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Isolated and combined organic acids in diets of broiler chickens challenged with *Eimeria acervulina*

[*Ácidos orgânicos isolados ou associados em dietas de frangos de corte desafiados com* Eimeria acervulina]

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ABSTRACT

This study aimed to evaluate the effect of isolated or combined citric and benzoic acids added to the diets of broiler chickens on performance, allometry of the digestive system organs, intestinal pH and quantity of microorganisms in the jejunum. A total of 840 male Cobb broiler chicks were utilized, distributed in a complete random design in 2 × 2 factorial scheme, supplemented or not with citric acid, and sodium benzoate, with seven replications. At 14 days old, 1mL of a solution containing 1×10^5 sporulated oocysts of *Eimeria acervulina* per bird was inoculated orally. There was no effect of the acids on the broiler's performance in the 1 to 21-day period. In the total period (1 to 42 days), the broilers fed with a blend of citric and benzoic acid presented greater feed intake, without increment in weight gain. The data of allometry of the digestive system organs and the jejunal pH values were not influenced by the treatments. The citric acid increased the bacterial quantity of gram-positive coccus and total anaerobes in the jejunum. Under the conditions of the present study, we conclude that the citric and benzoic acids, isolated or combined, do not benefit the nutrition of broilers challenged with *E. acervulina*.

Keywords: acidifying, additive, gastrointestinal tract, microbiota, performance, poultry

RESUMO

O objetivo deste trabalho foi avaliar os efeitos da inclusão isolada ou associada dos ácidos cítrico e benzoico na alimentação de frangos de corte sobre o desempenho, a alometria de órgãos do sistema digestório, o pH intestinal e a quantidade de microrganismos no jejuno. Foram utilizados 840 pintos de corte, machos, da linhagem Cobb, distribuídos num delineamento inteiramente ao acaso, em esquema fatorial 2 × 2, com suplementação ou não de ácido cítrico e suplementação ou não de benzoato de sódio, com sete repetições. Aos 14 dias de idade, foi inoculado, via oral, 1mL de solução contendo 1×10^5 *oocistos esporulados de* Eimeria acervulina *por ave. Não houve efeito dos ácidos sobre o desempenho dos frangos no período de um a 21 dias. No período total (um a 42 dias), os frangos alimentados com a mistura de ácidos cítrico e benzoico apresentaram maior consumo de ração, sem incremento no ganho de peso. Os dados de alometria dos órgãos do sistema digestório e os valores de pH do jejuno não foram influenciados pelos tratamentos. O ácido cítrico aumentou a quantidade de bactérias do gênero cocos Gram positivos e anaeróbios totais no jejuno. Nas condições do presente estudo, conclui-se que os ácidos cítrico e benzoico, isolados ou associados, não beneficiam a nutrição de frangos de corte desafiados com* E. acervulina.

Palavras-chave: acidificante, aditivo, ave, desempenho, microbiota, trato gastrintestinal

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INTRODUCTION

One of the main current challenges in poultry production is the search for balance between the intestinal microbiota and the host. Within this context, organic acids have been studied to model the intestinal microbial population, assisting the good functioning of the organism and the performance of broiler chickens (Barbosa *et al.*, 2005). The organic acids inhibit the development of fungi in raw materials and feeds, decreasing the proliferation of enterobacteria (*Salmonella* and *Escherichia coli*) in the intestine, reflecting in a greater nutritional utilization of rations (Gonzales and Sartori, 2001).

The inhibition of the microbial growth by organic acids is explained by the ability of these acids to go through the membrane, dissociating in the cellular interior, acidifying the cytoplasm (Van Immerseel *et al*.; 2006). The excessive export of protons by the bacteria to control the intracellular pH demands intake of adenosine triphosphate (ATP), which results in the depression of cellular energy (Ricke 2003), retarding its growth or even causing the microorganism to die. The benefits of acids on the control of pathogenic microorganism were reported by Flores *et al*. (2012) who observed a decrease in the presence of *Salmonella* in broiler chickens treated with organic acids.

Many studies investigate the effect of organic acids on broiler chickens' performance (Waldroup *et al*.; 1995; Rezende *et al*.; 2008; Chowdhury *et al*.; 2009; Adil *et al*.; 2010; Barbieri *et al*.; 2015). However, the published results are controversial, which does not allow a safe recommendation regarding the utilization and concentration of these products. The lack of consistency in the results of organic acids could have resulted from a lack of control in the intervening variables such as pH in the digestive tract, buffer capacity of diet ingredients, hygiene conditions in the production environment, heterogeneity of the intestinal microbiota, besides the inherent resistance of the microorganisms. Another important point is to investigate the effects of organic aids on *Eimeria*, which is an intestinal pathogen that causes the most damages in poultry production (Williams, 1999).

Therefore, this research study aimed to evaluate the supplementation of citric and benzoic acids, isolated or combined, in broiler chickens challenged with *Eimeria acervulina* on the performance, allometry of the digestive system organs, length of small and large intestines, pH of the intestinal content, and on the development of the microbiota found in the gastrointestinal tract (GIT).

MATERIAL AND METHODS

The study was carried out at the São Paulo State University (UNESP), College of Agricultural and Technological Sciences, Dracena, with the following geographical coordinates: latitude 21º 28' 57" south, longitude 51º 31' 58'' west and average altitude of 421 meters. The climate in the region is subtropical (warm and dry winter is followed by very hot summers) with average annual temperature of 23.6ºC according to the technical manual developed by Tremocoldi, Brunini (2008). 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), College of Agricultural and Technological Sciences, Dracena (Registration No. 38/2012).

The broiler chicks were housed in floor pens and raised on the floor with new wood shaving litter. The management practices followed the technical orientation of the *Cobb* manual. The initial heating of the chicks was done through 250-watt infrared lamps. Along the experiment, the control of the temperature and ventilation was manually done by managing the lateral curtains of the experimental rearing house and utilizing fans. The lighting was constant with 60-watt incandescent light bulbs.

A total of 840 1-day-old male Cobb broiler chicks were utilized from a commercial incubator, previously vaccinated against infectious bursal disease, Marek's disease, and Bouba's disease. The chicks were distributed in a completely random design in a 2x2 factorial scheme, with or without supplementation of citric acid and sodium benzoate with seven replications per treatment, resulting in 28 floor pens of $2.5m^2$, with 30 birds per floor pen (experimental unit), at initial density of 12 birds/m^2. The treatments were: basal feed (control treatment) - without addition of additives, basal feed + addition of 0.500% of citric acid, basal feed + addition of 0.242% of sodium benzoate, and basal feed + addition of a blend of citric acid and sodium benzoate (total of 0.742%

of acids). The addition of acids started from the substitution of inert material (kaolin). Both citric and sodium benzoate acids were in powder form and were incorporated to the feed at the time of processing.

The feeding program was divided into four phases: pre-starter, 1 to 7 days; starter, 8 to 21 days; grower, 22 to 33 days; and finisher, 34 to 42 days (Table 1). The diets were formulated with corn and soybean meal, according to the recommendations by Rostagno *et al*. (2011), without the addition of any type of antibiotic or anticoccidial. Water and feed were supplied *ad libitum*. Initial drinkers and feeders were utilized and substituted for bell drinkers at three days old, and for tubular feeders at 14 days old. The total period of poultry production was from 1 to 42 days old.

Table 1. Composition and calculated values of the experimental diets

Ingredients, %	Diets[1]			
	Pre-starter	Starter	Grower	Finisher
Corn	53.94	58.00	60.58	65.40
Soybean meal	38.37	34.97	31.78	27.53
Soybean oil	2.58	2.57	3.57	3.36
Choline chloride 60	0.072	0.064	0.058	0.043
Salt[2]	0.508/0.409	0.482/0.384	0.457/0.359	0.444/0.346
Dicalcium-phosphate	1.900	1.532	1.341	1.076
Limestone	0.918	0.908	0.820	0.766
L-lysine	0.284	0.212	0.187	0.229
DL-methionine	0.357	0.285	0.254	0.238
L-threonine	0.106	0.058	0.039	0.048
L-valine	0.075	0.024	0.015	0.030
Mineral premix[3]	0.050	0.050	0.050	0.050
Vitamin premix[4, 5, 6 and 7]	0.100	0.100	0.100	0.050
Kaolin[8]	0.742	0.742	0.742	0.742
Sum	100	100	100	100
	Calculated values			
AMEn, kcal/kg	2,950	3,000	3,100	3,150
CP, %	22.20	22.80	19.50	18.00
Lysine[9], %	1.310	1.174	1.078	1.010
Methionine[9], %	0.646	0.562	0.518	0.486
Methionine+cystine[9], %	0.944	0.846	0.787	0.737
Threonine[9], %	0.852	0.763	0.701	0.656
Valine[9], %	1.010	0.904	0.841	0.788
Calcium, %	0.920	0.819	0.732	0.638
Phosphorus[9], %	0.395	0.343	0.313	0.273
Sodium, %	0.220	0.210	0.200	0.195
Choline, mg/kg	375.0	330.0	300.0	225.0
Linoleic acid, %	2.73	2.77	3.32	3.26

[1]Pre-starter, 1 to 7 d-old; starter, 8 to 21 d-old; Grower, 22 to 33 d-old; Finisher, 34 to 42 d-old. [2]There was a reduction in the NaCl content of diets with inclusion of sodium benzoate to compensate the sodium present in the additive.[3]Mineral premix provided per kg of feed: Cu, 8; Fe, 50; Mn, 70; Zn, 50; I, 1.2; and Se, 0.2mg. [4]Vitamin premix provided per kg of feed in pre-starter diet (1–7 d): vitamin A, 8,000.00IU; vitamin D3, 2,400.00IU; vitamin E, 12.00IU; vitamin K3, 2.00mg; vitamin B1, 2.40mg; vitamin B2, 6.00mg; vitamin B6, 4.00mg; vitamin B12, 14.00mcg; folic acid, 1.00mg; pantothenic acid, 15.00mg; niacin, 40mg. [5]Vitamin premix provided per kg of feed in starter diet (8–21d): vitamin A, 7,000.00IU; vitamin D3, 2,200.00IU; vitamin E, 11.00IU; vitamin K3, 1.60mg; vitamin B1, 2.00mg; vitamin B2, 5.00mg; vitamin B6, 3.00mg; vitamin B12, 12.00mcg; folic acid, 0.80mg; pantothenic acid, 13.00mg; niacin, 35mg.[6]Vitamin premix provided per kg of feed in grower diet (22–33d): vitamin A, 6,000.00IU; vitamin D3, 2,000.00IU; vitamin E, 10.00IU; vitamin K3, 1.60mg; vitamin B1, 1.40mg; vitamin B2, 4.00mg; vitamin B6, 2.00mg; vitamin B12, 10.00mcg; folic acid, 0.60mg; pantothenic acid, 11.00mg; niacin, 30mg.[7]Vitamin premix provided per kg of feed in finisher diet (34–42d): vitamin A, 5,000.00IU; vitamin D3, 1,000.00IU; vitamin E, 8.00IU; vitamin K3, 1.60mg; vitamin B2, 2.00mg; vitamin B12, 5.00mcg; pantothenic acid, 9.00mg; niacin, 20mg.[8]Treatments were obtained by replacing kaolin with additives: Diets with citric acid, 0.500%; diets with sodium benzoate: 0.242%; diets with citric acid + sodium benzoate: 0.742%.[9]Digestible values.

In order to provide microbiological challenge to the birds, at 14 days old, 1mL of solution containing 1×10^5 of sporulated oocysts of *E. acervulina* was inoculated per bird. The broilers were manually contained and orally inoculated using an automatic pipette. The oocysts were acquired from the Laboratory of Molecular Biology of the School of Veterinary Medicine of USP – campus of São Paulo.

The performance data were obtained for the accumulated periods of 1 to 21 and 1 to 42 days old. They were: body weight (weight of the birds for each floor pen in the housing, at 21 and at 42 days old), weight gain (difference between the weight at the end of each period and the initial weight in the housing), feed intake (difference between the total provided feed and the leftovers collected at the end of each period, based on the average number of chickens), feed to gain ratio (ratio between the total consumed feed and the weight gain, corrected by the weight of the dead chickens), viability (100 – mortality that was recorded daily and expressed in percentage, by the relation between the initial number of birds and the number of dead birds in the period), and production factor (average daily weight gain × viability) / feed to gain ratio) / 10).

At 21 and 42 days old, seven birds per treatment were withdrawn (one per replication) and slaughtered by cervical dislocation followed by bleeding to determine the relative weight of the organs, the length of the small and large intestines, and the pH of the intestinal content in order to understand the mechanisms that affect the digestion of nutrients in the evaluated diets. The following organs were collected and weighed: spleen, pancreas, gizzard, proventriculus, liver, small intestine and large intestine. The relative weight of each organ was determined in relation to the birds' weight. The measurement of the small intestine was done where the duodenum emerges from the gizzard up to the beginning of the cecum. The measurement of the large intestine was calculated by the colon and rectum length added to the result of the caecum length.

To determine pH, 1g of the intestinal content from the initial portion of the jejunum was collected and mixed to 30mL of distilled water in plastic flasks (universal collector). The flasks were agitated and left to rest for a minute and next the reading was done using a portable pH meter (Coon *et al*.; 1990).

These microbiological analyses were done in the Laboratory of Animal Hygiene of São Paulo University - USP - campus of Pirassununga. At 42 days, a second bird was sacrificed to collect samples from the intestinal content to perform the microbiological analysis in the GIT. The samples were collected from the birds' jejunum and kept under refrigeration and they were posteriorly analyzed regarding the presence of total enterobacteria, gram-positive cocci and total anaerobes, according to the method described by Danicke *et al*. (1999).

Data analysis was done by Statistical Analysis System software (SAS/STAT, 2008). Firstly, the analyses of residue normality and variance homogeneity were done. Following the prerequisites, the data were submitted to analysis of variance (ANOVA) with probability at 5% of significance. When needed, the differences between the treatments were studied by comparing the averages of the minimum squares calculated by LSMEANS command with Tukey's test.

RESULTS AND DISCUSSION

The initial average weight of the chicks was 41.90g. There was no effect of the treatments on the performance of broiler chickens in the period of 1 to 21 days old (Table 2).

The antimicrobial efficiency of the acid depends on its dissociation constant (pKa). This value represents the pH point of the means in which there is balance between the dissociated and non-dissociated form of the acid. According to Partanen's adapted table (2002), the citric and benzoic acids are considered acids with average pKa with values of 3.13/4.76/6.40 for citric acid and 4.19 for the benzoic acid. For example, in a medium with pH 4.19, the benzoic acid will be half in the dissociated form and half in the non-dissociated one. Therefore, the greater the pKa of an acid is, the more efficient it will be.

Table 2. Performance of broilers at 21-d-old fed diets with or without organic acids

Variables[1]	Citric Acid	Benzoic Acid -	Benzoic Acid +	Means	Probability Citric	Probability Benzoic	Probability Interaction	CV[2]
BW gain	-	871	861	866	0.0511	0.6167	0.1363	2.90
	+	838	857	847				
	Means	854	859					
FI	-	1,032	1,033	1,032	0.2254	0.6963	0.7381	2.67
	+	1,015	1,023	1,019				
	Means	1,024	1,028					
F:G	-	1.188	1.207	1.197	0.0561	0.6874	0.1510	2.28
	+	1.222	1.212	1.217				
	Means	1.205	1.209					
L	-	98.59	98.90	98.74	0.6121	0.2039	0.1017	2.16
	+	99.53	97.14	98.34				
	Means	99.06	98.02					

[1]BW, body weight (g); FI, feed intake (g); F:G feed:gain (g:g); L, livability (%). [2]CV, coefficient of variation (%).

However, despite the characteristics that the citric and benzoic acids present, it was not possible to observe an increment in the initial performance. The absence of interaction also indicated that there was no potentiation with the combination of both acids. In the literature there is evidence that the organic acids included in the isolated form do not produce an effect on the performance, only the *blend* of several acids improved the feed to gain ratio (Polycarpo *et al*.; 2017).

It is possible that the action of several acids together present synergy among themselves, although in this study this was not observed. Thus, it is suggested that new combinations be evaluated, testing blends that contain more than two acids.

The results found here corroborate previous studies that did not observe improvement in broilers' performance with acid blends. Salazar *et al*. (2008) evaluated the isolated and combined use of butyric and lactic acids and did not observe increment of the broilers' performance. Vale *et al*. (2004) evaluated doses of the blend of formic and propionic acids and observed a decrease in the body weight (worsening of the performance), probably due to the feed intake reduction with high doses of acids.

Regarding the dose of organic acids, it is an important point since high doses damage the birds' intake and consequently their performance (Leeson *et al*.; 2005; Józefiak *et al*.; 2007; Islam *et al*.; 2008; Khosravinia *et al*.; 2015; Khan and Iqbal, 2016).

In studies carried out with isolated acids, it was also verified that acids do not affect performance. Campos *et al*. (2004) did not observe the benefits of the increasing doses of fumaric acid in low energy diets for broilers. Leeson *et al*. (2005) did not verify an improvement in the performance with increasing doses of butyric acid. The performance results of the period of 1 to 42 days are shown in Table 3.

There was interaction of the citric acid with the benzoic acid on the feed intake (FI) (Figure 1). In the interaction study, it is observed that the association of both acids increased the feed intake regarding the isolated use. However, despite the greater intake, the weight gain and feed to gain ratio were not altered by the treatments.

The increase of feed intake can be interpreted as something positive when it is followed by the increase in weight gain, but when it does not change, the increase in the feed intake is not interesting anymore because the cost with feed increases and the production does not alter.

Table 3. Performance of broilers at 42-d-old fed diets with or without organic acids

Variables[1]	Citric Acid	Benzoic Acid -	Benzoic Acid +	Means	Probability Citric	Probability Benzoic	Probability Interaction	CV[2]
	-	2,648	2,686	2,667				
BW gain	+	2,625	2,656	2,641	0.5371	0.4206	0.9370	4.07
	Means	2,637	2,671					
	-	3,800Aa[3]	3,811Ba	3,806				
FI	+	3,763Ab	3,906Aa	3,835	0.2296	0.0033	0.0101	2.09
	Means	3,781	3,859					
	-	1.627	1.628	1.628				
F:G	+	1.618	1.673	1.645	0.4792	0.2768	0.2892	3.98
	Means	1.623	1.650					
	-	80.49	77.22	78.85				
L	+	82.39	79.98	81.18	0.2280	0.1460	0.8200	6.07
	Means	81.44	78.60					
	-	312.3	296.9	304.6				
PEI	+	318.9	290.2	304.6	0.9993	0.1648	0.6706	13.18
	Means	315.6	293.6					

[1]BW, body weight (g); FI, feed intake (g); F:G feed:gain (g:g); L, livability (%); PEI, index of production efficiency (daily weight gain*L)/F:G ratio)/10). [2]CV, coefficient of variation (%). [3]Different letters, lowercase on the lines and capital on the columns, differ by Tukey test (P<0.05).

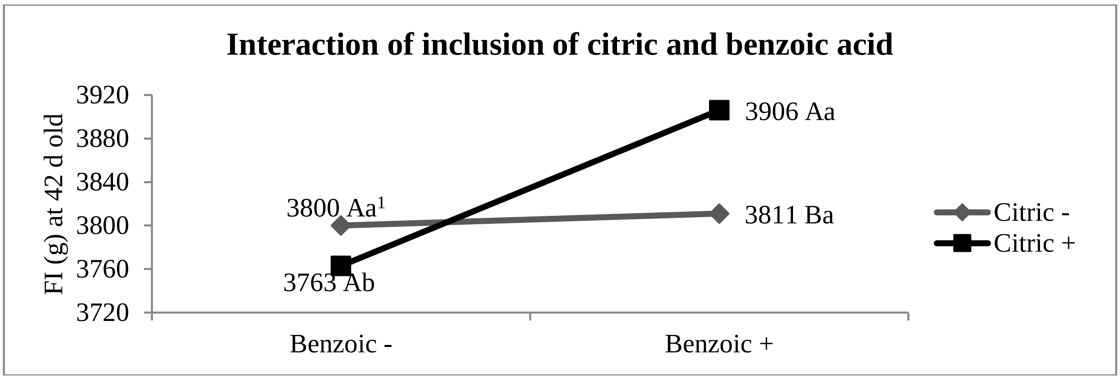

Figure 1. Interaction effects of citric and benzoic acid for feed intake at 42 d-old.
[1]Different letters, capital on the column and lowercase on the line, differ by Tukey test (P<0.05).

Garrido *et al*. (2004) worked with a blend of two acids (formic + propionic), and in the grower phase observed an increase in feed intake without change in the weight gain. Ribeiro *et al*. (2008) evaluated a blend of lactic, fumaric and citric acids and did not observe an improvement in the performance. The absence of effect of organic acids can be a result of a series of factors, among them the microbial challenge is a fundamental point to be considered.

Because the benefits of organic acids are mainly related with its antimicrobial effect, it is necessary that there be a microbiological challenge in the experiment for an appropriate evaluation. Since 1950, when the evaluations with antibiotics as growth promoters started, the need for a microbial challenge was evident in Lillie *et al*. (1953) and Coates *et al*. (1963), because there is no meaning in evaluating an antimicrobial in an environment without or with little microorganism challenge. In this study, the broilers were challenged with *E. acervulina*, which causes coccidiosis that is one of the diseases that most causes losses to poultry production. However, facing the results observed in this study, it can be inferred that the absence of increment in the performance by organic acids can be a result of the low bactericidal activity against this pathogen. *Eimeria* is a protozoa that parasites the intestinal region and it is possible that the evaluated acids reached this region partially or even totally dissociated, which can explain the absence of results.

The data of viability and index of production efficiency were not influenced by the treatments. The low average values of these two variables related to performance occurred due to the birds' high mortality. This occurred because of the high temperatures recorded in the season of the year in which the experiment was developed (summer), which generated caloric stress and, consequently, an increase in the birds' mortality.

The allometry results of GIT organs are shown in Table 4 and 5. At 21 as well as at 42 days old, the addition of organic acids do not change the relative weight of organs and the length of intestines. Although organic acids have several additional effects that go beyond those of antibiotics, such as reduction in digesta pH, increased pancreatic secretion, and trophic effects on the gastrointestinal mucosa (Dibner and Buttin, 2002), in this study, alterations on the weight and length of broilers' intestine were not observed.

On the other hand, Viola *et al*. (2008) found greater relative weight and length of the small intestine in diets containing blends of organic acids. In rats, it was demonstrated that the presence of short-chain acids stimulate the release of mucus in the colon (Shimotoyodome *et al*.; 2000). The release of alkaline mucus in the intestine seems to be associated to damages that acids cause in the mucosa (Vattay *et al*.; 1988). The effect of acids, therefore, can attack the intestinal mucosa and trigger defense reactions, which alter the structure and consequently the intestine' size, although the results observed here do not make evident the effects on the weight and length of the intestines.

Table 4. Organ relative weight and small and large intestine length of broilers at 21-d-old fed diets with or without organic acids

Variables[1]	Citric Acid	Benzoic Acid		Means	Probability			CV[2]
		-	+		Citric	Benzoic	Interaction	
	-	0.105	0.112	0.108				
Spleen	+	0.098	0.109	0.104	0.5321	0.2319	0.7801	25.79
	Means	0.102	0.110					
	-	0.415	0.360	0.387				
Pancreas	+	0.360	0.368	0.364	0.3530	0.3428	0.2093	24.81
	Means	0.387	0.364					
	-	2.21	2.35	2.28				
Gizzard	+	2.32	2.21	2.27	0.8349	0.7748	0.0820	11.15
	Means	2.27	2.29					
	-	2.71	2.93	2.82				
Liver	+	2.91	2.83	2.87	0.5421	0.4512	0.0829	11.09
	Means	2.81	2.88					
	-	0.575	0.564	0.570				
Proventriculus	+	0.525	0.504	0.514	0.0882	0.6170	0.8681	22.06
	Means	0.550	0.534					
	-	7.10	7.78	7.44				
SI (%)	+	8.04	7.73	7.89	0.0767	0.4582	0.0502	12.27
	Means	7.57	7.75					
	-	1.64	1.81	1.73				
LI (%)	+	1.87	1.62	1.74	0.9141	0.7344	0.0942	25.87
	Means	1.76	1.72					
	-	151.50	159.21	155.36				
SI (cm)	+	151.79	155.92	153.86	0.7390	0.1912	0.6916	10.74
	Means	151.64	157.57					
	-	39.14	42.93	41.04				
LI (cm)	+	41.14	41.00	41.07	0.9871	0.4096	0.3741	19.69
	Means	40.14	41.96					

[1]SI, small intestine; LI, large intestine. [2]CV, coefficient of variation (%).

Table 5. Organ relative weight and small and large intestine length of broilers at 42-d-old fed diets with or without organic acids

Variables[1]	Citric Acid	Benzoic Acid		Means	Probability			CV[2]
		-	+		Citric	Benzoic	Interaction	
	-	0.088	0.080	0.084				
Spleen	+	0.090	0.090	0.090	0.3375	0.5405	0.5177	25.96
	Means	0.089	0.085					
	-	0.200	0.192	0.196				
Pancreas	+	0.198	0.186	0.192	0.7742	0.4708	0.9186	18.90
	Means	0.199	0.189					
	-	1.29	1.27	1.28				
Gizzard	+	1.29	1.30	1.29	0.7408	0.8919	0.7520	12.30
	Means	1.29	1.28					
	-	1.81	1.74	1.77				
Liver	+	1.71	1.78	1.74	0.6127	0.9789	0.2361	12.14
	Means	1.76	1.76					
	-	0.26	0.26	0.26				
Proventriculus	+	0.25	0.26	0.26	0.5244	0.7035	0.8221	10.98
	Means	0.26	0.26					
	-	4.58	4.04	4.30				
SI (%)	+	4.27	4.54	4.41	0.7561	0.6810	0.2050	18.71
	Means	4.42	4.29					
	-	0.88	0.79	0.83				
LI (%)	+	0.87	0.87	0.87	0.4908	0.3798	0.2957	22.11
	Means	0.87	0.83					
	-	189.14	192.00	190.57				
SI (cm)	+	195.29	200.00	197.64	0.3087	0.5828	0.8925	8.99
	Means	192.21	196.00					
	-	45.29	43.50	44.39				
LI (cm)	+	43.50	44.50	44.00	0.7791	0.7791	0.3221	11.60
	Means	44.39	44.00					

[1]SI, small intestine; LI, large intestine. [2]CV, coefficient of variation (%).

In the analyses of pH at 21 and 42 days, the effect of organic acids was not observed (Table 6). The measurement of intestinal pH was done in the jejunum because most of the nutrients are absorbed in this segment.

The absence of effect of acids on the pH confirms the hypothesis that acids be dissociated in the proximal parts of GIT, not influencing the intestine. El-Ghany *et al*. (2016) found a reduction in the pH values of intestines with the utilization of organic acids, and as a result, bacterial growth was disturbed.

The greater amount of combined acids may have favored the selection of acid pH microorganisms in the proximal portions, reflecting in the microbial population and lower pH values in most distal regions of the intestine.

There was no interaction of acids on the total counting of the bacteria in the samples from the jejunum to 42 days (Table 7). The citric acid, regardless of being associated to benzoic acid, favored the increase of gram-positive cocci and total anaerobes in the jejunum. The benzoic acid did not change the results of the microbiological analyses. There was not growth and formation of total enterobacterial colonies, but it is important to emphasize that the lack of enterobacterial colonies does not mean that bacteria of this kind were not found in the birds' intestine. These results indicated that the amount of total enterobacteria was low, not reaching the limit for analysis detection.

Table 6. Intestinal pH of broilers at 21 and 42-d-old fed diets with or without organic acids

Variables	Citric Acid	Benzoic Acid		Means	Probability			CV[1]
		-	+		Citric	Benzoic	Interaction	
pH 21dias	-	6.39	6.51	6.45	0.2988	0.1872	0.3767	2.22
	+	6.38	6.41	6.39				
	Means	6.39	6.46					
pH 42dias	-	6.93	7.00	6.96	0.9893	0.8296	0.0974	1.30
	+	6.99	6.94	6.96				
	Means	6.96	6.97					

[1]CV, coefficient of variation (%).

Table 7. Total count of bacterial in jejunum from broilers at 42-d-old fed diets with or without organic acids

Bacteria[1] (CFU g/ jejunum)	Citric Acid	Benzoic Acid		Means	Probability			CV[2]
		-	+		Citric	Benzoic	Interaction	
GPC	-	3.21×10^2	3.67×10^2	3.42×10^2	0.0019	0.064 1	0.1036	68.05
	+	6.50×10^2	13.07×10^2	10.04×10^2				
	Means	4.73×10^2	8.73×10^2					
TA	-	27.14×10^2	44.71×10^2	35.93×10^2	0.0279	0.3993	0.1422	43.16
	+	55.71×10^2	50.83×10^2	53.46×10^2				
	Means	41.43×10^2	47.54×10^2					

[1]CFU/g, colony forming units per g of sample. Data submitted to log transformation; GPC, gram-positive cocci; TA, total anaerobes. [2]CV, coefficient of variation (%).

The results on the intestinal microbiota contradict the ones found by Polycarpo *et al*. (2016), who did not observe alterations of gram-positive cocci and total anaerobes with addition of organic acids to the diets. Aydin *et al*. (2010) observed that the addition of 3% of citric acid to the diet did not alter the intestinal microbiota, and also argued that this result is due to the action mode of citric acid, which mainly occurs in the upper part of GIT. The divergence between the results of this study and the ones found in the literature, suggests that more research be done to extend the reflections of citric acid supplementation in broilers' intestinal microbiota.

CONCLUSION

Citric and benzoic acids, isolated or combined, do not provide improvement in the performance of broiler chickens. The feed intake increases with the inclusion of the blend of citric and benzoic acids, without increment in the weight gain. Citric and benzoic acids do not modify the allometry of the digestive system organs as well as the intestinal pH. Only the citric acid favors greater concentrations of gram-positive bacteria and total anaerobes in chicken's jejunum. With this study, it was concluded that citric and benzoic acids do not benefit the nutrition of broilers challenged with *E. acervulina*.

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