PAPER

Effect of phytase supplementation on rumen fermentation characteristics and phosphorus balance in lactating dairy cows

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Abstract

This study aimed to evaluate the effects of exogenous phytase on rumen fermentation characteristics, the phosphorus (P)-flow at the duodenum and the P-balance in lactating dairy cows. For this purpose ruminal and duodenally fistulated cows were assigned to one of three dietary treatments: high P (HP) diet (n=7) provided a total of 45 g/d of P, archived by a supplementation of dicalcium phosphate to the diet; low P (LP) diet (n=5) provided 34 g/d of P without supplementation; LP+phytase (LP+PHY) diet (n=5) provided 34 g/d of P supplemented with an exogenous phytase. Dry matter intake and milk yield were recorded daily. In the first week of a sampling period P-balance was determined. Samples of ruminal fluid were taken and duodenal chyme was collected in the second sampling week. Ruminal pH and the concentration of volatile fatty acids were not different between the treatments. The HP-group shows a higher P-flow at the duodenum than other groups. No differences in apparent total tract P-digestibility were found between the treatments. The P-balance in the HP-group (2.6 g/d) was higher compared to the LP (-3.2 g/d) and LP+PHY (-3.0 g/d) group. Overall, phytase supplementation had no effect on P-digestibility in lactating dairy cows.

Introduction

Phytase releases phosphorus (P) from phytate [myo-inositol hexakisphosphate ($InsP_6$)] and lower inositol phosphates by dephosphorylation and hydrolysis (Suttle, 2010). In ruminants the microbial community produces phytase which is responsible for $InsP_6$ degradation (Morse *et al*., 1992). A study by Clark *et al*. (1986) pointed out that 98% of dietary $InsP_6$ was hydrolysed to inorganic P (P_i) in the gastrointestinal tract of dairy cows. However, *in vitro* investigations by Godoy and Meschy (2001) showed that in specific situations the ruminal phytase hydrolyses not all P from $InsP_6$. They carried out an experiment with a semi-continuous culture system, infusing P_i or a phytate source into the system. The results showed that only 67% P from the phytate source were available. These results are in contrast to the mentioned study of Clark *et al*. (1986) and were supported by an *in vivo* study by Kincaid *et al*. (2005), which showed values of phytate hydrolysis of approximately 80% irrespective of the dietary grain sources barley and corn. In both dietary situations (26% barley or 26% corn in the diet) an exogenous phytase supplementation increased the $InsP_6$ hydrolysis from approximately 80 to 85% (Kincaid *et al*., 2005). The authors attributed the incomplete hydrolysis of $InsP_6$ to an increased ruminal passage rate. Consequential a phytase supplementation could increase P-supply to the microbes. An insufficient P-supply to the microbes reduces organic matter (OM) fermentation and microbial protein synthesis rates in the rumen (Kincaid and Rodehutscord, 2005). We hypothesized that exogenous phytase increases the P-supply to the cow and rumen microbes, caused by increased ruminal degradation of $InsP_6$, in dairy cows fed a highly digestible diet with increased passage kinetics based on corn silage (70%) and concentrate (30%).

For this purpose, two P reduced diets, one of the two supplemented with exogenous phytase, were compared to a diet supplemented with dicalciumphosphate to meet the P requirement for dairy cows and rumen microbes. The objective of the experiment was to examine the effects of exogenous phytase on rumen fermentation characteristics, the P-flow at the duodenum and the P-balance in lactating dairy cows.

Materials and methods

Animal treatments and experimental design

The study was conducted in accordance with the German Animal Welfare Act with the approval of the Lower Saxony State Office for Consumer Protection and Food Safety, Oldenburg, Germany. The experiment with a total of nine multiparous German Holstein dairy cows was carried out at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Federal Research Institute for Animal Health, in Braunschweig, Germany. The cows were fitted with large rubber cannulas in the dorsal sac of the rumen (inner diameter: 10 cm) and T-shaped cannulas at the proximal duodenum close to the pylorus (inner diameter: 2 cm). At the beginning of the trial the average milk yield of the cows was 20.6±0.2 kg/d, the animals had an average body weight of 558±57 kg and the animals were on average in their 3.6±1.4 lactation. The cows were kept in a tethered stall with neck straps and individual troughs with free access to water. Cows were milked daily at 5:30 and 15:30 h.

The cows received three diets differing in the concentration of P and phytase supplementation. The P concentration of the high P (HP) diet was calculated to cover the recommendations for a dairy cow with a milk yield of 20 kg/d and a feed intake of 16 kg dry matter (DM)/d as given by the German Society of Nutrition Physiology (GfE, 2001). The basal low P (LP) diet was intended to contain 80% (2.6 g P/kg DM) of the P of the diet for group HP (3.3 g P/kg DM). The animals of group LP+phytase

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Key words: Dairy cows, P-balance, Phytase, Rumen.

Acknowledgments: the authors would like to thank DSM-Nutritional Products Ltd for financial support. The assistance of the co-workers of the Institute of Animal Nutrition and the Experimental Station of the Friedrich-Loeffler-Institut (FLI) in Braunschweig, Germany, in performing the experiment and analysis is gratefully acknowledged.

Received for publication: 23 June 2014.
Accepted for publication: 5 December 2014.

Italian Journal of Animal Science 2015; 14:3539
doi:10.4081/ijas.2015.3539

(LP+PHY) got the same concentrate as group LP, but supplemented with an experimental phytase (DSM-Nutritional Products Ltd, Basel, Switzerland and Novozymes A/S, Bagsvaerd, Denmark). The composition of concentrates is given in Table 1. As intended, the P concentration in the concentrates manifested variations. The proportion of $InsP_6$ in the total P of the concentrates was 21 and 27% in the LP and LP+PHY-group. Because of the supplementation of P_i in the HP-group, the percentage of $InsP_6$ declined to 12%. The concentrated feed of group LP and HP showed no phytase activity, while the concentrate LP+PHY showed an analysed phytase activity of 5859±15 phytase unit (FTU)/kg. The diets were intended to cover the energy and protein requirements of the cows according to the recommendations of the GfE (2001). To ensure the intended corn silage/concentrate ratio (70:30% on a DM basis), the DM of corn silage was determined twice a week. Corn silage and concentrates were given in two equal portions at 5:30 and 15:00 h. The pelleted concentrates were hand-mixed with the silage in the trough. During three periods the cows were assigned to three different experimental groups. Each experimental period consisted of three weeks of adaption and two weeks of sample collection. During the three weeks of adaption the animals became accustomed to the feed and the barn. The mean lactation day (calculated for the beginning of the first sampling week of each period) of the cows were on average 131 d (±69) in period one, 140 d (±29) in period two and 140 d (±56) in period three. Due to different calving dates, not every cow could be used in all periods. In period one each of the three treatments was fed to two cows. In the second period two cows received the LP, two cows the HP and one cow the LP+PHY diet. In period three one cow received the LP, three cows HP and two cows the LP+PHY-diet. No cow received the same treatment twice.

Measurements and sampling procedure

In the first and second sampling week, samples of corn silage and concentrate, as well as feed refusals, if any, were collected daily and pooled on a weekly basis. Feed samples and refusals were dried at 60°C before analysis.

In the first week of the sampling period, urine and faeces were collected completely. For that purpose the cows were equipped with urine devices, which were fitted around the vulva and allowed a separate collection of urine and faeces. Urine was piped from the urine device through a tube into a canister with 500 mL of sulphuric acid (10%, v/v). The amount was recorded every day and a subsample was taken and stored at -18°C. Faeces were homogenised and weighed daily. An aliquot of two percent was taken daily, pooled on a weekly basis and freeze dried. Milk yields were recorded daily. Milk samples were taken twice a week during morning and evening milking in the first sampling week to determine fat, protein, lactose, urea and somatic cell count (SCC). For this, a sample of 50 mL from each milking was conserved with bronopol and kept at 8°C until analysis. For the determination of milk urea, aliquots of the two daily milk samples were mixed and frozen at -18°C. Furthermore during one day of the first sampling week, samples of ruminal fluid (approximately 100 mL) were withdrawn from the ventral sac through the rumen cannula using a hand vacuum pump. Fluid was taken before the first feeding at 5:30 a.m. and 60, 120 and 300 minutes afterwards. In the second sampling week, duodenal chyme was collected for five consecutive days every two hours. At each sampling, four 100 mL samples were taken through the duodenal cannula from each cow. The pH-value was measured in each sample immediately. A glass electrode (pH525; WTW, Weilheim, Germany) was used to measure the pH and the sample with the lowest value was added to the daily pooled sample from each cow to get the sample with the lowest contamination by endogenous secretion (Rohr *et al*., 1984) and stored at -18°C. To estimate the digesta flow, a chromium oxide (Cr_2O_3) marker [19.8% Cr_2O_3, 79.1% wheat flour, and 0.67% aluminium sulphate (Al_2SO_4)] was used. The marker was given in two portions of 50 g at 5:15 a.m. and 5:15 p.m. into the rumen beginning ten days before starting the duodenal chyme collection. One day before and then during the sampling period, 25 g were given into the rumen every six hours at 5:45 a.m.; 11:45 a.m., 5:45 p.m. and 11:45 p.m.

Analysis

Samples of feedstuffs, refusals, duodenal chyme and faeces were analysed according to the methods of the Association of German Agricultural Analytic and Research Institutes (VDLUFA, 1997). Samples were dried at 60°C for 72 hours and ground through a 1-mm screen. Analysis of acid (ADF) and neutral

Table 1. Ingredients of concentrates and chemical composition of the diet components used during the trial.

Variable	Corn silage	Concentrate		
		HP	LP	LP+PHY
Ingredients, %				
Corn		35.0	35.0	34.2
Wheat gluten		10.0	10.0	10.0
Dried sugar beet pulp		48.0	48.5	48.5
Urea		3.0	3.0	3.0
Sodium chloride		0.2	0.2	0.2
Mineral premix°		2.0	2.0	2.0
Premix with phytase#		-	-	0.83
Dicalcium phospate		1.8	-	-
Calcium carbonate		-	1.3	1.3
Chemical composition, g/kg DM				
Nutrients				
OM	955	915	917	924
CP	83	258	254	256
EE	32	28	27	26
ADF	263	112	118	120
NDF	501	265	269	280
Minerals				
P	2.74	4.89	1.88	1.96
$InsP_6$	-	0.57	0.39	0.53

HP, high phosphorus (diet added with dicalcium phosphate); LP, low phosphorus (diet without phosphorus and phytase supplementation); PHY, phytase (diet without phosphorus-supplementation, but added with an exogenous phytase); DM, dry matter; OM, organic matter; CP; crude protein; EE, ether extract; ADF, acid detergent fibre; NDF, neutral detergent fibre; P, phosphorus; $InsP_6$, myo-inositol hexakisphosphate. °Composition (per kg): calcium, 200 g; sodium, 120 g; magnesium, 40 g; vitamin A (E672), 1,000,000 U; vitamin D_3 (E671), 100,000 U; vitamin E (alpha tocopherolacetate), 1500 mg; manganese [manganese (II)sulphate, monohydrate E5], 5.4 g; zinc (zincoxide E6), 6 g; copper (copper sulphate pentahydrate E4), 1 g; iod (calcium jodate, waterfree E2), 100 mg; selenium (sodium selenate E8), 40 mg; cobalt (cobalt sulphate, monohydrate, E3), 25 mg. #Composition (per kg): 0.952 kg corn grain added with 0.048 kg experimental phytase.

detergent fibre (NDF) was conducted following the methods of VDLUFA (1997). Samples of morning and evening milk were pooled according to their milk yields and freeze dried for analysis of P. Faeces samples taken during the first experimental weeks were also freeze dried for the determination of nutrients.

The phytase content of the concentrates was determined via the phytase activity and expressed as FTU/kg feed (Engelen *et al*., 1994). Phosphorus in diet, milk, urine and faeces was analysed with an optical emissions spectrometer with inductively coupled plasma [(ICP-OES) Quantima; GBC Scientific Equipment Pty Ltd, Braeside, Australia] according to VDLUFA (1997).

Myo-inositol hexakisphosphate was analysed in the feedstuffs, ruminal fluid, duodenal chyme and feaces using high-performance ion chromatography according to the method of Brejnholt *et al*. (2011). The $InsP_6$ content in ruminal fluid, duodenal chyme and feaces was on a very low level. Therefore the determination of $InsP_6$ in these samples was not possible and no data are available. Similar problems for analysis in digesta samples were reported by Brask-Pedersen *et al*. (2013). The milk samples were analysed for fat, protein, lactose, urea and the SCC with Fourier transform infrared spectroscopy and flow cytometric measurement system (Milkoscan FT 6000 combined with a Fossomatic 5000; Foss Electric, Hillerød, Denmark).

Volatile fatty acids (VFA) in rumen fluid were analysed using a gas chromatograph (HP5890II; Hewlett Packard, Böblingen, Germany) equipped with an automatic injector (HP7673 II; Hewlett Packard), a flame ionization detector and an integrator (HP 3396 II; Hewlett Packard). For sample preparation samples were centrifuged at 40,000 g at 4°C. One mL of the supernatant was mixed with 0.1 mL formic acid (98%) and then centrifuged again at 40,000 g for 10 minutes. A self-packed glass column (length 1.8 m, inner diameter 2 mm) filled with Chromosorb WAW 80/100 mesh with 20% Neopentyl-Glycol-Succinate and two percent ortho-phosphoricacid (Analyt, Mühlheim, Germany) was used for separation of VFA. Flow rates of the flame ionisation detector combustion gases hydrogen and synthetic air were 30 and 420 mL/min, respectively. Nitrogen was used as a carrier gas with a flow rate of 25 mL/min. Isothermal separation was carried out at an oven temperature of 130°C. The injection temperature was 220°C and the detection temperature 250°C.

Ammonia-N (NH_3-N) in rumen fluid and duodenal chyme was analysed according to DIN 38406-E5-2 (Beuth, 1998). The following analyses were carried out in the freeze dried and ground duodenal samples. The DM and ash contents of duodenal chyme were analysed in the daily pooled samples with the same methods as the feedstuffs. The proportion of microbial-N of the non-ammonia-N (NAN) in duodenal chyme was estimated using near infrared spectroscopy according to Lebzien and Paul (1997). Cr_2O_3 in duodenal chyme was measured using an ICP-OES (Quantima) after sample preparation according to Williams *et al*. (1962). The chromium concentration was used to calculate the daily duodenal DM flow. According to the daily duodenal DM flows on the 5 sampling days, one aliquot pooled sample was generated per cow per 5 sampling days. In the pooled samples NDF and ADF were analysed by the same methods as the feedstuff.

Calculations

The metabolisable energy (ME) and net energy for lactation (NEL) content of the diets were calculated using the regression equations given by the GfE (2001). Gross energy (GE), crude protein (CP), ether extract (EE), nitrogen free extract (NfE) were obtained from analyses of the feedstuffs, while digestible EE (DEE), digestible crude fibre (DCF) and digestible OM (DOM) were obtained from the digestion trial:

GE (MJ)=0.0239×g CP+0.0398 ×g EE+0.0201 ×g CF+0.0175 ×g NfE

ME (MJ)=0.0312 g DEE+0.0136 g DCF+0.0147 g (DOM-DEE-DCF)+0.00234 g CP

NEL (MJ)=(0.4632+0.0024×*q*)×ME (MJ), where *q* is ME/GE

Fat corrected milk (4% FCM) was calculated according to Gaines (1928):

FCM (kg/d)=[(% milk fat×0.15)+0.4]*milk yield (kg/d)

P balance was calculated with the following equation:

P Balance (g/d)=P intake (g/d)-faecal P (g/d)-urinary P (g/d)-milk P (g/d)

The native phytase activity for the corn silage is calculated according to tabulated values by Eeckhout and Depaepe (1994). This results in a phytase activity of 12 FTU/kg DM. Daily duodenal DM flow (DMF) was calculated as follows:

$$\text{DMF (kg/d)} = \frac{\text{Chromium application (mg/d)}}{\text{Duodenal chromium concentration (mg/g DM)}} /1000$$

The daily duodenal flows of OM and nutrients were estimated by multiplication of their respective concentrations in duodenal digesta with DMF. The utilisable CP (uCP) at the duodenum was estimated following Lebzien and Voigt (1999):

uCP (g/d)=CP-flow at the duodenum (g/d)-NH_3-N×6.25 (g/d)-endogenous CP (g/d)

The endogenous CP (EP) was estimated following Brandt and Rohr (1981) using DMF at the duodenum:

EP (g/d)=(3.6×kg DMF)×6.25

The ruminal nitrogen balance (RNB), ruminally undegraded feed CP (RUP), ruminally degraded CP (RDP) and ruminally fermented OM (FOM) were calculated with the following equations:

RNB (g/d)=[CP-intake (g/d)-uCP (g/d)]/6.25

RUP (g/d)=6.25 [(NAN at the duodenum (g/d)-microbial N (g/d)]-EP (g/d)

RDP (g/d)=CP-intake (g/d)-RUP (g/d)

FOM (kg/d)=OM intake (kg/d)-[duodenal OM flow (kg/d)-microbial OM (kg/d)]

The microbial OM was calculated according to Schafft *et al*. (1983):

Microbial OM (kg/d)=11.8×microbial N (kg/d)

Total tract digestibility was calculated with the following equation:

Total tract digestibility (%)=[nutrient intake (g/d)-nutrient in feaces (g/d)/ nutrient intake (g/d)]×100

The ME of the diets was calculated using the results from the sampling period and turned out to be 10.2±0.2, 10.4±0.2 and 10.1±0.2 MJ/kg DM for group LP, HP and LP+PHY. The NEL of the diets was 6.1±0.1, 5.9±0.2 and 5.9±0.2 MJ/kg DM for group HP, LP and LP+PHY.

Statistical analyses

The statistical analysis was carried out with the SAS-software package Version 9.1.3 (SAS, 2004). The procedure MIXED was used to analyse the data of intake, P concentration in milk, duodenal chyme, and faeces as well as rumen and duodenal variables. For repeated measures in ruminal fluid (pH-value, NH_3-N and VFA) an autoregressive covariance structure was modelled using sampling time relative to feeding as the repeated effect. The models contained the treatment group as a fixed factor and the fact that each cow was used in several periods for different treatments was considered using a random statement for the individual animal effect. Variances were evaluated with the restricted maximum likelihood method and degrees of freedom were calculated according to the Kenward-Roger method. The pdiff option was used to determine significant effects between the least square means and Tukey-Kramer test was applied for *post-hoc* analysis. The results of the trial are presented as least squares (LS) means±standard error (SE). Effects are graded as significant with $P<0.05$, a trend was considered if $P<0.10$ and $P\geq0.05$.

Results

The intended P concentrations in the diets of groups HP, LP and LP+PHY as well as the desired difference of 20% between the groups with or without P-supplementation were achieved. The average milk yield of 20.6 kg/d across all groups is required according to the GfE recommendations (2001) of 3.3 g P/kg DM in the diet. This content is equal to the analysed content in the HP diet. In LP and LP+PHY groups the dietary P content was reduced by 25.6 and 24.7%, respectively. The feed ingredients including corn silage, corn, wheat gluten and dried sugar beet pulp contributed to this comparatively low dietary P concentration as compared with other diets commonly used for dairy cows. The P concentration of the corn silage was on average 2.74 g/kg DM and the mean P concentration of the unsupplemented concentrates was 1.92 g/kg DM. There were no treatment effects on daily nutrient intakes (Table 2). The corn silage intake of the cows during the sampling period amounted on average to 9.8 kg DM/d. The mean DM intake from concentrate was 3.84±0.02, 3.85±0.03 and 3.83±0.03 kg/d in groups HP, LP and LP+PHY, respectively. The mean refusal weights were 0.05±0.2, 0.36±0.2 and 0.46±0.2 kg DM/d (P=0.436) for groups

Table 2. Nutrient intakes by the fistulated cows during the sampling period (least squares means±standard error).

	Experimental diets			P
	HP	LP	LP+PHY	
Animals/group	7	5	5	
Intakes, kg/d				
DM	13.6±0.15	13.3±0.18	13.6±0.18	0.414
OM	12.7±0.13	12.6±0.15	12.8±0.15	0.709
CP	1.8±0.17	1.8±0.20	1.8±0.20	0.602
EE	0.4±0.01	0.4±0.01	0.4±0.01	0.335
N	0.3±0.003	0.3±0.003	0.3±0.003	0.602
ADF	3.0±0.05	3.0±0.06	3.1±0.06	0.337
NDF	6.0±0.09	5.8±0.11	6.1±0.11	0.318

HP, high phosphorus (diet added with dicalcium phosphate); LP, low phosphorus (diet without phosphorus and phytase supplementation); PHY, phytase (diet without phosphorus-supplementation, but added with an exogenous phytase); DM, dry matter; OM, organic matter; CP; crude protein; EE, ether extract; N, nitrogen; ADF, acid detergent fibre; NDF, neutral detergent fibre.

Table 3. Effects of supplemental phosphorus and phytase on rumen fermentation parameters in dairy cows (least squares means±standard error).

	Experimental diets			P
	HP	LP	LP+PHY	
Animals/group	7	5	5	
pH	6.7±0.1	6.5±0.1	6.7±0.1	0.242
NH_3-N, mg/100 g	13.1±2.4	13.6±3.2	15.3±2.6	0.829
Total VFA, mmol/L	85.2±3.8	76.0±5.0	75.2±4.0	0.149
Acetic acid, mol %	59.4±1.1	60.0±1.4	61.3±1.2	0.439
Propionic acid, mol %	27.7±1.1	26.9±1.4	26.4±1.1	0.595
Butyric acid, mol %	11.9±0.5	12.1±0.6	11.5±0.5	0.732
Acetic:propionic acid	2.2±0.1:1	2.3±0.2:1	2.4±0.1:1	0.428

HP, high phosphorus (diet added with dicalcium phosphate); LP, low phosphorus (diet without phosphorus and phytase supplementation); PHY, phytase (diet without phosphorus-supplementation, but added with an exogenous phytase); VFA, volatile fatty acids.

Table 4. Effects of supplemental phosphorus and phytase on nutrient and phosphorus flows at the duodenum and amount of fermented organic matter in the rumen (least squares means±standard error).

	Experimental diets			P
	HP	LP	LP+PHY	
Animals/group	7	5	5	
OM				
kg/d	6.7±0.12	6.8±0.17	6.8±0.15	0.810
% of intake	52.9±1.12	53.2±1.54	53.5±1.36	0.952
NDF				
kg/d	3.0±0.12	2.9±0.16	3.2±0.13	0.389
% of intake	51.2±2.0	50.1±3.0	54.3±3.0	0.561
ADF				
kg/d	1.7±0.06	1.6±0.09	1.8±0.07	0.361
% of intake	56.4±2.0	55.9±3.0	59.5±3.0	0.670
FOM				
kg/d	7.5±0.15	7.4±0.20	7.5±0.18	0.854
% of intake	59.3±0.77	58.5±1.02	58.3±0.92	0.635
P				
g/d	65.0±2.06^{b}	58.9±2.40^{a}	55.1±2.13^{a}	0.001
% of intake	143.5±6.79^{a}	172.5±8.96^{b}	163.8±8.02^{ab}	0.025

HP, high phosphorus (diet added with dicalcium phosphate); LP, low phosphorus (diet without phosphorus and phytase supplementation); PHY, phytase (diet without phosphorus-supplementation, but added with an exogenous phytase); OM, organic matter; NDF, neutral detergent fibre; ADF, acid detergent fibre; FOM, fermented organic matter; P, phosphorus. ^{a,b}Different letters in the same row show significant differences ($P<0.05$).

HP, LP and LP+PHY.

Treatments did not affect rumen pH and ammonia-N concentration in rumen fluid (Table 3). The concentration of total VFA in rumen fluid was unchanged. No effects for molar percentage of acetic acid, propionic acid and butyric acid were observed among the three treatments. The acetic acid to propionic acid ratio was about 2.2 in the study and did not differ among the treatments. Treatments had no effect on the amount of FOM and the portion of OM intake fermented in the rumen. The ADF and NDF flow and its portion of intake showed no differences. The P-flow at the duodenum was higher in the HP-group (65 g/d) compared to the LP (58.9 g/d) and LP+PHY (55.1 g/d) group (P=0.001) (Table 4). The supplementation of feed with P and phytase had no effects on N flow at the duodenum, the rumen degradable and undegradable protein and the microbial protein synthesis (Table 5). There were no differences among the treatments for OM, CP and EE in faeces. The mean P concentration in urine was 0.05±0.04 g/kg DM. There were no differences among the treatments in the P concentration of urine and faeces (Table 6). Group LP showed a trend for a higher N concentration in urine (P=0.099). The treatments had no effect on total tract digestibility of OM, EE, NDF and ADF (Table 6). Milk yield amounted to 20.6±0.2 kg/d on average. No differences were observed in milk yield and milk composition among different treatments (Table 7). The mean P concentration in milk was 0.90 g P/kg milk and did not differ among the groups. The SCC showed no differences among the groups as well.

As intended by the experimental design, the P-intake differed among the groups. Group LP and LP+PHY had nearly the same intake (33.5 *vs* 34.1 g P/d), group HP had a higher intake (45.3 g P/d) (P<0.0001). The P-excretion with faeces tended to be higher in group HP than in either other group (P=0.057) (Table 8). There was no influence of treatment on the secretion of P with milk during the sampling period. Urinary P-excretion showed a higher value in the HP-group (P=0.014). Group HP is the only group which showed a positive P-balance and differed compared to the LP and LP+PHY-groups (P=0.01). However, there was no influence of treatment on the apparent total tract digestibility of P which averaged 47.5%.

Discussion

Maenz (2001) investigated the occurrence of phytic acid in plants and found that cereals and grain legumes that are commonly used as feed ingredients have phytate levels, approximating 0.25% of DM. The $InsP_6$ concentration in the concentrates was 0.57 g/kg DM in the LP+PHY group and on average 0.46 g/kg DM in the LP and LP+PHY group. Brask-Pedersen *et al*. (2013) found that an increase about four supplementation levels of exogenous phytase increased the ruminal degradation of $InsP_6$. This degradation of $InsP_6$ occurred mainly in the rumen and decreased $InsP_6$ content in the duodenal chyme samples in animals fed phytase. This indicates that the supply of phytase increases the ruminal phytase activity. The phytase concentration in the diets of the current study are similar to the highly supple-

Table 5. Effects of supplemental phosphorus and phytase on nitrogen flow at the duodenum as well as microbial protein synthesis and feed protein degradation in the rumen (least squares means±standard error).

	Experimental diets			P
	HP	LP	LP+PHY	
Animals/group	7	5	5	
N, g/d	228±10	223±12	219±11	0.775
Non-NH_3-N, g/d	216±8.9	212±10.9	210±10.1	0.855
Microbial CP				
g/d	821±36.6	802±44.8	799±41.5	0.847
g/kg FOM	109±6.2	109±7.8	108±7.1	0.985
g/MJ ME	5.8±0.33	6.2±0.39	5.9±0.36	0.583
g/g rumen degradable protein	0.51±0.03	0.48±0.03	0.49±0.03	0.644
Rumen undegradable protein				
g/d	355±18.0	339±21.1	328±20.0	0.400
% of feed CP	20±0.97	19±1.10	18±1.10	0.441
Rumen degradable protein, g/d	1429±20.6	1434±27.2	1460±24.4	0.609
RNB, g/MJ ME	0.7±0.10	0.8±0.14	0.6±0.12	0.421
Utilisable CP, g/d	1177±51.7	1142±62.5	1127±58.3	0.679

HP, high phosphorus (diet added with dicalcium phosphate); LP, low phosphorus (diet without phosphorus and phytase supplementation); PHY, phytase (diet without phosphorus-supplementation, but added with an exogenous phytase); N, nitrogen; CP, crude protein; FOM, fermented organic matter; ME, metabolisable energy; RNB, ruminal nitrogen balance.

Table 6. Effects of supplemental phosphorus and phytase on nutrient and phosphorus concentration of faeces, phosphorus- and nitrogen-concentration of urine in dairy cows as well as apparent total tract digestibility (least squares means±standard error).

	Experimental diets			P
	HP	LP	LP+PHY	
Animals/group	7	5	5	
Faeces, g/kg DM				
OM	899±4.0	888±4.8	901±4.8	0.097
CP	132±2.4	130±2.8	135±2.8	0.280
EE	33±2.2	34±2.6	33±2.6	0.940
P	4.9±0.32	4.3±0.36	4.5±0.36	0.257
Urine				
P, g/kg DM	0.10±0.03	0.02±0.03	0.04±0.03	0.180
N, g/kg DM	5.58±1.09	6.60±1.18	3.86±1.19	0.099
Urine excretion, kg/d	24.5±5.1	21.5±5.8	29.9±5.8	0.497
Apparent total tract digestibility, %				
OM	69±1.4	68±1.6	68±1.6	0.589
EE	67±1.7	62±2.0	65±2.0	0.185
NDF	57±2.0	53±2.3	56±2.3	0.253
ADF	49±2.9	46±3.4	49±3.4	0.645

HP, high phosphorus (diet added with dicalcium phosphate); LP, low phosphorus (diet without phosphorus and phytase supplementation); PHY, phytase (diet without phosphorus-supplementation, but added with an exogenous phytase); DM, dry matter; OM, organic matter; CP; crude protein; EE, ether extract; P, phosphorus; N, nitrogen; NDF, neutral detergent fibre; ADF, acid detergent fibre.

mented group of the study by Brask-Pedersen *et al*. (2013). However, the results of the actual study for P-balance data with no differences between the LP and LP+PHY group indicate that $InsP_6$ degradation and absorption of released P in the duodenum as a result of ruminal phytase activity is not influenced by phytase supplementation. Dvorakova (1998) determined that the optimum of pH is 2.5 or 5.5 for *Aspergillus niger phytase,* while Brask-Pedersen *et al*. (2013) mentioned that the optimum pH for the efficiency of the phytase used in their study was 5.0 to 5.5. The pH-value of the rumen of the cows in the current study is on average 6.6. The time per day the ruminal pH spent below 5.6 was determined in a study by Lohölter *et al*. (2013) and was on average 291 min/d or 20% of the whole day. Times when the ruminal pH coincided with the pH-optimum of the phytase are the exception rather than the rule and maybe a possible reason for the absence of phytase effects on the P-balance in the current study. Post ruminal phytate degradation was not observed because of the unchanged apparent total tract digestibility.

Effects of dietary P deficiency with an insufficient P-supply to the rumen microbes on the microbial metabolism are reduced feed intake, OM digestibility and efficiency of microbial protein synthesis (Breves and Schröder, 1991; Kincaid and Rodehutscord, 2005). In the present study the effects on rumen fermentation characteristics were only marginal. Parameters of microbial protein synthesis (Table 5) and OM digestibility (Table 6) were not influenced in groups with reduced P-supply. This suggested that P recycling via saliva was sufficient to supply the requirements of the microbes, even though the groups experienced a P deficiency in the diet. The duodenal P-flow markedly higher than 100 percent of intake in all groups confirmed this suggestion. The unchanged ammonia concentrations in the rumen fluid indicated no effect of P or phytase supplementation on protein degradation in the rumen.

Values for P in the milk of lactating Holstein cows determined over the complete lactation range are between 0.85-0.94 g P/kg milk (Brintrup *et al*., 1993; Valk *et al*., 2002; Wu *et al*., 2000). In the present trial the values amounted to 0.90 g P/kg milk. They are similar to the mean concentration of 0.9 g P/kg milk given by Pfeffer *et al*. (2005) and to the mean concentration of 0.89 g P/kg milk given by Klop *et al*. (2013). The present results confirm the statement of Pfeffer *et al*. (2005) that the P-intake has no influence on the P-excretion with milk. In contrast to the P-excretion with milk, the P-excretion with faeces tended to be higher in the HP-group (P=0.057) and faecal P-excretion was unaffected by the exogenous phytase fed to cows. The HP-group excreted 22 g P/d, while the LP-group excreted 19 g and the LP+PHY-group excreted 18 g P/d. This results in a difference of 14%, resp. 18%, compared to the HP group. The present results confirm the statement of Pfeffer *et al*. (2005) and other authors who found a direct correspondence between P-intake and P-excretion in dairy cows (Knowlton and Herbein, 2002; Knowlton *et al*., 2004; Wu *et al*., 2001). Hill *et al*. (2008) observed that total P excreted with faeces is not very sensitive to supplemented phytase and is comparable to the results of the present study. In contrast, dietary P-supplementation has a positive effect on the P-balance in the current study. The P-balance for group LP and LP+PHY was negative, while it was positive for the HP-group. The HP-group showed a higher P-balance (P=0.010) compared with both other groups. In the current study, a mineral P-intake according to the GfE recommendations (2001) enabled the cows to retain more P. Valk *et al*. (2002) found comparable results for lactating dairy cows. The P-balance of cows calculated by Hill *et al*. (2008) became negative at a similar dietary P content, which is slightly less

Table 7. Effects of supplemental phosphorus and phytase on milk production and composition in dairy cows (least squares means±standard error).

	Experimental diets			P
	HP	LP	LP+PHY	
Animals/group	7	5	5	
Milk yield, kg/d	20.5±1.6	20.8±1.9	20.5±1.7	0.984
FCM, kg/d	19.4±1.31	19.3±1.60	20.0±1.45	0.934
Milk composition, %				
Fat	3.8±0.23	3.6±0.26	3.7±0.25	0.768
Protein	2.7±0.07	2.7±0.08	2.8±0.07	0.951
Lactose	4.8±0.08	4.8±0.09	4.8±0.09	0.982
Milk yield, g/d				
Fat	750±51.7	746±61.1	774±55.8	0.931
Protein	556±35.9	570±42.1	559±38.4	0.949
Lactose	978±80.1	975±95.6	988±86.5	0.993
Urea, ppm	115±19.0	132±21.6	117±20.0	0.696

HP, high phosphorus (diet added with dicalcium phosphate); LP, low phosphorus (diet without phosphorus and phytase supplementation); PHY, phytase (diet without phosphorus-supplementation, but added with an exogenous phytase); FCM, fat corrected milk.

Table 8. Mean phosphorus-intake and phosphorus secretion with milk, phosphorus secretion with faeces, urine as well as phosphorus-balance of the dairy cows during the sampling period (least squares means±standard error).

	Experimental diets			P
	HP	LP	LP+PHY	
Animals/group	7	5	5	
Intake				
P, g/d	45±0.7^{b}	34±0.8^{a}	34±0.8^{a}	<0.0001
Excretion with faeces				
P, g/d	22±1.0	19±1.2	18±1.2	0.057
Excretion with urine				
P, g/d	2.4±0.52^{b}	0.2±0.62^{a}	0.1±0.62^{a}	0.014
Secretion with milk				
P, g/d	19±1.5	19±1.8	19±1.8	0.984
Balance				
P, g/d	2.6±1.25^{b}	-3.2±1.48^{a}	-3.0±1.48^{a}	0.010
Apparent total tract digestibility				
P, %	52±2.5	44±3.1	47±3.1	0.126

HP, high phosphorus (diet added with dicalcium phosphate); LP, low phosphorus (diet without phosphorus and phytase supplementation); PHY, phytase (diet without phosphorus-supplementation, but added with an exogenous phytase); P, phosphorus. ^{a,b}Different letters in the same row show significant differences (P<0.05).

than the requirements set by the National Research Council (2001). Negative P-balance of the LP-group was exactly planned in this study to investigate the effect of the exogenous phytase in group LP+PHY. However phytase was not able to compensate the reduced P-intake with feed. The balance of group LP+PHY stayed negative. Elizondo Salazar *et al*. (2013) investigated the body P mobilization, deposition and balance during lactation in dairy cows with different dietary P concentrations over the whole lactation cycle. They maintained that the dynamic of P metabolism can have important implications for dietary P requirements and ration formulations. The study determined that the animals restore P in tissues and bones at the end of lactation. In the current study, the cows are almost into the second third of lactation (day 137 at the beginning of the sampling periods). The negative P-balance of the LP-groups could possibly be explained by the lactation period and by the respective milk yield.

When comparing the retention of P (P-balance) in the current study, it can be seen that there are differences among the treatments. The comparison of group LP+PHY and HP showed that the intake of these groups differs by about 11.2 g/d and the balance differs by about 5.6 g/d. Consequently 50% of the supplemented P in group HP was utilised. The desired positive effect of phytase with regard to a higher P-digestibility and P-balance did not occur.

Conclusions

The supplemented phytase had no effect on duodenal flow and apparent total tract digestibility of P of cows supplied with P slightly below the recommendations. The absence of phytase effects on P secretion with milk, urine and faeces resulted in an unaffected P balance. On the contrary, the supplementation with mineral P lead to an increased duodenal P flow, no higher P secretion with milk and a slightly higher excretion with faeces. This resulted in a higher P balance in the cows fed the P-supplemented diet. However, question remains whether a higher supplementation level of the exogenous phytase is more suitable or whether another exogenous phytase with a higher pH-optimum would be more efficient. In the future more investigations should be done for the effect of P and phytase on the ruminal fermentation and digestibility of P.

References

Beuth, 1998. DIN 38406-E5-2. Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung. Beuth Publ., Berlin, Germany.

Brandt, M., Rohr, K., 1981. The quantification of nitrogen metabolism in the forestomachs of dairy cows. 1. The examination of microbial nitrogen with 15 nitrogen as a marker. J. Anim. Physiol. An. N. 46:39-48.

Brask-Pedersen, D.N., Glitso, L.V., Skov, L.K., Lund, P., Sehested, J., 2013. Effect of exogenous phytase on degradation of inositol phosphate in dairy cows. J. Dairy Sci. 96:1691-1700.

Brejnholt, S.M., Dionisio, G., Glitsoe, V., Skov, L.K., Brinch-Pedersen, H., 2011. The degradation of phytate by microbial and wheat phytases is dependent on the phytate matrix and the phytase origin. J. Sci. Food Agr. 91:1398-1405.

Breves, G., Schröder, B., 1991. Comparative aspects of gastrointestinal phosphorus metabolism. Nutr. Res. Rev. 4:125-140.

Brintrup, R., Mooren, T., Meyer, U., Spiekers, H., Pfeffer, E., 1993. Effects of 2 levels of phosphorus intake on performance and fecal phosphorus excretion of dairy cows. J. Anim. Physiol. An. N. 69:29-36.

Clark, W.D., Wohlt, J.E., Gilbreath, R.L., Zajac, P.K., 1986. Phytate phosphorus intake and disappearance in the gastrointestinal-tract of high producing dairy cows. J. Dairy Sci. 69:3151-3155.

Dvorakova, J., 1998. Phytase: sources, preparation and exploitation. Folia Microbiol. 43:323-338.

Eeckhout, W., Depaepe, M., 1994. Total phosphorus, phytate-phosphorus and phytase activity in plant feedstuffs. Anim. Feed Sci. Tech. 47:19-29.

Elizondo Salazar, J.A., Ferguson, J.D., Beegle, D.B., Remsburg, D.W., Wu, Z., 2013. Body phosphorus mobilization and deposition during lactation in dairy cows. J. Anim. Physiol. An. N. 97:502-517.

Engelen, A.J., Vanderheeft, F.C., Randsdorp, P.H.G., Smit, E.L.C., 1994. Simple and rapid determination of phytase activity. J. AOAC Int. 77:760-764.

Gaines, W.L., 1928. The energy basis of measuring milk yield in dairy cows. Illinois Aes. Bull. 308:401-438.

GfE, 2001. Empfehlungen zur Energie- und Nährstoffversorgung der Milchkühe und Aufzuchtrinder. DLG Publ., Frankfurt am Main, Germany.

Godoy, S., Meschy, F., 2001. Utilisation of phytic P by rumen bacteria in a semi-continuous culture system (Rusitec) in lactating goats fed on different forage to concentrate ratios. Reprod. Nutr. Dev. 41:259-265.

Hill, S.R., Knowlton, K.F., Kebreab, E., France, J., Hanigan, M.D., 2008. A model of phosphorus digestion and metabolism in the lactating dairy cow. J. Dairy Sci. 91:2021-2032.

Kincaid, R.L., Garikipati, D.K., Nennich, T.D., Harrision, J.H., 2005. Effect of grain source and exogenous phytase on phosphorus digestibility in dairy cows. J. Dairy Sci. 88:2893-2902.

Kincaid, R.L., Rodehutscord, M., 2005. Phosphorus metabolism in the rumen. In: E. Pfeffer and A. Hristov (eds.) Nitrogen and phosphorus nutrition of cattle. CABI Publ., Wallingford, UK, pp 187-193.

Klop, G., Ellis, J.L., Bannink, A., Kebreab, E., France, J., Dijkstra, J., 2013. Meta-analysis of factors that affect the utilization efficiency of phosphorus in lactating dairy cows. J. Dairy Sci. 96:3936-3949.

Knowlton, K.F., Herbein, J.H., 2002. Phosphorus partitioning during early lactation in dairy cows fed diets varying in phosphorus content. J. Dairy Sci. 85:1227-1236.

Knowlton, K.F., Radcliffe, J.S., Novak, C.L., Emmerson, D.A., 2004. Animal management to reduce phosphorus losses to the environment. J. Anim. Sci. 82:173-195.

Lebzien, P., Paul, C., 1997. Use of near-infrared reflectance spectroscopy for the estimation of the microbial portion of non-ammonia-nitrogen in the duodenum of dairy cows. Anim. Feed Sci. Tech. 68:225-233.

Lebzien, P., Voigt, J., 1999. Calculation of utilizable crude protein at the duodenum of cattle by two different approaches. Arch. Anim. Nutr. 52:363-369.

Lohölter, M., Meyer, U., Rauls, C., Rehage, J., Dänicke, S., 2013. Effects of niacin supplementation and dietary concentrate proportion on body temperature, ruminal pH and milk performance of primiparous dairy cows. Arch. Anim. Nutr. 67:202-218.

Maenz, D.D., 2001. Enzymatic characteristics of phytase as they relate to their use in animal feeds. In: M.R. Bedford and G.G. Partridge (eds.), Enzymes in farm animal nutrition. CABI Publ., Wallingford, UK, pp 61-84.

Morse, D., Head, H.H., Wilcox, C.J., 1992. Disappearance of phosphorus in phytate from concentrates in vitro and from rations fed to lactating dairy cows. J. Dairy Sci. 75:1979-1986.

National Research Council, 2001. Nutrient

requirements of dairy cattle. 7th rev. ed. National Academy Press, Washington, DC, USA.

Pfeffer, E., Beede, D.K., Valk, H., 2005. Phosphorus metabolism in ruminants and requirements of cattle. In: E. Pfeffer and A. Hristov (eds.) Nitrogen and phosphorus nutrition of cattle. CABI Publ., Wallingford, UK, pp 195-231.

Rohr, K., Brandt, M., Lebzien, P., Schafft, H., 1984. Measurement of duodenal flow in dairy cows by either total collection or spot sampling, using a special cannula. Can. J. Anim. Sci. 64:116-117.

SAS, 2004. SAS/STAT 9.1 User's guide. SAS Inst. Inc., Cary, NC, USA.

Schafft, H., Rohr, K., Lebzien, P., 1983. Investigations on the intraruminal decomposition of hay and grain protein. J. Anim. Physiol. An. N. 50:34-35.

Suttle, N.F., 2010. Mineral nutrition of livestock. CABI Publ., Wallingford, UK.

Valk, H., Sebek, L.B.J., Beynen, A.C., 2002. Influence of phosphorus intake on excretion and blood plasma and saliva concentrations of phosphorus in dairy cows. J. Dairy Sci. 85:2642-2649.

VDLUFA, 1997. Handbuch der Landwirtschaftlichen Versuchs- und Untersuchungsmethodik (VDLUFA-Methodenbuch). Die chemische Untersuchung von Futtermitteln. VDLUFA Publ., Darmstadt, Germany.

Williams, C.H., Iismaa, O., David, D.J., 1962. Determination of chromic oxide in faeces samples by atomic absorption spectrophotometry. J. Agr. Sci. 59:381-385.

Wu, Z., Satter, L.D., Blohowiak, A.J., Stauffacher, R.H., Wilson, J.H., 2001. Milk production, estimated phosphorus excretion, and bone characteristics of dairy cows fed different amounts of phosphorus for two or three years. J. Dairy Sci. 84:1738-1748.

Wu, Z., Satter, L.D., Sojo, R., 2000. Milk production, reproductive performance, and fecal excretion of phosphorus by dairy cows fed three amounts of phosphorus. J. Dairy Sci. 83:1028-1041.