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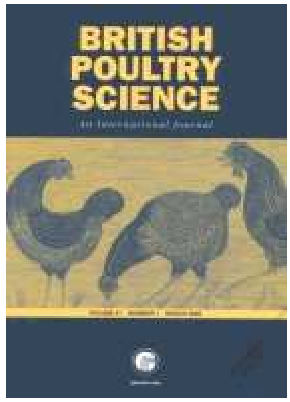

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Journal:	*British Poultry Science*
Manuscript ID:	CBPS-2006-186.R1
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	13-Dec-2006
Complete List of Authors:	Brenes, Agustín; Instituto del Frío, Spanish National Research Council (CSIC), Metabolism and Nutrition Centeno, Carmen; Instituto del Frío, Spanish National Research Council (CSIC), Metabolism and Nutrition Arija, Ignacio; Veterinary Faculty, Complutense University, Animal Production Viveros, Agustín; Veterinary Faculty, Complutense University, Animal Production
Keywords:	Amino acids, phytase, citric acid, chickens

CBPS-2006-186
ed. MacLeod, May 2007

Effects of citric acid and microbial phytase on amino acid digestibility in broiler chickens

C. CENTENO, I. ARIJA[1], A. VIVEROS[1] AND A. BRENES

Department of Metabolism and Nutrition. Instituto del Frío, Spanish National Research Council (CSIC) and [1]Department of Animal Production, Veterinary Faculty, Complutense University, 28040 Madrid, Spain

RUNNING TITLE: **PHYTASE, CITRIC ACID AND DIGESTIBILITY**

Correspondence to: Agustín Brenes. Departamento de Metabolismo y Nutrición. Instituto del Frío (CSIC), 28040 Madrid, Spain.

Tel: +34-91-5434545

Fax: +34-91-5434545

E-mail: abrenes@if.csic.es

Accepted for publication 29th March 2007

Abstract 1. Two experiments with growing chickens were carried out to study the effects of the inclusion of a microbial phytase (Natuphos® 5000) and citric acid (CA) in maize-soybean based diets on the performance and apparent ileal digestibility (AID) of crude protein (CP) and amino acids (AA). In both experiments the diets were formulated to contain the same amounts of energy and protein.

2. In the first experiment, data were analysed as a 2 x 2 factorial arrangement with two concentrations of available phosphorus (AP) from 1 d to 3 weeks of age (3.5 and 2.2 g/kg) and for 3 to 6 weeks (2.7 and 1.4 g/kg), and two inclusions of commercial phytase (0 and 500 FTU/kg) in each period. The AID of CP and dispensable and indispensable AA were not modified by the AP content of the diet. Addition of phytase improved the AID of CP and dispensable and indispensable AA only at low AP levels.

3. In the second experiment, data were analysed as a 3 x 2 factorial arrangement with three concentrations of citric acid (0, 20 and 50 g/kg) and two inclusions of commercial phytase (0 and 750 FTU/kg). Diets were formulated with deficient contents of AP (2.5 g/kg). Performance was not affected by commercial phytase addition. The addition of CA reduced the weight gain but did not modify the feed intake and gain:feed. In general, the AID of CP and dispensable and indispensable AA were not affected by CA addition. Commercial phytase increased the apparent ileal digestibility of crude protein but had no effect on AID of dispensable and indispensable AA.

4. In conclusion, the present work showed that microbial phytase enhanced AA digestibility in maize-soy-based diet only at very low AP concentrations, and that CA had no affect on the AID of CP and dispensable and indispensable AA. No synergism between CA and microbial phytase was detected.

INTRODUCTION

The capacity of phytic acid to bind minerals reduces the digestion and absorption of phosphorus, calcium, magnesium and zinc from plant-derived ingredients by monogastric animals (Jonbloed *et al*., 1996; Ravindran *et al*., 1999; Viveros *et al*., 2002). Many reports have indicated that the addition of microbial phytase to poultry diets can release inorganic phosphate from the phytate complex, thus improving phosphorus availability, and thereby reducing phosphorus excretion (Ravindran *et al*., 2000; Viveros *et al*., 2002). The effect of phytate on the utilisation by poultry and swine of nutrients other than P, however, has received little attention until recently. The basis for these effects probably lies in the strong chelating potential of phytic acid by its ability to form protein complexes (Gifford and Clydesdale, 1990; Selle *et al*., 2000), by its phytate-induced increases in endogenous amino acid flows (Ravindran *et al*., 1999; Cowieson *et al*., 2004) and by the inhibition or reduction of trypsin enzyme (Caldwell, 1992). The literature on phytate-mediated improvements in protein utilisation represents a conflicting base of information. The efficacy of phytase in improving protein utilisation has been inconsistent, data previously reported in several studies having suggested that microbial phytase can have beneficial effects on amino acid digestibility in individual feed ingredients (Ravindran *et al*., 1999) and in compound diets (Yi *et al*., 1996; Ravindran *et al*., 2000; Rutherfurd *et al*., 2002, 2004; Ravindran *et al*., 2006), whereas other investigators have not obtained statistically significant responses (Biehl and Baker, 1997; Peter and Baker, 2001; Auspurger and Baker, 2004; Onyango *et al*., 2005).

Citric acid (CA) has been reported to intensify phytate dephosphorylation *in vitro* (Zyla *et al*., 1995). More recent research has indicated that citric acid is also very efficacious in improving P utilisation in chickens fed on maize-soybean meal diets containing no supplemental P (Boling *et al.*, 2000; Brenes *et al.*, 2003; Snow *et al.*, 2004; Rafacz-Livingston *et al.*, 2005) and in one study reduced the amount of needed supplemental phosphorus by approximately 0.1% of the diet (Boling-Frankenbach *et al.*, 2001). In contrast, citric acid does not improve the utilisation of dietary P in laying hens given diets containing 38 g/kg Ca (Boling *et al.*, 2000; Snow *et al.*, 2004). There has been recent interest in combining citric acid and phytase to investigate possible interactive effects in poultry. Brenes *et al.* (2003) reported that the combination of phytase and citric acid did not improve phosphorus and calcium availability above that obtained with phytase alone. Since there is no clear consensus on the effects of microbial phytase addition on protein and amino acid digestibility in maize-soybean diets, and even less regarding the efficiency of phytase and citric acid on this measurement, the objective of the current study was to determine the effects of phytase and citric acid on the apparent amino acid digestibility in a maize-soybean diet fed to broiler chickens.

MATERIALS AND METHODS

General experimental procedures

Male chicks (Cobb) were obtained from a commercial hatchery and used in all experiments. Chicks were housed in electrically heated stainless steel battery brooders in an environmentally controlled room with 23-h constant overhead fluorescent lighting. Diets in pellet form (Experiment 1) and in mash form (Experiment 2) and water were provided for *ad libitum* consumption. Celite, a source of acid-insoluble ash (AIA) was added at 10 g/kg to all diets as an indigestible marker. Mortalities were recorded daily. At the end of each experimental period (6 weeks, Experiment 1 and 3 weeks, Experiment 2), birds were weighed and feed consumption was recorded for calculation of gain:feed ratio. Microbial phytase (Natuphos® 5000, BASF, Mount Olive, NJ 07828-

1234, USA) containing 5,000 phytase units (FTU)/g phytase activity was used in both experiments. One FTU is the quantity of enzyme that releases 1 µmol of inorganic P/min from 0.00015 mol/L sodium phytate at pH 5.5 at 37 ºC. Analysed dietary amino acids are shown in Table 2. All housing and handling were approved by the University Complutense of Madrid Animal Care and Ethics Committee in compliance with the Ministry of Agriculture, Fishery and Food for the Care and Use of Animals for Scientific Purposes.

Experiment 1

The objective of this experiment was to evaluate the effects of two available phosphorus (AP) concentrations and two inclusions of supplemental phytase on the apparent ileal protein and amino acid digestibilities. A total of 240 male 1-d-old broiler chickens were allocated to 30 pens, each pen containing eight chicks, to receive five dietary treatments of maize-soybean-based diets (Table 1) with six replicates of each treatment. At the end of 3 weeks, birds were moved from starter to grower finisher batteries for the remaining 3 weeks.

The experimental design was a 2 x 2 factorial with an additional diet (control diet) with two levels of phytase (0 and 500 FTU/kg) and 2 concentrations of AP (control minus 0.1 % and control minus 0.23 %) at each two phases (starter and grower). AP and Ca concentration were 4.5 and 9.9 g/kg (starter) and 3.7 and 8.7 g/kg (grower-finisher) in the control diet. The concentration of Ca was left the same when AP was changed. The Ca:tP ratio of the control, T_2-T_4 and T_3-T_5 was 1.39, 1.62 and 2.02 in the starter diet, and 1.40, 1.67 and 2.20 in the grower-finisher diet, respectively.

Tables 1 and 2 near here

Experiment 2

The objective of this experiment was to determine the effect of citric acid alone or in combination with phytase on performance and apparent ileal protein and amino acid digestibilities in P-deficient diets (2.5 g/kg AP). A total of 288 male 1-d-old broiler chickens were allocated to 36 pens, each pen containing 8 chicks, to receive 6 dietary treatments of maize-soybean-based diets (Table 1) with 6 replicates of each treatment.

The experiment consisted of a 3 x 2 factorial arrangement of the treatments with 3 concentrations of citric acid (Sigma-Aldrich Quimica, Tres Cantos, Spain) (0, 20 and 50g/kg) and two levels of supplemental phytase (0 and 750 FTU phytase/kg diet). Proportions of maize, soybean meal and sunflower oil were modified in diets to maintain ME (13.0 MJ/kg) and crude protein (230 g/kg) constant. The ME of citric acid was assumed to be 10.3 MJ/kg (FEDNA Tables). In both experiments, dietary concentrations were formulated to be below the current NRC (1994) to ensure maximum responses with phytase.

Collection of samples and measurements

At the end of the experimental period (6 weeks, Experiment 1 and 3 weeks, Experiment 2), 12 birds per treatment were weighed and sacrificed by cervical dislocation. The ileum was dissected within 5 min after killing. The ileum was defined as that portion of the small intestine extending from Meckel's diverticulum to a point of 40 mm proximal to the ileo-caecal junction. Ileal digesta of birds within a pen were pooled, resulting in 6 composite samples per dietary treatment. The digesta were frozen immediately after collection and subsequently freeze-dried. Digesta samples were ground to pass through a 0.5-mm sieve and stored in airtight containers at –4 C for chemical analyses.

Chemical analyses

Diets and ileal contents were analysed for crude protein (6.25 N) using a standard method (Association of Official Analytical Chemist, 1990). The AIA contents of diet and ileal digesta were measured after ashing the sample and treating the ash with boiling 4M hydrochloric acid (Siriwan *et al.*, 1993).

Amino acids were separated using a Beckman Model 6300 autoanalyser. Detection was carried out at 570 and 440 nm after postcolumn derivatisation of the amino acids with ninhydrin. Three replicates of all analyses were performed. The concentrate samples (100 mg) were hydrolysed with 6 N hydrochloric acid at 110ºC for 24 h in nitrogen atmosphere. The proportion of hydrochloric acid was 1 ml/mg of protein in the sample (Finley, 1985). Prior to acid hydrolysis, the samples were analysed by performic acid oxidation. The hydrolysate was filtered through Whatman 541 paper and the volume adjusted to 100 ml of solution with Milli-Q water. An amount of 2ml of solution was evaporated to dryness in a rotary evaporator at 40ºC. The hydrolysate residue was re-dissolved in 200 µL of 0.2 M citrate buffer (pH 2.2) containing norleucine as internal standard and diluted to 1000 µL with the same buffer, and 50 µL was injected into the autoanalyser. Determination of the amino acid tryptophan was not possible under the conditions of analysis employed.

Calculations and statistical analysis

Apparent ileal digestibilities (AID, %) of crude protein (CP) and amino acid (AA) were calculated using the following formula: 100 - [100 x (AIA concentration in feed ÷ AIA concentration in ileal digesta) x (CP or AA concentrations in ileal digesta ÷ CP or AA concentrations in feed)].

In experiment 1, data were analysed as a 2 x 2 factorial design with two concentrations of AP from 1 d of age to 3 weeks (3.5 and 2.2 g/kg) and for 3 to 6 weeks (2.7 and 1.4 g/kg), and two levels of phytase (0 and 500 FTU/kg) in each period.

Treatment 1 was considered as a positive control, adequate in AP and Ca; therefore, phytase was not added. This control treatment was excluded of statistical analysis. The statistical model used was: $Y_{ijk} = \mu + P_i + E_j + PE_{ij} + R_k + e_{ijk}$, where Y_{ijk} is the individual observation, μ is the experimental mean, P_i is the available phosphorus effect, E_j is the enzyme phytase effect, R_k is the replication effect, and e_{ijk} is the error term.

In experiment 2, data were analysed as a 3 x 2 factorial design with two levels of phytase (0 and 750 FTU/kg diet) and three concentrations of citric acid (0, 20 and 50 g/kg). The statistical model used was: $Y_{ijk} = \mu + E_i + C_j + EC_{ij} + R_k + e_{ijk}$, where Y_{ijk} is the individual observation, μ is the experimental mean, E_i is the enzyme phytase effect, C_j is the citric acid effect, R_k is the replication effect, and e_{ijk} is the error term. In both experiments, data were subjected to analysis of variance using the General Linear Models (GLM) procedures of SAS® (SAS Institute, 2001). Significant differences among treatment means were determined at *P*<0.05 by Duncan´s multiple range test. Comparisons between the diets with and without added phytase at each citric acid content were made using nonorthogonal contrast.

RESULTS

Experiment 1

Weight gain, feed consumption and gain:feed

The effects of AP concentrations and phytase supplementation on growth performance have been previously published by Viveros *et al.* (2002), indicating that the decrease in AP content in the diet depressed weight gain, feed consumption and gain:feed. Phytase had a favourable effect on weight gain at 3 and 6 weeks of age and on feed consumption only at 3 weeks. Gain:feed was not affected at any stage by addition of phytase.

AID of CP and dispensable amino acids

The influence of AP and microbial phytase on the AID coefficients of CP and indispensable (essential) amino acids (EAA) of male chickens are shown in Table 3. The main effects data indicated that the decrease in AP content in the diet did not affect the AID of CP and EAA of the diets. Likewise, the addition of phytase resulted in a significant increase (*P*<0.05) in AID of CP (6.2 %) and all of the EAA (Arg 3.0 %; His 4.8 %; Iso 5.2 %; Leu 4.4 %; Lys 4.7; Met 2.7 %; Phe 4.5 %; Thr 6.4 % and Val 5.3 %). Except for crude protein, a significant interaction (*P*<0.001) among AP concentrations and phytase was observed, indicating that phytase improved the amino acid digestibilities only at low AP levels.

Table 3 near here

AID of dispensable amino acids

The influence of AP and microbial phytase on the AID coefficients of dispensable (non essential) amino acids (NEAA) of male chickens is summarised in Table 4. The main effects data indicated that the decrease in AP content in the diet did not affect the AID of NEAA of the diets, except for Ala, which was reduced (7.6 %; *P*<0.01). Phytase addition increased (*P*<0.05 to 0.001) all of the NEAA (Asp 7.3%; Glu 3.3 %; Gly 5.8 %; Pro 4.5 %; Ser 7.6 %; Tyr 8.6 %, and Cys 6.9 %), except Ala, which was not affected. Except for Ala, a significant interaction (*P*<0.05 to 0.001) among AP concentration and phytase was observed, indicating that phytase improved the amino acid digestibilities only at low AP levels.

Table 4 near here

Experiment 2

Weight gain, feed consumption and gain:feed

Main effects. The effects of citric acid (CA) and phytase supplementation on growth performance are summarised in Table 5. The main effects data indicated that the increase in CA content reduced weight gain (up to 5%; *P*<0.01). However, the addition of CA did not significantly modify feed consumption and gain:feed. The performance of birds was not affected by phytase addition.

Contrast. The addition of phytase to each citric acid level did not influence weight gain, feed consumption and gain:feed.

Table 5 near here

AID of CP and dispensable amino acids

Main effects. The influence of CA and microbial phytase on the AID coefficients of CP and EAA of male chickens are shown in Table 6. The main effects data indicated that the increase in CA content in the diet did not affect the AID of CP and EAA of the diets, except Leu which was reduced (by up to 1.4%; $P<0.05$). The addition of phytase increased the AID of CP (2.8%; $P<0.001$) and Met (1 %; $P<0.05$). All two-way interactions were non-significant except in the case of Phe ($P<0.01$) and Thr ($P<0.05$), which indicates that the effect of phytase on digestibilities of these amino acids was dependent on CA content.

Contrast. When birds were given diets without CA, adding phytase diets increased crude protein ($P<0.001$), Met ($P<0.05$), Phe ($P<0.01$), and Thr (P<0.05) digestibilities. The addition of phytase to the 50 g/kg CA diets increased crude protein ($P<0.05$) digestibility.

Tables 6 and 7 near here

AID of dispensable amino acids

Main effects. The influence of CA and microbial phytase on the AID coefficients of NEAA of male chickens is shown in Table 7. The main effects data indicated that the increase in CA content in the diet improved the AID of Asp (up to 1.9; $P<0.05$) and Gly (up to 2.1 %; $P<0.05$) and decreased Pro (up to 5.8; $P<0.001$). The AID of NEAA was not affected by phytase addition. The two-way interaction of CA and phytase on Asp ($P<0.01$), Glu ($P<0.01$) Gly ($P<0.05$), Ser ($P<0.05$) and Tyr ($P<0.01$) digestibilities was also observed, which indicates that the effect of phytase on digestibilities of these amino acids was dependent on CA content.

Contrast. When birds were given diets without CA, adding phytase diets increased Asp (P<0.01), Gly (P <0.05), and Ser (P<0.05) digestibilities. The addition of phytase to the 20 g/kg CA diets decreased Asp (P<0.05), Glu (P<0.01), and Tyr (P<0.05) digestibilities. Amino acid digestibilities were not influenced by phytase at the 50 g/kg CA level.

DISCUSSION

The present study shows the effects of citric acid and microbial phytase supplementation on performance and AID of CP, EAA, and NEAA in male chickens fed a corn-soybean meal diet to 6 (Experiment 1) and 3 weeks. (Experiment 2) of age. The protein/amino acid effect of microbial phytase is of considerable practical significance and needs to be quantified to enable its inclusion in least-cost diet formulations. The efficacy of phytase in de-phosphorylating phytate in plant-derived ingredients and thereby improving its availability for pigs and poultry is established. However, the same cannot be said of protein and amino acid utilisation responses to microbial phytase due to a number of conflicting reports.

In the first experiment, results already published (Viveros *et al.*, 2002) demonstrated that the phytase supplementation improved the performance of the birds only in low-AP diets (1.4 g/kg), but not at higher AP concentration (2.7 g/kg). The second experiment confirms these results showing that microbial phytase supplementation failed to improve the performance of the chicks with similar AP levels (2.5 g/kg). These results were in agreement with those reported by Qian *et al.* (1996), which indicated also that microbial phytase seems to be more efficient in diets with little or no inorganic P supplementation. Um and Paik (1999) in laying hens, Ravindran *et al*. (2000) in chickens and Keshavarz (2000) in pullets also indicated that the birds have a greater ability to retain P from diets with lower rather than higher non-phytate phosphorus (nPP) content, which may influence performance.

Moreover, a decreased phytate-P digestibility in response to increasing the Ca:tP ratio has been reported in rats (Nelson and Kirby, 1979), poultry (Qian *et al*., 1996) and pigs (Lei *et al*., 1994). In fact, Qian *et al*. (1997) showed that the increase observed in P retention with phytase addition was negatively influenced by increasing the dietary Ca:total P ratio. The extra Ca may directly repress phytase activity by competing for the active sites of the enzymes (Pointillart *et al*., 1985). Therefore, the Ca:tP ratio of 2.2:1 used in our experiment may also explain the lack of effect of phytase on performance. However, Boling-Frankenbach *et al*. (2001) demonstrated that phytase was not affected for dietary Ca and non phytate P concentrations.

In experiment 2, the addition of the highest level of citric acid (50 g/kg) to low-AP diets depressed weight gain. These results are similar to those published by Brenes *et al*. (2003). The reason for a negative response or absence of response to citric acid is unclear. Boling-Frankenbach *et al*. (2001) found a negative effect on performance of chickens when adding 60 g/kg citric acid to diets containing adequate available phosphorus (4.5 g/kg). Boling *et al*. (2000) and Boling-Frankenbach *et al*. (2001) indicated that citric acid (20 to 60 g/kg) had a positive effect on performance only in low-AP diets (1.0 to 2.5 g/kg) and with a Ca:AP ratio similar to or greater than 4:1. Probably, as demonstrated by these authors, citrate addition in diets AP deficient and with low Ca:AP ratio caused an exacerbation of Ca deficiency by the release of additional available P. Therefore, the Ca:AP ratio of 3.2:1 used in our experiment may support this hypothesis.

In the first experiment, the addition of phytase (500 U/kg) increases the AID of CP and all of the EAA and NEAA only at low AP concentration (1.4 g/kg). These results are in general agreement with the findings of several studies which demonstrated positive effects of supplemental phytase on ileal digestibility of nitrogen, protein or amino acids in male (Ravindran *et al*., 1999, 2000, 2001; Rutherfurd *et al*., 2002), and female chickens (Sebastian *et al*., 1997), laying hens (van der Klis and Versteegh, 1991), and turkeys (Yi *et al*., 1996). The effectiveness of phytase was negatively related to the amount of inorganic phosphorus in the diet. Additional inorganic P and/or Ca compromised the capacity of exogenous phytase to enhance AA digestibility, presumably by reducing the extent of phytate hydrolysis (Ravindran *et al*., 2000). Lei and Stahl (2000) also argued that exogenous phytase is more efficacious in diets containing low levels of inorganic P because the catalytic activity of phytase is strongly inhibited by P, the hydrolytic end product of the reaction. Improvements in amino acid digestibility support the idea that phytate and protein can form binary complexes through electrostatic links of its charged phosphate groups with either the amino group on arginine and lysine residues present within protein or with the terminal amino group on proteins (Maenz, 2001). These complexes may be formed *de novo* at acidic pH in the gut from the protein bodies of oilseeds and in the protein-rich aleurone layers of cereal grains (Selle *et al*., 2000). The complexing of phytate with proteins can change the protein structure, which in turn decreases solubility, digestibility, and functionality of proteins (Yi *et al*., 1996). Another possible mechanism reported by Cowieson *et al.* (2004) is that phytate may interact with the gastrointestinal tract increasing N endogenous loss, mainly in the amino acids cystine, threonine, serine, and methionine (Selle *et al*., 2000).

In contrast, there are other studies in which dietary supplementation with phytase had no effect on apparent ileal digestibility of amino acids in male (Sebastian *et al*., 1997; Onyango *et al*., 2005; Martinez-Amezcua *et al*., 2006) and female chickens (Peter and Baker, 2001; Snow *et al*., 2003) and turkeys (Ledoux *et al*., 1999) or true amino acid digestibility in soybean meal intubated into caecectomised roosters (Biehl and Baker, 1997). In fact, in the second experiment the addition of phytase (750 FTU/kg) did not modify the AID of EAA and NEAA, except in the case of Met. Likewise, Peter *et al*. (2000) and Boling-Frankenbach *et al*. (2001) did not improve protein or amino acid utilisation by phytase addition in several feed ingredients in young chicks. Probably the lack of response of phytase in the second experiment may have been related to dietary AP content (2.5 g/kg), as in the first experiment (2.7 g/kg), or to the age of the birds (3 weeks). It is generally agreed that the ability of poultry to utilise phytate phosphorus increases with age (Edwards *et al*., 1989). In balance experiments investigating the hydrolysis of phytate by 4 and 9 week old broilers, Nelson *et al*. (1976) observed a slight increase in phosphorus utilisation by the older birds.

Another objective of the current experiment was to evaluate the efficacy of citric acid (CA) in improving the digestibilty of CP and AA and to observe whether phytase and CA may have some additive or synergistic effects. Since interactions between phytate and protein are mediated by cations (Gifford and Clydesdale, 1990), the addition of an organic acid, such as citric acid, may reduce the formation of this complex by chelating free cations. 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 acid. In fact, Maenz *et al*. (1999)

observed that competitive chelation by compounds such as EDTA, citric acid or phthalic acid has the potential to decrease enzyme-resistant forms of phytic acid and thereby improve the efficacy of microbial phytase in hydrolysing phytic acid.

In general, in the second experiment, the main effect data indicated that increasing the CA content of the diet did not affect the AID of CP, EAA and NEAA of the diets. Moreover, we did not observe a synergistic effect of microbial phytase and dietary acidification on amino acid digestibilities. The failure of citric acid to significantly improve digestibility of amino acids in chicks given phytase was unexpected. The reason for the absence of response is unknown. A possible explanation may be that the citric acid complexed with Ca and decreased its binding to phytate, increasing the susceptibility of the phytate to hydrolysis by enzyme. However, the additional P liberated could cause a decrease of the effectiveness of phytase as have already demonstrated Ballam *et al*. (1984), Yi *et al*. (1996) and Ravindran *et al*. (2000). In fact, in the second experiment, the AID of CP and the greatest part of AA were significantly increased in absence of CA, due probably to a lower concentration of P in the gut. In any case, there are no references in chicks on this subject. Similarly, Radcliffe *et al*. (1998) and Li *et al*. (1998) in pigs reported no synergistic effects with the combination of phytase and citric acid on performance and mineral digestibility.

In conclusion, apart from the positive effect on the digestibility of phosphorus and some other minerals, addition of phytase to maize-soybean diets resulted in a positive effect on the AIA of CP and AA only in low AP diets. Likewise, the AID of CP and dispensable and indispensable AA were unaffected by CA addition. In our experimental conditions, the combination of CA and phytase did not appear to be a practical solution to improving amino acid digestibilities. Further research is needed to study interactions between phytate, minerals, protein, phytase and citric acid, and the effects of these interactions on nutrient digestibilities.

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Table 1. *Composition of experimental diets (g/kg, as fed basis)*

	Experiment 1[a]						Experiment 2		
	Starter (0 to 3 wk)			Grower-Finisher (3 to 6 wk)			Starter (0 to 3 wk)		
Ingredients	**Control**	**T_2-T_4**	**T_3 – T_5**	**Control**	**T_2-T_4**	**T_3 – T_5**	**T_1 – T_2**	**T_3-T_4**	**T_5 – T_6**
Maize	522.1	523.5	526.8	610.0	611.7	613.6	516.2	484.1	435.6
Soybean meal	374.3	375.2	376.4	291.1	291.8	292.7	385.0	392.9	405.0
Sunflower oil	45.1	45.1	45.1	45.1	45.1	45.3	58.3	62.5	69.0
Calcium carbonate	11.2	14.7	19.4	10.5	14.1	18.6	13.3	13.3	13.3
Dicalcium phosphate	19.4	13.6	5.8	15.9	9.9	2.4	7.6	7.6	7.5
Salt	2.9	2.9	1.5	2.9	2.9	2.9	3.0	3.0	3.0
DL-methionine	1.5	1.5	1.5	1.0	1.0	1.0	1.6	1.6	1.6
Trace minerals mixture[b]	3.5	3.5	3.5	3.5	3.5	3.5	4.0	4.0	4.0
Vitamins mixture[c]	10.0	10.0	10.0	10.0	10.0	10.0	1.0	1.0	1.0
Celite[d]	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Citric acid	-	-	-	-	-	-	-	20.0	50.0
Natuphos® 5000[e]	-	- / +	- / +	-	- / +	- / +	- / +	- / +	- / +
Nutrient composition									
Calculated (g/kg)									
ME, MJ/kg[f]	12.6	12.6	12.6	12.7	12.7	12.7	13.0	13.0	13.0
Crude protein (N x 6.25)	223.0	223.0	223.0	190.0	190.0	190.0	230.0	230.0	230.0
Lysine	12.5	12.5	12.5	10.4	10.4	10.4	12.8	12.9	13.1
Methionine + cystine	8.8	8.8	8.8	7.6	7.6	7.6	9.0	9.0	9.0
Calcium (Ca)	9.9	9.9	9.9	8.7	8.7	8.7	8.0	8.0	8.0
Total phosphorus (tP)	7.1	6.1	4.9	6.2	5.2	4.0	6.1	6.0	6.0
Available phosphorus	4.5	3.5	2.2	3.7	2.7	1.4	2.5	2.5	2.5
Ca:tP ratio	1.39:1	1.62:1	2.02:1	1.40:1	1.67:1	2.2:1	1.31:1	1.33:1	1.33:1
Analysed (g/kg)									
Crude protein (N x 6.25)	221.0	220.8	221.9	186.6	188.8	186.7	231.0	229.0	230.0
Calcium	12.9	11.9	11.9	11.4	10.4	11.2	8.3	8.1	8.4
Phosphorus, total	8.1	7.1	5.8	7.6	6.4	5.2	6.3	6.0	6.6

[a] In experiment 1, birds in the control group (T_1) were given 4.5 g/kg available P (AP) from 1 d of age to 3 wk (starter diets) and 3.7 g/kg AP from 3 to 6 wk (grower-finisher diets) without enzyme added. The AP level was reduced by 1 g/kg in each period in T_2 and T_4 and by 2.3 g/kg in each period in T_3 and T_5.

[b] Mineral mix supplied the following per kg of diet: Mn, 55 mg; Zn, 50 mg; Fe, 80 mg; Cu, 5 mg; Se, 0.1 mg; I, 0.18 mg.

[c] Vitamin mix supplied the following per kilogram of diet: retinol, 2.5 mg; cholecalciferol, 25 µg; α-tocopherol acetate, 7.34 mg; menadione, 1.1 mg; cyanocobalamin, 11.5 µg; riboflavin, 5.5 mg; Ca panthotenate, 11 mg; niacin, 53.3 mg; choline chloride, 1,020 mg; folic acid, 0.75 mg; biotin, 0,25 mg; delaquin, 125 mg; DL-methionine, 500 mg.

[d] Celite Corp., Lompoc, CA 93436.

[e] Natuphos® 5000 (BASF Corp., Mt. Olive, NJ 07828-1234) was used as the source of microbial phytase to provide 500 (T_4 and T_5, experiment 1) and 750 (T_2, T_4 and T_6; experiment 2) units phytase /kg diet.

[f] Metabolisable energy (ME) was estimated using FEDNA Tables (2003).

Table 2. *Analysed amino acid composition of the diets (g/kg dry matter)*

	Experiment 1 (3 to 6 weeks)			Experiment 2 (1 d to 3 weeks)		
	Available phosphorus (g/kg)			Citric acid (g/kg)		
	3.7	2.7	1.4	0	20	50
Indispensable						
Arginine	13.6	13.6	13.3	14.6	16.1	16.0
Histidine	5.4	5.5	5.3	5.8	6.3	6.2
Isoleucine	7.8	7.7	7.5	8.8	9.0	9.1
Leucine	17.4	17.5	16.9	20.2	19.6	19.4
Lysine	10.8	10.8	10.1	12.1	13.0	13.0
Methionine	3.6	3.6	3.6	3.9	4.5	4.3
Phenylalanine	10.2	10.1	9.8	12.2	12.1	12.1
Threonine	6.9	7.7	7.4	8.0	8.9	8.9
Valine	8.5	8.3	8.1	8.8	9.3	9.2
Dispensable						
Alanine	12.5	12.0	10.7	10.5	10.7	10.7
Aspartate	17.4	19.9	19.1	22.1	24.5	24.8
Glutamate	30.0	32.1	28.0	39.4	41.7	41.8
Glycine	8.7	8.6	8.5	8.4	9.4	9.4
Proline	10.6	10.5	10.1	12.6	11.9	11.7
Serine	9.5	10.3	10.0	11.5	11.9	12.0
Tyrosine	7.3	7.6	7.4	8.0	8.5	8.2
Cystine	2.3	2.3	2.3	2.4	2.7	2.6

Table 3. *Effect of available phosphorus (AP) level with and without microbial phytase on apparent ileal digestibility coefficient of protein and indispensable amino acids in 42-d-old male chickens[1]. Experiment 1.*

			Digestibility coefficients									
Treatment	AP	Phytase	CP	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Val
	(g/kg)	(U/Kg)										
1 (Control)	3.7	-	0.794^{a}	0.834^{b}	0.791^{c}	0.774^{bc}	0.790^{c}	0.787^{bc}	0.823^{c}	0.779^{bc}	0.686^{b}	0.763^{bc}
2	2.7	-	0.784^{ab}	0.878^{a}	0.835^{ab}	0.818^{ab}	0.838^{ab}	0.836^{ab}	0.880^{ab}	0.821^{ab}	0.748^{a}	0.804^{ab}
3	1.4	-	0.749^{b}	0.836^{b}	0.776^{c}	0.754^{c}	0.777^{c}	0.755^{c}	0.838^{bc}	0.764^{c}	0.683^{b}	0.741^{c}
4	2.7	500	0.806^{a}	0.864^{ab}	0.817^{bc}	0.799^{bc}	0.817^{bc}	0.808^{abc}	0.858^{bc}	0.797^{bc}	0.729^{b}	0.784^{bc}
5	1.4	500	822^{a}	0.900^{a}	0.867^{a}	0.850^{a}	0.865^{a}	0.855^{a}	0.902^{a}	0.854^{a}	0.789^{a}	0.839^{a}
Pooled SEM			0.034	0.031	0.033	0.039	0.036	0.045	0.033	0.038	0.047	0.039
Main effects[2]												
AP												
2.7			0.795	0.872	0.827	0.809	0.828	0.823	0.870	0.810	0.740	0.795
1.4			0.786	0.868	0.821	0.802	0.821	0.805	0.870	0.809	0.736	0.790
Phytase												
0			0.767	0.857	0.806	0.786	0.807	0.796	0.859	0.792	0.716	0.773
500			0.814	0.883	0.844	0.827	0.843	0.833	0.882	0.828	0.762	0.814
Statistical significance			Probabilities									
AP effect			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Phytase effect			**	*	**	*	*	*	*	*	*	*
AP x phytase			NS	***	***	***	***	***	***	***	**	***

[a-c] Means in columns with no common superscript differ significantly (P<0.05).
[1] Values are means of 6 replications of 8 chicks each.
[2] Data analysed as a 2 x 2 factorial design, excluding the control group.
*** P<0.001; ** P<0.01; * P<0.05; NS not significant.

Table 4. *Effect of available phosphorus (AP) level with and without microbial phytase on apparent ileal digestibility coefficient of dispensable amino acids in 42-d-old male chickens[1]. Experiment 1.*

			Digestibility coefficients							
Treatment	AP	Phytase	Ala	Asp	Glu	Gly	Prol	Ser	Tyr	Cys
	(g/kg)	(U/Kg)								
1 (Control)	3.7	-	0.806[ab]	0.739[c]	0.808[c]	0.745[bc]	0.788[bc]	0.747[bc]	0.674[ab]	0.713[bc]
2	2.7	-	0.841[a]	0.769[bc]	0.870[ab]	0.779[ab]	0.827[ab]	0.802[ab]	0.713[ab]	0.745[ab]
3	1.4	-	0.750[b]	0.757[bc]	0.838[bc]	0.711[c]	0.774[c]	0.705[c]	0.650[b]	0.670[c]
4	2.7	500	0.811[ab]	0.787[b]	0.861[ab]	0.751[bc]	0.809[bc]	0.779[ab]	0.723[ab]	0.714[bc]
5	1.4	500	0.779[ab]	0.845[a]	0.900[a]	0.820[a]	0.861[a]	0.837[a]	0.755[a]	0.791[a]
Pooled SEM			0.049	0.035	0.035	0.038	0.030	0.052	0.067	0.038
Main effects[2]										
AP										
2.7			0.827	0.778	0.866	0.766	0.819	0.792	0.718	0.731
1.4			0.764	0.801	0.869	0.765	0.817	0.771	0.703	0.730
Phytase										
0			0.795	0.763	0.854	0.745	0.801	0.753	0.682	0.707
500			0.794	0.819	0.883	0.788	0.837	0.811	0.740	0.756
Statistical significance			Probabilities							
AP effect			**	NS	NS	NS	NS	NS	NS	NS
Phytase effect			NS	***	*	*	*	*	*	*
AP x phytase			NS	*	*	***	***	**	*	***

[a-c] Means in columns with no common superscript differ significantly (*P*<0.05).
[1] Values are means of 6 replications of 8 chicks each.
[2] Data analysed as a 2 x 2 factorial design, excluding the control group.
[***] *P*<0.001; [**] *P*<0.01; [*] *P*<0.05; NS not significant.

Table 5. *Effect of citric acid (CA) and phytase on weight gain, feed consumption, and gain:feed in 21-d-old male chickens[a]. Experiment 2*

Treatment	CA	Phytase[3]	Weight gain (g)	Feed intake (g)	Gain:feed (g/g)
	(g/kg)	(U/Kg)			
1	0	0	583[a]	782	1.36
2	0	750	565[abc]	778	1.38
3	20	0	567[ab]	775	1.37
4	20	750	557[abc]	750	1.33
5	50	0	533[c]	729	1.37
6	50	750	540[bc]	756	1.37
Pooled SEM			67.95	50.35	0.04
Main effects[2]					
CA					
0			574[a]	780	1.37
20			562[a]	763	1.35
50			537[b]	742	1.37
Phytase					
0			561	762	1.36
750			554	761	1.36
Statistical significance					
CA effect			**	NS	NS
Phytase effect			NS	NS	NS
CA x phytase			NS	NS	NS
Contrast Diet 1 vs 2			NS	NS	NS
Contrast Diet 3 vs 4			NS	NS	NS
Contrast Diet 5 vs 6			NS	NS	NS

[a-c] Means in columns with no common superscript differ significantly (P<0.05).
[1] Values are means of 6 replications of 8 chicks each.
[2] Data analysed as a 3 x 2 factorial design.
[***] P<0.001; [**] P<0.01; [*] P<0.05; NS not significant.

Table 6. *Effect of citric acid (CA) and phytase on apparent ileal digestibility coefficient of protein and indispensable amino acids in 21-d-old male chickens[1]. Experiment 2*

Treatment	CA	Phytase	Digestibility coefficients									
			CP	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Val
	(g/kg)	(U/Kg)										
1	0	0	0.855^{c}	0.918^{b}	0.877^{b}	0.889	0.911^{ab}	0.916	0.925^{b}	0.887^{b}	0.899^{b}	0.884
2	0	750	0.885^{a}	0.936^{a}	0.910^{a}	0.907	0.921^{a}	0.932	0.948^{a}	0.912^{a}	0.856^{ab}	0.894
3	20	0	0.862^{bc}	0.942^{a}	0.919^{a}	0.904	0.912^{ab}	0.931	0.942^{a}	0.903^{ab}	0.868^{a}	0.895
4	20	750	0.881^{a}	0.931^{ab}	0.905^{ab}	0.890	0.902^{ab}	0.916	0.943^{a}	0.887^{b}	0.844^{ab}	0.876
5	50	0	0.856^{c}	0.933^{ab}	0.900^{ab}	0.894	0.897^{b}	0.918	0.932^{ab}	0.884^{b}	0.837^{b}	0.876
6	50	750	0.876^{ab}	0.937^{a}	0.905^{ab}	0.891	0.902^{ab}	0.920	0.936^{ab}	0.891^{b}	0.845^{ab}	0.873
Pooled SEM			0.012	0.014	2.37	0.015	0.016	0.014	0.012	0.015	0.020	0.024
Main effects[2]												
CA												
0			0.870	0.927	0.893	0.899	0.916^{a}	0.924	0.938	0.899	0.842	0.889
20			0.873	0.936	0.911	0.896	0.906^{ab}	0.923	0.943	0.895	0.855	0.885
50			0.866	0.935	0.903	0.892	0.900^{b}	0.919	0.934	0.888	0.841	0.874
Phytase												
0			0.857	0.930	0.898	0.896	0.906	0.921	0.933	0.891	0.843	0.885
750			0.881	0.935	0.907	0.896	0.908	0.923	0.943	0.897	0.848	0.881
Statistical significance												
CA effect			NS	NS	NS	NS	*	NS	NS	NS	NS	NS
Phytase effect			***	NS	NS	NS	NS	NS	*	NS	NS	NS
CA x phytase			NS	NS	NS	NS	NS	NS	NS	**	*	NS
Contrast Diet 1 vs 2			***	NS	NS	NS	NS	NS	*	**	*	NS
Contrast Diet 3 vs 4			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Contrast Diet 5 vs 6			*	NS	NS	NS	NS	NS	NS	NS	NS	NS

[a-c] Means in columns with no common superscript differ significantly (P<0.05).
[1] Values are means of 6 replications of 8 chicks each.
[2] Data analysed as a 3 x 2 factorial design.
[***] P<0.001; [**] P<0.01; [*] P<0.05; NS not significant.

Table 7. *Effect of citric acid (CA) and phytase on apparent ileal digestibility coefficient of dispensable amino acids in 21-d-old male chickens*[1]*. Experiment 2*

Treatment	CA	Phytase	Digestibility coefficients							
			Ala	Asp	Glu	Gly	Prol	Ser	Tyr	Cys
	(g/kg)	(U/Kg)								
1	0	0	0.871	0.872^{c}	0.932^{bc}	0.833^{b}	0.892^{ab}	0.869^{b}	0.778^{b}	0.799^{ab}
2	0	750	0.891	0.894^{ab}	0.940^{ab}	0.860^{a}	0.913^{a}	0894^{a}	0.809^{ab}	0.822^{ab}
3	20	0	0.875	0.909^{a}	0.950^{a}	0.874^{a}	0.872^{bc}	0.888^{ab}	0.831^{a}	0.843^{a}
4	20	750	0.874	0.890^{bc}	0.931^{bc}	0.856^{ab}	0.851^{c}	0.871^{b}	0.777^{b}	0.809^{ab}
5	50	0	0.881	0.886^{bc}	0.923^{c}	0.856^{ab}	0.845^{c}	0.866^{b}	0.766^{b}	0.783^{b}
6	50	750	0.864	0.895^{ab}	0.938^{abc}	0.858^{ab}	0.857^{c}	0.875^{ab}	0.809^{ab}	0.813^{ab}
Pooled SEM			0.020	0.014	0.011	0.019	0.024	0.017	0.035	0.034
Main effects[2]										
CA										
0			0.881	0.883^{a}	0.936	0.847^{b}	0.903^{a}	0.881	0.793	0.811
20			0.874	0.898^{a}	0.940	0.864^{a}	0.861^{b}	0.879	0.801	0.824
50			0.872	0.890^{ab}	0.930	0.857^{ab}	0.851^{b}	0.870	0.788	0.798
Phytase										
0			0.875	0.888	0.934	0.853	0.870	0.873	0.789	0.807
750			0.877	0.890	0.936	0.858	0.873	0.880	0.798	0.814
Statistical significance										
CA			NS	*	NS	*	***	NS	NS	NS
Phytase			NS	NS	NS	NS	NS	NS	NS	NS
CA x phytase			NS	**	**	*	NS	*	**	NS
Contrast Diet 1 vs 2			NS	**	NS	*	NS	*	NS	NS
Contrast Diet 3 vs 4			NS	*	**	NS	NS	NS	*	NS
Contrast Diet 5 vs 6			NS	NS	NS	NS	NS	NS	NS	NS

[a-c] Means in columns with no common superscript differ significantly (*P*<0.05).
[1] Values are means of 6 replications of 8 chicks each.
[2] Data analysed as a 3 x 2 factorial design.
[***] *P*<0.001; [**] *P*<0.01; [*] *P*<0.05; NS not significant.