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308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

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Effect of Probiotic and Organic Acids in an Attempt to Replace the Antibiotics in Diets of Broiler Chickens Challenged with *Eimeria* spp.

Adriano Barbieri[1], Gustavo do Valle Polycarpo[2], Robert Guaracy Aparecido Cardoso[1], Kelry Mayara da Silva[1], Julio Cezar Dadalt[2], Alda Maria Backx Noronha Madeira[3], Ricardo Luiz Moro de Sousa[4], Ricardo de Albuquerque[2] and Valquíria Cação Cruz-Polycarpo[1]
[1]College of Technology and Agricultural Sciences, UNESP-Universty Estadual Paulista-Campus of Dracena, Rod. Cmte., João Ribeiro de Barros, km 651, Dracena-SP, 17900-000, Brazil
[2]Department of Animal Nutrition and Production (VNP), University of São Paulo (FMVZ-USP), Av. Duque de Caxias Norte, 225, Pirassununga-SP, 13635-900, Brazil
[3]Department of Parasitology, University of São Paulo (Biomedical Science Institute-USP), Av. Prof. Lineu Prestes, 1374, ICBII, São Paulo-SP, 05508-000, Brazil
[4]Department of Veterinary Medicine, University of São Paulo (FZEA-USP), Av. Duque de Caxias Norte, 225, Pirassununga-SP, 13635-900, Brazil

Abstract: The effect of probiotic (*Bacillus amyloliquefaciens*) and organic acids (lactic, acetic and butyric acid), isolated or combined, in an attempt to replace the antibiotics (avilamycin+sodium monensin) on intestinal anaerobic bacteria, allometric growth of digestive organs, intestinal morphometric and performance of broilers challenged by *Eimeria* (*acervulina*, *maxima* and *tenella*) were studied in a 1 to 21-d experiment. A total of 900 male Cobb chicks were distributed in a completely randomized design in a 2 x 2 + 1 factorial arrangement, presence or absence of probiotic and organic acids more a positive control with antibiotics, with six replicates. The probiotic promoted some changes on total anaerobic microorganisms throughout the small intestine in both periods ($p<0.02$), whereas the antibiotics decreased this counting only until 14 days ($p<0.04$). Antibiotics increased liver weight at 14 days ($p<0.01$) and reduced the relative weight of the pro-ventricle, gizzard and all segments of the small intestine at 21 days ($p<0.01$). At 14 days, the alternative additives reduced the villus height and crypt depth ($p<0.05$), whereas the probiotic increased the width of the villus base ($p<0.01$). Antibiotics reduced the crypt depth and width of the villi in the small intestine in both periods ($p<0.05$). Although some changes in the intestinal microbiota and morphology, the alternative additives, isolated or combined, did not change the birds' performance ($p>0.05$), only the antibiotics provided better results on BW gain and feed: gain in 1 to 14 and 1 to 21 d-old ($p<0.05$) of broilers challenged with *Eimeria* spp.

Key words: Acidifier, avilamycin, *Bacillus amyloliquefaciens*, sodium monensin

INTRODUCTION

The addition of antibiotics in sub therapeutic doses is broadly utilized in broiler chickens' diets to improve performance indexes. However, the utilization of antibiotics as growth promoters have been questioned more and more by public organs and consumers worried about safe food. Antibiotics used in animal feeding contribute to the dissemination of bacterial resistance throughout the food chain (Sorum and Sunde, 2001; Roe and Pillai, 2003), increasing the speculation of a relationship between the utilization of antibiotics in animal production and resistance to antibiotics in human infections. Regarding this, in 2006, as a preventive measure, the European Union decided to ban the use of antibiotics in animal feed (Langhout, 2000; Huyghebaert *et al*., 2011). This measure has generated serious concerns related to enteritis like coccidiosis which causes significant losses in poultry's performance (Kipper *et al*., 2013).

Probiotics are additives that consist of live microorganisms with beneficial action on the host, modulating intestinal microbiota balance (Mountzouris *et al*., 2007; Sanders, 2008; Kabir, 2009). On the other hand, organic acids are molecules with antimicrobial effect (Byrd *et al*., 2001) that have the capacity to dissociate ions and reduce bacterial cell pH (Ricke, 2003). Organic acids have species-specific action on microorganisms (Alakomi *et al*., 2000; Van Immerseel *et al*., 2006), hypothesizing that there are benefits in the association with probiotics through the joint action of these two additives in the reduction of undesirable bacteria.

Corresponding Author: Valquíria Cação Cruz-Polycarpo, College of Technology and Agricultural Sciences, UNESP-Universty Estadual Paulista-Campus of Dracena, Rod. Cmte., João Ribeiro de Barros, km 651, Dracena-SP, 17900-000, Brazil

Therefore, this study aimed to evaluate the effect of probiotic and organic acids addition in an attempt to replace the antibiotics in initial diets of broiler chickens experimentally challenged by *Eimeria* spp. on the intestinal anaerobic bacteria, allometric growth of organs of the digestive system, intestinal morphometry and growth performance.

MATERIALS AND METHODS

The experiment was carried out according to the principles and regulations of the Ethics Committee for the Use of Animals-CEUA, São Paulo State University-UNESP, Dracena campus (Registration No. 26, 2013).

Birds, design and experimental diets: Nine hundred male Cobb® broiler chicks were housed in floor pens with new wood shaving litter and raised until 21 days old. The birds were distributed in a completely randomized design in a 2 x 2 + 1 factorial scheme in which the variation of two factors was the presence or absence of probiotic and organic acids and a positive control treatment with addition of antibiotic + anticoccidial. Six replications with 30 birds were used. The utilized probiotic consisted of *Bacillus amyloliquefaciens* (1 x 10^9 CFU/g) with addition of 1 kg/t. The blend of organic acids was made up of lactic acid (40%), acetic acid (7%) and butyric acid (1%) with addition of 8 kg/t. The utilized antibiotic was avilamycin 20% added with 50 g/t and the anticoccidial was sodium monensin 40% added with 300 g/t, allotting 10 and 120 ppm of active principle, respectively.

Water and ration were provided *ad libitum*. The feeding program was divided into two phases: pre-starter (1 to 7 days) and starter (8 to 21 days). The rations were isoenergetic and isoaminoacidic, formulated with corn and soybean meal according to the recommendations by Rostagno *et al*. (2011) (Table 1). The additives were included "on top" in replacement of the inert material according to each treatment. Antibiotics and coccidiostats were not added in the mineral-vitamin supplement to avoid any interference with the proposed additives.

***Eimeria* spp. challenge:** At day 10, each bird was individually inoculated orally with 1 mL of solution of 2 x 10^5 sporulated oocysts/mL of *Eimeria acervulina* and 2 × 10^4 sporulated oocysts/mL of *E. maxima* and *E. tenella*. These three species were chosen because of the importance they have in broiler chicken production (Shirley *et al*., 2004), due to the high incidence and economic losses they may cause (Williams, 1999, 2005).

Response variables

Microbiology: Samples of the content of the duodenum, jejunum and ileum of one bird per replication were collected for microbiological analyses. The total counting of anaerobic bacteria was done by diluting 1 g of the sample in 9 mL of autoclaved peptone water (dilution/10). Later, the content was homogenized and 1 ml of the solution was transferred to another tube with 9 mL of peptone water. The content serial dilution was utilized to find the concentration that allowed the counting of colonies within pre-established values, which were up to 250 colonies.

After the dilution, 100 µL of each concentration were pipetted in disposable petri dishes with agar brewer cultivation medium. The content was spread using a Drigalski handle in circular movements until complete absorption of the liquid by the cultivation medium. The dishes were prepared in duplicates, placed in an anaerobiosis jar and incubated at 37°C for 48 h. After the incubation period, the counting of the total number of colonies in each dish was done.

Allometry of the digestive system organs: One bird from each replication was anesthetized and slaughtered to remove the pancreas, gizzard, pro-ventricle, liver, small intestine and large intestine. The organs were weighed to determine relative weight: ((organ weight, g x 100)/bird weight, g). The length of the intestines was measured to obtain relative length: (size of intestine, cm x 100)/bird weight, g).

The small intestine was segmented into duodenum, jejunum and ileum. The duodenum was considered the beginning of the small intestine up to the end of the duodenal loop. The jejunal portion started in the duodenal loop up to Meckel diverticulum. Finally, the ileum was established as the end of the jejunum up to the caecum insertion. The measurement of the large intestine was calculated by the colon and rectum length added to the result of the caecum length.

Morphometry: The morphological study was carried out by light microscopy. One bird per replication was slaughtered at 14 and 21 days to collect two 3 cm segments of the duodenum and two 3 cm segments of the jejunum. The segments were washed in physiological solution, opened by the mesenteric border, extended by the serum tunica, fixed in formaldehyde 10% during 24 h and stored in alcohol 70%. Later, the samples were reduced and dehydrated in alcohol, diaphonized in xylol and inserted in histologic paraffin. Five-micrometer (µm) cuts were done to prepare slides which were stained with Hematoxylin- Eosin (HE). 15 measurements of villus height and width and crypt depth were done using an objective lens 5x of an optical microscope coupled to an image analyzed system by Leica (Image-Pro Plus version 1.0.0.1). Villus height and width were measured from the basal region to the apex and from one lateral end to the other, respectively. The crypts were measured from the base to the crypt: villus transition region.

Performance: The analyzed performance variables were: BW gain, feed intake, feed: gain and viability. The viability of each experimental unity was obtained by the subtraction: 100-mortality. Mortality was calculated in percentage.

Statistical analysis: Data analysis was done by Statistical Analysis System software (SAS Institute, 2012) at 5% of probability. Residue normality analyses were done by Shapiro-Wilk Test (UNIVARIATE procedure). The microbiological values were submitted to logarithmic transformation. All data were submitted to analysis of variance by MIXED procedure through orthogonal contrasts. The effects of alternative additives were assessed comparing diets without antibiotics: main effect of probiotic (contrast 1: diets with probiotic vs. diets without probiotics), main effect of organic acids (contrast 2: diets with organic acids vs. diets without organic acids) and the effect of their interaction (contrast 3: contrast 1 x contrast 2). The fourth contrast compared the effect of the treatment with antibiotics vs. all the other treatments.

RESULTS

The probiotic and organic acids did not present interaction on the counting of total anaerobic microorganisms (Table 2). At 14 days, the probiotic increased the amount of total anaerobic microorganisms throughout the small intestine, whereas antibiotics decreased this counting. At 21 days there was lower counting of total anaerobic microorganisms added with probiotic in the jejunum. The organic acids did not influence the number of microorganisms in both periods.

The relative weight and length of the digestive system organs were not changed by the isolated or associated inclusion of alternative additives (Table 3 and 4). At 14 days, the antibiotics increased liver weight. At 21 days, the antibiotics showed a great effect on the digestive system organs, reducing the relative weight of the pro-ventricle, gizzard and all segments of the small intestine. There was an interaction between the probiotic and the organic acids on the jejunum crypt depth (Table 5), which presented smaller values in the isolated presence of additives. At 14 days, probiotic addition increased the width and decreased the height of villi in the duodenum and jejunum, respectively. In the same period, the organic acids decrease the crypts depth of the duodenum, whereas the antibiotics reduced the crypt depth in the duodenum and jejunum in both periods (14 and 21 d-old). Still, at 14 days, the antibiotics reduced the width of the apex and the base of the duodenal villi and the apex width of jejunum villi. At 21 days, antibiotics also reduced the apex width of jejunum villi.

Table 1: Composition and calculated values of the experimental diets

	Diets[1]	
Ingredients (%)	Pre-starter	Starter
Corn	53.61	57.67
Soybean meal (46% CP)	38.43	35.03
Soybean oil	2.687	2.682
choline chloride 60	0.072	0.064
Salt	0.508	0.482
Dicalcium-phosphate	1.902	1.533
Limestone	0.917	0.907
L-lysine	0.283	0.210
DL-methionine	0.357	0.285
L-Threonine	0.106	0.058
L-Valine	0.075	0.024
Mineral premix[2]	0.050	0.050
Vitamin premix[3]	0.100	0.100
Kaolin[4]	0.900	0.900
Sum	100.0	100.0
Calculated values		
AMEn (kcal/kg)	2,950	3,000
CP (%)	22.20	20.80
Methionine+cystine[5] (%)	0.944	0.846
Lysine[5] (%)	1.310	1.174
Threonine[5] (%)	0.852	0.763
Valine[5] (%)	1.009	0.904
Calcium (%)	0.920	0.819
Phosphorus[5] (%)	0.395	0.343
Sodium (%)	0.220	0.210
Choline (mg/kg)	375.0	330.0
Linoleic acid (%)	2.72	2.77

[1]Pre-starter, 1 to 7 d-old; starter, 8 to 21 d-old.
[2]Mineral premix provided per kg of feed: Cu, 9; I, 1; Zn, 60; Fe, 30; Mn and 60 mg.
[3]Vitamin premix provided per kg of feed: vitamin A, 11,000.00 IU; vitamin D3, 2,000.00 IU; vitamin E, 16.00 IU; vitamin K3, 1.50 mg; vitamin B1, 1.20 mg; vitamin B2, 4.50 mg; vitamin B6, 2.00 mg; vitamin B12, 16.00 mcg; folic acid, 0.40 mg; pantothenic acid, 9.20 mg; biotin, 0.06 mg; niacin, 0.035 mg; Se, 0.25 mg.
[4]Treatments were obtained by replacement of kaolin by additives: Diet without additive, 0.9% of kaolin. Diet with probiotic, 0.1% probiotic + 0.8% kaolin. Diet with organic acids, 0.8% organic acids + 0.1% kaolin. Diet with probiotic + organic acids, 0.1% probiotic + 0.8% organic acids. Diet with antibiotics, 0.005% avilamycin + 0.03% monensin sodium + 0.865% kaolin.
[5]Digestible values.

The inclusion of alternative additives did not change the birds' performance (Table 6). Only the antibiotics provided better results, increasing BW gain and improving feed: gain in the period of 1 to 14 days. The effect of antibiotics was even greater in the period of 1 to 21 days, increasing feed intake, BW gain and feed: gain ratio.

DISCUSSION

The importance of microbiology challenge in the evaluation of antimicrobials was reported in the very first studies on antibiotics added to feed as growth promoters (Lillie *et al*., 1953; Coates *et al*., 1963). The significant reduction of 23.51% in BW gain at 21 days between the treatment with antibiotics and the group without them shows that the challenge utilized in this study was very expressive.

Table 2: Duodenum, jejunum and ileum microbiology of broilers at 14 and 21 d-old

	Total anaerobes, CFU/g[2]					
	14 d-old			21 d-old		
Effects[1]	D[3]	J	I	D	J	I
Prob						
With	7.427	7.949	7.914	6.388	5.598	7.139
Without	5.839	5.733	5.952	6.155	6.467	7.019
OA						
+	6.882	6.698	6.955	6.651	5.940	7.421
-	6.384	6.983	6.911	5.892	6.125	6.737
Prob x OA						
With+	7.920	7.820	8.175	7.060	5.331	7.143
Without+	5.844	5.577	5.735	6.242	6.548	7.698
With-	6.933	8.077	7.653	5.716	5.865	7.134
Without-	5.834	5.888	6.169	6.067	6.385	6.339
Antib						
Presence	5.170	5.676	5.567	6.390	6.710	6.831
Absence	6.633	6.841	6.933	6.272	6.032	7.079
SEM	0.3022	0.2523	0.2448	0.1925	0.1555	0.1751
Source of variation	Probability					
Probiotic	0.0117	<0.0001	<0.0001	0.5820	0.0076	0.7500
Organic acids	0.4017	0.4315	0.9030	0.0818	0.5413	0.0781
Prob x OA	0.4104	0.9409	0.1886	0.1755	0.2541	0.0820
Antibiotics	0.0342	0.0071	0.0020	0.8023	0.0534	0.5580

[1]Prob: Probiotic, OA: Organic acids, Antib: Antibiotics, With: Presence of probiotic, Without: Absence of probiotic, +: Presence of organic acids, -: Absence of organic acids.

[2]CFU/g, colony forming units per g of sample. Data submitted to log transformation.

[3]D: Duodenum, J: Jejunum, I: Ileum

Despite the evident possibility of complementary effect between the probiotic and the organic acids (Neal-McKinney *et al*., 2012), it was not possible to demonstrate relevant interactions with the combined utilization of both additives in this study. The lactic acid, which resulted from the metabolism of *Bacillus amyloliquefaciens*, could complement the exogenous inclusion of organic acids. On the other hand, the organic acids could provide appropriate conditions for the growth and proliferation of the utilized probiotic. The efficiency of the combination of probiotic and organic acids was observed in the control of *Salmonella enteritidis* in a previous study conducted by Wolfenden *et al*. (2007). These authors pointed out that the utilization of probiotic + organic acids can be more effective than their isolated use due to the fact that the action of these two additives happens markedly in different regions of the gastrointestinal tract. Organic acids suffer metabolization throughout the gastrointestinal tract, so their potential occurs mainly in the crop. In turn, probiotics have most pronounced effect in the caecum and cecal tonsils.

The increase of total anaerobic counting at 14 days in the small intestine of chickens fed with probiotics may have occurred because *Bacillus amyloliquefaciens* is a facultative anaerobic bacterium. The *Lactobacillus* population may also have been benefited, as it was observed in the caecum of chickens that received *Bacillus amyloliquefaciens* (Lei *et al*., 2015). However, at 21 days, the probiotic presented inverse result, reducing the amount of total anaerobic bacteria. Ahmed *et al*. (2014) observed the reduction of *Escherichia coli* in the caecum of chickens fed with increasing levels of *Bacillus amyloliquefaciens*, corroborating the smaller amounts of total anaerobic found at 21 days in the birds' jejunum. This microbiota modulation shows that probiotic can change microbial populations in the small intestine and suggests that other investigations with specific techniques that identify which microorganism are benefited or depressed by *Bacillus amyloliquefaciens* be carried out. The organic acids did not modify the intestinal microbiota, disagreeing with previous studies that observed the significant effect of organic acids on the microbiology of the gastrointestinal tract (Gunal *et al*., 2006; Hassan *et al*., 2010; Skanseng *et al*., 2010; Menconi *et al*., 2013). The data at 14 days made the inhibiting effect of antibiotics on the bacterial growth of proximal and distal portions of the intestine evident and in accordance with previous studies that observed smaller amounts of pathogens with the use of antibiotics (Engberg *et al*., 2000; Knarreborg *et al*., 2002). However, it is worth to mention that, despite the depressing effect of antibiotics on pathogenic bacteria, antibiotics can increase the growth of some bacteria of the genus *Lactobacillus* that can have probiotic potential (Dumonceaux *et al*., 2006).

The reduction of the bacterial load by the use of antibiotics resulted in smaller weight and intestinal length. Recently, similar results with antibiotics on the intestinal parameters were reviewed by Miles *et al*. (2006). For years, it has been known that the bacterial control provided by antibiotics improves intestinal health

Table 3: Relative weight and length of digestive system of broilers at 14 d-old

	Weight (%)[2]									Length (%)[3]				
Effects[1]	Panc[4]	Prove	Gizzard	Liver	S	D	J	I	L	S	D	J	I	L
Prob														
With	0.420	0.677	2.60	3.01	9.01	1.69	3.57	3.74	1.44	0.229	0.0454	0.0916	0.0922	0.0500
Without	0.425	0.632	2.74	2.99	9.21	1.70	4.11	3.38	1.25	0.232	0.0437	0.0980	0.0906	0.0473
OA														
+	0.432	0.672	2.70	2.96	8.82	1.63	3.65	3.53	1.32	0.233	0.0457	0.0946	0.0924	0.0481
-	0.413	0.637	2.63	3.03	9.40	1.76	4.04	3.59	1.38	0.229	0.0434	0.0949	0.0904	0.0493
Prob x OA														
With+	0.421	0.697	2.64	2.92	8.99	1.60	3.52	3.87	1.40	0.237	0.0470	0.0925	0.0973	0.0497
Without+	0.443	0.646	2.76	3.01	8.66	1.67	3.78	3.19	1.24	0.229	0.0444	0.0967	0.0874	0.0464
With-	0.419	0.657	2.55	3.09	9.03	1.78	3.63	3.60	1.49	0.221	0.0438	0.0906	0.0870	0.0503
Without-	0.406	0.617	2.71	2.98	9.77	1.74	4.44	3.57	1.27	0.236	0.0430	0.0992	0.0938	0.0482
Antib														
Presence	0.490	0.647	2.52	3.43	8.78	1.58	3.90	3.29	1.38	0.220	0.0421	0.0939	0.0836	0.0454
Absence	0.422	0.654	2.67	3.00	9.11	1.70	3.84	3.56	1.35	0.231	0.0445	0.0948	0.0914	0.0486
SEM	0.0138	0.0140	0.0707	0.0620	0.2033	0.0587	0.1278	0.1047	0.0490	0.0043	0.0010	0.0020	0.0021	0.0009
Source of variation	Probability													
Probiotic	0.8798	0.1722	0.3981	0.8989	0.6688	0.9208	0.0599	0.1338	0.1009	0.7579	0.4378	0.1806	0.7391	0.2225
Organic acids	0.5432	0.2875	0.6693	0.5627	0.2211	0.3627	0.1645	0.8114	0.5599	0.6870	0.2975	0.9540	0.6805	0.5824
Prob×OA	0.5611	0.8645	0.8932	0.4145	0.2540	0.6523	0.3222	0.1664	0.7835	0.2561	0.6777	0.6406	0.0866	0.7825
Antibiotics	0.0579	0.8605	0.4411	0.0044	0.5202	0.4452	0.8560	0.2997	0.7804	0.3251	0.3294	0.8723	0.1494	0.1914

[1]Prob: Probiotic, OA: Organic acids, Antib: Antibiotics, With: Presence of probiotic, Without: Absence of probiotic, +: Presence of organic acids, -: Absence of organic acids.
[2][(organ weight, g × 100) / broiler weight, g]. [3][(Intestine length, cm × 100) / broiler weight, g].
[4]Panc, pancreas; Prove: Pro-ventricle, S: Small intestine, D: Duodenum, J: Jejunum, I: Ileum, L: Large intestine

Table 4: Relative weight and length of digestive system of broilers at 21 d-old

	Weight (%)[2]									Length (%)[3]				
Effects[1]	Panc[4]	Prove	Gizzard	Liver	S	D	J	I	L	S	D	J	I	L
Prob														
With	0.366	0.619	2.11	3.03	9.34	1.74	4.26	3.33	1.08	0.188	0.0356	0.0796	0.0722	0.0315
Without	0.405	0.596	2.26	3.14	9.29	1.74	4.25	3.29	0.97	0.187	0.0343	0.0813	0.0711	0.0296
OA														
+	0.406	0.616	2.28	3.25	9.56	1.78	4.43	3.33	1.06	0.192	0.0359	0.0815	0.0750	0.0319
-	0.366	0.600	2.10	2.92	9.07	1.69	4.08	3.29	0.99	0.182	0.0340	0.0795	0.0684	0.0293
Prob × OA														
With+	0.408	0.617	2.16	3.12	9.29	1.75	4.35	3.18	1.10	0.192	0.0366	0.0808	0.0749	0.0337
Without+	0.404	0.614	2.39	3.38	9.83	1.81	4.52	3.48	1.03	0.192	0.0353	0.0821	0.0751	0.0300
With-	0.324	0.623	2.07	2.94	9.39	1.72	4.18	3.49	1.06	0.183	0.0347	0.0785	0.0695	0.0293
Without-	0.407	0.578	2.13	2.91	8.75	1.66	3.98	3.09	0.92	0.181	0.0333	0.0806	0.0672	0.0292
Antib														
Presence	0.324	0.507	1.80	2.82	6.64	1.12	3.10	2.41	0.90	0.131	0.0246	0.0551	0.0515	0.0269
Absence	0.386	0.608	2.19	3.09	9.31	1.74	4.26	3.31	1.03	0.187	0.0349	0.0805	0.0717	0.0306
SEM	0.0155	0.0146	0.0597	0.0895	0.2744	0.0561	0.1190	0.1178	0.0389	0.0062	0.0012	0.0026	0.0027	0.0011

Table 4: Continued

Source of variation	Probability													
Probiotic	0.2398	0.4224	0.2117	0.5754	0.9005	0.9999	0.9638	0.8298	0.2442	0.9454	0.5254	0.7083	0.8333	0.4163
Organic acids	0.2379	0.6108	0.1411	0.1056	0.2650	0.2451	0.0608	0.8530	0.4085	0.3399	0.3511	0.6726	0.2179	0.2695
Prob×OA	0.1969	0.4959	0.4981	0.4760	0.1795	0.4237	0.3139	0.1319	0.7032	0.9324	0.9747	0.9348	0.8164	0.4604
Antibiotics	0.1052	0.0050	0.0061	0.2336	<0.0001	<0.0001	<0.0001	0.0013	0.2075	0.0001	0.0002	<0.0001	0.0021	0.1694

[1]Prob: Probiotic, OA: Organic acids, Antib: Antibiotics, With: Presence of probiotic, Without: Absence of probiotic, +: Presence of organic acids, -: Absence of organic acids.
[2][(organ weight, g × 100) / broiler weight, g]. [3][(Intestine length, cm × 100) / broiler weight, g]

Table 5: Morphometric intestinal of broilers at 14 and 21 d-old

	Morphometric[2] (μm)															
	14 d-old								21 d-old							
	Duodenum				Jejunum				Duodenum				Jejunum			
Effects[1]	Villus	Crypt	WVA	WVB	Villus	Crypt	WVA	WVB	Villus	Crypt	WVA	WVB	Villus	Crypt	WVA	WVB
Prob																
With	989	246	143	112	675	149	104	81	1,568	336	153	119	825	216	127	95
Without	1097	263	135	100	764	148	97	81	1,600	321	158	116	841	217	135	94
OA																
+	1117	231	138	107	715	148	101	85	1,547	331	154	117	813	215	129	92
-	970	277	139	105	724	149	100	77	1,621	325	157	118	852	218	134	97
Prob × OA																
With+	1073	235	140	114	692	169	107	82	1,528	326	151	114	784	213	127	95
Without+	1160	227	135	100	738	127	95	88	1,567	337	157	120	843	216	130	89
With-	906	256	145	109	658	128	101	80	1,608	345	155	124	866	219	127	95
Without-	1034	298	134	100	790	169	98	73	1,633	305	159	112	838	217	140	100
Antib																
Presence	1028	168	111	92	696	119	90	70	1,744	218	142	108	779	134	109	88
Absence	1043	254	139	106	720	148	100	81	1,584	328	155	118	833	216	131	95
SEM	40.02	11.87	3.59	2.03	18.87	5.99	2.16	2.53	32.73	13.28	3.11	2.79	25.18	9.81	2.66	2.44
Source of variation	Probability															
Probiotic	0.2369	0.4382	0.2481	0.0037	0.0376	0.9529	0.0948	0.9746	0.6606	0.5605	0.5201	0.6417	0.7948	0.9672	0.1145	0.8873
Organic acids	0.1128	0.0431	0.7992	0.4766	0.8176	0.9286	0.7728	0.1281	0.3160	0.7989	0.6527	0.8786	0.5129	0.8768	0.3324	0.3434
Prob×OA	0.8201	0.2605	0.6236	0.5306	0.3011	0.0005	0.2912	0.2300	0.9233	0.3056	0.8639	0.1586	0.4672	0.8991	0.3351	0.3504
Antibiotics	0.8778	0.0015	0.0011	0.0017	0.6042	0.0174	0.0426	0.0897	0.0561	0.0004	0.0925	0.1862	0.4169	0.0005	0.0002	0.2662

[1]Prob, probiotic; OA, organic acids; Antib, antibiotics; with, presence of probiotic; without, absence of probiotic; +, presence of organic acids; -, absence of organic acids.
[2]VH: villus height, Crypt: crypt depth, WVA: width of the villus apex, WVB: width of the villus base

Table 6: Performance of broilers fed with experimental diets

	Performance								
	1-14 d-old					1-21 d			
Effects[1]	BW initial	FI	BW gain	F:G ratio	Viability	FI	BW gain	F:G ratio	Viability
Prob									
With	39.86	537.0	432.0	1.243	99.16	1,045.9	711.7	1.456	98.61
Without	39.94	542.0	433.5	1.251	99.16	1,062.5	722.5	1.471	99.16
OA									
+	39.83	544.6	434.7	1.253	98.88	1,061.9	720.4	1.461	98.60
-	39.97	534.5	430.8	1.241	99.45	1,046.6	713.9	1.466	99.17
Prob × OA									
With+	39.78	536.4	429.4	1.250	98.89	1,050.2	713.8	1.444	98.33
Without+	39.89	552.8	440.0	1.257	98.87	1,073.5	727.0	1.478	98.87
With-	39.94	537.6	434.6	1.237	99.44	1,041.6	709.7	1.468	98.89
Without-	40.00	531.3	427.0	1.244	99.46	1,051.6	718.1	1.465	99.46
Antib									
Presence	39.67	551.7	449.8	1.227	100.00	1,253.5	937.5	1.338	98.33
Absence	39.90	539.5	432.8	1.247	99.16	1,054.2	717.1	1.464	98.89
SEM	0.1057	2.888	2.701	0.004	0.248	15.727	16.928	0.011	0.338
Source of variation	Probability								
Probiotic	0.7404	0.3892	0.7899	0.3781	0.9977	0.1745	0.2965	0.3074	0.4918
Organic acids	0.5813	0.0936	0.4759	0.1346	0.3208	0.2095	0.5287	0.7366	0.4765
Prob × OA	0.9123	0.0612	0.1036	0.9795	0.9744	0.5816	0.8167	0.2222	0.9817
Antibiotics	0.4039	0.0721	0.0092	0.0314	0.1998	<0.0001	<0.0001	<0.0001	0.5364

[1]Prob: Probiotic, OA: Organic acids, Antib: Antibiotics, With: Presence of probiotic, Without: Absence of probiotic, +: Presence of organic acids, -: Absence of organic acids

and decreases epithelial desquamation and the need of cellular proliferation (Krinke and Jamroz, 1996), resulting in the thinning of the intestinal wall (Jukes *et al*., 1956). Morphometric data confirmed the reductive effect that antibiotics cause in the intestine. These results occur mainly because the antibiotics decrease the microbial population. In agreement, previously study observed significantly reduction on villus area, crypt depth and lamina propria area in germ-free chicks (Cook and Bird, 1973). Alternative additives presented less marked results in variables of weight, length and intestinal morphology. However, Abdel-Fattah *et al*. (2008) observed an increase in relative weight, length and density of the small intestine with the utilization of organic acids. Adil *et al*. (2010) verified an increase in the height of intestinal villi with the use of organic acids. Likewise, Gunal *et al*. (2006) and Lei *et al*. (2015) observed an increase of intestinal villi with the use of a probiotic blend and *Bacillus amyloliquefaciens*, respectively. These data obtained in the literature suggest that alternative additives may generate an opposing response to antibiotics, acting not only on the microbial population but also on the intestinal development. Through the observed results in this study, it can be inferred that the challenge imposed with the inoculation of *Eimerias* was greater than the effect offered by the alternative additives.

The microbial reduction related to the decrease of cellular proliferation and the mucosal thinning in conjunction with less lamina propria in the intestine are some of the attributes of antibiotics that are related to the increase of absorptive surface area. Moreover, the lower energy demand to maintain tissues makes the use of energy for poultry's growth viable, improving performance as it has been observed in chickens fed with antibiotics. The efficiency of organic acids and probiotics as alternative additives to antibiotics on chickens' performance has been proved by several studies (Yeo and Kim, 1997; Mountzouris *et al*., 2007; Ashayerizadeh *et al*., 2009; Chowdhury *et al*., 2009; Hassan *et al*., 2010); however, the results presented here do not corroborate these findings. Abbas *et al*. (2011) demonstrated that citric acid in drinking water can control coccidiosis in chickens challenged with *E. tenella*. Denli *et al*. (2003) and Gunal *et al*. (2006) observed the same performance in chickens fed with probiotics and organic acids in comparison to antibiotics; however, antibiotics do not differentiate themselves from control diet (without additives), demonstrating that all additives had the same effect due to the lack of sanitary challenge. In assessments of feed additives, it is important to consider the challenge that is imposed to birds, because it is fundamental to understand the responses that are generated. This question requires meta-analytic studies that take into consideration the conditions of each additive is evaluated to help understand the contradictory effects.

Conclusion: In conclusion, the probiotic and organic acids, isolated or associated, did not present satisfactory results to substitute growth-promoting antibiotics in chickens challenged with *E. acervulina*, *E. maxima* and *E. tenella*.

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