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The Effects of Combination of Citric Acid and Microbial Phytase on the Concentration of Some Minerals of Serum and Parameters of Mineralization of Tibia in Commercial Laying Hens

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Abstract: This experiment was conducted to evaluate the combined effect of citric acid and microbial phytase on the concentration of minerals of serum and parameters of mineralization of tibia in Hy-line commercial layers (W-36) in 53-64 weeks of age. One hundred and ninety two of laying hens were tested. The experimental design was completely randomized design with a 3×2 factorial arrangement with three levels (0, 2 and 4%) of citric acid and two levels (0.0 and 300 FTU kg^{-1}) of microbial phytase in low available phosphorus diets with 6 treatments, 4 replicates and 8 hens in each replicate. The concentration of zinc, copper and manganese of serum and ash, calcium and phosphorus of tibia was evaluated. The results showed that interaction between citric acid and microbial phytase significantly affected the concentration of copper in serum, tibia ash and tibia ash calcium percentage ($p<0.01$). Using 300 FTU kg^{-1} microbial phytase into low available phosphorus diets increased tibia ash phosphorus percentage ($p<0.05$).

Key words: Citric acid, microbial phytase, laying hens, zinc, copper, manganese

INTRODUCTION

Phosphorus is an essential and costly mineral material in poultry nutrition. Animal feed additives are rich of phosphorus and the availability of this mineral material in these foods supposed 100% approximately, while the availability of phosphorus in plant feed additives is only 30% (National Research Council, 1994).

The portion of phosphorus of the grain, grain by products and plant feed additives available phosphorus are in form of acid phytic or it's salt form (phytate) that restrict the phosphorus besides could combine with proteins, carbohydrates and minerals and decrease digestibility and bioavailability of them because of so many eclectic charge. There is low availability of phosphorus in phytate because of low activity of phytase enzyme in poultry digestive system. Thus the noticeable amount of phosphorus available in poultry foods excretes to environment. From previous studies deduced that organic acids may increase the utilities of phosphorus. Adding the mixture of citric acid and sodium citrate in (1:1 ratio) to rat diets with lack of calcium and phosphorus inhibit from rickets diseases (Shohl, 1937).

Pileggi *et al*. (1956) study is confirmed the previous studies (Shohl, 1937) and showed that the best results of citrate is observed when the rat is feed with phytate containing diets and there is no useful response after adding of citrate to diets with lack of phytate. Boling *et al*. (2000) reported that in lying hens that feed with corn-soybean meal the citric acid didn't affect utility of phosphorus.

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Snow *et al*. (2004) showed that there are additive effects of citric acid, phytase, 1-α-hydroxycholecalciferol in response to tibia ash and phosphorus utility in broilers generally and combination of citric acid with phytase and 1-α-hydroxycholecalciferol with phytase could have much interaction effect on phytate phosphorus utility. Therefore deduced that the citric acid may decrease the ph of digestive juices in small intestine and prevent to phytate insoluble salts complex which resist to interior phytase enzymes hydrolysis due to be ready of phytic acid to hydrolysis with interior and exterior phytase enzymes (Maenz *et al*., 1999; Applegate *et al*., 2003).

Boling *et al*. (2000) resulted that attached the calcium itself comparative and decrease its connection to phytate and due to prevent insoluble complex of calcium-phytate and ready the diet phytate to endogenous phytase. It seems that microbial phytase hydrolysis ester bound (ligands) between phytic acid and citric acid. It decreases pH in gastrointestinal tract and blocks formation of insoluble mineral-phytate complexes, consequently, will increase bioavailability of minerals.

The aim of this study, although, is to evaluate the effects of combination of citric acid and microbial phytase on the concentration of some of minerals of serum and ash, calcium and phosphorus of tibia in commercial laying hens which fed low available phosphorus corn-soybean meal based diets.

MATERIALS AND METHODS

One hundred and ninety two, 53 week old Hy-Line (W-36) laying hens were examined in this study in age of we used 53 weeks to 64 week. Hens were distributed in completely randomized design included 6 treatments with 4 replications and 8 hens in each replicates. Microbial phytase, was the product of BASF company (Natuphos® 500, BASF Crop., Mt. Olive, NJ) and including 10000 unit active phytase per gram. This product was informed of white granules which derived from *Aspergillus niger* and citric acid is a monohydrated granule. The citric acid used in this experiment was monohydrate 92%, which was added to the diets after calculating purity percentage. The ME of citric acid was assumed to be 2600 kcal kg^{-1} (Boling *et al*., 2000).

The 6 experimental diets were:

- Control (C) with 0.1% available phosphorus (C)
- C+300 FTU kg^{-1} of microbial phytase
- C+2% citric acid
- C+2% citric acid + 300 FTU kg^{-1} of microbial phytase
- C+4% citric acid
- C+4% citric acid + 300 FTU kg^{-1} of microbial phytase

The diets had similar nutrient level except of phosphorus were regulated with National Research Council (1994) recommendation. The ingredients of diets are showed in Table 1. The used cages had 50 cm length, 50 cm wide and 50 cm height. Four hens were kept in each cage and every 2 cage were assumed as experimental unit. The experiment was done is 6 period, each 15 days sequential period.

Average temperature in all 6 periods was constant (19°C). In order to evaluate the condition of flock, first data collecting supplied in 1 month before starting the experiment and it was found that there were no differences in performance of treatments before the experiment. The hens fed *ad libitum* and exposed to the 16 h light and 8 h darkness during a day. In order to adaptation to new diets they were fed during 1 week before the experiment.

At the end of experiment, two birds were selected from each replication and 5 mL blood was taken from wing puncture. Blood samples centrifuged for 15 min (3000 rpm min^{-1}) and serum was separated.

Table 1: Ingredients and nutrient composition (g kg^{-1}) of experimental diets during laying (53-64) week of age

	Treatments					
Ingredients	1	2	3	4	5	6
Corn	664.4	663.8	632.7	632.1	601.1	600.5
Soybean meal (44%)	211.2	211.3	211.7	217.3	223.4	223.5
Soybean oil	12.1	12.3	15.7	19.9	19.4	16.6
Calcium carbonate	81.2	81.2	81.2	81.2	81.2	81.2
Oyster shell	20.0	20.0	20.0	20.0	20.0	20.0
Salt	1.5	1.5	1.5	1.5	1.5	1.5
Sodium bicarbonate	3.6	3.6	3.6	3.6	3.6	3.6
Premix[a]	5.0	5.0	5.0	5.0	5.0	5.0
DL-methionine	1.0	1.0	1.0	1.0	1.0	1.0
Citric acid (92%)	-	-	21.9	21.9	43.8	43.8
Phytase[b]	-	0.3	-	0.3		0.3
Calculated analysis (data on dry matter)						
ME (kcal kg^{-1})	2817.0	2817.0	2817.0	2817.0	2817.0	2817.0
Crude protein (g kg^{-1})	150.0	150.0	150.0	150.0	150.0	150.0
Available P (g kg^{-1})	1.0	1.0	1.0	1.0	1.0	1.0
Total P (g kg^{-1})	3.2	3.2	3.2	3.2	3.2	3.2
Calcium (g kg^{-1})	38.0	38.0	38.0	38.0	38.0	38.0
Met+Cys (g kg^{-1})	6.0	6.0	6.0	6.0	6.0	6.0
Lysine (g kg^{-1})	7.4	7.4	7.4	7.4	7.4	7.4

[a]Vitamin and mineral mix supplied/kg diet: vitamin A, 9000 IU; vitamin D_3, 2000 IU; vitamin E, 18 IU; vitamin K_3, 2 mg; Vitamin B_1, 1.8 mg; Vitamin B_2, 6.6 mg; Vitamin B_5, 4 mg; vitamin B_{12}, 0.015 mg; Nicotinic acid, 35 mg; folic acid, 1 mg; biotin, 0.1 mg; choline chloride, 250 mg; ethoxyquin, 0.125; Mn, 100 mg; Zn, 10 mg; cu, 100 mg; Se, 0.22 mg; I, 1 mg; Fe, 50 mg. [b]Natuphos® (BASF Crop., Mt. Olive, NJ) was used to supply 300 FTU microbial phytase per kilogram of diet

The concentration of zinc, copper and manganese measured by using ICP (Inductively Coupled Plasma Emission Spectrometer, Model JY-24, Jobin Yvon, Longjumeau, Cedex, France). Then, hens killed by cervical dislocation and left tibia was taken. After collecting fat from soft tissue of tibia (Soxhlet method), it was kept in alcohol for about 15 min and dried in 100° C and weighted. Dried tibia was burned in muffle furnace for 8 h and in 550°C. Tibia ash was calculated as percentage of dry weight. Then the calcium and phosphorus content of samples were measured (Association of Official Analytical Chemists, 1995).

General Linear Models (GLM) procedures of SAS® (SAS, 1990) software was employed and significant differences between treatments were separated using Duncan's multiple range test (Duncan's, 1955).

RESULTS AND DISCUSSION

Citric acid and microbial phytase didn't affect zinc and manganese of serum but citric acid× microbial phytase interaction had significant effect on copper in serum with low available phosphorus diets ($p<0.0001$), despite different levels of citric acid didn't have similar performance in different levels of phytase (Table 2).

Adding different levels of citric acid to the diets which didn't supplemented with phytase and had phosphorus deficiency, increased copper content in serum ($p<0.05$). The concentration of copper in serum increased significantly in comparison to control group when microbial phytase was added to low available phosphorus diets ($p<0.05$), but adding citric acid didn't show any differences.

It seems that supplementing diets with citric acid didn't affect efficacy of microbial phytase. It seems that microbial phytase added to the diets, hydrolysis ester ligand in phytate and releases phosphorus and other minerals from it, then changes the concentration of these minerals in serum. Many studies reported an increase in availability of phosphorus and minerals when diet was supplemented with microbial phytase (Nelson, 1976; Kornegary and Qian, 1996; Mitchell and Edwards, 1996; Sebastian *et al*., 1996; Lei and Stahl, 2000).

Table 2: The effect of citric acid and microbial phytase on the concentration of zinc, copper and manganese of serum and ash, calcium and phosphorus of tibia in laying hens (53-64) at whole period

Treatments							
Citric acid (%)	Phytase (FTU kg^{-1})[1]	Zinc	Copper	Manganese	Tibia ash	Tibia ash calcium	Tibia ash phosphorus
		(μg L^{-1})			(%)		
0	Control	199.40	211.86^{e}	52.56	42.51^{c}	38.10^{b}	16.16
0	300	148.45	246.60^{ab}	49.72	46.59^{ab}	38.75^{b}	20.20
2	0	166.20	227.74^{d}	47.39	44.84^{b}	39.00^{b}	16.55
2	300	157.85	242.27^{b}	45.28	45.26^{b}	38.75^{b}	21.40
4	0	176.73	233.94^{c}	48.54	47.63^{a}	37.50^{b}	16.15
4	300	207.45	248.21^{a}	44.92	45.08^{b}	41.50^{a}	18.75
SEM pooled		15.12	1.77	2.35	0.59	0.54	0.78
Main effects							
Citric acid	0	173.93	229.23^{c}	51.14	44.55^{b}	38.42	18.40
	2	162.03	235.00^{b}	46.33	45.05^{b}	38.87	18.97
	4	192.09	241.07^{a}	46.73	46.35^{a}	39.50	17.45
Phytase	0	180.78	224.51^{b}	49.49	44.99	38.20^{b}	16.43^{b}
	300	171.25	245.69^{a}	46.64	45.64	39.66^{a}	20.11^{a}
Probabilities							
Citric acid		0.1637	0.0001	0.1046	0.0190	0.1630	0.1742
Phytase		0.4505	0.0001	0.1550	0.1913	0.0037	0.0001
Citric acid×Phytase		0.0568	0.0001	0.9501	0.0001	0.0023	0.3762

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

Citric acid×microbial phytase interaction had a significant effect on tibia ash in layers ($p<0.0001$). Results showed that adding different levels of citric acid to the low available phosphorus diets which didn't supplemented with microbial phytase, increased the percentage of tibia ash but it wasn't the same when citric acid added to the phytase supplemented diets. Tibia ash percent was higher in diets contain 4% citric acid and 300 FTU kg^{-1} microbial phytase ($p<0.05$).

Adding 4% citric acid and 300 FTU kg^{-1} microbial phytase decreased tibia ash percent in comparison to the diets which supplemented ouly with 4% citric acid but the reason in not realized. It likely is because of the effect of citric acid on the pH makes the intestine an unreasonable place for activation of microbial phytase. Nevertheless, citric acid is an organic acid which rapidly metabolizes in body and have very little effect on the pH of intestine (Brenes *et al*., 2003).

Citric acid×microbial phytase interaction affected calcium of tibia ash in layers which fed low available phosphorus diets ($p<0.0001$). Adding different levels of citric acid to the low available phosphorus diets which didn't supplemented with phytase, didn't have effect on the calcium of tibia ash, although, the percentage of tibia ash was the highest in diets with 4% citric acid and 300 FTU kg^{-1} microbial phytase ($p<0.05$).

Phytase had main significant effect on the percentage of tibia ash from layers which fed low available phosphorus diets ($p<0.05$). Adding 300 FTU kg^{-1} microbial phytase to the diets which had phosphorus deficiency, increased the 18.3% of phosphorus of tibia ash in comparison to the control group, but it wasn't statistically significant.

This indicates that adding 300 FTU kg^{-1} microbial phytase to low available phosphorus diets, increases utilization of phytate phosphorus and the availability of phosphorus. Leeson *et al*. (2000) and Brenes *et al*. (2003) reported the same results but Viveros *et al*. (2002) indicated different results. From this study it could be deduced that:

- Supplementing low available phosphorus diets with different levels of citric acid, increases the concentration of serum copper and the percentage of tibia ash in laying hens at 53-64 week age.

- Adding different levels of citric acid to the low available phosphorus diets which supplemented with microbial phytase, didn't improved the efficacy of microbial phytase in laying hens at 53-64 week age.
- Adding 300 FTU kg^{-1} microbial phytase to the corn-soybean meal based diets with 0.1% phosphorus, increases the concentration of serum copper, calcium and phosphorus of tibia ash in laying hens at 53-64 week age.

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