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Institute of Genetics and Animal Biotechnology
of the Polish Academy of Sciences, Jastrzębiec, Poland

Effect of probiotic lactic acid bacteria and citric acid on performance, serum biochemistry, organ weight and carcass characteristics of broiler chicks*

Arezoo Heidary[1], Diane Valganon de Neeff[2]**, Hosseinreza Shahbazi[3], Magdalena Solka[4]***, František Zigo[5], Alireza Seidavi[1], Joanna Marchewka[4], Patryk Sztandarski[4], Aneta Jaszczyk[4], Paweł Urbański[4], Cyprian Tomasik[4], Ewelina Zima-Kulisiewicz[4] Carlos Augusto Fernandes de Oliveira[2]***

[1] Department of Animal Science, Rasht Branch, Islamic Azad University, Rasht, Iran

[2] Department of Food Engineering, School of Animal Science and Food Engineering, University of São Paulo. Av. Duque de Caxias Norte, 225, CEP 13635-900, Pirassununga, SP, Brazil

[3] Department of Animal Science, Kermanshah Branch, Islamic Azad University, Kermanshah, Iran

[4] Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences, Jastrzębiec, 05-552 Magdalenka, Poland

[5] Department of Animal Nutrition and Husbandry, University of Veterinary Medicine and Pharmacy, 04181 Košice, Slovakia

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**A. Heidary and Diane V. de Neef contributed equally to this study.
***Corresponding authors: m.solka@igbzpan.pl; carlosaf@usp.br

In this work, the effects of the addition of probiotics and citric acid to drinking water on performance, serum biochemistry, relative organ weight and carcass characteristics of broiler chicks were evaluated. Two hundred forty-one-day-old chicks (female Ross 308) were divided into 6 groups and submitted to 6 different treatments for 42 days, with 4 replicates in each treatment, with 10 birds in each. Drinking water was supplemented with a commercial probiotic mixture (Lactofeed®, Takgene Co, Iran) at 0 (control), 0.1 g/L (day 1 to 21) and 0.05 g/L (day 22 to 42), and citric acid at concentrations of 0 (control), 0.2 and 0.4% (day 1 to 42). Compared with controls, no significant changes (*P*>0.05) were found in the performance, serum biochemistry parameters and relative organ weights of broiler chicks receiving water supplemented with probiotics or citric acid after 42 days of experiment. However, a significant (*P*<0.05) interaction effect was observed between probiotic and citric acid supplementation on feed intake within the first 21 days. Moreover, chicks receiving water supplemented with probiotic and 0.2% of citric acid had highest (*P*<0.05) body weight at 42 days of age, as well total carcass weight. Results indicate that water supplementation with Lactofeed® and 0.2% of citric acid are promising strategies to enhance growth performance of broilers.

KEYWORDS: broiler / probiotic / lactic acid bacteria / citric acid / performance / immunity.

Feed is one of the most expensive items in poultry production, and the increasing prices of feed ingredients is decreasing the profits in this activity. Therefore, from an economic standpoint, balanced and effective feeding is very important [Marchewka *et al.* 2023]. There are novel reports on positive effects of feed additives in human [Liu *et al*. 2023, Chuai *et al*. 2023, Zhen *et al*. 2024, Liang *et al*. 2024] and animals [Seidavi *et al*. 2017, Movahhedkhah *et al*. 2019, Chand *et al*. 2021, Wójcik *et al.* 2023]. Feed additives such as synthetic hormones and antibiotics have been extensively used as growth promoters to enhance poultry production [Jadhav *et al*. 2015, Pourreza *et al.* 2023]. However, limitations regarding the possible selection of antibiotic-resistant microorganisms as well as the potential carry-over of residues in meat and eggs have raised concerns about potential health hazards to consumers [Jadhav *et al*. 2015]. For this reason, organic acids and probiotics have been proposed in poultry production as safer feed additives [Islam 2012, Khan and Iqbal 2016].

Organic acids such as citric, lactic and propionic acids or their corresponding salts have been extensively used in broiler nutrition to enhance growth performance. These compounds also have applications as sanitizers in animal feeds to prevent issues like infections caused by *Salmonella* sp., although other beneficial effects may include the suppression of pathogens and improved digestion and mucosal immunity, as well as local effects on the brush border of intestine [Islam 2012]. Some organic acids modulate the digestion and absorption process of proteins by increasing hormone release, and pepsin secretion / proteolysis [Khan and Iqbal 2016]. In this context, citric acid is classified as a weak organic acid and a natural preservative, which exists in small concentrations in several types of citric fruits and other vegetables [Islam 2012]. The inclusion of citric acid in animal diets has beneficial effects including the reduction of pathogens colonization and in the biosynthesis of toxic metabolites, improvement in the bioavailability of proteins and minerals like P, Ca, Mg and Zn [Islam 2012].

Probiotics are defined as feed supplements made up by live microorganisms that positively affects the intestinal balance of the host [Jadhav *et al*. 2015]. The gastrointestinal tract is colonized by two types of microbiota, a beneficial one and a harmful one. A symbiotic relation is observed between the host and beneficial microbes that colonize gut surfaces, which are essential to maintain the body functions under normal physiological conditions [Pourakbari *et al*. 2016]. In adverse internal conditions, immune resistance of the animal is lowered, and the normal gastrointestinal microbiota is affected by the proliferation of pathogenic microorganisms, resulting reduced performance, diarrhea, and gastroenteritis, among other problems [Seidavi *et al*. 2017]. Besides acting as regular, beneficial microbiota, probiotics may also protect against carcass contamination by enteric pathogens during the evisceration process of broilers, also improving parameters of performance such as body weight gain and feed efficiency [Jadhav *et al*. 2015]. There are many reports about the individual effects of probiotics [Pourakbari *et al*. 2016, Pournazari *et al*. 2017, Seidavi *et al*. 2017] or organic acids [Fathi *et al*. 2016, Nosrati *et al*. 2017] on broiler productivity. However, reports describing the combined effects of probiotics and organic acids on broiler performance are scarce in the literature. Therefore, the aim of the present study was to explore the effects of supplementation of drinking water with commercial probiotics and different levels of citric acid on performance, serum biochemistry, relative weight of organs and carcass characteristics of broiler chicks.

Material and methods

Experimental design

A total of 240 one-day-old female Ross 308 chicks with similar body weights were divided into 24 groups, which were assigned to 6 treatments with 4 replicates in each treatment and 10 birds per replicate. All rearing conditions were based on Mojarrad *et al*. [2014] and were the same for all the groups. A vaccination scheme against Newcastle Clone C (10 and 20 day of age) and Gumboro D78 diseases (14 day of age) was adopted. This study was conducted according to the Guide for the Care and Use of the Islamic Azad University Ethics Committee, which were in accordance with the ethical standards laid down in the Declaration of Helsinki and its later amendments.

Lactofeed® (Takgene Co, Iran), a mixture of *Lactobacillus acidophilus*, *L. casei*, *Bifidobacterium bifidium*, *Enterococcus faecium* (2.5×10^7 colony forming units/g of each strain), with no genetically modified organisms, was used in the study. Lactofeed® was used as a supplement dissolved in broiler drinking water at concentrations of 0.1 g/L (from 1-2 days of age) and 0.05 g/L (from 22-42 days of age). Citric acid (BP/USP Co, China) was purchased locally and delivered in drinking water from 1 to 42 days of age. Citric acid was also supplemented in drinking water at levels of 0, 0.2 and 0.4%. A drinking system with automatic nipples was used to provide the drinking water at room temperature *ad libitum*, with all the supplements included according to the following treatments. Therefore, a randomized block design in a factorial scheme with

2 levels of Lactofeed® and 3 levels of citric acid was used, and the 6 treatments were: T1 (Control): water without probiotics (-) and citric acid (0%); T2: water without probiotics (-) and with citric acid at 0.2%; T3: water without probiotics (-) and with citric acid at 0.4%; T4: water with probiotics (+) and without citric acid (0%); T5: water with probiotics (+) and citric acid at 0.2%; T6: water with probiotics (+) and citric acid at 0.4%.

All diets were based on maize-soybean meal balanced according to commercial conditions of broiler farming in Iran [Seidavi *et al*. 2017], without antibiotic feed supplements. The diets were formulated in accordance with the recommendations in Ross 308 strain rearing catalogue, as presented in Table 1. Feed intake (FI) and body weight gain (BWG) were recorded weekly. Feed conversion ratio (FCR) was estimated based on a conventional protocol. The production index was determined by multiplying the liveability (calculated as the number of broilers at slaughter divided by the total number of broilers, multiplied by 100) by the average daily gain and dividing the result by the FCR multiplied by 10 [Sasaki *et al*. 2014].

Table 1. Composition and calculated analysis of the experimental diets used in the trial

Item	Pre-starter (1-7 d)	Starter (8-15 d)	Grower (16-30 d)	Finisher 1 (31-40 d)	Finisher 2 (41-42 d)
Ingredients (%)					
maize	527.3	527.9	484.7	567.8	559
soybean meal	380	379	370	290	260
wheat meal	20	40	100	100	100
soybean oil	26	15	12	10	10
dicalcium phosphate	19	15	12	11	9
oyster shells	11	12	10	10.5	11.5
salt	4	3.5	3.4	3	3
sodium bicarbonate (NaHCO3)	1	-	-	-	-
DL-methionine	3	3	2	1.8	1.5
L-lysine HCL	2.5	1.6	0.5	0.5	0.6
threonine	0.8	0.6	-	-	-
enzymes[1]	0.4	0.4	0.4	0.4	0.4
vitamin mixture[2]	2.5	2.5	2.5	2.5	2.5
mineral mixture[3]	2.5	2.5	2.5	2.5	2.5
Calculated analysis[4]					
metabolizable energy (MJ/kg)	2980	2940	2870	2920	2970
crude protein (%)	22.5	21.5	20.5	18.5	17.5
calcium (%)	1.03	1.0	0.93	0.85	0.81
available phosphorus (%)	0.5	0.48	0.46	0.44	0.43
sodium (%)	0.17	0.17	0.16	0.16	0.16
threonine (%)	0.88	0.86	0.75	0.71	0.69
lysine (%)	1.28	1.2	1.10	0.95	0.91
methionine (%)	0.63	0.59	0.54	0.48	0.45
methionine + cysteine (%)	1.01	0.91	0.83	0.75	0.71

[1] Kemzyme MAP Dry (Kemin Industries, Inc., U.S.A.).
[2] Supplied per kilogram of feed - Vitamin A: 9000 IU; vitamin D_3: 2000 IU; vitamin E: 18 IU; vitamin K_3: 2 mg; thiamine: 1.8 mg; riboflavin: 6.6 mg; calcium pantothenate: 10 mg; niacin: 30 mg; pyridoxine: 3 mg; folic acid: 1 mg; vitamin B12: 0.015 mg; biotin: 0.1 mg; choline: 25 mg.
[3] Supplied per kilogram of feed - Mn: 99.2 mg; Fe: 50 mg; Zn: 84.7 mg; Cu: 10 mg; I: 1 mg; Se: 0.2 mg.
[4] According to National Research Council [1994].

Serum biochemistry analysis and relative weight of organs

Blood collection was performed after withdrawn of feeds from all birds for about 4 hours, to ensure stabilization of plasma components. To further lessen inconsistency of plasma components, all blood samples were collected in the morning. During the 42 days of life, 5-mL samples of venous blood were obtained with a syringe from the ulnar vein of two birds in each replicate (in a total of eight birds per treatment). The most representative birds were carefully selected based on body weight in comparison with the mean body weight value obtained in the replicate group. Blood samples were placed in tubes containing 10 mg of ethylene diamine tetra acetic acid (EDTA) and submitted to centrifugation at 3,000 rpm for 20 min. to ensure plasma separation. Samples were kept at -20°C until the moment of analysis. Glucose oxidase kits (TeifAzmoon Pars, Co., Tehran, Iran) were employed to measure plasma glucose according to the oxidase-peroxidase procedure.

Two broilers from each replicate (eight per treatment) were weighed and killed by cervical dislocation at the last day of the experiment to assess the relative weight of organs related to immunity (Bursa of Fabricius, spleen, liver, and thymus).

Statistical analysis

Data were subjected to two-way analysis of variance as a 2×3 factorial design with 2 levels of probiotic and 3 concentrations of citric, using the General Linear Models procedure in the SAS® software [SAS 2003]. The statistical model used was:

$$Y_{ijk} = \mu + A_i + B_j + AB_{ij} + e_{ijk}$$

where:

μ – overall mean;

A_i – fixed effect of probiotic;

B_j – fixed effect of citric acid;

AB_{ij} – random error;

e_{ijk} – dependent variable.

The Duncan *post hoc* test was used if the initial test result was significant at $P<0.05$.

Results and discussion

Tables 2 and 3 present the effect of water supplementation with probiotics and citric acid at different levels on productive performance (FCR, FI, and BWG) in starter (from day 1 to 21) and grower (from day 22 to 42) Ross 308 broilers, respectively. Although the individual supplementation with probiotics or citric acid did not affect ($P>0.05$) FCR, FI, and BWG in any period, interaction effects ($P<0.05$) between probiotic and 0.2 or 0.4% of citric acid were observed for FI during the starter period

Table 2. Performance parameters at the at the starter period (1^{st}-3^{rd} week of age) of Ross 308 broilers receiving drinking water containing the different levels of probiotics and citric acid

Treatment	Feed conversion ratio	Weight gain (g/day)	Feed intake (g/day)
Probiotics			
-	1.11	48.83	53.31
+	1.10	49.18	53.21
standard error of mean	0.01	0.47	0.40
P value	0.127	0.607	0.863
Citric acid (%)			
0	1.11	48.24	53.59
0.2	1.10	49.77	53.86
0.4	1.11	49.01	53.33
standard error of mean	0.01	0.57	0.49
P value	0.545	0.197	0.207
Interaction effects			
probiotics (+) and citric acid (0.2%)	1.10	49.36	54.36
probiotics (+) and citric acid (0.4%)	1.10	48.62	54.32
standard error of mean	0.01	0.81	0.69
P value	0.334	0.134	0.057

Table 3. Performance parameters at the at the grower period (4^{th}-6^{th} week of age) of Ross 308 broilers receiving drinking water containing the different levels of probiotics and citric acid

Treatment	Feed conversion ratio	Weight gain (g/day)	Feed intake (g/day)
Probiotics			
-	2.04	86.79	176.99
+	2.04	86.90	177.15
standard error of mean	0.02	0.79	0.23
P value	0.959	0.922	0.623
Citric acid (%)			
0	2.06	86.12	176.83
0.2	2.02	87.74	177.31
0.4	2.04	86.67	177.06
standard error of mean	0.02	0.97	0.29
P value	0.489	0.496	0.494
Interaction effects			
probiotics (+) and citric acid (0.2%)	2.05	86.32	176.99
probiotics (+) and citric acid (0.4%)	2.07	85.76	177.06
standard error of mean	0.03	1.37	0.40
P value	0.076	0.095	0.279

(Tab. 2). In addition, no effects ($P>0.05$) of water supplementation with probiotics and/or citric acid were observed on the production index. However, at 42 days of age, the body weight of broilers receiving probiotic and 0.2 of citric acid was higher ($P<0.05$) than the other individual or combined treatments, except for birds supplemented with 0.2% of citric acid alone (Tab. 4).

The beneficial effects of probiotics on performance parameters of broilers have been demonstrated in previous reports. Jahanbani *et al*. [2016] investigated the effect of *Enterococcus facium* isolated from the intestine of *Coracias garrulus* and

Table 4. Production index and body weight of broilers at 42 days of age of Ross 308 broilers receiving drinking water containing the different levels of probiotics and citric acid

Treatment	Production index	Body weight at 42nd day of age (g)
Probiotics		
-	404.0	2891.7
+	405.9	2897.5
standard error of mean	6.3	25.6
P value	0.829	0.874
Citric acid (%)		
0	398.0	2865.0[b]
0.2	413.3	2928.7[ab]
0.4	403.6	2890.0[b]
standard error of mean	7.7	31.3
P value	0.386	0.037
Interaction effects		
probiotics (+) and citric acid (0.2%)	403.3	2987.5 [a]
probiotics (+) and citric acid (0.4%)	397.3	2860.0 [b]
standard error of mean	10.9	44.3
P value	0.111	0.033

[ab]Means within each column with no common superscript letters differ significantly at $P<0.05$.

Lactofeed® probiotic on the carcass characteristics and performance indicators of broilers. Body weight were higher in broilers receiving *E. facium* and Lactofeed®, and FCR was lower in birds that received probiotics in drinking water compared to controls. However, BWG and FCR in the present experiment showed a significant influence of treatment only in week 5 (data not shown). In addition, Jahanbani *et al*. [2016] observed that *E. faecium* and Lactofeed® decreased serum cholesterol and triglycerides, also increasing the antibody response against sheep red blood cells. The authors postulated that the ingestion of *E. facium* isolates or Lactofeed® probiotic in drinking water or by spraying increases the body weight and FCR, also improving the immune system function and the intestinal microbiota of broilers.

Poorghasemi *et al*. [2017] evaluated the effect of Lactofeed® probiotic addition in combination with different fat sources in the feed of broilers on performance, carcass characteristics, and lipid concentrations. Similar to our study, the authors observed no significant changes in BWG, FI, and FCR between controls and supplemented groups. Also, as found in this work, there were no significant changes between controls and probiotic-only supplemented groups in the fat content of breast, thigh, liver, heart, and abdomen. The complete digestion and absorption process of nutrients is fundamental for higher performance, and intestinal microbiota has an essential role in these activities. In this context, probiotics exert positive effects on the host health, as they improve gut microbiota by reducing the activity of pathogenic bacteria and, consequently, improving the FCR values [Poorghasemi *et al*. 2017]. According to a review by Jadhav *et al*. [2015], several studies showed probiotics effectiveness in improving growth rates and body weight gain in broilers. They also observed positive effects on feed intake, as reported in this study. Other important findings were decreased mortality and possible reduction in cholesterol in the serum and in meat. However, the appropriate dose of probiotic is still a matter of discussion, considering the contradictory results on probiotics' effects on carcass characteristics of broilers [Jadhav *et al*. 2015].

Islam *et al*. [2008] observed that the addition of 0.5% citric acid increased the BWG of broilers, when compared with the controls during weeks 0-5 of age, also increasing feed consumption at 2nd and 3rd weeks of age and providing higher feed conversion during weeks 0-5 of age. However, this treatment did not affect carcass characteristics, as observed in our study. The authors concluded that the dietary inclusion of 0.5% may increase the live weight gain and improve the feed conversion of broilers. In the present experiment, we found significant influence at week 1 for BWG and FCR in chickens receiving water supplemented only with citric acid. Citric acid can be an excellent alternative to growth promoters based on antibiotics, as it may improve the gut health, growth rate, feed efficiency, bone mineral content and density, and carcass quality [Islam, 2012], although more studies are necessary to clarify its mode of action and the best concentrations to be applied in commercial broiler operations. Islam [2012] reported that up to 6% citric acid may be added to broiler diets without hampering performance. Best performance parameters were observed with 0.5 and 0.75% citric acid in mash and commercial pelleted diets, respectively. Citric acid may also enhance non-specific immunity as well as antibody titers against Newcastle disease. Although addition of citric acid in drinking water may not affect performance, low doses may improve the intestinal health by decreasing the load of pathogenic bacteria and increasing mineral availability to birds. These effects can also compensate for the lower performance of broilers fed diets with reduced protein and energy levels. Accordingly, Khan and Iqbal [2016] found that ingestion of citric acid, whether in association with other organic acids, presented antimicrobial activity and good effect on the poultry gastrointestinal tract, also improving the digestibility of major nutrients, and stimulating the natural immune response.

The results on serum biochemistry parameters at 42 days of age are shown in Table 5. Albumin, total protein, and glucose were not affected (P>0.05) by any treatment, and no interaction effects were observed in the trial. Table 6 presents the relative weight of organs from birds in the different treatments. No effect (P>0.05) was observed in the relative weights of Bursa of Fabricius, spleen, liver, or thymus, indicating that water supplementation with probiotics and/or citric acid did not affect the weight of organs related to immunity in Ross 308 broilers. Similarly, water supplementation did not affect (P>0.05) the carcass characteristics (percentage of eviscerated carcass, empty abdomen carcass weight, full abdomen carcass weight and defeather body weight) of Ross 308 broilers after 42 days, as shown in Table 7. However, the total carcass weight was higher (P<0.05) in the group receiving probiotic and 0.2% of citric acid.

Fascina *et al*. [2012] included phytogenic additives and organic acids in the diet of broiler chick, and evaluated their effects on the digestibility of nutrients, performance, and carcass characteristics of broilers. The authors also observed that organic acids

Table 5. Serum biochemical parameters at 42 days of age of Ross 308 broilers receiving drinking water containing the different levels of probiotics and citric acid

Treatment	Albumin (g/dL)	Total protein (g/dL)	Glucose (mg/dL)
Probiotics			
-	1.40	3.87	231.67
+	1.34	3.70	237.00
standard error of mean	0.05	0.14	5.17
P value	0.399	0.396	0.475
Citric acid (%)			
0	1.37	3.76	238.25
0.2	1.41	4.04	226.00
0.4	1.33	3.55	238.75
standard error of mean	0.06	0.17	6.33
P value	0.579	0.144	0.297
Interaction effects			
probiotics (+) and citric acid (0.2%)	1.40	4.07	231.25
probiotics (+) and citric acid (0.4%)	1.30	3.43	236.25
standard error of mean	0.08	0.23	8.96
P value	0.831	0.357	0.548

Table 6. Relative weight of organs (g/100 g bodyweight) from Ross 308 broilers receiving drinking water containing the different levels of probiotics and citric acid

Treatment	Bursa of Fabricius	Spleen	Liver	Thymus
Probiotics				
-	0.05	0.11	2.17	0.22
+	0.06	0.09	2.11	0.21
standard error of mean	0.01	0.01	0.09	0.03
P value	0.355	0.425	0.657	0.865
Citric acid (%)				
0	0.05	0.09	2.27	0.19
0.2	0.06	0.11	2.10	0.24
0.4	0.06	0.10	2.05	0.23
standard error of mean	0.01	0.01	0.11	0.03
P value	0.724	0.682	0.403	0.505
Interaction effects				
probiotics (+) and citric acid (0.2%)	0.07	0.10	2.10	0.23
probiotics (+) and citric acid (0.4%)	0.07	0.11	1.87	0.27
standard error of mean	0.01	0.01	0.16	0.05
P value	0.738	0.378	0.446	0.445

alone or in combination with phytogenic additives improved broiler performance, when compared with broilers submitted to diets containing an antibiotic enhancer at 42 days of age, and improved carcass characteristics. According to Araujo *et al*. [2019], the lack of effect of the probiotic and/or organic acids added to feed can be associated with proper environmental conditions, good management practices, and quality of feeds. However, there is evidence that the inclusion of organic acids in the feed of broiler may influence gastrointestinal bacteria, reduce the counts of pathogens, and mostly control the development of some competitive bacterial species [Hassan *et al*. 2010]. Therefore, the acidification of the diet inhibits this competition for nutritional factors and may possibly reduce the production toxic metabolites by pathogenic bacteria and increase the weight gain. Inhibition of potential growth of pathogenic bacteria in the diet and in the gastrointestinal lumen are also beneficial to host health [Hassan *et al*. 2010].

In the present experiment, supplementation with probiotics and citric acid did not affect any of the carcass characteristics, except for the total carcass weight of birds receiving probiotic and 0.2% of citric acid. Previous studies did not describe any effects of probiotics and/or organic acids on the carcass parameters evaluated. Barbieri *et al*. [2015] analyzed the effect of a probiotics and/or organic acids on the performance of broilers challenged by three *Eimeria* species. The authors found mild alterations in the microbiota and morphology of the intestines of broilers fed these supplements, but there was no significant change in the performance at slaughtering. In another report, the inclusion of organic acid significantly increased BWG, although no effect on this variable was observed on feed consumption and feed efficiency in different growth phases [Agboola *et al*. 2015]. Moreover, a lower feed : gain ratio was observed in the negative control diet, although this parameter was significantly improved by organic acid and probiotics in the initial growth phase. In contrast, Araujo *et al*. [2019] concluded that supplementation with probiotic and/or organic acids resulted in lower performance of broilers challenged by *Eimeria* species (*E. acervulina*, *E. maxima,* and *E. tenella*), when compared with chickens receiving antibiotics.

Table 7 Carcass characteristics of Ross 308 broilers receiving drinking water containing the different levels of probiotics and citric acid

Treatment	Eviscerated carcass (%)	Empty abdomen carcass weight (g)	Full abdomen carcass weight (g)	Defeather carcass weight (g)	Total carcass weight (g)
Probiotics					
-	80.64	1900.8	2356.7	2516.7	2882.1
+	79.91	1899.6	2373.3	2528.3	2892.5
standard error of mean	0.37	19.5	22.1	27.3	20.3
P value	0.18	0.964	0.601	0.766	0.721
Citric acid (%)					
0	79.59	1881.3	2363.7	2522.5	2850.6[b]
0.2	80.54	1921.9	2386.3	2545.0	2920.6[a]
0.4	80.70	1897.5	2345.0	2500.0	2890.6[b]
standard error of mean	0.46	23.9	27.1	33.5	24.8
P value	0.210	0.496	0.570	0.644	0.016
Interaction effects					
probiotics (+) and citric acid (0.2%)	80.19	1886.3	2352.5	2517.5	2983.7[a]
probiotics (+) and citric acid (0.4%)	79.93	1882.5	2342.5	2482.5	2862.5[b]
standard error of mean	0.65	33.9	38.3	47.3	35.1
P value	0.289	0.200	0.220	0.411	0.020

[ab]Means within each column with no common superscript letters differ significantly at $P<0.05$.

In conclusion, supplementation of drinking water with probiotic Lactofeed® and 0.2% of citric acid significantly increased feed intake of Ross 308 broilers within the first 21 days, as well as their body weight after 42 days and total carcass weight at slaughtering. Although no significant changes were observed in other performance parameters, serum biochemistry and relative weight of organs of broiler from the treatments tested, water supplementation with probiotic and 0.2% of citric acid is a promising strategy to enhance growth performance in broilers.

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