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Probiotic and synbiotic in broiler diet: performance and *Enterobacteriaceae*

[*Probiótico e simbiótico em ração para frangos: desempenho e Enterobacteriaceae*]

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ABSTRACT

The objective of this study was to evaluate the effects of probiotics and synbiotics on the performance and *Enterobacteriaceae* count of broiler chickens. A total of 640 one-day-old male broiler chicks were distributed in a completely randomized design with four treatments and eight replicates with 20 birds each. The treatments were: ration with performance enhancer (zinc bacitracin; positive control); ration without performance enhancer and probiotic/synbiotic (negative control); ration with probiotics; and ration with synbiotics. At 35 days, five birds from each treatment were euthanized and intestinal contents were harvested for determining the *Enterobacteriaceae* count. The performance data and average colony-forming units (CFUs) transformed as log CFU/g were subjected to analysis of variance and Tukey's test. The effects of probiotics and synbiotics were observed in the initial phase, with supplemented birds exhibiting comparable weight gain to those supplemented with bacitracin. No effect of the treatment on broiler performance was observed after 42 days. The enterobacterial count was comparable among all experimental treatments. Supplementation with probiotics and synbiotics did not compromise the performance of broilers and did not alter the *Enterobacteriaceae* count.

Keywords: bacitracin, *Escherichia coli*, microbiota

RESUMO

Objetivou-se avaliar o efeito do probiótico e do simbiótico sobre o desempenho e a contagem de Enterobacteriaceae *em frangos. Foram utilizados 640 pintos de corte, machos, de um dia de idade, distribuídos em delineamento inteiramente ao acaso, com quatro tratamentos, oito repetições com 20 aves cada. Os tratamentos foram: ração com melhorador de desempenho (bacitracina de zinco) (controle positivo); ração sem melhorador de desempenho e sem probiótico/simbiótico (controle negativo); ração com probiótico e ração com simbiótico. Aos 35 dias, cinco aves por tratamento foram eutanasiadas para retirada de conteúdo intestinal e determinação de* Enterobacteriaceae. *As médias das unidades formadoras de colônias, transformadas em log/UFC/g, e de desempenho foram submetidas à análise de variância e comparadas pelo teste Tukey. Foi observado efeito do probiótico e do simbiótico na fase inicial, sendo que aves apresentaram os mesmos resultados de ganho de peso e de peso corporal que o grupo de aves alimentado com bacitracina. Aos 42 dias, não houve efeito dos tratamentos sobre o desempenho. Aves que não receberam nenhum aditivo não apresentaram maior contagem de enterobactérias, sendo semelhantes aos demais tratamentos. A adição do probiótico e do simbiótico não compromete o desempenho dos frangos e não altera a contagem de* Enterobacteriaceae.

Palavras-chave: bacitracina, Escherichia coli*, microbiota*

INTRODUCTION

The use of antimicrobials has provided better feed conversion and weight gain in poultry for more than 50 years. However, because of the emergence of resistant bacteria, the number of antimicrobials permitted for use in animal husbandry has become increasingly limited.

Colistin sulfate was banned in 2016, followed by a more recent prohibition of tylosin, lincomycin, and tiamulin, by the Brazilian Ministry of Agriculture.

Thus, with the decreasing number of permitted antimicrobials, it has become necessary to study alternative additives that can achieve similar results, maintain intestinal health and immune system of birds, and improve nutrient utilization without inducing bacterial resistance, particularly probiotics and prebiotics (Wang *et al.*, 2016). Probiotics consist of one or more live microorganisms that beneficially affect the endogenous microbiota and may include species of *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and yeast (Al-Khalaifa *et al.*, 2019), whereas prebiotics are defined as ingredients that are non-digestible by animals and serve as substrates for beneficial bacteria. The combination of probiotics and prebiotics yields is called synbiotics, e.g., fructooligosaccharides (FOS) combined with bifidobacterial, which exert a synergistic effect and enhance bird performance, without leaving residues in the carcass (Aziz-Mousavi *et al.*, 2015; Beski and Al-Sardary, 2015; Reis and Vieites, 2019).

The addition of probiotics to chicken feed ameliorates feed conversion and body weight, which may be attributed to higher absorption of nutrients and improved bird performance and immunity, as reported by Royan (2018) using the probiotic species *Enterococcus faecium*. He *et al.* (2019) reported better performance and greater production of immunoglobulins in broilers fed diets containing *Bacillus subtilis*. Similarly, Ramlucken *et al.* (2020) reported that *B. subtilis* and *Bacillus velezensis* produce exogenous enzymes, improving nutrient utilization and feed conversion in supplemented animals.

Similar to probiotics, synbiotics can improve nutrient absorption because of the contained prebiotics, as stated by Aziz-Mousavi *et al.* (2015), who used a synbiotic combination of *E. faecium* and fructooligosaccharides in chicken feed and noted greater weight gain, better feed conversion, and higher protein consumption in supplemented birds. Cheng *et al.* (2017), Shokri *et al.* (2017), and Kridtayopas *et al.* (2019) also reported better bird performance on addition of synbiotics to chicken diet.

In addition, probiotics and synbiotics promote multiplication of beneficial bacteria to the detriment of pathogenic bacteria, as demonstrated by Fonseca *et al.* (2010) and Zhang *et al.* (2014). Śliżewska *et al.* (2020) reported higher numbers of *Bifidobacterium* spp. and *Lactobacillus* spp., and lower numbers of *Clostridium* spp. and *Escherichia coli* in the intestine of broilers supplemented with synbiotics. Kridtayopas *et al.* (2019), Mora *et al.* (2019), and Chen and Yu (2020) also reported an improvement in the population of beneficial bacteria along with pathogen control by synbiotics.

Ateya *et al.* (2019) observed that synbiotics combined with organic acids weakened the inflammatory response in the intestine of chickens challenged with *E. coli*, resulting in better performance, and lower mortality and bacterial elimination in the environment. In summary, replacement of performance enhancers by pro- and synbiotics has varying effects on the performance and intestinal microbiota of poultry. The best response depends on several factors, such as product composition, microorganism survival, dosage, and the challenge or stress conditions to which birds are exposed (Reis and Vieites, 2019; Shanmugasundaram *et al.*, 2019; Chen and Yu, 2020). Therefore, the objective of this study was to determine the effects of probiotic and synbiotics on the performance and *Enterobacteriaceae* count of chickens challenged with reused litter and untreated water.

MATERIAL AND METHODS

The experiment was approved by the Animal Use Ethics Commission (CEUA)- IF Goiano, under protocol number 7572161018. Experiments were performed using 640 one-day-old male Cobb broiler chicks (40.0 ± 4.0g) distributed in four treatment groups, with eight replicates each and 20 birds per replicate, following a completely randomized design. The experimental diets (Table 1) were formulated according to the recommendations of Rostagno *et al.* (2017) based on the nutritional requirements during the different breeding phases, and consisted of pre-starter (1 to 7 days), starter (8 to 21 days), grower (22 to 35 days), and finisher (36 to 42 days) diets.

The addition of the probiotics or synbiotics were added based on the manufacturer's recommendations, and the treatments were

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designed as follows: Treatment 1 - feed with performance enhancer (zinc bacitracin; positive control); Treatment 2 - feed without performance enhancer and probiotic\synbiotic (negative control); Treatment 3 - feed supplemented with probiotic (150g/ton feed). Treatment 4 - feed supplemented with synbiotic (100g/ton of feed).

The probiotics added in Treatment 3 included the following species, expressed in colony-forming units (CFU)/g: *Lactobacillus plantarum* (1.26×10^8 CFU/g), *Lactobacillus bulgarius* (2.06×10^8 CFU/g), *Lactobacillus acidophilus* (2.06×10^8 CFU/g), *Lactobacillus rhamnosus* (2.06×10^8 CFU/g), *Bifidobacterium bifidum* (2.00×10^8 CFU/g), *Streptococcus thermophilus* (4.10×10^8 CFU/g), and *E. faecium* (6.46×10^8 CFU/g), whereas the added synbiotic (Treatment 4) was composed of anaerobic bacteria (10^4 CFU/g), lactic acid-producing bacteria (10^4 CFU/g), and mannan-oligosaccharides (370g/kg). The bacterial species included in the synbiotic were not specified by the manufacturer.

Table 1. Percentage and calculated composition of diets for the different breeding phases

Ingredients	Pre-Starter	Starter	Grower	Finisher
Corn	56.119	59.916	62.429	67.311
Soybean meal, 45%	37.619	34.254	31.491	27.174
Soybean oil	2.165	2.239	2.926	2.716
Bicalcium phosphate	1.914	1.505	1.255	1.040
Limestone	0,816	0.840	0.809	0.731
DL-Methionine 98%	0.364	0.300	0.255	0.239
L-lysine HCL 99%	0.339	0.233	0.193	0.237
Salt (NaCl)	0.300	0.400	0.350	0.350
L-Threonine 99%	0.122	0.071	0.050	0.052
Vitamin premix[1]	0.100	0.100	0.100	0.100
Mineral premix[2]	0.050	0.050	0.050	0.050
Anticoccidian[3]	0.055	0.055	0.055	-------
Zinc bacitracin	0.037	0.037	0.037	-------
Total	100.0	100.0	100.0	100.0
Nutritional composition				
Metabolizable energy (kcal/kg)	2.950	3.000	3.100	3.150
Crude protein (%)	22.20	20.80	19.50	18.00
Calcium (%)	0.920	0.819	0.732	0.638
Available phosphorus (%)	0.470	0.391	0.342	0.298
Available Lysine (%)	1.333	1.174	1.078	1.01
Available Methionine + Cystine (%)	0.944	0.853	0.787	0.737
Available Methionine (%)	0.647	0.571	0.519	0.485
Available Threonine (%)	0.852	0.763	0.701	0.656

[1]Vitamin premix containing: Folic acid (min) 700mg, Pantoathenic Acid (min) 8000mg, Biotin (min) 60mg, Niacin (min) 30g, Selenium (min) 400mg, Vit. A (min), 8.000.000 IU, Vit. B1 (min) 3000mg, Vit. B12 (min) 10.000 cg, Vit. B2 (min), 4,000mg, Vit. B6 (min) 2000mg, vit. D3 (min) 2.000.000 IU, Vit. E, (min) 15.000 IU, Vit. K3 (min) 2000mg. [2] Mineral pre-mix containing: zinc, (min) 125g copper (min) 12,6g. Iodine, (min) 2,.520mg. Iron (min) 105g. Manganese (min) 126g. [3] Anticoccidian: salinomycin sodium (min) 12g.

Birds were challenged by reusing the same litter without treatment, cleaning pendular drinkers only once every week, and offering water without chlorine treatment. The performance of the birds was evaluated weekly and at the end of each phase. Feed consumption, weightgain, feed conversion, and final weight were measured. At 35 days of age, five birds from each treatment were euthanized by cervical dislocation and the number of bacteria (*Enterobacteriaceae*) in the small intestine was determined.

The intestinal contents were harvested, homogenized, and pooled to form a composite sample. Subsequently, 0.5g of the sample was transferred to test tubes containing 4.5mL of 0.1% sterile buffered saline, resulting in a 10^{-1} dilution, and successive dilutions up to 10^{-4} were prepared. To determine the number of enterobacteria,

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0.1mL each of the 10^{-2} and 10^{-4} dilutions was placed into duplicate Petri dishes containing MacConkey agar. The averages of the CFUs were transformed into log CFU\g and submitted, as well as the performance data, to an analysis of variance and compared pairwise using Tukey's test at 5% significance, using the statistical program R.

RESULTS AND DISCUSSION

The diets with the addition of probiotics and synbiotics provided broilers with similar weight gain and final weight results as those fed diets containing zinc bacitracin (Table 2).

Table 2. Performance of Cobb broilers supplemented with probiotics and synbiotics from day 1 to 21

Treatment	Body weight gain (kg)	Feed intake (kg)	Feed conversion ratio	Body weight (kg)
Positive control	0.966[a]	1.509	1.499	1.006 [a]
Negative control	0.914[b]	1.456	1.525	0.954[b]
Probiotic	0.969[a]	1.501	1.486	1.010[a]
Synbiotic	0.963[a]	1.495	1.490	1.003[a]
C.V* (%)	3.24	4.07	3.21	3.11
P value	0.003	0.337	0.3897	0.003

*coefficient of variation. Values with different superscript letters within a column are significantly different (5%, Tukey's test).

It is thus evident that the alternative additives did not compromise the weight of broilers in the starter phase, as opposed to broilers that did not receive any additives, which presented lower body weight. This is consistent with the results of He *et al*. (2019), Min *et al*. (2016), Ramlucken *et al*. (2020), and Zang *et al*. (2014), who reported beneficial effects of probiotics and synbiotics on the performance of broilers in the starter phase. It must be noted that the imposed sanitary challenge, i.e., reusing litter without treatment, only weekly cleaning of pendular drinkers, and providing water without chlorine, affected birds in the starter phase, which represents the period when the animal immune system is still developing.

The reused litter without treatment may contain numerous microorganisms and pathogens. Roll *et al*. (2011) emphasized the importance of litter treatment for controlling *Salmonella* spp. Vieira *et al*. (2015) detected *E. coli* in reused litter samples, whereas Wang *et al*. (2016) identified several genera of bacteria in reused chicken litter, including *Escherichia*, *Staphylococcus*, and *Corynebacterium*.

Regarding water quality, it is common practice to provide chlorinated water and to clean pendular drinkers at least twice every day. Amoroso *et al*. (2015) reported a higher number of coliforms and *E. coli* in unfiltered water with possible fecal contamination. *E. coli* can result in poor performance of chickens, which can be prevented by supplementation with probiotic and synbiotics,

as these additives weaken the inflammatory response to bacteria (Ateya *et al.* 2019).

However, the sanitary challenge did not impair broiler performance, which may be due to the strengthening of the immune system by the added probiotics and synbiotics, as reported by Luoma *et al*. (2017), Al-Khalaifa *et al*. (2019), and He *et al*. (2019). Zhang *et al*. (2014) challenged 7-day-old chicks with *E. coli* and reported higher immunoglobulin production in birds fed diets containing probiotics based on *Clostridium butyricum*, 14 days after pathogen challenge, and attributed the better performance of birds to said immunological reaction.

The better performance of birds in the starter phase may be attributed to improved nutrient utilization on supplementation with probiotics and synbiotics because some microorganisms used as probiotics, such as *Bacillus* spp., produce exogenous enzymes that promote degradation and more efficient use of nutrients. This was reflected in the higher feed conversion and greater weight gain of birds at 21 days of age, which was consistent with the results of He *et al*. (2019) and Ramlucken *et al*. (2020). During the entire breeding period, i.e., from day 1 to 42 (Table 3), the performance of birds fed diets without antimicrobial supplementation (Treatment 2) and with added probiotics and synbiotics (Treatments 3 and 4) was comparable to that of birds supplemented with a conventional antimicrobial (zinc bacitracin; Treatment 1).

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Table 3. Performance of Cobb broilers supplemented with probiotics and synbiotics from day 1 to 42

Treatment	Body weight gain (kg)	Feed intake (kg)	Feed conversion ratio	Body weight (kg)
Positive control	2.837	4.747	1.649	2.877
Negative control	2.709	4.593	1.670	2.750
Probiotic	2.731	4.562	1.647	2.772
Synbiotic	2.761	4.669	1.666	2.801
C.V (%)	4.25	6.06	6.64	4.18
P value	0.169	0.564	0.947	0.172

This was consistent with the results of Souza *et al.* (2018), who did not observe greater weight gain or better feed conversion in chickens supplemented with probiotics containing *B. subtilis*, *Bifidobacterium bifidum*, and *E. faecium* for 42 days. According to these authors, the sanitary challenge (reused litter with treatment and cleaning of drinking fountains every 2 days) to which birds were subjected may not have been sufficient to verify the beneficial effects of probiotics. However, birds were exposed to a more intense sanitary challenge in the present study, in the form of reused litter without treatment and cleaning of the pendular drinkers only once per week, which resulted in lower weight and poor feed conversion after 42 days.

Mora *et al.* (2019) did not observe any effect of synbiotic administration via water on feed conversion and weight gain of broilers challenged with *Salmonella* Typhimurium and *Clostridium perfringens* after 42 days. The authors attributed the differences in the results of probiotic- and synbiotic supplementation to the condition of the experimental birds, species included in the probiotic, and the type of fiber contained in the prebiotic.

In general, our study presented satisfactory results for probiotics and synbiotic supplementation. During both the starter phase and the total breeding period, the performance of broilers supplemented with the alternative additives was comparable to that of birds that received zinc bacitracin, which is a traditional antimicrobial for poultry. It should be noted that when considering the performance of birds supplemented with probiotics or synbiotics, several factors, such as probiotic composition, dosage, route of administration, and type and viability of microorganisms in the gastrointestinal tract, must be taken into account in addition to the imposed health challenge, as emphasized by Mora *et al.* (2019), Reis and Vieites (2019), and Shanmugasundaram *et al.* (2019).

The treatments did not affect the total *Enterobacteriaceae* count in the small intestine (Table 4). A higher *Enterobacteriaceae* count was expected in birds that did not receive any additives in the feed because both zinc bacitracin and probiotics can decrease the population of enterobacteria, coliforms, and *E. coli* in the intestine, as reported by Engberg *et al.* (2000), Fonseca *et al.* (2010), Boostani *et al.* (2013), and Carrasco *et al.* (2018).

In addition, birds subjected to the sanitary challenge likely ingested a greater amount of coliforms, because of the poor quality of the water provided (without chlorine and containing organic matter and/or excreta because of unclean drinkers). According to Amoroso *et al.* (2015), untreated and poor-quality water contains a higher number of coliforms, including *E. coli*.

Table 4. *Enterobacteriaceae* count in the small intestine of Cobb broilers supplemented with probiotics and synbiotics for 35 days

Treatment	Second dilution log (CFU/g)	Fourth dilution log (CFU/g)
Positive control	4.626	5.777
Negative control	3.742	5.595
Probiotic	4.047	5.369
Synbiotic	4.260	5.675
C.V. (%)	16.96	12.09
P value	0.284	0.805

However, the additives did not alter the *Enterobacteriaceae* population, which was consistent with the observations of Sherief *et al.* (2012), who did not report any effect of probiotics and synbiotics on the total count of coliforms, *Escherichia coli*, or aerobic bacteria. Our results were also in agreement with those of Torok *et al.* (2011), who did not observe any change in the enterobacterial population on supplementation with zinc bacitracin. Moreover, Mora *et al.* (2019) and Śliżewska *et al.* (2020) did not report any

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change in the *Enterobacteriaceae* count in the intestine of broilers supplemented with synbiotics. Conversely, Dibaji *et al.* (2014) reported a reduction in the *E. coli* and total coliform count in the cecum of broilers supplemented with synbiotics (*E. faecium* and fructooligosaccharides). Ateya *et al.* (2019) noted a reduction in the lipopolysaccharide receptor (TLR4), associated with the inflammatory process, in the intestine of chickens supplemented with probiotic and synbiotics, indicating a lower inflammatory response on challenge with *E. coli.*

In contrast, the use of probiotics or synbiotics has been reported to alter the microbiota in terms of the numbers of beneficial gram-positive bacteria, such as *Lactobacillus* sp. and *Bacillus* sp., which were shown to multiply and produce acids, thus lowering the intestinal pH and likely controlling pathogenic microorganisms (Sherief *et al.*, 2012). Chen and Yu (2020) analyzed the fecal microbiota of chickens supplemented with *Bacillus licheniformis* and noted a greater amount of *Lactobacillus* on probiotic addition to feed (3g/kg), demonstrating a positive correlation between the additive and higher body weight of birds.

Synbiotic supplementation significantly increased the lactic acid level and total short chain fatty acids (i.e., acetic, propionic, valeric, butyric, and formic acids). Short chain fatty acids play important roles in pH regulation, increase calcium, iron, and magnesium absorption, and positively affect hepatic metabolism of glucose and proteins. These fatty acids are very important in the maintenance of the structure, function, and integrity of the intestine for inhibiting *Salmonella*, *E. coli*, and *Campylobacter (*Śliżewska *et al.*, 2020).

According to He *et al.* (2019) and Ramlucken *et al.* (2020), the probiotic composition is important because certain bacteria, such as *B. subtilis*, *B. velezensis*, and *B. licheniformis*, are aerobic and provide a suitable environment for lactobacilli and bifidobacteria under anaerobic conditions, which subsequently produce acids and lower the intestinal pH, preventing the growth of pathogenic microorganisms. *Bacillus*, *Lactobacillus*, and *Bifidobacterium* aregram-positivegenera and were not investigated in the present study because the culture medium used, i.e., MacConkey agar, specifically selects gram-negative bacteria.

The results of the present study and those of other studies on the effect of additives on enteric microbiota evidence the microorganismal diversity and indicate that changes in the enterobacterial population depend on multiple factors. Thus, the handling of litter, food offered, type and composition of the probiotics\synbiotics, method of enterobacterial determination, included microorganisms, and intestinal location for the *Enterobacteriaceae* count must be considered. Furthermore, it is important to test new probiotic strains, including some fungal species, for potential poultry applications.

CONCLUSION

The addition of probiotics and synbiotics did not compromise the performance of broilers and did not alter the intestinal *Enterobacteriaceae* count.

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