrointestinal tract, the PRO treatment increased the relative weight and modified the pH levels in the crop as compared to GPA treatment but not to those fed with CON (P<0.05). Interestingly, the relative weight of the gizzard is decreased by GPA and PRO treatments compared to CON (P<0.05). Moreover, GPA treatment significantly reduces the relative weight of the duodenum compared to PRO treatment and CON (P<0.05). Conversely, PRO treatment significantly increased the relative weight of the large intestine as compared to GPA and CON (P<0.05).

On the other hand, no statistically significant change was observed in relative weight in visceral organs. When concerning reproductive organs, PRO treatment modified the pH levels in the infundibulum and magnum as compared to the CON group (P<0.05). Finally, no significant changes in these organs' relative weight were observed due to the treatments.

Several functional ingredients have been studied extensively during the recent decades in the diets of laying hens to replace growth-promoting antibiotics. These include antioxidants, probiotics, prebiotics, herbal extracts, and essential oils that can affect laying performance and egg quality; however, many studies have evaluated probiotic strains in the performance of laying hens (Hajiaghapour and Rezaeipour, 2018; Jha *et al.* 2020). It has been shown that the addition of probiotics in the early stages of poultry growth shows a beneficial effect on the constitution of the intestinal microbiota and, therefore, in the laying stage (Lutful Kabir, 2009; Hassanein and Soliman, 2010). In this study, during the last phase, the inclusion of 0.1% of *B. animalis* improved the FCR, while the amount of daily feed intake was reduced significantly as compared to those fed the antibiotics and control diets. According to current results, Mikulski *et al.* (2012) showed that a dietary probiotic *Pediococcus acidilactici* strain at doses of 100 mg/kg feed for 222 Hy-Line Brown laying hens improved the feed efficiency ratio per kilogram of eggs. Conversely, Forte *et al.* (2016) found that 0.5% *Lactobacillus acidophilus* and 1% *Bacillus subtilis* as additives in Hy line White laying hens' diet for 20 weeks had no significant effect on feed efficiency, only the color of the yolk showed significant differences. Concerning egg quality, in the present study, PRO treatment showed reduced air cell height, yolk volume, and yellowness of yolk color (b*), and improved the diameter of thick and thin albumen with respect to GPA and CON in the early stage of laying. In this context, eggs' reduced air cell size indicates higher quality. However, no modified egg and shell parameters were observed. In this regard, it has been reported that in younger laying hens, the efficiency in absorbing Ca is less; therefore, some parameters of egg quality do not improve (Abdelqader *et al.* 2013).

Table 2 Effects of *B. animalis*, growth promoter antibiotic, and control diets on performance of 20 to 32-week-old laying hens during 90 days of treatments

Item	20-24-week-old day				25-28-week-old				29-32-week-old			
	CON	GPA	PRO	SEM	CON	GPA	PRO	SEM	CON	GPA	PRO	SEM
Feed intake (g/hen/day)	69.6	68.6	70.4	1.28	88.7	92.1	69.7	1.17	106.2^{a**}	105.9^{a**}	99.6^{b}	0.74
Hen-day egg production (%)	34.2	36.6	31.9	1.65	48.2	50.6	43.1	2.04	65.4	63.1	58.0	2.39
Egg weight (g)	52.8	52.6	53.3	0.93	58.6	57.3	58.3	0.96	61.0	61.3	62.5	0.53
Feed conversion ratio (kg feed/kg egg)	1.22	1.22	1.24	0.02	1.47	1.54	1.47	0.01	$1.74^{a}*$	1.72^{a**}	1.64^{b}	0.0

CON: basal diet; GPA: basal diet supplemented with 0.5% of COLI-ZIN and PRO: basal diet supplemented with 0.1% of *Bifidobacterium animalis* ssp. lactis.
* (P<0.05) and ** (P<0.001).
SEM: standard error of the means.

Table 3 Effects of *B. animalis* (PRO), growth promoter antibiotic (GPA), and control (CON) diets on egg quality traits of laying hens at the end of the experimental period

Items[1]	CON	GPA	PRO	SEM
Egg width (mm)	43.8	43.7	43.9	2.27
Egg length (mm)	57.6	57.8	57.6	3.94
Shell thickness (mm)	0.30	0.35	0.32	0.01
Air cell height (mm)	1.5^{a}	1.6^{a}	0.9^{b*}	0.14
Thick albumen diameter (mm)	82.9^{a}	83.8^{a}	103.1^{b**}	2.77
Thin albumen diameter (mm)	61.1^{a}	66.3^{a}	77.8^{b*}	2.28
Albumen index	0.14	0.13	0.14	0.002
Yolk volume (cm^3)	16.6^{a}	17.4^{a}	13.7^{b*}	0.56
L*	64.3	65.1	66.2	0.58
a*	-0.9	-0.7	-1.0	0.37
b*	32.1^{a}	28.6^{ab}	25.5^{b*}	1.09
Egg surface area (cm^2)	75.5	72.7	73.6	0.42
EQI	116.3	114.1	116.3	0.83
Shape index (%)	76.1	75.6	76.3	0.31

CON: basal diet; GPA: basal diet supplemented with 0.5% of COLI-ZIN and PRO: basal diet supplemented with 0.1% of *Bifidobacterium animalis* ssp.
L*: lightness; a*: chroma; b*: hue and EQI: egg quality index.
* (P<0.05) and ** (P<0.001).
SEM: standard error of the means.

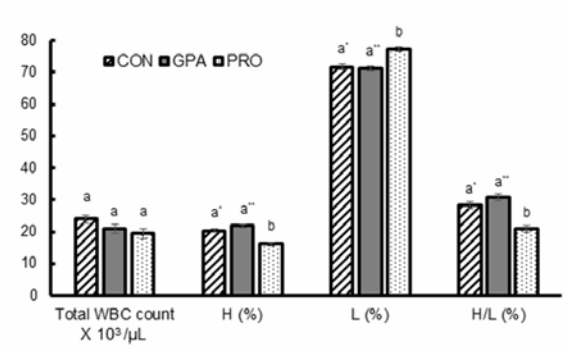

Figure 2 Effects of dietary treatments on total WBC count, heterophile (H), lymphocyte (L), and heterophile/lymphocyte ratio (H:L) of laying hens at 32 weeks of age corresponding to the end of the experimental period of 90 weeks
CON: basal diet; GPA: basal diet supplemented with 0.5 % of COLI-ZIN; PRO: basal diet supplemented with 0.1% of *Bifidobacterium animalis* ssp.

Moreover, Yan *et al.* (2019) reported that a commercial mixture of probiotics that contained (*Enterococcus faecium*, *Pediococcus acidilactici*, *Bifidobacterium animalis*, and *Lactobacillus reuteri*) with an inclusion level of 0, 0.5, 1.0, and 2.0 g/kg for seven weeks at 60-week-old White Leghorn hens decreases the percentage of broken eggs, but other performance parameters did not show relevant changes.

The mechanism by which probiotics act in poultry is not yet very clear; however, it has been postulated that it is due to the stimulation of the selective growth of beneficial bacteria that increase short-chain fatty acids like propionate, which is a precursor of gluconeogenesis, increase the availability of glucose; maturation, maintenance, and prevention of intestinal integrity reducing the probability of inflammation; modulation of the immune system by neutralization of enterotoxins, and improvement of digestion and metabolism due to the activity of bacterial strains (Lutful Kabir, 2009; Lan *et al.* 2016; Peng *et al.* 2016).

In this regard, the hematological parameters were determined to analyze the health status of the hens supplemented with *B. animalis*. It has been suggested that bioactive dietary components have immunomodulatory potential to prevent or mitigate disease infections in poultry (Kogut, 2009).

Table 4 Effects of *B. animalis* (PRO), growth promoter antibiotic supplementation (GPA) and control (CON) diets of relative weight and pH of various organs of the laying hens at 32 weeks of age

Organ	Treatments			
	CON	GPA	PRO	SEM
Gastrointestinal tract				
Crop				
Index (% BW)	0.54^{ab}	0.55^{a}	0.61^{b*}	0.03
pH	6.13^{a}	5.41^{b}	5.37^{b*}	0.10
Proventriculus				
Index (% BW)	0.54	0.55	0.45	0.03
pH	5.68	5.81	5.48	0.06
Gizzard				
Index (% BW)	1.48^{a}	1.24^{b}	1.21^{b*}	0.04
pH	5.63	5.5	5.3	0.07
Duodenum				
Index (% BW)	1.45^{a}	1.25^{b}	1.46^{a*}	0.03
pH	5.92	5.98	5.95	0.03
Jejunum				
Index (% BW)	1.05	0.87	0.92	0.05
pH	6.03	6.04	5.83	0.04
Ileum				
Index (% BW)	0.73	0.63	0.62	0.03
pH	6.03	6.16	6.05	0.04
Large intestine				
Index (%)	1.16^{a}	1.14^{a}	1.42^{b*}	0.04
pH	6.22	6.34	6.26	0.03
Ceca				
Index (% BW)	0.56	0.44	0.53	0.02
pH	6.52	6.52	6.6	0.03
Visceral organs				
Index (% BW)				
Heart	0.48	0.51	0.46	0.00
Liver	2.60	2.60	2.41	0.07
Spleen	2.60	2.60	2.41	0.00
Kidney	0.60	0.58	0.53	0.02
Lung	0.42	0.39	0.40	0.01
Brain	0.13	0.13	0.13	0.00
Reproductive organs				
Ovary				
Index (% BW)	2.57	3.01	2.71	0.17
pH	6.42	6.32	6.51	0.03
Infundibulum				
Index (% BW)	0.14	0.16	0.12	0.01
pH	6.35^{a}	6.45^{ab}	6.51^{b*}	0.02
Magnum				
Index (% BW)	1.79	1.93	2.38	0.11
pH	6.28	6.28	6.47	0.04
Isthmus				
Index (% BW)	0.41	0.45	0.48	0.01
pH	6.12^{a}	6.24^{ab}	6.32^{b*}	0.04
Uterus				
Index (% BW)	1.08	1.06	0.89	0.04
pH	6.30	6.24	6.23	0.02
Vagina				
Index (% BW)	0.13	0.17	0.15	0.02
pH	6.31	6.35	6.4	0.03

CON: basal diet; GPA: basal diet supplemented with 0.5% of COLI-ZIN and PRO: basal diet supplemented with 0.1% of *Bifidobacterium animalis* ssp.
* (P<0.05) and ** (P<0.001).
SEM: standard error of the means.

In this study, the inclusion of 0.1% *B. animalis* in the hen diet increases the percentage of lymphocytes (% L) and decreases the percentage of heterophils (% H), which participate in acute inflammation and phagocytosis of pathogens respectively, and the H:L ratio, which is an indicator of animal stress (Gross and Siegel, 1983).

Coinciding with our results, Tang *et al.* (2017) found that the dietary supplementation of a probiotic PrimaLac® in the diet of 20-52 weeks old laying hens increased the L% and decreased the H%, thus decreasing the H:L ratio accordingly. This suggests that probiotics are actively involved in immunological processes as immunomodulators, even in healthy animals that can be minimally stressed by handling and confinement.

On the other hand, in this study, a significant change in the relative weight of crop, gizzard, duodenum, and large intestine of hens with the inclusion of the probiotic was observed. Numerous studies indicated that the inclusion of probiotics in birds modifies the intestinal microbiota and intestinal histomorphology through the stimulation of the production of SCFAs (Awad *et al.* 2009; Song *et al.* 2014; Ricke, 2015; Ricke *et al.* 2020). Additionally, the inclusion of GPA reduced the cecum's relative weight. This is probably related to cecal microbiota modification (Danzeisen *et al.* 2011).

Moreover, the results showed that the addition of probiotics does not significantly affect visceral and reproductive organs. Although in the latter, the pH of the infundibulum and isthmus was slightly modified, perhaps due to the indirect effect on the modulation of the gastrointestinal microbiota. Probiotics have been reported to have the potential to prevent injury to the reproductive organs of laying hens, as they show antimicrobial and anti-inflammatory effects (Shini *et al.* 2013). However, the mechanisms of probiotics in the reproductive system of laying hens remain unclear.

CONCLUSION

It could be concluded that *Bifidobacterium animalis* ssp. lactis at 0.1% can be used as a dietary additive in the diets of early-stage laying hens as a substitute for growth-promoting antibiotics. This treatment can improve their production performance, and some egg quality traits include air cell height, thick albumen diameter, and thin albumen diameter, and other traits. Probiotic used in this study can be considered a good additive to the diets of laying hens without no adverse effects on organs.

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