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# Performance, Egg Quality and Mineral Utilization of Dokki-4 Laying Hens Fed Diets Containing Three Levels of Nonphytate Phosphorus in Absence or Presence of Microbial Phytase, Citric Acid or Both

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## ABSTRACT

The present study was conducted to determine the effects of single and combined addition of Citric Acid (CA) and Microbial Phytase (MP) on performance, egg quality and mineral utilization of hens fed graded levels of nonphytate phosphorus (NPP). The NPP levels were 0.40, 0.30 and 0.20% without and with MP (0.05%), CA (2.0%) or both. Twelve groups of both 28 weeks old hens and cockerels were fed their respective experimental diets. Criteria evaluated were feed intake, egg production, egg weight, egg mass, feed conversion ratio, egg components, shell thickness, Haugh units, yolk index, tibia ash, tibia Ca, P, Mg, Cu and Zn and apparent retention of these minerals. Feeding the 0.2% NPP diet adversely affected ($p<0.01$) egg production, egg weight, feed intake, egg mass, feed conversion, percent eggshell, yolk index, shell thickness, tibia ash content and retention of Cu and Zn compared with their positive control. Although birds fed 0.3% NPP gave comparable performance and egg quality to their positive controls, tibia bone ash, tibia contents of Ca and P and retention of P, Cu and Zn of the former were significantly lower than the latter. The poor productive performance and egg quality due to feeding 0.2% NPP diet were completely corrected by MP but CA was not effective. Added MP can effectively prevent the reductions in performance and egg quality and either microbial phytase or citric acid may partially alleviate the depression of mineral utilization in birds fed the low NPP diets, without a synergistic positive effect for their combination.

**Key words:** Dietary nonphytate P, microbial phytase, citric acid, productive performance, egg quality, mineral utilization, laying hens

## INTRODUCTION

It is generally accepted that about two-thirds of Phosphorus (P) found in plant feed ingredients is bound to phytic acid (phytate). This phytate-bound P is either unavailable to poultry (Ravindran *et al*., 1995) or poorly utilized by poultry (Marounek *et al*., 2008, 2010) due to insufficient endogenous phytase activity to break down the phytate effectively. In order to meet the requirements of laying hens for P, inorganic P sources and/or exogenous phytase are commonly included in their corn-soybean meal diets. But, the addition of inorganic phosphorus sources is not only expensive but also contributes to increasing the environmental pollution.

Several investigators reported that dietary supplementation with Microbial Phytase (MP) produced positive effects on egg production of laying hens fed low-nonphytate P diets (Boling *et al*., 2000a; Keshavarz, 2003; Lim *et al*., 2003; Roland *et al*., 2003; Wu *et al*., 2006; Ahmadi *et al*., 2008;

Hughes *et al*., 2008; Ziaei *et al*., 2009; Mohammed *et al*., 2010; Lucky *et al*., 2014) and eggshell quality (Lim *et al*., 2003; Hussein *et al*., 2006; Ziaei *et al*., 2009). Others failed to detect a beneficial effect for supplemental phytase on laying performance (Liebert *et al*., 2005; Al-Sharafat *et al*., 2009) or eggshell quality (Afsari *et al*., 2013; Lucky *et al*., 2014; Musilova *et al*., 2014). On the other hand, it is generally accepted that the efficacy of phytase to hydrolyze phytate P is incomplete (Van der Klis *et al*., 1997; Olukosi *et al*., 2007; Woyengo *et al*., 2008).

Positive effects for Citric Acid (CA) have been observed on phytate P utilization and growth performance of broiler chicks (Rafacz-Livingston *et al*., 2005; Chowdhury *et al*., 2009; Nourmohammadi and Afzali, 2013). However, the responses of laying hens to dietary CA supplementation are inconsistent, some authors observed no positive effect of CA on the utilization of dietary phosphorus or egg production of laying hens (Boling *et al*., 2000b; Nezhad *et al*., 2007; Vargas-Rodriguez *et al*., 2015) but others found that feed conversion ratio and tibia bone phosphorus content of laying hens were improved due to dietary CA supplementation (Al-Sharafat *et al*., 2009). The present study was undertaken to investigate the effects of single and combined addition of citric acid and microbial phytase on the performance of laying hen fed diets containing three levels of nonphytate phosphorus.

## MATERIALS AND METHODS

The field work of this experiment was carried out at Sakha Poultry Farm (Kafr El-Sheikh Governorate, Egypt) while chemical analyses of the experimental diets, excreta and tibia bone ash were undertaken at the Laboratories of Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture, Egypt, during the period from August-September, 2013.

**Experimental birds and management:** Three hundred and sixty, 28 weeks old Dokki-4 hens were used in this study. Birds were randomly distributed into 12 experimental groups, each consisted of thirty pullets plus three cockerels. Each experimental group was subdivided to three equal replications and kept at litter-floored pens in an open-sided laying house. Another group of 36, 28 weeks old cockerels was divided into twelve equal groups, kept in individual cages and fed their respective experimental diets. All birds were subjected to a daily photoperiod of 16 h and had free access to feed and water and managed similarly.

**Experimental diets:** Three corn and soybean meal-based diets were formulated to contain graded levels of non-phytate phosphorus (NPP: 0.40, 0.30 and 0.20%), the diet containing 0.4% NPP was served as a positive control while those containing 0.3 and 0.2% NPP were considered as negative controls. Microbial phytase at a level of 0.5 g $kg^{-1}$, citric acid (2.0% of the diet) or both were added to these diets, thus twelve experimental diets were formulated and used from 28-44 weeks of bird's age. With the exception of NPP level and the presence or absence of phytase and/or citric acid, all diets were isocaloric (ME of about 2750 kcal $kg^{-1}$) and isonitrogenous (CP of about 16.35%) and contained similar levels of other nutrients to meet the nutrient requirements of laying hens (NRC., 1994). The microbial phytase (Natuphos®), used herein, is a product of Gist-Brocades, Netherlands and BASF, Germany, which is made from *Aspergillus niger* and contains 1000 phytase units per gram. Citric acid was supplied as monohydrate citric acid, with a purity of 92%. Composition and chemical analysis of the experimental diets are shown in Table 1.

**Productive performance and egg quality:** The productive performance of Dokki-4 laying hens were evaluated as Daily Feed Intake (DFI), hen-day Egg Production Rate (EPR),

Table 1: Ingredient composition and analysis of the experimental diets containing three levels of nonphytate phosphorus in absence or presence of microbial phytase, citric acid or their combination (CO)

| | NPP | | | NPP+MP | | | NPP+CA | | | NPP+CO | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ingredients (%) | 0.4 | 0.3 | 0.2 | 0.4 | 0.3 | 0.2 | 0.4 | 0.3 | 0.2 | 0.4 | 0.3 | 0.2 |
| Yellow corn | 66.00 | 66.00 | 66.00 | 66.00 | 66.00 | 66.00 | 61.10 | 61.10 | 61.10 | 61.05 | 61.10 | 61.10 |
| SBM (44% CP) | 24.00 | 24.00 | 24.00 | 24.00 | 24.00 | 24.00 | 25.50 | 25.50 | 25.50 | 25.50 | 25.50 | 25.50 |
| Sunflower oil | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.51 | 1.51 | 1.51 | 1.51 | 1.51 | 1.51 |
| Limestone | 7.75 | 8.00 | 8.30 | 7.75 | 8.00 | 8.30 | 7.74 | 8.00 | 8.30 | 7.74 | 8.00 | 8.30 |
| DCP | 1.44 | 0.92 | 0.38 | 1.44 | 0.92 | 0.38 | 1.45 | 0.92 | 0.38 | 1.45 | 0.92 | 0.38 |
| Vit. Min. Prem. | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 |
| NaCl | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 |
| DL-Methionine | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| MP | 0.00 | 0.00 | 0.00 | 0.05 | 0.05 | 0.05 | 0.00 | 0.00 | 0.00 | 0.05 | 0.05 | 0.05 |
| CA | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 |
| Sand | 0.11 | 0.38 | 0.62 | 0.06 | 0.33 | 0.57 | 0.00 | 0.27 | 0.51 | 0.00 | 0.22 | 0.46 |
| Total | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| **Calculated analysis (As fed basis: NRC., 1994)** | | | | | | | | | | | | |
| ME (kcal $kg^{-1}$) | 2750.00 | 2750.00 | 2750.00 | 2750.00 | 2750.00 | 2750.00 | 2765.00 | 2765.00 | 2765.00 | 2765.00 | 2765.00 | 2765.00 |
| CP (%) | 16.23 | 16.23 | 16.23 | 16.23 | 16.23 | 16.23 | 16.47 | 16.47 | 16.47 | 16.47 | 16.47 | 16.47 |
| Ca (%) | 3.33 | 3.32 | 3.32 | 3.33 | 3.32 | 3.32 | 3.33 | 3.32 | 3.32 | 3.33 | 3.33 | 3.32 |
| NPP (%) | 0.39 | 0.29 | 0.19 | 0.39 | 0.29 | 0.19 | 0.39 | 0.29 | 0.19 | 0.39 | 0.29 | 0.19 |
| Total P (%) | 0.61 | 0.51 | 0.41 | 0.61 | 0.51 | 0.41 | 0.61 | 0.51 | 0.41 | 0.61 | 0.51 | 0.41 |
| Methionine (%) | 0.37 | 0.37 | 0.37 | 0.37 | 0.37 | 0.37 | 0.37 | 0.37 | 0.37 | 0.37 | 0.37 | 0.37 |
| Lysine (%) | 0.81 | 0.82 | 0.82 | 0.82 | 0.82 | 0.82 | 0.84 | 0.84 | 0.84 | 0.84 | 0.84 | 0.84 |
| **Determined analysis (Dry matter basis: AOAC., 1990)** | | | | | | | | | | | | |
| Dry matter (%) | 90.12 | 90.13 | 90.12 | 90.12 | 90.13 | 90.12 | 89.85 | 89.86 | 89.86 | 89.95 | 89.88 | 89.86 |
| CP (%) | 18.01 | 18.00 | 18.03 | 18.03 | 18.02 | 18.04 | 18.25 | 18.22 | 18.20 | 18.27 | 18.24 | 18.23 |
| Ca (%) | 3.65 | 3.70 | 3.73 | 3.67 | 3.72 | 3.77 | 3.71 | 3.68 | 3.69 | 3.72 | 3.70 | 3.66 |
| Total P (%) | 0.67 | 0.55 | 0.44 | 0.66 | 0.56 | 0.45 | 0.68 | 0.57 | 0.46 | 0.68 | 0.56 | 0.44 |

¶Supplied per kg of diet; Vitamin A: 10,000 IU, Vitamin $D_3$: 2,000 IU, Vitamin E: 10 mg, Vitamin $K_3$: 1.0 mg, Vitamin $B_1$: 1.0 mg, Vitamin $B_2$: 5.0 mg, vitamin $B_6$: 1.5 mg, vitamin $B_{12}$: 10 µg, Niacin: 30 mg, Pantothenic acid: 10 mg, Folic acid: 1.0 mg, Biotin: 50 µg, Choline: 260 mg, Cu: 4.0 mg, Fe: 30 mg, Mn: 60 mg, Zn: 50 mg, I: 1.3 mg, Se: 0.1 mg, Co: 0.1 mg, NPP: Nonphytate phosphorus, MP: Microbial phytase, CA: Citric acid

Egg Weight (EW), Daily Egg Mass (DEM) and Feed Conversion Ratio (FCR) for the entire experimental period. Daily records on egg production and egg weight were maintained for each replicate group on a 28 day period basis, throughout the experimental period. Feed intake, egg mass and feed conversion ratio (g feed: g egg) were determined on a replicate group basis. When the birds were 32 weeks of age, 360 freshly collected eggs (30 per treatment) were broken out and examined for egg quality measurements, at three consecutive days. Egg quality parameters included egg weight, egg components, Egg Shell Thickness (EST), Haugh units (HU; Haugh, 1937) and yolk index (YI). Shell thickness, as an average of two measures at corresponding positions on the equator of egg shell, was determined by a special micrometer.

**Digestion trials:** At the last week of study, digestion trials were carried out on individually caged Dokki-4 cockerels in order to determine apparent retention of Ca, P, Mg, Cu and Zn. Apparent retention rate of each mineral (Ca, P, Mg, Cu and Zn) was calculated as the percentage of mineral consumed minus mineral excreted divided by mineral intake.

**Slaughter test:** In order to measure bone mineralization, three hens per treatment were slaughtered post-oviposition, at the end of experiment (44 weeks old). After slaughtering, the left tibia of each hen was removed, cleaned of adhering flesh and dried at 100°C for 24 h, crushed and defatted using the Soxhlet extractor and dried again prior to ashing in a muffle furnace at 600°C overnight.

**Chemical analyses:** Tibia bone contents of ash, Ca, P, Mg, Cu and Zn were determined according to the methods of Association of Official Analytical Chemists (AOAC., 1990). The experimental diets and excreta were also analyzed for Dry Matter (DM), Crude Protein (CP), calcium and total P by using the official methods of analysis (AOAC., 1990).

**Statistical analysis:** A completely randomized design was used in this study. Data were statistically processed using the Statgraphics Program (Statistical Graphics Corporation, 1991) by one-way analysis of variance. The significant differences among means of different variables were separated by LSD-multiple range test of Quattro Program (Borland International Inc., 1990) at $p \leq 0.05$.

## RESULTS AND DISCUSSION

**Productive performance of laying hens:** Data on productive performance of Dokki-4 laying hens (Egyptian evolved chickens) fed the experimental diets containing three levels of Nonphytate Phosphorus (NPP) in absence or presence of Microbial Phytase (MP), Citric Acid (CA) or both from 28-44 weeks of age are given in Table 2. As presented in Table 2, reducing dietary NPP from 0.4-0.2% adversely affected ($p<0.01$) hen-day egg production rate, egg weight, daily feed intake, daily egg mass and feed conversion of laying hens but hens fed 0.3% NPP exhibited no significant differences in these parameters. It was observed that hens fed the lowest NPP diet plus MP achieved comparable productive performance to their positive controls for the entire experimental period. But dietary supplementation with 2.0% CA produced no positive effect ($p>0.05$) on the productive performance of laying hens. The combined addition of MP and CA to layer diets restored the above parameters of productive performance of laying hens fed the lowest NPP diet to the levels of the positive control birds.

There is no doubt that the reduction in daily feed intake and thereby in daily NPP intake for hens fed the lowest dietary level of NPP are the main reasons for their decreased egg production, egg weight and daily egg mass but their poor efficiency of feed utilization is mainly attributable to the reduced daily egg mass. It should be noted that supplemental MP, used in the present study, could effectively correct the adverse effect of feeding the low NPP-diets on the productive performance of hens. It is reasonable to attribute the observed improvements in egg production rate, egg weight and feed conversion in response to the combined addition of MP and CA to layer

Table 2: Productive performance of Dokki-4 laying hens fed experimental diets containing three levels of nonphytate phosphorus in absence or presence of microbial phytase, citric acid or both from 28 to 44 weeks of age

| Dietary treatments | EPR (%) | EW (g) | DEM (g) | DFI (g) | FCR (g:g) |
|---|---|---|---|---|---|
| 0.4% NPP | 62.64$^{ab}$ | 49.16$^{b}$ | 30.80$^{bc}$ | 94.24$^{a}$ | 3.05$^{bc}$ |
| 0.3% NPP | 60.02$^{b}$ | 49.28$^{b}$ | 29.58$^{c}$ | 92.29$^{bc}$ | 3.12$^{b}$ |
| 0.2% NPP | 47.23$^{d}$ | 48.76$^{c}$ | 23.03$^{e}$ | 90.39$^{d}$ | 3.94$^{a}$ |
| 0.4% NPP+0.05% MP | 63.06$^{ab}$ | 50.19$^{a}$ | 31.65$^{ab}$ | 94.65$^{a}$ | 2.99$^{bc}$ |
| 0.3% NPP+0.05% MP | 62.41$^{ab}$ | 50.05$^{a}$ | 31.23$^{b}$ | 92.68$^{bc}$ | 2.96$^{bc}$ |
| 0.2% NPP+0.05% MP | 61.45$^{b}$ | 49.01$^{bc}$ | 30.12$^{bc}$ | 92.66$^{bc}$ | 3.07$^{bc}$ |
| 0.4% NPP+2.0% CA | 63.00$^{ab}$ | 50.03$^{a}$ | 31.52$^{ab}$ | 94.56$^{a}$ | 3.00$^{bc}$ |
| 0.3% NPP+2.0% CA | 61.00$^{b}$ | 49.31$^{b}$ | 30.08$^{bc}$ | 93.03$^{b}$ | 3.09$^{b}$ |
| 0.2% NPP+2.0% CA | 50.35$^{c}$ | 48.71$^{c}$ | 24.52$^{d}$ | 91.66$^{c}$ | 3.75$^{a}$ |
| 0.4% NPP+0.05% MP+2.0% CA | 65.47$^{a}$ | 50.12$^{a}$ | 32.81$^{a}$ | 93.68$^{b}$ | 2.85$^{c}$ |
| 0.3% NPP+0.05% MP+2.0% CA | 63.24$^{ab}$ | 49.96$^{a}$ | 31.60$^{ab}$ | 93.51$^{b}$ | 2.95$^{bc}$ |
| 0.2% NPP+0.05% MP+2.0% CA | 61.25$^{b}$ | 49.35$^{b}$ | 30.22$^{bc}$ | 92.99$^{bc}$ | 3.06$^{bc}$ |
| Pooled SEM | 0.910 | 10.202 | 0.486 | 0.213 | 0.056 |
| Significance | ** | ** | ** | ** | ** |

$^{a-e}$Means in each column bearing different superscripts differ significantly ($p \leq 0.05$), EPR: Egg production rate, EW: Egg weight, DEM: Daily egg mass, DFI: Daily feed intake, FCR: Feed conversion ratio, NPP: Nonphytate phosphorus, MP: Microbial phytase, CA: Citric acid diets to their synergistic effect, since CA can change the intestinal pH and improve the activity of phytase, thus improving phytate-P utilization. The reduced performance of laying hens, reported herein, in response to decreasing dietary NPP from 0.4-0.2% is in disagreement with the results obtained by Gordon and Roland (1997) who found that decreasing the dietary NPP level from 0.5-0.2% gave no adverse effect on laying performance, from 21-38 weeks of age and concluded that the supplementation of these diets with microbial phytase gave no further improvement in laying hens performance. Similar results were also obtained by Punna and Roland (1999), who found that reduction of available P in laying hen diets from 0.4-0.2% had no effect on feed intake, egg production or egg weight during an experimental period from 21-48 weeks of age. In addition, Hussein *et al*. (2006) reported that neither dietary NPP nor MP supplementation had a positive effect on laying performance of Mamourah laying hens.

The present results, however, harmonize also with those of Carlos and Edwards (1998), who observed a significant increase in egg production of laying hens when phytase (Natuphos) was added to an available phosphorus-deficient diet. In accordance with the present results, Roland *et al*. (2003) reported that decreasing the dietary available phosphorus level from 0.4-0.1% reduced egg production, egg weight and feed consumption of laying hens. They also found that adding nongenetically modified phytase to a phosphorus-deficient diet alleviated the drop in egg production and egg weight. The current results are also in line with the finding of Liebert *et al*. (2005) that microbial phytase in low-P corn-soybean meal diet significantly improved feed conversion ratio of laying hens. Similar results were also obtained by Nezhad *et al*. (2007), who found that microbial phytase supplementation to low available-P diets significantly improved egg production and restored it to a level similar to that of the positive control laying hens. In addition, Ahmadi *et al*. (2008) indicated that dietary phytase supplementation positively affected egg production, egg weight and feed intake of laying hens. Moreover, Lucky *et al*. (2014) demonstrated that dietary phytase supplementation significantly improved egg production and feed conversion of aged laying hens.

On the other hand, the lack of positive effect of CA on the productive performance of laying hens, observed herein, concurs with the results of Boling *et al*. (2000b), who failed to detect a beneficial effect of CA on the utilization of dietary phosphorus by laying hens fed a corn-soybean meal diet containing 3.8% calcium. The present results agree also with the findings of Nezhad *et al*. (2007) and Vargas-Rodriguez *et al*. (2015), who found no effect of CA on the productive performance of laying hens. However, Al-Sharafat *et al*. (2009) found that feed conversion ratio of laying hens was improved due to dietary CA supplementation. Working with broiler chickens, Rafacz-Livingston *et al*. (2005) indicated that CA caused a significant improvement in weight gain and tibia ash of birds. Similarly, Chowdhury *et al*. (2009) reported that dietary supplementation with CA exerted positive effects on growth, feed intake and feed efficiency. In addition, Nourmohammadi and Afzali (2013) found that inclusion of CA in the diet of broiler chickens caused a positive effect on the morphology of small intestines; the intestinal epithelial thickness was significantly decreased which can facilitate the transport of nutrients across the enterocytes.

**Egg quality of laying hens:** Data on egg quality parameters of 32 weeks old Dokki-4 laying hens fed experimental diets containing three levels of nonphytate phosphorus (NPP) in absence or presence of Microbial Phytase (MP), Citric Acid (CA) or both, are presented in Table 3.

These results indicate that when dietary NPP level was reduced from 0.4-0.3% only percent egg shell ($p<0.05$) and shell thickness ($p<0.01$) were negatively affected but had no effect on the other

Table 3: Egg quality parameters of 32 weeks old Dokki-4 laying hens fed experimental diets containing three levels of nonphytate phosphorus in absence or presence of microbial phytase, citric acid or both

| Dietary treatments | Egg weight (g) | Egg components (%) Shell | Yolk | Albumen | Yolk index (%) | HU[¶] | Shell thickness (mm) |
|---|---|---|---|---|---|---|---|
| 0.4% NPP | $47.99^{bc}$ | $10.25^{abc}$ | $32.24^{f}$ | $57.51^{a}$ | $46.35^{ab}$ | 77.33 | $0.359^{abc}$ |
| 0.3% NPP | $47.63^{cd}$ | $10.08^{bc}$ | $32.88^{ef}$ | $57.04^{a}$ | $45.60^{ab}$ | 77.37 | $0.340^{ef}$ |
| 0.2% NPP | $47.14^{d}$ | $9.55^{d}$ | $33.18^{e}$ | $57.28^{a}$ | $43.86^{e}$ | 77.50 | $0.334^{f}$ |
| 0.4% NPP+0.05% MP | $47.77^{c}$ | $10.40^{ab}$ | $35.78^{bc}$ | $53.82^{cd}$ | $46.86^{a}$ | 77.26 | $0.362^{a}$ |
| 0.3% NPP+0.05% MP | $47.73^{c}$ | $10.37^{ab}$ | $35.16^{cd}$ | $54.47^{bc}$ | $46.26^{ab}$ | 78.06 | $0.360^{ab}$ |
| 0.2% NPP+0.05% MP | $47.96^{bc}$ | $9.92^{cd}$ | $35.34^{cd}$ | $54.73^{b}$ | $45.95^{ab}$ | 77.62 | $0.346^{de}$ |
| 0.4% NPP+2.0% CA | $48.42^{ab}$ | $10.22^{abc}$ | $36.49^{b}$ | $53.28^{d}$ | $45.81^{ab}$ | 77.31 | $0.359^{abc}$ |
| 0.3% NPP+2.0% CA | $47.98^{bc}$ | $10.22^{abc}$ | $34.97^{d}$ | $54.81^{b}$ | $45.26^{bc}$ | 77.35 | $0.352^{bcd}$ |
| 0.2% NPP+2.0% CA | $47.48^{cd}$ | $10.15^{abc}$ | $32.98^{e}$ | $56.87^{a}$ | $44.12^{cd}$ | 77.01 | $0.339^{ef}$ |
| 0.4% NPP+0.05% MP+2.0% CA | $48.80^{a}$ | $10.47^{ab}$ | $37.27^{a}$ | $52.25^{e}$ | $46.24^{ab}$ | 77.11 | $0.352^{abcd}$ |
| 0.3% NPP+0.05% MP+2.0% CA | $48.10^{bc}$ | $10.35^{a}$ | $36.42^{b}$ | $53.22^{d}$ | $45.90^{ab}$ | 77.61 | $0.350^{abcd}$ |
| 0.2% NPP+0.05% MP+2.0% CA | $48.56^{ab}$ | $9.88^{cd}$ | $36.57^{b}$ | $53.54^{cd}$ | $46.14^{ab}$ | 77.68 | $0.349^{cde}$ |
| Pooled SEM | 0.066 | 0.039 | 0.069 | 0.087 | 0.141 | 0.072 | 0.001 |
| Significance | ** | ** | ** | ** | ** | ns | ** |

[a-e]Means in each column bearing different superscripts differ significantly ($p \le 0.05$), HU: Haugh units, NPP: Nonphytate phosphorus, MP: Microbial phytase, CA: Citric acid

traits of egg quality examined. With the level of 0.2% NPP significant reductions ($p<0.01$) were observed in egg weight, percent egg shell, yolk index and shell thickness but percent egg yolk was slightly increased while percent egg albumen and Haugh units were not affected. Dietary supplementation with MP produced improvements in percentages of egg shell ($p<0.05$) and egg yolk ($p<0.01$) but decreased egg albumen while Haugh units was not affected. Addition of MP, applied in the present study, also corrected the negative impact of feeding the lowest NPP-diet on egg weight and shell thickness. Dietary addition of CA had a positive effect on the percentages of egg shell and egg yolk and yolk index for hens fed the lowest NPP-diet as compared to those of hens fed the negative control diet. Further improvements were observed in egg weight, percent egg yolk, yolk index and shell thickness in response to feeding the diets supplemented with CA plus MP.

The observed positive effects of MP on egg weight and shell thickness, particularly for hens fed the lowest NPP-diet might have been attributed to an increased degradation of phytate P and improved Ca and P utilization by laying hens, since they may need higher requirements of these two minerals during the early phase of egg production cycle. The present findings are in harmony with the results obtained by Hussein *et al.* (2006), who found that supplemental MP significantly improved eggshell quality. Similar results were obtained by Ahmadi *et al.* (2008), who reported that dietary phytase supplementation improved shell weight and shell thickness but egg weight was not affected. In line also with the present results, Lim *et al.* (2003) observed that phytase supplementation decreased the percentage of broken and soft-shell eggs. In contrast to the present results, Panda *et al.* (2005) stated that neither MP supplementation nor decreasing NPP level (from 0.30-0.18%) in laying hen diets had a significant effect on egg quality traits (egg weight, Haugh units, shell weight, shell thickness, shell strength or egg specific gravity). Other investigators also failed to find positive effects of MP on percentages of egg yolk and egg albumen (Mohammed *et al.*, 2010), shell thickness, egg specific gravity, yolk index and Haugh units (Afsari *et al.*, 2013), eggshell quality (Musilova *et al.*, 2014) or on all egg quality traits investigated (Lucky *et al.*, 2014). Inconsistencies exist in the scientific literature regarding the responses of laying hens to dietary phytase supplementation might have been due to multi-factors such as age and strain of birds, duration of experiment, dietary levels of calcium and NPP, feed ingredients, source and level of phytase used and differences in the experimental protocols.

The positive effect of CA supplementation on the percentages of egg shell and egg yolk and yolk index for hens fed the lowest NPP-diet as compared to those of hens fed the negative control diet, reported in this study, harmonizes with the findings of Soltan (2008), who found that dietary supplementation with organic acids improved eggshell quality and yolk index of laying hens. It is reasonable to attribute such a positive effect of CA to an increased bioavailability and utilization of nutrients. In this regard, Vargas-Rodriguez *et al*. (2015) reported that dietary supplementation with CA increased the digestibility of Ca, P and N and reduced their excretion in laying hens. Another possible reason is that added dietary CA perhaps could modify the microbial balance within the gastrointestinal tract in favor of the host laying hens. But Boling *et al*. (2000b) found that CA did not improve the utilization of dietary phosphorus of laying hens.

**Apparent mineral retention in cockerels:** Results on apparent mineral retention of 43 weeks old Dokki-4 cockerels fed experimental diets containing three levels of nonphytate phosphorus (NPP) in absence or presence of Microbial Phytase (MP), Citric Acid (CA) or both, are introduced in Table 4. These results clearly indicate that dietary treatments had significant effects ($p<0.01$) on the apparent retention of P, Cu and Zn in Dokki-4 cockerels but retention of Ca and Mg was not affected. It was observed that decreasing dietary NPP from 0.4-0.2% led to a significant increase in P retention in cockerels and but retention of Cu and Zn was negatively affected (Table 4). Dietary MP supplementation positively affected the retention of P and Zn in cockerels whatever was the dietary NPP level. When cockerels were fed diets containing 0.3% NPP plus MP their Cu retention was significantly higher than that of the unsupplemented birds. Feeding the lowest level of NPP plus MP to cockerels resulted in significantly lower Cu retention as compared to that of the unsupplemented ones. The above results also indicate that dietary CA supplementation exerted a beneficial effect on the retention of P and Zn in Dokki-4 cockerels, particularly with the higher dietary NPP levels (0.3 and 0.4%) but values of Cu retention were inconsistent. The combined addition of MP and CA to layer diets containing graded levels of NPP could partially alleviate the adverse effect of feeding the lowest NPP diet without any supplement or with MP or CA alone on the retention of copper and zinc.

The reason for the increased retention of P in cockerels fed the lowest level of NPP (0.2%), observed in the present study as compared to their positive control counterparts, is not clear but

Table 4: Apparent mineral retention of 43 weeks old Dokki-4 cockerels fed experimental diets containing three levels of nonphytate phosphorus in absence or presence of microbial phytase, citric acid or both

| Dietary treatments | Ca (%) | P (%) | Mg (%) | Cu (%) | Zn (%) |
|---|---|---|---|---|---|
| 0.4% NPP | 56.33 | $52.71^{d}$ | 33.88 | $64.74^{ab}$ | $21.33^{bc}$ |
| 0.3% NPP | 55.40 | $53.20^{bc}$ | 34.00 | $62.50^{ab}$ | $20.00^{cd}$ |
| 0.2% NPP | 56.00 | $54.00^{bc}$ | 33.00 | $60.48^{bc}$ | $18.85^{d}$ |
| 0.4% NPP+0.05% MP | 55.60 | $55.00^{ab}$ | 33.60 | $63.80^{ab}$ | $22.50^{ab}$ |
| 0.3% NPP+0.05% MP | 56.17 | $55.00^{ab}$ | 35.13 | $66.21^{a}$ | $22.87^{ab}$ |
| 0.2% NPP+0.05% MP | 55.01 | $55.30^{ab}$ | 34.05 | $57.63^{c}$ | $20.00^{cd}$ |
| 0.4% NPP+2.0% CA | 56.00 | $55.10^{ab}$ | 36.00 | $64.00^{ab}$ | $22.20^{ab}$ |
| 0.3% NPP+2.0% CA | 54.80 | $55.30^{ab}$ | 34.00 | $65.00^{ab}$ | $23.40^{a}$ |
| 0.2% NPP+2.0% CA | 55.00 | $54.80^{abc}$ | 35.00 | $56.80^{c}$ | $19.16^{d}$ |
| 0.4% NPP+0.05% MP+2.0% CA | 56.00 | $55.20^{ab}$ | 35.80 | $63.00^{ab}$ | $21.90^{ab}$ |
| 0.3% NPP+0.05% MP+2.0% CA | 55.20 | $56.50^{a}$ | 36.00 | $66.50^{a}$ | $23.50^{a}$ |
| 0.2% NPP+0.05% MP+2.0% CA | 54.95 | $55.00^{ab}$ | 34.76 | $60.80^{bc}$ | $20.00^{cd}$ |
| Pooled SEM | 0.347 | 0.410 | 0.458 | 0.802 | 0.523 |
| Significance | ns | * | ns | ** | ** |

[d]Means in each column bearing different superscripts differ significantly ($p\leq 0.05$), **: $p<0.01$, NPP: Nonphytate phosphorus, MP: Microbial phytase, CA: Citric acid

might be due to an enhanced P digestibility and absorbability or to decreased excretion of P. In line with this suggestion, Godwin *et al.* (2005) found that decreased dietary level of available P resulted in decreased total fecal P and water-soluble fecal P in turkey breeder hens. In agreement with the present result, Brenes *et al.* (2003) found that decreasing available P content in the diet led to a significant increase in the retention of Ca, P and Mg but decreased Zn retention in broiler chickens. However, Lim *et al.* (2003) reported that high NPP increased retention of P and Fe by laying hens.

The negative effect of reducing dietary NPP level on apparent retention of Cu and Zn, reported herein, may be linked to reduced digestibility or increased excretion of both minerals. The positive effect of MP on the apparent retention of P, Zn and Cu, achieved by cockerels in the present study, harmonizes with the findings of Um and Paik (1999) that retention of dry matter, ash, Ca, Mg, Fe and Zn were significantly greater but excretion of ash, P and Cu were lesser in the phytase-supplemented laying hens than their control counterparts. Hughes *et al.* (2009) reported that phytate digestibility in laying hens was significantly increased due to addition of Quantum phytase to low NPP diet. In addition, Abudabos (2012) also reported an improvement in phytate P utilization in laying hens in response to dietary phytase supplementation. With broiler chickens, Zanini and Sazzad (1999) found that phytase supplementation significantly improved the utilization of N, P, Ca and Zn and significantly reduced the amount of P in the excreta. In a later study conducted by Woyengo *et al.* (2010), dietary supplementation with phytase caused an improvement in ileal digestibility of P from 29.5-43%. Also, exogenous phytase supplementation has been reported to improve P retention in broiler chicks (Deepa *et al.*, 2011). Recently, Bougouin *et al.* (2014) demonstrated that phytase supplementation had a significant positive effect on P-retention in both broilers and laying hens. On the other hand, Nourmohammadi and Afzali (2013) found that dietary phytase supplementation produced a positive impact on morphometery of small intestine, toward facilitating the nutrient absorption and reducing the metabolic demands of the intestinal tract in broiler chickens.

The beneficial impact of CA supplementation on the apparent retention of P and Zn in cockerels, observed in this study, is in accordance with the results of Vargas-Rodriguez *et al.* (2015), who demonstrated that CA supplementation increased the digestibility of P, Ca and N and decreased their excretion rates in laying hens. The present results also agree with those obtained by Brenes *et al.* (2003), who reported that dietary CA addition increased Ca, P and Zn retention in broiler chicks. Also, Demirel *et al.* (2012) reported that P retention as a percentage of P intake increased significantly with addition of CA to diet of broiler chickens.

**Tibia bone mineralization of laying hens:** Results on tibia ash and mineral contents of 44 weeks old Dokki-4 laying hens fed experimental diets containing three levels of nonphytate phosphorus (NPP) in absence or presence of Microbial Phytase (MP), Citric Acid (CA) or both are given in Table 5.

These results indicate that tibia bone ash percentage was significantly decreased ($p<0.01$) due to decreasing the level of NPP from 0.4-0.2% but tibia contents of Mg and Zn were not affected. Addition of MP to the lowest NPP diet significantly improved ($p<0.01$) tibia ash percentage of laying hens but negatively affected tibia bone contents of Ca, P and Cu. However, tibia bone mineralization of laying hens did not respond to MP addition in the diets containing 0.3 or 0.4% NPP. Dietary CA supplementation exerted no positive effect on tibia ash percentage and adversely affected the tibia bone contents of Ca, P and Cu while tibia contents of Mg and Zn were not affected.

Table 5: Tibia ash and mineral contents of 44 weeks old Dokki-4 laying hens fed experimental diets containing three levels of nonphytate phosphorus in absence or presence of microbial phytase, citric acid or both

| | | Mineral contents of tibia ash | | | | |
|---|---|---|---|---|---|---|
| Dietary treatments | Tibia ash (%) | Ca (%) | P (%) | Mg (%) | Cu ($\mu g\ g^{-1}$) | Zn ($\mu g\ g^{-1}$) |
| 0.4% NPP | 63.91$^{ab}$ | 31.80$^{a}$ | 15.00$^{ab}$ | 0.565 | 8.09$^{a}$ | 467 |
| 0.3% NPP | 63.00$^{abc}$ | 30.76$^{ab}$ | 14.70$^{ab}$ | 0.562 | 8.00$^{ab}$ | 469 |
| 0.2% NPP | 60.76$^{d}$ | 30.80$^{ab}$ | 14.50$^{ab}$ | 0.566 | 7.90$^{ab}$ | 463 |
| 0.4% NPP+0.05% MP | 64.10$^{ab}$ | 31.73$^{a}$ | 14.85$^{ab}$ | 0.650 | 7.66$^{abc}$ | 483 |
| 0.3% NPP+0.05% MP | 63.21$^{ab}$ | 31.65$^{a}$ | 14.54$^{ab}$ | 0.643 | 8.00$^{ab}$ | 485 |
| 0.2% NPP+0.05% MP | 62.86$^{abcd}$ | 28.95$^{bc}$ | 12.35$^{c}$ | 0.593 | 6.64$^{bc}$ | 474 |
| 0.4% NPP+2.0% CA | 64.00$^{ab}$ | 32.00$^{a}$ | 15.00$^{ab}$ | 0.580 | 7.50$^{abc}$ | 480 |
| 0.3% NPP+2.0% CA | 63.52$^{ab}$ | 31.70$^{a}$ | 14.60$^{ab}$ | 0.560 | 8.05$^{ab}$ | 477 |
| 0.2% NPP+2.0% CA | 61.00$^{cd}$ | 28.00$^{c}$ | 12.30$^{c}$ | 0.570 | 6.50$^{c}$ | 470 |
| 0.4% NPP+0.05% MP+2.0% CA | 64.60$^{a}$ | 32.00$^{a}$ | 15.60$^{a}$ | 0.580 | 8.00$^{ab}$ | 496 |
| 0.3% NPP+0.05% MP+2.0% CA | 63.50$^{ab}$ | 31.80$^{a}$ | 15.08$^{ab}$ | 0.590 | 7.00$^{abc}$ | 480 |
| 0.2% NPP+0.05% MP+2.0% CA | 62.17$^{bcd}$ | 30.70$^{ab}$ | 13.45$^{bc}$ | 0.586 | 6.85$^{abc}$ | 475 |
| Pooled SEM | 0.751 | 0.412 | 0.264 | 0.013 | 0.201 | 3.768 |
| Significance | ** | ** | ** | ns | ** | ns |

$^{a-d}$Means in each column bearing different superscripts differ significantly ($p \leq 0.05$), NPP: Nonphytate phosphorus, MP: Microbial phytase, CA: Citric acid

The combined addition of MP and CA slightly improved tibia ash percentage of laying hens fed the lowest level of NPP but negatively affected tibia bone contents of P and Cu while tibia contents of Ca, Mg and Zn were not affected. Dietary supplementation with MP plus CA failed to exert a positive effect on tibia bone mineralization of laying hens fed the higher levels of NPP (0.3 and 0.4%).

The negative effect of reducing dietary NPP level on tibia bone mineralization, reported herein, is in harmony with the results of Brenes *et al.* (2003), who reported that decreasing dietary available P content caused a reduction in tibia contents of ash and Zn in broiler chickens. On the contrary, Keshavarz (2000) reported that tibia ash of laying hens was not influenced by dietary NPP levels (0.15, 0.20, 0.25, 0.30, 0.35 or 0.40%).

The positive effect for the addition MP to the lowest NPP diet on tibia ash percentage of laying hens is consistent with the findings obtained by Musapuor *et al.* (2005), who found that dietary phytase supplementation caused a significant increase in tibia ash percentage and tibia P in laying hens. In addition, Hughes *et al.* (2009) observed significantly higher tibia bone ash percentage in laying hens in response to feeding low NPP diet plus microbial phytase. Similarly, phytate P utilization significantly increased due to dietary phytase supplementation in laying hens, as reported by Abudabos (2012). Similar results were also achieved by Brenes *et al.* (2003), who reported that phytase supplementation increased tibia bone ash and tibia contents of Ca, P and Zn in broiler chickens.

The lack of positive effect for CA addition on tibia bone ash percentage of laying hens in the present study is in agreement with the finding of Boling *et al.* (2000b) that citric acid did not improve the utilization of dietary P in laying hens fed a corn-SBM diet containing 3.8% Ca. In contrast to the present results, Al-Sharafat *et al.* (2009) observed that dietary CA supplementation improved tibia bone phosphorus content of laying hens. In this regard, Nezhad *et al.* (2007) concluded that CA could not enhance phytase effectiveness in laying hen, probably due to high level of calcium in laying hens diets. Similarly, Demirel *et al.* (2012) found a significant interaction of phytase and citric acid supplementation with tibia ash percentage, in which the percent tibia ash increased due to the combined addition of CA and MP.

## CONCLUSION

Results of this study indicate that microbial phytase can effectively prevent the reductions in performance and egg quality but either MP or citric acid may partially alleviate the depression of mineral utilization in birds fed the low NPP diets, without a synergistic positive effect for their combination.

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