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Effects of Calcium, Citric Acid, Ascorbic Acid, Vitamin D_3 on the Efficacy of Microbial Phytase in Broiler Starters Fed Wheat-Based Diets I. Performance, Bone Mineralization and Ileal Digestibility

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Abstract: A study was conducted to determine the additive effects of calcium (Ca) levels and the enzyme phytase, organic acids (citric, ascorbic acid), vitamin D_3 on broiler performance and nutrient digestibility (d 1 to 21) in wheat-based diet. Broilers were fed the following diets at either 7.9, or 9 g/kg of dietary Ca with: 1) a negative control wheat-based diet, 3.15 g/kg available phosphorus (P) (NC); 2) NC + 500 units Ronozyme™ P phytase/kg diet; 3) phytase + 20 g/kg citric acid; 4) phytase + citric acid + 200 mg/kg diet ascorbic acid; 5) phytase + citric acid + ascorbic acid + 200 µg/kg diet vitamin D_3; 6) NC plus 1.35 g/kg available P. These 12 diets were supplemented with 50 mg/kg of xylanase (Ronozyme™ Wx, Roche) and fed to four replicates of 20 birds each. Phytase addition at the high Ca level increased body weight (BW), feed intake (FI), and tibia ash. Increased protein and P digestibility at the lower level of Ca and both levels of Ca, respectively was also present. Subsequent addition of citric and ascorbic acid to the low Ca diets increased BW and protein digestibility by 11.1 and 23%, respectively over the values obtained from the control diet. The BW of chicks that received the positive control diet were similar to those that received the phytase, organic acids and vitamin D_3 supplemented low-Ca, low-P diet. The addition of phytase with citric acid, ascorbic acid, and vitamin D_3 to the low Ca control diet improved BW and P digestibility by 18 and 60%, respectively. The data from this study shows that for broiler chickens, at low-P (3.15 g/kg), low-Ca (7.9 g/kg) diets supplemented with microbial phytase, organic acids and vitamin D_3 improved feed conversion ratio (FCR) and gave a similar BW as a Ca- and P- adequate diets. These low P and Ca diets can result in a considerable reduction in the amount of excreted phosphorus and nitrogen.

Key words: Calcium (Ca) levels, enzyme phytase, organic acids

Introduction

The dietary phytase activity suggested for maximum phytate-P utilization varies widely. This obvious source of variation would be compounded by any factors that influence the efficacy of phytase. Dietary levels of both Ca and P may influence the hydrolysis of phytate (Ballam *et al.,* 1984). This factor is probably illustrated by the adverse effects of dicalcium phosphate on the amino acid digestibility response to phytase in broiler chicks (Ravindran *et al.,* 2000). The effect of the calcium (Ca) to total phosphorus (P) ratio on phytate P utilization has been reviewed by Wise (1983). A high calcium level or a Ca to total P ratio of 2:1 resulted in lowered digestion of phytate (Nelson, 1967). Harms *et al.* (1962) demonstrated that narrowing the Ca to P ratio from 2:1 to 1:1 improved the availability of P from phytic acid.

It is widely held that high levels of inorganic Ca (or wide Ca:P values) have a negative influence on the efficacy of phytase. It has been proposed that the poor solubility of Ca-phytate (Wise, 1983) and other mineral-phytate complexes (Maenz *et al.,* 1999) renders phytate resistant to phytase activity. This mineral-phytate complexes are usually formed at a pH that is above, or at the upper end of the activity spectrum of microbial phytase. Thus, the prevailing pH in the gut may have an important influence on the efficacy of phytase. The bi-phasic pH profile of microbial phytase activity (Simons *et al.,* 1990) indicates that subtle changes in pH of the upper digestive tract by addition of organic acids such as citric and ascorbic acids could influence the activity of microbial enzyme. In addition, certain compounds with chelation capacity (such as ascorbic acid and citric acid) have been shown to increase mineral availability when included in plant-based diets fed to animals and may act as chelating agents. Citric acid has been known to be a chelator for Ca (Hastings *et al.,* 1934) and ascorbic acid increases iron (Fe) bioavailability in soy-based infant formulas (Davidsson *et al.,* 1994). As a consequence, the effectiveness of microbial phytase may be enhanced by feeding it in combination with an organic acid.

Cholecalciferol (vitamin D) plays a role in Ca and P absorption, and therefore influences their utilization. The data of Edwards (1993) suggest that vitamin D and dietary phytase activity may function synergistically to enhance phytate phosphorus utilization. Improving the efficiency of phytase could lead to reduce feed costs and to a greater use of phytase which would be of environmental importance.

The overall objectives of this study was to assess the effects of: (1) organic acids on phytase activity in low P diets and (2) the additive effects of phytase and vitamin D_3 under low or high Ca:P ratios in diets supplemented with organic acids fed to broiler chickens. The criteria used to assess the efficacy of phytase were: growth performance, tibia ash, protein and P digestibility.

Materials and Methods

Housing and diets: A total of 960 1-d-old (Ross×Ross) broiler chickens were used in this experiment. All chicks were randomly distributed into 48 floor pens consisting of 20 chicks per pen. Each floor pen contained one-bell shaped waterer, one hand–filled hanging feeders, one brooding light, and approximately 10 cm of dry-wood shaving litter. The temperature was maintained at 32±1°C in the first week and reduced by 3°C per week to 21°C. Lighting was continuous, water and feed were provided *ad-libitum*.

The experimental design consisted of a 6×2 factorial arrangement of dietary treatments with four pen replicates. The experimental diets consisted of 12 wheat-based diets. The basal diets were formulated to contain low available P (3.15 g/kg) (Table 1). With the exceptions of Ca and P content, all treatments were isocaloric, isonitrogenous and formulated to meet or exceed all nutrient requirements according to the National Research Council (1994). Inaccordance, the broilers were fed the following basal diets at either 7.9 or 9 g/kg of dietary Ca: 1) a negative control, wheat-based diet, 3.15 g/kg available P (NC); 2) NC + 500 units Ronozyme™ P-phytase (FYT)/kg diet; 3) NC + 500 FYT/kg diet and, 20 g/kg citric acid; 4) NC + 500 FYT/kg diet, 20 g/kg citric acid, and 200 mg/kg ascorbic acid; 5) NC + 500 FYT/kg diet, 20 g/kg citric acid, 200 mg/kg ascorbic acid, and 200 µg/kg diet vitamin D_3; 6) and a positive control diet, NC + 1.35 g/kg available P (from dicalcium phosphate). Ronozyme™ P is a granulated phytase and a product of Roche corporation (Hoffmann-La Roche Inc. Vitamins and Fine Chemical Division CH-4070 Basel (Switzerland)). One phytase unit is the activity of phytase that generates 1 micromole of inorganic phosphorus per minute from an excess of sodium phytate at pH 5.5 and 37°C .

The concentration of nonphytate phosphorus (NPP) in Diet 6 was calculated to meet the total P content of the test diets. Thus, the experimental diets would have the same nutrient content and the same Ca:NPP ratios as the positive control diet only if total dephosphorylation of feed phytates took place in gastrointestinal tract. To avoid the confounding effect caused by the presence of non-starch polysaccharides in wheat on broiler performance (Ravindran *et al*., 1999), all diets were supplemented with a xylanase (Ronozyme Wx is a granulated heat stable) at levels recommended by the manufacturer (50mg/kg). This product contained 1000 endoxylanase units/g xylanase activity as a main activity (Hoffmann-La Roche Inc. Nutley, NJ 07110-1199). Prior to feeding all of experiment diets were stored in a cool place.

Table 1: Composition and nutrient content of the low-P, negative control, basal diet[1]

Ingredient	(g/kg) of diet
Wheat	560.4
Soybean meal	344.0
Sunflower oil	54.50
Alfalfa meal	10.00
Oyster shell	11.80
DL-methionine	2.0
Salt	3.2
Dicalcium phosphate	9.1
Vitamins and minerals[2]	5.0
Composition	
Metabolizable energy(MJ/kg)	12.42
Crude protein(g/kg)	225
Analyzed CP (N×6.25)(g/kg)	228
Available P(g/kg)	3.15
Total analyzed P(g/kg)	5.00
Ca(g/kg)	7.90
Methionine(g/kg)	4.60
Lysine(g/kg)	11.10

[1]Additionally, the low-P negative control diets that contained 9 g/kg of Ca were formulated from the above diet by increasing oyster shell concentration at the expense of wheat. At each level of Ca, the following experimental diets were formulated: 1) a negative control (NC) diet, 3.15g/kg available P; 2) NC+500 phytase units / kg diet ; 3)NC plus 500 phytase units and 20 g citric per kg diet 4) NC plus 500 phytase units/kg diet, 20 g citric acid /kg diet and 200 mg ascorbic acid /kg diet; 5) NC plus 500 phytase units/kg diet, 20 g citric acid /kg diet, 200 mg ascorbic acid /kg diet and 200 µg vitamin D_3/kg diet; and 6) NC plus 1.25 g/kg available P(from dicalcium phosphate). Dietary additions were added at the expense of wheat and metabolizable energy was adjusted by increasing amounts of vegetable oil. One phytase unit is the activity of phytase that generates 1 micromole of inorganic phosphorus per minute from an excess of sodium phytate at pH 5.5 and 37 degrees Celsius.

[2]Supplied per kilogram of diet: Vitamin A, 9,000 IU; Cholecalciferol, 3,000 IU; vitamin E; 18 IU, vitamin K_3, 2 mg; vitamin B_{12}, 0.015 mg; thiamin, 1.8 mg; riboflavin, 6.6 mg; folic acid, 1 mg; biotin, 0.10; niacin, 35mg; pyridoxine, 4 mg; choline chloride, 250 mg; ethoxyquine, 0.125; manganese sulfate, 100 mg; copper sulfate, 10 mg; selenium (sodium selenate), 0.2 mg; iodine (EEI), 1mg; zinc sulfate, 100 mg; Fe, 50mg.

Table 2: Performance of broilers fed wheat-based diets with different dietary addition (phytase, citric, ascorbic acid and vitamin D_3) and different calcium concentration from 0 to 21 d of age

Supplements to basal diet (3.15 g/kg AP)[1]	Calcium concentration in the diet (g/kg)								
	Body weight (g)[2] 21 d			Feed intake 0-21 d (g/ bird/d)[2]			Feed conversion ratio 0-21 d (g:g)[2]		
	Total	7.9	9.0	Total	7.9	9.0	Total	7.9	9.0
		466	462		33.6	33.8		1.52	1.54
None	416^{d}	425^{ef}	407^{f}	32.0^{b}	33.7^{cde}	30.4^{f}	1.62^{a}	1.66	1.57
PHYT	459^{c}	440de	477^{bc}	33.1^{b}	31.4^{ef}	34.8^{bc}	1.52^{bcd}	1.50	1.54
PHYT+ CA	457^{c}	451^{cde}	463^{cd}	33.4^{b}	32.3^{cdef}	34.5^{bcd}	1.55^{abc}	1.54	1.57
PHYT+ CA+ AA	469^{bc}	472^{bc}	467^{cd}	32.8^{b}	33.2^{cde}	32.4^{cdef}	1.47^{cd}	1.48	1.46
PHYT+CA+AA+VITD$_3$	481^{b}	500^{ab}	461^{cd}	33.1^{b}	34.2^{cd}	32.2^{def}	1.45^{d}	1.44	1.47
1.35 g/kg AP	504^{a}	508^{a}	500^{ab}	37.7^{a}	36.8^{ab}	38.5^{a}	1.58^{ab}	1.53	1.63
Model	P>F			P>F			P>F		
Dietary addition (Da)	**			**			**		
Calcium (Ca)	0.49			0.65			0.54		
Da x Ca	**			**			0.32		

*, ** Indicate significance (P<0.05 and P<0.01, respectively) for main effects and interactions. abcd Different letters beside mean values indicate significant differences between mean values. [1]Supplements were the following: PHYT = phytase, 500 units (FYT)/kg; CA = citric acid, 20 g/kg, AA = ascorbic acid, 200 mg/kg, VITD$_3$ = vitamin D_3, 200 ìg/kg. One phytase unit is the activity of phytase that generates 1 micromole of inorganic phosphorus per minute from an excess of sodium phytate at pH 5.5 and 37 degrees Celsius. [2]The overall polled SEM was 2.7g, 0.23g/bird/d, and .012g/g for body weight, feed intake, and feed conversion ratio, respectively.

Sample collection and assays: Bird weights were calculated on a by pen basis at 0 and 21 days of age and feed consumption was measured throughout the experiment. Birds that died during the experiment were recorded daily and used to adjust feed conversion data. Feed conversion was corrected for mortality by adding body weights to the total pen weight at the end of observation period. At 16 days of age, chromic oxide (Cr_2O_3) (30 g/kg) was fed for five days in all diets and was used as an analytical marker for determination of nutrient digestibility. At 21 days of age, six birds from each pen were killed by cervical dislocation and the small intestine was immediately exposed. The contents of the lower ileum were expressed by finger pressure into plastic containers. The ileum was defined as that portion of the small intestine extending from the vitelline diverticulum to a point 40 mm proximal to the ileo-caecal junction. The ileum was divided into 2 halves and the digesta were collected from the lower half towards the ileo-caecal junction. Digesta from birds within a pen were pooled, resulting in 4 samples per dietary treatment. The digesta samples were frozen immediately after collection and subsequently lyophilized. Tibia samples were obtained by severing the left tibia. The tibias of 3 birds within a pen were pooled. The tibia bones were boiled to remove any traces of flesh, solvent-extracted to remove fat, then dried to a constant weight at 100^{o}C and then ashed in a muffle furnace at 600^{o}C for 6h. Tibia ash was expressed as a percentage of dry weight.

Excreta was dried and ground to pass through a 1 mm sieve and feed samples were also ground to pass through a 1 mm sieve. Phosphorus concentrations in feed and excreta were determined colorimetrically by the molybdo vanadate method (AOAC, 965.17 photometric method, 1995). The protein content (N×6.25) of diet and individual samples of excreta was determined by the Kjeldahl method after acid digestion. Diet and feces chromium (Cr) were analyzed by the procedure described by Fenton and Fenton (1979), using spectrophotometry. Phosphorus and protein digestibility were calculated using the following formula:

100%-[100% × (Cr concentration in feed ÷Cr concentration in excreta) × (P or protein concentration in excreta ÷ P or protein concentration in feed)]

Statistical analysis: A two-way analysis of variance was employed to determine the main effects (different basal diets, and calcium) and their interaction by using the ANOVA procedure of the SAS statistical package (Littell *et al.,* 1991). Separation of means was by the Duncan's multiple range test. Differences were considered significant at P<0.05 and P<0.01 levels.

Results

The effect of Ca level by dietary additions on BW is summarized in Table 2. Body weight was significantly improved (P<0.01) when 1.35 g/kg available P was added to the basal diet, irrespective of Ca concentration. For the low Ca diets, a significant increase (P<0.01) in BW compared to the negative control occurred with phytase and the addition of citric and ascorbic acids. Further increases occurred when vitamin D_3 was added. This latter diet compares favourably with the positive control diet.

For the high Ca diets, BW was significantly increased (P<0.01) when phytase alone was added. No further improvements to BW were found with the other additives

(slight decrease). All the additives together lead to a significantly lower BW than the positive control.

There were significant interactions between dietary additions and Ca for FI (Table 2). Feed intake was significantly improved (P<0.01) when 1.35 g available P/kg feed was added to the basal diet, irrespective of the Ca concentration. For the low Ca diets, a significant increase (P<0.01) in FI occurred with the phytase with citric and ascorbic acids and vitamin D_3 additions compared to phytase alone. Conversely in the high Ca diets, a decrease in FI occurred when phytase was added together with citric and ascorbic acids and vitamin D_3, compared to phytase alone.

For the whole experimental period, feed conversion ratio (FCR) was influenced by dietary additions (Table 2). Compared to the low P diets, FCR was significantly improved by the addition of phytase alone, irrespective of the Ca concentration. Further improvements occurred with phytase plus citric acid, ascorbic acid and vitamin D_3.

The effects of Ca level on dietary addition on tibia ash were significant (Table 3). For the low Ca diets, tibia ash was not affected by "dietary addition". For the high Ca diets, the addition of 500 phytase to the low P basal diet, resulted in a significant increase (P<0.01) in tibia ash, but no further improvement when citric acid, ascorbic acid and vitamin D3 are added.

Phosphorus digestibility in both Ca treatments were significantly greater with the addition of phytase. The further addition of citric and ascorbic acids did not significantly increase P digestibility except in the low Ca diet when vitamin D_3 was also added. In the low Ca diets, phosphorus digestibility was decreased when 1.35 g/kg AP was added to the basal diet. This reduction was offset by increased dietary Ca (Table 3).

In the low Ca diets, phytase alone significantly increased protein digestibility (P<0.01) compared to the negative and positive controls. Further improvements in protein digestibility occurred with the combination of phytase, citric, and ascorbic acids but there was no further increase in protein digestibility with vitamin D_3 (Table 3).

Discussion

As expected, decreasing AP in the diet caused a negative effect on the performance of chicks. These results were in agreement with those reported by Qian *et al.* (1996) and Punna and Roland (1999). Similarly, the positive effect of phytase on performance in broiler chicken diets has already been reported by Rama Rao *et al.* (1999) and Ahmad *et al.* (2000). Qian *et al.* (1996) furthermore indicated that microbial phytase seems to be more efficient in diets with none or low levels of inorganic P supplementation in both pigs and poultry.

In this current study, birds with the high Ca diets, with the addition of 500 U phytase to the low-AP basal diet, exhibited increased BW and FI. However, in low Ca diets, phytase did not modify bird BW and FI. The ability of phytase to significantly improve the performance at the chick stage was expected in the high Ca diets. A possible explanation could be that the additional P liberated by phytase would cause a greater Ca:AP ratio balance in the high Ca diets that could justify positive effect in the performance. However, for the low Ca diets, phytase addition to basal diets along with low Ca:AP ratio possibly caused an exacerbation of Ca deficiency by the release of additional available P.

Decreased feed intake resulting from low levels of available phosphorus in diets fed to poultry is a well-known phenomenon (Kiiskinen *et al.,* 1994). In the current study, however, this effect was partly compensated for the high Ca diets supplemented with phytase.

On the other hand, data also has shown that FCR was positively affected by the addition of phytase, probably as a result of the increase in weight gain. These results were similar to what other authors have reported, improved feed efficiency occurs with supplemental phytase (Rama Rao *et al.,* 1999). In contrast, other authors have reported that FCR was not affected by the addition of phytase (Ahmad *et al.,* 2000).

An objective of our experiment was to investigate whether the combination of phytase, citric and ascorbic acid may have some additive or synergistic effects on performance. Given that microbial phytase is most active at pH 2.5 and 5.5 (Simons *et al.,* 1990), and that some intestinal sections have different pH values (Dänicke *et al.,* 1999), the effectiveness of microbial phytase may be enhanced, at least in theory, by feeding it in combination with an organic acids. However, in our experiment, in the low Ca diets, when phytase altogether with citric acid and ascorbic acid was added, BW was enhanced by 11.1% over the value obtained from the negative control diet. The positive effect of citric and ascorbic acid with phytase to significantly improve performance in chicks was expected. A possible explanation could be that the organic acids complexed with minerals and decrease additional P liberated by phytase in the intestine, thereby increasing the Ca:AP ratio causing an imbalance in the low Ca diets and promote BW. In any case, there are no references for chicks on this subject. However, these results were similar to those reported by Boling *et al.* (2001), who indicated that citric acid (20 to 60 g/kg) only had a positive effect on performance in low-AP diets. In contrast, other authors in pigs reported no synergistic effects with the combination of phytase and citric acid on performance (Radcliffe *et al.,* 1998).

Body weight was further increased when vitamin D_3 together with phytase and organic acids were added to low Ca diets. The multifaceted effects of phytase in practical diets are being increasingly appreciated, and it is possible that the observed body weight responses

Table 3: Effect of different dietary addition (phytase, citric, ascorbic acid and vitamin D_3) and different calcium concentration on the tibia ash and phosphorus and protein digestibility of broilers aged 21 d

[2]Supplements to basal diet (3.15 g/kg AP)[1]	Calcium concentration in the diet (g/kg)								
	Tibia ash (g/kg)[2]			Phosphorus digestibility (% of intake)[2]			Protein digestibility (% of intake)		
	Total	7.9	9.0	Total	7.9	9.0	Total	7.9	9.0
		473	495	54.6	53.0	68.6^{a}	65.6^{b}		
None	464	490^{abc}	437^{c}	43.6^{c}	45.3^{c}	42.0^{c}	64.4^{c}	63.9^{def}	64.8^{cde}
PHYT	497	457^{bc}	537^{a}	59.3^{b}	60.0^{b}	58.6^{b}	67.8^{b}	68.1^{bc}	67.6^{bcd}
PHYT+ CA	484	464^{bc}	504^{ab}	56.1^{b}	57.3^{b}	54.9^{b}	68.1^{b}	70.7^{b}	65.5^{cd}
PHYT+ CA+ AA	475	460^{bc}	490^{abc}	58.1^{b}	57.8^{b}	58.3^{b}	72.7^{a}	78.4^{a}	67.0^{bcd}
PHYT+CA+AA+VITD$_3$	491	492^{abc}	491^{abc}	66.0^{a}	72.5^{a}	59.5^{b}	68.8^{b}	70.2^{b}	67.5^{bcd}
1.35 g/kg AP	492	475^{abc}	510^{ab}	40.0^{c}	34.9^{d}	45.0^{c}	61.0^{d}	60.6^{f}	61.5^{ef}
Model	P>F			P>F			P>F		
Dietary addition (Da)	0.53			**			**		
Calcium (Ca)	0.06			0.25			**		
Da x Ca	**			**			**		

*, ** Indicate significance (P<0.05 and P<0.01, respectively) for main effects and interactions. abcd Different letters beside mean values indicate significant differences between mean values. [1]Supplements were the following: PHYT = phytase, 500 units (FYT)/kg; CA = citric acid, 20 g/kg, AA = ascorbic acid, 200 mg/kg, VITD$_3$ = vitamin D_3, 200 ìg/kg. One phytase unit is the activity of phytase that generates 1 micromole of inorganic phosphorus per minute from an excess of sodium phytate at pH 5.5 and 37 degrees Celsius.
[2]The overall polled SEM was 5.5 g/kg, 0.69%, and 0.35% for tibia ash, phosphorus, and protein digestibility, respectively.

with vitamin D_3 may reflect the release of P, and improve FI and FCR by the additives. Plausibly, vitamin D_3 may improve the apparent efficacy of supplemental dietary phytase by increasing Ca uptake from the gut, thereby facilitating utilization of digested P either by increasing its transport rate and/or by increasing the solubility of phytate in the small intestine, thereby influencing accessibility to phytase (Mitchell and Edwards, 1996). Body weight was adversely affected when vitamin D_3 is fed with a high calcium diet. The mechanism for this phenomenon is uncertain, however, increased calcium absorption attributable to vitamin D_3 content may have caused a negative side effects on growth such as hypercalcaemia (Roberson and Edwards, 1994).

In the low Ca diets, tibia ash was not influenced by phytase alone or with all additives. The absence of a significant influence of all additives on tibia ash compare to control diet indicates that the diets contained adequate amounts of nonphytate P to support bone mineralization. Keshavarz (2000) observed in pullets and Rama Rao *et al.* (1999) in chickens that tibia ash was not influenced by phytase in the diets. However, in high Ca diets, phytase supplementation to low-P basal diet significantly increased tibia ash. The increase in tibia ash has been reported by several authors in chickens and considered to be a good indicator of bone mineralization (Ahmad *et al.,* 2000; Leeson *et al.,* 2000). This improvement in ash percentage in tibia can be related to the increase in minerals retention.

In the current study, phytase supplementation to the low-AP basal diets increased P digestibility, irrespective of Ca concentration. An increase in P retention by phytase addition has already been reported by Qian *et al.* (1997) and Ravindran *et al.* (2000). Ahmad *et al.* (2000) indicated that P excretion on a low-P diet decreased with the addition of phytase and this might have increased the availability of P. The results of the current study also indicated that the addition of vitamin D_3 to low Ca diets increased P digestibility. This experiment confirms previous studies reported by Mohammad *et al.* (1991) and Ravindran *et al.* (1995). The mechanism by which vitamin D_3 increases the P digestibility is to enhance Ca and P absorption. Increased Ca absorption could decrease Ca-phytate formation in the digesta, which could increase the efficacy of phytate hydrolysis (Ravindran *et al.,* 1995). Vitamin D has been shown to induce chick's intestinal mucosal phytase activity (Davies *et al.,* 1970).

Protein digestibility was significantly improved when phytase was added to low-P and Ca diets. These data agree with the study of Mroz *et al.* (1994) who reported improved protein digestibility as a results of phytase supplementation. Phytic acid has been reported to form complexes with protein (Gifford and Clydesdale, 1990) and to inhibit the activity of trypsin and pepsin (caldwell, 1992). Although specific studies relating to phytic acid are lacking, evidence with other anti-nutritive factors (non starch polysaccharides) indicate that these components can significantly increase endogenous N flows in poultry (Angkanaporn *et al.,* 1994). Therefore, the improvement in protein digestibility with phytase possibly is mediated through a reduction in endogenous losses.

Protein digestibility was significantly improved by the addition of citric and ascorbic acid to phytase (23 and 15% as compared negative control and phytase alone, respectively). The improvement in protein digestibility with citric and ascorbic acid is in agreement with previous studies using fumaric acid. In these studies it was suggested that the reduction in gastric pH which occurs following organic acid feeding may increase

pepsin activity (Kirchgessner and Roth, 1982). However, peptides arising from pepsin proteolysis trigger the release of hormones, including gastrin and cholecystokinin, which regulate the digestion and absorption of protein (Hersey, 1987). In addition, it seems that acidifying the diet (data not shown) by adding citric and ascorbic acids increase the solubility of wheat phytates, a phenomenon known to occur during seed germination (Gasprovic *et al.,* 1997). Acidification provides a better environment for phytase to reduce the amount of phytate in the digesta flowing into small intestine, thus largely preventing the formation protein-mineral-phytate complexes. Antrim and Solheim (1994) observed stabilizing effects of anti-oxidants on "-amylase under extreme values of pH and temperature. It may be hypothesized that similar effects took place in the small intestine of chicks fed diets enriched with antioxidant properties of ascorbic acid, because phytase is prone to oxidative damage in the small intestines as the pH increases. So it is possible that the observed protein digestibility and body weight response may reflect the probable reduction in nitrogen loss that caused by the added organic acids along with phytase. In conclusion, the data from this experiment showed that for broiler chickens, a low-P (3.15 g/kg), low-Ca (7.9 g/kg) wheat-based diet supplemented with microbial phytase (500 FYT/kg), organic acid and vitamin D_3 improved feed efficiency and gave a similar body weight as a Ca-and P-adequate diet. The presented study clearly indicated that supplementation of poultry diets with Ca and P is not necessary when adequate activities of phytase enzyme are provided and the feed is supplemented with appropriate concentrations of Ca, organic acid (citric or ascorbic acid) and vitamin D_3. This may lead to considerable reductions in the amounts of excreted phosphorus, without compromising the performance, and nutritive status of birds.

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