African Journal of Biotechnology Vol. 10(66), pp. 15089-15093, 26 October, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2553

Full Length Research Paper

Effect of combination of citric acid and microbial phytase on digestibility of calcium, phosphorous and mineralization parameters of tibia bone in broilers

Y. Ebrahim Nezhad*, J. Ghyasi Gale-Kandi, T. Farahvash and A. R. Yeganeh

Animal and Poultry Science, Faculty of Agriculture, Islamic Azad University, Shabestar Branch, Shabestar, East Azerbaijan, 5381637181, Iran.

Accepted 7 September, 2011

This experiment was conducted to evaluate the combined effects of citric acid (CA) and microbial phytase (MP) on digestibility of calcium, phosphorus and mineralization parameters of tibia bone in broilers chicks. A total of 360 Ross-308 male broiler chicks were used in a completely randomized design with a 3 × 2 factorial arrangement (0, 2.5 and 5% CA and 0 and 500 FTU MP). Four replicate of 15 chicks each were fed dietary treatments including: (i) P-deficient basal diet [0.2% available phosphorus (aP)] (NC); (ii) NC + 500 FTU MP per kilogram of diet; (iii) NC + 2.5% CA per kg of diet; (iv) NC + 2.5% CA + 500 FTU MP per kg of diet; (v) NC + 5% CA per kg; and (vi) NC + 5% CA + 500 FTU MP per kg of diet. The content of calcium, phosphorus and length of tibia bone and digestibility of calcium and phosphorus was evaluated. The results show that interaction effect of CA × MP on tibia calcium content and low available phosphorus diets was significant ($p<0.01$). Adding of CA to P-deficient diets increased tibia phosphorus content of broilers when compared with the control group ($p<0.01$). Adding MP to P-deficient diets based on corn-soybean meal, cause increased digestibility of calcium, phosphorus and also, length of tibia in broilers ($p<0.01$). Also, adding of CA to broiler diets deficient in available phosphorus, significantly increased digestibility of phosphorus ($p<0.01$) and length of tibia ($p<0.001$) when compared with the control group. From this study, it could be deduced that adding MP to low available phosphorus diets can cause improvement of utilization of phytate phosphorus. Also, adding CA as a chelator to diet can improve tibia mineralization parameters in broilers.

Key words: Available phosphorus, calcium, citric acid, microbial phytase.

INTRODUCTION

The environment contamination with phosphorus which is caused by animals recently, has been an important issue. Mono gastric animals consume diets based on oil seed meals and crops. These diets contain high amounts of phosphorus in phytase or phytic acid forms. Commonly, phytase, which has known activity in the intestine of poultry, is not available (Nelson, 1976). Various feed additives are used in order to increase the use of phosphorus and decrease the excretion of phosphorus in poultry and swine. It is known that the phytase (Edwards, 1993; Biehl et al., 1995; Biehl and Baker, 1996; Gordon and Roland, 1997), vitamin D and it's products (Edwards 1993; Biehl et al., 1995; Angel et al., 2001; Edwards, 2002; Snow et al., 2004), and citric acid (Boling et al., 2000; Boling-Frankenbach et al., 2001; Rafacz et al.,2003; Snow et al., 2004) can be affectively used to develop the availabilities of phytate in mono gastric animals.

There is little information on whether organic acids (except for citric acid) can improve the availability of phytate phosphorus in poultry. The EDTA is an organic acid which has similar potential with citric acid, and it increases availability of some minerals. EDTA is a strong chelator and it improves the absorption rate of minerals of diets in poultry.

Previous studies indicated that supplementing diets which contain plant protein with EDTA, improved absorption of (Zn^{++}) in turkey chicks (Kratzer et al., 1959)

*Corresponding author. E-mail: ebrahimnezhad@iaushab.ac.ir.

Table 1. Composition and nutrient content of the diet during starter (0 to 21 days) period.

Ingredient (%)	Treatment					
	1	2	3	4	5	6
Corn	62.32	62.22	58.36	58.26	54.41	54.31
Soybean meal (44%)	33.72	33.74	34.48	34.5	35.24	35.26
Soybean oil	0.29	0.32	0.75	0.78	1.21	1.24
Oyster shell	2.26	2.26	2.25	2.25	2.24	2.24
Dicalcium phosphate	0.3	0.3	0.31	0.31	0.32	0.32
Common salt	0.41	0.41	0.41	0.41	0.41	0.41
Premix[a]	0.5	0.5	0.5	0.5	0.5	0.5
DL-Met	0.2	0.2	0.2	0.2	0.21	0.21
Citric acid (92%)	-	-	2.73	2.73	5.47	5.47
Phytase[b]	-	0.05	-	0.05	-	0.05
Nutrients (calculated)						
ME (Kcal/kg)	2875	2875	2875	2875	2875	2875
CP (%)	20.25	20.25	20.25	20.25	20.25	20.25
Available P (%)	0.2	0.2	0.2	0.2	0.2	0.2
T.P (%)	0.45	0.45	0.45	0.45	0.45	0.45
Ca (%)	0.9	0.9	0.9	0.9	0.9	0.9
Met + Cys (%)	0.85	0.85	0.85	0.85	0.85	0.85
Lysine (%)	1.07	1.07	1.07	1.07	1.07	1.07

[a]Supplied per kilogram of diet: vitamin A, 9000 IU; Cholecalciferol, 3000 IU; vitamin E, 18 IU; vitamin K3, 2 mg; vitamin B12, 0.015 mg; thiamin, 1.8 mg; riboflavin, 6.6 mg; folic acid, 1 mg; biotin, 0.10; niacin, 35 mg; pyridoxine, 4 mg; choline chloride, 250 mg; ethoxyquine; manganese sulphate, 100 mg; copper sulphate, 10 mg; selenium (sodium selenate), 0.2 mg; iodine (EEI), 1 mg; zinc sulfate, 100 mg; Fe, 50 mg. [b]Natuphos® (BASF Crop., Mt. Olive, NJ) was used to supply 500 FTU microbial phytase per kilogram of diet.

and chicks (O'Dell et al., 1964). Maenz et al. (1999) showed that EDTA increased the hydrolyzation of phytate phosphorus from canola meal when associated with microbial phytase in *in vitro* experiments. It seems that EDTA comparatively links to the calcium and decreases it's ligand to the phytate. Consequently, it bounds the formation of insoluble calcium-phytate complexes and makes phytate of the diet sensitive to the endogenous and exogenous phytase.

The objective of this research was to study the combined effect of citric acid and microbial phytase on digestibility of calcium, phosphorus and mineralization parameters of tibia bone in broilers chicks and its effect on efficacy of microbial phytase in corn-soybean diets with low available phosphorus level.

MATERIALS AND METHODS

A total of 360 feather sexed male day old Ross 308 broiler chicks were used in this experiment. Chicks were weighed individually and randomly assigned to battery pens so that pens had equal initial weight and weight distribution. The experiment was carried out using a completely randomized design with a 3 × 2 factorial arrangement (0, 2.5 and 5% CA and 0 and 500 FTU MP). Four replicates of 15 chicks each were fed dietary treatments including: (i) P-deficient basal diet [0.2% available phosphorus (aP)] (NC); (ii) NC + 500 FTU MP per kilogram of diet; (iii) NC + 2.5% CA per kilogram of diet; (iv) NC + 2.5% CA + 500 FTU MP per kilogram of diet; (v) NC + 5% CA per kilogram; and (vi) NC + 5% CA + 500 FTU MP per kilogram of diet. All diets meet or exceeded NRC (1994) recommendation, except for aP (Table 1). The same ingredients were used for formulation of diets during 0 to 21 and 21 to 49 days of age (diet composition for period of 21 to 49 days of age are not presented). The supplied MP (Natuphos-500; BASF, Mt. Live, Nj) had 1000 FTU active phytase per gram. The citric acid used in this experiment was monohydrate 92%, which was added to the diets after calculating purity percentage.

At day 44, chrome oxide (Cr_2O_3) was added for all diets at 0.1% level as a detectable marker for specifying zinc (Zn^{++}), copper (Cu^{++}), manganese (Mn^{++}) and also apparent metabolizable energy. To determine the digestibility of minerals, special sacs fastened to back of two chickens (their weights were close to mean weight of cage and their feces) were collected for three days. Samples of digested materials were frozen immediately after collection and were then dried in oven at 60°C. After drying samples of digested materials, they were grinded and 1 mm pore filter was used to homogenize them. Also, samples of foods were grind and filtered in this leach. Gross energy of feed samples and feces samples were measured by automatic colorimeter (Automatic Colorimeter, AC-300, and Model 789-400) in nutrition laboratory of Tabriz University and then apparent metabolizable energy were calculated. Minerals concentration for feeds and feces samples was measured by atomic absorption in nutrition in Islamic Azad University Shabestar Branch, and was expressed as percent. Chrome (Cr) concentration in feeds and feces samples were measured by method explained by Fenton and Fenton (1979) and by using spectrophotometer. Digestibility of minerals was calculated by using the following formula (Ravindran et al., 2000):

$$\text{Digestibility of nutrients (\%)} = 100 - \left(100 \times \frac{\text{Chrome concentration in feed (\%)}}{\text{Chrome concentration in feces (\%)}} \times \frac{\text{Nutrient concentration in feces (\%)}}{\text{Nutrient concentration in feed (\%)}}\right)$$

At the end of experiment, two birds were selected from each replication and five milliliter blood was taken from wing puncture. Blood samples centrifuged for 15 min (3000 rpm/min) and serum was separated. The concentration of zinc, copper and manganese was measured by using ICP (Inductively Coupled Plasma Emission Spectrometer, Model JY-24, Jobin Yvon, Longjumeau, Cedex, France).

Data were statistically evaluated by the analysis of variance procedure of SAS software (SAS Institute, 1990), involving a factorial arrangement of main factor (citric acid and microbial phytase levels) in a completely randomized design. Significant differences between mean values were separated by the GLM procedure of SAS software (SAS Institute, 1990). Statistical significance was considered at $p < 0.05$.

RESULTS AND DISCUSSION

The effect of CA and MP on the percentage of calcium (Ca) and phosphorus (P) content of left tibia bone and also on the digestibility of Ca, P, dry matter, protein and the length of left tibia bone in broilers are presented in Table 2. Results indicate a significant interaction between CA and MP on the left tibia bone Ca in broiler chicks which were fed low available P-deficient diets (p<0041), so that Ca content of tibia bone increased when 2.5 and 5% CA was added to low available P-deficient diets but supplementing 2.5 and 5% CA did not significantly increase Ca content of left tibia bone. Diets with 500 units/kg MP which had available P deficiency, improved numerically (38.8 versus 37.3) Ca of left tibia bone when supplemented with 2.5% citric acid, but adding 5% citric acid, decreased Ca content of left tibia bone in comparison to other treatments (p<0.05). It is likely that because adding 5% citric acid, dramatically declined pH intestines, consequently, MP and phosphatase enzymes could not work correctly. This would result in decreasing hydrolyzation of phytic acid and decline released of P, which causes imbalanced Ca : P ratio (4.5:1) when compared with the usual diet with Ca : P ratio (2:1). Consequently, the excretion of Ca increased and Ca content of tibia declined. That phytase activity in the gastrointestinal tract is affected by factors such as phytase sources, pH and the presence of metal ions should be considered. MPs are active in broader range of pH in comparison with plantal phytases, then, they are more active in gastrointestinal tract (Ravindran et al., 1995). Maximum activity of these enzymes occurs in pH 2.5 and 5 (Ullah and Phillippy, 1994). It is suggested that phytases are activated by different bivalent cations such as Ca, magnesium, zinc and vitamin D (Ravindran et al., 1995) and it seems that high levels of CA in diet, causes strong bond with Ca in intestine, which is an essential cofactor for activating MP. This decreases the efficiency of MP and causes imbalanced Ca : P ratio (mentioned earlier), and this is due to decreased tibia bone Ca. Adding 2.5 and 5% CA to low available P-deficient diets improved P content of tibia about approximately 20% (p<0.0084), but there were no differences between different levels of citric acid. Also, it is reported that supplementing diets with low available P-deficient with 2.5 and 5% citric acid, increased digestibility of P (Table 2) and utilization of phytate P, and consequently, increased P content of tibia. These results are in agreement with other researcher's data (Boling et al., 2000; Boling-Frankenbach et al., 2001; Brenes et al., 2003). Also, Brenes et al. (2003) reported that reducing available level of dietary P (from 0.35 to 0.25%), increased phosphorous content of tibia.

Interaction effect between CA and MP influenced digestibility of Ca, supplementing 2.5% CA to low available P-deficient diets, increased digestibility of Ca in comparisons with diets without citric acid. Adding 2.5 and 5% CA to low available P-deficient diets supplemented with MP, did not have significant influence on digestibility of Ca. It seems that adding 2.5% CA to low available P-deficient diets, prevented binding between Ca and phytic acid in intestine. It is because of the chelating effect of Ca, and it causes Ca retention in the intestine. These results are in agreement with Erdman (1979) and Brenes et al. (2003). Furthermore, since acidic pH provides better absorption for minerals, so, CA may increase intestinal absorption of Ca. CA significantly influenced digestibility of P (p<0.0057), when low available P-deficient diets were supplemented with 2.5 and 5% CA there was increase of P (32.36 and 25.22 respectively) but the difference was not significant between 2.5 and 5%. It is because of the fact that citric acid, improved utilization of phytate P, which is reported by other researchers (Boling et al., 2000; Boling-Frankenbach et al., 2001; Brenes et al., 2003). Also, adding MP to diets containing low available P, increased digestibility of P (3.11%) (p<0.0016). Increase in P retention by adding MP to the diets have been reported by various researchers (Sebastian et al., 1996; Qian et al., 1996; Selle et al., 2000; Ravindran et al., 2000; Viveros et al., 2002; Brenes et al., 2003). Perney et al. (1993) and Ahmad et al. (2000) showed that the excretion of P in Dietscontaining low available P, decreased when supplemented with MP, it might be because of the increase availability in P, as a result of release from phytic acid complex. It seems that excretion of P decreases when P is a limiting factor for maintaining physiological functions, more P remains in the body and, consequently, less P is excreted through feces. The results show that in adding 5% CA to the diets containing low available P, left tibia length of broilers increased (p<0.0002), whereas between 2.5% CA in the diet and

Table 2. Effects of CA and MP digestibility of calcium and phosphorus and mineralization parameters of tibia bone on broilers (at 49 days, finisher period).

Treatment		Tibia calcium (%)	Tibia phosphorous (%)	Digestible calcium (%)	Digestible phosphorous (%)	Digestible dry matter (%)	Digestible protein (%)	Tibia length (cm)
CA (%)	MP (FTU kg^{-1})							
0	0	35.5[c]	13.2	44.7[c]	20.2	79.8	54.2	9.7
0	500	39.2[ab]	13.9	83.6[a]	35.8	81.7	68.7	10.2
2.5	0	38[ab]	17	77[ab]	35.9	85.8	71.8	9.9
2.5	500	39.6[a]	17.7	70.5[ab]	46.8	84.8	70.1	10.1
5	0	39.5[a]	17.1	60.9[bc]	34.1	78.1	58.8	10.3
5	500	36.8[bc]	17.6	73.1[ab]	41.5	80.5	67.8	10.8
SEM pooled		0.8	1.1	5.9	3.4	2.9	6.4	0.1
Main effects								
CA	0	37.3	13.6 [b]	64.1	28[b]	80.8	61.5	9.9[b]
	2.5	38.8	17.4 [a]	73.8	41.4[a]	85.3	71	10[b]
	5	38.1	17.4 [a]	67	37.8[a]	79.3	63.3	10.5[a]
MP	0	37.6	15.7	60.9[b]	30.1[b]	81.2	61.6	9.9[b]
	500	38.5	16.4	75.7[a]	41.3[a]	82.4	68.9	10.3[a]
P-value								
CA		0.1867	0.0084	0.2841	0.0057	0.1448	0.3292	0.0002
MP		0.1795	0.4697	0.0095	0.0016	0.6399	0.1905	0.0007
CA × MP		0.0041	0.9950	0.0078	0.4949	0.8316	0.4652	0.2940

Means in columns with no common superscript differ significantly (P<0.05); [1]Natuphos® (BASF Crop., Mt. Olive, NJ) was used to supply 300 FTU microbial phytase per kilogram of diet.

group without CA, significant differences were not observed. It seems that lengthening of tibia in broilers fed diets containing low available P, is as a result of the following reasons:

1. CA is a strong chelator and can increase Ca absorption from the intestine, consequently, increase the bone growth.
2. Use of CA increases the utilization of phytate P and releases P from the phytic acid and thus modifies the balance of Ca to P and provides P for more bone growth trend and bone synthesis.

Adding 500 units/kg of MP to diets containing low available P, increased the length of tibia in broilers (p<0.0007). Since MP released P from phytate and because P is one of the components of bone, therefore, it helps in bone synthesis process in the body. Also, it improves the Ca : P ratio, consequently, affects the length of tibia. Adding different levels of CA and MP to diets containing low available P had no effect on dry matter and protein digestibility of broiler chicks at the finisher period. From this study it could be deduced that:

1. Adding 2.5% CA to the diets containing low available P diets based on corn-soybean meal diets increases the P content and length of tibia and P digestibility in broiler chicks.
2. It seems that adding CA to the diets containing low available P diets based on corn-soybean meal diets which is supplemented MP, did not have effect on the efficiency of MP.

3. Adding 500 units/kg MP to the diets containing available P and low P (containing 0.2% low available P) based on corn-soybean meal diets, increases the digestibility of Ca and P and length of the tibia in broiler chicks.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial and technical assistance of Shabestar Islamic Azad University and Shahid Sardari's Educational and Research Center of Agriculture, Jahad Ministry of East Azerbaijan.

REFERENCES

Ahmad T, Rasool S, Sarwar M, Haq A, Zia-ul H (2000). Effect of microbial phytase produced from a fungus *Aspergillus Niger* on bioavailability of phosphorus and calcium in broiler chicken. Anim. Feed Sci. Technol. 83: 103-114.

Angel R, Dhandu AS, Applegate TJ, Christman M (2001). Phosphorus sparing effect of phytase, 25-hydroxycholecalciferol and citric acid when fed to broiler chicks. Poult. Sci. 80 (Suppl.): 133-134. (Abstr.).

Biehl RR, Baker DH (1996). Efficacy of supplemental 1-α-hydroxycholecalciferol and microbial phytase for young pigs fed phosphorus or amino acid deficient corn-soybean meal diets. J. Anim. Sci. 74: 2960-2966.

Biehl RR, Baker DH, Deluca HF (1995). 1-α-hydroxylatedcholecalciferol compounds act additively with microbial phytase to improve phosphorous, zinc, and manganese utilization in chicks fed soy-based diets. J. Nutr. 125: 2407-2416.

Boling SD, Webel DM, Marromichalis I, Parsons CM, Baker DH (2000). The effects of citric acid on phytate phosphorus utilization in young chicks and pigs. J. Anim. Sci. 78: 682-689.

Boling-Frankenbach SD, Snow JL, Parsons CM, Baker DH (2001). The effect of citric acid on the calcium and phosphorus requirements of chicks fed corn-soybean meal diets. Poult. Sci. 80: 783-788.

Brenes A, Viveros A, Arija I, Centeno C, Pizarro M, Braro C (2003). The effect of citric acid and microbial phytase on mineral utilization in broiler chicks. Anim. Feed Sci. Technol. 110: 201-219.

Edwards Jr. HM (1993). Dietary 1, 25-dihydroxycholecalciferol supplementation increases natural phytate phosphorous utilization in chickens. J. Nutr. 123: 567-577.

Edwards Jr. HM (2002). Studies on the efficacy of cholecalciferol and derivatives for stimulating phytate utilization in broiler. Poult. Sci. 81: 1026-1031.

Erdman JW, Jr (1979). Oilseed phytates: Nutritional implications. J. Am. Oil Chem. Soc. 56: 736-741.

Fenton TW, Fenton M (1979). Determination of chromic oxide in feed and feces. Can. J. Anim. Sci. 58: 631-634.

Gordon RW, Roland Sr. DA (1997). Performance of commercial laying hens fed various phosphorus levels, with and without supplemental phytase. Poult. Sci. 76: 1172-1177.

Kratzer FH, Alfred JB, Davis PN, Marshall BJ, Vohra P (1959). The effect of autoclaving soybean protein and the addition of ethylene di amine tetra acetic acid on biological availability zinc for turkey poults. J. Nutr. 68: 313-316.

Maenz DD, Engele-Schann CM, Newkirk RW, Classen HL (1999). The effect of minerals and mineral chelators on the formation of phytate-resistant and phytase-susceptible forms of phytic acid in solution and in slurry of canola meal. Anim. Feed Sci. Technol. 77: 177-192.

National Research Council (1994). Nutrient requirements of poultry. 9th edition National Academy Press Washington DC, U.S.A.

Nelson TS (1976). The hydrolysis of phytate phosphorus by chicks and laying hens. Poult. Sci. 55: 2262-2267.

O'Dell BL, Yohe JM, Savage JE (1964). Zinc availability in the chick as affected by phytate, calcium and ethylene di amine tetra acetate. Poult. Sci. 43: 415-419.

Perney KM, Cantor AH, Straw ML, Herkelman KL (1993). The effect of dietary phytase on growth performance and phosphorus utilization of broiler chickens. Poult. Sci. 72: 2106-2114.

Qian H, Kornegay ET, Ravindran V, Denbow DM (1996). Effects of supplemental phytase and phosphorus on histological and others tibial bone characteristics and performances of broilers fed semi-purified diets. Poult. Sci. 75: 618-626.

Rafacz KA, Martinez C, Snow JL, Baker DH, Parsons CM (2003). Citric acid improves phytate phosphorus utilization in two breeds of chicks fed a corn-soybean meal diets. Poult. Sci. 82: (Suppl.1): 142. (Abstr.).

Ravindran V, Bryden WL, Kornegay ET (1995). Phytates: occurrence, bioavailability and implications in poultry nutrition. Poult. Avian Biol .Rev. 6: 125-143.

Ravindran V, Cabahug S, Selle PH, Bryden WL (2000). Response of broiler chickens to microbial phytase supplementation as influenced by dietary phytic acid and non-phytate phosphorus levels. II. Effects on apparent metabolizable energy, nutrient digestibility and nutrient retention. Br. Poult. Sci. 41: 193-200.

SAS Institute (1990). SAS/STAT User's Guide: Statistics. Release 6.04 ed. SAS Institute Inc., Cary, NC.

Sebastian S, Touchburm SP, Chavez ER, Lague PC (1996). The effect of supplemental microbial phytase on the performance and utilization of dietary calcium, phosphorus, copper and zinc in broiler chickens fed a corn-soybean diets. Poult. Sci. 75: 729-736.

Selle PH, Ravindran V, Caldwell RA, Bryden WL (2000). phytate and phytase: consequences for protein utilization. Nutr. Res. Rev. 13: 255-278.

Snow JL, Baker DH, Parsons CM (2004). Phytase, citric acid, and 1-α-hydroxycholecalciferol improve phytate phosphorus utilization in chicks fed a corn-soybean meal diet. Poult. Sci. 83: 1187-1192.

Ullah AHL, Phillippy BQ (1994). Substrate selectivity in *Aspergillus Ficuum* phytase and acid phosphatase using myo-inositol phosphates.J. Agric. Food Chem. 42: 423-425.

Viveros A, Brenes A, Arija I, Centeno C (2002). Effects of microbial phytase supplementation on mineral utilization and serum enzyme activities in broiler chicks fed different levels of phosphorus. Poult. Sci. 81: 1172-1183.