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Effect of Citric Acid, Phytase and Calcium in Diets of Laying Hens on Productive Performance, Digestibility and Mineral Excretion

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Abstract: The objective of this experiment was to evaluate the effect of citric acid (CA), phytase and calcium in diets of laying hens on the productive performances and digestibility and excretion of phosphorus (P), calcium and nitrogen (N). An experiment was designed using 24 week old laying hens, which were fed a diet with a base of sorghum and soybean meal containing 2,700 kcal ME/kg, 15% crude protein, 3.25% calcium and 0.25% available phosphorus. The following were also added to the diets of hens depending on the treatment groups: 0.0, 0.6 and 1.2% citric acid, 0.0 and 300 units of phytase/kg of diet (FTU) and 3.00 and 3.25% calcium. The phytase was added as an ingredient into to diets, contributed to 0.1% of phosphorus and 0.3% of calcium levels. The experiment involved 12 treatments in a 3 x 2 x 2 factorial design. The treatment 0.6% citric acid, 300 FTU and 3% calcium increased the digestibility of phosphorus, resulting in its decreased excretion ($p<0.05$). Citric acid decreased the excretion of calcium and N linearly ($p<0.01$), increased ($p<0.05$) their digestibility and had effect on the response of the phytase ($p<0.05$). The treatment of 1.2% citric acid, 300 FTU and 3.25% calcium increased ($p<0.05$) the digestibility of N quantitativaly. It can be concluded that citric acid reduces the excretion levels and increases the digestibility of P, N and calcium and has effect on the response of the phytase to affect the excretions of P and N.

Key words: Citric acid, phytase, calcium, laying hens, digestibility

INTRODUCTION

The total production of excreta by laying hens is estimated to be 33,600 kg for 1,000 birds per production cycle, the nitrogen (N) is the most prominent component at a concentration of 14 kg/t, followed by P at 10 kg/t. This poses potential environmental risks because excess phosphate and nitrogen are frequently incorporated into shallow and subterranean aquifers, polluting lakes and rivers and affecting aquatic life (Boersma, 2001), ecosystems and ultimately the planet as a whole.

The efficacy of microbial phytase in improving the availability of phosphorus has been previously demonstrated (Simons *et al*., 1990; Scott *et al*., 1999; Walk *et al*., 2012), which allows for the partial reduction of inorganic phosphorus in the diet.

On the other hand, the phytic acid present in the vegetable feed is soluble at acidic pH levels, as the food advances through the gastrointestinal tracts of the birds, it encounters increased pH levels in some areas that favour the precipitation of the phytic acid with certain minerals, mainly calcium, (Erdman, 1979). Alkaline pH levels significantly reduce the solubility of these minerals (Gifford and Clydesdale, 1990; Kornegay and Quian, 1996).

In vitro studies show that the highest activity of microbial phytase takes place between pH 2.5 and 5 (Simons *et al*., 1990). The main site of phytase's action is the proventricle (Yi *et al*., 1996) whose pH 4.4, when an acid is added to diets, gastric pH will be lower (Giesting and Esaster, 1995) and therefore phytase's activity will be better.

On the other hand, *in vitro* studies with citric acid (CA) has been demonstrated to increase the availability of phytic phosphorus because it relieves the effect inhibiting of calcium in phytic acid hydrolysis (Zyla *et al*., 1995) and it improves the retention of phosphorus, calcium and proteins (Gentesse *et al*., 1994; Vargas-Rodriguez *et al*., 2002; Rafacz-Livingston *et al*., 2005a,b). Very little research has been conducted to evaluate the effects of the supplementation of citric acid in the diets of laying hens (Boling *et al*., 2000; Vargas-Rodriguez *et al*., 2002) and no integrated studies assessing citric acid, phytase and calcium levels have been attempted to date. Vargas-Rodriguez *et al*. (2002), in a study of the effects of citric acid and phytase in 80-week-old laying hens, found that 2% CA+600 FTU reduces the excretion of P and increases egg weight and that 2% CA

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reduces the excretion of N and increases the calcium concentrations of the shells.

Therefore, we conducted this investigation with the following objectives: (a) To evaluate the effects of CA in the response of the phytase and the productive performance, excretion rates and digestibility of P, calcium and N, in the diets of laying hens from the ages of 24 to 39 weeks and (b) To evaluate the effects of the diets on the productive performances, excretion rates and digestibility of P, calcium and N in laying hens aged 24-39 weeks.

MATERIALS AND METHODS

The investigation was carried out at the Campo Experimental "Valle de México," at SAGARPA's National Institute of Forestry, Agricultural and Husbandry in Chapingo, Mexico. All chemical analyses and ingredient, feed and excreta sampling were conducted at the laboratory of the Livestock Production Program at the Institute of Genetic Resources and Productivity of the Postgraduate College in Montecillo, Texcoco, Mexico. One hundred and forty-four Hy-line W98 24-week-old hens were housed in individual metal cages with automatic feed and water troughs. They were exposed to 12 h of natural light in addition to a sufficient duration of artificial light to attain 17 h of total light during the peak laying period. The base ingredients of the feed consisted of sorghum and soybean meal and contained 15% protein, 0.25% available phosphorus and 3.25% calcium and a total of 2,700 Kcal EM/kg (Table 1).

Prior to the feed's formulation, the ingredients to be used in the diets were analysed as follows: N levels were measured by the Kjeldahl method, P levels by spectrophotometry (Genesys 10S UV-Vis. Madison, WI, US.) and calcium levels by atomic absorption (Varian Spectra. Inc., AA 240 FS. Palo Alto, CA, US.) (A.O.A.C., 1990). All feed was prepared at the beginning of the experiment and stored in a well-ventilated shed at room temperature.

Sixty grams of phytase/t was added to the diet. Phytase was considered as a diet's ingredient based on the concentration of 300 FTU (phytase unit) used and on its potential product, Natuphos 5000 (BASF. Mexico. D.F). One phytase unit (FTU) is defined as the amount of enzyme activity that liberates 1 mmol of inorganic P/minute from a 0.5 mM Na-phytate solution at pH 5.5 and 37.5°C. The inclusion rate of phytase determined the assigned values of calcium and P. Phytase has been demonstrated to release approximately 0.1% P and 0.3% calcium (Parr, 1996).

Food-grade anhydrous citric acid was added to the experimental diets. The pH levels of these diets were determined by a potentiometer using a 20 g sample at the start, midway point and final stage of the experiment. They were 12 treatments, 4 replicates per treatment and 3 birds per replicate. Each replicate consisted of 3 cages, with one hen per cage, the hen was the experimental unit, which were distributed randomly.

Table 1: Composition of the basal diet fed to laying hens from 21 to 39 weeks of age[1]

Ingredient	Percentage
Sorghum	73.5700
Soybean meal	14.5600
Calcium carbonate	9.1300
Dicalcium phosphate	1.8000
Salt	0.3500
DL-methionine	0.1500
Vitamin premix[2]	0.1000
Mineral premix[3]	0.1000
Soybean oil	0.1000
L-lysine	0.1400
Natural pigment	0.0015
Calculated nutrients	
Crude protein	15.00
Metabolisable energy (Kcal/kg)	2.700
Lysine	0.690
Methionine+cysteine	0.580
Threonine	0.470
Calcium	3.250
Nonphytate phosphorus (AP)	0.250
Total phosphorus (TP)	0.500

[1]Dietary treatments were: 0.0% CA, 0.0 FTU, 3.25% Ca; 0.0% CA, 0.0 FTU, 3.00% Ca; 0.0% AC, 300 FTU, 3.00% Ca; 0.0% CA, 300.0 FTU, 3.25% Ca; 0.6% CA, 0.0 FTU, 3.00% Ca; 0.6% CA, 0.0 FTU, 3.25% Ca; 0.6% CA, 300 FTU, 3.00% Ca; 0.6% CA, 300 FTU, 3.25% Ca; 1.2% CA, 0.0 FTU, 3.00% Ca; 1.2% CA, 0.0 FTU, 3.25% Ca; 1.2% CA, 300 FTU, 300% Ca and 1.2% CA, 300 FTU, 3.25% Ca

Citric acid and phytase were added at the expense of sorghum. Phytase was added as a dietary ingredient to relevant treatments based on the dose of 300 FTU and on its potential product, Natuphos 5000 (BASF, Mexico, D.F). For diets containing phytase, the P and Ca levels were subtracted from the orthophosphate concentrations.Phytase leads to the release of approximately 0.1% P and 0.3% calcium (Parr, 1996)

[2]Vitamin premix per kg of feed: A (retinol), 12,000 IU; D (cholecalciferol), 2,400 IU; E (DL-alpha-tocopherol), 20 IU; K (menadione), 1.2 mg; thiamine, 1.6 mg; riboflavin, 8.0 mg; niacin, 32 mg; pyridoxine 3 mg; pantothenic acid, 11.2 mg; cyanocobalamin, 16 µg; folic acid, 1.6 mg; choline, 250 mg

[3]Mineral premix per kg of feed: Mn, 60 mg; Zn, 50 mg; Fe, 30 mg, Cu, 5 mg; I, 1.0 mg; Se, 0.1 mg

The dietary treatments were as follows: 0.0% CA, 0.0 FTU, 3.25% Ca; 0.0% CA, 0.0 FTU, 3.00% Ca; 0.0% CA, 300 FTU, 3.00% Ca; 0.0% CA, 300.0 FTU, 3.25% Ca; 0.6% CA, 0.0 FTU, 3.00% Ca; 0.6% CA, 0.0 FTU, 3.25% Ca; 0.6% CA, 300 FTU, 3.00% Ca; 0.6% CA, 300 FTU, 3.25% Ca; 1.2% CA, 0.0 FTU, 3.00% Ca; 1.2% CA, 0.0 FTU, 3.25% Ca; 1.2% CA, 300 FTU, 300% Ca and 1.2% CA, 300 FTU, 3.25% Ca.

Measured variables: Feed consumption and feed conversion rates were recorded every two weeks and production rates and egg weights were measured daily. To 110 days of experimentation were selected four hens per treatment, one hen per replicate, to evaluate the P,

Ca and N levels in the excreta of the birds. The trays under each cage were lined with plastic to facilitate the collection of the materials.

The trial was conducted over the course of three consecutive days, after which the excreta were immediately stored in plastic bags, frozen at -20°C and ultimately dried at 55°C. They were also weighed and sifted using 1 mm mesh for later analyses. During the digestibility assay, the feed consumption rates were recorded daily.

The endogenous material collections were carried out at the end of the experiment, when these same treated birds were submitted to 24 h fasts. The endogenous materials were collected in trays that were placed under each cage for a period of 24 h.

The phosphorus concentrations of the diets and excreta were determined by spectrophotometry, those of calcium were measured by atomic absorption spectrometry and those of nitrogen were assessed by the Kjeldahl method (A.O.A.C., 1990).

To determine the P and calcium levels, once the sample ashes were collected, they were mixed with an HCl solution at 50 and 10% and evaporated to minimal volumes of 10 ml. The solutions were transferred to 50 ml volumetric flasks for later dilutions. The samples were diluted in molybdic acid and p-methylaminophenol, dilutions were made depending of the sample measured. Next, the absorbances of the samples were measured using an ultraviolet light spectrophotometer at 660 nm.

The samples were diluted in lanthanum oxide to determine the calcium levels by measuring their absorbance by atomic absorption spectrophotometry at 425 nm.

The digestibility of P, calcium and N were determined according to Ammerman *et al.* (1995).

Statistical analysis: The data were analysed using a factorial design (3 x 2 x 2); there were three levels of citric acid (0, 0.6 and 1.2%), 2 levels of phytase (0 and 300 FTU) and 2 levels of calcium (3.0 and 3.25%) with four repetitions per treatment and three birds per repetition. Averages were compared using Tukey's test (Steel and Torrie, 1988). All data obtained describing the variable rates of egg production and nutrient digestibility were transformed according to the arc sin function $\sqrt{x}$. The data were analysed using the SAS procedure (SAS, 1990).

RESULTS AND DISCUSSION

Productive performance: Table 2 shows the average production variables of the laying hens that were 24 to 39 weeks of age. All data showed that CA, phytase and calcium and their interactions had similar effects on productive performance, which included feed consumption, feed conversion and egg production ($p>0.05$).

Phytase was added to the diets, which provided 0.1% P and 0.3 % calcium, demonstrating that birds consuming phytase-enriched feed have similar productive performances as those of other types of poultry; thus, the addition of orthophosphate to their diets can be reduced by more than 50% and replaced by phytase without negatively affecting their productive performances. Similar findings have been reported by other studies (Van der Klis *et al.*, 1997; Keshavarz, 2000; Scott *et al.*, 2001; Watson *et al.*, 2006).

Furthermore, Simons *et al.* (1990), Schoner *et al.* (1991) and Um and Paik (1999) showed that phytase replaces inorganic P at a level of approximately 0.1%.

CA was found to have no effect on productive performance, these results are in agreement with the reported by Woyengo *et al.* (2010) in broiler chickens and Boling *et al.* (2000) in laying hens. The latter authors found that CA did not improve productive behaviours with the addition of high levels of calcium, 3.8%, to their diets. In contrast, Snow *et al.* (2004) and Rafacz-Livingston *et al.* (2005b) found that the addition of 3% CA to the diets of broiler chickens affected their productive performances, allowing for improved weight gain.

In another study, Vargas-Rodriguez *et al.* (2002) found that diets including CA and phytase increased egg weights and 2% CA increased shell calcium levels.

Factors such as age, physiological and production stages, dietary CA levels, dietary levels of calcium and P and relation calcium:P affect the birds' responses.

Digestibility and excretion of phosphorus: In terms of P digestibility, the effects of the interaction of 0.6% AC x 300 FTU x 3.0% calcium on phosphorus digestibility was significant ($p<0.05$) (Table 3); this treatment led to the increased digestibility of phosphorus by 63.25% and with 3.25% calcium caused digestibility to be reduced to 40.0%. In the absence of CA or absence of phytase and 3% calcium, the digestibility decreased by 30.0 and 41.84%, respectively. This interaction was significant because of the response of the phytase combined with CA and 3% calcium, increased P digestibility versus with 3.25% of calcium or phytase or CA alone. In the absence of CA or absence of phytase, P digestibility was reduced quantitatively.

With respect to P excretion, the interaction of 0.6% CA x 300 FTU x 3.0% calcium was significant ($p<0.05$) and reduced the excretion of P to 0.25 g, while with 3.25% calcium led to an increase in P excretion to 0.36 g and the absence of CA or absence of phytase and 3% calcium caused P excretion to increase to 0.41 g and 0.39 g, respectively. This interaction was significant because of the response of the phytase combined with 0.6% CA and 3% calcium, which drastically reduced the excretion of P in contrast with 3.25% of calcium, or phytase or CA alone. 0.6% citric acid x 300 FTU x 3.0% calcium indicated an approximately 50% reduction in the excretion of P.

Table 2: Effects of citric acid, phytase and calcium on productive performances of laying hens from 24 to 39 weeks of age[1]

Treatments		----- Egg weight (g) -----		-- Egg production (%) --		Feed cons. (g/bird/day)		Feed conversion (kg/kg)	
Citric acid (%)	Phytase[2] (FTU/kg)	---------- Calcium (%) ----------							
		3.0	3.25	3.0	3.25	3.0	3.25	3.0	3.25
0	0	58.59	58.20	87.19	87.95	116.2	116.0	2.32	2.25
	300	57.79	58.48	88.26	89.60	115.8	115.4	2.29	2.19
0	0.6	59.49	57.62	88.13	89.73	115.4	115.4	2.20	2.27
	300	59.47	58.45	87.11	87.66	115.2	115.2	2.29	2.16
0	1.2	59.25	58.49	87.13	88.40	115.8	116.2	2.23	2.27
	300	56.84	59.64	89.20	86.47	114.0	116.3	2.24	2.28
SEM		---------- 0.72 ----------		---------- 1.48 ----------		---------- 0.80 ----------		---------- 0.05 ----------	

[1]There was no interaction effect. [2]Natuphos 5000. BASF, México, D.F, Cons: Consumption

Table 3: Effects of citric acid, phytase and calcium on excretion (g/hen/day) and digestibility (%) of phosphorus (P), calcium (Ca) and nitrogen (N) in laying hens from 24 to 39 weeks of age

			---------- Excretion ----------			---------- Digestibility ----------		
Variable			P	Ca	N	P	Ca	N
Citric acid (%)								
0.0			0.426^{a}	1.06^{a}	1.28^{a}	28.89^{b}	68.84^{b}	65.83^{b}
0.6			0.347^{b}	0.93^{ab}	1.05^{ab}	49.32^{a}	72.55^{ab}	78.17^{a}
1.2			0.354^{b}	0.88^{b}	1.00^{b}	47.14^{a}	75.95^{a}	88.26^{a}
Phytase(FTU/kg)								
0			0.416^{a}	0.95	1.13	39.54^{b}	71.89	75.88
300			0.335^{b}	0.97	1.07	44.48^{a}	72.54	76.93
CA x phytase								
0.0	0		0.483^{a}	1.041	1.213^{ab}	23.11^{b}	68.66	69.31^{ab}
0.0	300		0.379^{b}	1.087	1.372^{a}	33.70^{b}	68.99	61.48^{b}
0.6	0		0.386^{b}	0.992	1.146^{ab}	46.96^{a}	69.25	73.88^{ab}
0.6	300		0.308^{c}	0.875	0.974^{ab}	51.68^{a}	75.76	81.74^{a}
1.2	0		0.391^{b}	0.838	1.050^{ab}	45.81^{a}	76.74	83.04^{a}
1.2	300		0.317^{c}	0.924	0.955^{b}	48.07^{a}	73.42	83.53^{a}
CaxphytasexCa								
0.0	0.0	3.00	0.47^{a}	1.02	1.14	25.94^{de}	68.88	71.75^{ab}
0.0	0.0	3.25	0.49^{ab}	1.05	1.26	21.23^{e}	68.52	67.68^{ab}
0.0	300	3.00	0.41^{bcde}	1.08	1.42	30.0^{cde}	69.63	59.71^{b}
0.0	300	3.25	0.35^{def}	1.09	1.32	37.70^{bcde}	68.36	63.25^{b}
0.6	0.0	3.00	0.39^{cdef}	1.01	1.13	41.84^{bcd}	67.28	71.49^{ab}
0.6	0.0	3.25	0.38^{cdef}	0.96	1.15	52.09^{abc}	72.21	75.48^{ab}
0.6	300	3.00	0.25^{g}	0.79	0.93	63.35^{a}	76.99	81.83^{ab}
0.6	300	3.25	0.36^{cdef}	0.99	1.01	40.0^{bcde}	73.92	81.65^{ab}
1.2	0.0	3.00	0.38^{abc}	0.96	0.86	45.29^{bcd}	73.73	83.97^{ab}
1.2	0.0	3.25	0.39^{abcd}	0.71	1.24	46.33^{abc}	79.84	82.10^{ab}
1.2	300	3.00	0.29^{fg}	1.00	1.07	53.58^{ab}	77.36	77.67^{ab}
1.2	300	3.25	0.33^{efg}	0.85	0.78	43.00^{bcd}	64.97	92.32^{a}
SEM			0.02	0.10	0.13	2.19	3.5	12.2

$^{a,b,c,...}$Averages with different superscript letters within columns differ, Tukey ($p<0.05$). *Linear effects ($p<0.01$)

As levels of calcium in the diet rose to 3.25%, it could have acted as a buffer, interfering with the acidification of the feed and the absorption of P and calcium, as was observed with the addition of 0.6% CA, phytase and 3.25% calcium, confirming that excess calcium in the diet reduces the full utilisation of phytic phosphorus and likely other minerals (Applegate *et al*., 2003). Boling *et al.* (2000) in a study whit CA to laying hens' diets reported that it did not improve the utilisation of P due to the high levels of calcium in the diet (3.8%), that likely inhibited the activity of CA. High levels of calcium cause CA to bind to non-phytic calcium, consequently rendering it unavailable for binding to the calcium-phytate complex. However, when intestinal pH levels change from neutral to alkaline, calcium acquires a high chelation rate because it bonds with phytic acid, which makes this absorption unavailable (Quian *et al*., 1997). pH levels of 5.5 have been shown to significantly decrease the solubility of calcium (Gifford and Clydesdale, 1990). During the present investigation, diets containing CA, 0.6% CA, 300 FTU and 3% calcium led to the lowest rate of excretion; similar results were found by Boling *et al.* (2001), Vargas-Rodriguez *et al.* (2002) and Rafacz-Livingston *et al.* (2005a), who showed that CA significantly improves the utilisation of phosphorus. Rafacz-Livingston *et al.* (2005b) reported that the supplementation of 3% CA to the diets of broiler chickens leads to the release of between 0.05 and 0.1% P and Snow *et al.* (2004) found that 3% CA causes the release of 0.03% P.

Digestibility and excretion of calcium: In terms of calcium excretion, CA presented a linear response ($p<0.01$) (Table 3). The average values for the excretion

of calcium were 1.06 vs. 0.93 and 0.88 g for the birds that were provided 0, 0.6 and 1.2% CA, respectively, 1.2% CA scored the lowest value of excretion and 0.0% CA highest value. A reduction of up to 25% in calcium excretion was observed.

The average digestibility values for calcium were 68.84 vs. 72.55 and 75.95% for the birds that were provided 0, 0.6 and 1.2% CA, respectively ($p<0.05$). Neither phytase nor calcium affected the digestibility and excretion of calcium. No interaction was observed for the treatment with CA x calcium x phytase.

In the intestines, high molar ratios of calcium lead to the formation of insoluble calcium-phytate complexes, the solubility of which depend on the chemical forms of the calcium salts and of the pH in the intestinal regions (Bronner, 1998). The present investigation showed that the dietary supplementation of CA reduced pH levels from 5.4 (control group) to 4.7 and 4.3 in the birds that were provided 0.6 and 1.2% CA, respectively. This dietary acidification may have maintained the acidities in the stomachs of the birds that consumed CA, which could have favoured the greater absorption of calcium and its reduced excretion. These findings are similar to those reported by Gentesse *et al.* (1994), Mroz *et al.* (2000) and Vargas-Rodriguez *et al.* (2002). However, acidic pH levels reduce the inhibitory effects of some minerals on phytate hydrolysis due to the protonation of the phosphate groups of weak acids, which replaces the minerals (Maenz *et al.*, 2000). CA could have acted as a proton donor, preventing the formation of calcium phytate salts.

Digestibility and excretion of nitrogen: In terms of excretion of nitrogen, CA presented a linear response ($p<0.01$) (Table 3). The average values for N excretion were 1.28, 1.05 and 1.00 for the birds that were fed 0, 0.6 and 1.2% CA, respectively. 1.2% CA scored the lowest value of excretion and 0.0% CA highest value. Phytase and calcium had no effects on N excretion.

The interaction of AC x phytase was significant ($p<0.05$) and led to the reduced excretion of nitrogen. A total of 0.955 g were excreted when the birds were fed 1.2% AC and 300 FTU and the absence of CA led to an increase in excretion to 1.372 g. This interaction was significant due to the effects of the CA combined with phytase, which considerably increased digestibility, compared with the phytase alone. No interactions were observed with CA x phytase x calcium.

The average values for N digestibility were 65.83 vs. 78.17 and 88.26% for the birds that were provided 0, 0.6 and 1.2% CA, respectively ($p<0.05$). 1.2% CA scored the highest value of digestibility and 0.0% CA lowest value. Phytase and calcium had no effects on N digestibility.

The interaction of 1.2% AC x 300 FTU x 3.25% calcium led to the increased digestibility of N by 92.32%, but in the absence of CA, N digestibility was reduced. This interaction was significant due to the effects of the CA combined with phytase and 3.25%, which considerably increased digestibility, compared with phytase alone with 3.25% calcium.

Birds that were fed diets containing CA, the proteolytic activities in their stomachs were stimulated, leading to improved digestibility and the decreased excretion of N. At acidic pH levels, dietary protein is degraded efficiently, improving its digestion (Blank *et al.*, 1999). The addition of acidifiers to the diets allows the maintenance of optimal acidic pH levels, creating healthy intestinal environments and thus enabling the regulation of beneficial flora (Vargas-Rodriguez *et al.*, 2013) optimising the activities of digestive enzymes. These findings are similar to those reported by Blank *et al.* (1999) and Mroz *et al.* (2000) in study of swine and Vargas-Rodriguez *et al.* (2002) in study of laying hens, who reported reduced levels of N excretion and improved N digestibility.

The results found in this study suggest that the addition of 0.6% CA, 300 FTU and 3% calcium to hens' diets improves the digestibility and significantly reduces the excretion of P; the addition of CA increases the digestibility and reduces the excretion of calcium and N. CA has effect on the response of the phytase to affect the excretions of P and N.

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