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# Influence of different levels of calcium, non-phytate phosphorus and phytase on apparent metabolizable energy, nutrient utilization, plasma mineral concentration and digestive enzyme activities of broiler chickens

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**ABSTRACT**

The study investigated the effect of different levels of dietary Ca, non-phytate phosphorus (NPP) and phytase on the nutrient utilization of broiler chickens. A total of 576 day-old broiler chicks (Ross 308) were offered diets containing 3 concentrations of Ca (6, 8 or 10 g/kg diet), 2 concentrations of NPP (3 or 4 g/kg) and 2 concentrations of exogenous microbial phytase (0 or 500 FTU/kg). Diets with high Ca (10 g/kg) reduced ($p < .001$) the ileal digestibilities of P and protein. The digestibility of Ca and P was negatively ($p < .001$) affected by high Ca and low NPP (3 g/kg) diet supplemented with phytase. Phytase improved ($p < .001$) the digestibility of protein and P and also the retention of P and Ca. Phytase supplemented to diets containing 8 g Ca/kg improved ($p < .030$) the tissue protein content of jejunal mucosa, but reduced ($p < .009$) the Ca-ATPase activity. Supplemental phytase elevated the plasma P level, especially in birds fed diets containing 6 g/kg Ca and 4 g/kg NPP. In conclusion, phytase supplementation of diets reduced the excretion of minerals by improving their digestibilities; however, this response was dependent on the concentration of dietary Ca and NPP.

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Broilers; phytase; calcium; non-phytate P; digestibility; plasma

## 1. Introduction

Poultry diets comprise mainly cereals and oilseed cakes and contain variable amounts of phosphorus in the form of phytic acid or its salt, phytate. In addition to binding phosphorus, phytic acid also forms complexes with other minerals and nutrients in the gastrointestinal tract (GIT) of poultry. Hydrolysis of phytate by phytase is essential to liberate the bound nutrients in the GIT for absorption (Cowieson et al. 2004). Poultry diets are commonly supplemented with exogenous microbial phytase for effective dephosphorylation of phytate because of the low endogenous phytase activity (Selle et al. 2000). In general, adding microbial phytase to diets enhances the bird's growth performance and mineral digestibility, and reduces nutrient excretion to the environment (Selle & Ravindran 2007).

It has been reported that microbial phytase supplementation may influence the protein or amino acid digestibility in poultry by releasing the phytate-bound protein and increasing their utilization (Ravindran et al. 2000). In contrast, Adeola and Sands (2003) reported no effect of phytase on protein utilization. Similarly, controversy exists in the literature on the effect of phytase on dietary apparent metabolisable energy (AME).

This variation in the effect of phytase on energy and protein utilization could be the result of different influential factors, such as the source and concentration of phytate and protein in diet, protein digestibility and concentration of Ca and P in diets (Selle et al. 2000). Several studies have revealed an improvement in amino acid and protein digestibility when phytase is added to low non-phytate phosphorus (NPP) diet (Yi et al. 1996a, Ravindran et al. 2000). In contrast, Boling-Frankenbach et al. (2001) found no significant effect of NPP and Ca on phytase efficacy in relation to protein digestibility. High dietary Ca concentration tends to increase the pH of the gizzard and proventriculus and facilitates the formation of highly insoluble phytase-resistant Ca–phytate complexes (Gifford & Clydesdale 1990). This increases the risk of undigested phytate in the intestine binding to protein and resulting in lower protein digestion. Moreover, there is some evidence regarding the formation of ternary protein–phytate complexes in the presence of divalent ions, especially Ca, under the alkaline pH condition of the small intestine, but the effects of these complexes on protein digestion is not clearly evident (Selle et al. 2000).

From the foregoing, it can be deduced that low Ca (from low limestone) is reported to slightly reduce gastric pH on account of there being less limestone and, therefore, a lower acid-binding effect; but at higher concentration, it negatively affects phytase activity due to the formation of Ca–phytate complexes. Therefore, the present study was conducted to examine the influence of three levels of Ca and two levels of NPP on the efficacy of microbial phytase, and their impact on AME, ileal digestibilities of protein and minerals, and the retention of minerals by broiler chickens.

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## 2. Materials and methods

### 2.1. Animal ethics

All experimental procedures were approved by the University of New England Animal Care and Ethics Committee (Approval No: AEC13–167).

### 2.2. Experimental design and birds' management

A total of 576 day-old male Ross 308 broiler chicks were distributed to 72 cages in six multi-tier brooder units, located in two environmentally controlled rooms. There were 6 replicate cages (8 birds/cage) per dietary treatment. Every cage was equipped with a stainless steel feeder and two nipple drinkers. The cage floor (wire mesh) was covered with a soft plastic mesh during the first 10 days. During the first three days, room temperature was maintained at 35°C and then was reduced gradually to 24 ± 1°C at 21 days of age and maintained at this temperature until the end of the experiment. Twenty-three hours of lighting per day was provided for the first 3 days and then 18 hours per day was maintained for the rest of the trial period. Birds were provided feed and water *ad libitum* throughout the experimental period. Body weight (BW) and feed leftover were recorded on day 24 on a cage basis for the determination of body weight gain (BWG) and feed intake (FI). Mortality was recorded as it occurred and feed conversion ratio (FCR; FI/BWG) was corrected for mortality.

### 2.3. Dietary treatments

The experiment was conducted in a 3 × 2 × 2 factorial arrangement. Twelve experimental diets were formulated with three levels of Ca (6, 8 or 10 g/kg diet), two levels of NPP (3 or 4 g/kg diet) and two levels of exogenous microbial phytase (0 or 500 U/kg diet). The Ca, NPP and Na levels in the phytase-supplemented diets were calculated to include the mineral matrix (1.5 g/kg NPP, 1.65 g/kg Ca and 0.35 g/kg Na) of the commercial phytase product (Quantum Blue, AB Vista, Marlborough, UK) derived from *Escherichia coli*. The phytase activity of the product was 5000 U/g, where a unit (U) is defined as the quantity of enzyme that liberates 1 µmol of inorganic P per minute from sodium phytate at pH 5.5 and 37°C. The dietary treatments were as follows: (1) 6 g Ca + 3 g NPP (T1), (2) 6 g Ca + 4 g NPP (T2), (3) 8 g Ca + 3 g NPP (T3), (4) 8 g Ca + 4 g NPP (T4), (5) 10 g Ca + 3 g NPP (T5), (6) 10 g Ca + 4 g NPP (T6), (7) T1 + phytase (T7), (8) T2 + phytase (T8), (9) T3 + phytase (T9), (10) T4 + phytase (T10), (11) T5 + phytase (T11) and (12) T6 + phytase (T12). Titanium dioxide (5 g/kg) was added to all grower diets as an indigestible marker for digestibility analysis. After mixing, the diets were pelleted at 65°C. The diets were formulated to be iso-energetic and iso-nitrogenous, and were fed as starter (0–10 days) and grower (11–24 days). The ingredient composition and nutrient specifications of diets are presented in Tables 1 and 2. All the diets were formulated to either meet or exceed the Aviagen (2009) nutrient recommendations and breed standards, with the exception of Ca and NPP.

### 2.4. Collection and processing of samples

On day 21, the excreta trays in each cage were cleaned and aluminium foil was placed on the trays. Droppings from each cage were collected from 22 to 24 days. The excreta samples were then mixed thoroughly and subsamples were collected in plastic containers, weighed and kept at −20°C until further analysis. On day 24, approximately 10 ml of blood from two birds from each cage were collected in a heparinized vacutainer

**Table 1.** Ingredient composition and nutrient specification of the starter diets (0–10 days).

| Ingredient composition (g/kg) | T1 | T2 | T3 | T4 | T5 | T6 | T7 | T8 | T9 | T10 | T11 | T12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Maize | 587.6 | 595.1 | 590.7 | 585.9 | 580.6 | 576.3 | 596.6 | 588.7 | 586.4 | 589.8 | 576.9 | 592.5 |
| Soybean meal | 352.7 | 336.4 | 335.8 | 336.3 | 336.9 | 337.4 | 359.5 | 364.9 | 362.4 | 352.1 | 362.9 | 335.6 |
| Meat meal | 11.4 | 22.4 | 23.4 | 23.7 | 24 | 24.2 | 5.2 | 2.2 | 4.4 | 11.5 | 5.4 | 23.3 |
| Canola oil | 20.0 | 18.2 | 19.7 | 21.3 | 23.0 | 24.4 | 16.7 | 19.1 | 20 | 19.3 | 23.1 | 19.1 |
| Limestone | 6.6 | 2.6 | 10.7 | 7.7 | 15.9 | 12.9 | 7.4 | 4.7 | 12.7 | 9.0 | 17.9 | 13.2 |
| DCP | 6.9 | 10.2 | 4.6 | 9.9 | 4.5 | 9.8 | 0 | 5.9 | 0.2 | 4.2 | 0 | 2.0 |
| Salt | 2.0 | 2.0 | 2.0 | 2.0 | 1.9 | 1.8 | 2.0 | 2.0 | 1.4 | 1.2 | 1.4 | 1.0 |
| $NaHCO_3$ | 2.0 | 2.0 | 1.8 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| Premix[1] | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Choline-Cl | 0.8 | 0.8 | 0.9 | 0.9 | 0.9 | 0.9 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.9 |
| L-lysine HCl | 2.8 | 3.1 | 3.1 | 3.0 | 3.0 | 3.0 | 2.7 | 2.7 | 2.7 | 2.8 | 2.7 | 3.1 |
| DL-methionine | 4.0 | 4.0 | 4.1 | 4.1 | 4.1 | 4.1 | 3.9 | 3.9 | 3.9 | 4 | 3.9 | 4.1 |
| L-threonine | 1.8 | 1.9 | 1.9 | 1.9 | 1.9 | 1.9 | 1.8 | 1.7 | 1.8 | 1.8 | 1.8 | 1.9 |
| Phytase (U/kg) | – | – | – | – | – | – | 500 | 500 | 500 | 500 | 500 | 500 |
| Calculated analysis[2] | | | | | | | | | | | | |
| Calcium | 6 | 6 | 8 | 8 | 10 | 10 | 6 | 6 | 8 | 8 | 10 | 10 |
| NPP | 3 | 4 | 3 | 4 | 3 | 4 | 3 | 4 | 3 | 4 | 3 | 4 |
| Total P | 5.4 | 6.4 | 5.4 | 6.4 | 5.4 | 6.4 | 4 | 5 | 3.9 | 4.9 | 3.9 | 4.9 |
| Analysed values | | | | | | | | | | | | |
| Calcium | 6.8 | 7.9 | 10 | 9 | 10 | 13 | 6.5 | 6.8 | 8.1 | 8.3 | 10 | 9 |
| Total P | 6 | 7 | 6 | 7 | 7 | 7 | 4 | 5 | 5 | 6 | 4 | 6 |
| Fe (mg/kg) | 104.2 | 103.1 | 104.3 | 102.9 | 105.8 | 105.2 | 105.2 | 105.4 | 102.5 | 102.7 | 102.1 | 103.8 |
| Zn (mg/kg) | 98 | 98.9 | 99.1 | 101.4 | 102.1 | 100 | 98.6 | 98.3 | 100.4 | 103.1 | 99.6 | 101.8 |
| Phytase | 45 | 40 | 30 | 45 | 40 | 42 | 510 | 526 | 531 | 550 | 545 | 550 |

[1]Supplied per kg of diet (g): 3.6 mg vitamin A (as all-trans retinol); 0.09 mg cholecalciferol; 44.7 mg vitamin E (as d-α-tocopherol); 2 mg vitamin K3; 2 mg thiamine; 6 mg riboflavin; 5 mg pyridoxine hydrochloride; 0.2 mg vitamin B12; 0.1 mg biotin; 50 mg niacin; 12 mg D-calcium pantothenate; 2 mg folic acid; 80 mg Mn; 60 mg Fe; 8 mg Cu; 1 mg I; 0.3 mg Co; 1 mg Mo, DCP = Dicalcium phosphate; $NaHCO_3$ = Sodium bicarbonate.

[2]All diets were formulated to contain 12.7 MJ/kg metabolizable energy; 220 g/kg crude protein; 6.9 g/kg digestible methionine; 12.7 g/kg digestible lysine; 9.4 g/kg digestible methionine + cysteine; 8.3 g/kg digestible threonine; 13.7 g/kg digestible arginine.

**Table 2.** Ingredient composition of the grower diets (11–24 days).

| Ingredient composition (g/kg) | T1 | T2 | T3 | T4 | T5 | T6 | T7 | T8 | T9 | T10 | T11 | T12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Maize | 619.1 | 615 | 608.6 | 605 | 598.1 | 594.4 | 588 | 615.3 | 577.4 | 606.2 | 566.9 | 594.2 |
| Soybean meal | 279.5 | 279.7 | 281.3 | 280.7 | 283.1 | 282.7 | 343.1 | 299.6 | 344.9 | 301.1 | 346.7 | 303.2 |
| Meat meal | 49.0 | 49.4 | 49.1 | 50 | 49.2 | 50.0 | 6.3 | 34.7 | 6.4 | 34.8 | 6.6 | 34.9 |
| Canola oil | 31.9 | 33.3 | 35.3 | 36.5 | 38.7 | 40 | 39.4 | 32.3 | 42.8 | 35.3 | 46.2 | 39.1 |
| Limestone | 3.0 | 0 | 8.3 | 5.2 | 13.5 | 10.4 | 7.3 | 1.6 | 12.5 | 6.8 | 17.7 | 12.0 |
| DCP | 0 | 5.3 | 0 | 5.2 | 0 | 5.2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Salt | 1.5 | 1.4 | 1.5 | 1.4 | 1.5 | 1.4 | 1.5 | 1.0 | 1.5 | 2.0 | 1.5 | 1.0 |
| $NaHCO_3$ | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0.3 | 2 | 2 |
| $TiO_2$ | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| Premix[1] | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Choline Cl | 1 | 1 | 1 | 1 | 1 | 1 | 0.9 | 1 | 0.9 | 1 | 0.9 | 1 |
| L-lysine HCl | 2.0 | 2.0 | 1.9 | 1.9 | 1.9 | 1.9 | 1.1 | 1.7 | 1.1 | 1.7 | 1.0 | 1.6 |
| DL-methionine | 3.4 | 3.4 | 3.4 | 3.4 | 3.4 | 3.4 | 3.1 | 3.3 | 3.1 | 3.3 | 3.1 | 3.3 |
| L-threonine | 1.4 | 1.4 | 1.4 | 1.4 | 1.4 | 1.4 | 1.0 | 1.2 | 1.0 | 1.2 | 1.0 | 1.2 |
| Phytase (U/kg) | – | – | – | – | – | – | 500 | 500 | 500 | 500 | 500 | 500 |
| Calculated values[2] | | | | | | | | | | | | |
| Calcium | 6 | 6 | 8 | 8 | 10 | 10 | 6 | 6 | 8 | 8 | 10 | 10 |
| NPP | 3 | 4 | 3 | 4 | 3 | 4 | 3 | 4 | 3 | 4 | 3 | 4 |
| Total P | 5.3 | 5.8 | 5.3 | 6.3 | 5.3 | 6.2 | 3.9 | 4.8 | 3.9 | 4.8 | 3.9 | 4.8 |
| Analysed values | | | | | | | | | | | | |
| Calcium | 7.1 | 7.5 | 9.1 | 8.9 | 10.4 | 10.2 | 6.0 | 6.0 | 8.6 | 8.3 | 10.1 | 10.0 |
| Total P | 5.6 | 6.8 | 5.4 | 7.3 | 6.5 | 6.3 | 4.2 | 5.3 | 4.6 | 5.3 | 4.6 | 5.0 |
| Fe (mg/kg) | 106.2 | 105.1 | 106.3 | 104.9 | 107.8 | 107.2 | 107.2 | 107.4 | 104.5 | 104.7 | 104.1 | 105.8 |
| Zn (mg/kg) | 100.0 | 100.9 | 101.1 | 103.4 | 104.1 | 102 | 100.6 | 100.3 | 102.4 | 105.1 | 101.6 | 103.8 |
| Phytase(U/kg) | 40 | 41 | 30 | 45 | 39 | 42 | 550 | 526 | 538 | 550 | 565 | 559 |

[1]Diet composition as in Table 1; DCP = Dicalcium phosphate; $NaHCO_3$ = Sodium bicarbonate.
[2]Diets were formulated to contain 13.2 MJ/kg metabolizable energy; 210 g/kg crude protein; 6.1 g/kg digestible methionine; 11.0 g/kg digestible lysine; 8.4 g/kg digestible methionine + cysteine; 7.3 g/kg digestible threonine; 12.6 g/kg digestible arginine.

tube, placed in an ice bath and immediately sent to the laboratory for harvesting of plasma. Two birds from each cage were randomly selected, weighed and then slaughtered by cervical dislocation. The abdominal cavity was opened and the small intestine was removed. Immediately after cervical dislocation, a part (around 4 mm) of the proximal jejunum was excised and opened to expose the mucosa, which was rinsed with normal saline (0.9% NaCl). The sample was carefully wrapped in aluminium foil, labelled with a permanent marker, then snap-frozen in liquid nitrogen and stored at −20°C for enzyme analysis. The contents from the ileum were collected by gently squeezing into separate, labelled plastic containers. The ileum was considered as that part of the small intestine extending from Meckel's diverticulum to the ileocaecal junction. Ileal digesta from the two birds from each cage were pooled and stored at −20°C. Ileal digesta and excreta samples were freeze-dried (Martin Christ Gerfriertrocknungsanlagen, GmbH, Osterode am Harz, Germany). Dry matter (DM) content was determined according to AOAC (1994). Diets, dried ileal digesta and excreta samples were then ground to pass through a 0.5-mm sieve, by using a stainless steel blade grinder, and stored at 4°C until analysis.

## 2.5. Chemical analysis

### 2.5.1. Mineral analysis

Samples of diets, freeze-dried excreta and ileal digesta were analysed for mineral contents using the inductively coupled plasma (ICP) method (Vista MPX, Melbourne, Australia) according to the protocol of Anderson and Henderson (1986). The sealed chamber digest method was used for Ca, P, S, K, Na, Mg and trace elements (Fe and Zn). This digest is appropriate for ICP analyses, in which final oxidation occurs in the high-temperature plasma of the ICP. Phytase activity of the diets was analysed by the method of Kim and Lei (2005).

### 2.5.2. Measurement of apparent nutrient digestibility

The $TiO_2$ concentration of diet, freeze-dried ileal digesta and excreta samples was measured as per the method of Short et al. (1996). The diets, ileal digesta and excreta samples were analysed for gross energy using an IKA bomb calorimeter (IKA-WERKE, C7000, Staufen, Germany). The nitrogen contents of the digesta, diets and excreta were determined according to the Dumas combustion technique as described by Sweeney (1989), using a LECO FP-2000 automatic nitrogen analyser (Leco Corp., St. Joseph, MI, USA), and then converted to crude protein usually by multiplying by 6.25. These data were then used to calculate nutrient digestibility.

$$\text{Digestibility coefficient} = 1 - \frac{\text{Digesta nutrient (g/kg)/Digesta TiO2 (g/kg)}}{\text{Diet nutrient(g/kg)/Diet TiO2(g/kg)}}. \quad (1)$$

The AME was calculated using the following equation:

$$\text{AME}_{\text{diet}}(\text{MJ/kg}) = \text{GE diet} - [\text{GE}_{\text{excreta}}(\text{TiO}_{2\text{diet}}/\text{TiO}_{2\text{excreta}})]. \quad (2)$$

Total nutrient retention (TNR) was calculated based on the following equation of Viveros et al. (2002):

$$100\% - [100\% \times (\text{TiO}_2 \text{ in diet}/\text{TiO}_2 \text{ in excreta})/(\text{Nutrient in excreta}/\text{Nutrient in diet})] \quad (3)$$

(Equations 1, 2 and 3 related to the nutrient digestibility measurement supplied in a separate file).

### 2.5.3. Tissue protein and digestive enzyme analysis

The jejunal tissue was processed as described by Shirazi-Beechey et al. (1991) for the assessment of total tissue protein

and digestive enzyme activities. The frozen tissue was weighed and cut into an ice-cold buffer (100 mM mannitol, 2 mM HEPES/Tris, pH 7.1). The mucosa was then stripped into the buffer using a vortex mixer at high speed for one min. After filtration through a Buchner funnel, the mixture was homogenized at medium speed (No. 2, 13,000 rpm) for 30 s using an Ultra Turrax T 25 Basic Homogenizer (IKA® Works, Wilmington, NC, USA). Subsamples of the homogenate were transferred into Eppendorf tubes (Eppendorf South Pacific, North Ryde, Australia) and stored in a freezer (–20°C) for enzyme analysis.

The specific activities of jejunal enzymes were assessed by incubation with fixed substrate concentrations as standardized for poultry by Iji et al. (2001). On the jejunal homogenates, assays were conducted for mucosal protein content and activity of alkaline phosphatase (AP; EC 3.1.3.1). The concentration of protein in the jejunal tissue was measured by using the Coomassie dye-binding procedure described by Bradford (1976). All of the raw data for protein concentration were processed through the Lowry software (Mcpherson 1985), before statistical analysis. The specific activity of AP was measured according to the method previously described for other species (Holdsworth 1970), after standardization for poultry. Ca-Mg ATPase, Ca-ATPase and Mg-ATPase activities were analysed according to the method described by Qin et al. (1993).

#### 2.5.4. Blood mineral contents and enzyme activities

Blood samples were collected on day 24 and centrifuged at 2000×*g* for 10 min at 4°C, 6 hours after collection. Plasma was then transferred to Eppendorf tubes and stored at −20°C until analysis. Minerals and enzymes in plasma were measured with a clinical analyser (Siemens Dimension plus auto analyser, Newark, USA) according to the instructions provided by the manufacturer.

## 3. Statistical analysis

The data were analysed as a 3 × 2 × 2 factorial ANOVA using the general linear model procedure of Minitab software (Minitab 2010). The statistical model included Ca, NPP, phytase levels and their interaction effects. Differences within a significant effect were separated using Tukey's honest significant difference test. Significant differences between the diets were tested using Fisher's least significance difference test at $p \leq .05$.

## 4. Results and discussion

### 4.1. Gross response

The influence of Ca, NPP and phytase levels on the growth performances of broilers is presented in Table 3. From day 0 to 24, a 3-way interaction of Ca, NPP and phytase was observed for FI, where phytase supplementation to diets with 8–10 g Ca and 3 g NPP/kg reduced ($p < .035$) the FI of broilers. A reduction in FI (Ca × phytase, $p < .005$) was observed in the birds that consumed phytase-supplemented diets containing 8–10 g Ca/kg. A similar trend was observed for FI when phytase was supplemented to diets containing 3 g NPP/kg (NPP × phytase, $p < .005$). FI tended ($p = .057$) to reduce in birds fed high Ca and low-NPP diets. High Ca diets with low NPP (Ca × NPP, $p < .001$) reduced the BWG of birds. Additionally, diets with high Ca (10 g/kg) and low NPP (3 g/kg) reduced (Ca × NPP, $p < .001$) the BWG of birds. Phytase supplementation to diets containing 3 g NPP/kg reduced the FI ($p < .005$) and BWG ($p < .035$) and subsequently resulted in poor FCR ($p < .020$). Supplementation of phytase increased ($p < .017$) the BWG but had no effect on FCR.

**Table 3.** Effect of dietary Ca and NPP with and without microbial phytase on FI, weight gain and FCR of broilers at day 0–24[1].

| Ca (g/kg) | NPP (g/kg) | Phytase[2] | Feed intake (g/bird) | Weight gain (g/bird) | FCR |
|---|---|---|---|---|---|
| 6 | 3 | 0 | 1640$^{ab}$ | 1297 | 1.26 |
| | | 500 | 1649$^{ab}$ | 1322 | 1.25 |
| | 4 | 0 | 1593$^{bc}$ | 1283 | 1.25 |
| | | 500 | 1728$^{a}$ | 1347 | 1.28 |
| 8 | 3 | 0 | 1624$^{bc}$ | 1207 | 1.35 |
| | | 500 | 1510$^{c}$ | 1261 | 1.20 |
| | 4 | 0 | 1614$^{abc}$ | 1318 | 1.22 |
| | | 500 | 1542$^{bc}$ | 1261 | 1.21 |
| 10 | 3 | 0 | 1380$^{d}$ | 1030 | 1.35 |
| | | 500 | 1539$^{bc}$ | 1210 | 1.27 |
| | 4 | 0 | 1592$^{bc}$ | 1307 | 1.22 |
| | | 500 | 1621$^{abc}$ | 1303 | 1.24 |
| SEM | | | 58.6 | 46.3 | 0.05 |
| Ca (g/kg) | | | | | |
| 6 | | | 1647$^{a}$ | 1312$^{a}$ | 1.26 |
| 8 | | | 1573$^{b}$ | 1266$^{ab}$ | 1.25 |
| 10 | | | 1533$^{b}$ | 1212$^{b}$ | 1.27 |
| | NPP (g/kg) | | | | |
| | 3 | | 1552$^{b}$ | 1222$^{b}$ | 1.28 |
| | 4 | | 1617$^{a}$ | 1305$^{a}$ | 1.24 |
| | | Phytase | | | |
| | | 0 | 1574 | 1240$^{b}$ | 1.28 |
| | | 500 | 1595 | 1287$^{a}$ | 1.24 |
| Sources of variation | | | | | |
| Ca | | | 0.001 | 0.001 | 0.631 |
| NPP | | | 0.008 | 0.001 | 0.123 |
| Phytase | | | 0.388 | 0.017 | 0.102 |
| Ca × NPP | | | 0.057 | 0.001 | 0.105 |
| Ca × phytase | | | 0.005 | 0.233 | 0.259 |
| NPP × phytase | | | 0.005 | 0.035 | 0.020 |
| Ca × NPP × phytase | | | 0.043 | 0.076 | 0.927 |

[a–d]Means with different superscripts within the columns are different ($p < .05$).
[1]Each value represents the mean of six replicates (six birds per replicate).
[2]U/kg diet, U = Phytase unit.

The study showed that increasing Ca (8–10 g/kg) levels in phytase-supplemented diets, especially with low NPP (3 g/kg), had an adverse effect on the FI of birds. These groups of birds also showed a marginal reduction ($p = .076$) in weight gain. Birds that consumed diet with high Ca and low NPP also showed reduced the FI and BWG. Consumption of diets with high Ca and low NPP may lead to the formation of the Ca–phytate complex or Ca-pyro or orthophosphate in the intestine due to the imbalance between Ca and NPP or wide Ca: NPP ratio, which could prevent the phytate hydrolysis by phytase and consequently reduce the P availability and performance of birds (Sebastian et al. 1996). It is also noted that the negative effect of increased Ca (8–10 g/kg) level on phytase response was mostly counterbalanced when the NPP level increased up to 4 g/kg. These results indicate that maintaining a proper balance between Ca and NPP in diets is also important to optimize the phytase response. The significant two-way interactions between NPP and phytase for FI, BWG and FCR implies that even with phytase supplementation, at least 4 g NPP/kg is needed to optimize the growth performance, especially when the *p*-value of phytase is considered in diet formulation. Dietary P partly

regulates the birds' attitude towards FI; therefore, consuming low-NPP diets for a constant period of time results in reduced FI and substantial BWG, which cannot be countered even with phytase supplementation (Schöner et al. 1994). The phytase-induced improvement in weight gain, especially in birds fed diets with low Ca and high NPP, can be explained by increased FI as there was no significant effect on FCR.

### 4.2. Apparent ileal digestibility of nutrients

Table 4 summarizes the effects of different levels of Ca, NPP and phytase on ileal nutrient digestibility. A significant three-way (Ca × NPP × phytase) interaction was observed for Ca and P digestibility. Phytase supplementation to diets with high Ca and low NPP reduced ($p < .001$) the ileal digestibility of Ca and P. Birds that consumed the diet with high Ca and low NPP showed decreased (Ca × NPP, $p < .001$) digestibility of protein and Ca. High Ca diets supplemented with phytase decreased (Ca × phytase, $p < .006$) the Ca digestibility. Phytase supplemented to 3 g NPP/kg reduced (NPP × phytase, $p < .001$) the ileal digestibility of protein, while a similar trend was observed for Ca digestibility, but with 4 g Ca/kg diet. Phytase supplementation improved the protein, P, Fe ($p < .001$) and Ca ($p < .036$) digestibility. Mg digestibility was reduced ($p < .001$) in birds fed diet with 8–10 g Ca/kg. None of the interaction effects were significant for Mg and Fe digestibility.

High Ca diet with low NPP reduced the ileal digestibility of protein and Ca. Regardless of phytase supplementation, increasing Ca (10 g/kg) concentration in the diet with low NPP decreased the ileal digestibility of Ca and P. The decreased digestibility of Ca and P could be explained by the fact that increased dietary Ca relative to NPP could trigger the negative interaction between these minerals and result in poor digestibility of Ca and P due to the possible formation of calcium–phosphate or Ca–phytate complexes (Maenz et al. 1999, Olukosi & Fru-Nji 2014). The two-way interaction between Ca and P influenced the ileal protein digestibility, and the best ileal protein digestibility was observed in birds fed diet with low Ca and low NPP. This result partly agrees with the findings of Cardoso Júnior et al. (2010) who reported that feeding birds with diet containing 6.5 g/kg of Ca and 3.25 g/kg NPP increased the protein digestibility. These authors also suggested that the Ca and P levels in phytase-supplemented diets can be reduced concomitantly without influencing the performance, provided that the Ca and avP ratio is maintained at 2:1.

On the other hand, improvement in the apparent ileal digestibility of P in birds on the phytase-supplemented diets containing low Ca and high NPP is in line with increased FI and BWG. Phytase-induced degradation and subsequent release of bound P from phytate are one of the main reasons behind improved P digestibility. However, there is speculation that excess excretion of absorbed P in birds fed diets with low Ca reduces the utilization of P for bone formation and other metabolic processes. In that condition, birds consume more feed in an effort to maximize their Ca level. This is believed to be induced by reduced plasma Ca. Although the present study did not reveal any 23 change in plasma Ca, maximum feed consumption was recorded in birds fed low Ca diets.

**Table 4.** Effect of dietary Ca and NPP levels with or without microbial phytase on ileal nutrient digestibility at 24 days of age[1].

| Ca (g/kg) | NPP (g/kg) | Phytase[2] | Protein | Ca | P | Mg | Fe |
|---|---|---|---|---|---|---|---|
| 6 | 3 | 0 | 0.82 | $0.52^{cd}$ | $0.53^{e}$ | 0.30 | 0.45 |
| | | 500 | 0.85 | $0.62^{ab}$ | $0.67^{a}$ | 0.32 | 0.53 |
| | 4 | 0 | 0.80 | $0.54^{cd}$ | $0.60^{bc}$ | 0.31 | 0.37 |
| | | 500 | 0.82 | $0.51^{d}$ | $0.65^{ab}$ | 0.25 | 0.47 |
| 8 | 3 | 0 | 0.76 | $0.50^{de}$ | $0.45^{f}$ | 0.16 | 0.23 |
| | | 500 | 0.83 | $0.65^{a}$ | $0.59^{cd}$ | 0.22 | 0.46 |
| | 4 | 0 | 0.80 | $0.58^{bc}$ | $0.52^{e}$ | 0.25 | 0.33 |
| | | 500 | 0.83 | $0.50^{de}$ | $0.52^{e}$ | 0.23 | 0.46 |
| 10 | 3 | 0 | 0.76 | $0.42^{fg}$ | $0.40^{f}$ | 0.24 | 0.34 |
| | | 500 | 0.74 | $0.42^{fg}$ | $0.42^{f}$ | 0.23 | 0.59 |
| | 4 | 0 | 0.81 | $0.43^{fg}$ | $0.54^{de}$ | 0.25 | 0.34 |
| | | 500 | 0.78 | $0.47^{de}$ | $0.39^{f}$ | 0.23 | 0.48 |
| SEM | | | 0.002 | 0.01 | 0.01 | 0.01 | 0.08 |
| Main effects | | | | | | | |
| Ca (g/kg) | | | | | | | |
| 6 | | | $0.82^{a}$ | $0.52^{a}$ | $0.61^{a}$ | $0.29^{a}$ | 0.46 |
| 8 | | | $0.80^{b}$ | $0.53^{a}$ | $0.52^{b}$ | $0.21^{b}$ | 0.37 |
| 10 | | | $0.78^{c}$ | $0.43^{b}$ | $0.44^{c}$ | $0.24^{b}$ | 0.44 |
| | NPP (g/kg) | | | | | | |
| | 3 | | 0.81 | $0.52^{a}$ | $0.51^{b}$ | 0.24 | 0.43 |
| | 4 | | 0.82 | $0.47^{b}$ | $0.54^{a}$ | 0.25 | 0.41 |
| | | Phytase | | | | | |
| | | 0 | $0.79^{b}$ | $0.48^{b}$ | $0.48^{b}$ | 0.25 | $0.34^{b}$ |
| | | 500 | $0.82^{a}$ | $0.51^{a}$ | $0.56^{a}$ | 0.25 | $0.50^{a}$ |
| Source of variation | | | | | | | |
| Ca | | | 0.001 | 0.001 | 0.001 | 0.001 | 0.064 |
| NPP | | | 0.532 | 0.001 | 0.023 | 0.714 | 0.377 |
| Phytase | | | 0.001 | 0.036 | 0.001 | 0.855 | 0.001 |
| Ca × NPP | | | 0.001 | 0.001 | 0.220 | 0.081 | 0.231 |
| Ca × phytase | | | 0.099 | 0.006 | 0.639 | 0.543 | 0.338 |
| NPP × phytase | | | 0.001 | 0.001 | 0.077 | 0.081 | 0.287 |
| Ca × NPP × phytase | | | 0.065 | 0.001 | 0.001 | 0.584 | 0.705 |

[a–g]Means with different superscripts within the columns are different ($p < .05$).
[1]Each value represents the mean of six replicates (two birds per replicate).
[2]U/kg diet, U = Phytase unit.

### 4.3. Apparent metabolizable energy and total tract nutrient retention

Dietary AME and total tract retention of P were not influenced ($p > .05$) by Ca, NPP and their interaction effect (Table 5). The three-way interaction of Ca, NPP and phytase was significant for Mg and Fe. Birds which received phytase-supplemented diet containing 6 g Ca and 4 g NPP/kg showed the lowest ($p < .008$) retention of Fe, while the same trend ($p < .006$) was observed for Mg retention when the enzyme was supplemented to diets with 8 g Ca and 3 g NPP. Phytase supplemented to 6 g Ca/kg reduced (Ca × phytase, $p < .001$) the Fe retention. Increasing the Ca level (10 g/kg) in diets reduced the retention of Ca ($p < .009$) and Mg ($p < .010$). Phytase supplementation improved the retention of Ca ($p < .003$) and P ($p < .011$) compared to the non-supplemented diets. Total retention of Fe was improved ($p < .007$) in birds fed low-NPP diet, while Ca and phytase levels had no effect.

The improved total tract retention of Ca and P due to phytase supplementation is consistent with the results of Ravindran et al. (2000). This finding further demonstrates the beneficial effect of exogenous phytase in lowering mineral excretion into the environment and improving broiler performance. Phytase supplementation did not improve the retention of Mg and Fe, a finding that supports the work of Sebastian et al. (1997), but the reason behind this is still not clear. In contrast, Viveros et al. (2002) reported that phytase

**Table 5.** Effect of dietary Ca and NPP levels with and without microbial phytase on total tract nutrient retention at 24 days of age[1].

| Ca (g/kg) | NPP (g/kg) | Phytase[2] | AME | Ca | P | Mg | Fe |
|---|---|---|---|---|---|---|---|
| 6 | 3 | 0 | 14.4 | 0.85 | 0.82 | 0.91$^{ab}$ | 0.46$^{ab}$ |
| | | 500 | 15.0 | 0.90 | 0.89 | 0.91$^{ab}$ | 0.38$^{ab}$ |
| | 4 | 0 | 15.4 | 0.88 | 0.77 | 0.92$^{a}$ | 0.42$^{ab}$ |
| | | 500 | 14.3 | 0.92 | 0.81 | 0.89$^{bcd}$ | 0.11$^{c}$ |
| 8 | 3 | 0 | 14.4 | 0.85 | 0.78 | 0.90$^{abc}$ | 0.45$^{ab}$ |
| | | 500 | 14.3 | 0.84 | 0.82 | 0.87$^{d}$ | 0.28$^{bc}$ |
| | 4 | 0 | 14.7 | 0.85 | 0.74 | 0.88$^{cd}$ | 0.29$^{bc}$ |
| | | 500 | 14.8 | 0.88 | 0.81 | 0.90$^{abc}$ | 0.50$^{ab}$ |
| 10 | 3 | 0 | 14.5 | 0.74 | 0.76 | 0.91$^{ab}$ | 0.45$^{ab}$ |
| | | 500 | 15.2 | 0.88 | 0.87 | 0.90$^{abc}$ | 0.58$^{a}$ |
| | 4 | 0 | 14.0 | 0.82 | 0.77 | 0.90$^{abcd}$ | 0.22$^{bc}$ |
| | | 500 | 14.2 | 0.85 | 0.83 | 0.90$^{abcd}$ | 0.43$^{ab}$ |
| SEM | | | 0.51 | 0.04 | 0.06 | 0.01 | 0.09 |
| Main effects | | | | | | | |
| Ca (g/kg) | | | | | | | |
| 6 | | | 13.0 | 0.89$^{a}$ | 0.82 | 0.91$^{a}$ | 0.34 |
| 8 | | | 12.8 | 0.85$^{ab}$ | 0.79 | 0.89$^{b}$ | 0.38 |
| 10 | | | 12.8 | 0.82$^{b}$ | 0.80 | 0.90$^{ab}$ | 0.42 |
| | NPP (g/kg) | | | | | | |
| | 3 | | 12.9 | 0.84 | 0.82 | 0.90 | 0.43$^{a}$ |
| | 4 | | 12.8 | 0.86 | 0.79 | 0.90 | 0.33$^{b}$ |
| | | Phytase | | | | | |
| | | 0 | 12.9 | 0.83$^{b}$ | 0.77$^{b}$ | 0.90 | 0.38 |
| | | 500 | 12.8 | 0.88$^{a}$ | 0.84$^{a}$ | 0.90 | 0.38 |
| Source of variation | | | | | | | |
| Ca | | | 0.577 | 0.009 | 0.468 | 0.010 | 0.249 |
| NPP | | | 0.808 | 0.202 | 0.150 | 0.439 | 0.007 |
| Phytase | | | 0.878 | 0.003 | 0.011 | 0.202 | 0.980 |
| Ca × NPP | | | 0.140 | 0.997 | 0.534 | 0.595 | 0.055 |
| Ca × phytase | | | 0.561 | 0.160 | 0.831 | 0.773 | 0.001 |
| NPP × phytase | | | 0.077 | 0.395 | 0.787 | 0.549 | 0.354 |
| Ca × NPP × phytase | | | 0.212 | 0.203 | 0.747 | 0.008 | 0.006 |

[a–d] Means with different superscripts within the columns are different ($p < .05$).
[1]Each value represents the mean of six replicates (six birds per replicate).
[2]U/kg diet, U = Phytase unit.

supplementation increased Mg retention, but the authors also assumed that this improvement could be the result of reduced excretion or endogenous loss of this mineral.

Diets with 8 g Ca/kg reduced the ileal digestibility and total tract retention of Mg. Although none of the interactions were significant for ileal Mg digestibility, the Ca × NPP × phytase interaction influenced the Mg retention, which is difficult to explain due its irregular pattern. Phytase supplemented to low-NPP diets with 8 g Ca/kg showed the lowest Mg retention than with high NPP diets or diets with low or high Ca. These results indicate that dietary Ca or NPP rather than phytase may have an influential effect on Mg retention. Previous study reported that consumption of high Ca can reduce the Mg absorption in rat due to the formation of Mg–Ca–phosphate complexes in the intestinal lumen (Brink et al. 1992) or competition of Ca and Mg for the same transport system in the ileum (Ross et al. 1984). It has been suggested that the formation of insoluble Ca–Mg–phosphate complexes is critically dependent on the dietary phosphate: magnesium ratio. According to Brink et al. (1992), at high dietary phosphate: magnesium ratio (6:1), increasing dietary Ca triggered the formation of abundant amounts of Ca–Mg–phosphate complexes in the rat intestine and subsequently reduced the Mg absorption (Brink et al. 1992). In the present study, the phosphate: magnesium ratio in phytase-supplemented low-NPP diets with 8 g Ca/kg was higher (2.65:1) than that in diets with 6 or 10 g Ca/kg, which partly justifies the low retention of Mg in the aforementioned diets. Besides, Favus et al. (2006) suggested that phosphate depletion or hypercalcemia could reduce the tubular reabsorption of Mg and resulted in low Mg retention. In contrast, Palacios et al. (2013) observed no effect of dietary Ca on urinary or faecal Mg excretion in humans. However, hypercalcemia may not be the case in the present study as plasma Ca level was not significantly different in the aforementioned diets.

Notably, in the current study, birds were killed by cervical dislocation and ileal digesta was collected by manual squeezing. These methods have been suggested to increase the chance of shedding of intestinal mucosa and subsequent mixing with ileal digesta (Nandha et al. 2013). Therefore, it is possible that both the procedures may have some bearing on the nutrient digestibility and utilization results reported in the present study.

There was no effect of varying levels of dietary Ca on AME. This finding is in agreement with Mutucumarana et al. (2014). It has been reported that Ca has a significant negative effect on AME at a concentration of more than 12 g/kg of diet (Shafey & McDonald 1991). This justifies the lack of a Ca effect on AME because all of the diets in the present study contained Ca levels from 6 to 10 g/kg. There was no effect of phytase on AME, which is an agreement with Cowieson et al. (2014), but not with Ravindran et al. (2000). Discrepancies in the phytase effect on AME value could be due to several factors (Cowieson et al. 2014). Importantly, Olukosi et al. (2008) observed a significant phytase-related improvement in net energy for production (NEp) in broilers, but no improvement in AME. This is because an amount of energy remains bound as protein and fat in the body and this is not reflected in the AME value, but rather in the net energy (NE) value. This further indicates that measuring NE could be more appropriate in evaluating the energy effect of phytase.

### 4.4. Protein content and enzyme activities at the jejunum

Different levels of dietary Ca had no effect on jejunal mucosal protein content, while low (3 g/kg) NPP increased ($p < .005$) the protein content at day 24 (Table 6). Phytase supplementation increased tissue protein content ($p < .030$) and Ca-Mg-ATPase activity ($p < .001$), but decreased ($p < .001$) the activity of Ca-ATPase in the jejunal mucosa. The interaction of Ca and NPP significantly influenced the activities of all measured enzymes in the jejunum. The diet with high Ca and low NPP increased the activity of AP ($p < .001$), while the reverse ($p < .001$) was the case for Ca-Mg-ATPase and Mg-ATPase activity. On the other hand, the Ca × NPP, Ca × phytase and NPP × phytase interactions were significant for Ca-ATPase activity, which indicates that Ca-ATPase activity in jejunal mucosa was highest ($p < .05$) in birds that received diet containing low Ca (6 g/kg) and high NPP, regardless of phytase supplementation. The protein content in the jejunal mucosa increased (Ca × phytase, $p < .015$) with 8 g Ca/kg of diet supplemented with phytase. The significant two-way interaction of Ca × phytase and NPP × phytase indicated that phytase supplemented to high Ca diet or low-NPP diet reduced ($p < .001$) the AP activity. The activities of Ca-Mg-ATPase ($p < .003$), Ca-ATPase ($p < .012$) and Mg-ATPase ($p < .001$) were influenced by the interaction between NPP and phytase, where phytase supplementation to the diet with 4 g NPP/kg increased the activities of these

**Table 6.** Effect of different dietary Ca and NPP levels with or without microbial phytase on the TP content, and activities of membrane-bound enzymes in the jejunal mucosa.[1]

| Ca (g/kg) | NPP (g/kg) | Phytase[2] | Protein | AP | Ca-Mg-ATPase | Ca-ATPase | Mg- ATPase |
|---|---|---|---|---|---|---|---|
| | | | mg/g | μM/mg protein/min | nmol/mg protein/min | | |
| 6 | 3 | 0 | 61.2 | 5.1$^{b}$ | 200.2$^{ab}$ | 195.0 | 204.6$^{d}$ |
| | | 500 | 65.0 | 5.6$^{ab}$ | 195.2$^{ab}$ | 195.3 | 201.7$^{de}$ |
| | 4 | 0 | 58.0 | 2.6$^{f}$ | 201.3$^{ab}$ | 259.3 | 201.0$^{de}$ |
| | | 500 | 56.0 | 3.2$^{e}$ | 216.0$^{a}$ | 226.0 | 229.6$^{c}$ |
| 8 | 3 | 0 | 65.0 | 4.3$^{cd}$ | 196.0$^{ab}$ | 195.0 | 234.5$^{bc}$ |
| | | 500 | 68.4 | 4.5$^{c}$ | 203.0$^{ab}$ | 201.0 | 197.6$^{de}$ |
| | 4 | 0 | 61.0 | 4.0$^{d}$ | 197.0$^{ab}$ | 200.0 | 242.1$^{b}$ |
| | | 500 | 72.2 | 5.3$^{b}$ | 198.2$^{ab}$ | 206.0 | 257.4$^{a}$ |
| 10 | 3 | 0 | 56.0 | 5.8$^{a}$ | 184.2$^{b}$ | 214.1 | 191.5$^{ef}$ |
| | | 500 | 56.4 | 3.2$^{e}$ | 198.2$^{ab}$ | 204.0 | 161.5$^{g}$ |
| | 4 | 0 | 55.0 | 3.5$^{e}$ | 193.0$^{ab}$ | 235.1 | 165.0$^{g}$ |
| | | 500 | 65.0 | 3.4$^{e}$ | 215.0$^{a}$ | 194.1 | 184.1$^{f}$ |
| SEM | | | 0.84 | 0.05 | 2.06 | 1.69 | 1.78 |
| Main effects | | | | | | | |
| Ca (g/kg) | | | | | | | |
| 6 | | | 60.0 | 4.1$^{ab}$ | 188.6 | 218.8$^{a}$ | 209.2$^{b}$ |
| 8 | | | 63.0 | 4.5$^{a}$ | 195.0 | 200.3$^{b}$ | 220.8$^{a}$ |
| 10 | | | 62.0 | 3.9$^{b}$ | 197.6 | 211.8$^{ab}$ | 188.2$^{c}$ |
| | NPP (g/kg) | | | | | | |
| | 3 | | 65.0$^{a}$ | 4.7$^{a}$ | 196.0 | 200.6$^{b}$ | 207.0 |
| | 4 | | 58.4$^{b}$ | 3.6$^{b}$ | 191.4 | 219.9$^{a}$ | 204.8 |
| | | Phytase | | | | | |
| | | 0 | 59.2$^{b}$ | 4.2 | 183.3$^{b}$ | 216.4$^{a}$ | 206.4 |
| | | 500 | 64.0$^{a}$ | 4.2 | 204.2$^{a}$ | 204.2$^{b}$ | 205.6 |
| Sources of variation | | | | | | | |
| Ca | | | 0.557 | 0.003 | 0.334 | 0.002 | 0.001 |
| NPP | | | 0.005 | 0.001 | 0.371 | 0.001 | 0.380 |
| Phytase | | | 0.030 | 0.995 | 0.001 | 0.005 | 0.650 |
| Ca × NPP | | | 0.716 | 0.001 | 0.047 | 0.001 | 0.001 |
| Ca × phytase | | | 0.015 | 0.001 | 0.163 | 0.009 | 0.001 |
| NPP × phytase | | | 0.108 | 0.001 | 0.003 | 0.012 | 0.001 |
| Ca × NPP × phytase | | | 0.971 | 0.001 | 0.007 | 0.188 | 0.001 |

Note: AP = Alkaline phosphatase.
[a-g]Means with different superscripts within the columns are different ($p < .05$).
[1]Each value represents the mean of six replicates (two birds per replicate).
[2]U/kg diet, U = Phytase unit.

enzymes. There were significant three-way (Ca × NPP × phytase) interaction effects on AP, Ca-Mg-ATPase and Mg-ATPase. Phytase supplemented to high Ca and low-NPP diets reduced ($p < .001$) the activity of AP and Mg-ATPase. On the other hand, phytase supplemented to diets with 6 or 10 g Ca and 4 g NPP/kg increased ($p < .007$) the Ca-Mg-ATPase activity than diets without phytase.

The enzymatic activities of AP and Mg-ATPase were inhibited by an increased dietary Ca level, particularly with low NPP, irrespective of phytase supplementation, and this is in accordance with the findings of Wang and Gilles-Baillen (1993). Although there is controversy regarding the role of AP in Ca absorption, it has been suggested that the entrance of Ca into enterocytes for absorption is assisted by AP, an enzyme of the jejunal brush border which hydrolyses organic pyro- or ortho-phosphates and impairs the formation of Ca pyro- or orthophosphate (Bhatti 1998). This mechanism increases the concentration of intraluminal free Ca, which is ready to be absorbed through the intestine, and also increases AP activity. In the current study, phytase supplementation of diets containing 6 g Ca/kg and 4 g NPP/kg improved the Ca-ATPase activity, which is in support with Bronner (1987). According to this author, low dietary Ca intake increased the Ca-ATPase-dependent active transport mechanism of Ca absorption from the intestine of chickens. The reduced activity of AP and Mg-ATPase in phytase-supplemented diets with high Ca and low NPP, or improved activities of Ca-Mg-ATPase due to phytase supplementation to diets with low or high Ca and high NPP are in line with the gross performance and nutrient utilization data. The improved activities of Ca-Mg-ATPase, Ca-ATPase and Mg-ATPase in birds fed diet with high NPP supplemented with phytase are also consistent with better performance and nutrient utilization. The Ca × phytase interaction influenced the protein concentration in the jejunal mucosa, but the pattern was not consistent. The highest concentration of protein in the jejunal mucosa was observed when 8 g/kg Ca was included in the diet. It has been suggested that an increase in the intestinal mucosal protein content of birds reflects the activities of digestive enzymes and absorptive capacity (Swatson et al. 2003). This could possibly be related to the improved digestibility and performance traits of the aforementioned diet groups.

### 4.5. Plasma mineral content and enzyme activities

Table 7 summarizes the results of mineral content and enzyme activities in plasma. All the interactions except Ca × phytase were significant for plasma Ca levels. The three-way interaction of Ca, NPP and phytase for the plasma Ca level can be explained by the increased ($p < .010$) plasma Ca concentration in phytase-supplemented diets with high Ca and NPP. Diets with increased Ca and low-NPP levels showed the highest (Ca × NPP, $p < .013$) plasma Ca concentration. Phytase supplementation to high NPP

**Table 7.** Effect of different dietary Ca and NPP levels with or without microbial phytase on mineral and enzyme profiles of the plasma at 24 day of age.[1]

| Ca (g/kg) | NPP (g/kg) | Phytase[2] | Ca | P | Zn | TP | AST | LDH | ALT |
|---|---|---|---|---|---|---|---|---|---|
| | | | (mg/100 ml) | | (µg/100 ml) | g/L | | (U/L) | |
| 6 | 3 | 0 | 5.4$^{cd}$ | 6.1 | 220.5 | 3.1 | 228.3 | 1049 | 2.4 |
| | | 500 | 5.5$^{bcd}$ | 5.1 | 244.9 | 2.9 | 227.8 | 1048 | 2.3 |
| | 4 | 0 | 5.1$^{cd}$ | 7.0 | 232.9 | 2.7 | 208.7 | 970 | 2.8 |
| | | 500 | 5.1$^{cd}$ | 6.5 | 223.9 | 3.0 | 236.0 | 955 | 3.2 |
| 8 | 3 | 0 | 5.7$^{bc}$ | 5.2 | 239.0 | 3.0 | 221.0 | 1033 | 3.7 |
| | | 500 | 5.4$^{cd}$ | 4.8 | 203.6 | 2.9 | 206.7 | 997 | 2.5 |
| | 4 | 0 | 4.8$^{d}$ | 5.6 | 192.5 | 2.8 | 218.0 | 1149 | 3.5 |
| | | 500 | 5.5$^{bc}$ | 6.1 | 220.9 | 3.0 | 204.5 | 914 | 2.4 |
| 10 | 3 | 0 | 6.1$^{b}$ | 3.8 | 219.7 | 2.9 | 206.2 | 758 | 2.5 |
| | | 500 | 5.3$^{cd}$ | 3.4 | 230.2 | 2. | 214.0 | 973 | 2.5 |
| | 4 | 0 | 5.6$^{bc}$ | 5.7 | 240.6 | 2.9 | 237.5 | 964 | 3.5 |
| | | 500 | 6.9$^{a}$ | 4.8 | 222.5 | 2.8 | 203.2 | 905 | 2.6 |
| SEM | | | 0.34 | 0.43 | 28.54 | 0.15 | 21.38 | 121.3 | 0.82 |
| Main effects | | | | | | | | | |
| Ca (g/kg) | | | | | | | | | |
| 6 | | | 5.3$^{b}$ | 6.2$^{a}$ | 230.6 | 2.9 | 225.2 | 1005$^{ab}$ | 2.7 |
| 8 | | | 5.4$^{b}$ | 5.7$^{a}$ | 214.0 | 2.9 | 212.2 | 1023$^{a}$ | 3.0 |
| 10 | | | 6.0$^{a}$ | 4.5$^{b}$ | 228.3 | 2.8 | 215.2 | 875$^{b}$ | 2.8 |
| | NPP (g/kg) | | | | | | | | |
| | 3 | | 5.6 | 4.8$^{b}$ | 226.3 | 2.9 | 217.3 | 976 | 2.7 |
| | 4 | | 5.5 | 6.0$^{a}$ | 222.2 | 2.9 | 218.0 | 959 | 3.0 |
| | | Phytase | | | | | | | |
| | | 0 | 5.6 | 5.7$^{a}$ | 224.2 | 2.9 | 220.7 | 970 | 3.1 |
| | | 500 | 5.5 | 5.1$^{b}$ | 224.4 | 2.9 | 215.4 | 965 | 2.6 |
| Source of variation | | | | | | | | | |
| Ca | | | 0.001 | 0.001 | 0.459 | 0.185 | 0.463 | 0.035 | 0.705 |
| NPP | | | 0.504 | 0.001 | 0.726 | 0.768 | 0.942 | 0.737 | 0.301 |
| Phytase | | | 0.273 | 0.001 | 0.989 | 0.594 | 0.601 | 0.922 | 0.155 |
| Ca × NPP | | | 0.013 | 0.450 | 0.760 | 0.397 | 0.732 | 0.617 | 0.595 |
| Ca × phytase | | | 0.902 | 0.712 | 0.900 | 0.248 | 0.351 | 0.103 | 0.311 |
| NPP × phytase | | | 0.001 | 0.967 | 0.980 | 0.023 | 0.797 | 0.197 | 0.830 |
| Ca × NPP × phytase | | | 0.010 | 0.550 | 0.167 | 0.721 | 0.264 | 0.709 | 0.715 |

Note: TP = Total protein, AST = Aspartate transaminase, LDH = Lactate dehydrogenase, ALT = Alanine transaminase.
[a,b]Means with different superscripts within the columns are different ($p < .05$).
[1]Each value represents the mean of six replicates (two birds per replicate).
[2]U/kg diet, U = Phytase unit.

diets also elevated (NPP × phytase, $p < .001$) the plasma Ca level. High level of Ca (10 g/kg) significantly ($p < .001$) increased plasma Ca and decreased P concentrations. On the other hand, plasma P concentration was elevated ($p < .001$) by high NPP. Although phytase supplementation had no effect on plasma Ca and Zn concentrations, it reduced ($p < .001$) P concentration. Zinc concentration in the plasma was not affected by Ca, NPP, phytase and their interactions. Dietary Ca, NPP, phytase and their interactions also had no effect ($p > .05$) on plasma total protein (TP) or enzymes, except plasma lactate dehydrogenase (LDH) activity, which was reduced ($p < .035$) by high dietary Ca (10 g/kg) content.

In the recent study, plasma Ca concentration was influenced by the Ca × NPP × phytase and Ca × NPP interactions. Increasing Ca (10 g/kg) levels in diets with low NPP elevated the plasma Ca concentration in diets without phytase, while the opposite effect was observed in phytase-supplemented diets. The increased plasma Ca due to low dietary NPP was also reported by previous studies (Sebastian et al. 1996, Viveros et al. 2002). This is because offering birds low-NPP diets could increase the ionized Ca concentration in plasma, which depresses the release of parathyroid hormone (PTH). Thus, a reduction in PTH in plasma due to feeding low-NPP diets consequently inhibited the tubular reabsorption of phosphate, leading to increased urinary excretion of the absorbed Ca from the gut (Taylor & Dacke 1984). Moreover, the lowest Ca retention (not significant) in the birds fed high Ca and low-NPP diets partly justifies the reason for high plasma Ca concentration in the same group of birds. On the other hand, in the case of phytase-supplemented high Ca and low-NPP diets, it is possible that a relatively low amount of dietary Ca was absorbed from the gut due to the formation of Ca–phytate complexes, which subsequently resulted in low Ca concentration in plasma. The reduced FI of the same group of birds can be another possible cause of lower plasma Ca concentration.

The interactions of NPP and phytase influenced the plasma TP concentration in the current study. The lowest plasma TP concentration was observed in phytase-supplemented diets with low NPP, especially at high dietary Ca content. It is possible that the presence of a protein–phytate complex in the intestine of chickens could affect the protein digestibility and subsequent change in the plasma TP (Selle et al. 2000). In the present study, plasma LDH activity was reduced at a high dietary Ca level, irrespective of phytase supplementation. Birds on a high Ca diet were apparently healthy, but, due to lower FI and BWG, it is possible that there may have been underlying disorders or underdevelopment of muscles, internal organs or soft tissue, and this may have affected the plasma LDH activity.

## 5. Conclusions

In conclusion, the present study showed that high dietary Ca could depress phytase activity and reduce phytate hydrolysis and subsequently nutrient utilization. Low NPP (3 g/kg)

exaggerated the negative effect of high Ca. Supplementation with microbial phytase improved protein and mineral digestibilities when added to diets containing 6–8 g Ca/kg diet. Measurement of endogenous intestinal enzymes in this study supported the gross responses, but some trends and mechanisms remain unclear, warranting further investigations.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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## References

Adeola O, Sands JS. 2003. Does supplemental dietary microbial phytase improve amino acid utilization? A perspective that it does not. J Anim Sci. 81:78–85.

Anderson DL, Henderson LJ. 1986. Sealed chamber digestion for plant nutrient analysis. Agro J. 78:937–938.

AOAC. 1994. Association of official analytical chemists. Official method of analysis. 16th ed. Washington, DC: AOAC International.

Aviage. 2009. Ross 308 broiler nutrition specification. [cited 2014]. Available from: http://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross_Nutrition_Supplement.pdf

Bhatti MS. 1998. Intestinal calcium transport in the chickens [PhD Thesis]. The University of British Columbia, Vancouver, Canada.

Boling-Frankenbach SD, Peter CM, Douglas MW, Snow JL, Parsons CM, Baker DH. 2001. Efficacy of phytase for increasing protein efficiency ratio values of feed ingredients. Poult Sci. 80:1578–1584.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72:248–254.

Brink EJ, Beynen AC, Dekker PR, van Beresteijn EC, van der Meer R. 1992. Interaction of calcium and phosphate decreases ileal magnesium solubility and apparent magnesium absorption in rats. J Nutr. 122:580–586.

Bronner F. 1987. Intestinal calcium absorption: mechanisms and applications. J Nutr. 117:1347–1352.

Cardoso Júnior A, Rodrigues PB, Bertechini AG, Freitas RTFD, Lima RRD, Lima GFR. 2010. Levels of available phosphorus and calcium for broilers from 8 to 35 days of age fed rations containing phytase. R Bras Zootec. 39:1237–1245.

Cowieson AJ, Acamovic T, Bedford MR. 2004. The effects of phytase and phytic acid on the loss of endogenous amino acids and minerals from broiler chickens. Br Poult Sci. 45:101–108.

Cowieson AJ, Aureli R, Guggenbuhl P, Fru-Nji F. 2014. Possible involvement of myo-inositol in the physiological response of broilers to high doses of microbial phytase. Anim Prod Sci. 55:710–719.

Favus MJ, Bushinsky DA, Lemann J. 2006. Regulation of calcium, magnesium, and phosphate metabolism. In: Favus MJ, editor. Primer on the metabolic bone diseases and disorders of mineral metabolism. 6th ed. Durham, NC: American Society for Bone and Mineral Research; p. 76–83.

Gifford SR, Clydesdale FM. 1990. Interactions among calcium, zinc and phytate with three protein sources. J Food Sci. 55:1720–1724.

Holdsworth ES. 1970. The effect of vitamin D on enzyme activities in the mucosal cells of the chick small intestine. J Membr Biol. 3:43–53.

Iji PA, Saki A, Tivey DR. 2001. Body and intestinal growth of broiler chicks on a commercial starter diet. 2. Development and characteristics of intestinal enzymes. Br Poult Sci. 42:514–522.

Kim TW, Lei XG. 2005. An improved method for a rapid determination of phytase activity in animal feed. J Anim Sci. 83:1062–1067.

Maenz DD, Engele-Schaan. CM, Newkirk RW, Classen HL. 1999. The effect of minerals and mineral chelators on the formation of phytase-resistant and phytase-susceptible forms of phytic acid in solution and in a slurry of canola meal. Anim Feed Sci Technol. 81:177–192.

Mcpherson, GA. 1985. *Lowry Program*, Cambridge, UK: Elsevier-BIOSOFT.

Minitab 16 statistical software. 2010. *State College*, Pennsylvania, USA: Minitab Inc. www.minitab.com.

Mutucumarana RK, Ravindran V, Ravindran G, Cowieson AJ. 2014. Influence of dietary calcium concentration on the digestion of nutrients along the intestinal tract of broiler chickens. J Poult Sci. 51:392–401.

Nandha NK, Woyengo TA, Payne RL, Nyachoti CM. 2013. Ileal digestibility of amino acids in pea protein isolates, wheat-corn distillers dried grains with solubles, and short-season corn fed to broiler chicks. Poult Sci. 92:184–191.

Olukosi OA, Cowieson AJ, Adeola O. 2008. Energy utilization and growth performance of broilers receiving diets supplemented with enzymes containing carbohydrase or phytase activity individually or in combination. Bri J Nutr. 99:682–690.

Olukosi OA, Fru-Nji F. 2014. The interplay of dietary nutrient level and varying calcium to phosphorus ratios on efficacy of a bacterial phytase: 2. Ileal and total tract nutrient utilization. Poult Sci. 93:3044–3052.

Palacios C, Wigertz K, Braun M, Martin BR, McCabe GP, McCabe L, Pratt JH, Peacock M, Weaver CM. 2013. Magnesium retention from metabolic-balance studies in female adolescents: impact of race, dietary salt, and calcium. Am J Clin Nutr. 97:1014–1019.

Qin X, Klandorf H, Porter DW, Holt SB, Martin WG. 1993. Estrogen enhancement of Ca-, Mg-, and Ca- Mg stimulated adenosine triphosphatase activity in chick shell gland. Gen Comp Endocrinol. 89:4–10.

Ravindran V, Cabahug S, Ravindra G, Selle PH, Bryden WL. 2000. Response of broiler chickens to microbial phytase supplementation as influenced by dietary phytic acid and non-phytate phosphorous levels. II. Effects on apparent metabolisable energy, nutrient digestibility and nutrient retention. Br Poult Sci. 41:193–200.

Ross RD, Cromwell GL, Stahly TS. 1984. Effects of source and particle size on the biological availability of calcium in calcium supplements for growing pigs. J Anim Sci. 59:125–134.

Schöner FJ, Schwarz G, Hoppe PP, Wiesche H. 1994. Effect of microbial phytase on Ca-availability in broilers. In: Proceedings of the Third Conference of Pig and Poultry Nutrition. Germany: University of Halle.

Sebastian S, Touchburn SP, Chavez ER, Lague PC. 1996. Efficacy of supplemental microbial phytase at different dietary calcium levels on growth performance and mineral utilization of broiler chickens. Poult Sci. 75:1516–1523.

Sebastian S, Touchburn SP, Chavez ER, Laguë PC. 1997. Apparent digestibility of protein and amino acids in broiler chickens fed a corn-soybean diet supplemented with microbial phytase. Poult Sci. 76:1760–1769.

Selle PH, Ravindran V. 2007. Microbial phytase in poultry nutrition. Anim Feed Sci Technol. 135:1–41.

Selle PH, Ravindran V, Caldwell RA, Bryden WL. 2000. Phytate and phytase: consequences for protein utilisation. Nutr Res Rev. 13:255–278.

Shafey TM, McDonald MW. 1991. The effects of dietary calcium, phosphorus, and protein on the performance and nutrient utilization of broiler chickens. Poult Sci. 70:48–553.

Shirazi-Beechey SP, Smith MW, Wang Y, James PS. 1991. Postnatal development of lamb intestinal digestive enzymes is not regulated by diet. J Physiol. 437:691–698.

Short FJ, Gorton P, Wiseman J, Boorman KN. 1996. Determination of titanium dioxide added as an inert marker in chicken digestibility studies. Anim Feed Sci Technol. 59:215–221.

Swatson HK, Iji PA, Gous RM. 2003. Body growth, visceral organ weight and intestinal digestive enzyme of chickens on diets varying energy and protein contents. J Anim Vet Adv. 2:305–311.

Sweeney RA. 1989. Generic combustion method for determination of crude protein in feeds: Collaborative study. J. AOAC Int. 72: 770–774.

Taylor TG, Dacke CG. 1984. Calcium metabolism and its regulation. In: Physiology and biochemistry of the domestic fowl. London: Academic Press; 126–170.

Viveros A, Brenes A, Arija I, Centeno C. 2002. Effects of microbial phytase supplementation on mineral utilization and serum enzyme activities in broiler chicks fed different levels of phosphorus. Poult Sci. 81:1172–1183.

Wang H, Gilles-Bailllen M. 1993. Ca2+-ATPase and Mg2+-ATPase activities distinct from alkaline phosphatase in rat jejunal brush-border membranes. Arch Physiol Biochem. 101:387–393.

Yi Z, Kornegay ET, Denbow DM. 1996a. Supplemental microbial phytase improves zinc utilization in broilers. Poult Sci. 75:540–546.