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Effect of physical feed form, phytase super dosing, and/or citric acid on growth performance, phosphorus digestibility, and intestinal microbial population of broiler chickens

[*Efeito da forma física da ração, da superdosagem de fitase e/ou do ácido cítrico sobre o desempenho de crescimento, a digestibilidade do fósforo e a população microbiana intestinal microbiana intestinal de frangos de corte*]

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ABSTRACT

We investigated the effects of feed form, phytase super dosing, and citric acid on growth performance, phosphorus digestibility, and intestinal microbial population of broiler chickens. For this purpose, 1000 newly hatched, straight-run Cobb 500 broiler chicks were assigned to 8 experimental treatments with five replicate pens each and 25 broiler chickens per replicate in a 2x2x2 factorial arrangement with two forms of feed (pellets or mash), two phytase concentrations (0 or 2000 FTU per kg), and two citric acid levels (0 or 0.4% of diet) for 42 days. Birds fed pelleted feed showed higher weight gain and feed intake than those fed mash form ($p<0.05$). Adding citric acid and phytase to the pelleted diets compared to the mash diets resulted in elevated body weight gain in chickens ($p<0.05$). Compared to the mash diets, the addition of phytase to the pelleted diet reduced the cecal aerobic bacterial population ($p<0.05$). The addition of both phytase and citric acid to the pelleted diets enhanced the phosphorus and calcium digestibility ($P<0.05$). In general, the use of pellet form with a mixture of citric acid and phytase was better than other treatments to improve performance and digestibility of phosphorus and calcium.

Keywords: broiler chickens, citric acid, feed form, phytase

RESUMO

Investigamos os efeitos da forma da ração, da superdosagem de fitase e do ácido cítrico sobre o desempenho do crescimento, a digestibilidade do fósforo e a população microbiana intestinal de frangos de corte. Para isso, 1.000 pintinhos de corte Cobb 500 recém-eclodidos e de crescimento direto foram distribuídos em 8 tratamentos experimentais com cinco baias de réplica cada e 25 frangos de corte por réplica em um arranjo fatorial 2x2x2 com duas formas de ração (pellets ou purê), duas concentrações de fitase (0 ou 2.000 FTU por kg) e dois níveis de ácido cítrico (0 ou 0,4% da dieta) por 42 dias. As aves alimentadas com ração peletizada apresentaram maior ganho de peso e consumo de ração do que aquelas alimentadas com a forma de purê (p<0,05). A adição de ácido cítrico e fitase às dietas peletizadas, em comparação com as dietas à base de purê, resultou em maior ganho de peso corporal dos frangos (p<0,05). Em comparação com as dietas de purê, a adição de fitase à dieta peletizada reduziu a população bacteriana aeróbica cecal (p<0,05). A adição de fitase e ácido cítrico às dietas peletizadas aumentou a digestibilidade do fósforo e do cálcio (p<0,05). Em geral, o uso da forma de pellets com uma mistura de ácido cítrico e fitase foi melhor do que outros tratamentos para melhorar o desempenho e a digestibilidade do fósforo e do cálcio.

Palavras-chave: frangos de corte, ácido cítrico, forma de ração, fitase

INTRODUCTION

Offering pelleted feed compared to mash feed to broiler chickens positively affects growth performance. Physiological changes in the development of the digestive tract of broiler chickens fed pellets have been reported (Engberg *et al*., 2002). The decreased size and relative weight of the gizzard and its elevated pH during pellet feeding have been associated with decreased grinding activity due to the fine particles in pellet processing (Abdollahi *et al*.,

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2011). Moreover, according to some reports, the feed form affects the microbial profile and digestive enzymatic activity (Engberg *et al*., 2002).

Gizzard volume and pre-gastric secretions are closely and reciprocally related in poultry where the effects of pepsin and hydrochloric acid on food digestion fluctuate tailored to gizzard contractions and food retention time. Excessive gizzard activity in poultry leads to more pre-gastric hydrochloric acid secretion, and an increase in the acid level guarantees the health and sterilization of the intestine in poultry (Poorghasemi *et al*., 2017).

Research findings indicate that gizzard volume in poultry can be increased by using large grains or insoluble fibers in the diet. So, diet modifications can not only enlarge gizzard but also increase its capacity to keep food materials. Previous research has delineated that pellet food can recuperate technical food processing, and on the other hand, by mimicking large food particles, improve the activity of digestive enzymes in gizzard (Poorghasemi *et al*., 2015).

Nowadays, using phytase in broiler diets is a common practice intended to eliminate the anti-nutritional effects of phytate (phytic acid), improving phosphorus availability, and decreasing the phosphorus excretion rate. Given the lack of phytase secretion in the poultry's digestive tract, supplementing the diet with phytase elevates the phytate phosphorus digestibility, and eventually enhances the birds' performance and enhances production (Cowieson *et al*., 2012). In recent years, using super dosing of phytase has been recommended with the objective of enhancing the availability of phytate phosphorus in broiler chickens (Cowieson *et al*., 2012). Higher phytase doses result in higher efficiency, providing a balanced Ca:P ratio and confirming the phytase extra phosphoric effect (Marchal *et al*., 2020). Gonzalez-Uarquin *et al*. (2020) reported that the liberation of myo-inositol from phytate into the gastrointestinal tract results in extra-phosphoric effects. An increase in plasma myo-inositol or an increase in myo-inositol content in the plasma, digestive tract, and tissue of young birds enhanced the levels of inositol and Myo-inositol phosphate in erythrocytes of hatchlings from a breeder flock fed phytase the use of super dose phytase when reducing phosphorus level in the diet has been associated with improved digestibility of amino acids, minerals, calcium, and energy in broiler chickens (Gonzalez-Uarquin *et al*., 2020; Whitfield *et al*., 2022).

Previous studies with broilers showed that organic acids increased the use of phytate-bound phosphorus (Liem *et al*., 2008). One of the organic acids used in poultry, citric acid, has been shown to chelate free calcium ions, leading to a decrease in the generation of insoluble calcium-phytate complexes, with a much lower affinity to phytase compared to the phytic acid form (Vargas-Rodriguez *et al*. 2015). Inconsistent results have been reported in studies on the combination of citric acid and phytase in the diet of broiler chickens regarding their effect on phosphorus utilization and growth performance (Brenes *et al*., 2003; Aydin *et al*., 2010). Ramigani *et al*. (2015) declared that the addition of organic acids to the diet decreased the pH of the craw (crop) and duodenum, as well as the count of *E. coli*. Also, in a test by Vikram Reddy *et al*. (2017), they reported that body weight increased and feed consumption and conversion ratio decreased in the groups with organic acids supplemented in the pre-starter and starter periods.

Given the low pH in the initial part of the digestive tract, it experiences a high activity of phytase, indicating suitable conditions for phytate decomposition. Therefore, using organic acids in the diet may be associated with positive effects on phytase activity owing to the decreased pH of the digestive tract. Since studies have mainly been carried out using phytase or organic acids separately, not as much attention has been paid to the combination of these two supplements or to their use in diets with two different physical forms, pellets or mash. Thus, the present broiler chicken experiment was aimed at investigating the effects of the addition of organic acids, phytase, or their interaction in broiler chicken diets presented in either of two physical forms, pellets or mash, on performance, digestibility of calcium and phosphorus, and intestinal microbial population.

MATERIALS AND METHODS

The experiment was conducted in Karaj, Iran (35°49'58" N, 50°59'30" E, 1345 m altitude). Research was approved by the Institutional Committee on Animal Use (approval number: IR-AREEO-AEC 018-2022).

In the present study, 1000 newly hatched, straight-run Cobb 500 broiler chicks were assigned to 8 experimental treatments with five replicate pens each and 25 broiler chickens per replicate in a 2x2x2 factorial arrangement with two forms of feed (pellets or mash), two phytase concentrations (0 or 2000 FTU per kg), and two citric acid levels (0 or 0.4% of diet) for 42 days. At the onset of the experiment, broiler chickens were weighed and those with approximately the same average weight were distributed. Initial ambient temperature was 35°C for the first 3 days and decreased to 4 ◦C each week until reaching 23 ◦C at the beginning of week four. The chickens were reared under the same continuous lighting schedule (23 h lighting and 1 h dark).

The feeds were used according to soybean and corn meal. The diets were formulated based on the Cobb 500 management guide with starter (1-10), grower (11-24), and finisher (25-42 days) periods (Table 1). Before formulating the diets, samples of soybean meal, corn, and corn gluten meal were analyzed by the near-infrared (NIR) spectrometry method of Evonik Company (AminoNIR, Evonik Industries AG, Essen, Germany). All experimental diets had similar nutrient concentrations. Dietary treatments included a basal diet based on corn and soybean meal in mash or pellet form, each supplemented with phytase (0 and 2000 FYT/kg) or citric acid (0 and 0.4 %).

The phytase (DSM Nutritional Products, RONOZYME HiPhos, Switzerland) was formulated into the diets as recommended at 2000 FYT/kg. The nutrient release or uplift values for phosphorus (0.18%), calcium (0.2%), and amino acids (Lys, 0.031; Met+Cys, 0.031, Thr, 0.041, Trp, 0.045; Arg, 0.022; Ile 0.031 and Val, 0.035%), energy (93 kcal/kg), and sodium (0.02%) were used in the formulation. In acid-containing treatments, food-grade citric acid was used at a dose of 0.4%.

To gain weight, poultry were weighed at the beginning and end of each rearing period. Also, feed intake was determined during each period. Finally, the feed conversion ratio was calculated by dividing the feed intake by weight gain. Daily losses were recorded, and appropriate corrections were applied on the feed intake (Ahmadi *et al*. 2019). The subjects had feed and water during the 42-d experiment.

To measure the apparent digestibility of calcium and phosphorus, on day 38 two birds from each pen (10 birds for each replicate) were selected and placed in a new pen. The birds were fed experimental diets containing chromium oxide (0.3%) for 4 days, and on day 42, excreta samples were collected. The digestibility of each sample was separately obtained using the following equation (Ramigani *et al*., 2015):

$$Y=100-100\times[(Cr_2O_3\ \text{diet}\times \text{P digesta}) / (Cr_2O_3\ \text{digesta}\times \text{Pdiet})]$$

where Y is digestibility percentage, Cr_2O_3 is chromium oxide content in diet and intestinal contents, P digesta is phosphorus content in intestinal contents, and P diet is phosphorus content in the diet.

To assess carcasses' characteristics, two birds were selected from each run at the end of the experimental period, and after at least 6 hours of starvation, the birds were slaughtered, and carcasses were dissected. The weight and parts of the carcass, such as breast, thigh, heart, gizzard, intestine, liver, and spleen were weighed using a digital scale to the level of 0.01 gram. Then by dividing the weight of each part by the pre-slaughter weight, the percentage of each parameter was estimated (Poorghasemi *et al*., 2017).

In each repetition, two birds were chosen on days 22^{nd} and 42^{nd} and slaughtered for obtaining samples from the digestive system. For this purpose, one gram of different parts of the digestive system (crop, gizzard, and ileum) was taken and poured into 9 mL of deionized water. After that, pH was read using a pH meter (Metrohm, 747) (Al-Natour and Alshawabkeh, 2005).

Table 1. Ingredients and calculated nutrient composition of experimental diets

Ingredients (%)	Starter (1-10 d)		Grower (11-24 d)		Finisher (25-42 d)	
	-Phytase	+Phytase	-Phytase	+Phytase	-Phytase	+Phytase
Corn	52.30	56.06	56.73	61.75	59.20	66.06
Soybean meal (44 % CP)	37.80	38.40	35.70	32.80	31.20	28.20
Corn gluten meal (60% CP)	2.60	0	0	0	0	0
Sand	0	1.00	0	1.20	0.80	0.80
Soybean oil	2.60	0.90	3.20	0.90	4.80	1.90
MC phosphate	1.50	0.70	1.40	0.60	1.26	0.45
CaCO3	1.40	1.25	1.35	1.15	1.20	1.05
Salt	0.24	0.19	0.24	0.17	0.24	0.14
Sodium bicarbonate	0.19	0.19	0.19	0.23	0.19	0.27
*Vitamin premix	0.30	0.30	0.25	0.25	0.25	0.25
*Mineral premix	0.30	0.30	0.25	0.25	0.25	0.25
DL-Met	0.32	0.32	0.32	0.30	0.29	0.26
L-Lys HCL	0.26	0.21	0.20	0.23	0.18	0.22
L-Thr	0.13	0.10	0.09	0.07	0.07	0.06
Choline HCL 60%	0.08	0.08	0.08	0.08	0.07	0.07
Phytase*	0	200	0	200	0	200
Calculated nutrient composition (%)						
ME (Kcal/kg)	2905	2915	2976	2981	3111	3107
CP	22	22	20	20	18.20	18.20
Ca	0.89	0.89	0.85	0.84	0.76	0.76
Total P	0.75	0.58	0.71	0.54	0.67	0.48
AvP	0.45	0.45	0.42	0.42	0.38	0.38
Na	0.17	0.17	0.17	0.17	0.17	0.17
Lys	1.22	1.22	1.05	1.05	0.95	0.95
Met	0.45	0.45	0.42	0.42	0.39	0.39
Met+cys	0.88	0.88	0.80	0.80	0.74	0.74
Thr	0.77	0.77	0.69	0.69	0.65	0.65
Trp	0.18	0.18	0.17	0.17	0.17	0.17
Arg	1.24	1.24	1.10	1.10	1.03	1.03
Val	0.89	0.89	0.81	0.81	0.73	0.73

*Vitamin and mineral concentrations per kilogram of diet: retinol 11,000 IU, cholecalciferol 4,000IU, α-tocopherol acetate 11 IU, vitamin K1 2.2 mg, thiamine 1.5 mg, riboflavin 4 mg, niacin 35 mg, pantothenic acid 8 mg, pyridoxine 2.5 mg, folic acid 0.5 mg, vitamin B12 0.01mg, biotin 0.15 mg, betaine 190 mg, Zn 65 mg, Mn 75 mg, Se 0.2 mg, Cu 6 mg, and Fe 75 mg.
*2,000 FTU phytase per each kg.
Citric acid treatments contain 0.4 % citric acid.

For microbial count, samples were taken from the contents of the cecum of two birds killed at the age of 42 days. One gram of each specimen was diluted 1:9 (wt/vol) in sterile saline (154 mmol/L) to count coliforms, aerobes, and *Lactobacillus* bacteria. Ten sequential dilutions 1:9 were applied to all specimens (vol/vol). Coating of plates was done evenly using 0.1 mL of each of the final four dilutions with lactic acid and incubated at 37°C for 24 hours on MacConkey agar to assess the total plate colony counts, and then *Lactobacillus* colonies were determined on MRS agar medium for 48 hours at 37° C. To estimate the log CFU for each gram of cecal content, the plates containing countable colonies were enumerated and averaged.

Data analysis was done by the GLM procedures of SAS in a randomized complete block design with a 2×2×2 factorial (SAS, 1990). The main factors considered in this analysis were two levels each of dietary phytase (0 and 2,000 FYT per kg), citric acid (0 and 0.4%), and two feed forms (mash vs. pellet). The proposed mathematical model was as follows:

$$y_{ijkl} = \mu + A_i + P_j + F_k + A_i{*}P_j + A_i{*}F_k + P_i{*}F_k + P_i{*}A_i{*}F_k + e_{ijkl}$$

In this model, Y_{ijkl} was the value of each observation for the studied trait, μ was the average of observations, A_i was the citric acid effect, P_j was the phytase effect, F_k was the feed form effect, ($A_i{*}P_j$) was the effect of the combined use of citric acid and phytase, ($A_i{*}F_k$) was the effect of the combined use of citric acid and feed form, ($P_i{*}F_k$) was the effect of the combined use of phytase and feed form, ($P_i{*}A_i{*}F_k$) was the effect of the combined use of phytase, citric acid, and feed form and e_{ijkl} was the effect of experimental error. Means were compared by Tukey's test and at 5%.

RESULTS

In the starter period, feed intake was not influenced by the feed form factor, also the impact of citric acid, phytase (PHY), and their interactions was not significant (Table 2). The factor of feed form and the interaction effects of CA×FF, PHY×FF, and FF×CA× PHY significantly affected the body weight gain so that supplementing the pellet diet with phytase, citric acid, or citric acid-phytase, and without additives increased the body weight compared to the mash form. In the starter period, the experimental treatments did not affect the feed conversion ratio.

Table 2. Effect of super dose of phytase and citric acid on growth performance of broilers

Treatment		Starter (1-10 d)			Grower (11-24 d)			Finisher (25-42 d)			Total period (1-42 d)		
	Additive	FI	BWG	FCR	FI	BWG	FCR	FI	BWG	FCR	FI	BW	FCR
Mash	None	299.8	269.0[bc]	1.11	1416.2[c]	974.2[c]	1.54	2635.8[b]	1329.6[d]	1.98	4368.6[c]	2537.6[d]	1.72
	CA	303.2	277.6[b]	1.09	1423.6[c]	961.6[c]	1.48	2668.2[b]	1357.6[d]	1.96	4394.0[bc]	2597.0[cd]	1.69
	PHY	299.4	265.0[c]	1.12	1443.0[c]	934.0[d]	1.45	2647.5[b]	1370.2[cd]	1.93	4362.8[c]	2613.8[cd]	1.67
	CA+PHY	307.1	267.4[bc]	1.14	1464.2[c]	973.7[c]	1.50	2686.1[b]	1416.3[c]	1.89	4457.4[bc]	2667.4[bc]	1.67
Pellet	None	306.1	296.8[a]	1.03	1549.8[b]	975.1[c]	1.59	2710.1[a]	1425.1[bc]	1.90	4566.0[b]	2697.0[bc]	1.69
	CA	305.3	296.2[a]	1.03	1599.4[a]	989.4[bc]	1.60	2723.0[a]	1483.3[b]	1.84	4637.6[a]	2766.9[b]	1.68
	PHY	301.9	290.8[a]	1.04	1609.0[a]	1021.3[b]	1.57	2749.7[a]	1472.5[b]	1.87	4660.6[a]	2808.8[cd]	1.66
	CA+PHY	307.2	298.4[a]	1.02	1593.4[a]	1109.4[a]	1.44	2796.6[a]	1545.4[a]	1.81	4695.2[a]	2963.2[a]	1.58
	SEM	4.70	5.30	0.06	20.8	18.8	0.09	46.9	29.7	0.11	53.0	41.4	0.08
P-values													
FF		0.05	0.01	0.21	0.01	0.03	0.22	0.31	0.05	0.14	0.02	0.01	0.57
CA		0.43	0.27	0.18	0.04	0.04	0.69	0.02	0.03	0.21	0.03	0.05	0.54
PHY		0.74	0.16	0.43	0.04	0.02	0.24	0.47	0.03	0.11	0.04	0.02	0.23
CA×PHY		0.18	0.15	0.21	0.32	0.63	0.18	0.14	0.87	0.11	0.09	0.01	0.07
CA ×FF		0.38	0.05	0.34	0.03	0.14	0.23	0.04	0.04	0.09	0.04	0.04	0.28
PHY ×FF		0.77	0.02	0.11	0.05	0.05	0.23	0.05	0.05	0.11	0.02	0.04	0.31
FF×CA× PHY		0.33	0.03	0.39	0.02	0.03	0.30	0.03	0.01	0.14	0.01	0.02	0.19

a-b Means with different superscripts in a column differ.
FF is Feed Form; CA is Citric Acid; PHY is Phytase; BWG is Body Weight Gain; FI is Feed Intake; and FCR is Feed Conversion Ratio.

In the grower period, adding citric acid, phytase, and citrate + phytase to chickens fed with pellet form increased feed intake than mash form ($p<0.05$). The addition of citrate-phytase in pellet diets enhanced the body weight gain of broiler chickens than other experimental treatments ($p<0.05$). Supplementing mash diets with phytase had lower body weight gain than other experimental treatments ($p<0.05$). The experimental treatments did not affect the feed conversion ratio.

In the finisher period, the addition of citrate, phytase, or phytase-citrateenzyme to the diet of broiler chickens with pellet form increased feed intake compared to mash form ($p<0.05$). Using phytase + citrate enzyme in the pelleted diets caused an increase in the body weight gain of broiler chickens compared to other experimental treatments, whereas the addition of citrate to the mash diet decreased the body weight gain of broiler chickens. The experimental treatments did not affect the feed conversion ratio.

Total feed intake showed a significant increase in the groups using pellet feed in association with citric acid, phytase, and their mixture compared to other groups (P<0.05). Also, the highest statistically significant weight gain was observed in the groups consuming pellet feed with a mixture of citric acid and phytase compared to other groups (P<0.05).

Chickens fed pelleted diets showed higher calcium digestibility compared to mash diets (P<0.05) (Table 3). The addition of citric acid in pelleted diets enhanced calcium digestibility compared to chickens fed mash diets (p<0.05). The use of phytase and phytase + citric acid in pelleted or mash diets did not have a significant difference in calcium digestibility. The physical form of the feed did not affect phosphorus digestibility. However, adding citric acid, phytase, or citric acid + phytase in the pelleted diets enhanced the digestibility of phosphorus compared to the mash diets (p<0.05).

Table 3. Effect of super dose of phytase and citric acid on digestibility of Ca and P

Treatment		Ca (mg/dL)	P (mg/dL)
Mash	None	58.4^{c}	65.1^{cd}
	CA	62.4^{c}	62.3^{d}
	PHY	68.9^{b}	66.4^{cd}
	CA+PHY	72.2^{ab}	69.6^{c}
Pellet	None	68.7^{b}	74.8^{bc}
	CA	69.3^{b}	76.3^{b}
	PHY	73.4^{ab}	78.8^{ab}
	CA+PHY	77.8^{a}	82.9^{a}
SEM		3.1	3.1
P-value			
FF		0.04	0.04
CA		0.12	0.21
PHY		0.03	0.03
CA×PHY		0.08	0.35
CA×FF		0.01	0.01
PHY×FF		0.03	0.04
FF×CA×PHY		0.03	0.04

[a-b] Mean values with different superscripts in a column differ.
FF: feed form; CA: citric acid; PHY: phytase

As shown in results, no significant effect was observed by adding different levels of phytase and citric acid in pelleted or mash diets on the carcass thigh, breast, heart, gizzard intestine, liver, or spleen (Table 4).

Also as shown in results, at 22 days the pH of crop and gizzard contents in broilers fed pelleted diets increased significantly than the mash diets (Table 5). At 42 days, the pH of the crop and ileum was higher in chickens receiving pelleted rather than mash diets (P<0.05). At 22 and 42 days, the addition of citric acid in the pelleted or mash diets had no significant effect on the pH of the digestive contents of broiler chickens. At 21 days old, using phytase in pelleted diets increased the pH of the gizzard compared to mash diets treated with phytase (p<0.05); but the addition of phytase in the pelleted or mash diets did not significantly affect the pH of the crop and ileum of broiler at 22 and 42 days. At 42 days, supplementing pelleted diets with phytase + citric acid increased ileum pH compared to mash diets with the same additive. At 22 and 42 days, there were no significant differences in the crop and gizzard pH between these two treatments.

Supplementing the pelleted diets of broiler chickens with citric acid, phytase, or citric acid + phytase reduced the population of aerobes B compared with other experimental treatments except the treatment of mash diet + citric acid (Table 6).

The population of *Coliform* bacteria was affected by the FF and CA factors and by the interaction effects of CA×PHY, CA×FF, and FF×CA×PHY. Supplementing the diet (pellet form) with citrate + phytase and without additives reduced bacteria of coliform (p<0.05). Also, adding citrate to the diet with mash form also reduced the population of *Coliform* (p<0.05). As can be seen, the use of phytase in pellet and mashflour diets could not have a significant effect on the number of *Coliform* bacteria.

The phytase factor and the interaction effect of FF×CA×PHY significantly affected the population of *Lactobacillus* bacteria (p<0.05), but other factors did not significant difference in the population of *Lactobacillus*. The addition of phytase, citrate, or citrate + phytase in the mash or pelleted diets reduced the population of *Lactobacillus* bacteria compared to chickens fed with mash diets without additives (p<0.05).

Table 4. Effect of super dose of phytase or citric acid on carcass characteristics of broilers

Treatment		Carcass characteristics (%)							
		Carcass	Thigh	Breast	Heart	Gizzard	Intestine	Liver	Spleen
Mash	None	71.49	17.78	21.56	0.43	3.49	5.09	1.93	0.06
	CA	78.87	17.67	22.75	0.48	3.46	5.78	2.10	0.08
	PHY	76.87	17.30	23.92	0.44	3.94	5.48	2.33	0.06
	CA+PHY	75.52	18.42	25.48	0.44	3.66	5.25	2.31	0.07
Pellet	None	72.79	17.21	23.47	0.47	3.31	5.43	1.93	0.08
	CA	74.04	17.65	24.35	0.51	3.62	5.24	2.10	0.08
	PHY	76.20	17.88	25.78	0.46	3.32	5.37	2.01	0.09
	CA+PHY	75.56	18.48	24.91	0.47	3.68	4.75	2.02	0.07
SEM		2.68	0.79	1.06	0.09	0.32	0.58	0.17	0.03
P-value									
FF		0.35	0.08	0.15	0.08	0.24	0.23	0.16	0.19
CA		0.12	0.24	0.96	0.28	0.70	0.30	0.17	0.07
PHY		0.32	0.18	0.89	0.22	0.38	0.61	0.47	0.58
CA×PHY		0.28	0.42	0.14	0.93	0.13	0.53	0.15	0.50
CA×FF		0.17	0.98	0.18	0.98	0.99	0.06	0.93	0.20
PHY×FF		0.39	0.37	0.29	0.30	0.10	0.09	0.43	0.39
FF×CA×PHY		0.47	0.48	0.12	0.74	0.34	0.47	0.93	0.87

a-b Means with different superscripts in a column differ.
FF: feed form; CA: citric acid; PHY: phytase

Table 5. Effect of super dose of phytase and citric acid on digestive tract pH of broilers

Treatment		22 days			42 days		
		Crop	Gizzard	Ileum	Crop	Gizzard	Ileum
Mash	None	4.76^{b}	4.08^{c}	5.28^{ab}	5.34^{b}	4.04^{ab}	6.12^{b}
	CA	4.48^{b}	3.94^{c}	5.02^{b}	5.32^{b}	3.88^{b}	6.02^{b}
	PHY	4.70^{b}	4.09^{c}	5.28^{ab}	5.42^{ab}	4.18^{ab}	6.04^{b}
	CA+PHY	4.82^{ab}	4.18^{b}	5.24^{ab}	5.36^{b}	4.04^{ab}	6.14^{b}
Pellet	None	5.10^{a}	4.62^{a}	5.34^{a}	5.58^{a}	4.44^{a}	6.38^{a}
	CA	4.90^{ab}	4.16^{bc}	5.16^{ab}	5.32b	4.08^{ab}	6.18^{b}
	PHY	4.98^{ab}	4.37^{b}	5.12^{ab}	5.42^{ab}	4.42^{a}	6.22^{ab}
	CA+PHY	5.00^{a}	4.56^{ab}	5.38^{a}	5.48^{ab}	4.36^{a}	6.40^{a}
SEM		0.14	0.10	0.15	0.09	0.09	0.10
P-value							
FF		0.96	0.18	0.18	0.37	0.04	0.96
CA		0.17	0.03	0.97	0.46	0.65	0.03
PHY		0.56	0.38	0.69	0.46	0.70	0.05
CA×PHY		0.04	0.03	0.03	0.01	0.03	0.01
CA×FF		0.05	0.03	0.04	0.03	0.04	0.05
PHY×FF		0.05	0.04	0.05	0.01	0.01	0.05
FF×CA×PHY		0.01	0.03	0.01	0.01	0.02	0.01

a-b Means with different superscripts in a column differ.
FF: feed form; CA: citric acid; PHY: phytase

Table 6. Effect of super dose of phytase and citric acid on bacterial population (Log CFU/g)

Treatment		Aerobes B	*Coliform*	*Lactobacillus*
Mash	None	8.66^{a}	5.85^{a}	8.75^{a}
	CA	8.09^{ab}	5.32^{b}	8.22^{b}
	PHY	8.60^{a}	6.00^{a}	8.18^{b}
	CA+PHY	8.56^{a}	5.67^{a}	8.00^{b}
Pellet	None	7.49^{b}	5.21^{b}	8.30^{ab}
	CA	7.47^{b}	5.49^{ab}	7.87^{bc}
	PHY	7.93^{b}	5.65^{a}	8.12^{b}
	CA+PHY	7.70^{b}	4.72^{c}	7.69^{c}
SEM		0.35	0.30	0.25
FF		0.04	0.03	0.30
CA		0.01	0.04	0.16
PHY		0.33	0.27	0.03
CA×PHY		0.12	0.04	0.24
CA×FF		0.09	0.05	0.16
PHY×FF		0.05	0.12	0.46
FF×CA×PHY		0.05	0.02	0.04

[a-b] Mean values with various superscripts in a column differ significantly.
FF is Feed Form; CA is Citric Acid; and PHY is phytase.

DISCUSSION

Based on our results, in the starter and finisher periods, the use of pellet form compared to mash form increased the body weight gain of broilers. In the grower and finisher periods, the feed intake of broilers fed pelleted diets was higher compared to that of mash diets (Table 2). In this regard, Azizian and Saky (2020) reported that using pelleted diets compared to mash diets improved the body weight, feed intake, and feed conversion ratio of broiler chickens. The improved growth performance of chickens receiving pelleted diets compared to mash diets is thought to be related to reducing selective feed consumption, reducing the cost and energy for feed consumption, reducing feed waste, and improving palatability (McKinney and Teeter, 2004). Furthermore, proper thermal processing in pelleting alters the physical structure of feed components such as starch, decreases pathogens and harmful anti-nutritive substances, and increases feed density, particularly energy and protein, resulting in improved performance (Goliyart, 2005). Better performance may also be associated with the positive effect on peristalsis, increased consumption of dietary nutrients, increased digestibility, and reduced time and energy spent eating feed (Guo and Lua, 2001). However, the feed's physical form is not regarded as the only criterion for proper performance as other factors like pellet quality, higher density of nutrients, digestibility, and reduced amount of feed consumption (better efficiency) are important as well (Lemme *et al*., 2006).

In the present study, the addition of a super dose of phytase in mash diets did not improve the growth performance of broilers compared to mash diets without addition. Supplementing pelleted diets with a super dose of phytase significantly affected the growth performance of broiler chickens than pelleted diets without additives. The addition of phytase to the pelleted diets compared to the mash diets improved the growth performance of broiler chickens (Table 2). In this regard, using phytase supplementation in diets with low phosphorus improved broiler chicken weight gain during the growout (Cowieson *et al*., 2012). In a meta-analysis, Bougouin *et al*. (2014) reported that using microbial phytase with low phosphorus in the diet enhanced the growth performance of broiler chickens and reduced the excretion of phosphorus by 30%. Moradi *et al*. (2023) examined the impact of bacterial phytase on growth performance in broilers fed pelleted diets and reported that the phytase supplementation in the negative control diets linearly could improve body weight gain and FCR from hatch to day 35. Dersjant-Li and Kwakernaak (2019) declared that at 1000 FTU/kg average daily gain was similar to the positive control, but FCR showed an improvement by 2.11% on d 42. Marchal *et al*. (2020) stated that adding phytase in a diet decreased Ca (−0.2 to −0.3% points) and total replacement of Pi at 1000 FTU/kg preserved growth performance similar to a nutritionally adequate diet in all growth stages. The bird's improved performance supports the hypothesis of the release of phytase phosphate groups in the inositol ring. Phytate-bound proteins are released and made available to digestive enzymes (Biehl and Baker, 1997). By including 1000 or 1500 FTU/kg phytase in the diet, Walk *et al*. (2014) indicated that the concentration of inositol increased in the birds' gizzard, and this was related to better live performance. Generally, improved weight gain occurred in groups with high levels of phytase in their diet compared to groups fed a diet with low levels of phosphorus.

In the present research, adding citric acid to the pelleted or mash diets had no positive impact on the growth performance of broiler chickens. In the grower period only, feed intake increased by adding citric acid to the pelleted diets (Table 2). In this regard, Abdel-Fattah *et al*. (2008) stated that the addition of citric acid to the diet increased broiler chickens' feed intake and body weight. Nezhad *et al*. (2007) showed that citric acid did not significantly affect feed consumption of broiler chickens. According to Afsharmanesh and Pourreza (2005), adding 0.5% citric acid enhanced body weight gain and feed intake. According to the report of Garcia *et al*. (2007), the acid chemical form, bacterial species, pKa value, animal species, and the acid action site affect performance. Also, environmental conditions, management, type of acid additive, and bird characteristics such as age, species, and stage of production also affect performance (Vargas-Rodriguez *et al*. 2015).

Using pelleted diets in broiler chickens increased calcium digestibility than mash diets, but the digestibility of phosphorus was not influenced by the feed's physical form (Table 3). In this regard, Abdollahi *et al*. (2011) stated that chickens fed with pellets had higher digestibility of calcium and phosphorus compared to the mash diet. Also, they investigated the effects of the physical form of feed in broiler chickens and showed that the use of pelleted or mash diets did not significantly affect the apparent digestibility of phosphorus at 21 and 42 days of age.

Calcium digestibility increased by adding phytase to mash diets, but the digestibility of phosphorus was not affected by phytase. The addition of phytase and citric acid to the pelleted diets had no effect on the digestibility of phosphorus or calcium compared to the pelleted diets without additives. However, the interaction effect of citric acid and phytase increased the digestibility of calcium and phosphorus in pelleted diets compared to mash diets (Table 3). Ravindran *et al*. (2000) reported that adding phytase to broiler chicken diets enhanced the digestibility of phosphorus. Sebastian (1996) stated that phytase increased the utilization of calcium bound to phytate in broilers. Phytic acid can form an insoluble complex with calcium ions, thus decreasing the bioavailability of calcium for intestinal absorption. Under these conditions, the phytase increases calcium availability by releasing calcium bound to the phytate-calcium complex (Sebastian, 1996). Nourmohammadi *et al*. (2012) stated that citric acid and phytase in the diet increased phosphorus digestibility compared to the controls, but calcium digestibility was not affected. Liem *et al*. (2008) declared that adding citric acid to the diet increased the ileal digestibility of phosphorus.

No clear results have been reported regarding the interaction of acids and enzymes; however, adding acid to the broiler chickens' diet appears to provide optimal conditions for the phytase function by acidifying (lowering the pH of) the diet and digestible materials (Han and Lei, 1999). Citric acid alone or together with phytase results in increased phytate hydrolysis, contributing to improved broiler chicken performance (Brenes *et al*., 2003). The application of organic acids provides suitable acidic conditions in the digestive tract and consequently improves the phytase activity thus increasing the dissolution of phytic acid, ultimately improving the availability of nutrients (Aydin *et al*., 2010).

In our research, superdose phytase had no effect on the carcass traits of broiler chickens (Table 4). Kidd *et al*. (2001) stated that the use of an enzyme significantly reduced intestinal weight. Attia *et al*. (2003) reported that the multienzymes and phytase had no significant effect on carcass weight or internal organs of broilers.

The carcass characteristics of broiler chickens were not affected by citric acid. In this regard, Mohammad Bagheri and Najafi (2014) showed that adding citric acid to the diet had no significant effect on the weight of breast, thigh, or broiler chickens' carcass yield.

Our results show that due to the physical form of the diet, the pH of the digestive tract changed during the finisher period (25-42 days); specifically, broilers fed pellets showed a higher pH of the digestive tract (Table 5). Consistent with the results of this study Gonzalez-Uarquin *et al*. (2020) stated that the mash form of the diet decreased the pH of gizzard contents compared to the pellet form. Nonetheless, Naderinejad *et al*. (2015) stated that aggregate pH in response to feed particle size only occurs in pelleted diets.

In our study, supplementing the pelleted or mash diets with citric acid did not significantly affect the pH of the digestive tract. Ghahri *et al*. (2006) investigated various levels of organic acids and stated that the use of different levels of organic acids causes a significant decrease in the pH of the digestive tract. Also, Waldroup and Kanis (1995) stated that the use of different levels of acid in poultry diets significantly reduced the pH of the digestive tract. Banupriya *et al*. (2016) reported that Organic acids can be added to poultry feed at 2.5–3.0 kg/ton for *Salmonella* control and pH decrease and 0.5 kg/ton for mold control. Hesabi Hesabi *et al*. (2021) investigated the effects of citric acid-based acidifiers produced commercially and reported that the addition of organic acid to the diet reduced the intestinal pH of broiler chickens.

Based on our results, the use of pelleted diets reduced the number of aerobes and *Coliform* bacteria compared with mash diets, but there were no significant differences regarding the number of *Lactobacillus* between the two treatments (Table 6). Azizian *et al*. (2018) compared three forms of feed (mash, pelleted, or extruded) in broiler chickens and reported that the total number of aerobic bacteria was not affected by the feed's physical form. They also concluded that in broiler chickens fed on mash diets, the count of lactobacillus increased while that of bifidobacteria decreased (Azizian *et al*., 2018). According to Abadi *et al*. (2019) birds receiving coarse particle size diets showed significantly higher lactic acid bacteria populations in the ceca. Also, broilers receiving pelleted diets showed a lower cecal *Clostridium* spp. bacterial population. In another study, Engberg *et al*. (2002) compared mash *vs*. pelleted diets and found that consuming coarse grains increased the bacteria population. The feed in birds receiving fine particles seems to be less exposed to gizzard protease and low pH, and the digested feed enters the duodenum earlier (Boroojeni *et al*., 2016). Coarse feed particles influence the intestine's microbial profile via two mechanisms including stimulation of the development of calculus and increased secretion of gastric acid, plus decreased pH and consequently, antimicrobial effects on pathogenic bacteria present in the terminal part of the digestive tract (Engberg *et al*., 2002). On the other hand, the colonization of co-food bacteria may stimulate competitive elimination, leading to the reduced colonization of harmful bacteria (Bjerrum *et al*., 2005).

The use of citric acid in the broilers' mash diets reduced the *Coliform* and lactobacillus bacteria of the ceca compared to the mash diets without additives. Adding citric acid to the pelleted diets did not influence the bacterial population compared to the pelleted diets without additives. Al-Khalaifah (2018) reported that adding acid to the diet reduces the population of pathogenic bacteria. In the present research, the total count of gastrointestinal bacteria decreased, and lactic acid bacteria increased. The bacterial population is under the influence of the organic acids through their affecting fungi growth and preventing the formation of mycotoxins, besides improving the broiler chicken performance by competitive elimination of bacteria and positively affecting the birds' immune system (Ghahri *et al*., 2006). Using phytase in mash diets decreased the amount of Lactobacillus. In contrast, the number of *Coliform* bacteria increased with the addition of phytase in the pelleted diets. In this regard, Bougouin *et al*. (2014) stated that supplementation of the diet of broilers with microbial phytase had no effect on the number of coliform bacteria, aerobic bacteria, or lactobacillus.

CONCLUSION

In general, application of phytase or citric acid [use alone or, use together] improved performance, digestibility of calcium and phosphorus, as well as intestinal microbial population of broilers. In the current test, adding phytase to the pelleted diets indicated a greater effect compared to that in the mash diet, the effect that synergistically increased after the addition of acid.

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