Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

ISSN 1516-635X 2023 / v.25 / n.1 / 001-010

http://dx.doi.org/10.1590/1806-9061-2021-1608

Original Article

# Performance, Gut Integrity, Enterobacteria Content in Ceca of Broiler Fed Different Eubiotic Additives

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■Keywords

Antibiotics, Bacteriophages, Probiotic, Symbiotic, Poultry Nutrition.



Submitted: 06/December/2021
Approved: 01/November/2022

## ABSTRACT

An experiment was carried out to study the effect of different eubiotics on productive characteristics, intestinal integrity, as well as the content of enterobacteria in the cecum of broiler chickens. A completely randomized design with five treatments and 8 replicates of 25 birds each was used. In total 1000 mixed broiler chickens from Ross308 strain, one day old were obtained from a commercial hatchery. The birds were housed on concrete floors in a conventional house. A sorghum+soybean meal control diet was used, to which the additives under study were added. The treatments were distributed as follows: T1 = Control diet without antibiotic or eubiotic; T2 = T1 + bacteriophages; T3 = T1 + antibiotic; T4 = T1 + probiotic; T5 = T1 + symbiotic. The results obtained at 49 days of age for weight gain and feed conversion rate improved ($p$<0.05) with the addition of the antibiotic and eubiotics. A lower ($p$<0.05) intestinal density was observed with the probiotic. The height, width, and area of villi in duodenum was higher ($p$<0.05) when antibiotic and eubiotics were included. In the histological score, in duodenum, the antibiotic and eubiotics resulted with a higher score ($p$<0.05), associated to a physiological and controlled inflammation response that allowed improving productivity. Finally, the relative expression of enterobacteria, such as *Lactobacillus salivarius*, allowed associating positive changes in the microbiome and better productive parameters when including the symbiotic, with comparable results to the antibiotic when including the eubiotics.

## INTRODUCTION

Currently, the development of feed additives as alternatives to the use of antibiotic growth promoters (AGP) in broiler diets and other productive species is still under investigation. Although several studies show that it is complicated to match the productive and economic results obtained with the use of antibiotics, the implications of continuing to use them are also known; environmental contamination, risks to aquatic organisms and, of course, antimicrobial resistance, which implies a global challenge in the control of infectious diseases (Sethiya, 2016; Al-Khalaifah, 2018; Oviedo-Rondón, 2019; Selaledi *et al.*, 2020). The topic of intestinal health has maintained interest in those additives classified as biomodulators of the intestinal microbiota or also called eubiotics, derived from eubiosis, understood as a balance of the intestinal microbial ecosystem (Iebba *et al.*, 2016; Oviedo-Rondón, 2019) and that their use promotes among other things animal welfare and food safety (Sethiya, 2016; Oviedo-Rondón, 2019). Some of these additives have been known for several decades, such as probiotics, prebiotics, organic acids, phytobiotics, enzymes (Caly *et al.*, 2015; Sethiya, 2016) and recently the use of new commercial alternatives, such as bacteriophages. Among them, probiotics and symbiotics (probiotic+prebiotic), are

widely used as AGP alternatives in poultry production, for its low production cost (Oviedo-Rondón, 2019). These additives promote the proliferation of desirable bacteria in the gut by competitive exclusion, competing for nutrients, immunomodulation, production of antimicrobial compounds and of course, growth of the probiotic organism, by providing a substrate available to the probiotic fermentation in case of the prebiotic (Roberts *et al.*, 2015; Sethiya, 2016; Bajagai *et al.*, 2016; Oviedo-Rondón, 2019). As for bacteriofages, models have already been used to investigate the dynamics of the phage-bacteria ecosystem (killing, lysogenization, passage of the bacteriophage from one strain to another),as described by De Paepe (2014). However, despite the research done on these alternatives there is much to be understood and tested regarding their effects on intestinal health and integrity, by reduction of the inflammatory response and better immune response against pathogenic bacteria, without affecting productive performance (M'Sadeq *et al.*, 2015; Sethiya, 2016; Tarradas *et al.*, 2020). Therefore, within eubiotic nutrition, what is sought is the combination of additives for each circumstance, which allows promoting the presence of a balanced and "healthy" intestinal microbiome (Yasar *et al.*, 2017) as well as the integrity of the mucosal barrier, optimization of gut morphology and digestibility, reduction in nutrient excretion and intestinal immunomodulation that help control inflammation (Oviedo-Rondón, 2019; Tarradas *et al.*, 2020) and prevent the transition from physiological to pathological inflammation, which normally reduces productivity. Therefore, the present study was conducted to evaluate the effect of adding eubiotics to the diet of broilers on productive performance, carcass characteristics, intestinal integrity, and the count of some enterobacteria in ceca.

## MATERIALS AND METHODS

The research was carried out at the Center for Teaching, Research, Extension and Poultry Production of the Faculty of Veterinary Medicine and Zootechnics of the UNAM. In total 1000 mixed broiler chickens from Ross308 strain, one day old were obtained from a commercial hatchery. All chicks were received with an average initial weight of 43 g, vaccinated against Gumboro-Marek's disease and later against Newcastle disease at 10 days of age. The birds were randomly distributed in 40 compartments; they were housed for 7 weeks in a natural environment house with cement floor pens, wood-shavings litter, with a density of 10 birds/$m^2$ and tunnel breeding. They were kept under a natural light program, with an average of 11 hours of light per day. All animal care and technical procedures were approved by the Institutional Subcommittee for the Care and Use of Experimental Animals (protocol DC-2018/2-5) of the faculty mentioned above.

### Experimental design and diets

A completely randomized design was used with five treatments with 8 replicates of 25 birds each. A sorghum+soybean meal control diet was used to which the additives under study were added, considering 3 feeding phases: Initiation (1-21 days of age), Growing (22-35 days of age) and Finishing (36-49 days of age). The treatments were distributed as follows: T1 = control diet without antibiotic or eubiotic; T2 = T1 + bacteriophage (500 g/Ton); T3 = T1 + antibiotic (300 g/Ton); T4 = T1 + probiotic (100 g/Ton); T5 = T1 + symbiotic (500 g/Ton). The composition of the experimental control diet is shown in Table 1.

**Table 1 –** Composition of the control diet used in the experiment.

| | Initiation | Growing | Finishing |
|---|---|---|---|
| | 1 - 21 d | 22 - 35 d | 36 - 49 d |
| Ingredient | 21% | 19% | 17% |
| Sorghum | 583.0 | 624.15 | 675.31 |
| Soybeanmeal | 328.7 | 277.50 | 226.80 |
| Vegetable oil | 35.08 | 45.71 | 48.23 |
| Ortophosphate | 18.79 | 16.61 | 15.41 |
| Calcium carbonate | 16.79 | 14.01 | 13.47 |
| Salt | 4.350 | 3.840 | 3.860 |
| DL-Methionine 80% | 4.130 | 4.005 | 3.610 |
| L-Lysine HCl 78% | 4.110 | 3.460 | 2.940 |
| L-Threonine | 1.900 | 1.560 | 1.220 |
| Cholinechloride + vit*/min** | 2.500 | 2.500 | 2.500 |
| Nicarbazin | 0.500 | 0 | 0 |
| Salinomicin | 0 | 0.500 | 0.500 |
| Antioxidant | 0.150 | 0.150 | 0.150 |
| Pigment | 0 | 6.00 | 6.00 |
| Total (kg) | 1000 | 1000 | 1000 |
| Nutrientcomposition | | | |
| ME, Kcal/kg | 2988 | 3176 | 3176 |
| Protein % | 21.00 | 18.00 | 17.00 |
| Lysine % | 1.22 | 1.04 | 0.99 |
| Met+Cis % | 0.91 | 0.82 | 0.78 |
| Threonine % | 0.93 | 0.72 | 0.71 |
| Tryptophan % | 0.27 | 0.23 | 0.22 |
| Arginine % | 1.34 | 1.13 | 1.03 |
| Calcium % | 1.05 | 0.90 | 0.85 |
| Phosphorus disp.. % | 0.50 | 0.45 | 0.42 |
| Na % | 0.22 | 0.19 | 0.18 |
| Cl % | 0.20 | 0.20 | 0.20 |

*Vitamin A (12,000,000 IU), vitamin D3 (2,500,000 UIP), vitamin E (15,000 IU), vitamin K (2.0g), vitamin B1 (2.25g), vitamin B2 (7.5g), vitamin B6 (3.5g), vitamin B12 (20mg), folicacid (1.5g), biotin (125mg), pantothenicacid (12.5g), niacin (45g); **Iron (50g), zinc (50g), manganese (110g), copper (12g), iodine (0.30g), selenium (0.20g), cobalt (0.20g).

Water and feed were supplied *ad libitum*. All additives were commercial products in powder form and were added to the diets at the levels recommended by the manufacturers. The bacteriophage-containing product was obtained from an additive manufacturing company (CTC Bio Inc.) that includes a cocktail of lyophilized bacteriophages specific for *Salmonella enterica* serovars Typhimurium, Enteritidis, Cholerasuis and Derby, *Staphylococcus aureus*, *Escherichia coli* (k88, k99 and f41) and *Clostridium perfringens* type A and C. The titers of each bacteriophage in the bacteriophage cocktail are $10^9$ pfu/g cocktail. The antimicrobial product was bacitracin-zinc and was added at 30 ppm. The probiotic contained $10^7$ CFU/g of *Bacillus Subtilis* (BaymixGrobig®). The symbiotic product (PoultryStar® ME, BIOMIN), consisted of the sum of multispecies probiotic ($1.3x10^{11}$ CFU/g *Enterococcus faecium*, $5.0x10^{10}$ CFU/g *Pediococcusacidilactici*, $2.1x10^{10}$ CFU/g *Bifidobacterium animalis*, $5.0x10^9$ CFU/g *Lactobacillus reuteri*, $5.0x10^9$ CFU/g *Lactobacillus salivarius*) and a prebiotic (Inulin).

## Performance and Carcass parameters

At the end of each week, the body weight gain (WG) and feed intake (FI) of the birds of each treatment was obtained, as well as the feed conversion ratio (FCR) and percentage of general mortality. At 49 days of age, 26 birds per treatment were selected, identified, weighed, and slaughtered after an 8-hour fast. Each bird was subjected to the slaughter protocol of the processing plant of the aforementioned center: 1) hanging and electrically stunned, under the parameters of 25 V, 0.25 A and 460 Hz of direct current, pulsed type; 2) the slaughter was performed by unilateral neck cutting in order to be bled out for2 minutes; 3) scalded in water at 53°C for one minute; 4) mechanical plucking and manual evisceration. The weights of the carcass, abdominal fat, breast muscle (*Pectoralis major*) and legs (thighs and drumsticks) were obtained. For each case, the yields of carcass, abdominal fat and primal cuts were determined on a live weight basis. Skin pigmentation (yellowness) was measured in the lateral apterium region, in the live chicken and in the carcass, using a Minolta CR-400 reflectance colorimeter.

## Histological preparation

At 49 days, from each treatment, one chicken per replicate (8 birds per treatment) was selected and the intestine was dissected entirely. Intestinal segment samples approximately 2 cm long were obtained from the duodenum (the midpoint of the pancreatic loop). All samples were washed with 0.9% saline and fixed in 10% neutral-buffered formalin solution for a minimum period of 24 hours at 4°C; they were subsequently processed by routine histological techniques (Laudadio *et al.*, 2012); paraffin embedding and cross-sectioning of the segments at 5 µm thickness. Subsequently, two stains were used; hematoxicillin-eosin (HE) and Alcianblue (AB)/periodic acid-Schiff (PAS); HE staining was used for gut morphological measurements and ISI histological analysis, while PAS-AB staining was used in the goblet cell counts of duodenum in accordance to Setiawan *et al.* (2018). Ten well-oriented villi and 10 crypts of Lieberkühn were measured per cross-section of each intestinal segment. All the observations and measurements were performed with an optical microscope (LEICA MC170HD®).

## Intestinal morphometry measurement

The small intestine (from the end of the gizzard to 1 cm above the ileocecal junction) of each bird was excised and weighed. The length of the small intestine was obtained with a tape measure. Intestinal mass per unit of length defined as "intestinal density" was calculated as the ratio between absolute weight in grams and length in centimeters of the small intestine (g/cm) according to Alshamy *et al.* (2018) and Riahi *et al.* (2020). In the duodenum, the following morphometric variables were determined: height (µm) and width (µm) of intestinal villi, crypt depth (µm) and goblet cell count. Villus height (LV) was measured as the distance from the tip of the villus to the transition region of the crypt and villus. Measurement of intestinal crypt depth was taken from the base of the villus to the submucosa. The apparent villus surface area ($mm^2$) was calculated using the following formula: [(villus width at one-third + villus width at two-thirds) × $2^{-1}$× villus height] used by Laudadio *et al.* (2012). Villus:crypt ratio was calculated dividing villus height by crypt depth. The number of goblet cells per 100 intestinal epithelial cells in intestinal sections was determined (Sun *et al.*, 2013).

## Intestinal histological changes

Three histological parameters were evaluated (epithelial hyperplasia, goblet cell hyperplasia and inflammatory cell infiltration of the lamina propria by lymphocytes) that were adapted to that described by Kraieski *et al.* (2017), in their ISI methodology (I See Inside). In this methodology, an impact factor (IF) is defined for each histological alteration in the intestine, according to the reduction in functional capacity, considering 3 features with impact factor 1,

2 and 3: Epithelial cell hyperplasia (IF = 1), Goblet cell hyperplasia (IF = 2), Inflammatory cell infiltration (IF = 3). IF = 3 has the greatest impact on organ function. Likewise, the degree of intensity or frequency observed was considered, designated as score of the alteration in each tissue qualifying from 0 to 3: 0 (absence of alteration or frequency observed), 1 (alteration up to 25% of the area or frequency observed), 2 (alteration from 25 to 50% of the area or frequency observed) and 3 (alteration extended in more than 50% of the area or frequency observed). To obtain the final value of the ISI index, the IF of each alteration is multiplied by the respective score number, and the result of all alterations are summed according to the formula ISI = ∑ (IF*S), where IF = impact factor and S = Score. For our evaluation scale the total score ranges from 0 to 18.

## Protocol for DNA extraction in cecal content

At day 49, 8 chickens per treatment were sacrificed and cecal content samples were collected from each chicken. The samples were placed in sterile 2 ml eppendorf vials and refrigerated for a maximum of 1.5 h. then, were frozen at -80°C until DNA extraction, whose procedure was an adaptation of the method of Wilson (1997). For this, 200 mg of frozen cecal contents were suspended in 1 mL of PBS (phosphate buffered saline), homogenized, and centrifuged at 389 × g for 10 min at room temperature. The supernatant was discarded, and the pellet was suspended in 567 µL of TE (Tris/EDTA) buffer and 50 µL of lysozyme (10 mg/mL) was added and incubated at 37°C for 30 min. After this time, 30 µL of 10% SDS (sodium dodecyl sulfate) and 4 µL of proteinase K (20 mg/mL) were added and incubated for 1 h at 56°C. After this time, 100 µL of 5 M NACl was added followed by 100 µL of CTAB/NaCl solution and incubated for 10 min at 65°C, then 80 µL of chloroform/isoamyl alcohol was added and centrifuged at 19064 × g for 10 min. The supernatant was taken, leaving the interphase behind. The volume was equalized with phenol/chloroform/isoamyl alcohol (PCIA). After centrifugation (19064 × g for 10 min), three phases were obtained: organic phase, interphase and aqueous phase (supernatant with DNA). Only the aqueous phase was taken. 200 µL of chloroform was added, mixed and incubated for 10 min at room temperature. Centrifuged at 19064 × g for 15 min and the supernatant was recovered. Then 500 µL of cold isopropanol was added, mixed and incubated in freezing for 24 h. It was then centrifuged at 19064 × g for 10 min and the isopropanol was decanted. Two washes were made with 300 µL of cold 70% ethanol to remove residual CTAB, centrifuging at 9726 × g for 5 min. All centrifugations were performed on Thermo Scientific Heraus Primo R. The ethanol was removed, and the DNA pellet was allowed to dry at room temperature for 30 min. Finally, the pellet was suspended in 100 µL of DNase/RNase-Free Water and frozen until DNA concentration was quantified using a spectrophotometer (NanoDrop2000, Thermo Scientific).

## Real-time PCR procedure

A total volume of 20 µL of reaction mixture was prepared containing 10 µL of reagent (Kappa Sybr® Fast DNA polymerase), 0.8 µL of lyophilized primers (forward and reverse), 2 µL of DNA and 7.2 µL of DNase/RNase-Free Water. Each DNA sample was performed in triplicate and once all the reaction samples were prepared, they were tested using a Rotor Gene Q® thermal cycler for the DNA amplification process. The amplification conditions were set according to the instructions of the KappaSybr® reagent commercial kit: Enzyme activation, 95°C × 3 min, followed by 40 cycles of denaturation, 95°C × 1-3 sec and Extension, 60°C × 10 sec. Primer sequences are presented in Table 2. Ct values obtained from amplification, were used to analyze relative gene expression using the $2^{-\Delta\Delta Ct}$method (Livak & Schmittgen, 2001; Tan, 2014).

## Statistical analysis

From the information collected, productive performance, carcass characteristics and histological examination were statistically analyzed by one-way ANOVA. Comparison of means was performed using Tukey's test, considering a significance level of 1% and 5%. Variables expressed as percentage, were transformed to arcsine before the analysis. The Mann-Whitney statistical test was used when the data were not normally distributed. All the statistical analysis was performed using SPSS software (version 17.0, Chicago, IL, USA) with assistance from GraphPad Prism software (version 4.00; GraphPad Software, San Diego, CA).

**Table 2 –** Primer used in bacterial quantification.

| Microorganism | Primer sequence 5'-3' | Reference |
|---|---|---|
| *Clostridium perfringens* | F-TTACCTTTGCTGCATAATCCC<br>R-ATAGATACTCCATATCATCCTGCT | Whelan *et al.,* 2019 |
| *Escherichia coli* | F-GTGTGATATCTACCCGCTTCGC<br>R-AGAACGCTTTGTGGTTAATCAGGA | Faseleh et al, 2017 |
| *Lactobacillus salivarius* | F-GATCGCATGATCCTTAGATGAA<br>R-GCCGATCAACCTCTCAGTTC | Torok *et al.,* 2013 |

# RESULTS

## Productive performance and carcass characteristics

Regarding productive data at 49 days of age, a higher BWG and a lower FCR ($p$<0.01) were obtained in the chickens that received eubiotics and the antibiotic. Analyzing FI and percentage of mortality (Table 3), no significant differences ($p$>0.05) were found between treatments. In carcass characteristics (Table 4),broiler chickens fed the antibiotic showed a higher ($p$<0.05) carcass yield than control diet and the others were similar. No statistical differences ($p$>0.05) were found in the yield of breast muscle, thighs+drumsticks, and yellowness between treatments.

**Table 3 -** Effect of eubiotics on productive performance in broiler chickens at 49 days of age.

| Treatment | Feed intake (g) | Weight gain (g) | FCR[3] (g/g) | Mortality (%) |
|---|---|---|---|---|
| Control | 5352 | 2902b | 1.844b | 5.5 |
| Bacteriophages | 5199 | 2993ab | 1.737a | 4.0 |
| Antibiotic | 5318 | 3054a | 1.742a | 7.0 |
| Probiotic | 5303 | 3059a | 1.734a | 9.8 |
| Symbiotic | 5273 | 3005a | 1.755a | 4.5 |
| P[1] | 0.387 | 0.001 | 0.0001 | 0.762 |
| SEM[2] | 5289±25 | 3003±14 | 1.762±0.01 | 6.1±0.92 |

Values with different letters (a, b) in the same row are statistically different (see probability). [1]probability; [2]Average and standar error of means; [3]feed conversion ratio.

## Intestinal morphology and histometry

In total intestinal density (g/cm), it was observed (Figure 1) that treatment with the control diet had higher intestinal density ($p$00.01) compared to the diet containing probiotic, and the other groups presented similar results. The measurements of intestinal villi (Table 5) in duodenum improved ($p$<0.01) with the use of eubiotics and antibiotic.

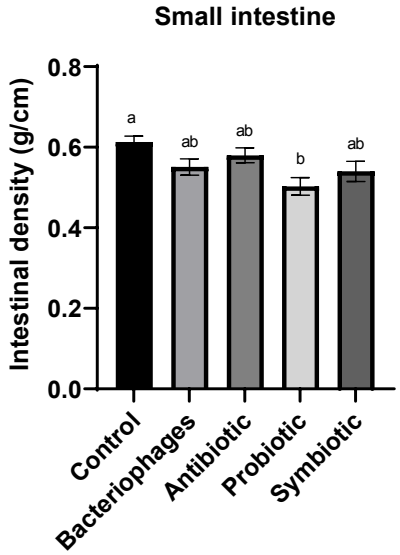


**Figure 1** – Effect of eubiotics on intestinal density (g/cm) in broiler chickens at 49 d. Vertical lines associated with histogram bars represent standard error of the mean for the total histologic score. [a,b,ab]Indicate significant differences ($p$<0.01).

## Intestinal histological changes

Using the histological ISI evaluation in duodenum (Figure 2), a higher ($p$<0.01) total ISI score considering epithelial changes and inflammatory changes was found when using the antibiotic and eubiotics when compared with the control group. The changes that

**Table 4 –** Effect of eubiotics on carcass characteristics in broiler chickens at 49 days of age.

| Treatment | Carcass % | Breast muscle[3] % | Thighs+Drumsticks % | Liver % | Abdominal fat % | Yelowness[4] |
|---|---|---|---|---|---|---|
| Control | 72.5b | 19.3 | 22.6 | 1.7 | 0.52 | 44.1 |
| Bacteriophages | 73.2ab | 19.2 | 22.1 | 1.7 | 0.45 | 45.4 |
| Antibiotic | 74.0a | 19.0 | 22.9 | 1.8 | 0.51 | 44.3 |
| Probiotic | 73.5ab | 19.2 | 22.8 | 1.8 | 0.44 | 44.6 |
| Symbiotic | 72.8ab | 18.8 | 22.8 | 1.9 | 0.64 | 45.2 |
| P[1] | 0.020 | 0.371 | 0.916 | 0.174 | 0.168 | 0.656 |
| SEM[2] | 73.2±0.15 | 19.1±0.1 | 22.7±0.1 | 1.8±0.02 | 0.51±0.02 | 44.7±0.3 |

Values with different letters (a, b) in the same row are statistically different (see probability). [1]probability; [2]standar error of means; [3]only *Pectoralis major*; [4]Skin b* values (yellowness).

**Table 5 –** Effect of eubiotics on histological measurements (duodenum) in broiler chickens at 49 days of age.

| | Control | Bacteriophages | Antibiotic | Probiotic | Symbiotic | P[3] | SEM[4] |
|---|---|---|---|---|---|---|---|
| Villus height, µm | 1965c | 2122bc | 2032bc | 2166b | 2441a | 0.001 | 2145±28 |
| Villus width, µm | 209b | 255a | 267a | 275a | 274a | 0.001 | 256±5.7 |
| Crypt depth, µm | 266ab | 234b | 260b | 264ab | 303a | 0.001 | 266±5 |
| Villus Surface Area, mm$^2$ | 0.410c | 0.536b | 0.545b | 0.596ab | 0.677a | 0.001 | .553±.016 |
| Villus: Crypt ratio[1] | 7.6b | 9.1a | 7.9ab | 8.3ab | 8.1ab | 0.021 | 8.2±0.1 |
| GC[2]:100 epithelial cells ratio | 21.2ab | 24.3a | 19.5b | 19.1b | 22.8ab | 0.001 | 21.3±0.5 |

Values with different letters (a, b, c) in the same row are statistically different (see probability). [1]Villus height: crypt depth ratio; [2]number of goblet cells; [3]probability; [4]standar error of means.

favored this higher total score, were in accordance with the obtained in goblet cell hyperplasia and inflammatory cell infiltration.

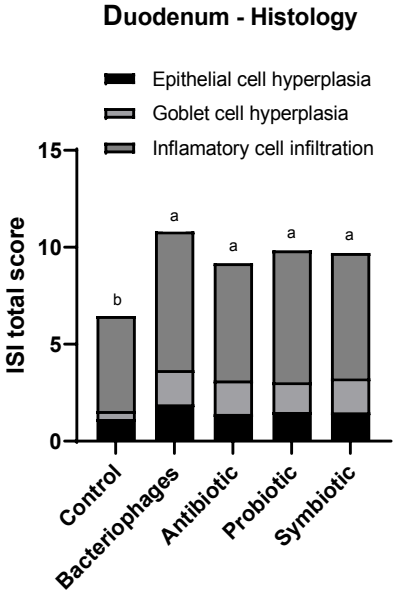


**Figure 2 –** Total sum of the histologic alteration score in the duodenum of broiler chickens supplemented with eubiotics. Epithelial hyperplasia (IF=1), Goblet cell hyperplasia (IF=2), Inflammatory infiltration in the lamina propria (IF=3). [ab]Indicate significant differences ($p$<0.01).

### Relative expression of enterobacteria

In relation to the fold change of relative quantification of enterobacteria determined by real time PCR can be found in Figure 3. In ceca, in the case of *E. coli*, an increase ($p$<0.05) of 2.9 times more in its expression level was obtained when using bacteriophages than the control group at 49 days of age. For *C. perfringens*, it was overexpressed 7.3 times ($p$<0.05) more when the probiotic *B. subtilis* was added than the control group in chickens at 49 days of age. Regarding *L. salivarius*, the use of the symbiotic allowed a 2-fold increase in its expression level compared to the control treatment; however, there was no significant difference ($p$>0.05).

## DISCUSSION

Different studies support that the broiler performance with the use of eubiotics is comparable to that obtained with AGP (Mountzouris *et al.*, 2010; Ghazanfari *et al.*, 2015; Gao *et al.*, 2017; Hussein *et al.*, 2020), although according to other authors, these additives do not cover the economic and productive benefits of an antibiotic (Al-Khalaifah, 2018; Oviedo-Rondón, 2019). In the present study, FCR was optimized by 5% when including the antibiotic and eubiotics in the diet, also obtaining a higher carcass yield when the antibiotic was used with respect to the control group

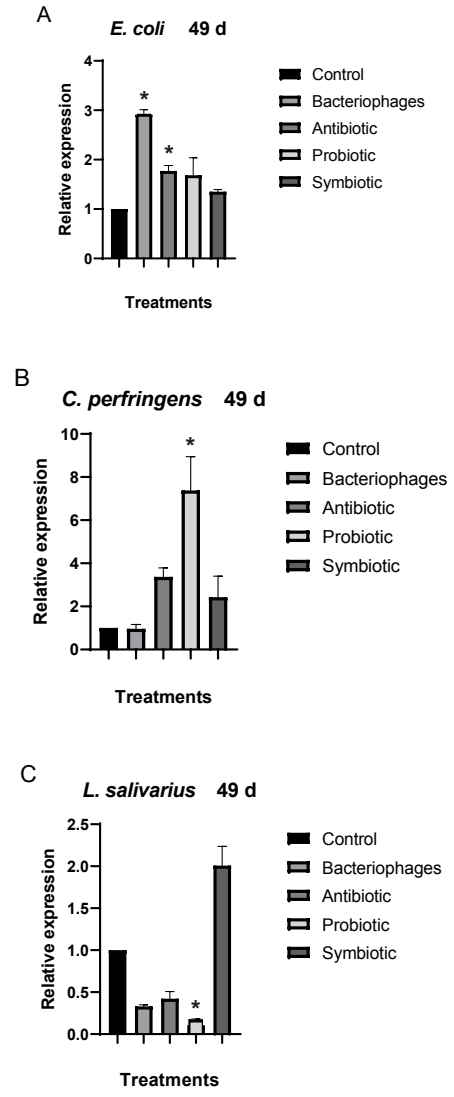


**Figure 3** – Effects of eubiotics in broiler chickens on the relative expression in cecum of A) *E. coli*, B) *C. perfringens* and C) *L. salivarius*. Error bars represent the standard error of the mean of the relative expressions. *Indicate significant differences ($p$<0.05).

(74% vs. 72.5%). Gao *et al.* (2017) reported a similar improvement (5.9%) in FCR when using a probiotic (*Lactobacillus plantarum*) compared with a combination of antibiotics, just like Hussein *et al.* (2020) who, using *Bacillus* spores (genera *subtilis* and *licheniformis*) and a phytobiotic compound to supplement diets of broiler chickens infected with *C. perfringens*, obtained a BWG, FCR and mortality comparable to the group treated with antibiotic (avilamycin). A study performed with bacteriophages (0.5 and 1 g/kg), showed a higher BWG than the control group, without effect in FCR (Upadhaya *et al.*,2021). In the gut morphological evaluation, the intestinal density (g/cm) was higher in the broiler chickens of the control group, with respect to the chickens fed *B. subtilis*-added diet. An increase in intestinal density may be related to changes in the

intestinal mucosa, which in some mammals represents more than 50% of the thickness of the intestine, in duodenum and jejunum (Di Donato *et al.*, 2013), and is possibly even greater in birds where the villi are usually higher than that of mammals (Smyth, 2016). On the other hand, it is known that, although intestinal weight and length in chickens decrease after the first week of age, this is compensated by an increase in intestinal density to maintain nutrient delivery function (Ravindran *et al.*, 2006), which may indicate that birds with higher intestinal mass have better nutrient utilization. However, Cardinal *et al.* (2019) when evaluating a reduced protein diet in broilers, observed that supplementation of a protease reduced the thickness of the lamina propria and epithelial surface, which was associated with improved nutrients transport and absorption, and thus higher productivity. In another study, when using a mycotoxin metabolite in broilers, it was observed a lower intestinal density derived, according to their conclusions, from a lower villi height (Riahi *et al.*, 2020). When changes in intestinal thickness result in villi atrophy and thinning of the tunica muscularis, macroscopically, the intestinal wall becomes more translucent, which can be used as an evaluation parameter for dysbacteriosis (Teirlynck *et al.*, 2011). This dysbacteriosis due to acute infection or tissue damage manifests as pathological inflammation, but when the immune response of the chickens is appropriate, the inflammation is of a physiological type (Kogut *et al.*, 2018). To this extent, it can be explained why the tendency to a lower intestinal density implied a better productive response, as obtained in this study. In the histometry of intestinal villi, a greater height and area of villi in duodenum was recorded, in chickens supplemented with the antibiotic and eubiotics, being this intestinal segment, important in the digestion and absorption of nutrients (Apajalahti & Vienola, 2016). Something similar was reported in other experiments, where using probiotics, prebiotics and phytobiotics, improved characteristics of the villi (Markovic *et al.*, 2009; Giannenas *et al.*, 2014; Hussein *et al.*, 2020). Elhassan *et al.*, (2018) found that the use of *B. subtilis* in chickens improved mucosal integrity in the duodenum, reporting an increase in villi height, as well as in the percentage of intact villi and a reduction of somatostatin immunoreactive cells. When compared with the use of an acidifier, it showed better results for jejunum and ileum. For the specific case of bacteriophages, there are data confirming that an increase in villi height is possible, in duodenum and jejunum, as shown by Kim *et al.* (2017) in weaned piglets and that it was possible to verify it in the present investigation, at least for duodenum. When analyzing the histological score based on epithelial changes and inflammatory cell infiltration of the lamina propria, the results of the research may be associated with a low-grade inflammation status, since from a total score of 18, maximum values of 10 were reached in the duodenum, with a higher score in the broiler chicken fed additives. The above, because of an increase in epithelial changes and inflammatory cell infiltration, which positively affected intestinal integrity, obtaining a better histometry of intestinal villi, which contributed to intestinal health. In contrast, Ghazanfari *et al.* (2015), when supplementing broiler diets with antibiotic and coriander essential oil, reported an increase in villus height and crypt depth, but at the same time, a decrease in epithelial layer thickness and number of goblet cells in the villi, which shows that epithelial cell hyperplasia plays a dynamic role in intestinal integrity and functionality. Cardinal *et al.* (2019) in their evaluation of intestinal health, recorded the best productive performance associated with a lower histological score, although the differences were only observed in three histological measurements; decrease in lamina propria thickness, epithelial thickness and enterocyte proliferation, without changes in goblet cells and inflammatory infiltration. Intestinal changes of this type were also reported by Hassan *et al.* (2014) who indicated that the use of probiotics favored a better performance due to the increase in villi height and size and depth of the crypts, as well as an increased amount of goblet cells in the epithelial layer of the crypts, revealing an active hyperplasia in the villi and crypts of Lieberkühn, which is comparable to what was obtained in the present investigation. In some cases, there are reports that the use of *B. subtilis* can induce epithelial hyperplasia and moderate metaplasia of the intestinal epithelium into goblet cells, registering intestinal epithelium regeneration (Hussein *et al.*, 2020),which can be compared with the results of current experiment suggesting that the goblet cell hyperplasia and histometry of the villi in the gut, favored intestinal functionality and performance, as well as the treatment with bacteriophages with a higher ratio of goblet cells per 100 epithelial cells also improved productive performance. It is known that this hyperplasia of goblet cells and mucus production can be induced by beneficial bacteria, as demonstrated by Huang *et al.* (2019), who reported an improvement in the relative mRNA expression of proteins that compose tight junctions, claudin and mucin 2 when using *E. faecium* in chickens challenged with *E. coli*, whereas a reduction

in *E. coli* counts was obtained from the third day post-infection. In the evaluation of the content of enterobacteria in the ceca, chickens that received diets supplemented with bacteriophages and bacitracin showed an increase in *E. coli* without changes in the expression of the other bacteria at 49 days of age. Engberg *et al.* (2000) indicated that bacitracin can decrease the population of *C. perfringens* and *L. salivarius*, although it didn't happen in this study. Respecting bacteriophages, Kim *et al.* (2017), observed that when they were added to piglets, a decrease in colonization by coliforms and *Clostridium* spp. was observed, as well as an increase in the population of *Lactobacillus* spp., different from what was observed in this study. *Lactobacillus salivarius* was found higher than the control group, this occurred when symbiotic was added to the diet, although no statistical difference was recorded. It is noteworthy that this symbiotic does contain *L. salivarius* as probiotic bacteria, and it was the only one in which a clear tendency was found in its increase. Something that is important to mention is that there was an under expression of *L. salivarius* in the ceca of the chickens treated with *B. subtilis*, and that according to previous studies it is known that this probiotic bacterium usually stimulates the presence of bacteria of the genus *Lactobacillus*, especially *L. reuteri*, because it produces subtilisin and catalase that facilitate its growth (Al-Khalaifah *et al.*, 2018), and therefore can stimulate a change in microbial composition and diversity in broilers by increasing beneficial microorganisms, contributing to protection against *Salmonella* infection (Oh *et al.*, 2017). The relationship between *B. subtilis* and *L. reuteri* could not be ascertained, so it is suggested to look for *L. reuteri* in the ceca in a next experiment. Also, the addition of probiotics such as *L. reuteri* and *Bifidobacterium* is related to the stimulation in the production of MUC2 and the increase in the thickness of the mucus layer (Paone & Cani, 2020) which may explain what was obtained in the present study, that, although a greater expression of *L. salivarius* was not demonstrated, there was an increase in the hyperplasia of goblet cells in the duodenum with the use of eubiotics. In general, what has been seen so far has shown that the use of multiespecie probiotics or symbiotics help to increase the population of *Lactobacillus* spp. in, ileum or ceca, as well as to decrease the population of bacteria such as *E. coli* and sometimes *C. perfringens* (Mountzouris *et al.*, 2010; Giannenas *et al.*, 2014; Dibaji *et al.*, 2014). The genus *Lactobacillus* are an effective tool to positively alter the microbiome, as quoted verbatim by Gao *et al.* (2017), who proved, that the maturation of the gut microbiome, was greatly accelerated with the use of a strain of *L. plantarum*, increasing the population of *Lactobacillus* spp., while the maturation was delayed when antibiotic were used, however, the productivity using the antibiotic was still possible, certainly because the antibiotic promoted the growth of beneficial bacteria. According to Tarradas *et al.* (2020), it has been seen that in some cases, the mechanisms of action of probiotics, such as *L. salivarius* and *B. animalis*, change in the presence of pathogens, which implies that the same changes may not always be observed, so in particular situations they will act as pro-inflammatory or as anti-inflammatory, being considered immuno-modulators, that is why Adedokun & Olojede (2019) do not recommend the use of these products in healthy birds, without a minimum of intestinal stress or challenge. In the case of bacteriophages, some authors claim that they can also modify the microbiome, even with the presence of temperate bacteriophages that do not lyse bacteria (De Paepe *et al.*, 2014). Upadhaya *et al.* (2021) using bacteriophages as alternative additives to APCs, indicated that the relative abundance of *L. salivarius* increased from 18.86 % (control group) to 37.80 and 40.13% with bacteriophages at 0.5 and 1% respectively in ileal mucosa of broilers at 35 days of age. It should be added that the use of bacteriophages, as commercial additives, requires more information to prove their mode of action and their effectiveness in the productive scale (Clavijo & Vives, 2018). Finally, the findings observed in the present study suggest that there was a physiological inflammation, which according to Cardoso *et al.* (2020), can be defined as a controlled intestinal inflammatory response, in which only positive intestinal histological changes were observed, evident in the duodenum by the use of the antibiotic and eubiotics, together with the optimal growth of intestinal villi, which can be interpreted as described by Kim & Ho (2010), as a balance and dynamic interactions between intestinal epithelial cells, increased digestive capacity and immunomodulation that is associated with the integrity and maintenance of the intestinal mucosa, promoted by the adequate colonization of beneficial bacteria, which could only be observed as a trend of colonization by *L. salivarius* when using the symbiotic.

## CONCLUSION

The addition of the antibiotic bacitracin and eubiotics used in the present study; bacteriophages, probiotic (*B. subtilis*) and the symbiotic (*L. salivarius, L. reuteri, E. faecium, B. animalis, P. acidolactici* and inulin), in

broiler diets, improved the productive performance at 49 days of age. This was congruent with what was reported in the histometric variables of the villi, as well as the histological changes that showed controlled inflammation, fundamental for the maintenance of intestinal integrity. The effect of bacteriophages showed that they promote benefits at the intestinal level as well as probiotics and symbiotics, however, it is important to continue investigating the mechanisms of action that make this possible. Considering the variability in the results of enterobacterial counts, it is recommended to contemplate the microbiome in each study, since, as a dynamic system, more research is required to understand the relationship of eubiosis with productivity. For the time being, the use of eubiotic additives, including bacteriophages, indicated that they are an alternative to the use of the growth-promoting antibiotic Bacitracin.

## ACKNOWLEDGMENTS

The authors would like to acknowledge the financial support of CTCBIO DE MÉXICO S. DE R.L. DE C.V. and Elanco Salud Animal, S.A. de C.V.

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