Effect of probiotic *Bifidobacterium animalis* as an alternative to growth-promoting antibiotics on performance, egg quality, and health parameters in young laying hens

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Abstract

Probiotics are live microorganisms that promote the selective growth of beneficial bacteria in birds and can be an effective alternative to growth-promoting antibiotics (GPAs) in poultry. The present study was conducted to determine the effects of dietary probiotics in the diet of early phase laying hens on laying performance, egg quality, blood parameters, and organs morphological characteristics. Seventy-two 13-week-old Lohman White hens were randomized into three dietary groups feed diets with GPA (0.5% of COLI-ZIN), probiotic (PRO; 0.1% *Bifidobacterium animalis* ssp lactis) or without additive (control; CON) for a 90-days feeding trial. Significant differences were observed in feed intake and feed conversion ratio in PRO with respect to GPA and CON at 90-days treatment ($p < 0.05$). PRO-treated group improved egg weight compared with the GPA and CON groups, but with no statistically significant difference. The air cell height, yolk volume, and yellowness of yolk color were lower, and thick and thin albumen were greatest in PRO than CON and GPA groups ($p < 0.05$). Dietary PRO decreased the number of heterophils and increased the number of lymphocytes, improving the H/L index significantly ($p < 0.05$). PRO treatment increases the relative weight of crop and duodenum compared to GPA ($p < 0.05$). No significant changes in the relative weight of reproductive organs were observed. Based on the results of the present study, it can be concluded that, *Bifidobacterium animalis* ssp lactis supplementation can be used as a safe alternative in young laying hens.

Introduction

Due to the high demand in poultry production, growth-promoting antibiotics (GPAs) are usually used to improve feed conversion, reduce mortality of production birds, accelerate their development and production, increase weight gain (Suresh et al. 2018; Muhammad et al. 2020). Since the prohibition of GPAs in 2006 in the European Union due to the emergence of bacterial resistance and that have been found as 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 establishing guidelines for the prohibition of the use of GPA. Moreover, South Korea, New Zealand, and Mexico and others, have reduced its use (McNamee et al. 2013; Mund et al. 2017; Vieco-Saiz et al. 2019).

In this regard, one of the most recommended alternatives that can contribute to reducing bacterial resistance to antimicrobials is the use of probiotics. Probiotics are known as living microorganisms that provide substantial benefits to the health of the host, after their administration in optimal and safe amounts. There is a wide variety of strains, the most used are *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Bacillus*, or *Bifidobacterium* alone or in combination and administered in powder form dissolved in water or incorporated into food (Forte et al. 2016; Alagawany et al. 2018; Yan et al. 2019).

It has been shown that the addition of probiotics in the early stages of poultry growth shows a very useful effect on the constitution of the intestinal microbiota and therefore in the laying stage (Kabir, 2009; Hassanein & Soliman, 2010). 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 (Kabir, 2009; Lan et al. 2016; Peng et al. 2016). 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 compared with the effect of the use of a growth-promoting antibiotic (Colizin) and without.

Material And Methods

Animals, management, 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), Guanajuato, Mexico. A total of 72 hens of the 13-week-old Lohmann White strain were purchased from a local commercial layer farm (Jalisco, Mexico). All birds were divided into wire cages (25 cm x 35 cm x 40 cm) with a floor slope of 12° and individual feed trough and water trough and were housed under controlled environmental conditions with a room temperature keep at 25-27 °C with exposure to 6 hours continuous artificial lighting and 8 hours darkness. The birds were fed with a balanced diet for 2 weeks previous weeks of adaptation and during for 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 laying hens were randomly assigned into three dietary treatment groups with 24 birds in each group. The diets were supplemented with 0.5 % of COLI-ZIN (zinc bacitracin and colistin sulfate) as a growth-promoting antibiotic (GPA) and 0.1% of *Bifidobacterium animalis* ssp lactis as probiotic (PRO), or without (control; CON). The *Bifidobacterium animalis* ssp lactis strain showed the viability of 3×10^{11} /g (Bi-07 300B, FloraFIT®, Danisco, USA) (figure 1).

[TABLE 1]

[FIGURE 1]

Figure 1. Scanning electron microscopy micrographs of the *Bifidobacterium animalis* sub. Lactis.

Sample collection and laying performance

All birds were weighed once a week throughout the experimental phase. Feed intakes were recorded daily, and egg production was recorded at three times (30, 60, and 90 days) of the feeding period. The eggs were collected daily twice at 8:00 a.m. and 3:00 p.m. and weighed. Egg production 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 feed per kilograms of egg (Tapingkae *et al.* 2017).

Egg quality parameters

Egg quality was evaluated at the end of the experiment at 26 weeks of animal 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, thin albumen, 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 was calculated using the formula: $ES\ (cm^2) = 4.68\ W^{2/3}$; were: W= egg weight (Sauveur, 1988). Yolk volume, Improved Albumen Index (Ali) used as an alternative to Haugh units, and Egg Quality Index (EQI) was determined by Narushin *et al.* 2021. Egg yolk color was determined by the color profile L* (lightness value), a* (redness value), b* (yellowness value) using Hunterlab Colour Flex (Hunterlab, USA).

Sampling and heterophile and lymphocyte index

Ten animals per treatment were selected for the blood collection, which was 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. To the total count of leukocytes were used a Neubauer chamber diluting the sample 1:20 in Natt and Herrick solution (Natt & Herrick, 1952). Then, the nine quadrants of the entire chamber were counted (40X) and multiplied by 20 and divided by 1000, reporting the number of leukocytes x

10³/μL (Acevedo *et al.* 2012). To perform leukocyte differential counts we used the Wright stain. To calculate the H:L index, which is a direct indicator of animal stress, we divide the number of heterophils by the number of lymphocytes (Gross & Siegel, 1983).

Determination of weight and pH of various organs

At the end of the experiment, ten hens of each treatment were weighed and slaughtered according to guidelines of NOM-033-ZOO-1995, Mexico. 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 major organs was performed.

Statistical Analysis

Data were analyzed by one-way ANOVA using Statistica software (version 8.0, Statsoft, USA). The analysis of the comparison between means was performed using Duncan’s multiple range test considering a $p \leq 0.05$ as significant.

Results

Laying hen performance and egg quality

Table 2 shows the effect of CON and GPA and PRO treatments on the performance of the laying hens three times (30, 60, and 90 days) during 13 to 26 weeks of age. No significant differences were observed in feed intake between treatments in two first times in response to the different functional ingredients. However, in the last period, PRO treatment decreases significantly feed intake compared to GPA and CON groups ($p<0.001$). On the other hand, no significant differences were observed hen-day egg production and egg weight between treatments during three experimental periods. Interestingly, PRO treatment decreases significantly feed conversion ratio compared to GPA and CON groups in the third period ($p<0.001$).

[TABLE 2]

Egg quality

Table 3 presents the effects of quality parameters of eggs collected at the end of the experimental period (13 weeks). No significant differences were observed in width, length, surface, EQI, shape index, or shell thickness of eggs between treatments. However, the air cell height was lower in PRO treatment compared to GPA treatment and CON groups ($p<0.05$). Moreover, the thick and thin albumen were greatest in PRO than CON and GPA treatment ($p<0.05$). Furthermore, we observed that PRO treatment significantly decreases yolk volume with respect to CON and GPA groups ($p<0.05$). The yellowness of the yolk color was lower in PRO compared to CON groups ($p<0.05$).

[TABLE 3]

Leucocyte count

Figure 2 showed the number of total leucocytes, percent of heterophils and lymphocytes, and H/L index of laying hens at 26 weeks of age. No statistically significant change was observed in a total leucocytes count. However, the number of heterophils was significantly lower in PRO compared to GPA treatment and CON ($p<0.05$). Conversely, the number of

lymphocytes was significantly increasing in PRO compared to GPA and CON groups ($p<0.05$). Therefore, we can observe that the H/L ratio decreased significantly with respect to GPA and CON groups ($p<0.05$).

[FIGURE 2]

Figure 2. Effects of dietary treatments on total WBC count, heterophile (H), lymphocyte (L), and heterophile/lymphocyte ratio (H/L) of laying hens at 90 days of the experimental period.

Weight and pH of various organs

Table 4 presents the weight and pH of the gastrointestinal tract and visceral and reproductive organs in hens at 26 weeks of age. Regarding the gastrointestinal tract, the PRO treatment increases relative weight and modified the pH levels in the crop compared to the GPA treatment but not to CON ($p<0.05$). Interestingly, the relative weight of the gizzard is modified 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 increases the relative weight of the large intestine compared to GPA treatment and CON ($p<0.05$). On the other hand, no statistically significant change was observed in relative weight in visceral organs. Regarding reproductive organs, PRO treatment modified pH levels in the infundibulum and magnum compared to the CON group ($p<0.05$). Finally, no significant changes in the relative weight of these organs were observed due to the effect of the treatments.

[TABLE 4]

Discussion

Functional ingredients have been studied in recent decades as feed additives in the diet of laying hens to replace growth-promoting antibiotics. These include antioxidants, probiotics, prebiotics, herbal extracts, and oils that can affect laying performance and egg quality; however, few studies have evaluated probiotic strains in the performance of laying hens (Hajiaghapour & Rezaeipour, 2018; Jha et al. 2020). In this study, the inclusion of 0.1% of *B. animalis* improved the feed conversion ratio while the feed intake was decreases compared to the antibiotics and control diets in the last period in early laying hens. According to our results, Mikulski et al. (2012), showed that a dietary probiotic *Pediococcus acidilactici* strain at doses of 100 mg/kg feed at 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 to the diet of 900 Hy line White hens for 20 weeks, no significant effect on productive parameters, only the color of the yolk showed significant differences. Moreover, Yan et al. (2019), reported that a commercial mixture of probiotics which 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 7 weeks at 60-week-old White Leghorn hens decreases the percentage of broken eggs but other performance parameters did not show relevant changes.

Concerning egg quality, in the present study, PRO treatment decrease air cell height, yolk volume, and yellowness of yolk color, and improved the thick and thin albumen with respect to GPA and CON in the early stage of laying. However, no modified egg and shell parameters. 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).

To analyze the health status of the hens during supplementation with *B. animalis* we determined the hematological parameters. It has been suggested that dietary bioactive components have immunomodulatory potential which can prevent or mitigate diseases infections in poultry (Kogut 2009). We found that 0.1% *B. animalis* increases the percentage of lymphocytes (%L) and decrease the percentage of heterophils (%), which participate in acute

inflammation and phagocytosis of pathogens respectively, and the H: L ratio, which is an indicator of animal stress (Gross & Siegel 1983). Coinciding with our results, the study of Tang et al. (2017), found that dietary supplementation of a probiotic PrimaLac® in the diet of 20-52 weeks old laying hens increases the %L and decreases the %H, decreasing accordingly the H/L ratio. This suggests that probiotics are actively involved in immunological processes, even in healthy animals.

On the other hand, in this study, we observed a significant change in the relative weight of crop, gizzard, duodenum, and large intestine of hens with the inclusion of the probiotic. Numerous studies indicate 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, we observed that the inclusion of GPA decreases the relative weight of the cecum which may be related to the modification of the cecal microbiota (Danzeisen et al. 2011). The results showed that the addition of probiotics does not have a significant effect on visceral organs 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), although 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 without the inclusion of growth-promoting antibiotics. This treatment can improve the quality characteristics of the egg and have an immunomodulatory effect in laying hens decreases physiological stress; therefore, it can be considered a good additive for the diets of early laying hens without negative effects on organs.

Declarations

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Compliance with ethical standards

Conflict of interest

The authors have no competing interests to declare that are relevant to the content of this article.

Ethical standards

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The protocol of bird experiments was approved by the Institutional Committee of Bioethics in Research of the University of Guanajuato (CIBIUG-P36-

2020). The authors confirm that they have followed Mexican standards for the protection of animals used for scientific purposes (NOM-033-Z00-1995; NOM-062-Z00-1999).

Authors's contribution

Elena Franco-Robles conceptualization, supervision, writing – review and editing. Cristina D. Balcón-Pacheco and Fabiola D. Coronel-Gómez methodology, analysis, performed the experiment. César Ozuna, Maribel Jiménez-Fernández and César Angel-Sahagún reviewed and criticised the draft manuscript.

Data availability

Data available on request from the corresponding author.

Declaration of funding

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Tables

Table 1. Ingredients and nutritional composition of the experimental diets

Ingredients (%)	Treatments		
	CON	AGP	PRO
Sorghum grain	37.5	37.3	37.2
Soya bean meal	20.4	20.4	20.4
Corn	28.5	28.5	28.7
Fish flour	10.4	10.4	10.4
Soya bean oil	1.1	1.1	1.1
Calcium carbonate	1.5	1.5	1.5
Orthophosphate	0.5	0.5	0.5
Vitamins and minerals premix ¹	0.09	0.09	0.09
L- threonine	0.05	0.05	0.05
L- tryptophan	0.05	0.05	0.05
Colizin	0	0.2	0
<i>Bifidobacterium animalis</i> 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 (%)	2.01	2.01	2.01
Phosphorus (%)	0.6	0.6	0.6

¹.Supplied per kilogram of diet: vitamin A 39,378 IU, vitamin D3 5,358 IU, vitamin B1 8.17 mg, B2 21.60 mg, B6 16.66 mg, B12 108 mcg, pantothenic acid 82.5 mg, folic acid 5.3 mg, copper 36 mg, biotin 7 mg, selenium 0.75 mg, zinc 360 mg.

Table 2. Effects of *B. animalis*, growth promoter antibiotic and control diet on performance of laying hens during 90 days of treatments.

Item	0-30 day				30-60 day				60-90 day			
	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

Data are presented as the mean and their SEM. Superscript letters indicate significant differences between the groups by Duncan's test. *p <0.05, **p <0.001. CON: basal diet; GPA: basal diet supplemented with 0.5 % of COLI-ZIN; PRO: basal diet supplemented with 0.1% of *Bifidobacterium animalis* ssp lactis.

Table 3. Effects of *B. animalis*, growth promoter antibiotic and control diet on egg quality characteristics of laying hens at the end of experimental period.

Items	CON	GPA	PRO	SEM	<i>p</i> -value
Egg width (mm)	43.8	43.7	43.9	2.27	0.89
Egg length (mm)	57.6	57.8	57.6	3.94	0.10
Egg surface (cm ²)	75.5	72.7	73.6	0.42	0.50
EQI	116.3	114.1	116.3	0.83	0.48
Shape index (%)	76.1	75.6	76.3	0.31	0.69
Shell thickness (mm)	0.30	0.35	0.32	0.01	0.28
Air cell height (mm)	1.5 ^a	1.6 ^a	0.9 ^b	0.14	0.04
Thick albumen (mm)	82.9 ^a	83.8 ^a	103.1 ^b	2.77	0.0003
Thin albumen (mm)	61.1 ^a	66.3 ^a	77.8 ^b	2.28	0.002
Ali	0.14	0.13	0.14	0.002	0.45
Yolk volume (cm ³)	16.6 ^a	17.4 ^a	13.7 ^b	0.56	0.01
L	64.3	65.1	66.2	0.58	0.43
a	-0.9	-0.7	-1.0	0.37	0.84
b	32.1 ^a	28.6 ^{ab}	25.5 ^b	1.09	0.03

Note: Data are presented as the mean and their SEM (n=6). Superscript letters indicate significant differences between the groups by Duncan's test ($p < 0.05$). CON: basal diet; GPA: basal diet supplemented with 0.5 % of COLIZIN; PRO: basal diet supplemented with 0.1% of *Bifidobacterium animalis* ssp lactis. EQI: Egg Quality Index. Ali: Improved Albumen Index.

Table 4. Effects of *B. animalis*, growth promoter antibiotic supplementation and control diet of relative weight and pH of various organs of the laying hens at 26 weeks age.

Organ	Treatments				<i>p</i> value
	CON	GPA	PRO	SEM	
Gastrointestinal tract					
Crop					
Index (% BW)	0.54 ^{ab}	0.55 ^a	0.61 ^b	0.03	0.045
pH	6.13 ^a	5.41 ^b	5.37 ^b	0.10	0.001
Proventriculus					
Index (% BW)	0.54	0.55	0.45	0.03	0.420
pH	5.68	5.81	5.48	0.06	0.115
Gizzard					
Index (% BW)	1.48 ^a	1.24 ^b	1.21 ^b	0.04	0.028
pH	5.63	5.5	5.3	0.07	0.229
Duodenum					
Index (% BW)	1.45 ^a	1.25 ^b	1.46 ^a	0.03	0.040
pH	5.92	5.98	5.95	0.03	0.829
Jejunum					
Index (% BW)	1.05	0.87	0.92	0.05	0.343
pH	6.03	6.04	5.83	0.04	0.105
Ileum					
Index (% BW)	0.73	0.63	0.62	0.03	0.442
pH	6.03	6.16	6.05	0.04	0.513
Large intestine					
Index (%)	1.16 ^a	1.14 ^a	1.42 ^b	0.04	0.014
pH	6.22	6.34	6.26	0.03	0.455
Ceca					
Index (% BW)	0.56	0.44	0.53	0.02	0.129
pH	6.52	6.52	6.6	0.03	0.547
Visceral organs					
Index (% BW)					
Heart	0.48	0.51	0.46	0.00	0.164
Liver	2.60	2.60	2.41	0.07	0.518

Spleen	2.60	2.60	2.41	0.00	0.521
Kidney	0.60	0.58	0.53	0.02	0.571
Lung	0.42	0.39	0.40	0.01	0.602
Brain	0.13	0.13	0.13	0.00	0.988
Reproductive organs					
Ovary					
Index (% BW)	2.57	3.01	2.71	0.17	0.731
pH	6.42	6.32	6.51	0.03	0.139
Infundibulum					
Index (% BW)	0.14	0.16	0.12	0.01	0.398
pH	6.35 ^a	6.45 ^{ab}	6.51 ^b	0.02	0.032
Magnum					
Index (% BW)	1.79	1.93	2.38	0.11	0.276
pH	6.28	6.28	6.47	0.04	0.122
Isthmus					
Index (% BW)	0.41	0.45	0.48	0.01	0.742
pH	6.12 ^a	6.24 ^{ab}	6.32 ^b	0.04	0.004
Uterus					
Index (% BW)	1.08	1.06	0.89	0.04	0.178
pH	6.30	6.24	6.23	0.02	0.636
Vagina					
Index (% BW)	0.13	0.17	0.15	0.02	0.326
pH	6.31	6.35	6.4	0.03	0.523

Data are presented as the mean $\pm$ SEM. Index (%): organ weight/ body weight. Superscript letters indicate significant differences between the groups by Duncan's test ($p < 0.05$). CON: basal diet (n=10); GPA: basal diet supplemented with 0.5 % of COLI-ZIN (n=10); PRO: basal diet supplemented with 0.1% of *Bifidobacterium animalis* ssp lactis (n=10).

Figures

Figure 1

Scanning electron microscopy micrographs of the *Bifidobacterium animalis* sub. Lactis.

Figure 2

Effects of dietary treatments on total WBC count, heterophile (H), lymphocyte (L), and heterophile/lymphocyte ratio (H/L) of laying hens at 90 days of the experimental period.