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Efficacy of bacterial phytase, citric acid and their combination in broiler fed inorganic phosphorus free diet

S Munmun, MA Rahman, KMS Islam and R Chowdhury ✉

Department of Animal Nutrition, Faculty of Animal Husbandry, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

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Correspondence:
Dr. Rakhi Chowdhury ✉: rakhich03_bau@bau.edu.bd

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ABSTRACT

Present study was conducted to assess the efficacy of *Escherichia Coli* derived phytase alone or with citric acid (CA) given inorganic phosphorus (P) free broiler diet. Ninety six one-day old COBB-500 male broilers were divided into four groups and fed one of the following diets for a period of 35 days: positive control (PC) diet formulated based on the NRC (1994) recommendations, negative control (NC) diet (inorganic P free diet; containing 0.20% lower P than that in the PC diet), and two other diets were formulated by adding only phytase (500 FTU/kg of feed) or phytase with CA (500 FTU/kg of feed with 2 % CA) in the NC diet. Growth performance, serum minerals concentration, tibia and shank characteristics, and nutrient retention were measured. Impaired growth performance (final BW, BW gain, and FCR) in broilers (NC group) fed inorganic P free diet was restored with the addition of phytase in NC diet. Best FCR (1.83) was recorded in phytase added group and worst in NC group (2.03), however, further addition of CA with phytase did not show any significant variation. Lowest concentration of serum P (mg/dl), tibia P (%) and shank P (%) 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 (%) in broilers given phytase added diet was comparable with broilers given PC diet, although the former diet contained lower level of available P in diet than the later one. Addition of CA further increased this retention (%) numerically. Broilers fed inorganic P free diet deficient in available P, with *Escherichia Coli* derived phytase showed the growth performance (P<0.05) and relative retention of P comparable with broilers fed NRC (1994) recommended one. Therefore, relying on *Escherichia Coli* derived phytase alone in available P deficient diet may be viable to ensure sufficient supply of P in broilers diets.

Introduction

Reduction of nutrient excretion into the environment has become one of the most important issues in modern broiler production. Among the nutrients, nitrogen (N) from protein and phosphorus (P) from mineral are two most concerned elements in broiler excreta, when used as fertilizer or dumped into soil, can lead to contaminate soil as well as water (Lin *et al*., 2017). Broilers are basically reared on plant-based feed ingredients which are rich in phytic acid. It is the primary storage form of phosphorus in plants, exists as 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). Maize and soybean meal are two commonly used feed ingredients in broiler diets, contain approximately 0.40 % phytate P which is poorly digested by them due to the insufficient endogenous phytase enzyme, and leads to induce an environmental

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pollutant through P excretion (Tahir *et al*., 2012; Abbasi *et al*., 2019). Additionally, phytate makes complexes with mineral and protein, inhibit pepsin activity thus reduce protein digestion and increase N excretion (Knuckles *et al*., 2006; Dersjant-Li *et al*., 2015).

To solve this problem two approaches are recently becoming dominate: 1) adding phytase enzyme that hydrolyze phytate and 2) using genetically modified ingredients with low phytate P content (Li *et al*., 2000; Waldroup *et al*., 2000). Although the former one is now well practiced, the later one is still under consideration because of the cost involving issue. Normally, P from inorganic sources such as mono-calcium phosphate or di-calcium phosphate used in broiler diets to meet up the requirements, so when phytase also added in such diets it makes dietary phytate P available to broilers as well as increase the net amount of available P for broilers than their requirement. Negative feedback mechanisms by excessive amount of dietary P on P availability are reported by several researchers (Simons *et al*., 1990; Ankra-Badu *et al*., 2004). Consequently, spare P is excreted through feces and nearly 50 % of excreted P is likely to be of inorganic origin (Waldroup, 2000).

Considering these circumstances, it may be applicable to conduct research on broilers fed inorganic P free diets, but phytase. Several studies have been conducted using phytase in broilers diet, but in most cases, diets contained inorganic P (Ebrahimnezhad *et al*., 2008; Nezhad *et al*., 2011; Nourmohammadi *et al*., 2010, 2012; Mohammadagheri *et al*., 2016; Demirel *et al*., 2021; Anwar *et al*., 2022). Besides, organic acid such as citric acid (CA) is a well-recognized feed additive that is recently used in broiler diets to increase nutrient digestibility, mineral retention (Chowdhury *et al*., 2009; Islam *et al*, 2012) as well as enhance phytase activity (Vieira *et al*., 2018). Some studies (Vieira *et al*., 2018; Nourmohammadi, *et al*., 2012) suggested positive effects of organic acid on phytase activity; nevertheless, controversial results still prevent specific recommendations for the combined use of these additives. Thus, the objective of this study was to assess the efficacy of phytase alone or in combination with CA on performance, bone characteristics and nutrient retention in broilers fed inorganic P free diets.

MATERIALS AND METHODS

The experimental protocol for bird handling, slaughtering, sample collection was approved by the Animal Welfare and Experimentation Ethics Committee; AWEEC/BAU/2022 (61).

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. Upon arrival of chicks, weight was taken and then chicks were placed into different dietary groups (there was three replications per dietary group: eight birds per replication). Birds were housed in cage in electrically heated brooders for the first week. 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 mash diets were used in this study. The diets included - a positive control (PC), formulated according to the NRC (1994) recommendations, a negative control (NC) diet formulated without inorganic P (contain 0.20% lower non-phytate P than the PC diet), NC diet containing phytase (OptiPhos®, *Escherichia Coli* derived phytase), NC diet containing phytase and 2 % CA (Table 1). 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. Phytase was added at the top of the ration (500 FTU/kg of feed). Starter diet contained 22.3% CP and 3,020 kcal of ME/kg and was 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.

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 in a freezer 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; 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 (HERMLE Z 306) at 3421 rpm 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

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 + Escherichia coli derived phytase; NC + Phytase + CA, Negative control + Escherichia coli derived phytase + Citric acid; Pro. C, Protein concentrate; CP, Crude protein; CF, Crude fibre; Ca, Calcium; P, Phosphorus; ME, Metabolizable energy. Phytase and citric acid 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).

kit by colorimetric method (Bioanalyzer Urit-810). 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.

Chemical Analyses

Samples of diets and excreta were analyzed for proximate composition following the standard

methods (AOAC, 2005). Total P and phytate P of the samples were measured according to ISO (1998) and Haug and Lantzsch (1983), respectively: 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 Analysis

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 and Version 20).

Table 2. The effects of phytase with and without citric acid on growth performance of broilers fed inorganic phosphorus free diets[1]

Parameters	Groups				p-value
	PC	NC	NC + Phytase	NC + Phytase + CA	
Initial BW (g/b)	40.33±0.95	41.00±0.50	40.92±1.61	40.30±0.93	0.782
Final BW (g/b)	1605.42^{b}±28.10	1511.58^{c}±45.45	1706.83^{a}±22.14	1713.42^{a}±35.58	<0.001
Live WG (g/b)	1565.08^{b}±27.31	1470.58^{c}±45.10	1665.92^{a}±21.41	1673.12^{a}±34.67	<0.001
Total FI (g/b)	3044.66±46.43	2981.67±69.30	3046.33±37.22	3130.33±81.71	0.097
FCR	1.95^{b}±0.01	2.03^{a}±0.02	1.83^{c}±0.01	1.87^{c}±0.03	<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 + Escherichia coli derived phytase; NC + Phytase + CA, Negative control + Escherichia coli derived phytase + Citric acid; BW, Body weight; WG, Weight gain; FI, Feed intake; FCR, Feed conversion ratio; g, gram; b, broiler;[1]Values for each parameter represent mean±standard deviation values with twenty four observations.

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

Parameters	Groups				p-value
	PC	NC	NC + Phytase	NC + Phytase + CA	
Serum concentration (mg/dl)					
Calcium	9.27^{a}±0.33	8.55^{b}±0.19	9.32^{a}±0.31	9.37^{a}±0.20	0.038
Phosphorus	5.87^{a}±0.33	4.62^{b}±0.55	6.19^{a}±0.18	6.25^{a}±0.15	<0.001
Nutrient retention (%)					
Dry matter	5.56^{a}±0.11	5.12^{b}±0.15	5.65^{a}±0.22	5.79^{a}±0.34	0.030
Phosphorus	47.00^{a}±1.28	42.77^{b}±1.50	46.83^{a}±1.27	48.07^{a}±1.22	0.005
Nitrogen	44.27^{a}±0.55	42.08^{b}±0.87	45.10^{a}±0.93	45.64^{a}±0.83	0.003

$^{a-b}$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 + Escherichia coli derived phytase; NC + Phytase + CA, Negative control + Escherichia coli derived phytase + Citric acid.[1]Values for each parameter represent mean±standard deviation values with seven observations.

Results and Discussions

Growth Performance

Growth performance parameters were presented in Table 2. Broilers given diet (PC diet) containing NRC recommended amount of available P showed the final BW around 1,605 g, this value was further decreased by about 5 % in the NC group given available P deficient diet, but increased approximately 6 % 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. The deteriorated growth performance in the NC group was restored by the addition of phytase, suggesting that broilers completed the

shortage of P in the NC diet with P released from phytate P by the action of phytase. 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 with CA added group. Worse FCR was noted in NC group (2.03) and best in phytase added group (1.83) group. Similar observations were reported by several researchers (Żyła *et al*. 2001; Ebrahimnezhad *et al*., 2008; Tahir *et al.,* 2012). Although, CA is very efficacious in improving P utilization in chickens fed corn-soybean meal based diets containing limited supplemental P (Soliman and Al-Youseef, 2020), current results did not show any booster effect of CA with phytase.

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

Parameters	Groups				*P*-value
	PC	NC	NC + Phytase	NC + Phytase + CA	
Tibia characteristics					
Dry weight (g)	5.56±.07	5.05±.08	5.95±.02	5.79±.85	0.068
Ash (%)	43.82±2.28	41.24±1.50	42.97±1.40	42.52±1.41	0.073
P (%)	3.86^{a}±0.13	3.42^{b}±0.18	3.83^{a}±0.16	3.88^{a}±0.22	0.036
Shank characteristics					
Dry weight (g)	3.48±0.91	2.78±0.34	3.19±0.35	3.20±0.42	0.306
Ash (%)	39.0^{b}±1.55	37.22^{b}±1.50	41.61^{a}±0.43	42.30^{a}±0.41	0.001
P (%)	3.47^{a}±0.12	3.02^{b}±0.13	3.51^{a}±0.16	3.55^{a}±0.20	0.009

$^{a-b}$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 + Escherichia coli derived phytase; NC + Phytase + CA, Negative control + Escherichia coli derived phytase + Citric acid; P, Phosphorus.[1]Values for each parameter represent mean±standard deviation values with seven observations.

Serum minerals concentration and nutrient retention

Data of serum minerals concentration and nutrient retention were presented in Table 3. Serum analysis showed a significant reduction of Ca and P concentration in NC group compared with PC group. According to Selle *et al*. (2009) normal metabolism of Ca and P may be disturbed by their wide ratio in broiler diets. However, the concentration restored (P<0.05) with the addition of phytase, and the values further increased numerically in phytase with CA group. 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 broilers fed available P deficient diet with phytase. In addition, several researchers (Vieira *et al*., 2018; Akter *et al*., 2018) reported about the ability of phytase as well as CA to release and increase the availability of Ca from Ca-phytate complex in broilers.

Dietary reduction of available P showed noticeable effect on P and N retention (P<0.05). Decreased retention of P in NC group restored with the addition of phytase. Further addition of CA numerically increased the retention value of P. Retention of P in phytase added groups fed lower level of available P were comparable with PC group fed NRC recommended one. When diets contain low available 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 inorganic 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 inorganic P source, it is assumed that P released from phytate P by the action of phytase alone or with CA was completely available to broilers. Significantly higher P retention in phytase added groups were the confirmation result of above assumption. On the other hand, although all diets were isonitrogenous, nitrogen retention varied considerably among the groups. Lowest (P<0.05) retention (42.77%) was recorded in NC group and highest (P>0.05) in phytase with CA group (48.07%). Addition of phytase increase the digestibility of amino acid and the flow in the of endogenous amino acid gastrointestinal tract in

broilers by splitting phytate-protein complex (Selle *et al*., 2003; Selle and Ravindran, 2007).

Tibia and shank characteristics

Data of tibia and shank characteristics were presented in Table 4. Non-significant variation was observed among the groups in terms tibia dry weight and ash content, but tibia P. However, in case of shank, diets showed its effects (P<0.05) on ash content as well as P. Decreased P content in both tibia and shank in broilers given NC diet was restored with the addition of phytase. Comparing the values, the degree of restoration was greater (P>0.05) in phytase with CA group. Phytate can form salt with essential minerals thus reducing their solubility as well as absorption (Vieira *et al*., 2018; Sandberg and Svanberg, 1991); whereas phytase are able to hydrolyze phytate salt and release minerals (Wodzinski and Ullah, 1996). Moreover, several researchers reported that dietary addition of CA increase the availability of P in broilers (Chowdhury *et al*., 2009; Islam *et al*., 2012).

Conclusion

This study demonstrated that addition of *Escherichia Coli* derived phytase in diets without inorganic P can improve the efficiency of utilization of P as well as other nutrients, reduce the quantity of excreted P from broilers without any adverse effect on performances. This strategy can help to reduce negative environments effects. Further addition of CA with *Escherichia Coli* derived phytase did not show significant beneficial effects in current study. However, more studies are needed to refine the application of this strategy in the future.

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Department of Animal Nutrition, Faculty of Animal Husbandry, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

Conflict of Interest

There is no conflict of interest.

Authors Contribution:

S Munmun: Design, formulation and supervision of experiment, data analysis and writing of manuscript. MA Rahman: Data analysis, writing of manuscript. KMS Islam: Supervision of experiments. R Chowdhury: Supervision of experiments and review of manuscript.

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