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Original Article

Effect of a Feed Additive Based on Organic Acids and Tangerine Wort (Citrus reticulata) as Growth Promoter for Broiler Chickens

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ABSTRACT

Feed additive alternatives to antibiotics, such as organic acids, and substances rich in polyphenols, such as tangerine wort, can promote improved intestinal health in broilers by modulating the microbial population and improving nutrient utilization. In this work, a product which combines organic acids (fumaric acid 0.5%, lactic acid 5.13%, citric acid 5.44% and ascorbic acid 1.2%) and tangerine wort (*Citrus reticulata*) 8.36% was studied. To determine the effect and the most appropriate level of inclusion of product in the diet of broilers, an experiment was carried out with 1400-day-old male chicks, in a conventional poultry house, evaluating the performance until 42 days of age. The birds were housed in RCB design with 5 treatments and 7 replicates of 40 birds each, and the diets with the additive inclusions were evaluated: A250 (250 mg/kg), A500 (500 mg/kg), A1000 (1000 mg/kg), a negative control (NC, not supplemented), and a positive control (PC, 10 mg/kg of enramycin). The diets were formulated based on corn and soybean meal, containing added phytase and without anticoccidial; the additives replaced an inert in the basal diet. Performance characteristics, microbiota count, morphometry and jejunum morphology were evaluated. Considering the overall experimental period, the inclusion of the alternative additive based on organic acids and tangerine wort at different levels (250, 500 and 1000 mg/kg) did not result in difference from the negative control diet or the positive control with the inclusion of the antibiotic enramycin for performance traits ($p>0.05$), as well as for the microbiota count, morphology, jejunal morphometry and viability. Considering the period of 29-35 days alone, treatment with 500 mg/kg of alternative additive improved weight gain and feed intake of the chickens ($p<0.05$), but had no effect on feed conversion.

INTRODUTION

In face of concerns related to human health, restrictions and prohibitions on the use of antibiotics as growth promoters in animal feed have been adopted worldwide, since residues of these compounds in food could give rise to super-resistant microbiological organisms (Fleming-Dutra *et al.*, 2016).

Alternatives to these compounds are being studied in order to promote animal performance without the risk to human and animal health, by modulating the microbiota and improving digestion and nutrient absorption (Santos, 2005).

Among the additives with a growth promoting effect, organic acids have been developed; these are chemical substances of natural origin with a general R-COOH structure and acidic properties (Menten, 2014; Kim *et al.*, 2015). The resulting acidification of the contents of the gastrointestinal tract is considered the mode of action of these compounds, impairing the development of pathogenic microorganisms.

Additionally, substances rich in polyphenols such as the wort of some fruits, like citrus and grapes, can have a growth promoting effect. Some polyphenols such as hesperidin and rutin, which are aromatic substances composed of phenolic rings and present in tangerine, have effects on the intestinal microbiota. The supposed mode of action is based on the cleavage of aromatic rings for energy production by some microorganisms, which, in turn, generates metabolites with bactericidal effect (Iqbal *et al.* 2020).

The association of different methods of modulating the intestinal microbiota, such as organic acids with polyphenols, may favor the performance of broilers (Fascina *et al.* 2017). Therefore, the objective of this work was to evaluate the effect of a commercial alternative additive that combines the organic acids: fumaric acid, lactic acid, citric acid and ascorbic acid with tangerine wort rich in the polyphenols rutin and hesperidin, in three different supplementation levels on the performance and intestinal health of broilers raised in a conventional environment.

MATERIAL AND METHODS

The experimental procedures performed in this work were approved by the institutional animal use and care committee.

Animals and Facilities

The experiment was carried out in the Department of Animal Science, USP, Piracicaba, SP, Brazil, in a traditional open-sided poultry house with floor pens, with electric heating, automated ventilation system (fans and foggers), tubular feeders, nipple drinkers, side curtains and reused litter composed of rice husk and wood-shavings on 4.0 m2 pens.

For the experiment, 1,400 male day-old Cobb broiler chicks were purchased from a commercial hatchery, originating from breeders of 51 weeks of age, vaccinated in the hatchery against Marek's disease and avian bronchitis. The average individual initial weight was 47.0 grams. The birds were distributed in a randomized complete block design considering 5 treatments and 7 replications that were randomized within the blocks, totaling 35 pens, each with 40 animals.

The temperature in the shed was controlled through the automatic system of electric heaters, fans, nebulizers and manual curtain handling. The mean temperature in the initial period (1-21 days) was 27.6 °C, in the growth period (22-35 days) was 26.3 °C and in the final period (36-42 days) was 26.0 ° C.

Alternative Additive and Experimental Diets

The diets were based on corn and soybean meal and formulated according to the nutritional specifications for male broilers of regular performance according to the Brazilian Tables for Poultry and Swine (Rostagno *et al.* 2017). A basal diet was formulated for each phase (pre-starter, starter, grower and finisher) to contain an inert and the additives were supplemented replacing the inert (Table 1). The diets contained phytase, did not contain anticoccidial additive, and kaolin was used as the inert material.

Table 1 – Percentage composition and calculated values of the basal diets of the experiment, as-fed basis.

Ingredients	Un.	0 -7 days	8 -21 days	22-35 days	36-42 days
Corn	%	48.25	50.56	57.28	63.52
Soybean meal	%	44.35	41.84	35.25	29.95
Soybean oil	%	3.73	4.35	4.62	4.18
Dicalcium phosphate	%	1.35	1.02	0.83	0.44
Limestone	%	1.01	0.94	0.81	0.78
Salt	%	0.53	0.52	0.49	0.47
DL-Methionine	%	0.3325	0.3159	0.2730	0.2320
L- Lysine. HCl	%	0.1146	0.1255	0.1547	0.1712
Choline chloride 70%	%	0.0800	0.0800	0.0600	0.0400
Mineral supplement*	%	0.0500	0.0500	0.0500	0.0500
Vitamin supplement	%	0.0500**	0.0500**	0.0400***	0.0400***
L-Threonine	%	0.0491	0.0466	0.0403	0.0293
Phytase	%	0.0050	0.0050	0.0050	0.0050
Inert / Additive****	%	0.1000	0.1000	0.1000	0.1000
Calculated nutritional composition					
Metabolizable energy	kcal / kg	2975	3050	3150	3200
Crude protein	%	24.27	23.31	20.84	18.91
Calcium	%	0.871	0.758	0.638	0.514
Available Phosphorus	%	0.363	0.299	0.254	0.176
Sodium	%	0.225	0.218	0.208	0.197
Digestible lysine	%	1.307	1,256	1,124	1.014
Dig. methionine + cysteine	%	0.967	0.929	0.832	0.750
Digestible threonine	%	0.863	0.829	0.742	0.669

* Salus Mineral Premix, provided per kilogram of diet: Manganese, 80 mg ; Zinc, 70 mg; Iron, 50 mg; Copper, 10 mg and Iodine, 1 mg.

** Salus Vitamin Premix , supplied per kilogram of diet: Vitamin A, 8,500 IU; Vitamin D_3, 3000 IU; Vitamin E, 18 IU; Vitamin K_3, 2.5 mg ; Vitamin B_1, 2 mg; Vitamin B_2, 6 mg; Vitamin B_6, 3 mg; Vitamin B_{12}, 14 μg; Vitamin B_5, 14 mg; Folic acid, 1.2 mg; Biotin, 0.08 mg and Selenium, 0.5 mg.

*** Salus Vitamin Premix , supplied per kilogram of diet: Vitamin A, 6,800 IU; Vitamin D_3, 2400 IU; Vitamin E, 14 IU; Vitamin K_3, 2.0 mg ; Vitamin B_1, 1.6 mg; Vitamin B_2, 4.8 mg; Vitamin B_6, 2.4 mg; Vitamin B_{12}, 11 μg; Vitamin B_5, 14 Mg; Folic acid, 1.0 mg; Biotin, 0.06 mg and Selenium, 0.4 mg.

**** According to each treatment (Table 2).

The alternative additive used is a commercial product that consists of a blend of organic acids: fumaric acid (0.5%), lactic acid (5.13%), citric acid (5.44%) and ascorbic acid (1.2%), in addition to tangerine wort rich in polyphenols (8.36%) and vehicle (79.37%). The inclusion of the product consisted of three levels, starting from a basal diet + 250 mg /kg (A250), a basal diet + 500 mg/kg (A500), standard dose recommended by the manufacturer, and a basal diet + 1000 mg/kg (A1000); a positive control treatment, basal diet + inclusion of 10 mg /kg enramycin as a growth promoter (PC), and a negative control (NC) without additives (basal diet) were also included.

Table 2 – Inclusion of alternative additive and enramycin according to each treatment.

Ingredients	Un.	A250	A500	A1000	PC	NC
Inert	%	0.0750	0.0500	0.0000	0.0875	0.1000
Alternative additive	%	0.0250	0.0500	0.1000	0.0000	0.0000
Enramycin 8%	%	0.0000	0.0000	0.0000	0.0125	0.0000

Measurements and methods of analysis.

To measure the performance of the animals, weekly weighing of the birds of each box was carried out from 1 to 42 days, and the average weight gain (WG) for each week was obtained. The ration was weighed weekly before and after the offer to obtain the data of individual feed intake (FI). Feed conversion ratio (FCR) was obtained from the ratio between FI and WG for each pen.

At the age of 42 d, one animal from each pen was sacrificed by cervical dislocation, without previous fasting, to collect the contents of the jejunum region for microbiota analysis and a tissue sample of the same region for morphology and morphometric analysis. These determinations were performed in a private laboratory. (Integral Poultry and Animal Pathology – AVIPA, Campinas, SP).

The methodology used for morphology and morphometry of the intestine was adapted from the ISI methodology, in patent process (INPI BR 1020150036019) described by (Kraieski *et al.* 2017). The morphometric measurements were done in jejunum where the samples were embedded in paraffin and sections of 5 µm were mounted on slides and stained with hematoxylin and eosin plus Alcian blue; one slide per bird was evaluated and the villi were divided into fused and normal. Ten intestinal villi and ten crypts per bird were evaluated proportionally to the morphological distribution (fused and normal), in a 10X objective (40X objective was also used to confirm changes) of an optical microscope.

Using the ISI methodology, the morphological assessment is performed based on alterations or lesions in the intestinal mucosa, which receive a score from 0 to 3, where 0 is the absence of alteration and 3 is the severe alteration, and each alteration receives an impact factor (IF) for organ function ranging from 1 to 3, where 3 is the most impactful. For the evaluation of the jejunum, Inflammatory Infiltrates (IF=3), Congestion (IF=3), Desquamation (IF=2), Coccidiosis (IF=3), Bacterial clumps (IF=3), Rods (IF=3), Cystic Crypts (IF=3), Mucus (IF=1), Necrosis (IF=3) and Edema (IF=2) were considered. The sum of the scores multiplied by their IF generates an overall score for the morphology of the intestine.

For microbiota, the CFU/g (Colony Forming Units) count of mesophylls and enterobacteria were evaluated through plating of the jejunal contents; after counting, the logarithm of the values observed was applied before statistical analyses.

Statistical analysis

The performance data were submitted to the ANOVA test by PROC MIXED of SAS 9.4 (SAS, 2013) in which the block factor was considered as a random effect, while the others as fixed effects in the model. When a significant effect was verified, the variables were subjected to a comparison of means by the Tukey-Kramer test considering an alpha of 5% of significance.

RESULTS AND DISCUSSION

Performance

The results of performance obtained in the experiment are shown in Table 3. The WG and FI in the starter period (1-21 days) were significantly higher ($p<0.05$) for the treatment with the addition of enramycin (PC) compared to all others. Compared to the NC, WG was improved by 10%, FI increased 6% and the FCR was improved by 4%, indicating that environmental challenges were present under the experimental conditions. The antibiotic treatment was effective as growth promoter, while the three doses of the alternative additive (A250, A500 and A1000) did not result in improvement in performance of the birds in the starter period.

For the period of 1-35 days, it was found that the PC treatment obtained better results, with 165 g greater WG, 143 g increased FI and an improvement of 0.031 in the FCR when compared to the NC ($p<0.05$). At this age, A500 was the only treatment containing the alternative additive that had WG and FI not differing from PC (*p*>0.05), but still the FCR was worse (*p*<0.05). This treatment resulted in an increase of 54 g in WG and 90 g in FI compared to NC, although the difference was not significant. The treatment A500 was partially able to overcome the health challenge imposed to the chickens. Birds receiving the additive at dosages A250 and A1000 had the performance parameters inferior (*p*<0.05) to the birds in the PC treatment.

The last week of the trial (36-42 days) week was marked by maximum temperatures above 30 °C, which may have interfered with the performance of the animals and the effect of the diets in this period; according to Paulino *et al.* (2019) the thermal comfort temperature for commercial broilers at this age and weight range is below 28 °C. There were no significant differences among treatments (*p*>0.05) for WG, FI and FCR (*p*>0.05) on that week. A higher experimental error during this last week of the trial was noted, indicated by high values of the standard errors of the mean of the characteristics observed during this period, which were greater than those observed in the rest of the experiment.

In the total period of the experiment (1-42 days), there was no significant difference in WG for PC, NC, A250 and A500 (*p*>0.05), with only A1000 being worse than PC, a difference of 209 g (*p*<0.01). The average live weight of birds in the total period was 3443 g; also, with the exception of the difference between PC, with 3586 g, and A1000 with 3376 g, the other treatments (A250, A500 and CN) did not differ from PC (*p*>0.05). The FI of the total period did not differ statistically (*p*>0.05), although higher intakes were observed for PC and A500 (5114 g and 5084 g, respectively) compared to the other treatments. FCR was significantly (*p*<0.05) improved for PC (1.447) compared to A500 (1,493) but did not differ from the other treatments. It can be noted that the relative increase in weight gain of 40 g obtained with the intermediate (recommended) dose of the additive compared to NC was achieved due to a numerically higher FI (109 g) and not by improving feed efficiency.

Table 3 – Performance results for broilers fed increasing levels of the alternative additive from 1-42 days.

	Weight Gain (WG)			Live Weight (LW)			Feed Intake (FI)			Feed conversion rate (FCR)		
Treat.	1-21	1-35	1-42	1-21	1-35	1-42	1-21	1-35	1-42	1-21	1-35	1-42
A250	962 ^{b}	2498 ^{b}	3334 ^{ab}	1016 ^{b}	2547 ^{b}	3386 ^{ab}	1211 ^{b}	3437 ^{b}	4947	1.257 ^{b}	1.376 ^{b}	1.484 ^{ab}
A500	989 ^{b}	2588 ^{ab}	3407 ^{ab}	1036 ^{b}	2634 ^{ab}	3452 ^{ab}	1256 ^{b}	3565 ^{ab}	5084	1.270 ^{b}	1.381 ^{b}	1.493 ^{b}
A1000	968 ^{b}	2526 ^{b}	3327 ^{b}	1015 ^{b}	2574 ^{b}	3376 ^{b}	1213 ^{b}	3449 ^{b}	4915	1.253 ^{b}	1.367 ^{b}	1.478 ^{ab}
NC	1001 ^{b}	2535 ^{b}	3367 ^{ab}	1048 ^{b}	2577 ^{b}	3414 ^{ab}	1251 ^{b}	3475 ^{b}	4975	1.248 ^{b}	1.371 ^{b}	1.478 ^{ab}
PC	1109 ^{a}	2700 ^{a}	3536 ^{a}	1156 ^{a}	2744 ^{a}	3586 ^{a}	1333 ^{a}	3618 ^{a}	5114	1.203 ^{a}	1.338 ^{a}	1.447 ^{a}
p-value	<.0001	0.0002	0.0003	<.0001	0.0002	0.0385	0.0003	0.0073	0.0792	<.0001	<.0001	0.0274
SEM	14.58	28.78	52.43	14.36	28.28	52.79	17.35	37.2	56.38	0.010	0.006	0.011

Treatments: NC= negative control; PC= NC + addition of enramycin 10 mg /kg; A250 = NC+ 250 mg /kg alternative additive; A500 = NC+ 500 mg /kg alternative additive; A1000 = NC+ 1000 mg /kg alternative additive,

SEM: standard error of the mean,

$^{a, b}$ Mean values within a column with different superscripts are statistically different by Tukey's test (p<0.05).

Similar results were found by Fascina *et al.* (2017) with chickens fed diets containing natural sources of polyphenols and organic acids (30% lactic acid, 25% benzoic acid, 7% formic acid, 8% citric acid and 6.5% % acetic acid). They showed lower body weight gain and worse feed conversion compared to the diet with the antibiotic avilamycin and did not differ statistically from the control treatment. Furthermore, in the period of 22-35 days there were no differences between treatments for performance. In studies carried out by Isabel & Santos (2009) with formic acid and propionic acid, by Talebi *et al.* (2010) with organic acids (citric, benzoic and tartaric acids) and by Kopecký *et al.* (2012) with acetic and citric acid, there were also no significant differences in the performance of broilers.

Adil *et al.* (2011), using a basal diet supplemented with butyric acid, fumaric acid and lactic acid, found that birds fed diets supplemented with organic acids had significantly greater body weight gains than the control treatment and also improved feed conversion. In a study carried out by Simitzis *et al.* (2011) no effect of dietary hesperidin supplementation (1.5-3.0 g/kg) on final body weight, body weight gain and feed conversion ratio were observed. In contrast, Hassan *et al.* (2018) found a positive effect of the use of rutin on the weight gain of broilers fed with 0.25, 0.5 or 1 g/kg of supplementation.

Microbiota, Morphology and Morphometry of the Jejunum

The microbiological quantitation of bacterial groups (Table 4), considering mesophiles and enterobacteria, did not show very clear differences among treatments; PC showed lower counts of mesophiles compared to

A250 ($p<0.05$), but not to the other treatments. The counts for PC was numerically lower than NC, but not significant ($p>0.05$). For enterobacteria the statistics did not detect differences between treatments ($p>0.05$).

Villus height and crypt depth in the jejunum also did not differ statistically for the different diets ($p>0.05$); compared to NC, the enramycin-treated birds had lower values for villus height and crypt depth, with no change in villus-crypt ratio. The results obtained with ISI methodology did not show significant treatment effect on this index ($p>0.05$). However, it should be pointed out that the three treatments containing the alternative additive had lower values than the negative or positive controls, which may indicate the additive may promote a better gut health in chickens.

Table 4 – Analysis of jejunum microbiota, morphometry and morphology of broilers fed the alternative additive from 1-42 days.

Parameters	A250	A500	A1000	NC	PC	*p*-value	SEM
Mesophiles (log_{10} *CFU/g)	4.92^{b}	4.32^{ab}	4.35^{ab}	4.57^{ab}	4.11^{a}	0.0457	0.21
Enterobacteria (log_{10} *CFU/g)	5.19	5.74	6.35	6.07	5.76	0.2944	0.47
Villus height (µm)	1446	1255	1199	1485	1291	0.0936	85.78
Crypt depth (µm)	150	150	158	163	147	0.8367	11.78
Villus-Crypt ratio	9.86	8.55	7.66	9.27	9.35	0.3352	0.78
Gut morphology	4	3.71	3.71	6.00	5.29	0.7221	1.26

Treatments: NC= negative control; PC= NC + addition of enramycin 10 mg /kg; A250 = NC+ 250 mg/kg Alternative Additive; A500 = NC+ 500 mg/kg Alternative Additive; A1000 = NC+ 1000 mg/kg Alternative Additive.
SEM: standard error of the mean.
[a, b] Mean values within a line with different superscripts are statistically different by Tukey's test ($p<0.05$).

Mustafa *et al.* (2021) also did not observe changes in jejunal morphology in birds supplemented with organic acids compared to the control treatment. Polycarpo *et al.* (2016) found no significant differences in the microbiota, in particular enterobacteria, of the jejunum in birds supplemented with a mixture of lactic acid, acetic acid and butyric acid, as well as no differences in performance.

In a study by Yang *et al.* (2018) using a negative control group, without additives, an antibiotic group with 150 mg/kg of enramycin and a group with the addition of 300 mg/kg of encapsulated organic acids and essential oils, the group with organic acids showed a better feed conversion ratio, although the diet with enramycin was more efficient in decreasing the microbiological count. Supplementation with organic acids tended to reduce the pH of jejunal digesta, thus improving feed conversion.

Viability

For viability (Table 5) there were no significant differences for any of the scenarios evaluated ($p>0.05$), although lower viability means were observed for the CP treatment containing enramycin in the total period from 1 to 42 days. Mortality and culling losses were higher than 5% only for the treatment supplemented with enramycin, which may be related to the faster weight gain of birds in this treatment. The standard error of the mean considering the total period of the experiment was also greater than in the other periods, indicating an increase in the experimental error in the last experimental week.

Table 5 – Viability of broilers supplemented with an alternative additive based on organic acids and polyphenols.

Parameters	Period		
	1-21 days	1-35 days	1-42 days
A250	99.29	98.21	97.50
A500	98.93	97.50	96.78
A1000	99.29	98.21	97.50
NC	97.86	97.85	96.78
PC	98.21	96.07	94.64
SEM	0.57	0.90	1.21
p-value	0.2972	0.4428	0.4626

Treatments: NC= negative control; PC= NC + addition of enramycin 10 mg /kg; A250 = NC+ 250 mg/kg Alternative Additive; A500 = NC+ 500 mg/kg Alternative Additive; A1000 = NC+ 1000 mg/kg Alternative Additive.
SEM: standard error of the mean.

CONCLUSION

The inclusion of the alternative additive based on organic acids and tangerine wort at different levels (250, 500 and 1000 mg/kg) showed no effect as a performance enhancer for broilers compared to the negative control diet and the positive control with inclusion of the antibiotic enramycin considering the total period. The additive also did not promote changes in microorganisms counts, jejunal morphology, morphometry and viability.

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