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RESEARCH ARTICLE

Influence of phytase enzyme on ruminal biogas production and fermentative digestion towards reducing environmental contamination

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Abstract

Environmental impact of livestock production has received a considerable public scrutiny because of the adverse effects of nutrient run-offs, primarily N and P, from agricultural land harboring intensive energy livestock operations. Hence, this study was designed to determine the efficacy of dietary phytase supplementation on fermentation of a sorghum grain–based total mixed ration (TMR) using a ruminal in vitro digestion approach. Phytase was supplemented at three doses: 0 (control), 540 (P540), and 720 (P720) g/t dry matter, equivalent to 0, 2.7×10^6, and 3.6×10^6 CFU/t DM, respectively. Compared to P720 and the control, gas production was higher for P540 after 12 h ($P = 0.02$) and 24 h ($P = 0.03$) of fermentation suggesting a higher microbial activity in response to phytase supplementation at lower phytase levels. Correspondingly, dry matter degradability was found to have improved in P540 and P720 compared to the control by 13 and 11% after 24 h of incubation ($P = 0.05$). For ammonia nitrogen (NH_3-N), a tendency towards lower values was only observed for P540 at 24 h of fermentation ($P = 0.07$), while minimal treatment effects were observed at other fermentation times. The concentrations of total volatile fatty acids (VFA) were higher ($P < 0.05$) after 48 h of fermentation for P540 and P720 compared to the control ($P = 0.03$) by 10% and 14%, respectively. Ruminal acetate tended towards higher values in the presence of phytase after 12 h of fermentation ($P = 0.10$), but towards lower values after 24 h of fermentation ($P = 0.02$), irrespective of the phytase dose applied. A trend towards lower ruminal propionate levels was observed in the presence of phytase after 6 h ($P = 0.10$) and 12 h ($P = 0.06$) of fermentation, while no effects were found at other fermentation times. In conclusion, phytase supplementation has the potential to improve metabolic energy activity of rumen microorganisms and the use of feed constituents. Thus, phytase supplementation could help to reduce environmental contamination in areas of ruminant production.

Keywords Biogas · Biodegradability · Environment · Phytase · Ruminal fermentation

Introduction

Phosphorus (P) is considered to be an important nutrient in livestock production systems. This is because of its presence as a structural component in animals (Tayyab and McLean 2015) and diet (cereal) being a primary source of P for animals. Recently, environmental impact of livestock production practices has received greater attention because of adverse effects of nutrient run-offs from agricultural land harboring intensive livestock operations. P run-offs from agricultural systems, for example, leads to higher vegetative growth resulting in a depletion of available oxygen (eutrophication) in water bodies adjacent agricultural land (Steinfeld and Wassenaar 2007). Furthermore, increasing competition between man, animal, and industry for raw materials has and

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would continue to increase the food, feed, and fuel crisis. Furthermore, the environmental footprint of livestock such as phosphorus (P), nitrogen (N), carbon dioxide (CO_2), and methane (CH_4) would continue to be a debate in human history until a sustainable/lasting solution is found. This study has a potential to address both cases. Similarly, there is a need to shift the focus on agricultural industry as just food provider. Can agricultural industry reduce their contribution to environmental pollution and start being an energy provider?

It is now important to establish agricultural industry as being capable/having the potential to provide renewable and sustainable energy while also reducing its greenhouse gases footprint.

Strategies to reduce P intake and increase P digestibility are considered most effective in reducing P excretion and accumulation on livestock farms (Kincaid et al. 2005). Phytase is a hydrolytic enzyme capable of initiating the stepwise dephosphorylation of phytate (Guyton et al. 2003; Applegate and Angel 2008; Abdel-Megeed and Tahir 2015). Hence, it is paramount to explore mitigation options for reducing P losses from livestock operations. Phytase as feed additive improves the nutritional value of feeds by enhancing the availability of dietary amino acids and minerals to the animal (Knowlton et al. 2005). Furthermore, phytase supplementation has the potential to improve P digestibility. However, the efficacy of phytase in improving P availability varies with forage content and grain source.

The use of phytase could help to reduce the excretion of phosphorous to the environment while the manure generated from such animals could be co-digested with other agricultural or organic matter for biogas production through anaerobic digestion. Green energy production involves the development of new catalysts and the development and use of renewable resources (e.g., solar energy and biomass). Promising approaches towards cleaner, more energy-efficient, and more cost-effective processes, including catalysts, reaction media, reactors, and separators, were developed to meet the increasing needs of both society and the environment (Chen 2017).

Sorghum is widely used in Mexico for ruminant nutrition. However, about 60 to 80% of its total P is present in form of phytate (Eeckhout and De Paepe 1994). Hence, phytase supplementation might be an effective strategy to increase P availability from sorghum-based diets. Khullar et al. (2011) observed an increase in ethanol production from the use of phytase with grain. From the energy yield perspective, the specific methane yield per hectare of sorghum is similar to maize (Wannasek et al. 2017). Methane potential of Maize has an estimate of 1660–12,150 methane yield (m^3/ha) while sorghum has an estimate of 2124–8370 methane yield (m^3/ha) (Murphy et al. 2011). Due to the limited knowledge on the efficacy of exogenous phytase in sorghum grain–based total mixed ration, the effects of addition of two levels of exogenous phytase to a sorghum-based ration on ruminal fermentation using an in vitro approach was studied. It was hypothesized that exogenous phytase supplementation enhances P availability to rumen microorganisms resulting in an improvement of dry matter degradability along with other rumen fermentation parameters including ammonia-N, gas production, and total volatile fatty acids (VFA). Thus, a reduction in the contamination of the agriculture environment in areas of ruminant production is expected.

Materials and methods

Experimental substrate and treatments

Sorghum grain–based TMR was used as a substrate for the in vitro fermentation studies (Table 1) and phytase RONOZYME® HiPhos (DSM Nutritional Products Ltd., Kaiseraugst, Switzerland) containing 5000-phtyase unit (FTU)/g according to the supplier was applied. Besides, a control (0 g phytase/t DM) and two phytase levels were studied: 540 g phytase/t DM corresponding to 2.7×10^6 FTU/t (P540) and 720 g phytase/t DM corresponding to 3.6×10^6 FTU/t (P720).

Ruminal fluid collection and batch culture incubation

Two ruminal (2.5-cm internal cannula diameter) and duodenal (0.8-cm internal cannula diameter) fistulated Suffolk rams (60 ± 2.8-kg body weight) were used as a source of rumen contents. The animals were treated according to the guidelines of the Mexican Official Standard of technical specifications for production, care, and use of animals (NOM-062-ZOO-1999). The

Table 1 Ingredients and chemical composition of the total mixed ration used as substrate for the in vitro fermentation study

Ingredient (g/kg dry matter basis)	
Ground sorghum grain	700
Corn gluten	169
Alfalfa hay	120
Calcium carbonate	11
Chemical composition (g/kg dry matter basis)	
Dry matter (g/kg wet material)	937
Organic material	963
Crude protein	183
Neutral detergent fiber	198
Acid detergent fiber	88
Calcium	6.4
Phosphorus	3.1
Phytatephosphorus	2.1

The basal diet was supplemented with 0 (control treatment), 2.7×10^6 (P540 treatment), and 3.6×10^6 (P720 treatment) FTU/t RONOZYME® HiPhos (DSM Nutritional Products Ltd., Kaiseraugst, Switzerland)

rams were housed in a corral equipped with steel-valved automatic waterers and fed the control diet. Both animals were fed ad libitum twice daily (08:00 and 16:00 h), and rumen contents were collected after adaptation to the diets for 14 days.

Rumen contents were collected from the ventral sac 2 h after the morning feeding. The pooled rumen contents from the two Suffolk rams were filtered using four gauze layers. The filtrate was collected in an insulated thermal container preheated to 39 °C. The rumen liquid was immediately transported to the lab. There, it was kept at 39 °C in a water bath and continuously flushed with CO_2 to mimic the ruminal milieu and maintain anaerobic conditions.

One volume of rumen fluid was mixed with three volumes of prewarmed artificial saliva (39 °C) (Goering and van Soest 1970) without addition of trypticase. Forty milliliters of the buffered ruminal fluid were added to 0.5 g of substrate in 120-mL glass bottles. The headspace of bottles was flushed with CO_2 for 30 s, and the bottles were closed using neoprene plugs and crimp-sealed to avoid leakage of gases during fermentation. Sealed glass bottles were incubated in a water bath at 39 °C for 3, 6, 9, 12, 24, and 48 h. Four independent incubations were performed with three replicates per sampling time.

Gas production was measured using a water displacement apparatus as described by Fedorak and Hrudey (1983) equipped with a conical funnel, a 100-mL burette, and two latex hoses of 0.5 and 1 m in length and 3/8″ in diameter. The vials were punctured with a 16-gauge needle placed at the end of the hose, and gas production (mL) was determined by displacement of water in the burette. Thereafter, the vials were placed in a freezer (− 4 °C) for 5 min in order to stop the fermentation process.

After measurement of gas production, the fermentation vials were opened and pH values of the media were measured using pH meter (Thermo Scientific, Orion Star™ A121, Beverly, MA, USA).

Dry matter degradability

Dry matter (DM) degradability was determined by collecting the residues after fermentation using a filtration system connected to a vacuum pump. To ensure complete recovery of the residues, the vials were rinsed three times with hot water. The residues were collected in ANKOM® bags (F57 bags, Ankom® Technologies), and the bags were dried in a forced air oven at 55 °C for 48 h to a constant weight. The dry matter digestibility (DMD) was expressed as the difference of the initial weight of the dried substrate and the weight of the dried residue.

Ammonia nitrogen and volatile fatty acids

For quantification of NH_3-N and volatile fatty acid (VFA), the fermentation liquid was collected by filtration. Ammonia-N was determined as described by Broderick and Kang (1980). In brief, aliquots of the filtrates were centrifuged at × 3000*g* for 10 min. One milliliter of phenol and 1 mL of hypochlorite were added to 20 μL of supernatants and mixed thoroughly. After an incubation at 39 °C for 30 min, 5 mL of distilled water were added and absorbance of the solution was read at 630 nm (Varian, model Cary 1E, CA, USA).

For volatile fatty acid quantification, 4 mL of the filtrate were mixed with 1 mL of 25% (*w*/*v*) metaphosphoric acid, shaken slightly, and placed in a freezer until further use. One and a half milliliters of the filtrate were centrifuged for 10 min at ×4000*g*, and the VFA profiles (acetic, propionic, and butyric acids) were determined in the supernatants by gas chromatography (Perkin Elmer, model Claurus 500, Beaconsfield, UK). The gas chromatograph was equipped with an autosampler and a flame ionization detector. Samples were analyzed by maintaining column temperature at 240 °C, the temperature of the flame ionization detector at 250 °C, and oven temperature at 140 °C. Hydrogen was used as carrier gas with a flow rate of 400 mL/min, while the flow rate for air was kept at 40 mL/min.

Chemical composition of experimental diets

The DM contents of the diets were determined by drying feed samples in a forced air oven at 55 °C for 48 h. The organic matter (OM) content of diets were quantified by carbonization of the samples in muffle furnace at 550 °C overnight and subtracting the obtained ash content from the total DM content of the same sample (AOAC 2000). The neutral detergent fiber (NDF) and acid detergent fiber (ADF) concentrations of TMR were determined using an ANKOM 200 Fiber Analyzer (ANKOM technology, Macedon, NY). NDF was quantified according to van Soest et al. (1991) and ADF using the procedure of the Association of Official Analytical Chemists (AOAC 2000). Calcium in TMR was quantified by atomic absorption spectrometry (Robinson 1975). A colorimetric method was used for the determination of the P content of TMR (AOAC 2000), whereas the ferric precipitation method was used to quantify phytate P (Raboy et al. 1984).

Experimental design and statistical analysis

PROC MIXED of SAS (2006) was used for the statistical analysis of the data. A randomized complete block design (four blocks/runs) of three treatments (level of phytase added) performed in triplicate for each phytase level was used for data analysis. The data of each one of the three runs (block) within the same sample (TMR) and phytase level were averaged and used as an experimental unit (Udén et al. 2012) according to the following model:

$$Y_{ijk} = \mu + T_i + B_j + (T \times B)_{ij} + E_{ijk} \quad (1)$$

whereby Y_{ijk} represents very observation of the jth block assigned to ith treatment, μ the overall mean, T_i the effect of the treatment, $(T \times B)_{ij}$ the interaction between treatment and block, and E_{ijk} the residual error.

For multiple comparisons of means, the Tukey test was applied in order to examine dose responses in respect to phytase supplementations. Polynomial (linear and quadratic) contrasts were used. Significance levels were chosen at $P < 0.05$ and trends at $P \leq 0.10$.

Results

Fermentation pH and biogas production

No treatment effects were observed for fermentation pH after 3 h ($P = 0.18$), 12 h ($P = 0.51$), 24 h ($P = 0.63$), and 48 h ($P = 0.74$) of fermentation. Fermentation pH, however, was found to be higher in the presence of phytase after 6 h ($P < 0.01$) and 9 h ($P = 0.03$) of fermentation. Overall, fermentation pH decreased during fermentation in the first 24 h; thereafter, fermentation pH increased irrespective of the treatment (Fig. 1).

The effects of phytase supplementation on gas production after different fermentation times are presented in Fig. 2. No treatment effects were observed on cumulative gas production after 3 h ($P = 0.61$), 6 h ($P = 0.67$), and 48 h ($P = 0.83$) of fermentation. Compared to the control, gas production tended towards higher values for P540 but lower values for P720 ($P = 0.06$) after 9 h of fermentation. Phytase supplemented at a lower level (P540) improved cumulative gas production after 12 h ($P = 0.02$) of fermentation when compared to P720 and the control. Compared to the control however, gas production was higher for both P540 and P720 after 24 h of fermentation ($P = 0.03$).

DM degradability

No effects on DMD were observed with phytase supplementation after 3 h ($P = 0.92$), 6 h ($P = 0.64$), 9 h ($P = 0.14$), 12 h ($P = 0.17$), and 48 h ($P = 0.90$) of fermentation (Fig. 3). Compared to the control, however, DMD was improved by 13 and 11% for P540 and P720, respectively, after 24 h of fermentation ($P = 0.05$). Irrespective of the treatment, average DMD was higher by 70, 109, 136, 220, and 275% at 6 h, 9 h, 12 h, 24 h, and 48 h, respectively, when compared to the baseline levels of DMD after 3 h of fermentation.

Ammonia-N and volatile fatty acid concentrations

The average NH_3-N concentrations observed during in vitro fermentation are given in Table 2. While no significant treatment effects were observed, NH_3-N tended towards lower concentrations for P540 after 24 h of fermentation ($P = 0.07$) compared to the control and P720.

Total VFA concentrations (Table 2) were expressed as the sum of the individual acetate, propionate, and butyrate concentrations (Fig. 4) after the respective incubation times. No treatment effects on total VFA were observed after 3 h, 6 h, 9 h, 12 h, and 24 h of fermentation. After 48 h, total VFA increased ($P < 0.01$) for P540 by 10% and for P720 by 14% compared to the control. No differences in total VFA were observed between P540 and P720. No treatment effects on the acetate-to-propionate ratios were found after 3 h ($P = 0.47$), 6 h ($P = 0.13$), 9 h ($P = 0.41$), 24 h ($P = 0.14$), and 48 h ($P = 0.18$) of fermentation. Phytase supplementation, irrespective of the dose applied, tended towards an increase in the acetate-to-propionate ratios after 12 h of fermentation ($P = 0.08$) (Table 2).

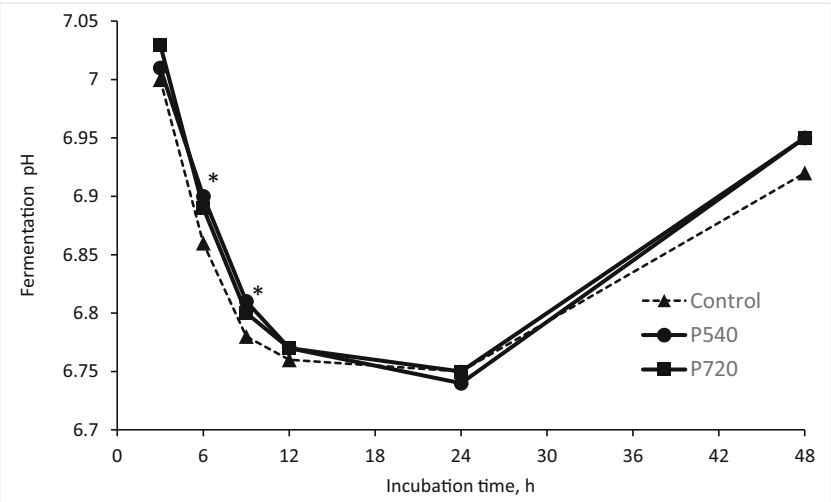

Fig. 1 Development of fermentation pH during in vitro fermentation of a sorghum grain–based total mixed ration with rumen liquid in the presence and absence of exogenous phytase. The basal diet was supplemented with 0 (control treatment), 2.7×10^6 (P540 treatment), and 3.6×10^6 (P720 treatment) FTU/t RONOZYME® HiPhos (DSM Nutritional Products Ltd., Kaiseraugst, Switzerland)

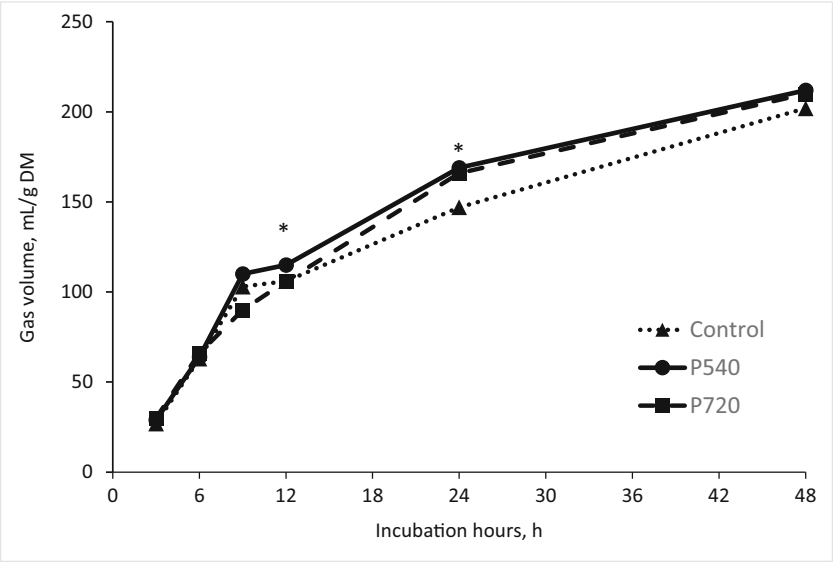

Fig. 2 Gas production during *in vitro* fermentation of a sorghum grain–based total mixed ration with rumen liquid in the presence and absence of exogenous phytase. The basal diet was supplemented with 0 (control treatment), 2.7×10^6 (P540 treatment), and 3.6×10^6 (P720 treatment) FTU/t RONOZYME® HiPhos (DSM Nutritional Products Ltd., Kaiseraugst, Switzerland)

Discussion

The effects of phytase supplementation on rumen fermentation were assessed using a sorghum grain–based total mixed ration (i.e., TMR) as a substrate with alfalfa hay being the sole source of forage. Based on the results obtained, it is evident that phytase supplementation had an effect on ruminal fermentation irrespective of the phytase dose applied. A higher gas production after 12 h and 24 h of fermentation and a higher dry matter degradability (i.e., DMD) after 24 h of fermentation was observed in the presence of phytase. No effect on ruminal pH, however, could be found when supplementing phytase.

Generally, fermentation is seen as an indicator for the degradation of dry matter resulting in the production of volatile fatty acids (VFA) and several gases, mainly CO_2, H_2, and CH_4 (Hernandez et al. 2017). The gas volume obtained during fermentation in the in vitro fermentation model applied, is a measure for the nutrient availability and metabolic activity of rumen microorganisms (Kholif et al. 2017). Furthermore, gas production profiles related to the composition of the diets (Menke et al. 1979). In contrast to the results reported by Ahmed and co-workers (Ahmed et al. 2014), a higher gas production was found to be associated with improved DMD in the presence of exogenous phytase. This might be attributed to an improved P availability to rumen microorganisms in the studied system after enzymatic release of phosphate residues from the *myo*-inositol ring of phytate—the major P source in sorghum grain–based diets (Eeckhout and De Paepe 1994). This is because the organically bound phosphate is not directly available to microorganisms; P might be the limiting factor for microbial activity in the in vitro system used. The effects of enzyme supplementation were not evident before 12 h of fermentation

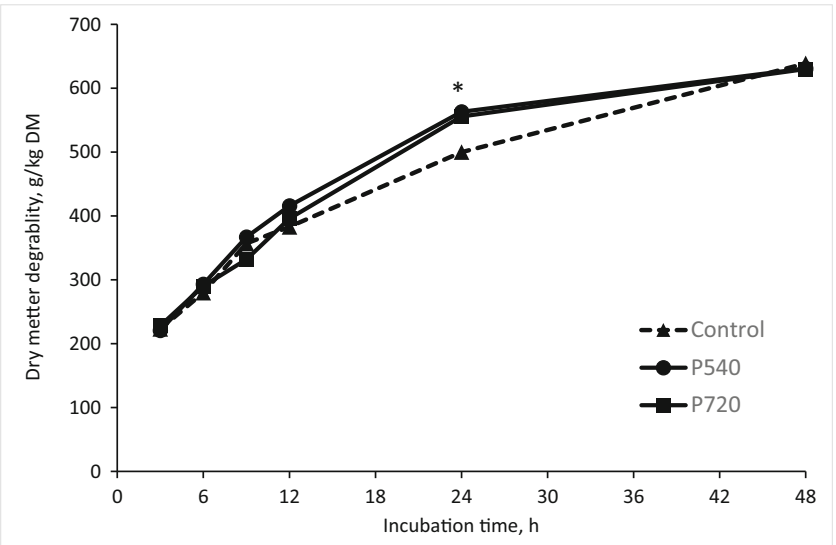

Fig. 3 Dry matter degradability (DMD) during *in vitro* fermentation of a sorghum grain–based total mixed ration with rumen liquid in the presence and absence of exogenous phytase. The basal diet was supplemented with 0 (control treatment), 2.7×10^6 (P540 treatment), and 3.6×10^6 (P720 treatment) FTU/t RONOZYME® HiPhos (DSM Nutritional Products Ltd., Kaiseraugst, Switzerland)

Table 2 Concentrations of ammonia-N (NH_3-N) and total volatile fatty acids (VFA) as well as the acetate-to-propionate ratio during in vitro fermentation of a sorghum grain–based total mixed ration with rumen liquid in the presence and absence of exogenous phytase

		Treatments[1]				*P* value		
	Incubation time (h)	Control	P540	P720	SEM	Treatment	Linear	Quadratic
NH_3-N (mg/dL)	3	35.7	33.7	33.0	0.64	0.23	0.01	0.44
	6	32.5	33.6	35.1	0.79	0.12	0.04	0.83
	9	34.2	35.0	35.2	0.79	0.65	0.39	0.78
	12	33.7	34.6	33.0	1.26	0.69	0.68	0.45
	24	39.6	38.1	40.0	0.55	0.070	0.60	0.03
	48	49.0	50.3	54.6	2.00	0.50	< 0.01	0.25
Total VFA (mmol)	3	40.0	42.5	41.7	1.47	0.49	0.32	0.51
	6	48.2	46.7	45.0	1.56	0.39	0.20	0.67
	9	47.6	47.6	47.9	1.28	0.98	0.89	0.89
	12	51.7	48.7	49.1	1.52	0.36	0.19	0.61
	24	55.6	54.3	59.8	2.32	0.25	0.38	0.16
	48	58.8^{b}	64.8^{a}	67.2^{a}	1.94	0.03	< 0.01	0.92
Acetate:propionate	3	4.69	4.63	4.54	0.08	0.47	0.27	0.62
	6	4.03	4.10	4.19	0.05	0.13	0.06	0.49
	9	3.76	3.77	3.74	0.01	0.41	0.57	0.24
	12	3.56	3.94	4.03	0.14	0.08	0.03	0.90
	24	4.07	4.01	3.96	0.04	0.14	0.06	0.71
	48	4.53	3.92	3.96	0.24	0.18	0.08	0.57

The basal diet was supplemented with 0, 2.7×10^6, and 3.6×10^6 FTU/t) g/t feed RONOZYME® HiPhos (DSM Nutritional Products Ltd., Kaiseraugst, Switzerland)

SEM standard error of the mean

[a, b] Means with differentdifferent alphabet are significantly ($P<0.05$) different

indicating that this time was required to alter microbial metabolism due to increased inorganic P concentrations.

The effects of phytase supplementation observed on DMD are in good agreement with previous studies showing greater DM degradability with prolonged exposure to exogenous phytases (Buendía et al. 2010; Brask-Pedersen et al. 2011). The increase in DM degradability could be due to a better solubility of minerals and/or a better protein digestibility after lowering phytate concentration in the system as well as a higher cell wall degrading activity of rumen microorganisms in the presence of higher inorganic phosphate concentrations (Komisarczuk-Bony and Durand 1991; Darabighane et al. 2018). The observed effects of phytase supplementation on gas production with phytase supplementation further support this assumption. The accumulation of gas in the rumen results from microbial fermentation of feed is highly correlated with the extent of carbohydrate fermentation since gas production from protein and fat conversion is negligible (Akinfemi et al. 2009).

The lack of treatment effects on the VFA profile during the initial 24 h of fermentation did not correlate well with the responses observed for gas production and DMD. This observation, however, is not essentially new. Ahmed et al. (2014) already reported a missing effect of phytase supplementation on ruminal VFA concentrations. Winter et al. (2015) observed only marginal effects of phytase addition on individual VFA in cows. In contrast to previous studies, however, total VFA concentrations were higher after 48 h of fermentation in the presence of exogenous phytase. The late phytase effect on VFA concentrations might be explained by the time needed to make the organic phosphate available for the rumen microorganisms (about 12 h). Due to a higher microbial activity after 12 h of fermentation, carbohydrates were degraded to their respective monomeric units (hexoses). Subsequently, hexoses were metabolized to VFA yielding energy required for overall microbial metabolism (van Lingen et al. 2016).

No effects of phytase supplementation on NH_3-N were identified. This is in agreement with previous studies (Ahmed et al. 2014; Winter et al. 2015). Taking an improvement of protein digestibility with lower phytate concentrations into account, an increase in NH_3-N concentrations would be expected when supplementing phytase. Thus, an improvement in protein digestibility might be dependent on the protein source used in the in vitro and in vivo studies.

From the biogas perspective, the improved digestibility shows that inclusion of phytase in animal diet would help to prevent phosphorus excretion into the environment.

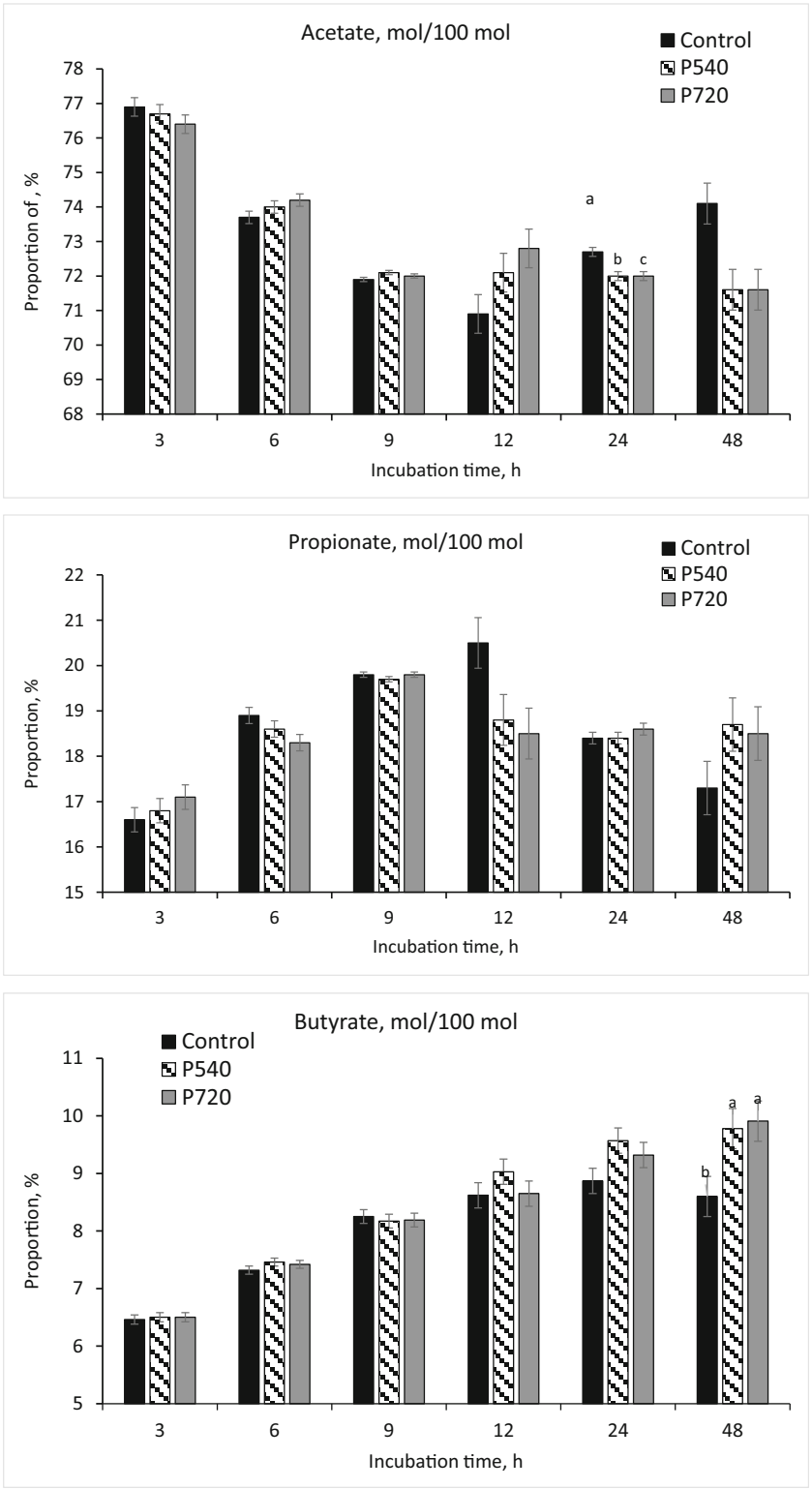

Fig. 4 Relative amounts of individual volatile fatty acids (acetate, propionate, butyrate) during *in vitro* fermentation of a sorghum grain–based total mixed ration with rumen liquid in the presence and absence of exogenous phytase. The basal diet was supplemented with 0 (control treatment), 2.7×10^6 (P540 treatment), and 3.6×10^6 (P720 treatment) FTU/t RONOZYME® HiPhos (DSM Nutritional Products Ltd., Kaiseraugst, Switzerland)

This implies that phytase aids energy/starch digestibility, which is the possible reason for the increase gas production. The non-effect of phytase on NH_3-N shows that the protein availability in the rumen might be because of low protein digestibility, hence a potential increase in crude protein availability in the feces. The probable increase in fecal crude protein could help to increase the nitrogen content in animal manure which if used in co-digestion with other waste product in biogas production could help to improve the C/N ratio.

Conclusions

Phytase supplementation resulted in higher gas production, an improved dry matter digestibility, and higher VFA concentrations in a ruminant in vitro digestion model. An effect on ammonia-N concentration was not observed. The observed effects has been shown to be dependent on the phytase dose applied and on the fermentation time. Further studies in animal models are required to confirm findings observed in the present study. The results obtained in this study, however, are promising in respect to the potential of phytase to reduce environmental pollution in intensive ruminant production systems. In addition, other ruminant/residual energy in feces could be converted through anaerobic digestion in order to find solution to the increasing food, feed, and fuel crisis.

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