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Effects of Phytogenic Additives and Organic Acids, alone or in combination, on the Performance, Intestinal Quality and Immune Responses of Broiler Chickens

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ABSTRACT

The present study was conducted to evaluate the effects of phytogenic additives (PA) and organic acids (OA), alone or in combination, on the performance, intestinal histomorphometry and lipid oxidation, and immune responses of broiler chickens. In this experiment, 820 one-day-old chicks were distributed according to a completely randomized design in a 2 × 2 + 1 factorial arrangement, with four replicates of 41 broilers each. The dietary treatments consisted of a control diet with no PA or OA (CD); CD with OA and no PA (CD+OA-PA); CD with PA and no OA (CD+PA-CD); CDwith both PA and OA (CD+PA+CD); and CD + avilamycin + monesin sodium. Broiler performance was not affected by the alternative feed additives, except from 1 to 21 days, when broilers fed the CD or CD+PA+OA diets showed higher body weight gain than those fed the CD with only OA. The broilers fed the diet containing avilamycin and monensin presented better performance. The supplementation of PA and OA increased bursalcortical area on21 and 42 days post-hatch. On 21 days post-hatch, broilers fed the AGP diet presented higher ileal villus height than those fed the control diet. The pH values of the jejunum content were reduced on the OA-fed chickens. Higher villus height and crypt depth were found in the alternative additive-fed chickens on 7 days post-hatch. On 42 days post-hatch, the percentage of the bursal cortex increased in PA-fed broilers; however, there was no increase in antibody production. The PA-fed chickens presented lower thiobarbituric acid reactive substances (TBARS) values in the small intestine. The dietary supplementation of phytogenic additives, individually or in combination associated with organic acids, does not affect broiler live performance or intestinal histomorphometry; however, it enhances immune responses and intestinal quality.

INTRODUCTION

The increasing demand for poultry meat in the world market has led poultry producers to use their facilities to their maximum capacity by applying high stocking densities, which in turn may increase the health challenge of these broiler flocks (Heckert *et al*., 2002; Muniz *et al*., 2006). High stocking density combined with stressful environmental factors can negatively influence the immune system of birds, increasing their susceptibility to pathogens, reducing vaccine responses, and resulting in higher carcass condemnation rates. Consequently, there is an increased health challenge, resulting in a higher usage of antibiotic growth promoters (AGP) and coccidiostats in order to improve the growth performance and suppress pathogen replication in the gut of birds.

In some countries, due to the human health concern on the use of antimicrobial agents and their effects on antimicrobial resistance in humans, certain AGP have already been banned, and there is a possibility of future restrictions on their use worldwide; therefore, non-antibiotic alternatives to control diseases and promote broiler growth, such as organic acids (Vieira *et al*., 2008), probiotics (Mountzouris *et al*., 2010), prebiotics (Patterson & Burkholder, 2003), phytogenic additives (Hernández *et al*., 2004), and essential oils (Basmacioğlu Malayoğlu *et al*., 2010),are of great interest. The use of phytogenic additives (PA) in poultry feeds has been investigated relative to their stimulation of digestive enzymes (Basmacioğlu Malayoğlu*et al*., 2010), antimicrobial activity (Mitsch *et al*., 2004), antifungal activity (Gowda*et al*., 2004), antiparasitical activity (Giannenas *et al*., 2003), antioxidant effects (Ciftci *et al*., 2010; Zhang *et al*., 2013), and immuno stimulant action (Chen *et al*., 2003).

Organic acids are added to poultry feeds to reduce pathogenic microbiota, although its use as an alternative to AGP has shown inconsistent effects on broiler growth performance. While some authors have demonstrated that organic acids may replace AGPs, others did not find any effects of dietary organic acids on performance. Organic acids can reduce gut pH, thereby suppressing pathogenic bacteria proliferation (Ao *et al*., 2009), which improves gut health and nutrient intake. In an undissociated form, organic acids can pass through bacteria and fungi walls, reducing intracellular pH, and causing their death (Ricke, 2003). Organic acids can also stimulate pancreatic juice secretion and improve gut morphology, such as villi height increase (Dibner & Buttin, 2002).

The aim of this study was to evaluate the effect of phytogenic additives and organic acids, individually or combined, on the intestinal histomorphometry and lipid oxidation, the immune-system responses, as well as on the performance and health of broiler chickens.

MATERIAL AND METHODS

All procedures were approved by the Ethics Committee on Animal Use (CEUA) of College of Veterinary Medicine and Animal Science, UNESP, under protocol number 183/2008-CEEA.

Experimental design, birds, and diets

A total of 820 one-day-old male Cobb 500 chicks, with average initial weight of 44 g ± 0.6 g, were allotted to a conventional house, which floor pens were covered with wood-shavings litter (reused from two previous broiler flocks and untreated), and equipped with a tube feeder and a bell drinker. The birds were vaccinated in the hatchery against Marek's disease, infectious bursal disease, and fowl pox. At 10 days post-hatch, the chicks were vaccinated against Newcastle disease (LaSota strain), and at 14 days post-hatch, they received another dose of vaccine against infectious bursal disease via drinking water.

The experimental design was completely randomized in a factorial arrangement with an additional treatment (2 × 2 + 1) (with or without phytogenic additives × with or without organic acids + control diet with AGP and coccidiostat). The phytogenic additive (PA) (Imunostart®+ Enterocox® - Phytosynthese) used was composed of turmeric extract (*Curcuma longa*), citrus extract, grape seed extract (*Vitis sp*.), Chinese cinnamon essential oil (*Cinnamomum zeylanicum*), Chilean boldo leaves (*Peumus boldus*),and fenugreek seeds (*Trigonella foenum-graecum*). The organic acid (OA) blend (Premium Sal-Ácido 8® - Nutriacid) was composed of 30% of lactic acid, 25% of benzoic acid, 7% of formic acid, 8% of citric acid, and 6.5% of acetic acid. The dietary inclusion levels of PA were: Imunostart®, 700 grams per ton of feed (1 to 10 days), 500 grams per ton of feed (11 to 21 days); Enterocox®, 300 grams per ton of feed (1 to 10 days), 1,000 grams per ton of feed (11 to 35 days) and 500 grams per ton of feed (36 to 42 days). The OA blend was included at 3.5 kilograms per ton of feed (1 to 21 days), 2.5 kilograms per ton of feed (22 to 35 days) and 1.5 kilograms per ton of feed (36 to 42 days). Avilamycin (10 grams per ton of feed) plus monensin sodium salt (100 grams per ton of feed) were used as AGP and coccidiostat, respectively.

The dietary treatments consisted of control diet (CD) -PA -OA; CD +OA -PA; CD +PA -OA; CD +PA+OA; and CD + avilamycin + monesin sodium. The diets were formulated based on corn and soybean meal and in order to meet or exceed the nutritional requirements recommended by Rostagno *et al.* (2005) (Table 1). The feed additives tested were included in the diets to replace inert material.

Growth performance, organ size and pH of jejunal digesta

Body weight gain, feed intake, and feed conversion ratio were determined on 21 and 42 days post-hatch, and mortality was monitored daily.

On 21 and 42 days post-hatch, two birds with pen's average weight were taken from each experimental unit, weighed, and, after fasting for two hours, were sacrificed by cervical dislocation in order to determine

Table 1 – Composition and nutritional levels of the experimental diets (as-fed basis).

Ingredients, g/kg	Pre-starter		Starter		Grower		Finisher	
	Control[1] or AGP or PA	OA[2] or OA plus PA	Control[1] or AGP or PA	OA[2] or OA plus PA	Control[1] or AGP or PA	OA[2] or OA plus PA	Control[1] or AGP or PA	OA[2] or OA plus PA
Corn	559.6	556.7	569.3	566.2	598.6	597.3	643.2	642.4
Soybean meal	373.2	373.5	355.5	356	319.5	319.7	278.7	278.8
Limestone	9.4	8.4	9.0	8.0	8.5	7.8	8.1	7.7
Dicalcium phosphate	19.5	19.5	18.4	18.4	17.0	17.0	15.4	15.4
Soybean oil	22.3	22.8	34.7	35.3	44.2	44.3	42.9	43.0
DL-Methionine	2.3	2.4	1.7	1.7	1.6	1.6	1.6	1.6
L-lysine	3.7	3.7	2.1	2.1	2.0	2.0	2.6	2.6
L-threonine	1.5	1.5	0.6	0.6	0.5	0.5	0.7	0.7
Choline chloride	0.6	0.6	0.5	0.5	0.5	0.5	0.4	0.4
Sodium bicarbonate	0.8	0.3	0.5	---	0.1	---	---	---
Salt	4.6	4.6	4.7	4.7	4.7	4.5	4.6	4.4
Inert material (kaolin)	1.0	4.5	1.5	5.0	1.5	3.5	0.8	2.0
Vitamin premix[3,4,5]	1.0	1.0	1.0	1.0	0.8	0.8	0.5	0.5
Mineral premix[6]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Calculated nutrient composition								
AME, kcal/kg	2960	2960	3050	3050	3150	3150	3200	3200
CP	221.1	221.1	211.4	211.4	197.3	197.3	183.1	183.1
Digestible Lys	13.6	13.6	11.9	11.9	11.0	11.0	10.5	10.5
Digestible Met	5.4	5.4	4.6	4.6	4.4	4.4	4.2	4.2
Digestible TSAA	8.4	8.4	7.5	7.5	7.1	7.1	6.8	6.8
Digestible Thr	8.8	8.8	7.7	7.7	7.1	7.1	6.8	6.8
Digestible Trp	2.4	2.4	2.3	2.3	2.1	2.1	1.9	1.9
Linoleic acid	24.8	24.9	31.5	31.7	36.9	36.9	36.7	36.8
Calcium	9.4	9.0	9.0	8.6	8.4	8.1	7.7	7.6
Av.Phosphorus	4.7	4.7	4.5	4.5	4.2	4.2	3.9	3.9
Potassium	8.4	8.4	8.1	8.1	7.5	7.5	6.9	6.9
Sodium	2.2	2.0	2.2	2.0	2.1	2.0	2.0	1.9
Chloride	3.2	3.2	3.2	3.2	3.2	3.1	3.1	3.1

[1]Control diet; AGP, antibiotic growth promoter (avilamycin and monensin); PA, phytogenic additive; OA, organic acids.

[2]Nutritional matrix of organic acids applied (Premium Sal-Ácido 8: AME: 1200 kcal/kg; Calcium: 11%; Sodium: 4,5%).

[3]Supplied per kilogram of feed, in pre-starter and starter phases: vit. A, 11,000 IU; vit. D_3, 2,000 IU; vit. E, 16 mg; folic acid, 0.4 mg; pantothenic acid, 10 mg; biotin, 0.06 mg; niacin, 35 mg; vit. B6, 2 mg; riboflavin, 4.5 mg; thiamine, 1.2 mg; vit. B12, 16 µg; Vit. K3, 1.5 mg.

[4]Supplied per kilogram of feed, in grower phase: vit. A, 8,800 IU; vit. D_3, 1,600 IU; vit. E, 12.8 mg; folic acid, 0.32 mg; pantothenic acid, 8 mg; biotin, 0.048 mg; niacin, 28 mg; vit. B6, 1.6 mg; riboflavin, 3.6 mg; thiamine, 0.96 mg; vit. B12, 12.8 µg; Vit. K3, 1.2 mg.

[5]Supplied per kilogram of feed, in finisher phase: vit. A, 3,000 IU; vit. D_3, 500 IU; vit. E, 5 mg; pantothenic acid, 4 mg; biotin, 0.015 mg; niacin, 5 mg; vit. B6, 0.4 mg; riboflavin, 1 mg; thiamine, 0.3 mg; vit. B12, 3 µg; vit. K3, 0.5 mg.

[6]Supplied per kilogram of feed: copper, 9 mg; zinc, 60 mg; iodine, 1 mg; iron, 30 mg; manganese, 60 mg; selenium, 0.25 mg.

the weights of the proventriculus plus gizzard, small and large intestines, liver, pancreas, spleen, thymus and bursa of Fabricius relative to whole body weight, and intestinal length.

The pH of the jejunal content was determined on 21 and 42 days post-hatch. Jejunal content was placed in a beaker with 30 mL of distilled water (standard pH = 7.0), resulting in a total volume of 60 mL, shaken, and allowed to stand for two hours at 8° C. The pH was measured using a standard pH meter (Marconi Model MA522), according to Coon *et al.* (1990).

Histological analysis

For histomorphometric analysis, small segments (~2 cm) of the duodenum, jejunum, and ileum were collected from the two birds per pen sacrificed on

day 21 post-hatch. Samples were fixed in Bouin's solution for 24 hours and then stored in 70% ethanol. The bursa of Fabricius from both birds per pen was collected and fixed in 10% neutral buffered formalin for further preparation of histological slides on 21 and 42 days post-hatch.

Intestinal and bursal tissue slides were examined by optical microscopy, at 10x magnification, and images were captured by a camera attached to the microscope and transferred to an image analyzer software (Leica®). Twenty readings of villus height and crypt depth were made per chick and per intestinal segment. Villus height was measured from the apical to basal region, which corresponds to the upper portion of the crypts. Crypt depth was measured from the base to the transition region between the crypt and villi.

The cortical lymphoid follicle area of the bursa was analyzed from a total of 12 full follicles per slide and only slides containing central-region follicles were considered for measurement. The selected follicles were surrounded by a line providing the total follicular area. Afterwards, the medullary portion of the same follicle was determined by drawing a line on the basal membrane that divides the cortical from the medullary area to calculate the percentage of follicular cortex.

Immunological parameters

On 21 and 42 days post-hatch, 5 mL of blood were collected from two birds per pen by ulnar vein puncture for the evaluation of serum titers of antibodies against Newcastle disease virus (NDV). The blood samples were placed in tubes, left at rest for clot formation, and subsequently centrifuged to separate the serum, which was collected and stored in 1.5 mL microtubes. The production of NDV antibodies was analyzed by an enzyme linked immunosorbent assay (ELISA) kit (Enzyme-Linked Immunosorbent Assay - Idexx®), in accordance to the methodology described by Purchase *et al*. (1989).

On 42 days post-hatch, blood samples were collected from one bird per pen and a total of 200 leukocytes were counted to determine the heterophil:lymphocyte (H:L) ratio.

Lipid oxidation of intestinal samples

In order to quantify the level of lipid oxidation in the small intestine, one bird per pen was sacrificed on 21 and 42 days post-hatch. The value of malonaldehyde (MDA) was determined according to the modified method described by Madsen *et al*. (1998). A 10-g sample of the small intestine was homogenized for 1 min in an Ultra-Turrax mixer with 50 mL of trichloroacetic acid solution (7.5%). Then, the mixed sample was filtered, and a 5 mL fraction was mixed with 5 mL of 2-thiobarbituric acid solution (0.02 mol/L) and placed in a water bath (100° C) for 10 minutes. The absorbance of samples was measured at 532 nm using a spectrophotometer. The determination of MDA was performed in duplicate and expressed in mg of MDA per kg of intestine, using a standard curve (concentration range from 0.1 nmol/L to 6 nmol/L) prepared with 1,1,3,3-tetraethoxypropane (Merck).

Statistical analysis

The data were analyzed by analysis of variance with two factors and additional treatment, and they were complemented by F test ($\alpha = 0.05$) for the treatment means of the factorial arrangement (2 × 2). In order to compare the factorial dietary treatment means with the additional treatment, the Dunnett test was performed ($\alpha = 0.05$) using the General Linear Model Procedure (GLM) of SAS statistical software (2002). Antibody against NDV data were transformed by $\log_2$ before analysis.

RESULTS

Growth Performance

On 21 days post-hatch, the chickens fed the diets containing OA showed lower body weight gain ($p<0.01$) compared with those fed the diets with or without the inclusion of both feed additives (PA and OA) (Table 2). The diet containing AGP promoted higher body weight gain when compared with the OA-supplemented diets. There was no interaction between PA and OA on broiler performance at 42 days post-hatch ($p>0.05$). Broilers fed the AGP diet showed higher bodyweight gain on 42 days post-hatch and better feed conversion ratio on 21 days post-hatch ($p<0.05$) compared with those fed diets with the alternative feed additives (PA or OA).

Organ size and jejunal digesta pH

There was an interaction between PA and OA on intestinal and pancreas weights at 21 days post-hatch ($p<0.05$) (Table 3). Broilers fed the diet with the PA and OA combination presented lower relative weights of the intestine and pancreas than those the OA diets. The combination of feed additives resulted in lower relative pancreas weight than the diets with no OA. The evaluated alternative feed additives did not affect the relative weights of the other organs on 21 days post-hatch.

Table 2 – Performance of broilers fed diets supplemented with phytogenic additives (PA) and organic acids (OA) in replacement of antibiotic growth promoters (AGP).

PA	OA	Body weight gain, g		Feed intake, g		FCR, g/g		Livability, %	
		1 - 21 d	1 - 42 d	1 - 21 d	1 - 42 d	1 - 21 d	1 - 42 d	1 - 21 d	1 - 42 d
Interaction effects									
-	-	873a	2447γ	1182	4038	1.30γ	1.70	98.9	97.8
-	+	855b,γ	2508γ	1154	4036	1.30γ	1.68	95.1	91.1
+	-	871ab	2518γ	1176	3993	1.29γ	1.66	97.6	92.7
+	+	885a	2504γ	1177	4000	1.30γ	1.68	96.0	88.7
AGP[1]		891	2648	1160	4097	1.26	1.64	95.7	90.3
Main effects									
	-	872	2483	1179	4016	1.29	1.68	98.2	95.2
	+	870	2506	1166	4018	1.30	1.68	95.5	89.9
-		864	2478	1168	4037	1.30	1.69	97.0	94.4
+		878	2511	1177	3997	1.29	1.66	96.7	90.7
Source of variation									
PA		0.017	0.116	0.573	0.401	0.219	0.159	0.865	0.160
OA		0.681	0.245	0.353	0.953	0.751	0.870	0.110	0.054
PA × OA		0.007	0.087	0.320	0.929	0.529	0.206	0.503	0.602
AGP		0.008	<0.001	0.500	0.523	0.012	0.069	0.437	0.252
SEM		3.797	17.951	5.775	2.275	0.004	0.007	0.692	1.344

[a-b]Means followed by different letters within the same column are statistically different ($p<0.05$).

[γ]Differ from diet with antibiotic growth promoter (AGP) ($p<0.05$).

SEM, standard error of means.

[1]Avilamycin and monensin sodium.

The relative weights of proventriculus plus gizzard, liver, bursa of Fabricius, and thymus were not affected ($p>0.05$) by the dietary treatments on 21-d and 42-d days post-hatch (data not shown).

There was no interaction between PA and OA dietary supplementation on the relative weights of digestive organs on 42 days post-hatch (Table 3). The dietary inclusion of PA resulted in higher relative intestine weight, and OA supplementation increased pancreas relative weight. There was an interaction ($p<0.05$) between PA and OA on the small intestine length of broilers on 42 days post-hatch. The broilers fed PA presented the longest intestine. On 21 days post-hatch, chickens fed diets with OA presented higher relative intestinal weight ($p<0.05$) than those fed the diet containing AGP, and on 42 days post-hatch, PAsupplementation increased ($p<0.01$) intestinal weight compared with the AGP diet, whereas the non-supplemented diet reduced pancreas weight ($p<0.05$) in comparison with the AGP diet. The dietary OA supplementation reduced total intestinal length on 42 days post-hatch.

There was no interaction between PA and OA on the relative weight of the immune organs on 21 days post-hatch. Chickens fed diets containing PA and OA showed higher relative weight of spleen than those not fed with these additives ($p<0.05$). Broilers fed the diets supplemented with alternative additives, alone or in combination, presented heavier ($p<0.01$) spleens than those fed AGP. No effect of dietary treatments on relative weight of immune organs was observed on 42 days post-hatch.

There was no interaction between PA and OA dietary inclusion on jejunal digesta pH on 21 days post-hatch (Table 3). However, PA supplementation increased jejunal digesta pH in comparison with PA-free diets, whereas OA dietary inclusion reduced jejunal digesta pH. Furthermore, chickens fed the diets containing OA presented lower jejunal digesta pH than those fed AGP. On 42 days post-hatch, there was an interaction ($p<0.01$) between PA and OA on jejunal digesta pH. The jejunal digesta pH was reduced in chickens fed diets with PA or OA alone in comparison with those fed the control diet or that supplemented with the PA and OA combination. However, when associated with PA, OA supplementation did not decrease jejunal pH. Broilers fed the diets with AGP presented higher jejunal digesta pH ($p<0.01$) than those fed PA.

Histological analysis

There was no interaction between PA and OA on small intestine histology on 21 days post-hatch (Table 4). Feed additives had no influence on intestinal villus development. When PA and OA diets, individually or in combination, and diets not supplemented with feed additives were compared with AGP diets,

Table 3 – Relative organ size and jejunal digesta pH of broiler chickens fed diets with phytogenic additives (PA) and organic acids (OA) in replacement of antibiotic growth promoters (AGP).

PA	OA	Intestines[2] (%)		Pancreas (%)		Spleen (%)		Jejunal digesta pH		Intestines[2] length, cm	
		21 d	42 d	21 d	42 d	21 d	42 d	21 d	42 d	21 d	42 d
Interaction effects											
-	-	7.00ab	4.20	0.31ab	0.15γ	0.10	0.09	6,21	6.19a	151.00	192.00
-	+	7.72a,γ	4,04	0.32a	0.19	0.12γ	0.09	6.08γ	5.93b	154.71	190.63γ
+	-	7.22ab	5.00γ	0.32a	0.18	0.12γ	0.10	6.37	5.77b,γ	152.50	222.60
+	+	6.72b	4.62	0.27b	0.19	0.13γ	0.10	6.19	5.99a	157.75	195.83
AGP[1]		6.37	4.33	0.31	0.17	0.09	0.08	6.32	6.16	153.50	212.50
Main effects											
	-	7.11	4.60	0.31	0.16b	0.11b	0.09	6.29a	5.98	151.75	207.30a
	+	7.22	4.33	0.29	0.19a	0.12a	0.10	6.13b	5.96	156.23	193.23b
-		7.36	4.12b	0.32	0.17	0.11b	0.09	6.14b	6.04	152.86	191.31b
+		6.97	4.81a	0.29	0.19	0.13a	0.10	6.28a	5.88	155.13	209.21a
Source of variation											
PA		0.181	<0.001	0.084	0.117	0.008	0.186	0.016	0.016	0.569	0.010
OA		0.703	0.157	0.125	0.027	0.047	0.542	0.007	0.769	0.264	0.037
PA × OA		0.041	0.558	0.017	0.250	0.709	0.727	0.644	0.002	0.846	0.058
AGP		0.020	0.005	0.084	0.035	<0.001	0.201	0.002	0.001	0.748	0.001
SEM		0.139	0.093	0.007	0.005	0,004	0,003	0,027	0,040	1.597	3.211

[a-b]Means followed by different letters within the same column are statistically different (p<0.05).

[γ]Differ from the diet with antibiotic growth promoter (AGP) (p<0.05).

SEM, standard error of means.

[1]Avilamycin and monensin.

[2]Small intestine + large intestine.

Table 4 – Histological parameters of small intestine and percentage of cortical area in the lymphoid follicles of the bursa of Fabricius of broiler chickens fed diets with phytogenic additives (PA) and organic acids (OA) in replacement of antibiotic growth promoters (AGP).

PA	OA	Duodenum		Jejunum		Ileum		Cortical area of Bursa (%)	
		Villus height (µm)	Crypt depth (µm)	Villus height (µm)	Crypt depth (µm)	Villus height (µm)	Crypt depth (µm)	21 d	42 d
Interaction effects									
-	-	1267	255	804	172	504γ	188	36.12	31.53b,γ
-	+	1328	291	910	176	563	175	40.25	36.44a
+	-	1332	274	904	155	536	164	38.34	38.73a
+	+	1208	250	853	179	545	162	41.69	35.53ab
AGP[1]		1303	253	834	175	657	203	36.07	37.58
Main effects									
	-	1299	265	804	164	520	176	37.23b	35.13
	+	1268	271	881	177	554	168	40.97a	35.98
-		1297	273	857	174	534	181	38.18	33.98
+		1270	262	829	167	541	163	40.01	35.53
Source of variation									
PA		0.716	0.540	0.406	0.589	0.815	0.185	0.228	0.021
OA		0.673	0.730	0.361	0.275	0.257	0.566	0.021	0.514
PA × OA		0.230	0.108	0.405	0.426	0.397	0.693	0.792	0.004
AGP		0.699	0.645	0.152	0.688	0.039	0.194	0.099	0.003
SEM		29.756	9.461	15.268	5.220	17.124	6.209	0.867	0.746

[a-b]Means followed by different letters within the same column are statistically different (p<0.05).

[γ]Differ from the diet with antibiotic growth promoter (AGP) (p<0.05).

SEM, standard error of means.

[1]Avilamycin and monensin.

no development differences of villi in the different segments of intestine were detected, except for ileal villus height. Broilers fed the AGP diet presented better intestinal development than those fed the control diet.

On 21 days post-hatch, no effect of PA supplementation on the bursa of Fabricius cortical area was detected. The diets supplemented with OA increased (p<0.05) bursal cortical area. On 42 days post-hatch, there was an interaction (p<0.05) between feed additives, when chickens fed the diets with PA or OA presented the largest bursal cortical area. The dietary inclusion of AGP diet resulted in larger (p<0.05) cortical area compared with the feed additive-free diets.

Antibodies against NDV

There was an interaction between PA and OA for the production of NDV antibodies on 21 days post-hatch (p<0.01) (Table 5), with antibody titers against NDV determined in the birds fed the diets with PA and OA inclusion. However, no differences in anti-NDV antibody titers were observed on 42 days post-hatch.

Lipid oxidation of intestinal samples

There was no interaction between feed additives for MDA values in the intestines of broilers on 21 days post-hatch (Table 5). On 21 days post-hatch, PA dietary supplementation reduced lipid intestinal oxidation. On 42 days post-hatch, there was an interaction between PA and OA (p<0.01), when the combined supplementation of PA and OA reduced gut MDA values compared with the non-supplemented diet. The broilers fed the OA-supplemented diets showed lower lipid oxidation values than those fed the additive-free diet on 42 days post-hatch, and those fed the non-supplemented diet presented higher values (p<0.05) than those fed the AGP diet on 21 days post-hatch. However, on 42 days post-hatch, the individual inclusion of PA or in combination with OA resulted in lower MDA levels (p<0.01) the AGP diet.

DISCUSSION

Research results on the use of dietary organic acids for broilers are contradictory and inconsistent. In the present study, the dietary inclusion of OA impaired broiler performance on 21 days post-hatch, which is in accordance with other studies that evaluated the effect of the addition of citric acid and/or gluconic acid (Biggs & Parsons, 2008; When *et al.*, 2009) and found a reduction in broiler body weight. Some authors who evaluated the effects of supplementing organic acids and plant extracts in broiler diets found no effects on broiler performance (Gunal *et al.*, 2006; Hashemi *et al.*, 2012; Naseri *et al.*, 2012), nor studies with citric

Table 5 – Antibody titers against Newcastle disease virus (NDV), lipid oxidation (TBARS values) in the small intestine and heterophil:lymphocyte ratio (H:L)of broiler chickens fed diets with phytogenic additives (PA) and organic acids (OA) in replacement of antibiotic growth promoters (AGP).

PA	OA	NDV (Log_2)		TBARS (mg/kg)		H:L
		21 d	42 d	21 d	42 d	
Interaction effects						
-	-	1.915b	2.588	0.198γ	0.220a	0.626
-	+	2.224a	2.869	0.114	0.150ab	0.655
+	-	2.183a	2.956	0.091	0.110b,γ	0.493
+	+	1.953b	3.111γ	0.109	0.103b,γ	0.539
AGP[1]		1.940	2.653	0.104	0.173	0.582
Main effects						
	-	2.061	2.772b	0.144	0.165	0.597
	+	2.089	2.990a	0.111	0.126	0.559
-		2.084	2.729b	0.156a	0.185	0.640
+		2.068	3.034a	0.100b	0.106	0.516
Source of variation						
PA		0.874	0.001	0.036	<0.001	0.218
OA		0.787	0.013	0.198	<0.001	0.699
PA × OA		0.012	0.442	0.052	<0.001	0.930
AGP		0.110	<0.001	0.028	<0.001	0.755
SEM		0.048	0.049	0,012	0,010	0.041

[a-b]Means followed by different letters within the same column are statistically different (p<0.05).

[γ]Differ from the diet with antibiotic growth promoter (AGP) (p<0.05).

SEM, standard error of means.

[1]Avilamycin and monensin.

acid and formic acid alone (Hernández *et al.*, 2006; Liem *et al.*, 2008). One possible explanation for the inconsistent results obtained with the use of OA in starter broiler diets may be the differences in the health challenges and management practices to which birds are exposed in the different trials. In the present study, broilers were submitted to challenging rearing conditions, such as high stocking density (17 birds/m²), poor environmental control systems, and reused litter. These factors may negatively affect the birds'immune system, resulting in the poorer performance observed in the broilers fed the OA diets. Another possible influence is water quality, because in areas where water pH is nearly alkaline, the effects of OA can be enhanced, whereas when drinking water pH is neutral, the effects of OA can be mitigated.

The weight gain improvement observed during the starter phase (14 days of age) in the broilers fed the PA diets may be attributed to the active compounds present in the evaluated PA, and demonstrate the potential effectiveness of its antimicrobial and antioxidant effects, as well as of their stimulation of the release of pancreatic enzymes, enhancing nutrient digestibility. The higher weight gain of the 21-d-old broilers fed the PA diets in the present study was also observed with the supplementation of black cumin seeds (Khalaji *et al*., 2011). On the other hand, the alternative feed additives evaluated in the present study did not affect feed intake or livability, which is in agreement with findings with fenugreek and parsley (Abbas, 2010), oregano extract and essential oil (Fukayama *et al.*, 2005; Basmacioğlu Malayoğlu *et al.*, 2010; Karimi *et al*., 2010), blend of oregano, cinnamon and pepper essential oils (Jamroz *et al*., 2005; Rizzo *et al*., 2010), and of oregano, thyme, rosemary and marjoram essential oils (Cross *et al.*, 2007).

The individual supplementation of PA provided a similar feed conversion ratio as the diet containing AGP. Such effect may be due to the improvement in nutrient metabolization and to the antimicrobial and antioxidant properties of phytogenic additives, as observed in other studies (Hernández *et al*., 2004; García *et al*., 2007; Amad *et al*., 2011; Fascina *et al*., 2012), demonstrating that on the first days post-hatch, the dietary supplementation with phytogenic products may provide the same beneficial effects as antibiotics.

The higher bodyweight gain of 42-d-old broilers fed the diet containing AGP demonstrates the superior effect of antibiotics on broiler performance, as they regulate the intestinal microbiota, allowing chickens to express their maximum growth potential, even when reared at high stocking densities. As birds age, excreta production increases and rearing space available is reduced (15.6 kg/m² at 21 days vs. 43.6 kg/m² at 42 days of age), increasing the health challenge for older broilers. This explains the lack of effects of the AGP used in the present study on broiler growth performance from 1 to 21 days of age, when the health challenge was lower in comparison with 42 days of age, when broilers fed diets the diet without or with alternative feed additives showed poorer performance than those fed diets with AGP. However, in other studies with broilers, the replacement of AGP with garlic extract, thyme and probiotics (Shams Shargh *et al*., 2012), green tea (Sarker *et al*., 2010) mixtures of essential oils and commercial plant extracts (Hernandez *et al*., 2004; Muhl & Liebert, 2007), formic acid (Hernandez *et al*., 2006) and a blend of formic and propionic acids and plant extract (Gunal *et al.*, 2006), resulted in poorer performance at starter phases.

The higher relative weight of the intestines and pancreas of the chickens fed the diets with OA in the starter phase compared with other diets may be attributed to their lower bodyweight and greater absolute weight of these organs. Although no bodyweight differences between the chickens fed phytogenic additives and the combination of additives were found, those fed only PA presented higher pancreas relative weight. This result could be related to a possible stimulation of the secretion of pancreatic and intestinal enzymes promoted by these plant extracts (Jamroz *et al*., 2005; Jang *et al*., 2007), which may can also improve nutrient metabolization (Fascina *et al*., 2012).

The chickens fed the PA diets showed better intestinal development, as shown by the longer and, thus, higher relative weight of their intestines, as also previously found in other studies with mixtures of phytogenic additives (Hernandez *et al*., 2004, Abbas, 2010) and garlic extract (Carrijo *et al*., 2005).

The supplementation of alternative feed additives did not affect the relative weight of the bursa and thymus; however, spleen relative weight during the starter phase was greater in the chickens fed the additives alone (PA or OA), in agreement with the results found by Basmacioğlu Malayoğlu *et al.* (2010) in chickens fed oregano essential oil and exogenous enzymes. Heavier spleens may indicate better development of the immune system. Li *et al*. (2009) observed a synergistic and positive effect of the dietary inclusion of a probiotic, composed by *Lactobacillus spp.* and *Bacillus cereus*, and *Astragalus membranaceus* extract

on the morphometry of the immune organs, with the production of high antibody levels. Although no synergistic effect was observed between the additives on the morphometry of immune organs in the present study, Li *et al*. (2009) that demonstrated that the dietary inclusion of probiotics and *Astragalus membranaceus* extract increased the weights of the spleen, thymus and bursa of broilers on 42 days post-hatch. A possible reason for the lack of response of alternative feed additives supplementation on the weight of immune organs, such as the bursa and the thymus, in the present study may have been the high stocking density applied. High stocking densities may trigger aneurohormonal mechanism, increasing the release of adrenocorticotropic hormone and corticosteroids that can suppress organs of the immune system (Hill, 1983). Some studies show that high stocking densities have negative effects on broiler growth performance and on their lymphoid organs, leading to immunosuppression of (Heckert *et al*., 2002; Muniz *et al*., 2006).

The low pH of the jejunal content determined on 42 days post-hatch in broilers fed the diets with OA can beneficially modify intestinal microbiota balance, as observed by Biggs & Parsons (2008) with the inclusion of citric acid, which reduced *E. coli* and *C. perfringens* counts. In other studies, supplementation of a mixture of ortho-phosphoric acid, formic acid, and propionic acid reduced the pH of the duodenum and gizzard, allowing a greater proliferation of *Lactobacillus* throughout the gastrointestinal tract and reducing that of pathogenic bacteria (Samanta *et al*., 2008).

There was no effect of feed additives on the intestinal histomorphometry on 21 days post-hatch, as previously found with the inclusion of oregano extract (Fukayama *et al*., 2005), garlic powder (Carrijo *et al*., 2005; Shams Shargh *et al*., 2012), a blend of plant extract with formic and propionic acids (Gunal *et al*., 2006), or lactic and butyric acids (Salazar*et al*., 2008). However, other studies showed increase in villus height when diets were supplemented with formic acid and plant extracts (García *et al*., 2007), a phytogenic additive composed of oregano, cinnamon, and pepper (Jamroz *et al*., 2005), a combination of lactic, acetic, citric, benzoic, formic, and orthophosphoric acids (Viola & Vieira, 2007), and of lactic, formic, and citric acids (Smulikowska *et al*., 2010).

These diverse and inconsistent results may be attributed to differences in the active compounds, dietary inclusion levels, and utilization form of the feed additives applied in those different studies. Another factor to be taken into account is that some active compounds of the extracts of essential oils and plants may damage the intestinal mucosa, impairing villus development (Viveros *et al*., 2011).

In this study, the follicular cortex area of the bursa was reduced as the birds aged, which was also observed by other researchers (Guimarães *et al*., 2003; Muniz *et al*., 2006). As broilers age, the number and intensity of stressors, such as reduced physical space and temperature variations, increase and may result in lower development of lymphoid tissue, cortical area reduction, and follicular degeneration due to an increased release of corticosterone. However, the results of the present study indicate that the inclusion of a PA with potential antioxidant and immunostimulatory activities may alleviate bursal atrophy and protect the follicles, and, consequently, maintain the production of antibodies and improve humoral immunity, as also observed in several studies with broilers submitted to health challenge or not (Qiu *et al*. 2007; Li *et al*. 2009; Srikhun *et al*. 2010). Citric acid may also increase the population of immunocompetent cells in the bursa follicles and cecal tonsils, improving the responsiveness of these organs (Chowdhury *et al*., 2009). Furthermore, the alternative feed additives evaluated in the present study may stimulate the phagocytic activity of unspecific immune cells, increasing the activity of neutrophils and monocytes (Faix *et al*., 2009), the proliferation of spleen lymphocytes (Lee *et al*., 2010), and the production of mucin and IgA in the gut (Klasing, 2007).

The improvement of the immune system components is mainly due to the better gut integrity, considering that a healthy intestine of broilers needs 20% of the daily energy requirement for 50% intestinal turnover (Cant *et al*., 1996). Reducing intestinal pathogen challenges increases the availability of nutrients and energy available for growth and also for the immune system, strengthening the birds' immune responses.

Despite the effects on the maturation area of B cells in the bursa, an increase in antibody titer against NDV was not observed, which demonstrates little or no immunostimulatory effect on broilers housed at high stocking density. These results are consistent with studies that did not find any differences in antibody production in broilers fed oregano (Basmacioğlu Malayoğlu*et al*., 2010), aniseed (Soltan *et al*., 2008),or tamarind polyphenols (Srikhun *et al*., 2010). However, other studies showed immunostimulatory effects of PA on broilers fed diets with turmeric extract (Ali*et al*., 2010; Lee*et al*., 2010), *Astragalus membranaceus* (Qiu *et al*., 2007; Li *et al*., 2009), and a phytogenic blend (Khodambashi Emami *et al*., 2012).

During the lipid oxidation process, there is a great release of free radicals and reactive oxygen species that degrade tissue cells, causing their death and increasing the production of radicals. The levels of malondialdehyde (MDA), the main free radical and final product of lipid oxidation, can be reduced by plant components that have antioxidant activity (Bagchi *et al*., 1997). According to Faix *et al*. (2009), the gastrointestinal tract is considered to be an important production site of free radicals, which can be absorbed into the bloodstream. In the present study, the active compounds of the plant extracts used in the diets presented antioxidant activity, as shown by the reduced MDA content in the intestine of the chickens. This result is in agreement with the findings of Faix *et al*. (2009) and Ciftci *et al*. (2010), who observed a reduction of MDA levels in the duodenal mucosa and blood, and increased glutathione peroxidase activity in chickens fed diets supplemented with cinnamon extract. These results demonstrate the antioxidant potential of plant extracts that help to improve the health of birds. In addition, ginger also reduces gut lipid peroxidation (Manju & Nalini, 2010). Therefore, the supplementation of PA in the diets in the present study was able to protect the gut against the lipid oxidation caused by intestinal pathogens, resulting in better intestinal integrity and nutrient absorption, and increased the activity of antioxidant enzymes that helped to enhance the birds'immune response.

In conclusion, the dietary supplementation of phytogenic additives alone or in combination with organic acids does not affect broiler performance or intestinal histomorphometry; however, it enhances immune responses and intestinal quality, as shown by the increase in bursal cortical area and in antibody titers against NDV, and by the reduction in gut lipid oxidation.

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