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Research Article

Phosphorus Availability in Broilers Given Phosphorus Deficient Diets Containing Phytase with and Without Citric Acid

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ABSTRACT

In the present study, the effects of phytase supplementation with and without citric acid (CA) on growth performance, carcass characteristics and serum minerals concentration, tibia and shank characteristics, and phosphorus (P) retention were determined in broilers fed non-phytate P deficient diets. Ninety six one day old male broilers (COBB-500) were divided into four groups (twenty four birds each, and were fed one of the following diets until they were 35 days old: positive control (PC) diet formulated based on the NRC (1994) recommendations, negative control (NC) diet containing 0.20 % lower non-phytate P than that in the PC diet, and two other diets were formulated by adding only phytase (*Aspergillus niger* derived, 500 FTU/kg of feed) or phytase with CA (*Aspergillus niger*, 500 FTU/kg of feed with 2 % CA). At the age of 36 day, birds were sacrificed, samples were collected, processed, and then analyzed. Compared with the PC group, NC group showed impaired growth performance (final BW, BW gain, and FCR), serum P concentration, tibia and shank characteristics (dry weight, contents of ash and P). However, in most cases, these impairments were ameliorated ($P<0.05$) by the addition of phytase, and the restoration magnitude was non-significantly greater in phytase with CA group. Retention of total P increased ($P<0.05$) in phytase added groups compared with NC group, and was comparable with PC group, although non-phytate P level was lower in those groups. In conclusion, broilers fed phytase added non-phytate P deficient diets (without any dietary synthetic source of P) showed the growth performance, mineral (P) concentration in blood and bone, and relative retention of P comparable with broilers fed recommended one. Addition of CA along with phytase in diet was a costlier affair without any significant beneficial effects.

Introduction

Broilers are basically reared on plant-based feed ingredients which are rich in phytic acid. Phytic acid is the primary storage form of phosphorus (P) in plants, exists as the phytate salt (myo-inositol 1,2,3,4,5,6-hexakisphosphate), and accounts for approximately 50 to 90% of the total P in cereals and legumes (Ravindran et al., 1995). It is well known that maize and soybean meal are two commonly used feed ingredients in broilers diet, contain approximately 0.40% phytate P which is poorly digested by them due to the insufficient endogenous phytase enzyme, and leads to induces an environmental pollutant through P excretion (Abbasi et al., 2019; Tahir et al., 2012). Dietary phytase is recognized as an important feed additive used to increase the availability of phytate P (Dersjant-Li et al., 2015; El-Hack et al., 2018).

On the other hand, P from synthetic source such as di-calcium phosphate is normally used in broilers diet to meet up the requirements, so when phytase also added in diet it makes dietary phytate P available to birds and increase the net amount of available P for birds than their requirement. As a result, spare P is excreted through feces and improper disposal of these feces leads to environmental pollution. Considering these circumstances, it may be applicable to conduct research on diet without synthetic P, but phytase in broilers. Besides, organic acid such as citric acid (CA) is a well-recognized feed additive that is recently used in broilers diet to increase nutrient digestibility as well as mineral retention (Islam et al, 2012; Chowdhury et al., 2009). Although studies have been conducted using phytase alone or with CA in broilers diet, in most cases diets

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contained synthetic P (Brens et al., 2007; Ebrahimnezhad et al., 2008; Nezhad et al., 2011; Deepa et al., 2011; Nourmohammadi et al., 2010, 2012; Demirel et al., 2012; Mohammadagheri et al., 2016; Anwar et al., 2022).

To investigate the efficacy of phytase alone or in combination with CA on the enhancement of P utilization in broilers, we measured the performance, bone quality, serum minerals, and nutrient retention in broilers reared on non-phytate P-deficient diets and discussed the efficacy of these additives in absence of dietary P from any synthetic sources.

Materials and Methods

Birds and Housing

A total of ninety-six, one day old male broiler chicks of COBB-500 strain were obtained from a commercial hatchery and were used in this experiment, which lasted for 35 days. Chicks were individually weighed upon arrival and divided into four dietary groups of 24 birds (eight birds / replication; three replications per group) each balanced for weight. Birds were housed in cage in electrically heated brooders. The brooder and room temperatures were set at 32°C and 29°C, respectively, during the first week. Light was provided for 24 h throughout the experiment.

Experimental Diets

Corn-soybean meal-based diets were used in this study. The diets (Table 1) included - a positive control (PC), formulated according to the NRC (1994) recommendations, a negative control (NC) diet formulated to contain 0.20% lower non-phytate P than the PC diet, NC diet containing phytase (*Aspergillus niger* phytase, Rena phytase, Reneta Limited, Bangladesh), NC diet containing phytase (500 FTU/kg of feed) and CA (2 %); Phytase and CA was added at the top of the ration. One phytase unit is defined as the amount of enzyme that liberates 1 µmol of inorganic P per minute from sodium phytate at a pH of 5.0 and temperature of 37°C. Starter diet contained 22.3% CP and 3,020 kcal of ME/kg and were used for 1-14 days of age. Next, grower diet with 21.1% CP and 3,140 kcal of ME/kg were provided for 15-35 d of age.

Table 1. Ingredients and chemical composition of the experimental diets[1] (%)

Ingredients	Diets							
	Starter diet (d 1-14)				Grower diet (d 15-35)			
	PC	NC	NC+ Phytase	NC+ Phytase + CA	PC	NC	NC+ Phytase	NC + Phytase + CA
Corn	42.5	43.2	43.2	43.2	47.0	47.7	47.7	47.7
Pro.C	15.0	15.0	15.0	15.0	11.0	11.0	11.0	11.0
SBM	32.0	32.0	32.0	32.0	31.0	31.0	31.0	31.0
Limestone	0.5	1.8	1.8	1.8	0.7	1.5	1.5	1.5
DCP	2.0	-	-	-	1.5	-	-	-
Oil	7.0	7.0	7.0	7.0	7.8	7.8	7.8	7.8
Vit-min premix[2]	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Methionine	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Salt	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Chemical composition (analyzed value %)								
CP	22.26	22.37	22.34	22.29	21.03	21.05	21.09	21.06
CF	4.80	4.81	4.81	4.81	4.64	4.13	4.09	4.65
Ca[3]	1.00	1.01	1.01	1.01	0.94	0.92	0.91	0.90
Total P	0.79	0.59	0.59	0.59	0.71	0.52	0.52	0.52
Phytate P	0.33	0.34	0.34	0.34	0.35	0.36	0.36	0.36
Non-phytate P[3]	0.46	0.25	0.25	0.25	0.36	0.16	0.16	0.16
ME (kcal/ kg)[4]	3019	3027	3027	3027	3139	3140	3140	3140

[1]PC= Positive Control, NC= Negative Control, NC + Phytase = Negative Control + *Aspergillus niger* derived phytase, NC + Phytase + CA = Negative Control + *Aspergillus niger* derived phytase + Citric Acid, Pro.C = Protein concentrate, P = Phosphorus. Phytase and CA was added at the top of the ration.

[2]Each kg premix contained: vitamin A palmitate, 6,600 IU; cholecalciferol, 2,200 IU; menadione dimethylpyridine bisulfite, 2.2 mg; riboflavin, 4.4 mg; pantothenic acid, 13 mg; niacin, 40 mg; choline chloride, 500 mg; biotin, 1 mg; vitamin B12, 22 µg; ethoxyquin,125 mg; iron, 50 mg; copper, 6 mg; zinc, 40 mg; manganese, 60 mg; selenium, 0.2 mg.

[3]Calculated value

[4]Calculated nutrient content was based on ingredient composition data from NRC (1994).

Experimental Procedure

Birds were kept in floor pens from 1 to 28 days of age and then transferred to wire-floor cages for excreta collection. Diets and water were provided *ad libitum* for the 35 days experimental period. Feed intake (FI) and body weight (BW) were recorded daily and weekly, respectively. Feed conversion ratio (FCR) was calculated at the end of trial as the ratio of FI to weight gain (g

feed/g gain). Excreta were collected from 33 to 35 d of age and stored at −20°C for determination of apparent nutrient retention. Care was taken during the collection of excreta samples to avoid contamination from feathers and other foreign materials. Frozen excreta samples were then thawed, homogenized, dried, and ground before analysis. On 36 d of age, seven birds per dietary group were killed by cervical dislocation and then subjected to scalding at about 55° C for 30 seconds. Feathers were removed, heads and shanks were separated and dressed weight was recorded. Evisceration was done by removing crop, gullet, trachea and preen glands. Eviscerated weight was recorded as the weight of carcass together with giblets (heart, liver and gizzard). Weight of giblets and their constituting organs were also taken separately. All the weights related to carcass traits were expressed as the percentage of live weight. Blood sample was collected from the live birds during slaughtering in falcon tube and quickly preserved in ice box to prevent blood clotting, then centrifuged (Z 306, Hermle, Germany) at 3421 rpm (6 × 50 ml) for 15 minutes to collect the blood serum and preserved at -20˚C for further analysis. Serum samples were analyzed to measure serum calcium (Ca) and P using specific kit by colorimetric method (Bioanalyzer Urit-810, Urit Medical Electronic Group Co. Ltd., China). Shank and tibia bones (2 shanks and 2 tibias per bird) were collected, measured for dry weight after drying at 100°C for 24 h, and then ashed at 600°C for 24 h (Chung and Baker, 1990). The percentage ash was determined relative to the dry weight of the bone.

Cost analysis

The cost of broiler production for each treatment group was calculated based on the market price of feed ingredients, price of chicks, test substances (phytase and CA), vaccination, medication and electricity.

Chemical and Statistical Analyses

Samples of diets and excreta were analyzed for proximate composition following the standard methods (AOAC, 2005). Total P was measured according to ISO (1998), in brief: feed material was ashed following digestion in hydrochloric acid; molybdovanadate reagent was added which results in a characteristics yellow colour after reaching with P and that was measured spectrophotometrically. Phytate P of the samples were measured according to Haug and Lantzsch (1983), in brief: phytic acid was precipitated with an acidic iron-III-solution of known iron content; the decrease of iron in the supernatant is a measure for the phytic acid content. Non-phytate P was calculated by subtracting the phytate P from total P. Retention of total P and nitrogen was the amount of these retained per bird per day, which was calculated based on their availability and feed intake (Um and Paik, 1999).

Statistical significances among the dietary groups were determined using Tukey's multiple comparison tests at a significance level of 5% after one-way ANOVA (Statistical Packages for the Social Sciences, IBM SPSS, Version 20).

Results

Growth performance

The effect of phytase supplementation with and without CA on final BW, weight gain (WG), total FI and FCR of broilers fed non-phytate P deficient diets are presented in Table 2. Final BW in the birds given PC diet (1,591 g) was slightly lower than the corresponding value of male COBB 500 broiler (2,392 g) (Cobb-Ventres.com 2018). This value was further decreased by about 5 % in the NC group but restored (increased approximately 9 % compared with PC group) with the addition of phytase alone as well as with CA in diets. BW gain showed a similar trend as the final BW. Non-significant variation was observed among the groups in terms of total FI; however, numerically lowest FI was recorded in NC group and highest in phytase added group. FCR in the PC group (1.84) was higher than the corresponding value of male COBB 500 broiler (1.48) (Cobb- Ventres.com 2018), which was deteriorated in the NC group (2.04). Although restoration of the FCR was observed in phytase added group (1.83), best FCR was observed in phytase with CA (1.78) group.

Table 2. The effects of phytase with and without citric acid on growth performance of broilers fed non-phytate phosphorus deficient diets[1]

Parameters	Groups				*P*-value
	PC	NC	NC + Phytase	NC + Phytase + CA	
Initial BW (g/b)	39.5±1.09	40.8±1.17	40.1±1.15	40.08±1.01	0.446
Final BW (g/b)	1591.4^{b}±18.9	1523.3^{c}±21.7	1731.5^{a}±18.7	1734.8^{a}±33.53	0.001
Live WG (g/b)	1651.9^{a}±27.98	1482.4^{c}±51.39	1691.4^{a}±17.95	1694.7^{a}±32.52	0.001
Total FI (g/b)	3030.9±49.25	2970.5±43.06	3096.2±28.26	3017.2±41.97	0.346
FCR	1.84^{b}±0.03	2.04^{a}±0.02	1.83^{c}±0.03	1.78^{c}±0.04	0.001

$^{a-c}$Mean values within the same row with different superscripts are significantly different ($P < 0.05$).
PC= Positive Control, NC= Negative Control, NC + Phytase = Negative Control + *Aspergillus niger* derived phytase, NC + Phytase + CA = Negative Control + *Aspergillus niger* derived phytase + Citric Acid, BW = Body weight, FI = Feed intake, FCR = Feed conversion ratio.

[1]Values for each parameter represent mean±standard deviation values with twenty-four observations.

Carcass characteristics and serum minerals concentration

The effect of phytase supplementation with and without CA on carcass characteristics and serum mineral concentration in broilers fed non-phytate P deficient diets are summarized in Table 3. Dressed weight as percentage of live weight was decreased in NC group, though, it was recovered with the addition of phytase. Addition of CA did not show any further effect in this case. Eviscerated and carcass weight remained almost similar in all groups. Reducing dietary non-phytate P depressed serum Ca and P level (mg/dl) concentration in broilers. Serum analysis showed a significant reduction of Ca and P concentration in NC group compared with PC group. However, the concentration restored with the addition of phytase, and the values further increased numerically in phytase with CA group.

Table 3. The effects of phytase with and without citric acid on carcass characteristics and serum minerals concentration in broilers fed non-phytate phosphorus deficient diets[1]

Parameters	Groups				*P*-value
	PC	NC	NC + Phytase	NC + Phytase + CA	
Carcass traits (% of live weight)					
Dressed wt.	81.21[a]± 0.13	79.11[b]±0.14	81.41[a]±0.24	81.31[a]±0.19	0.027
Eviscerated wt.	72.06±0.48	69.42±0.58	72.72±0.37	72.18±0.33	0.647
Giblets wt.	4.39±0.05	4.22±0.14	4.40±0.09	4.49±0.12	0.458
Serum concentration (mg/dl)					
Ca	9.37[a]±0.32	8.53[b]±0.23	9.02[a]±0.27	9.12[a]±0.19	0.038
P	5.76[a]±0.33	4.76[b]±0.55	6.03[a]±0.18	6.15[a]±0.15	0.001

[a-b]Mean values within the same row with different superscripts are significantly different ($P < 0.05$).

PC= Positive Control, NC= Negative Control, NC + Phytase = Negative Control + *Aspergillus niger* derived phytase, NC + Phytase + CA = Negative Control + *Aspergillus niger* derived phytase + Citric Acid Ca = Calcium, P = Phosphorus.

[1]Values for each parameter represent mean±standard deviation values with seven observations.

Tibia and shank characteristics

The effect of phytase supplementation with and without CA on tibia and shank characteristics in broilers fed non-phytate P deficient diets are summarized in Table 4. All values in tibia (dry weight, ash and P content) decreased significantly in the NC group compared with the PC group, and the values were restored with the addition of phytase. Comparing the values, the degree of restoration was greater ($P>0.05$) in phytase with CA group. Trend was almost similar in shank characteristics, but P content. Although significant effect of diets was observed in dry weight and ash content, P content of shank was nearly comparable in all groups.

Table 4. The effects of phytase with and without citric acid on tibia and shank characteristics in broilers fed non-phytate phosphorus deficient diets[1]

Parameters	Groups				*P*-value
	PC	NC	NC + Phytase	NC + Phytase + CA	
Tibia					
Dry wt. (g)	6.18[a]±.07	5.13[b]±.08	6.10[a]±.02	6.31[a]±.85	0.028
Ash (%)	42.82[a]±2.28	39.24[b]±1.50	42.97[a]±1.40	43.32[a]±1.41	0.013
P (%)	3.94[a]±0.03	3.33[b]±0.01	3.86[a]±0.01	4.07[a]±0.05	0.035
Shank					
Dry wt. (g)	3.76[a]±0.21	2.82[b]±0.32	3.39[a]±0.35	3.84[a]±0.42	0.031
Ash%	42.10[a]±1.55	35.22[b]±1.50	41.61[a]±0.43	42.30[a]±0.41	0.001
P %	3.56±0.12	3.47±0.15	3.50±0.09	3.55±0.18	0.078

[a-b]Mean values within the same row with different superscripts are significantly different ($P < 0.05$).

[1]PC= Positive Control, NC= Negative Control, NC + Phytase = Negative Control + *Aspergillus niger* derived phytase, NC + Phytase + CA = Negative Control + *Aspergillus niger* derived phytase + Citric Acid, P = Phosphorus.

[1]Values for each parameter represent mean±standard deviation values with seven observations.

Phosphorus and nitrogen retention

The effect of phytase supplementation with and without CA on the retention of P and nitrogen in broilers fed non-phytate P deficient diets are presented in figure (Figure 1- phosphorus and nitrogen retention). Retention of P varied between PC and other groups because of the varying dietary level of P. Interestingly, retention increased in phytase added groups compared with NC group, though these groups contained similar level of dietary P. Besides, P retention in phytase added groups were comparable with PC group. Highest ($P>0.05$) retention was observed in phytase with CA

group, and the value (48.37 %) was numerically higher than that in the PC group (46.85 %). On the other hand, nitrogen retention varied considerably among the groups although diets contained similar level of crude protein. Lowest (P<0.05) retention was recorded in NC group and highest (P>0.05) in phytase with CA group.

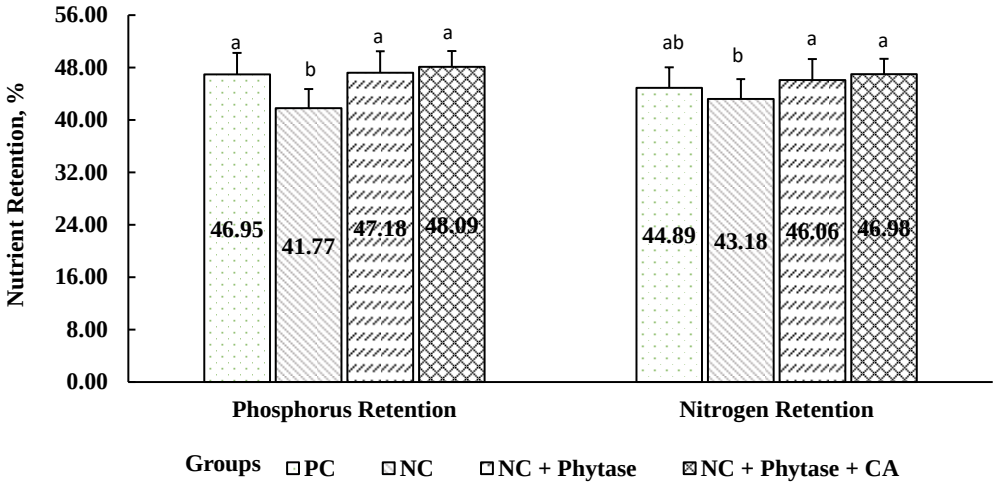

Figure 1. The effects of phytase with and without citric acid (CA) on phosphorus and nitrogen retention in broilers fed non-phytate phosphorus deficient diets.

Cost of production

The effect of phytase supplementation with and without CA on the cost of production are presented in Table 5. Costs were calculated considering costs of chicks, feed, test substances, medication, vaccination, and electricity. It was observed that the complete exclusion of synthetic P source reduced the feed cost in NC group. However, addition of phytase and CA to the NC diet increased (P<0.05) the cost of feed per bird. Production cost per kilogram of live weight gain of birds was lowest in phytase added group, though there were non-significant differences among control, phytase and phytase with CA groups.

Table 5. Analysis of cost [in BDT] of broilers fed phytase with and without citric acid in non-phytate phosphorus deficient diets [1]

Cost (35-d trial)	Groups				*P*-value
	PC	NC	NC + Phytase	NC + Phytase + CA	
Cost/kg feed[2]	54.30	53.67	53.73	56.31	
Cost (feed/broiler)	164.6^{b}	159.4^{c}	166.4^{ab}	169.9^{a}	0.003
Cost (feed + others[3])/broiler	227.1^{b}	221.9^{c}	228.8^{ab}	232.4^{a}	0.003
Cost (feed + others)/ kg of live weight gain	137.5^{b}	149.8^{a}	135.3^{b}	137.1^{b}	<0.001

$^{a-c}$Mean values within the same row with different superscripts are significantly different (P < 0.05).
[1]PC= Positive Control, NC= Negative Control, NC + Phytase = Negative Control + *Aspergillus niger* derived phytase, NC + Phytase + CA = Negative Control + *Aspergillus niger* derived phytase + Citric Acid.
[2]Cost of test substances included.
[3]Other includes costs of chicks, medication, vaccination, electricity.

Discussion

In the present study, the diets were formulated based on the NRC (1994) in which the nutrient specifications were slightly lower than those in "Cobb 500 nutrition specification". This may be one of the reasons of slightly decreased final BW in PC group compared with the Cobb 500 broilers (Cobb- Ventres.com 2018). Lowering non-phytate P level in NC diet decreased feed intake, weight gain and increased FCR: it is evident that maintaining optimum Ca: P ratio resulted in better performance of broilers. Similar observations were reported by several researchers (Zyla et al., 2001; Ebrahimnezhad et al., 2008; Chowdhury et al., 2018, 2019). The deteriorated growth performance in the NC group was restored by the addition of phytase, suggesting that birds completed the shortage of P in the NC diet with P released from phytate P by the action of phytase. The degree of restoration was greater in the phytase with CA group: the effects of organic acid such as CA on the phytase activity is associated with the common potential mechanism of lowering

gastrointestinal pH as well as its own nature that generally leads to better conditions for phytase activity (Vieira et al., 2018).

Serum Ca and P concentration decreased as dietary non-phytate P level reduced in NC diet. It is well known that a wide ratio of Ca: non-phytate P disturbed their normal metabolism in broilers, as well as Ca is chelated by phytic acid and forms phytate-mineral reducing Ca availability (Plumsted et al., 2008; Selle et al., 2009; Jianhui et al., 2012). However, the situation improved by the addition of phytase in NC diet: liberation of P from phytate salt (Sebastian et al., 1996) and utilization of myo-inositol, final product of phytate dephosphorylation (Simons et al., 1990) might be the possible mechanism to increase the serum P concentration in birds fed non-phytate P deficient diet with phytase. Moreover, positive influence of phytase as well as CA to increase Ca availability was also reported by several researchers (Woyengo et al., 2010; Nezhad et al., 2011; Qian et al., 2012).

Deteriorated characteristics of tibia and shank in the NC group was restored by the supplementation of phytase; CA addition further boosted the values. Such observation was also reported by other researchers (Brenes et al., 2003; Nourmahammadi et al., 2012; Islam et al., 2012). Phytate can form salt with essential minerals thus reducing their solubilities as well as absorption (Sandberg and Svanberg, 1991); whereas phytase are able to hydrolyze phytate salt and release minerals (Wodzinski and Ullah, 1996). Increased concentration of serum P, bone ash and retention in current study gives the evidence of phytate hydrolyzation by phytase and P availability in birds. The mentionable point here is that birds can completely recover their deficiency of non-phytate P by utilizing the P released just only from phytate P in their diet. When diets contain low non-phytate P, but synthetic P there is a possibility of increasing P retention by homeostasis mechanism of body that increase the absorption of P specially from synthetic sources as compared to normal phenomenon (Allen and Wood, 1994), and kept the phytate P unutilized or partially utilized which excreted through feces. But in current study, because of absence of synthetic P source, it is assumed that P released from phytate P by the action of phytase alone or with CA was completely available to birds. Significantly higher P retention in phytase added groups was the confirmation result of above assumption. In addition, nitrogen retention tended to increase in phytase added groups, although all diets were isonitrogenous. It has been reported that phytate depress the digestibility of amino acid and induce increases in endogenous amino acid flows in the gastrointestinal tract in birds (Ravindran et al., 1999; Selle et al., 2000; 2003), which may be partially countered by phytase supplementation (Selle and Ravindran, 2007).

It is noteworthy that, in most cases such as, live WG, FCR, serum P, carcass traits, tibia ash phytase alone can defeated (P<0.05) the deficiency of non-phytate P in diets, however, CA showed its booster effect (P>0.05) in few cases. Addition of CA may play a significant role by creating a favourable gut environment in getting full efficacy of phytase to hydrolyze phytate P (Enami et al, 2013; Vieira et al., 2018;). However, in current study addition of only phytase ameliorated all impairments of non-phytate P deficiency by increasing P availability, and further addition of CA with phytase showed limited (P>0.05) effects on studied parameters.

Conclusion

This study demonstrated that the addition of phytase substantially improved growth performance, serum minerals concentrations, tibia and shank characteristics in broilers given P deficient diets. Phytase alone showed its efficacy by restoring negative effect of P deficiency. This nutritional strategy may also be effective in terms of environmental aspect as it increased the retention of P and nitrogen. However, in current study addition of CA along with phytase in diet was a costlier affair without any significant beneficial effects.

Authors contribution

MAH was developed the concept, designed the experiment, collected data, analysed samples and contributed to writing manuscript. MAR contributed to recording the data, analyzed data statistically, prepared results and contributed to writing the manuscript. MA and RC contributed to revising the manuscript critically for important intellectual content. All authors read the article and approved the final version to be published.

Competing interests

The authors have declared that no competing interests exist.

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