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The effect of dietary organic acids on performance, carcass characteristics, immunity, blood constitutes and ileal microflora of broiler chickens*

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Organic acids have been proposed as food additives with great potential to replace growth-promoting antibiotics. The experiment was performed to evaluate the effect of the inclusion of different levels of formic acid and acetic acid on performance, carcass characteristics, immunity, blood constitutes, and ileal microflora of broiler chickens. 120-day-old male broiler chicks (Ross 308) were distributed into 4 treatments having 3 replicates of 10 birds each during 42 days of treatment. T1: control group without any dietary supplementation; T2 - 0.25% formic acid and 0.25% acetic acid; T3 - 0.5% formic acid and 0.5% acetic acid; T4 - 0.75% formic acid and 0.75% acetic acid. A significant improvement in the feed conversion ratio was observed in a total experimental period in T2, T3, and T4 with respect to the control (P<0.05). Organic acids increase significantly the total Lactobacilli count and reduce the *E. coli* count in ileum digesta (P<0.05). Organic acids can be used in the dietary supplementation of broilers as a replacement for growth-promoting antibiotics.

KEY WORDS: acetic acid / *Escherichia coli* / feed conversion ratio / formic acid / lactobacilli

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In recent decades the demand for chicken meat has increased thanks to its low cost, good nutritional profile, and suitability for further processing [Petracci *et al*. 2015]. It has been shown that growth-promoting antibiotics (GPA) can disrupt the protective barrier, leaving the host susceptible to colonization by pathogens [Ubeda and Pamer 2012], thus disturbing the intestinal ecosystem in broilers. Dietary interventions with feed additives in young chickens can impact their gastrointestinal integrity [Adedokun and Olojede 2019] and the development and responses of the immune system [Taha-Abdelaziz *et al.* 2018]. This is observed particularly in the case of organic acid supplementation as an alternative to GPA [Khan and Iqbal 2016]. Organic acids are a broad class of compounds with the general structure of R-COOH that include monocarboxylic acids such as formic acid (HCOOH, pKa 3.75) and acetic acid (CH_3COOH, pKa 4.76) exhibiting antimicrobial properties [Hajati 2018]. Several studies have shown that dietary organic acids at different levels can improve growth performance and favor the intestinal environment by lowering the pH of the upper part of the gastrointestinal tract in birds, thus, promoting the retention of nutrients and the reduction of pathogenic bacteria [Kim *et al.* 2015, Saleem *et al.* 2020]. Recent evidence has indicated that oral administration of organic acids alone, in combination with other feed additives, or encapsulated, modifies the morphometry of the gastrointestinal tract, induces the expression of tight junction proteins of the intestinal barrier, and has an impact on intestinal microbiota in broilers [Fascina *et al.* 2017, Yang *et al.* 2018, Aristimunha *et al.* 2020, Dai *et al.* 2020]. Therefore, the aim of this study was to evaluate the effect of dietary formic acid and acetic acid on performance, carcass characteristics, immunity, blood constitutes, and ileal microflora of broiler chickens.

Material and methods

Birds' management and treatments

The trial was conducted on an experimental poultry farm at the University of Guilan (Rasht, Iran). The procedures were approved by the authors' Institution's Ethics Committee (No. 991220), and care was taken to minimize the number of animals used. Before starting the experimental phase, the confinement area was washed, disinfected with 1% formalin, and fumigated 24 hours before introducing the birds, a similar procedure was applied for the cages (1m x 1.5 m x 1m) and drinkers.

120-day-old male Ross 308 chicks were obtained from a commercial poultry farm and were housed in their cages where they were administered an electrolyte solution in the drinking water (1:1000) before the application of a vaccination protocol (day 1 and day 14 of age bronchitis virus IBH 120; day 20 of age Gumboro virus IBD B78; day 10 and day 20 of age Newcastle disease B1, via drinking water). The experimental period consisted of 42 days under controlled temperature, humidity (55-65%), and light conditions (lighting was provided for 24 hours on the first day of age, in the 2-7 days 23 h, 8-35 days 20 h, at 36-41 days 20.5, 21, 21.5, 22, 22.5, 23 h, respectively and 41-42 days 23 h). The birds were randomly allocated into four dietary treatments, with 30 birds per treatment with 3 replicates per treatment. All birds were fed with an initial standard diet and finisher standard diet based on the broiler's nutritional requirements given in the Ross 308 strain rearing catalog (Tab. 1). Feed and water were supplied *ad libitum* throughout the experimental period of 42 days. The different treatments were administered from day 1 to day 42, as follows: treatment 1 (T1) - drinking water (control); treatment 2 (T2) - drinking water containing 0.25% formic acid and 0.25% acetic acid; treatment 3 (T3) - drinking water containing 0.5% formic acid and 0.5% acetic acid; treatment 4 (T4) - drinking water containing 0.75% formic acid and 0.75% acetic acid.

Table 1. Ingredients and nutrient analysis of used diets during the starter (1-21 days of age), and grower (22-42 days of age) periods

Ingredient (%)	Starter period (1-21 days of age)	Finisher period (22-42 days of age)
Corn	57.65	59.45
Soybean meal	33.10	30.00
Fish meal	3.40	3.50
Soybean oil	2.00	3.50
Ca%22P%18	1.55	1.10
Mineral oysters	1.03	1.18
DL-Methionine	0.01	0.01
Mineral premix[1]	0.50	0.50
Vitamin premix[2]	0.50	0.50
NaCl	0.26	0.26
Nutrient analysis		
metabolizable energy (kcal/kg)	2910	3030
crude protein (%)	20.10	19.00
fat (%)	4.60	6.14
calcium (%)	0.95	0.90
phosphorus (%)	1.23	1.06
available phosphorus (%)	0.45	0.36
methionine (%)	0.50	0.38
lysine (%)	1.01	1.00
methionine + cysteine (%)	0.83	0.71

[1]Calcium pantothenate – 4 mg/g; Cu – 3 mg/g; Zn – 15 mg/g; Mn – 20 mg/g; Fe – 10 mg/g; K – 0.3 mg/g.
[2]Vitamin A – 5000 IU/g; Vitamin D3 – 500 IU/g; Vitamin E – 3 mg/g; Vitamin K3 – 1.5 mg/g; Vitamin B2 – 1 mg/g; Niacin – 15 mg/g; Vitamin B6 – 13 mg/g.

Performance traits

Feed and weight gain were recorded using a precision A&D GF-300 digital balance (A&D Weighing Design and Manufacture, San Jose, CA) in starter (1-21 days of age) and grower (22-42 days of age) periods by pen. The feed conversion ratio was determined by dividing grams of feed consumed by weight gain at the starter and grower periods.

Blood parameters

At the end of the study, at 42 days of age, one bird per group, in total 3 birds per treatment, was selected for blood collection. Then, 1 mL of total blood per bird was collected from the wing veins into EDTA tubes. Samples were transferred to the laboratory for analysis within 2 hours of collection. Differential leukocyte counts were examined under a light microscope with three samples per treatment on Giemsa-stained blood smears. One hundred cells were counted and differentiated into heterophils (H) and lymphocytes (L). The mean H/L ratio was calculated from the number of H and L. The total blood was centrifuged at 3000 g for 10 minutes at room temperature to obtain plasma. Then, plasma was harvested to polypropylene tubes and stored at -20°C for further processing. Biochemical analyses of total protein, albumin, glucose, total cholesterol, triglycerides, LDL, VLDL, and HDL cholesterol were performed based on standard protocols using commercial laboratory kits.

Measurement of carcass characteristics and immune organs

The chickens were euthanized (stunned and slaughtered) after 42 days of experimental feeding and following the plucking operations the head and shanks were separated. The birds representing the body weight close to the mean of the group were chosen. The emptied stomach contents, carcass weight, breast, thigh, wings, abdominal fat, gizzard, and liver were dissected and weighed into the carcass. Weight measurements were recorded for the immune organs including the thymus, bursa of Fabricius, and spleen. The weights of the eviscerated carcass, breast, drumsticks (thighs), gizzard, liver, abdominal fat, and immune organs are expressed in % relative to body weight.

Culture conditions and bacterial count

At the end of the experimental period the ileum was removed. To determine bacterial growth and colony counts, the methodology previously described by Abbasi *et al.* [2015] was followed. Briefly, the samples of ileum content were streaked in agar plates and collected into sterile culture tubes previously weighed and autoclaved. Later, samples were transferred to the laboratory and the tubes were weighed again, with the difference between the two values constituting the amount of the sample. Serial dilutions in phosphate-buffered saline (PBS) were prepared from the collected samples and 100 µL of 10^{-4}, 10^{-5}, and 10^{-6} were taken to spread onto the agar plates. MRS agar (Man Rogosa Sharpe agar, 1.10660.500) and Eosin Methylene Blue Agar (EMB, 1.01347.0500), respectively, were used to culture and count Lactobacilli and *Escherichia coli*. Lactobacilli bacteria were incubated at 37ºC under anaerobic conditions (anaerobic jar) for 72 hours. Bacteria in Petri dishes were counted using a colony counter and the result was reported as the logarithm of CFU/g of the sample.

Statistical analysis

This study was conducted based on a completely randomized design (CRD) with 4 treatments and 3 replicates per treatment. Data were analyzed by the Statistical Analysis System (SAS Institute, Inc., 2000) using the generalized linear model (GLM) procedure and the statistical comparison was made using the Duncan test at the 95% probability level. We used the Kolmogorov-Smirnov test to determinate normality. Only the weights of the liver and gizzard, and albumin traits were not normally distributed. The transformation method used was the Square-Root Transformation.

Results and discussion

Growth parameters

The effects of organic acid supplementation on the performance of broilers are presented in Table 2. The formic acid and acetic acid supplementation at different levels had no significant effect on the body weight gain and feed intake in either the starter or finisher period, or overall compared to T1. However, for the total feed conversion ratio at the 42nd day of age there were statistical differences in T2, T3, and T4 to T1 (p<0.05). Several studies evaluated the performance of commercial broilers treated with diets differing in organic acids with positive effects in laying hens [Baghban-Kanani *et al.* 2019] and broilers [Fathi *et al.* 2016, Nosrati *et al.* 2017, Ghazvinian *et al.* 2018]. The results of BWG and FI were consistent with those of Akyurek *et al.* [2011], who observed that the addition of organic acids did not affect growth parameters. Conversely, Fathi *et al.* [2016] stated that formic and propionic acids ameliorate BWG and FCR in broilers. Moreover, Saleem *et al.* [2020] showed that 3 kg/ton of ammonium formate and propionate, and 4 kg/ton of calcium formate and propionate supplementation in diets of broiler chickens significantly improved their BWG and FCR. The differences in these results can be explained by the fact that broiler chickens are exposed to many stress factors inherent to management or environmental housing conditions (vaccination, feed withdrawal, and high ambient temperature, among others).

Table 2. Growth performance of Ross 308 broilers in starter period (1st-21st days of age) and finisher period (22nd-42nd days of age) fed diets containing different levels of organic acids

Parameters (g/bird/period)*	T1	T2	T3	T4	SEM	P-value
Weight gain in the starter period	625.27	657.00	665.85	657.55	5.49	0.08
Weight gain in the finisher period	1335.70	1380.15	1435.50	1372.35	27.65	0.94
Total weight gain	1960.97	2037.15	2101.35	2029.90	25.67	0.94
Feed intake in the starter period	945.20	955.25	966.60	960.20	13.47	0.35
Feed intake in the finisher period	2805.33	2774.30	2793.75	2771.25	44.85	0.05
Total feed intake	3750.53	3729.55	3760.35	3731.45	52.65	0.05
Feed conversion ratio in the starter period (g/g)	1.511	1.453	1.451	1.449	0.03	0.08
Feed conversion ratio in the finisher period (g/g)	2.100	2.010	1.950	2.020	0.03	0.05
Total feed conversion ratio (g/g)	1.910^{a}	1.840^{b}	1.790^{c}	1.840^{d}	0.02	0.01

*Means within each column of dietary treatments with no common superscript differ significantly at P<0.05.
Dietary treatments were as follows: T1 – control; T2 – 0.25% formic acid and 0.25% acetic acid; T3 – 0.5% formic acid and 0.5% acetic acid; T4 – 0.75% formic acid and 0.75% acetic acid.

Carcass characteristics relative to weight

Table 3 presents the effects of dietary formic acid and acetic acid supplementation on the relative weight of the eviscerated carcass, breast, drumsticks (thighs), gizzard, liver, and abdominal fat at 42 days of age. No significant differences were observed in carcass traits or giblets weight (gizzard, liver, abdominal fat) for T2, T3, or T4 with respect to T1 ($p>0.05$). These results are consistent with those of Garcia *et al.* [2007] and Kopecky *et al.* [2012]. Contrary to our findings, several studies showed that organic acids positively affect carcass yield, gizzard weight, and abdominal fat content by dietary acidification in broilers [Panda *et al.* 2009, Lakshmi and Sunder 2013, Sultan *et al.* 2015, Dehghani-Tafti and Jahanian 2016, Saleem *et al.* 2020].

Table 3. Carcass characteristics of Ross 308 broilers fed diets containing different levels of organic acids

Parameters (%, relative to body weight)*	T1	T2	T3	T4	SEM	P-value
Eviscerated carcass	58.80	59.82	64.58	64.35	1.56	0.48
Breast	31.20	32.64	33.98	33.69	1.15	0.92
Drumsticks (thighs)	29.11	30.69	31.01	31.37	0.53	0.52
Gizzard	1.00	1.03	1.05	1.08	0.03	0.90
Liver	2.52	2.55	2.58	2.65	0.05	0.91
Abdominal fat	1.12	1.08	1.02	0.99	0.05	0.83

*Means within each column of dietary treatments with no common superscript differ significantly at $P<0.05$. Dietary treatments were as follows: T1 – control; T2 – 0.25% formic acid and 0.25% acetic acid; T3 – 0.5% formic acid and 0.5% acetic acid; T4 – 0.75% formic acid and 0.75% acetic acid.

Immune parameters

The relative weight of immune organs as well as the number of heterophils and lymphocytes and the H:L ratio were expressed in percent and are presented in Table 4. The addition of formic acid and acetic acid to the diet did not modify immune parameters in broilers compared with the control group. Chronic stress can suppress the immune response leading to increased susceptibility to infection and decreased growth rate [Nelson *et al.* 2018, Nelson *et al.* 2020]. In this respect in our study we found no significant differences in immune organs or alterations in the number of

Table 4. Immunity related organ characteristics and humoral immunity of Ross 308 broilers fed diets containing different levels of organic acids

Parameters (%, relative to body weight)*	T1	T2	T3	T4	SEM	P-value
Thymus	0.11	0.11	0.15	0.13	0.017	0.22
Bursa of fabricius	0.21	0.22	0.26	0.28	0.10	0.32
Spleen	0.11	0.11	0.13	0.14	0.006	0.30
Heterophils	19.00	20.00	20.66	21.33	0.87	0.85
Lymphocytes	74.00	74.66	75.33	76.66	1.30	0.92
Heterophils:lymphocytes (%)	25.00	26.00	27.00	28.00	1.00	0.95

*Means within each column of dietary treatments with no common superscript differ significantly at $P<0.05$. Dietary treatments were as follows: T1 – control; T2 – 0.25% formic acid and 0.25% acetic acid; T3 – 0.5% formic acid and 0.5% acetic acid; T4 – 0.75% formic acid and 0.75% acetic acid.

heterophils and lymphocytes and the H:L ratio due to the effect of organic acids. The H:L ratio is altered in response to stress in chickens, which could not be observed in this study thanks to the controlled environmental conditions.

Blood metabolites

The plasma levels of total protein, albumin, glucose, total cholesterol, triglycerides, LDL cholesterol, VLDL cholesterol, and HDL cholesterol did not differ between the control birds and T2, T3, and T4 (p>0.05). The results of these parameters are given in Table 5. Our results are consistent with those reported by Salem *et al.* [2020] and El-Hakim *et al.* [2009], who observed that the levels of total protein, globulin, HDL, LDL, and albumin by the supplementation of organic acids in the diets of broilers are not modified by the treatment. Moreover, Khosravi *et al.* [2008] stated that the levels of total cholesterol and albumin are not modified by dietary supplementation of organic acids in broilers. Conversely, Zhang *et al.* [2011] administrated sodium butyrate at different levels to 120 1-day-old chickens exposed to *Escherichia coli* lipopolysaccharide, finding inhibitory effects in serum glucose and total protein concentrations at 20 d of age. They concluded that induction with LPS activates catabolism, decreases glucose and triglycerides concentrations and increases total protein concentrations.

Table 5. Plasma constituents of Ross 308 broilers at 42nd day of age fed diets containing different levels of organic acids

Parameters (mg/dL)*	T1	T2	T3	T4	SEM	P-value
Total protein	2.30	2.43	2.43	2.46	0.04	0.69
Albumin	0.93	1.00	1.03	1.06	0.02	0.20
Glucose	263.33	260.00	258.67	274.00	0.70	0.50
Total cholesterol	122.33	125.33	100.00	114.67	4.95	0.29
Triglycerides	106.67	94.33	108.33	121.00	0.85	0.51
LDL Cholesterol	21.33	25.00	19.66	22.00	0.87	0.17
VLDL Cholesterol	21.00	19.00	21.66	24.00	0.92	0.32
HDL Cholesterol	88.33	85.33	80.66	75.00	0.73	0.37

*Means within each column of dietary treatments with no common superscript differ significantly at P<0.05.
Dietary treatments were as follows: T1 – control; T2 – 0.25% formic acid and 0.25% acetic acid; T3 – 0.5% formic acid and 0.5% acetic acid; T4 – 0.75% formic acid and 0.75% acetic acid. LDL – low-density lipoprotein. VLDL – very low-density lipoprotein. HDL – high-density lipoprotein.

Bacterial cell count

Figure 1 shows the total count (log_{10} UFC/g) of Lactobacilli and *Escherichia coli* in the ileal digesta samples of broilers. Interestingly, there are significant differences in the counts of Lactobacilli and *E. coli* between all the treatments (p=0.001, p=0.001, respectively). The data for T4 show a significantly highest count of Lactobacilli (7.33 log cells/g), followed by T3 (7.15 log cells/g), T2 (6.93 log cells/g), and T1 (control, 6.67 log cells/g). Conversely, T4 shows a significant reduction in the count of *E. coli* cells (5.90 log cells/g) followed by T3 (5.98 log cells/g), T2 (6.25 log cells/g), and

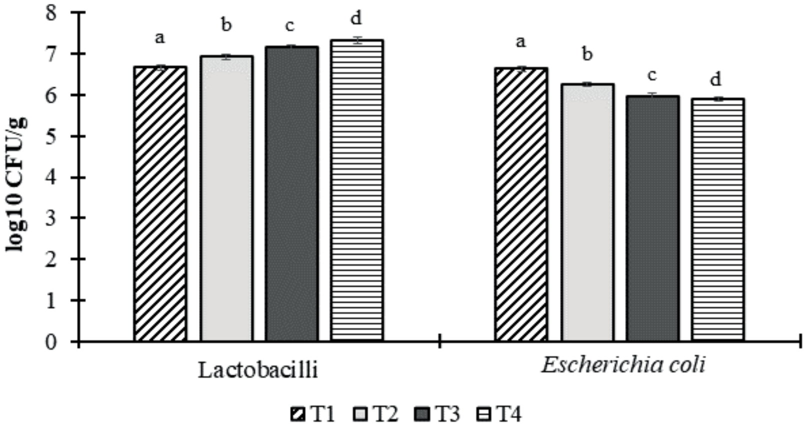

Fig. 1. Total counts of *Lactobacilli and Escherichia coli* in ileal contents of broilers (log10 CFU/g). Means within each column of dietary treatments with no common superscript differ significantly at $p<0.05$. Dietary treatments were as follows: T1 - control; T2 - 0.25% formic acid and 0.25% acetic acid; T3 - 0.5% formic acid and 0.5% acetic acid; T4 - 0.75% formic acid and 0.75% acetic acid.

T1 (6.63 log cells/g). Based on our results it has been shown that the concentration of organic acids in the ileal digesta of broilers was positively correlated with Lactobacillus and negatively correlated with *E. Coli* [Yang *et al.* 2019]. Moreover, Sabour *et al.* [2019] stated that dietary supplementation of 1g/kg of organic acid in a broiler diet increased counts of Lactobacillus bacteria. Khan and Iqbal [2016] observed that organic acid supplementation reduces pathogenic bacteria by disrupting normal physiology of bacteria cells. Lactobacilli have been considered beneficial for the host and have been used in broilers as an alternative to antibiotics and other banned additives [Wu *et al.* 2019].

In conclusion, our results showed that the combinations of 0.5% formic acid plus 0.5% acetic acid and 0.75% formic acid plus 0.75% acetic acid, respectively, reduce the feed conversion ratio. Moreover, dietary supplementation of combined 0.75% formic acid and 0.75% acetic acid in broilers in the current research trial increased the intestinal Lactobacillus bacteria population and decreased the *Escherichia coli* population, which suggests that this combination is ideal to be used as an additive in the diet of broilers, since it improves feed conversion and intestinal health.

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