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The Effect of Calcium Level, Microbial Phytase and Citric Acid on Performance Parameters and Eggshell Quality of Laying Hens Fed Corn Soybean Meal Diet

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Abstract: A 2×2×2 factorial experiment was conducted to determine the effect of phytase enzyme, citric acid or both at two levels of calcium on the performance and egg shell quality of Lohman Brown-Classic laying hens. Eight dietary Treatments (T) consisted of the corn-SBM basal diets were fed to seventy two birds from the 23rd to 38th week of age. T_1 (Treatment 1) contained 3.8% calcium, T_2 contained 3.8% Ca + 300 FTU, T_3 contained 3.8 Ca% + 2.5% citric acid, T_4 contained 3.8% Ca + 300 FTU + 2.5% citric acid, T_5 contained 2.6% calcium, T_6 contained 2.6% Ca + 300 FTU (T_7) contained 2.6% Ca + 2.5% citric acid and T_8 contained 2.6% Ca + 300 FTU + 2.5% citric acid. All diets were standardized at 0.11% NPP (total phosphorus: 0.33%). The performance criteria for evaluating the effect of dietary treatments were egg production, egg weight, egg mass, feed intake, feed conversion ratio, body weight, tibia ash, calcium in tibia ash, phosphorus in tibia ash, shell percentage and egg shell density. After the performance trial termination, the digest from the crop and the proximal small intestine was obtained from each hen and the total phosphorus disappearance was calculated using TiO_2 as an indigestible marker. All the investigated performance parameters except feed intake and phosphorus in tibia ash were depressed by feeding 2.6% calcium compared with 3.8% calcium. Feed conversion ratio and phosphorus in tibia ash were increased by adding citric acid, whereas, body weight and feed intake were decreased. Phytase supplementation significantly increased tibia ash and calcium in tibia ash. Microbial phytase in combination with citric acid has no effect on the performance parameters and egg shell quality of laying hens fed a corn-SBM diet containing 2.6% Calcium. High dietary calcium (3.8%) decreased phosphorus disappearance of the crop contents compared to 2.6% Ca. This study also indicated that the main site of microbial phytase activity in the digestive tract of laying hens is in the crop.

Key words: Calcium level, microbial phytase, citric acid, performance parameters, eggshell quality, laying hens

INTRODUCTION

Phytic acid is the major storage form of phosphorus in cereals, legumes and oil seeds. Minerals can readily bind to phytic acid and thus have the potential to form mineral-phytate complexes. The formation of insoluble phytate-mineral complexes in the intestinal tract prevents mineral absorption. It has been well established that supplementing animal feed with microbial phytase results in hydrolyses of phytate molecule found in plant seeds (Simons *et al*., 1990). This hydrolysis, in turn liberates phytate-bound minerals such as Ca, P, Mg, Cu, Zn, Fe and K (Sebastian *et al*., 1996). High amount of dietary calcium in diets of laying hens may reduce the efficiency of phytase enzyme to hydrolyze phytine and therefore, the releasing of calcium and other minerals, which bind to phytate molecule will be reduced. Erdman (1979) reported that citric acid, a strong chelator of Ca, decreases Ca binding to the phytate molecule, thus making it less stable and more soluble. Shol (1937) reported that dietary addition of citric acid/sodium citrate to rat diets (deficient in Ca, P or both) prevented rickets in rats. If the latter is correct, or at least partially true, it is possible that adding microbial phytase in the presence of citric acid may improve the efficiency of phytase enzyme to hydrolyze phytate and release Ca and other cations, which bind to phytate molecule.

The main objective of the current study was to determine the effect of microbial phytase, citric acid, or both on the performance parameters, egg shell quality and total phosphorus disappearance in the digestive tract of laying hens fed two different levels of dietary calcium.

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MATERIALS AND METHODS

Diets (Table 1) were factorially arranged in two levels of calcium (3.8 and 2.6%), two levels of phytase (0 and 300 FTU kg^{-1}) and two levels of citric acid (0 and 2.5%). Seventy two Lohman Brown-Classic laying hens. were randomly assigned to one of eight dietary treatments in an experiment that was conducted from 23-38 weeks of age, beginning April 2008. Each dietary treatment was fed to 9 hens. The hens were kept in individual cages with the stocking density of 6.9 hens m^{-2} (cage size: 0.4×0.36 m). The birds were housed in a completely enclosed, ventilated caged-layer building in which they were exposed to a 14 h light: 10 h dark daily lighting schedule.

The compositions of corn-SBM basal diets are shown in Table 2. Total P was kept constant with marginal level in all dietary treatments at 0.31% (0.11% NPP). Other dietary nutrients were formulated to meet nutrient requirements of laying hens (NRC, 1994). Energy, protein and amino acids were kept constant in all diets by maintaining the ratio between corn and soybean meal constant in all dietary treatments.

The diets were presented in mash form and feed and water were consumed *ad libitum* during the experimental period. Citric acid was added at the expense of wheat starch.

The microbial phytase RONOZYME-P5000®; Roche Vitamins Ltd., Basel (Switzerland) from *Peniophora lycii*, is a 6-phytase with an activity of 5000 units (FTU) g^{-1}.

Parameters examined: At the onset of the experiment, hens were weighed individually and the number of eggs daily recorded for each hen, the hens assigned to treatments in randomized design based on body weight and egg production rate, so that mean body weight and egg production rate were similar for hens on all treatments. Egg production and egg mass were daily measured. Feed intake was recorded weekly. At the end of the experiment, final body weight and feed conversion ratio were calculated. At the end of the experiment, the hens were slaughtered and the left tibias were removed and cleaned of all adhering tissue. The tibias were immersed in alcohol solution (chloroform: methanol; 2:1) in glass container and covered overnight for degreasing. Bones were then dried at 105°C for 2 days and weighed. Dry-ashed was carried out overnight in muffle furnace at 550°C; the ash cooled down in desiccator and weighed again to determine the weight of ash in tibia. P and Ca in tibia were analyzed. The Ca in tibia ash was determined using an atomic absorption spectrophotometer (Varian, Spectr AA) at a wavelength of 422.7 nm. P in tibia ash was analyzed according to VDLUFA method (Naumann and Bassler, 1976-1997).

Table 1: Design of the experiment

Diets	Ca (%)	Phytase supplemented* (FTU kg^{-1})	Citric acid (%)
T_1	3.8	0	0
T_2	3.8	300	0
T_3	3.8	0	2.5
T_4	3.8	300	2.5
T_5	2.6	0	0
T_6	2.6	300	0
T_7	2.6	0	2.5
T_8	2.6	300	2.5

*RONOZYME-P5000®

Table 2: Ingredients and nutrient composition of the experimental diets

	Ca (%)	
Ingredients (%)	3.8	2.6
Corn	57.230	57.23
Soybean meal (49)	24.150	24.15
Soybean oil	3.100	0.59
Wheat starch	2.620	8.56
$CaCO_3$	9.900	6.52
NaCl	0.050	0.05
Cellulose powder	1.850	1.80
DL-Methionine	0.100	0.10
Premix*	1.000	1.00
Nutrient composition (%)		
Dry matter	91.730	91.27
Crude protein	16.400	16.57
Crude fat	5.810	3.34
Crude fiber	3.340	3.34
Crude ash	12.730	9.48
Na	0.140	0.14
Calcium (total)	3.920	2.76
Phosphorus (total)	0.329	0.330
Phytate-P	0.220	0.24
Metabolizable energy (MJ)	11.560	11.56

*1 kg of Premix contains: 600.000 IU vitamin A, 100.000 IU vitamin D3, 1.850 mg vitamin E, 160 mg vitamin B1, 480 mg vitamin B2, 500 mg vitamin B6, 2.000 mcg vitamin B12, 200 mg vitamin k3, 2800 mg nicotinic acid, 1000 mg Ca-Pantothenate, 60 mg folic acid , 10000 mcg biotin, 80000 mg cholinchlorid, 2500 mg Fe, 1600 mg Cu, 8000 mg Mn, 8000 mg Zn, 120 mg I, 25 mg Se, 55 mg Co, 10000 mg BHT, 350 mg canthaxanthin

Egg shell quality (shell percentage and shell density) was measured every 2 weeks. After gathering the eggs from the stall, each egg was weighed and kept cool at -4°C. Shell weight for each egg with membrane was determined after breaking the egg and cleaning the shell from adhering albumen and drying the shell at room temperature for 24 h. Percentage shell was determined by dividing the dried shell weight by egg weight:

$$\text{Shell (\%)} = (\text{Dried shell weight/Egg weight}) \times 100$$

The egg shell density (shell weight per unit surface area mg cm^{-2}) was calculated by dividing the shell weight (mg) by the egg surface area (cm^2). Egg Surface area (S) was calculated from the fresh egg Weight (W) in gram by the equation of Mueller and Scott (Tyler and Geake, 1953):

$$S = 4.67 \times W^{\%}$$

where, 4.67 is a constant.

Phosphorus measurement in the digestive tract (titanium method): At the end of the experiment, titanium dioxide was added into the feed at a rate of 2 g kg^{-1} as an indigestible indicator substance. The feed was pelleted and fed to the hens 5 days before the slaughtering. The birds were starved over night for 18 h (12:00-06:00), given access to the feed (with titanium) for 4 h and slaughtered. They were then dissected to reveal the crop and small intestine. Digesta were squeezed manually from the crop and the proximal small intestine (Duodenum and Jejunum) into a small plastic container. Samples were weighed and kept frozen at -18°C until analysis. Samples of diets and freeze-dried gut contents were ground (1 mm sieve) to determine the total P and titanium. The procedure from death of the bird to completing the sample collection lasted approximately 5 min. Because Ca and P absorption are dependent on the progress of eggshell formation (Van der Klis *et al*., 1997), the slaughtering time was taken in to consideration to eliminate differences in mineral absorption due to the stage of eggshell formation between the treatments (Eight hens were randomly chosen from the eight different treatments (1 hen/1 treatment) and slaughtered at the same time, this slaughtering method was repeated until slaughtering all hens in the eight treatments). Phosphorus disappearance in the digestive tract was estimated by using the analyzed data of TiO_2 and phosphorus concentration in the diets and digesta.

The following equation was used to calculate the P hydrolyses:

$$D_P = 100 - ((TiO_2\%)_{diet} (TiO_2\%)_{digesta} \times (P\%)_{digesta} (P\%)_{diet} \times 100)$$

where:

D_P = P disappearance (%)
$(TiO_2\%)_{diet}$ = Concentration of titanium dioxide in the diet
$(TiO_2\%)_{digesta}$ = Concentration of titanium dioxide in the digesta
$(PP\%)_{digesta}$ = Concentration of phosphorus in the digesta
$(PP\%)_{diet}$ = Concentration of phosphorus in the diet

Statistical analyses: Data was subjected to ANOVA using the general linear models procedure of SPSS program (10.0). Significant differences among treatment means were assessed with the least significant difference test at $p \leq 0.05$.

RESULTS AND DISCUSSION

Egg production, egg mass, feed intake, feed conversion ratio and body weight: Egg production, egg mass, feed intake, feed conversion ratio and body weight for the entire 23rd-38th week period are shown in Table 3. No significant differences ($p \leq 0.05$) in performance were observed among treatments during the first 6 weeks of the experiment. By 29th week of age, the low calcium diets resulted in significantly lower performance of laying hens compared with hens, who consumed high calcium diets. Egg production was significantly reduced at low calcium level (2.6%) compared to hens fed high Ca level (3.8%). Abdallah *et al*. (1993) reported that low calcium diet decreased EP in laying hens, whereas, Frost and Roland (1991) indicated that feeding Ca levels in range between 2.5-3.1% had no effect on egg production. Egg mass was reduced at low dietary calcium. EP and EM were not affected by phytase enzyme and/or citric acid. However, Gordon and Roland (1998) reported that phytase enzyme improved the egg production and egg weight in hens fed low calcium diets. Feed intake was not affected by dietary calcium. Rama Rao *et al*. (2003) and Keshavarz and Nakajima (1993) reported that the feed intake was not affected by dietary calcium levels in layer diet. Rodehutscord *et al*. (2002) also reported that calcium level (2.8 and 3.7%) did not affect the feed intake of Lohman-Brown laying hens fed corn soybean meal diets. At both levels of calcium, adding citric acid decreased feed intake of laying hens ($p = 0.058$). This result is in agreement with earlier studies Angel *et al*. (2001) and Shellem and Angel (2002), who reported that citric acid depressed the feed intake in broiler chicks.

The decrease in feed intake from citric acid was probably due to decreasing the feed palatability. The body weight of hens fed high calcium diets (3.8%) was increased compared with hens fed low calcium diets (2.6%). Citric acid reduced hen body weight at both calcium levels. This reduction in body weight of hens fed citric acid was probably due to decreasing feed intake. This result with laying hens is in agreement with previous study with chicks (Boling-Frankenbach *et al*., 2001). They reported that the addition of citric acid resulted in depressions in chicks body weight at different calcium levels. It can be observed that at both levels of calcium the hens, which were supplemented with phytase (without citric acid) showed heavier body weight compared to hens who were not supplemented with phytase ($p = 0.083$) (T_2 vs. T_1 and T_6 vs. T_5). Low Ca diets decreased the feed conversion ratio compared to hens fed high Ca diets. At both calcium levels, adding citric acid significantly improved the feed conversion ratio (T_1 vs. T_3 and T_5 vs. T_7).

Ash, calcium and phosphorus in tibia: Tibia ash, calcium in tibia ash and phosphorus in tibia ash levels are shown

Table 3: The effect of calcium level, microbial phytase and citric acid on laying performance

Diets	Ca (%)	Phytase supplemented (FTU kg^{-1})	Citric acid (g)	BW[1] (%)	EP[2] (g day^{-1})	EM[3] (g day^{-1})	FI[4] (g day^{-1})	FCR[5]
T_1	3.8	0	0	1921^{ab}	96.58	58.07	114.1	1.96^{bc}
T_2	3.8	300	0	1983^{a}	98.18	60.94	117.3	1.92^{c}
T_3	3.8	0	2.5	1752^{cd}	97.43	61.63	110.0	1.78^{d}
T_4	3.8	300	2.5	1899^{ab}	97.33	59.31	111.2	1.87^{cd}
T_5	2.6	0	0	1782^{bcd}	92.55	56.01	114.8	2.06^{ab}
T_6	2.6	300	0	1860^{abc}	94.70	56.04	117.3	2.09^{a}
T_7	2.6	0	2.5	1647^{d}	93.12	55.80	107.6	1.93^{c}
T_8	2.6	300	2.5	1687^{cd}	97.03	58.16	115.2	1.98^{abc}
Main effects								
3.8% calcium	-	-	-	1889^{a}	97.38^{a}	60.0^{a}	113.16	1.88^{b}
2.6% calcium	-	-	-	1744^{b}	94.36^{b}	56.5^{b}	113.76	2.01^{a}
0 FTU kg^{-1}	-	-	-	1775	94.93	57.88	111.63	1.93
300 FTU kg^{-1}	-	-	-	1857	96.81	58.61	115.29	1.96
No citric acid	-	-	-	1887^{a}	95.51	57.77	115.9	2.01
2.5% citric acid	-	-	-	1746^{b}	96.23	58.72	111.0	1.89
Citric acid × phytase	-	-	-	1793	97.18	58.74	113.22	1.92
No citric acid × phytase	-	-	-	1922	96.44	58.49	117.36	2.01
Citric acid × no phytase	-	-	-	1699	95.27	58.71	108.8	1.86
Probabilities								
Ca	-	-	-	0.003	0.020	0.004	0.816	0.000
Citric acid	-	-	-	0.004	0.574	0.412	0.058	0.000
Phytase	-	-	-	0.083	0.142	0.527	0.155	0.325
Calcium × citric acid	-	-	-	0.771	0.575	0.997	0.934	0.936
Calcium × phytase	-	-	-	0.627	0.374	0.695	0.576	0.824
Citric acid × phytase	-	-	-	0.803	0.986	0.540	0.764	0.276
Calcium × citric acid × phytase	-	-	-	0.506	0.490	0.109	0.486	0.379
SE	-	-	-	92.91	2.53	2.3	5.05	0.062

[a, b,c]:Means within a column with no common superscript letters significantly different ($p<0.05$); [1]BW: Body Weight, [2]EP: Egg Production, [3]EM: Egg Mass, [4]FI: Feed Intake, [5]FCR: Feed Conversion Ratio

in Table 4. Tibia ash and calcium in tibia ash were significantly reduced in hens fed low calcium diet (2.6%) compared to hens fed 3.8% Ca. Supplementing diet with phytase enzyme resulted in an increased tibia ash. The increase in tibia ash was reported also by Gordon and Roland (1998) and considered to be a good indicator of bone mineralization. This increasing in tibia ash by adding microbial phytase was more pronounced at high calcium level (3.8%). Citric acid significantly increased calcium in tibia ash. Shol (1937) observed that dietary addition of citric acid/sodium citrate prevented rickets in rats fed deficient Ca diets. Whereas Sifri *et al.* (1977) reported that citric acid did not improve calcium in tibia ash of chicks. Adding microbial phytase improved calcium in tibia ash. The increase in Ca in tibia ash in this study by adding citric acid or phytase (without combination) was probably due mainly to increase Ca availability in the diet. The three-way interaction of calcium, citric acid and phytase on Ca in tibia ash was observed. Calcium in tibia ash was significantly increased in hens fed 3.8% dietary calcium level, phytase and without citric acid (T_2) compared to hens, which were fed the other dietary treatments.

P in tibia ash: Citric acid increased P in tibia ash, however this increase was more pronounced in hens fed low calcium diets (2.6%) than (3.8% Ca). The two way interaction of phytase×citric acid was observed on phosphorus in tibia ash. At 3.8% Ca, P in tibia ash of birds supplemented with phytase and citric acid was reduced compared to birds, which were supplemented only with phytase (T_4 vs. T_2). There are no references for laying hens on this subject (phytase×citric).

Microbial phytase improved the tibia ash and Ca in tibia ash at high dietary calcium level (T_2). In addition, due to least significant differences test between individual treatments, adding microbial phytase alone at the both levels of calcium increased P in tibia ash. A possible explanation for this improvement at 3.8% Ca by adding microbial phytase could be that additional phosphorus was liberated by adding phytase, would cause a greater Ca: available P ratio balance in the high calcium diets (Afsharmanesh and Pourreza, 2005).

The results indicated that at low calcium diet (2.6%), adding microbial phytase in combination with citric acid showed a further increase (but not significant) in egg production and egg mass than adding phytase or citric acid separately, however, phosphorus and calcium in tibia ash were significantly reduced (T_8) (Table 4). This decreasing in calcium and phosphorus in tibia ash might be due to the maximum egg production on this treatment which could necessitate a higher calcium and P for eggshell formation. The egg production of hens fed low

Table 4: The effect of calcium level, microbial phytase and citric acid on bone mineralization

Diets	Ca (%)	Phytase supplemented (FTU kg^{-1})	Citric acid (%)	Ash in tibia (%)	Ca in tibia ash (%)	P in tibia ash (%)
T_1	3.8	0	0	54.56^{bc}	33.13^{bcd}	15.70^{cd}
T_2	3.8	300	0	58.29^{a}	35.26^{a}	16.06^{a}
T_3	3.8	0	2.5	54.57^{bc}	33.60^{b}	15.95^{ab}
T_4	3.8	300	2.5	56.66^{ab}	33.00^{cd}	15.60^{cd}
T_5	2.6	0	0	52.88^{cd}	32.20^{e}	15.50^{d}
T_6	2.6	300	0	53.99^{bc}	33.39^{bc}	15.77^{bc}
T_7	2.6	0	2.5	50.14^{d}	32.82^{d}	16.09^{a}
T_8	2.6	300	2.5	54.58^{bc}	32.67^{de}	15.66^{cd}
Main effects						
3.8% calcium	-	-	-	56.02^{a}	33.75	15.82
2.6% calcium	-	-	-	52.90^{b}	32.77	15.76
0 FTU kg^{-1}	-	-	-	53.04^{b}	32.94	15.81
300 FTU kg^{-1}	-	-	-	55.88^{a}	33.58	15.77
No citric acid	-	-	-	54.93	33.50	15.76
2.5% citric acid	-	-	-	53.99	33.02	15.82
Citric acid × phytase	-	-	-	55.62	32.83	15.63
No citric acid × phytase	-	-	-	56.14	34.32	15.91
Citric acid × no phytase	-	-	-	52.35	33.21	16. 02
Probabilities						
Ca	-	-	-	0.000	0.000	0.258
Citric acid	-	-	-	0.256	0.001	0.270
Phytase	-	-	-	0.001	0.000	0.518
Calcium × citric acid	-	-	-	0.871	0.004	0.005
Calcium × phytase	-	-	-	0.939	0.389	0.501
Citric acid × phytase	-	-	-	0.611	0.000	0.000
Calcium × citric acid × phytase	-	-	-	0.137	0.017	0.993
SE	-	-	-	1.64	0.282	0.119

^{a,b,c}: Means within a column with no common superscript letters significantly different ($p<0.05$)

calcium diets (2.6%), supplemented with phytase and citric acid was 97%; however, the egg production of hens fed the same level of calcium (2.6%) and supplemented only with citric acid was 93% or with phytase was 94.7%.

Eggshell quality: The egg weight, eggshell density and the egg shell percentage were shown in Table 5.

Measuring egg shell weight is a more labor-intensive method for evaluating calcium metabolism in layer because it is related to shell thickness and therefore $CaCO_3$ deposition (Gordon and Roland, 1998). Increasing dietary calcium resulted in an increase in egg weight, percentage shell and shell density. Ousterhout (1980), Bar *et al.* (2002) and Chandramoni and Sinha (1989) reported eggshell quality was reduced in hens fed low calcium diet. Citric acid and/or phytase did not show any affect on the egg weight and the egg shell quality. Lim *et al.* (2003) and Van der Klis *et al.* (1997) reported that dietary phytase does not have any consistent effect on eggshell quality. Other investigators observed a beneficial effect on eggshell quality of phytase supplementation (Gordon and Roland, 1997; Punna and Roland, 1999).

Phosphorus disappearance in the digestive tract: Based on the known function of the crop it is unlikely that phosphorus could be absorbed into the organ (Table 6). Therefore, it was preferred to use disappearance as an expression rather than using absorption. A possible explanation for phosphorus disappearance of the crop content could be due to the feed particles size. Phosphorus, which is mainly in solution in the crop, might be emptied more rapidly than the larger feed particles. A similar explanation was given by Hurwitz and Bar (1965); they observed that the P and Ca concentration in the gizzard was lower than that in the feed. Phosphorus disappearance from the crop content was significantly higher at low calcium diets (2.6%) than 3.8% Ca. However, calcium level had no effect on phosphorus disappearance in the proximal small intestine. In contrast to this result, Van der Klis *et al.* (1997) reported that an increase in dietary calcium concentration from 30-40 g kg^{-1} lead to a decrease in the praecaecal phosphorus net absorption from 44-35%.

Microbial phytase increased the phosphorus disappearance from the crop content ($p = 0.08$). At high dietary calcium level (3.8%), without any supplementation, phosphorus disappearance was 0.83%, however, adding microbial phyase (without citric acid) substantially increased the phosphorus disappearance to 13.4% in laying hens fed the same level of calcium (T_1 vs. T_2). Also, at 2.6% dietary calcium level without any supplementation (T_5), the P disappearance of the crop contents increased from 9.5-11.3% in laying hens fed the same amount of calcium supplemented only with phytase (T_5 vs. T_6).

Table 5: The effect of calcium level, microbial phytase and citric on egg weight and eggshell quality

Diets	Ca (%)	Phytase supplemented (FTU kg^{-1})	Citric acid (%)	Egg weight (g)	Egg sell (%)	Egg shell density (mg cm^{-2})
T_1	3.8	0	0	60.13	9.20	77.37
T_2	3.8	300	0	62.06	9.08	76.94
T_3	3.8	0	2.5	63.31	8.95	76.70
T_4	3.8	300	2.5	60.96	8.97	75.97
T_5	2.6	0	0	60.17	8.60	72.37
T_6	2.6	300	0	59.22	8.37	70.15
T_7	2.6	0	2.5	59.92	8.15	68.37
T_8	2.6	300	2.5	59.9	8.04	67.54
Main effects						
3.8% calcium	-	-	-	61.62[a]	9.05[a]	76.74[a]
2.6% calcium	-	-	-	59.80[b]	8.29[b]	69.61[b]
0 FTU kg^{-1}	-	-	-	60.88	8.72	73.70
300 FTU kg^{-1}	-	-	-	60.53	8.61	72.65
No citric acid	-	-	-	60.40	8.81	74.21
2.5% citric acid	-	-	-	61.02	8.53	72.14
Citric acid × phytase	-	-	-	60.43	8.50	71.75
No citric acid × phytase	-	-	-	60.64	8.72	73.55
Citric acid × no phytase	-	-	-	61.62	8.55	72.53
Probabilities						
Ca	-	-	-	0.037	0.000	0.000
Citric acid	-	-	-	0.466	0.165	0.215
Phytase	-	-	-	0.685	0.583	0.525
Calcium × citric acid	-	-	-	0.632	0.607	0.453
Calcium × phytase	-	-	-	0.874	0.763	0.775
Citric acid × phytase	-	-	-	0.328	0.763	0.869
Calcium × citric acid × phytase	-	-	-	0.132	0.986	0.798
SE	-	-	-	1.700	0.400	3.280

[a, b,c]: Means within a column with no common superscript letters significantly different ($p<0.05$)

Table 6: The effect of calcium level, microbial phytase and citric acid on total P disappearance in the crop and the proximal small intestine

Diets	Ca (%)	Phytase supplemented (FTU kg^{-1})	Citric acid (%)	Crop (%)	SI (%)[1]
T_1	3.8	0	0	0.83[e]	22.59
T_2	3.8	300	0	13.44[a]	24.38
T_3	3.8	0	2.5	9.91[ab]	32.04
T_4	3.8	300	2.5	3.33[de]	16.72
T_5	2.6	0	0	9.56[bc]	-[2]
T_6	2.6	300	0	11.30[ab]	31.08
T_7	2.6	0	2.5	8.45[bc]	23.65
T_8	2.6	300	2.5	6.32[cd]	-[2]
Probability					
Calcium	-	-	-	0.021	0.832
Phytase	-	-	-	0.089	0.114
Citric acid	-	-	-	0.090	0.892
Calcium × phytase	-	-	-	0.059	-
Citric acid × phytase	-	-	-	0.000	0.052
Calcium × citric acid × phytase	-	-	-	0.000	-
SE	-	-	-	0.82	2.26

[a, b,c, d]: Means within a column with no common superscript letters significantly different ($p<0.05$); [1]Proximal Small Intestine; [2]Missed data (The digesta was not enough for laboratory analysis)

Liebert *et al.* (1993), reported in chickens that 69-86 of added microbial phytase activity was detected in the crop and 31-38% of added phytase activity was detected in the proventriculus. No phytase activity was detected in the small intestine. This improvement in phosphorus availability may be explained by the fact that phytate complexes were, to some extent, cleaved by phytase (Nair *et al.*, 1991). Microbial phytase had no effects on the phosphorus disappearance in the small intestine. In contrast to this result, Van der Klis *et al.* (1997) reported that microbial phytase significantly increased phosphorus absorption in the small intestine of laying hens. But, it is important to note that Van der Klis *et al.* (1997) used another sort of microbial phytase (Natuphos), which has wider pH range (2.5 and 5.5) than the microbial phytase sort which was used in this study (ronozyme). Ronozyme enzyme has one narrow optimal pH of 4.4. Zyla *et al.* (2004) reported that the 3-phytase (natuphos) appeared to be more effective than 6-phytase (ronozyme) in feed dephosphorylation and in Ca release at different calcium levels under the intestinal conditions. An explanation for this low effectiveness of microbial phytase on P

disappearance in the small intestine was also given by Wise (1983), who reported that the increase in pH of the digesta as it moves distally along the GIT causes the phytate molecule to be ionized and thus, more readily form complexes with divalent metal cations like Zn, Ca, Mg and Fe. The higher pH levels result in decreased solubility of the complex and therefore, decreased efficiency of the microbial phytase. Angel *et al.* (2002) also reported that the low phytase activity in the small intestine may be due to a much more basic pH (above 6), which is less favorable for high phytase activity.

At both levels of calcium, adding microbial phytase in combination with citric acid decreased the phosphorus disappearance in the crop content compared to the birds, which were supplemented only with phytase. No published reports were found about the effect of microbial phytase in combination with citric acid in laying hens. As a main effect, citric acid did not show a significant effect on the P disappearance in the crop ($p = 0.09$). No significant effect was also observed in the proximal small intestine. The results of Boling *et al.* (2000) indicated that citric acid does not improve the utilization of P in corn-SBM diets for laying hens. Boling *et al.* (2000) also reported that it is possible that the very high dietary Ca level in laying hens diet resulted in the supplemental citric acid being bound to nonphytate Ca. Consequently, there was still ample Ca available for binding to phytate and the citric acid would not have been available to bind to the Ca in the Ca-phytate complex.

The results of this study indicated that citric acid did not affect egg production, egg mass egg shell quality and phosphorus disappearance in the digestive tract. However feed efficiency, Ca and P in tibia ash were improved by adding citric acid. The current finding is in agreement with Boling *et al.* (2000), who reported that adding citric acid in laying hens diet did not improve egg production, egg mass and body weight; however, feed efficiency was similar to the positive control diet. Earlier study with chicks (Sifri *et al.*, 1977) reported that citric acid did not have any effect on calcium utilization. This study suggests that 0.33% TP (0.11% NPP) appears to be sufficient for maintaining egg production and eggshell quality in laying hens (Lohman Brown-Classic) fed 3.8% dietary calcium. Similar result was obtained by Liebert *et al.* (1993). Microbial phytase improved tibia ash and Ca and P in tibia ash. However, phytase has not shown a significant effect on laying performance. All performance parameters except feed intake and phosphorus in tibia ash were depressed by feeding 2.6% calcium compared with 3.8% calcium. The finding with calcium requirement for laying hens seems to be marginally higher than NRC (1994) recommendation. Adding microbial phytase in combination with citric acid numerically increased the egg production and egg mass (not significant), however, this increasing was at the expense of Ca and P in tibia ash.

CONCLUSION

An experiment was conducted to determine the effect of phytase enzyme, citric acid or both at two levels of calcium on the performance and egg shell quality of Lohman Brown-Classic laying hens. Several treatments consisted of the corn-SBM basal diets with two levels of calcium; citric acid and FTU were fed to 72 birds from the 23rd-38th weeks of age. Egg production, egg weight, egg mass, feed intake, feed conversion ratio, body weight, tibia ash, calcium in tibia ash, phosphorus in tibia ash, shell percentage and egg shell density were the main performance criteria used for evaluating the effect of dietary treatments. All the investigated performance parameters except feed intake and phosphorus in tibia ash were depressed by feeding 2.6% calcium compared with 3.8% calcium. Feed conversion ratio and phosphorus in tibia ash were increased by adding citric acid, whereas, body weight and feed intake were decreased. Phytase supplementation significantly increased tibia ash and calcium in tibia ash. Microbial phytase in combination with citric acid has no effect on the performance parameters and eggshell quality of laying hens fed a corn-SBM diet containing 2.6% Calcium. High dietary calcium (3.8%) decreased phosphorus disappearance of the crop contents compared to 2.6% Ca. The main site of microbial phytase activity in the digestive tract of laying hens is in the crop.

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