Research Article

Received: 17 January 2018 Revised: 22 March 2018 Accepted article published: 31 March 2018 Published online in Wiley Online Library: 20 May 2018

(wileyonlinelibrary.com) DOI 10.1002/jsfa.9047

The effect of exogenous phytase supplementation on nutrient digestibility, ruminal fermentation and phosphorous bioavailability in Rambouillet sheep

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Abstract

BACKGROUND: The effect of phytase supplementation with respect to a high sorghum grain diet on sheep voluntary feed intake, apparent nutrient digestibility, ruminal fermentation, phosphorus (P) excretion and blood serum P concentration was evaluated.

RESULTS: Phytase supplementation significantly decreased fecal P excretion ($P = 0.003$), resulting in a 26% decrease in relation to the phytase free diet. Dry matter intake, nutrient digestibility, ruminal butyrate and serum P were not ($P > 0.10$) affected by the phytase level. Neutral detergent digestibility showed a tendency to increase linearly ($P = 0.10$) with increasing phytase levels. Ruminal pH was lower for phytase supplemented sheep, with a significant decrease ($P = 0.007$) at 9 h post feeding, whereas ruminal ammonia-N at 3 h post feeding was lower ($P = 0.004$) for the phytase treatment groups, resulting in a decreasing linear response ($P = 0.001$) with an increasing phytase dose. Duodenal pH was significantly reduced at 6 h post feeding. Propionate tended ($P = 0.051$) to be increased linearly as the phytase supplementation level increased.

CONCLUSION: Exogenous phytase supplementation of high sorghum grain diets significantly decreased fecal P excretion in Rambouillet rams. Phytase supplementation appears to affect neutral detergent fiber digestibility, duodenal and ruminal pH, ammonia and propionate.

Keywords: digestibility; exogenous phytase; phosphorus excretion; Ruminal fermentation

INTRODUCTION

One of the major challenges confronting livestock production is the accumulation of phosphorus (P) in soils and the threat to surface water quality resulting from P losses to waterways, as a result of run-off or leaching. Inefficient P utilization by livestock results in the excretion of 60–80% of that consumed; therefore, a large portion of P imported to the farm in livestock diets is retained on the farm, rather than being transferred to meat or milk.[1] Although P metabolism in livestock has been investigated for many years, the present environmental focus has aroused research interest in this mineral.[2–4] The excess P excreted in feces represents both an unnecessary cost to farmers and a source of environmental pollution. Intensive animal production, as practised globally to safeguard animal protein supply to the teeming population, has been identified as a significant source of P contamination of receiving surface waters.

The nutritional manipulation of livestock diet to reduce the P content of their manure is regarded as a powerful, cost-effective approach for mitigating P losses from livestock farms and helping farmers to meet increasingly stringent environmental regulations.[1] In developed countries, environmental regulations regarding P and N excretion have increased consistently.[5] Diets based on cereal grains are used to promote the rapid growth of ruminants. However, the incorporation of a large amount of cereals in diets results in an increase in phytate P content, which makes the evaluation of phytate utilization by ruminants fed cereal-based diets imperative.[3,4] Although phytate P is partially hydrolyzed by phytase-producing rumen microbes, P excretion of finishing lambs fed with a high grain content diet is still considerably high.[6] However, supplementation of the diet with exogenous phytase may further increase P availability, enhance phytate

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utilization, improve digestibility and animal performance, and reduce P excretion.[3,4]

It was hypothesized that the addition of exogenous phytase would enhance phytate P utilization, resulting in a reduction of P excretion and an improvement in nutrient digestibility and animal performance. Therefore, the present study aimed to evaluate the effect of supplemental exogenous phytase at different application rates on nutrient digestibility, ruminal fermentations and phosphorus output in Rambouillet sheep.

MATERIALS AND METHODS

Study location

The experiment was conducted during the autumn, at the animal metabolic unit and the laboratory of animal nutrition of the Colegio de Postgraduados, Texcoco, Montecillo, Estado de México, México. The altitude was 2240 m a.s.l, with a moderately humid climate, an average temperature of 15–18 °C and an annual rainfall of 650 mm.

Animals, treatment and management

All procedures with respect to the handling of animals during the experimental period were conducted in accordance with the official Mexican standard of animal care (NOM-051-ZOO-1995). Six Rambouillet rams, weighing 39 ± 1.8 kg, fitted with permanent cannulas in the rumen (internal diameter 2.5 cm) and duodenum (T-type, internal diameter 0.8 cm), were used. The sheep were housed in individual metabolic cages equipped with high flow valve steel water bowls and were fed a basal diet containing 700 g of ground sorghum grain per ton dry matter (DM). The diet was balanced for minerals and vitamins and formulated to match the nutrient requirements of sheep in accordance with National Research Council[7] recommendations (Table 1). Adjustments were made to the diets to ensure the collection of orts. At the beginning of the experiment, sheep were treated with ivermectin (Ivomec-F-1, Duluth, Minnesota, USA; 1 mL 50 kg^{-1} body weight, subcutaneously), bacterin (Covexin, Intervet Inc., Madison, New Jersey, USA; 10 mL per animal; intramuscular) and vitamins A, D and E (Vigantol ADE, Germany; 1 mL per animal, intramuscularly). The experiment was laid out according to a 3 × 3 Latin square design with three treatments, three application rates of exogenous phytase enzyme (RONOZYME® HiPhos; DSM Nutritional Products Ltd, Kaiseraugst, Switzerland; contains 5000 units phytase g^{-1} of product), namely 0 g ton^{-1} DM (control treatment), 540 g ton^{-1} DM (P540 treatment) and 720 g ton^{-1} DM (P720 treatment) of the basal diet. In the first experimental period, the treatments were randomly assigned to the experimental units (sheep). Experimental periods consisted of 21 days, with an adaptation period of 15 days for the experimental diets and 7 days for measurements and sample collection. Sheep were fed *ad libitum* daily at 07.00 h. The exogenous phytase enzyme was added at the corresponding application rate and mixed with the diet individually before being fed. During the collection period, the amount of feed offered was recorded and orts were collected and weighed for determination of daily feed intake. Feeds were sampled daily, composited weekly, dried at 60 °C to constant weight and stored for chemical analysis.

Table 1. Ingredient and chemical composition of the dietary treatments

	Diets[a]		
	Control	P540	P720
Ingredient (g kg^{-1} DM basis)			
Ground sorghum grain	700	700	700
Corn gluten	169	169	169
Alfalfa hay	120	120	120
Calcium carbonate	11	11	11
Chemical composition (g kg^{-1} DM basis)			
Organic matter	963	964	969
Crude protein	183	183	183
Neutral detergent fiber	198	198	198
Acid detergent fiber	88	88	88
Calcium	6.4	6.4	6.4
Phosphorus	3.1	3.1	3.1
Phytate phosphorus	2.1	2.1	2.1

[a] The basal diet without (Control treatment) or with addition of RONOZYME® HiPhos at 540 (P540 treatment; contains 2 700 000 U ton^{-1}) or 720 (P720 treatment; contains 3 600 000 U ton^{-1}) g ton^{-1} feed (DM basis).

Nutrient digestibility

Total tract digestibility and fecal P excretion were determined by total fecal collection during the sample collection period of 7 days (from days 16 to 21 of each experimental period). Feces were collected twice a day from each ram at 07.00 h and 15.00 h in accordance with the methodology proposed by Peréz-Luna *et al.*[8] and stored at −10 °C for subsequent analysis. A sample of approximately 100 g of feces from each ram was taken daily and pooled for each animal within the entire period. The pooled samples were dried in a forced air oven at 65 °C for 72 h and then ground through a 1-mm screen using a Wiley mill grinder (Arthur H. Thomas, Philadelphia, PA, USA) and kept for subsequent determination of compositional analysis.

Collection of ruminal and duodenal samples

On days 17, 18 and 19 of the study period, duodenal digesta samples (approximately 5 mL) were collected from each sheep at 3, 6, 9, 12 and 24 h postprandially. Samples were obtained from the duodenal cannula, collected in a 100-mL amber vial, mixed with metaphosphoric acid (250 g L^{-1}) (1:4, v/v) and immediately frozen until analysis. On the same days (i.e. days 17, 18 and 19) of the study period, rumen samples were collected at 3, 6, 9, 12 and 24 h postprandially to determine pH, volatile fatty acids and ammonia-N (NH_3-N). Samples collected directly from rumen probe were filtered using a triple layer of gauze, and the pH was measured with a portable potentiometer (ORION, model SA 210; Thermo Scientific, Waltham, MA, USA). Then, 4 mL of ruminal liquid was placed in a test tube with 1 mL of metaphosphoric acid (250 g L^{-1}) to achieve a concentration of 4:1; the samples were frozen until analysis.

Serum phosphorous determination

On day 21 of each period, blood samples were taken by puncture of the jugular vein into vacutainer tubes without anticoagulant and centrifuged at 4000 × *g* for 10 min at 4 °C. Serum was separated and the supernatant kept frozen until P analysis.

Chemical composition

Dried feed, feed orts and fecal samples were ground through a Wiley mill (Arthur H. Thomas) using a 1-mm screen and analyzed for chemical composition. The DM (#930.15), ash (#942.05), ether

extract (EE; #920.39), nitrogen (#954.01) and acid detergent fiber (ADF; #973.18) were analyzed in accordance with the methods of AOAC,[9] and neutral detergent fiber (NDF) by the procedures of Van Soest *et al.*[10] using an Ankom (200/220) fiber analyzer unit (Ankom, Macedon, NY, USA). Organic matter content was calculated by difference. The concentration of NH_3-N was determined as described by McCullough.[11] Briefly, to determine the concentration of NH_3-N, the sample (2 mL) was thawed and centrifuged at $3000 \times g$ for 10 min. The supernatant (20 μL) was collected and deposited in 10-mL test tube. Next, 1 mL of phenol and 1 mL of hypochlorite sodium were added. Samples were incubated in water bath at 37 °C for 30 min, 5 mL of distilled water was added for sample dilution and the samples were stirred in a vortex (Genie 2 Model G-560; Scientific Industries Inc., Bohemia, NY, USA). Readings were performed in an ultraviolet light visible Cary 1-E spectrophotometer (Varian Inc., Palo Alto, CA, USA) at 630 nm. To determine the concentration of volatile fatty acids, the samples were thawed and an aliquot of 1.5 mL was centrifuged at $4000 \times g$ for 10 min. The supernatant was placed in a glass vial for chromatographic determination of the volatile fatty acids concentration in accordance with the proposed measurement by Erwin *et al.*,[12] using a gas chromatograph (model Claurus 500; Perkin Elmer, Waltham, MA, USA) with an auto sampler and equipped with a free fatty acid phase capillary column of length 15 m, an injector temperature of 240 °C, detector flame ionization of 250 °C and an oven at 140 °C with a gas flow of 400 mL min^{-1} for hydrogen and 40 mL min^{-1} for air. Phosphorous in the fecal samples content was determined by spectrophotometry. The serum phosphorus concentration was determined in a Selectra Junior chemical analyzer (Vital Scientific NV, Dieren, The Netherlands).

Statistical analysis

Data on feed intake, component digestibility, ruminal fermentation parameters (at each time post feeding) and blood parameters were analyzed as a 3×3 Latin square design with three periods and three experimental diets (Control, P540, P720) using PROC MIXED of SAS.[13] Two rams were used within each period and treatment. The statistical model was:

$$Y_{ijkl} = \mu + A_i + P_j + T_k + \epsilon_{ijkl}$$

where Y_{ijkl} is the observation for a given response variable, μ is the overall mean, A_i is the random effect of ram, P_j is the fixed effect of period, T_k is the fixed effect of rate of addition of enzyme and ϵ_{ijkl} is the residual error. When appropriate, data were subjected to one-way analysis of variance and Tukey's test was used for multiple comparisons of means. Polynomial contrasts (linear and quadratic effects) were fitted to the three rates of addition of the phytase enzyme. $P < 0.05$ was considered statistically significant. $P \leq 0.10$ was considered as a tendency approaching significance.

RESULTS

Feed intake and total tract digestibility

Dry matter intake (DMI) and nutrient digestibility were not affected ($P > 0.10$) by phytase level, period, and treatment × period interaction (Table 2). However, there was an increasing linear tendency ($P = 0.10$) for NDF digestibility as the phytase level increased in the diets. Phosphorus fecal excretion was lower at the P540 ($P = 0.061$) and P720 ($P = 0.003$) phytase doses (Fig. 1). By contrast, phytase supplementation had no effect on blood serum P level ($P > 0.10$).

Ruminal fermentation

Effects of phytase level, period, and treatment × period interaction were marginal ($P > 0.10$) for ruminal pH, except after 9 h of feeding (Table 3). Treatment ($P = 0.014$), period ($P = 0.005$), and treatment × period interaction ($P = 0.037$) had a pronounced effect on ruminal pH after 9 h of feeding. Phytase level affected ruminal pH (quadratic effect, $P = 0.007$). Period effect on ruminal pH tended toward significance after 12 h ($P = 0.096$). Treatment effect on ruminal NH_3-N was significant ($P = 0.004$) after 3 h of feeding, resulting in a linear decrease ($P = 0.001$) as the phytase levels increased, although it was marginal ($P > 0.10$) for the rest of the post feeding hours. Ammonia-N tended toward linear ($P = 0.065$) and quadratic ($P = 0.071$) trends as the phytase supplementation level increased in the diets. Ruminal NH_3-N was affected by period ($P < 0.05$) at 3, 12 and 24 h, tended to be affected ($P = 0.065$) at 9 h post feeding, and was not affected ($P > 0.10$) at 6 h post feeding. Treatment × period interaction affected ($P = 0.001$) ruminal NH_3-N at 3 h post feeding but had no effect ($P > 0.10$) for the rest of the post feeding hours. Period and treatment × period interaction affected ($P < 0.10$) ruminal acetate at 3 h post feeding. Period affected ruminal acetate at 2 h ($P = 0.0002$) and 48 h ($P = 0.009$). Treatment and treatment × period interaction did not affect ($P > 0.10$) ruminal propionate at all time points. Period effect on ruminal propionate was significant ($P = 0.026$) at 24 h, tended to be significant at 9 h ($P = 0.091$) and 12 h ($P = 0.059$), and had no effect ($P > 0.10$) at 3 and 6 h post feeding. Treatment and treatment × period interaction had no effect ($P > 0.10$) on ruminal butyrate, although there was a quadratic tendency ($P = 0.075$) as the phytase level increased at 48 h post feeding. Period affected ($P < 0.05$) ruminal butyrate at 3, 6, 9 and 12 h, and also tended to affect it at 24 h ($P = 0.051$) post feeding. Phytase level had no effect ($P > 0.10$) on duodenal NH_3-N, although there was a decreasing linear tendency ($P = 0.064$) after 6 h of feeding. Effect of period on duodenal NH_3-N was pronounced at 6 and12 h and also tended to be pronounced at 9 h ($P = 0.063$), although it had no effect ($P > 0.10$) at 3 and 24 h. Treatment × period interaction affected duodenal NH_3-N ($P = 0.007$) at 12 h and also tended to have an effect ($P < 0.10$) at 6 h, although it had no effect for the rest of the post feeding period.

DISCUSSION

There are inconsistencies in the reports of the effects of phytase supplementation on phosphorus excretion and serum P level in the literature. Phytase supplementation resulted in a significant decrease in fecal P excretion. This is in accordance with previous studies by Knowlton *et al.*,[14] Ahmed *et al.*[15] and Rodriguez *et al.*[16] but in contradiction to the study by Buendía *et al.*[5] The inconsistencies may be a result of animal factors, variation in diet composition and the levels of phytase administered. The significant effects of phytase supplementation on P excretion and serum P suggest that the available P concentrations in the diets did not exceed recommendation levels or that the endogenous phytase secretion by the animal's rumen bacteria was insufficient to hydrolyze all of the dietary phytate present. The data also suggest that there was a possible complementarity between the exogenous and endogenous phytase.

Ahmed *et al.*[15] reported no effect of phytase supplementation on the ruminal pH of sheep and the highest pH was observed before feeding (0 h post feeding). Most commercial enzyme products designed for application as monogastric feed supplements are highly active at low pH (pH ~3), as present in the stomach.[17]

Table 2. Feed intake and nutrient digestibility of diets with different levels of exogenous phytase and fed Rambouillet rams

	Diets[a]				*P* value				
	Control	P540	P720	SEM	Treatment	Period	Treatment × Period	Linear	Quadratic
DM intake (g day^{-1})	1206	1173	1300	53.1	0.320	0.592	0.874	0.281	0.286
Nutrient digestibility									
DM	736	745	757	18.73	0.331	0.598	0.596	0.162	0.910
Crude protein	691	721	720	13.6	0.316	0.437	0.759	0.206	0.411
Neutral detergent fiber	570	602	610	13.3	0.196	0.721	0.257	0.100	0.514
Acid detergent fiber	608	634	632	19.7	0.624	0.776	0.460	0.442	0.592

[a] The basal diet without (Control treatment) or with addition of RONOZYME® HiPhos at 540 (P540 treatment; contains 2 700 000 U ton^{-1}) or 720 (P720 treatment; contains 3 600 000 U ton^{-1}) g ton^{-1} feed (DM basis).

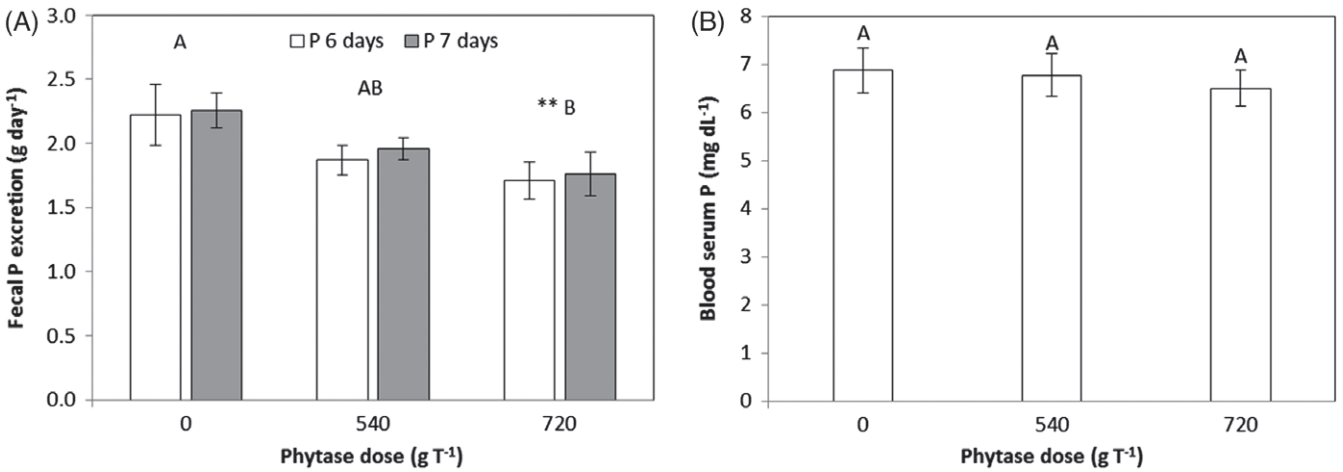

Figure 1. Fecal phosphorous excretion (g day^{-1}) (A) and blood serum phosphorus (mg dL^{-1}) (B) in Rambouillet rams fed diets with different levels of exogenous phytase. The basal diet treatments were without (0 g ton^{-1}; Control) or with addition of RONOZYME® HiPhos at 540 g ton^{-1} (contains 2 700 000 U ton^{-1}) or 720 g ton^{-1} (contains 3 600 000 U ton^{-1}) of feed (DM basis). Error bars correspond to the SEM and the same lowercase letters indicate that the treatments are not statistically different ($P < 0.05$, Tukey's test).

The phytase used (RONOZYME® HiPhos) has a typical bell-shaped pH-dependency of activity, with its highest activity at pH 4 and almost no activity at pH 6.5.[17] The rumen pHs observed in the present study imply a reduction of approximately 50% of the added activity at 3 h, without accounting for proteolysis and other sources of phytase activity losses. The high enzyme dose used may be sufficient to dephosphorylate the dietary phytate despite the pH-induced loss in activity. However, a prediction of the enzyme performance under *in vivo* conditions is not possible because, in addition to its pH profile, a variety of enzymatic properties need to be considered.[17] Although DMI was numerically highest for P720 and lowest for P540, there was no significant variation among diets, implying that phytase supplementation of ground sorghum grain based-diets fed to sheep did not affect feed intake. In earlier studies on lambs,[5,3,16] lactating cows[18,19] and finishing bulls,[20] no effects of phytase supplementation on DMI were observed. However, Winter *et al.*[21] reported a tendency towards a higher feed intake for bulls fed phytase supplemented diets containing varying P and Zn concentrations. According to Godoy and Meschy,[22] the inability of phytase supplementation to induce feed intake, body weight gain and feed conversion is a result of the saturation of its capability to hydrolyze the substrate. Although DM, crude protein, NDF and ADF digestibilities increased with an increasing supplemental phytase level, the increases were not significant. This is in disagreement with previous studies[5] where phytase supplementation resulted in significant linear increase in nutrient digestibility. However, Ahmed *et al.*[15] reported that phytase supplementation did not affect nutrient digestibility. The missing effect of phytase supplementation on nutrient digestibility in the present study may be attributed to a reduced digesta transit time through the gastrointestinal tract.

The decreased ruminal NH_3-N levels of phytase supplemented groups at 3 h post feeding could either be the result of a reduction in the proteolytic bacteria population or an increased incorporation of the NH_3-N into microbial protein as a result of improved microbial growth.[22] It is also possible that supplemental phytase reduced the ruminal degradability of dietary N. However, the effect of phytase supplementation on NH_3-N became marginal and insignificant with an increasing hours of feeding. Ahmed *et al.*[15] reported that phytase supplementation did not affect ruminal NH_3-N concentration. The lack of treatment effect on ruminal propionate and butyrate production suggests that supplemental phytase had no effect on gluconeogenesis and lipogenesis.[22] In accordance with a previous study,[15] phytase supplementation did not influence ruminal volatile fatty acids concentrations.

CONCLUSIONS

Exogenous phytase supplementation with respect to a high sorghum grain diet significantly decreased fecal P excretion

Table 3. Ruminal fermentation in Rambouillet rams fed diets with different levels of exogenous phytase

	Diets[a]				*P* value				
	Control	P540	P720	SEM	Treatment	Period	Treatment × Period	Linear	Quadratic
pH									
3 h	5.55	5.59	5.68	0.044	0.212	0.135	0.355	0.104	0.625
6 h	5.74	5.52	5.60	0.095	0.354	0.411	0.465	0.338	0.282
9 h	5.63	5.38	5.54	0.032	0.014	0.005	0.037	0.124	0.007
12 h	5.25	5.24	5.22	0.034	0.776	0.096	0.502	0.525	0.822
24 h	6.02	5.99	5.98	0.108	0.959	0.622	0.681	0.791	0.944
NH_3-N (g L^{-1})									
3 h	26.5	24.2	20.9	0.50	0.004	0.001	0.001	0.001	0.481
6 h	22.3	22.2	24.0	3.04	0.897	0.726	0.999	0.715	0.807
9 h	26.1	24.9	25.3	1.27	0.819	0.065	0.708	0.703	0.642
12 h	28.3	29.3	28.1	2.03	0.908	0.026	0.646	0.946	0.683
24 h	20.2	23.9	24.9	1.30	0.131	0.009	0.418	0.065	0.459
Acetate (mmol L^{-1})									
3 h	26.5	24.2	20.9	0.50	0.003	0.002	0.001	0.001	0.481
6 h	22.3	22.2	23.9	3.04	0.897	0.726	0.999	0.714	0.807
9 h	26.1	24.9	25.3	1.27	0.819	0.065	0.708	0.703	0.642
12 h	28.3	29.3	28.1	2.03	0.908	0.026	0.646	0.946	0.683
24 h	20.2	23.9	24.8	3.10	0.131	0.009	0.418	0.065	0.459
Propionate (mmol L^{-1})									
3 h	37.7	38.9	31.9	6.36	0.729	0.130	0.853	0.557	0.628
6 h	30.6	36.4	38.7	3.46	0.339	0.146	0.450	0.176	0.704
9 h	37.5	38.8	38.1	2.70	0.944	0.091	0.407	0.883	0.775
12 h	43.9	48.6	48.0	5.92	0.833	0.059	0.594	0.645	0.731
24 h	33.5	36.5	42.6	2.35	0.113	0.026	0.285	0.051	0.615
Butyrate (mmol L^{-1})									
3 h	12.0	12.5	12.0	1.64	0.960	0.030	0.272	0.999	0.788
6 h	11.9	10.9	14.4	1.11	0.192	0.011	0.356	0.187	0.182
9 h	15.1	13.7	15.5	2.01	0.813	0.049	0.434	0.903	0.552
12 h	19.8	22.6	22.8	3.92	0.843	0.045	0.459	0.623	0.798
24 h	16.4	17.1	13.5	2.46	0.596	0.051	0.246	0.453	0.521
Duodenal NH_3-N (g L^{-1})									
3 h	12.3	12.2	11.2	0.77	0.562	0.180	0.273	0.350	0.665
6 h	13.7	12.1	11.1	0.74	0.145	0.036	0.076	0.064	0.786
9 h	11.9	11.5	12.1	0.89	0.914	0.063	0.193	0.925	0.699
12 h	11.6	12.4	12.6	0.40	0.285	0.002	0.007	0.151	0.586
24 h	11.6	12.8	12.5	1.34	0.823	0.262	0.527	0.677	0.671

[a] The basal diet without (Control treatment) or with addition of RONOZYME® HiPhos at 540 (P540 treatment; contains 2 700 000 U ton^{-1}) or 720 (P720 treatment; contains 3 600 000 U ton^{-1}) g ton^{-1} feed (DM basis).

in Rambouillet rams. Phytase supplementation appears to affect neutral detergent fiber digestibility, duodenal pH, ruminal pH, ammonia and propionate. These effects of phytase on ruminal fermentation need to be explored in additional studies.

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