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Effect of citric acid and microbial phytase on serum enzyme activities and plasma minerals retention in broiler chicks

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An experiment was conducted to study the effect of microbial phytase supplementation and citric acid in broiler chicks fed corn-soybean meal base diets on enzyme activities and some blood parameters of serum and plasma minerals concentration in Ross 308 strain broilers. The data was analysed using a randomized complete block design with factorial arrangement of 3×3, three levels of citric acids (0, 3 and 6%) and three levels of phytase (0, 500 and 1000 enzyme unit per kg). There were three replicates for each treatment that total to 270 chicks for the whole experiment. The results indicated that addition of citric acid to diets caused significant decrease in alkaline phosphatase (P<0.05), lactate dehydrogenase (P<0.01) activities, cholesterol (P<0.05) and plasma phosphorus (P) (P<0.01) and Fe (P<0.05) concentrations. Microbial phytase caused significant decrease (P<0.01) in serum enzyme activities and plasma Fe concentration and significantly increased (P<0.01) aspartate aminotransferase activity, triglyceride and plasma P concentration. Microbial phytase and citric acid could modify some serum enzyme activities and increase the availability and use of minerals for growth and performance improvement of broilers. It is therefore necessary to re-evaluate mineral requirements of broiler chickens when a diet is supplemented with phytase and citric acid.

Key words: Citric acid, microbial phytase, plasma minerals, serum enzyme activity, broilers.

INTRODUCTION

About two-thirds of the total phosphorus (P) in plants, is in the form of phytate (Viveros et al., 2000) and is unavailable to be utilized by humans and monogastric animals. This unavailability is due to the very low phytase activity found in the digestive tract (Pallauf et al., 1994). Therefore, diets of monogastric animals are often supplemented with sources of inorganic P, which increases the cost of the diets and contributes to environmental pollution. Physical methods such as soaking, drying,

germination (Jongbloed et al., 1991), supplementation of diets with microbial phytase (MP) (Kornegey, 2001) and vitamin D (Mitchell and Edwards, 1996b) are effective methods in increasing phytate hydrolysis. Dietary supplementation with MP sources is an effective and practical method to improve phytate. MP hydrolyzes phytate to inositol and inorganic phosphate. Supplementation of diets with MP has been known to increase availability of phytate P and Zn in chicks (Sebastian et al., 1996a; Mohanna and Nys, 1999; Ravindran et al., 2000). MP increases availability and retention of Ca (Sebastian et al., 1996a), improves absorption and retention of Mg, Cu, and Fe (Pallauf et al., 1992; Sebastian et al., 1996a).

Previous research showed that the poultry digestive tract acidity is not desirable for complete hydrolyze or acceptive phytate by MP (Brenes et al., 2003). Citric acid (CA) may change the intestinal pH and improve MP activity, because the MP efficiency is correlated in both

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Abbreviations: ALP, Alkaline phosphatase; **ALT,** alanine aminotransferase; **AST,** aspartate aminotransferase; **LDH,** lactate dehydrogenase; **CA,** citric acid; **MP,** microbial phytase.

acidity and concentration of other free cations. Therefore, it might be used with the properties of chelated organic acids to enable intensification of MP efficiency. Kemme et al. (1998) reported that MP efficiency is related to plant or microbial sources, in hydrolyzed phytate and gut pH term and durability time. Therefore, CA and MP may have synergistic effect. This study was carried out to investigate the effect of supplementing diet with both MP and CA interaction on enzyme activity and some blood concentration of serum and plasma minerals concentration parameters.

MATERIALS AND METHODS

Animals and dietary treatments

A total of 270 feather sexed Ross 308 strain, day old broiler chicks were randomly assigned to 27 groups of 10 chicks per each replicate with similar body weight among groups mean. The experiment was carried out using a randomized complete block design (RCBD) with factorial arrangement of 3×3, three levels of CA (0, 3 and 6%) and three levels of MP (0, 500 and 1000 enzyme unit per kg). There were nine experimental diets, three replicates with ten chicks in each replicate. A basal diet (without MP or CA), was formulated with corn-soybean meal for grower (7 to 21 days) and finisher (22 to 42 day) periods according to the National Research Council (NRC, 1994) recommendations. Diets were provided in the mash form. Broiler chicks were fed with the following diets with equal energy and protein levels: (T1) basal diet, (T2) basal diet + 500 IU/kg of MP, (T3) basal diet + 1000 IU/kg of MP, (T4) basal diet + 3% CA, (T5) basal diet + 3% CA + 500 IU/kg of MP, (T6) basal diet + 3% CA + 1000 IU/kg of MP, (T7) basal diet + 6% CA, (T8) basal diet + 6% CA + 500 IU/kg of MP and (T9) basal diet + 6% CA + 1000 IU/kg of MP. MP was provided by BASF Group, Ludwigshafen, Germany (Natuphos® 10,000, BASF Corp., Mt. Live, Nj) which also had 10,000 active phytase unit per gram and CA supplied as monohydrate CA with 99.5% purity that was provided by Sigma-Aldrich Quimica, Tres Cantos, Spain. Also, MP and CA were in granulated forms.

Ingredient composition and the calculated nutrient composition are given in Table 1. The temperature was regulated at 32 ± 1°C in the first week and reduced by 3°C per week to receive 23°C in the fourth week. Feed and water were provided *ad libitum* and a continuous lighting schedule were used through out the experimental period.

Samples collection and analysis

At the end of the experimental period (42 d of age), two birds per replicate (six chicks per each treatments) were selected randomly and killed by cervical dislocation. Blood samples (approximately 10 ml) were collected in heparinized vacuitainer tube for measurement of plasma minerals concentration (Ca, P, Mg, Fe and Zn), some blood metabolites of serum (urea, cholesterol, triglycerides and total protein), serum enzyme activity of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH). Immediately after collection, tubes were placed in an ice bath and transferred to the laboratory. Plasma was harvested subsequently by centrifuging the whole blood samples at 3000 rpm for 15 min. The heparinized plasma samples were stored at -20°C in Eppendorf tubes and analyzed

subsequently. The experiment was performed by an automated chemistry analyzer of Zest Shimi Kit (Ziest Chem., Diagnostica, and Cat No.10-508. 5256).

Statistical analysis

The data were subjected to an analysis of variance (ANOVA) using the general linear models (GLM) procedures of SAS software (SAS institute, 1990) and the corresponding means were compared by Tukey-Krammer test at P <0.05.

RESULTS

The main effects data indicated that CA caused significant decrease in cholesterol (P < 0.05) but it had no significant effect on urea, triglycerides and total protein of serum concentrations (Figures 1 and 2). MP caused significant increase in cholesterol (P < 0.05) and triglycerides concentrations (P < 0.01), but had no significant effect on urea and total protein concentrations (Figures 3 and 4). The results show that CA significantly decreased enzyme activity of ALP (P < 0.05), also it increased the enzyme activity of lactate dehydrogenase which was shown as increase in enzyme activity of LDH (P < 0.01), but it had no significant effects on activities of AST (Figure 5) and ALT serum enzymes (Figure 6). Supplementation of diets with MP caused a decrease in ALP, ALT and LDH serum activities (P < 0.01) and increased activity of AST (P < 0.01) (Figures 7 and 8). According to the obtained results, CA caused a decrease in plasma P (P < 0.01) and Fe (P < 0.05) concentrations, but had no significant effect on plasma Ca, Zn and Mg concentrations (Figures 9 and 10). MP caused an increase in plasma P (P <0.01) (Figure 11), but was reduced in plasma Fe concentrations (P < 0.01) (Figure 12). However, there was no significant effect on plasma Ca, Mg and Zn concentrations.

DISCUSSION

Adding MP is shown to numerically decrease total protein and at the same time increase cholesterol in the serum of broilers (Mondal et al., 2007). These results are not in agreement with the observations in this study. Similar results to this study were found by Viveros et al. (2002) which assessed the effect of MP on broilers blood parameters and indicated that addition of 500 unit of MP caused numerical increase in total protein concentration. Abdel-Fattah et al. (2008) observed a non significant increase in total protein concentration and lipid metabolites reduction (cholesterol and triglycerides) by addition of different levels of CA (0, 1.5 and 3%). The decrease in serum TP could be due to a decrease in synthesis of protein caused by liver disorders, small intestinal malabsorption or increased loss proteinuria due

Table 1. Ingredients and nutrient composition of the experimental diets.

Ingredient (%)	Grower (7 to 21 days)			Finisher (22 to 42 days)		
	T_1-T_3	T_4-T_6	T_7-T_9	T_1-T_3	T_4-T_6	T_7-T_9
Corn	57.00	51.00	46.00	58.60	54.00	49.10
Soybean meal	33.10	34.40	35.10	30.00	30.30	30.60
Fish meal	3.40	3.20	3.50	3.50	3.80	4.00
Soybean oil	2.00	4.00	5.55	3.50	5.10	6.80
Dicalsium phosphate	1.55	1.57	1.55	1.10	1.10	1.10
Oyster shell	1.03	1.03	1.03	1.18	1.15	1.13
DL-Methoinine	0.01	0.01	0.01	0.01	0.01	0.01
Common salt	0.26	0.26	0.26	0.26	0.26	0.26
Sand	0.65	0.53	0.00	0.85	0.28	0.00
Trace minerals mix[1]	0.50	0.50	0.50	0.50	0.50	0.50
Vitamins mix[2]	0.50	0.50	0.50	0.50	0.50	0.50
Citric acid	0.00	3.00	6.00	0.00	3.00	6.00
Phytase[3]	-/+	-/+	-/+	-/+	-/+	-/+
Total	100.00	100.00	100.00	100.00	100.00	100.00
Nutrient composition (Calculated)						
ME (kcal/kg)	2910	2910	2910	3030	3030	3030
CP	20.10	20.10	20.10	19.00	19.00	19.00
Ca	0.95	0.95	0.95	0.90	0.90	0.90
Total P	1.23	1.23	1.23	1.06	1.06	1.06
nPP	0.45	0.45	0.45	0.36	0.36	0.36
Ca:nPP	2.11	2.11	2.11	2.50	2.50	2.50
Methionine	0.50	0.50	0.50	0.38	0.38	0.38
Lysine	1.10	1.10	1.10	1.00	1.00	1.00
Methionine+cystine	0.83	0.83	0.83	0.71	0.71	0.71

[1]Mineral mix supplied the following per kilogram of diet: Mn, 55 mg; Zn, 50 mg; Fe, 80 mg; Cu, 5 mg; Se, 0.1 mg; I, 0.18 mg.
[2]Vitamins mix supplied following per kg of diet: Vitamin A, 18000 IU; vitamin D_3, 4000 IU; vitamin E, 36 mg; vitamin K_3, 4 mg; vitamin B_{12}, 0.03 mg; thiamine, 1.8 mg; riboflavin, 13.2 mg; pyridoxine, 6 mg; niacin, 60 mg; calcium pantothenate, 20 mg; folic acid, 2 mg; biotin, 0.2 mg; choline chloride, 500 mg. [3]Natuphos 10,000 (BASF Crop., Mt. Olive, NJ) was used as the source of microbial phytase to provide 500 (T_2, T_5 and T_8) and 1000 (T_3, T_6 and T_9) microbial phytase unit per kilogram of diet.

to renal diseases or malnutrition (Zantop, 1997; Kaya and Tuncer, 2009), by organic acids addition to broiler diets that decreased total protein and triglycerides, and increased cholesterol concentration. The present study does not agree with the later observations.

Decrease in serum ALP activity is associated with supplementation of MP that might be reflected from the increase in the availability of phosphorus (Huff et al., 1998). This decrease could also be related to the increase observed from Zn retention. Zn has a specific role in the reactivation of chicken intestinal ALP after acid exposure. Total serum ALP measures several Zn metalloenzymes isoenzymes by cells in a number of organs, such as; liver, bone, muscle, small intestine and kidney (Moss, 1982). Brenes et al. (2003) reported that reduction of phosphorus level in diets, increases ALP enzyme activity. In addition, Roberson and Edwards (1994b) showed that plasma ALP activity was not

affected by MP in broiler chicks. Probably, difference in measuring methods of isoenzymes composition of serum ALP causes, were responsible for differences in the results. Campbell and Coles (1986) reported that the intestinal isoenzyme makes the largest contribution to serum ALP activity of birds. In contrast, Zantop (1997) indicated that increases of serum ALP is most related to liver diseases, even though the level of ALP activity in this organ is low.

Viveros et al. (2002) and Brenes et al. (2003) reported that decreasing P level of diet increased ALP activity, MP and CA through the mechanism mentioned above, facilitate liberation of phytate P and so increase plasma P concentration (as observed in our study) that resulted in decreased ALP activity. Viveros et al. (2002) found that addition of 500 IU/kg MP caused a significant decrease in LDH and ALT enzymes, and also, significant increase in AST activities. Elevated activity is usually an indication of
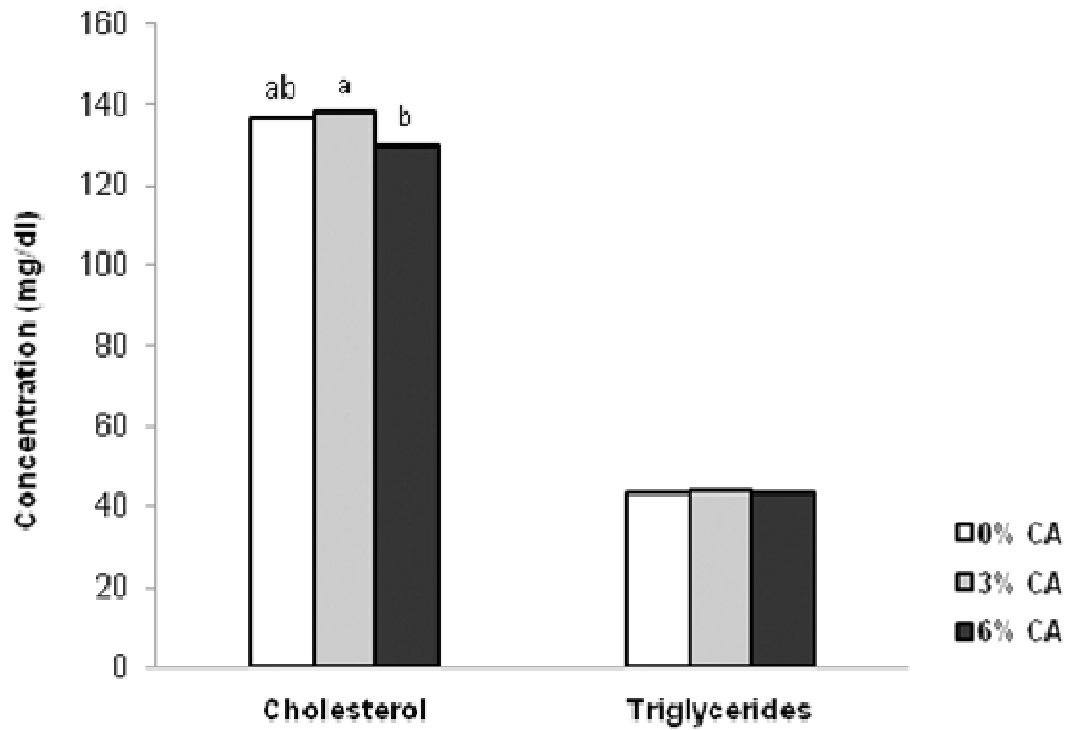
Figure 1. Effect of different levels of citric acid (CA) (0, 3 and 6%) on serum metabolites concentration (cholesterol and triglycerides). Values with different letters (a, b) are different at $P < 0.05$.
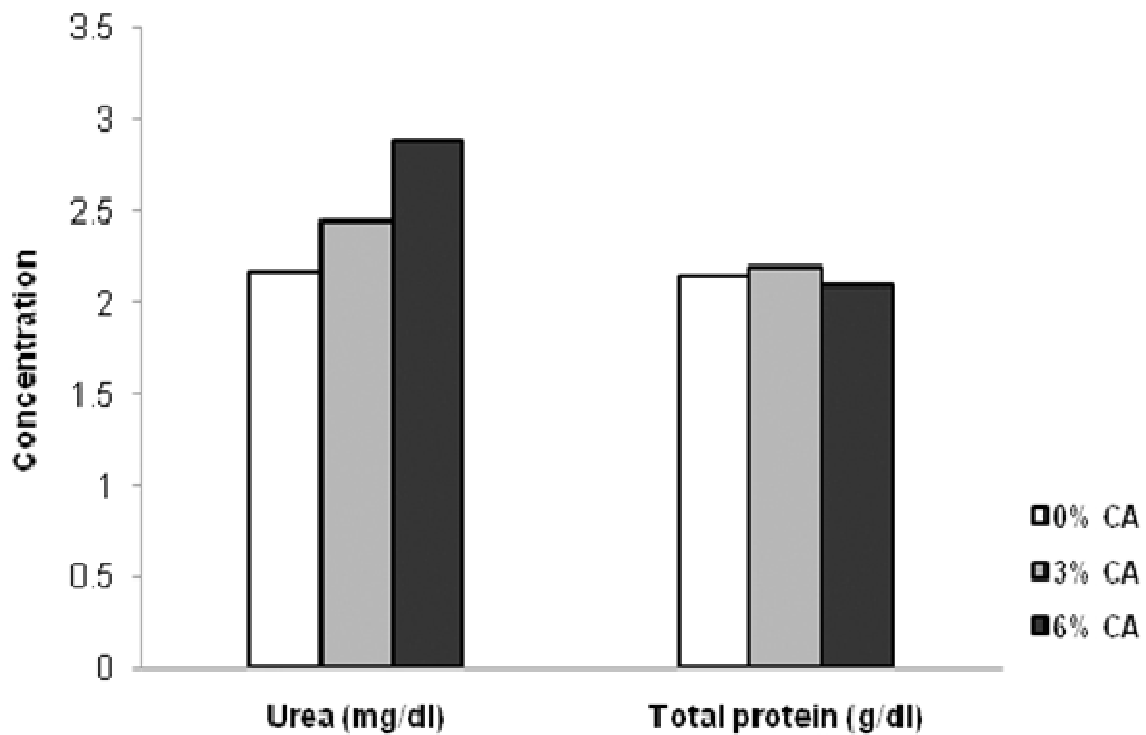

Figure 2. Effect of different levels of citric acid (CA) (0, 3 and 6%) on serum metabolites concentration (urea and total protein).

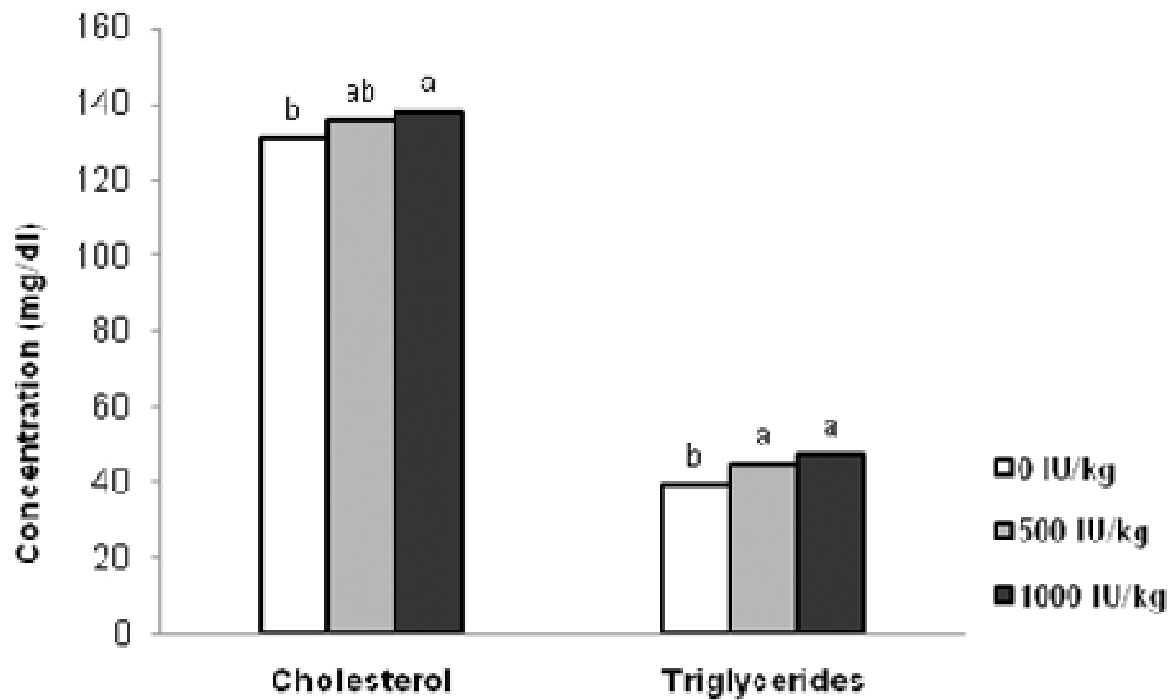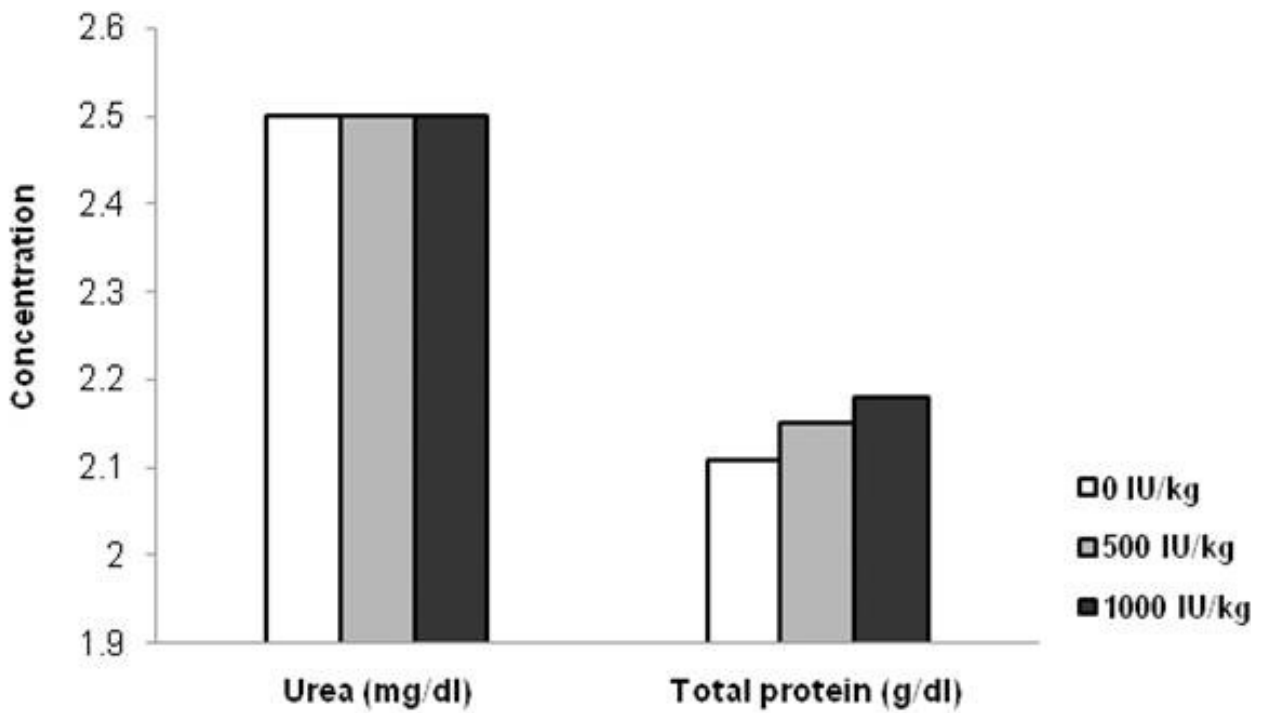**Figure 3.** Effect of different levels of microbial phytase (MP) (0, 500 and 1000 IU/kg) on serum metabolites concentration (cholesterol and triglycerides). Values with different letters (a, b) are different at $P < 0.05$.

Figure 4. Effect of different levels of microbial phytase (MP) (0, 500 and 1000 IU/kg) on serum metabolites concentration (urea and total protein).
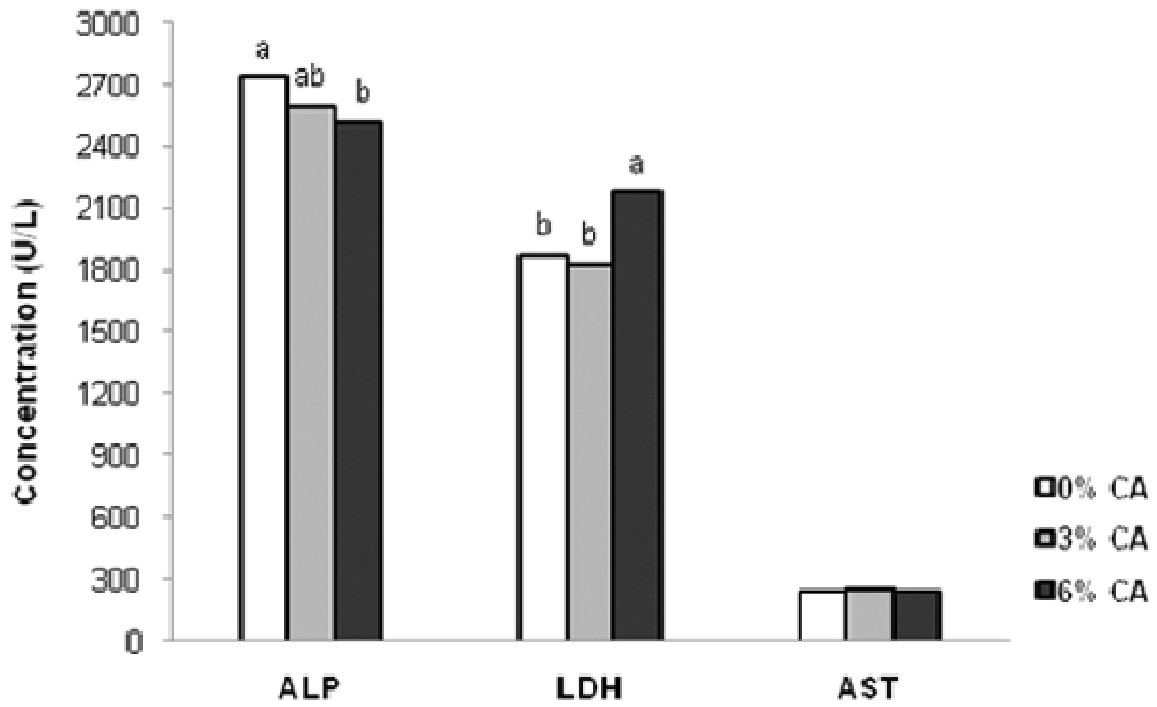
Figure 5. Effect of different levels of citric acid (CA) (0, 3 and 6%) on serum enzyme activity of alkaline ALP, LDH and AST. Values with different letters (a, b) are different at $P < 0.05$.
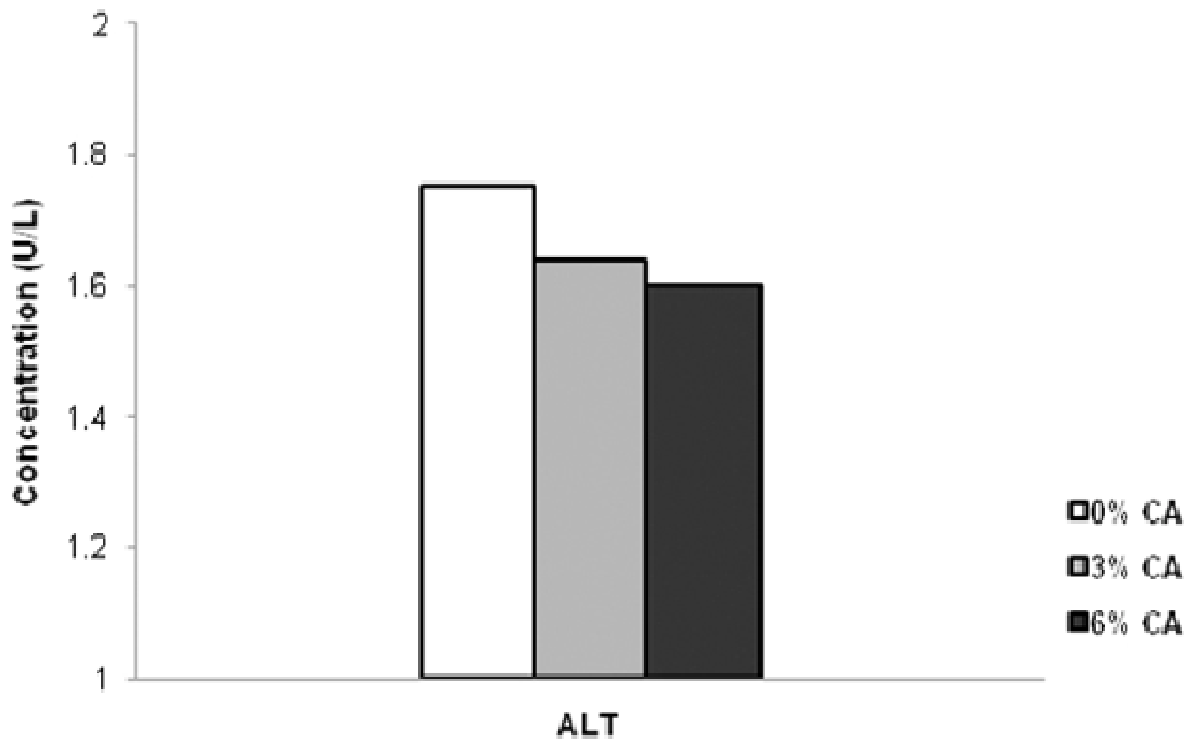

Figure 6. Effect of different levels of citric acid (CA) (0, 3 and 6%) on serum enzyme activity of alanine aminotransferase (ALT).
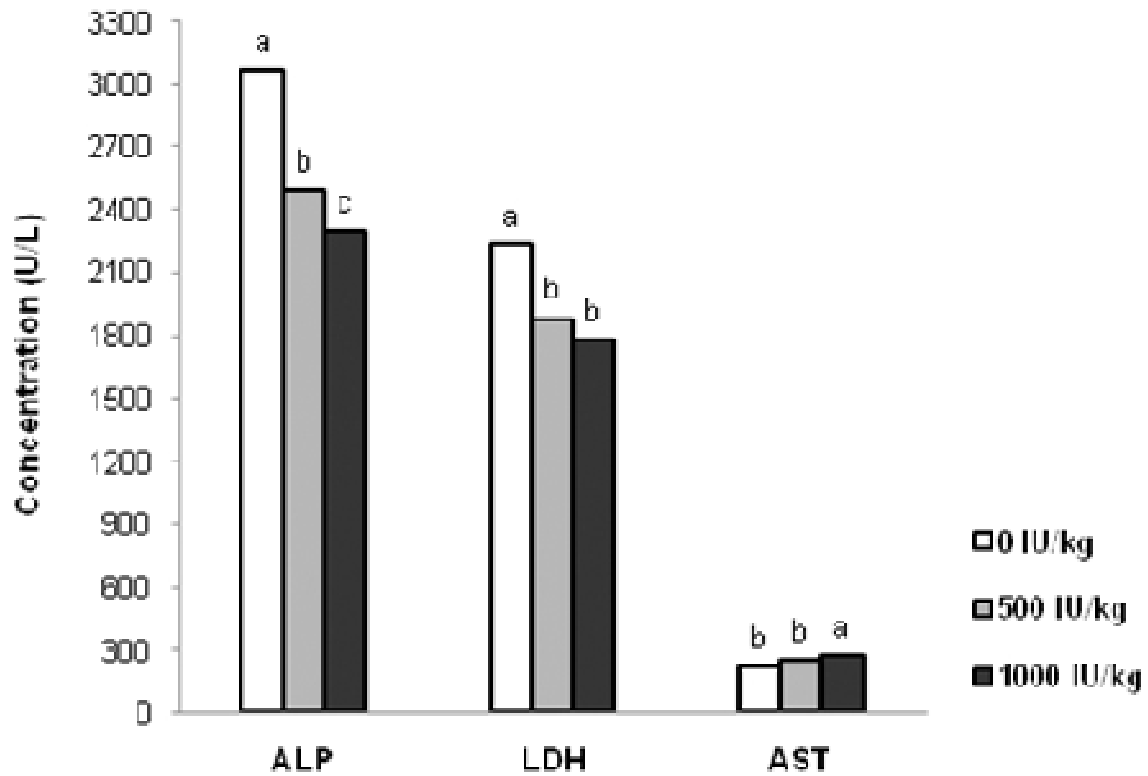
Figure 7. Effect of different levels of microbial phytase (MP) (0, 500 and 1000 IU/kg) on serum enzyme activity of ALP, LDH and (AST. Values with different letters (a-c) are different at P< 0.05.
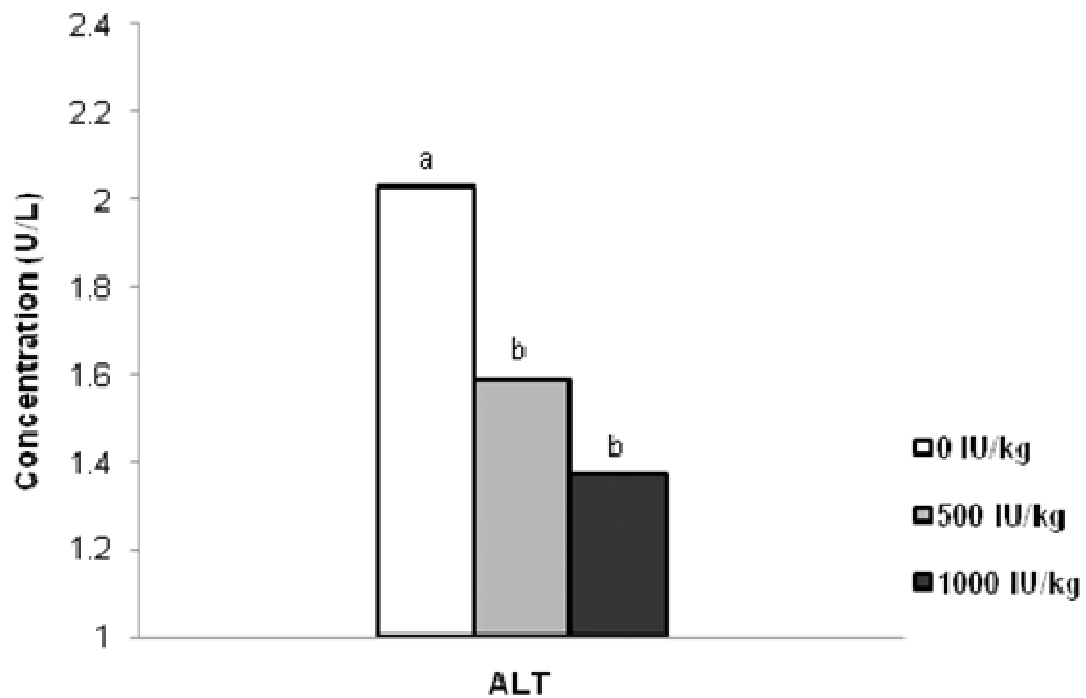

Figure 8. Effect of different levels of microbial phytase (MP) (0, 500 and 1000 IU/kg) on serum enzyme activity of ALT. Values with different letters (a, b) are different at P < 0.05.
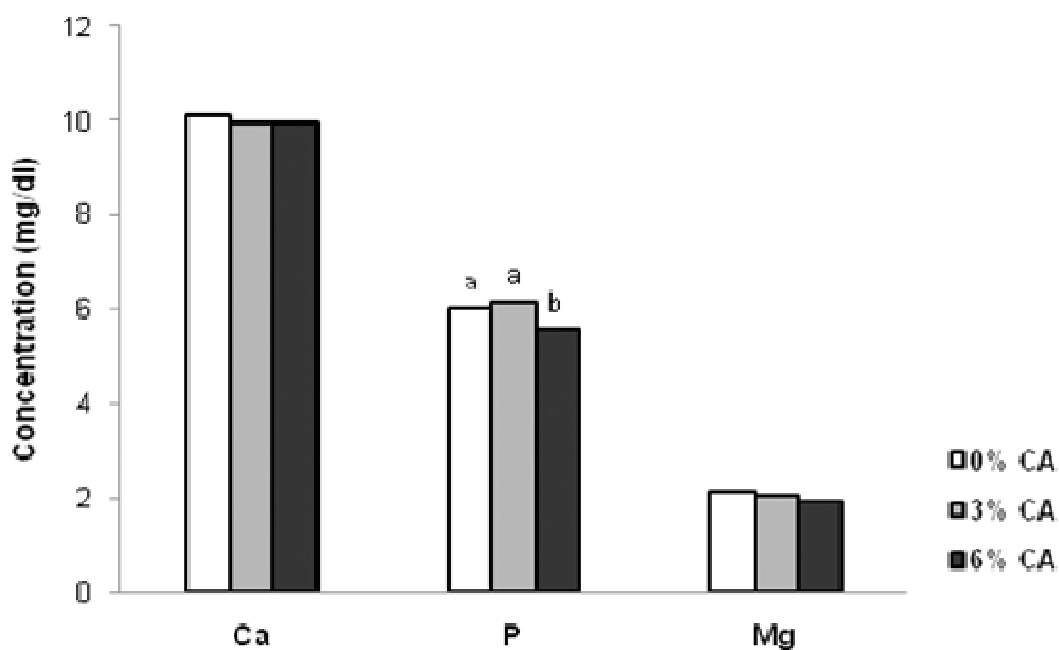
Figure 9. Effect of different levels of citric acid (CA) (0, 3 and 6%) on plasma minerals concentration (Ca, P and Mg). Values with different letters (a, b) are different at P < 0.05.
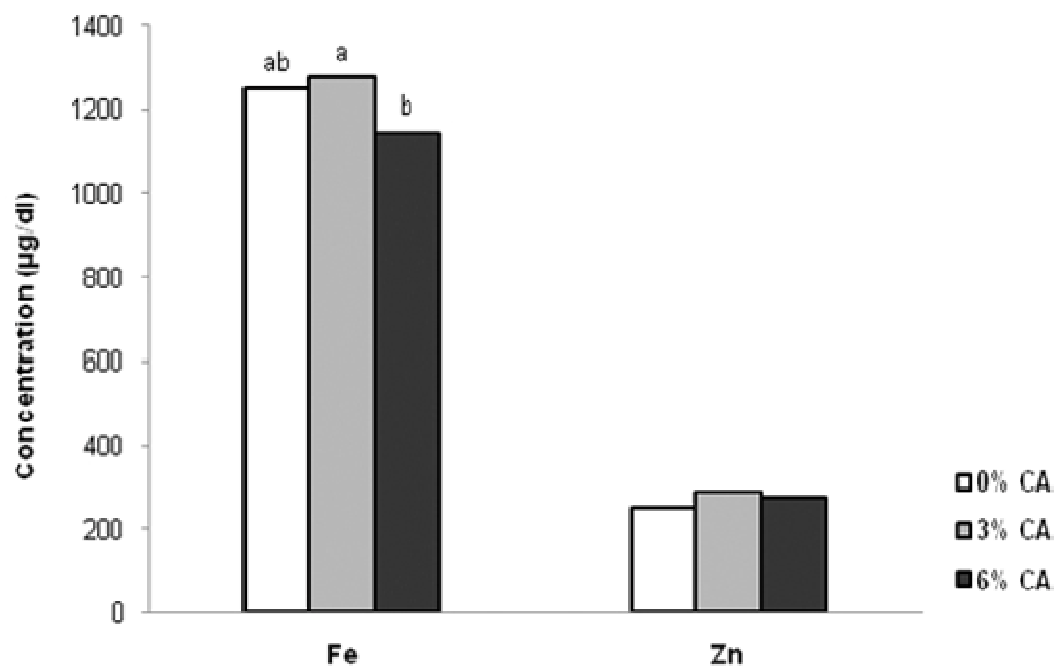

Figure 10. Effect of different levels of citric acid (CA) (0, 3 and 6%) on plasma minerals concentration (Fe and Zn). Values with different letters (a, b) are different at P < 0.05.

liver or muscle damage. Even though, ALT activity has been reported to be low in all tissues of chickens (Bogin and Israeli, 1976), ALT activities often are increased due to damage in many tissues (Zantop, 1997). Therefore, specific diagnostic value of these enzymes in birds is poor. In many cases, birds with severe liver damage have
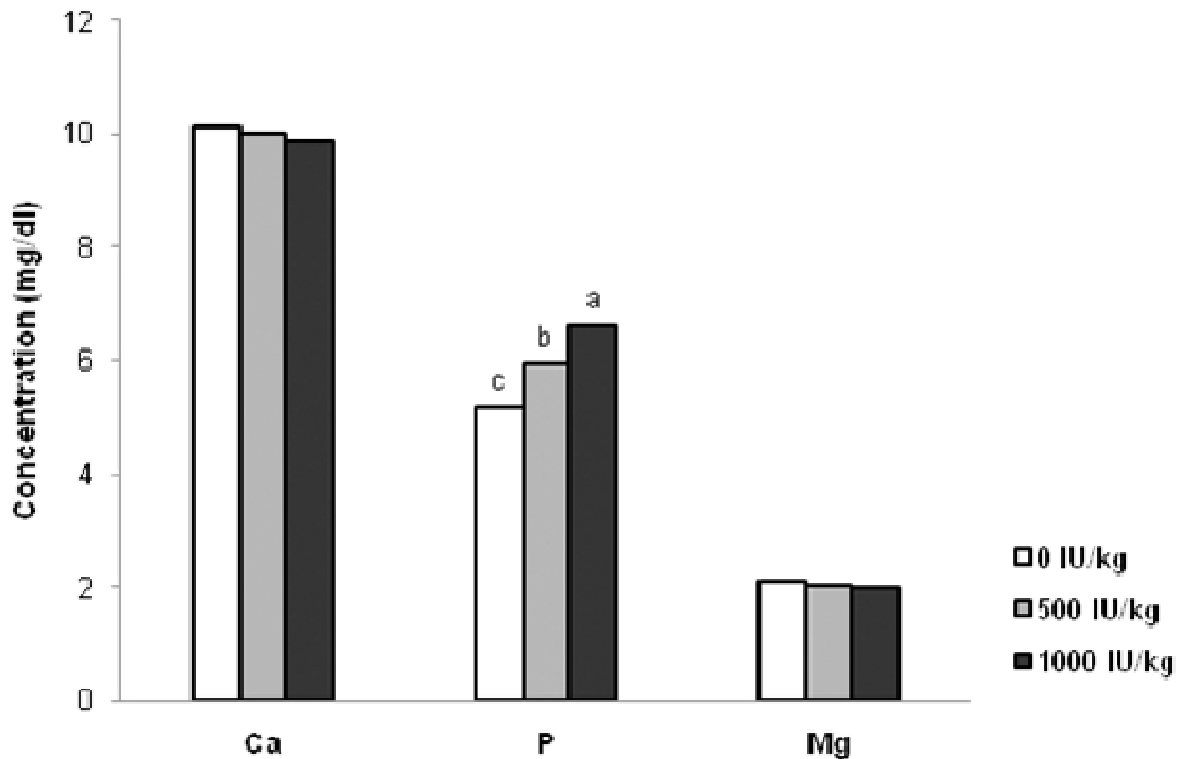
Figure 11. Effect of different levels of microbial phytase (MP) (0, 500 and 1000 IU/kg) on plasma minerals concentration (Ca, P and Mg). Values with different letters (a-c) are different at $P < 0.05$.

normal ALT activities. Moreover, there are five LDH isoenzymes in birds; each occuring in several tissues, including skeletal muscle, cardiac muscle, liver, kidney, bone and red blood cells that is found to decrease in LDH activity which could be related to liver diseases, because this enzyme decreases quickly as the disease progresses (Zantop, 1997). Mondal et al. (2007) showed that MP addition in levels of 250 and 500 IU/kg caused a decrease in AST and increased ALT activities. This study signifies that the birds were apparently healthy throughout the experimental period that could also be correlated to livability.

Viveros et al. (2002) reported that addition of 500 IU/kg MP to diets including different nonphytate phosphorus levels caused significant decrease on plasma Ca concentration, but significantly increased plasma P concentration. As suggested by Taylor and Dacke (1984), the low-P dietscause an ionized elevation of Ca in the plasma which depressed the release of parathyroid hormone. Thus, reducing inhibition of parathyroid hormone on tubular reabsorption of phosphate permits urinary excretion of the addition of absorbed Ca from the gut during feeding of a low-P diet. This effect has also been reported in chickens (Broz et al., 1994; Rama-Rao et al., 1999), ducks (Orban et al., 1999) and turkeys (Atia et al., 2000). Lei et al. (1994) showed that the increase

observed in plasma P was negatively influenced by increasing the dietary Ca : P ratio in pigs. Broz et al. (1994) and Sebastian et al. (1996a) found decreases in plasma Ca concentrations with MP addition which was not true in our study.

Roberson and Edwards (1994a) and Rama-Rao et al. (1999), did not observe MP effect on plasma Ca concentration, whereas Ebrahimnezhad et al. (2008) and Sebastian et al. (1996b) observed increase in plasma P concentration by MP addition. Onyango et al. (2004) showed that supplementation of diet with MP had no significant effect on plasma Ca concentration of broiler chicks blood at 22 day of age but significantly increased plasma P concentration. Also, Mitchell and Edwards (1996a) showed that supplementation of diets with 600 IU/kg of MP did not significantly influenced plasma Ca concentration of broilers at 21 day of age, but caused significant increase in plasma P concentration. All of these results are in agreement with the results of this study. Plasma Ca and P concentrations are controlled by the absorption of digestive tract, by storage and reabsorption by bone, and also by the stool and urinary excretion or reabsorption from renal system, where the body regulates Ca and P equilibrium with impressive vitamin D_3 and hormones such as; Parathormone and Calcitonin in small intestine, kidney and bone
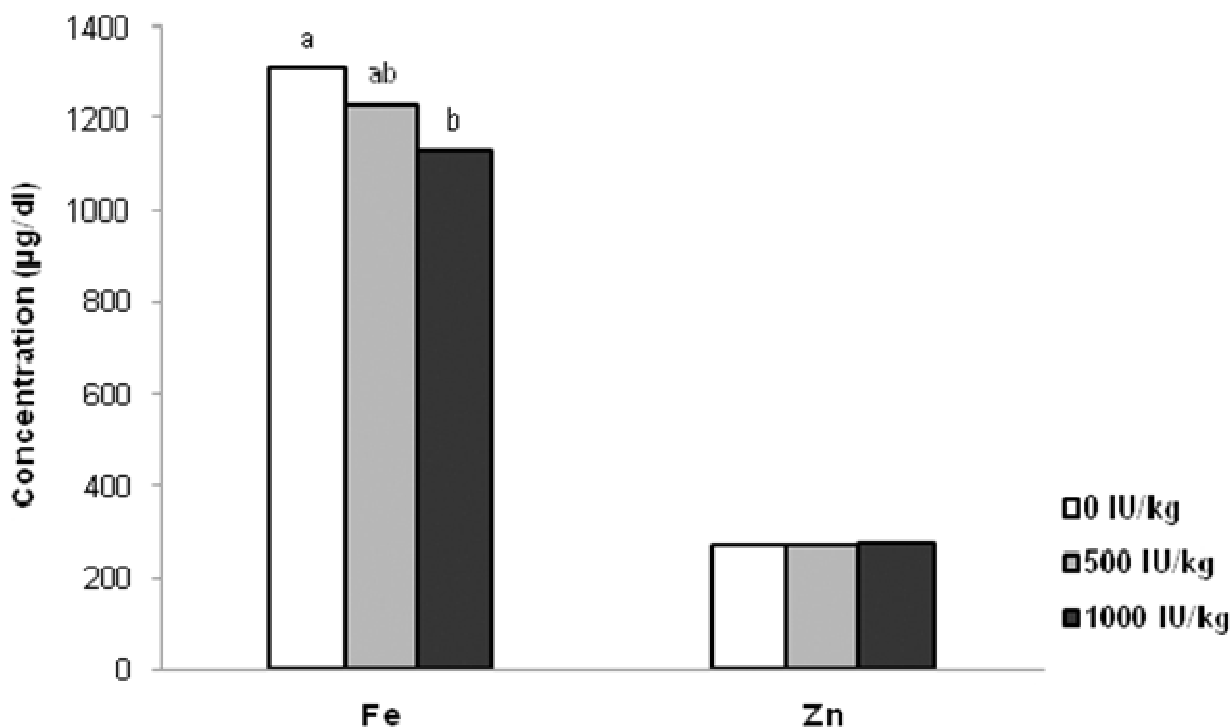
Figure 12. Effect of different levels of microbial phytase (MP) (0, 500 and 1000 IU/kg) on plasma minerals concentration (Fe and Zn). Values with different letters (a, b) are different at P < 0.05.

(Hassanabadi et al., 2007). Nonetheless, the observed mechanism in this study, could not supply enough phosphorus to poise this level of mineral in plasma, during the use of diets with different levels of CA and MP. With addition of MP to broiler diets, available P, and absorption of mineral increased and caused an increase in plasma P level that resulted in subsidiary weight gain and feed conversion ratio in treatments containing MP, better than treatments without enzyme. Abdel-Fattah et al. (2008) found that adding different levels of CA (1.5 and 3%) significantly changed blood Ca and P concentrations and caused an increase in minerals concentrations. The increase in Ca and P levels of blood serum produced by the addition of organic acids may be attributed to the lowering of GI-tract pH by using acids which increases the absorption of such minerals from the gut into the blood stream. Improving the utilization of calcium and phosphorus due to provision of organic acids was approved by Boling et al. (2001).

Also, Zeinb (2004) observed an increase in blood calcium of broiler chicks fed on dietary acidifier. In contrast, Ebrahimnezhad et al. (2008) observed increase in plasma Ca and P concentration by adding different levels of CA (2.5 and 5%). The authors survey the effect of CA on phytate phosphorus utilization and efficiency of MP in laying hen, and found that addition of CA in two levels (2 and 4%) did not have any effect on Ca

concentration. In agreement with these results, Viveros et al. (2002) reported that plasma Mg and Zn concentrations were affected by 500 IU/kg MP additions to diets with different levels of nonphytate phosphorus. It was later found that MP caused a decrease in Mg concentration and an increase in Zn concentration of plasma. Brink et al. (1991) also observed a lack of correlation between Mg retention and plasma Mg concentrations in rats. This lack of response was reported by Roberson and Edwards (1994a) and Sebastian et al. (1996a) in broiler chicks. These authors suggest that adequate Zn in a diet might be responsible for the lack of effect on plasma Zn. Most authors have reported increase in plasma Zn when Zn-deficient diets supplemented with phytase was fed (Pallauf et al., 1994; Mohanna and Nys, 1999). According to Pallauf et al. (1994), failure to observe increases in the circulation of Zn concentrations in conjunction with increases in Zn retention indicate that the maximum plasma Zn concentrations may not be a good indicator of absolute Zn availability.

Based on the results of this research, addition of MP supplementation to the diet could modify some serum enzyme activities and increase the availability and use of minerals for growth and performance improvement of broilers. Moreover, CA decreases serum enzyme activities and increases minerals retention. It is therefore necessary to re-evaluate mineral requirements of broiler

chickens when a diet is supplemented with MP and CA.

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