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Effects of addition of phytase, phosphorus and zinc to ruminant diets on rumen metabolism, digestion and performance

Dissertation

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Für Ben,

ich weiß Du wärst unsagbar stolz gewesen endlich Dr. Winter sagen zu dürfen…

Für Reinhard, Mama und Marius,

weil Ihr immer an mich geglaubt habt und für mich da gewesen seid…Danke.

Abbreviations

1,25-$(OH)_2D$	1,25-dihydroxyvitamin D
AD	Apparent total tract digestibility
ADF	Acid detergent fibre
AIA	Acid insoluble ash
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BHB	ß-hydroxybutyrate
BW	Body weight
BW	Body weight
$C_6H_{18}O_{24}P_6$	Phytic acid
Ca	Calcium
Ca^{2+}	Calcium ion
CEN	Comite Europeen de Normalisation
CF	Crude fibre
Co^{2+}	Cobalt ion
CP	Crude protein
Cr_2O_3	Chromium oxide
Cu^{2+}	Copper ion
CVB	Centraal Veevoederbureau voedernormen Landbouwhuidieren en voederwaarde veevoeders
d	day
DCF	Digestible crude fibre
DEE	Digestible ether extract
DIM	Days in milk
DIN	German Institute for Standardization
DLG	Deutsche Landwirtschaftsgesellschaft
DM	Dry matter
DMF	Dry matter flow
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
DOM	Digestible organic matter
EBW	Empty body weight

EE	Ether extract
EP	Endogenous crude protein
FCM	Fat corrected milk
Fe^{2+}	Ferric ion
FFA	Free fatty acid
FID	Flame ionization detector
FLI	Friedrich-Loeffler-Institut
FOM	Fermented organic matter
FTU	Unit for phytase activity
GE	Gross energy
GfE	Gesellschaft für Ernährungsphysiologie
GGT	Gamma glutamyltransferase
GLDH	Glutamate dehydrogenase
HCW	Hot carcass weight
ICP-OES	Optical emissions spectrometer with inductively coupled plasma
INRA	Institut national de la recherche agronomique
IP-6/5/4/3/2/1	Inositol phosphorus 6/5/4/3/2/1
ISO	International Organization for Standardization
JCBN	Joint Commission on Biochemical Nomenclature
K^+	Potassium ion
LSmeans	Least square means
M_{diet}	Mineral content of diet
ME	Metabolizable energy
M_{faeces}	Mineral content of faeces
Mn^{2+}	Magnesium ion
MTs	metallothioneins
Na^+	Sodium ion
NAN	Non ammonia nitrate
NC-IUBMB	Enzyme Nomenclature Committee of the International Union of Biochemistry and Molecular Biology
NDF	Neutral detergent fibre
N_{diet}	Nutrient concentration
NEL	Net energy for lactation

NfE	Nitrogen free extract
NH_3-N	Ammonia nitrogen
Ni^{2+}	Nickel ion
NPGS	Neopentyl-Glycol-Succinate
NRC	National Research Council
OM	Organic matter
P	Phosphorus
P_i	Inorganic phosphorus
P_i	Phosphorus inorganic
pKa	Negative decadic logarithm
PO_4^{3-}	Phosphate
RDP	Ruminally degraded crude protein
RNA	Ribonucleic acid
RNB	Ruminal nitrogen balance
RO-$PO(OH)_2$	Phosphate esters
RUP	Ruminally undegraded feed crude protein
SCC	Somatic cell count
SCC	Somatic cell count
SCFA	Short chain fatty acids
SE	Standard error
TMR	Total mixed ration
tsd	Thousand
U	Unit
UCP	Utilizable crude protein
v/v	Concentration of volume
VDLUFA	Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten
Zn	Zinc
Zn^{2+}	Zinc ion

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Background and general discussion

Paper I

Contents

1. Introduction

Phosphorus (P) is an essential nutrient and is generally indispensable for the metabolism, including the energy metabolism (Suttle, 2010). Animals as well as plants rely on a steady P accommodation (Flachowsky and Rodehutscord, 2008). The increasing specialization and concentration of livestock and crop production has led to the net export of nutrients from major crop-producing areas of the country to areas with a high concentration of animal agriculture. The livestock utilize P inefficiently, excreting 60 to 80% of the consumed matter. This results in the fact that the majority of P brought onto the farm in feed stays on the farm, rather than being exported in meat or milk (Knowlton et al., 2004). Global resources of phosphate are limited, the European Union is dependent on imports and prizes for mineral phosphate increased (Flachowsky and Rodehutscord, 2008). The unutilized inorganic P is excreted with manure and contributes to environmental problems by eutrophication of water resources (Brask-Pedersen et al., 2013). Therefore efforts have been undertaken to decrease the contamination of surface water (streams, lakes, rivers) by P. If agricultural practices continue as they have in the past, continued damage to water resources and a loss of fishing and recreational activity are almost inevitable (Knowlton et al., 2004). To secure supply and protect resources it is essential to consider possibilities for conservation. It is for this reason that the investigation to preserve P resources is made. The sustainable use of P in farm animals should be studied.

One way of study is the supply of exogenous phytase to the feed. Phytase releases phosphorus (P) from inositol phosphate (InsP) by hydrolysis (Suttle, 2010). P from phytate is suggested to be highly available to ruminants because of the microbial phytase activity in the rumen (Clark et al., 1986, Morse et al., 1992). Therefore, ruminal P-excretion with faeces seems to be linear to P-concentration in the diet of ruminants and it seems to be depending on the P-requirement and digestion (Call et al., 1987, Suttle, 2010). Although ruminants generally have the ability to use phytin-bound phosphorus through ruminal hydrolysis, different studies have showed that the dietary supplementation of exogenous phytase leads to reduced faecal excretion and an increased P-concentration in bones.

However, not only P is bound to phytin but also trace elements such as zinc (Zn) are known to be released by the action of exogenously added phytase in monogastric animals (Matsui,

2002). Whether such effects occur in ruminants is not known. Therefore, phytase mediated release of these trace elements may increase their bioavailability.

To investigate the effect of phytase on the metabolism of P in bulls and cows three experiments were carried out. An experiment with 24 dairy cows (Paper 1), a study with 48 fattening bulls (Paper 2) and an investigation with nine fistulated cows (Paper 3) was done.

2. Background

2.1 Chemical characteristics of the minerals P and Zn and of the enzyme phytase

Phosphorus

The element phosphorus (P) has an atomic number of 15 and an atomic weight of 30.97. It occurs in four modifications as white, red, black and violet P and exists naturally as the isotope ^{31}P. White P, with a melting point of 44.1°C has a specific gravity of 1.82g/cm³, red and black P, which are heavier, evince specific gravities of 2.2 and 2.69 g/cm^3, respectively. When P is exposed to ultraviolet light or vaporized at 250°C, it converts to the red modification which does not phosphoresce in air and is fairly stable. Elemental P does not occur alone in nature, but is linked with various other elements in form of inorganic minerals or as components of organic compounds. The biologically most important form of P is the pentavalent oxygen compound, phosphate (PO_4^{3-}) (Boyd L. and O´Dell, 1997).

The organic phosphate esters, $RO\text{-}PO(OH)_2$, and their salts play a major role in natural science. Phosphate exists in forms of mono-, di- and triphosphoesters. Each form has specific chemical properties leading to different biologic function (Boyd L. O´Dell, 1997). One salt of phosphate esters is phytate. Phytate is composed of phytic acid and several important cations such as Ca^{2+}, Fe^{2+}, Zn^{2+} and K^+.

Phytic acid (myo-inositol 1,2,3,4,5,6 hexakisphosphate) consists of a sugar molecule, called myo-inositol, with covalently linked phosphate groups (Lott, 1984; Morris and Ellis, 1976; O´Dell et al, 1977). The molecular formula of phytic acid is $C_6H_{18}O_{24}P_6$ and its molecular weight is 659.86 (Vohra and Satyanarayana, 2003). Structurally, phytic acid consists of a fully phosphorylated myo-inositol ring that exists in a chair confirmation in dilute solution (Johnson and Tete, 1969). Phytic acid readily forms complexes with multivalent cations, the most stable one is formed with Zn^{2+} followed by Cu^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+} and Fe^{2+} in decreasing order of stability (Cheryan, 1980). The phytate-mineral complex can exist as either

a soluble chelate or an insoluble complex depending on the concentrations of phytic acid and mineral and pH of the solution. Both parameters have effects on the structure of phytic acid and phytate hydrolysis. In plants, it is known to function as a $(PO_4)^{3-}$ storage depot and forerunner for other inositol phosphates (Maenz, 2001).

Zinc

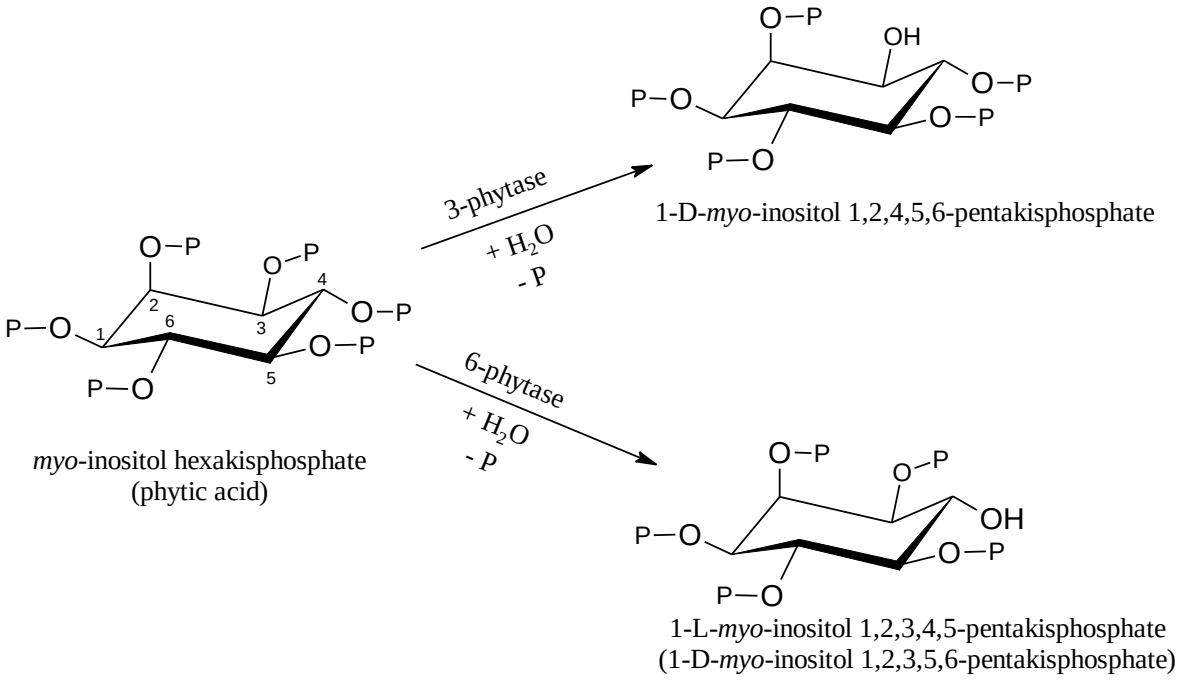
The element Zn is a trace element and possesses only one oxidation state in biological systems and is distinguished from the remaining cationic essential trace metals. The lack of unpaired electrons and a complete 3*d* shell makes the detection difficult by physicochemical techniques and consequently the importance of Zn in biology has been recently noticed. In biological systems the majority of Zn appears complexed with organic ligands. Zn is found in more than 100 specific enzymes and is often coordinated to amino acids, especially aspartic acid, glutamic acid, cysteine and histidine. Furthermore Zn has roles in the metabolism of RNA and DNA (Boyd L. O´Dell, 1997).

Phytase

The phytase enzyme (*myo*-inositol hexakisphosphate phosphohydrolase) occurs in two forms: 3-phytase class (typical for micro-organism) and the 6-phytase class (typical for plants). This classification is distinguished by the *Enzyme Nomenclature Committee of the International Union of Biochemistry and Molecular Biology* (NC-IUBMB) in consultation with the IUPAC-IUBMB *Joint Commission on Biochemical Nomenclature* (JCBN) based on the first phosphate group attacked by the enzyme. A 3-phytase (*myo*-inositol-hexakisphosphate 3-phosphohydrolase, EC 3.1.3.8) firstly attacks phytate at the 3-position, whereas 6-phytase (*myo*-inositol-hexakisphosphate 6-phosphohydrolase, EC 3.1.3.26) reacts at 6-position (Vohra and Satyanarayana, 2003).

P associated with phytic acid is not available for intestinal absorption unless the inorganic form of P (P_i) is hydrolyzed from the inositol ring by action of phytase (McCance and Widdowson, 1935; Nelson, 1967; Pointillart, 1987; Pointillart, 1991; Morse et al, 1992). Phytase belongs to the group of phosphoric monoester hydroleases. It catalyzes the hydrolysis of phytic acid to inorganic monophosphate and lower phosphoric esters of *myo*-inositol, or to *myo*-inositol itself (Dvorakova, 1998). Phytase can be found in plant material and is produced by many microorganisms, especially moduls of the *Aspergillus* type. Commercially available microbial phytases are non-specific phosphomonoesterases belonging to the group of acid phosphatases (Jongbloed et al., 1993). These enzymes catalyze dephosphorylation of myo-

inositol hexakiphosphates in a step-wise manner and produce five classes of intermediate products (myo-inositol pentakis-, tetrakis-, tris-, bis- and monophosphates) of variable stereochemistry (Frolich et al., 1986). A schematic illustration of phytate dephosphorylation is shown in **Figure 1**.



(McCleary, 2001)

Figure 1: Schematic representation of the hydrolysis of phytic acid by phytase

The phytate-degrading enzymes are also divided into two types based on their optimal pH-value. These are, on the one hand, acid phytate-degrading enzymes with a pH optimum around 5.0, and on the other hand, alkaline phytate-degrading enzymes belonging to the acid type having a pH-optimum of eight (Dvorakova, 1998). Acid phytases are able to catalyze phytate dephosphorylation to lower *myo*-inositol phosphates (multiple isomers of IP_4, IP_3 and IP_2) by alternative pathways of IP_5 hydrolysis, sometimes yielding free myo-inositol, whereas alkaline phytase is unable to catalyze dephosphorylation of IP_3. The characterization of a phytase cannot be considered completely until the structure of the produced pentaphosphate has been determined (Lim and Tate, 1973).

Phytase is successfully used as a nutritional additive to improve P availability in feed. Furthermore, phytase can influence the Zn-release from the phytate complexes, because it has

to be taken into account that metal cations, such as Zn form insoluble complexes with phytate P.

2.2 Role of the minerals P and Zn and the enzyme phytase in the nutrition and metabolism of cattle

Phosphorus

As is well known, P plays an important role in the metabolism and health of cattle (Puggaard et al., 2011). P is an essential mineral for the metabolism with diverse functions in the body. For example, P is a necessary constituent of bones and teeth, and takes a position in cell membrane structure (pospholipds), energy transfer (ATP) and in structure of DNA (Satter et al., 2002).

Functions of P

About 80% of body P is found in the bones and teeth of animals. The formation and maintenance of bones are quantitatively the most important functions of P, especially its requirement for the formation of the organic bone matrix as well as the mineralization of that matrix. The other 20% of body P is stored in the fluids and soft tissues of the body (Suttle, 2010). P occurs ubiquitously in the soft tissues and is essential for many enzymatic reactions, particularly those concerned with energy metabolism and transfer of genetic information (i.e. DNA and RNA). Furthermore, P is an important component of carbohydrate, amino acid, fat, muscle and nervous tissue (Ekelund, 2003). P also plays an essential role in maintenance and reproduction of ruminal micro-organisms (Breves and Schröder, 1991; NRC 1996).

P digestion and absorption

The P intake and excretion became a main issue in the international animal nutrition (Rodehutscord, 2008). Between 30% to 50% of feed P is transferred into body tissues and into milk. However the remaining 50% to 70% is excreted with faeces and urine of ruminants. To evaluate the total P excretion the excretion contents into milk, urine and faeces have to be taken into account (Brintrup et al., 1993).

Figure 2 represents an example for the P flows in dairy cows with a milk yield of 25 kg/d.

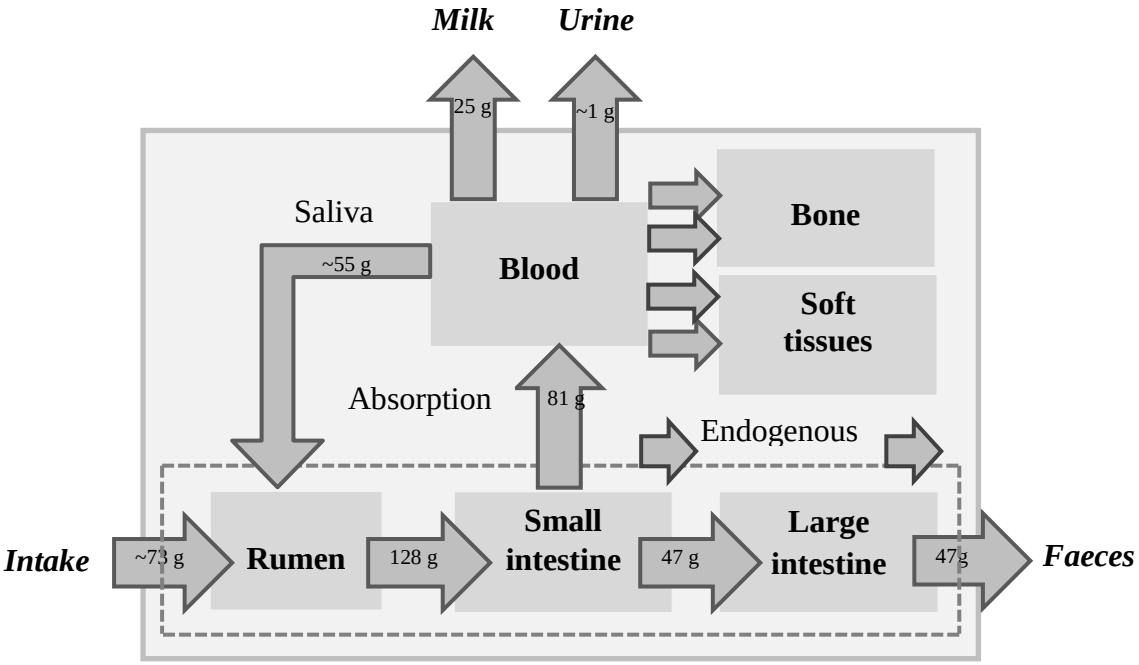


Figure 2: Schematic representation of daily P flows in dairy cow with a milk yield of 25 kg/d. *Words in italics outside the grey box indicate the P inflow and P outflow from the cow´s body. Words in boxes inside the grey box indicate pool sizes (g/d) of P in the various metabolic or physical compartments of the gut lumen or the body of the dairy cow that are involved in the dynamics of P flows and pool sizes according to* Hill et al. (2008) and Bannink et al. (2010)

P metabolism and balance

The supply of P with the feed is needed for the different physiological processes in cows. From the total supply, a part of fed P is distributed into the uterus of pregnant cows (**Figure 3**). During the first 150 days of pregnancy, P is not transferred through the placenta from the cow to the fetus. From day 150 to 200 approximately 0.6 g P, from days 200 to 250 2.7 g P and during the final period of pregnancy 7.4 g P per day are absorbed to the uterus (Moustgaa, 1972). Furthermore, another part of dietary P is excreted with the faeces.

Animals excrete P via faeces for one of three potential reasons (Pfeffer et al., 2005): first of all some fraction of P contained in feed may not be absorbable due to its chemical binding. This fractions might break down and catalyzed in the rumen or by the supply of exogenous phytase (Pfeffer et al., 2005) in order to increase the P-digestibility (Kincaid et al., 2005). The second reason alludes to the fact that some P is lost in faeces independent of P-intake from the diet. These kinds of losses are defined as inevitable, or obligatory, and it is assumed that they are caused by the physiology of the host animal and by its microbial organisms (Pfeffer et al.,

2005). The final potential reason for P-excretion via faeces is that the surplus of P will be excreted. Due to the fact that more than 90 percent of the P is excreted via faeces, the excretion via urine must be considered as marginal (Grace et al., 1974, Bertoni, 1976, Boxebeld et al., 1983, Braithwaite, 1984, 1985, Wylie et al., 1985, Martz et al., 1990, Khorasani and Armstrong, 1992, Bortolussi et al., 1996, Vitti et al., 2000).

Another part of the fed P enters the milk. 1.0 g P per kg milk is calculated for lacteal P-excretion according to the recommendations of GfE (2001). P-requirement for milk P-excretion is the largest proportion of total P quantity required by dairy cows. Concerning rising milk performance, an increasing quantity of P must be fed. However, a higher P-concentration than 4 g/kg DM is not necessary (Brintrup et al., 1993, Wu et al., 2000, Valk et al., 2002). Reference values for P in milk of lactating Holstein cows over the complete lactation cycle range between 0.85-0.94 g P/kg milk (Brintrup et al., 1993, Wu et al., 2000, Valk et al., 2002).

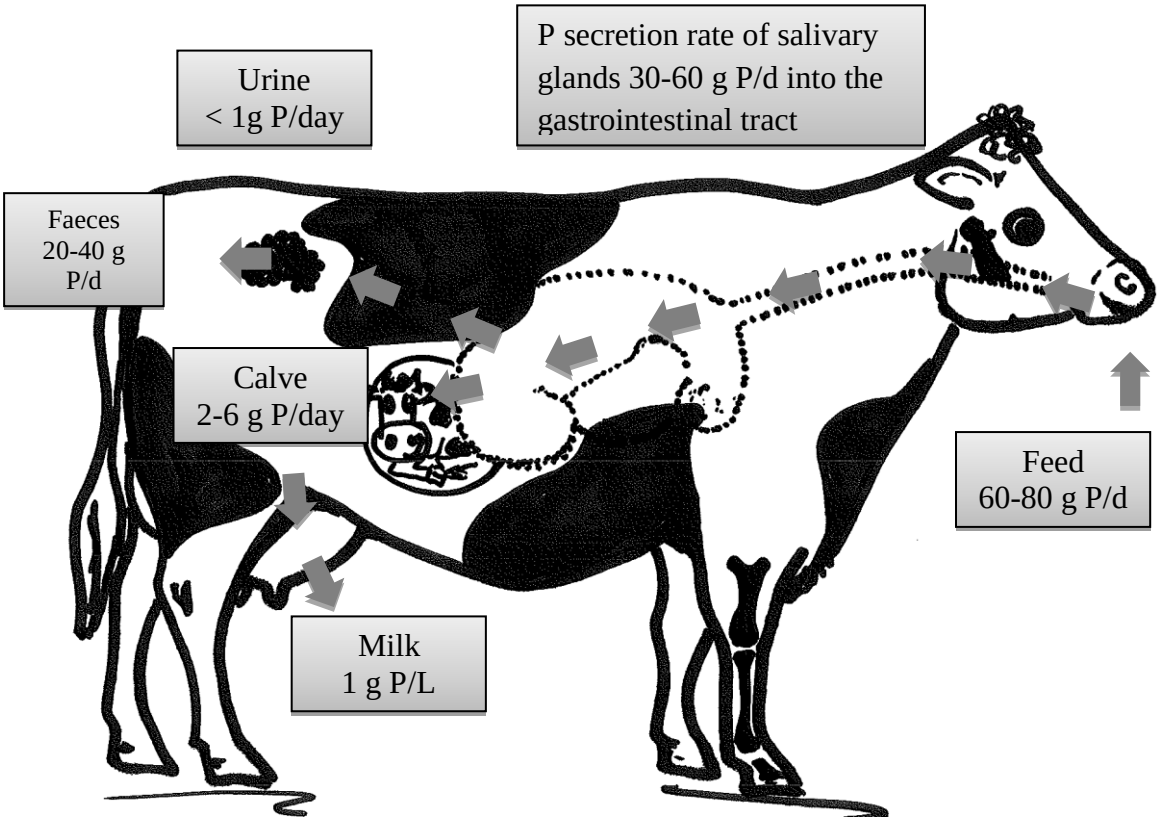


Figure 3: Schematic showing P-intake and P-excretion of dairy cows according to Kolb and Gürtler (1971) and Breves and Schröder (1991)

The measurement of P absorption in ruminants is complicated due to the copious secretion of P in saliva. The salivary P adds greatly to the flow of P into the rumen (Suttle, 2010). The

kinetics of P in the whole animal have been measured by a variety of techniques including the use of duodenal cannulas and ruminal fistulas and the short half-life isotope ^{35}P. In measuring gastrointestinal kinetics, the isotope has been widely used to mark endogenous P and to differentiate it from dietary P (Challa et al., 1989, Ternouth, 1997). Ternouth et al. (1985) have investigated the secretion of the inorganic phosphates by salivary glands. As a result, the glands have the ability to concentrate P approximately 10-fold during the secretion process, although this is dependent upon the rate of secretion in saliva (Bailey and Balch, 1960, Ternouth et al., 1985). Challa et al. (1989) have shown that the salivary P comprises 50% of P entering the rumen of calves fed normal diets and over 80% when with lower P intakes (<10 mg P/kg LW). Salivary P concentration is related to plasma P concentration whilst salivary P volume is related to dry matter intake (Karn, 2001). Total salivary P secretion is a product of salivary volume and P concentration. The mean daily influx of salivary P in the rumen is between 30 to 90 g in cows (Nikolic et al., 1978, Reinhardt et al., 1988). Salivary P occurs as inorganic P, a form that is highly available to the rumen microbes and is absorbed in the small intestine along with dietary P (Horst, 1986, Valk et al., 2002). Salivary P may be the main source of P for ruminal microbes especially when insoluble phosphates are consumed via the diet (Durand and Kawashima, 1980).

P is mainly absorbed in phosphate form from the small intestine of ruminants, especially in the duodenum and jejunum and is higher than the ruminal one (Breves and Schröder, 1991). In all species this process is largely unregulated. On the contrary the absorption is linearly related to P_i intake over wide ranges, with high coefficients of 0.68-0.80. Dephosphorylation and hydrolysis of P in ingested grains and seeds by microbial phytases and phosphatases releases PO_4^{3-} into the rumen (Reid, 1947, Nelson et al., 1976, Morse et al., 1992). This is followed by a comprehensive incorporation into microbial protein. However the degradation of phytin P can be far from complete. Microbial P is marginally less well absorbed than PO_4^{3-}, which is readily absorbed (Suttle, 2010). Only small quantities of P are absorbed by animals. It depends on the nature of the feed, the kind of P source, P-concentration in the diet, requirement of the animal, intestinal pH-value, diseases, parasites and the environment. Furthermore, the quantity of P is influenced by the age of an animal and the dietary levels of Ca, Fe, Al, Mg, potassium, manganese and fat (Ekelund, 2003; Suttle, 2010). P absorption occurs in two different ways; one active and one passive. The process of each way depends on the absorbable P in the lumen of the small intestine. On the one hand, the active process dominantes when animals are deficient in P; Vitamin D influenced that active absorption. The

biologically active form of Vitamin D is synthesized by the photochemical conversion of 7-dehydrocholesterol in the skin or by the photochemical conversion of ergoestrol in plants (Guyton, 2002). When Vitamin D enters the bloodstream it is transformed into 1,25-dihydroxyvitamin D (1,25-$(OH)_2$D) by microsomal enzymes located in the microsomes and mitchochondria. In the case of P deficiency, 1,25-$(OH)_2$D-synthesis is activated, which in turn causes an increase in the efficiency of P absorption in the small intestine (Guyton, 2002). On the other hand P absorption by the small intestine is a passive process. In ruminants it becomes dominant when P is sufficiently available for absorption from the small intestine. This process is not subject to interactions of hormones or vitamins as with the active process and takes place when ruminants consume normal to high P quantities of P through their diet or when the amount of P in the plasma is elevated (Guyton, 2002). To summarize, a relationship between the passive P absorption and the amount of P in the small intestine lumen and blood P-concentration exists (NRC, 2001). Most organic P, which is not hydrolyzed in the rumen, becomes solubilized by the low pH of the abomasum (Breves and Schröder, 1991).

Inorganic P uses an active co-transport mechanism with Na^+ to overcome the border membrane of enterocytes of the small intestine. The Na^+ flow against the electrochemical gradient helps the accumulation of P access the enterocytes of the small intestines. The Na^+ gradient and subsequent accumulation of P in the cell are maintained by the Na^+, K^+ adenosine triphosphate (ATPase) pump (Guyton, 2002, Suttle, 2010).

In the large intestine of cattle, phytate P is partly degraded by phytase activity from intact bacterial cells although this released P is not available for ruminants as little or even no P is absorbed from the large intestine (Pfeffer et al., 1970).

P is required for many physiological functions and there are numerous signs of P deficiency in animals. If P deficient diets are fed for longer periods, feed intake decreases and results in negative effects on metabolic activity of body cells. This deficiency can influence the satiety center and can decrease bone P concentration (McDowell, 1992; Wu et al , 2001). During short periods of insufficient dietary P intake, the resulting P deficiency can be compensated by P recycling and mobilization from bone (McDowell, 1992).

P requirement

For optimization of P concentration in feed, information concerning P requirement is needed. Depending upon the country or scientific society, calculations for requirements vary. In the current studies, the German recommendations are used. The GfE (2001) calculates the P-requirement by taking feed intake, milk yield and digestibility into account. The P-concentration of the diet is calculated according to the recommendations of the Society of Nutrition Physiology (GfE, 1995, GfE, 2001) as follows:

$$\mathrm{P-recommendation\ (g/d)} = \frac{\left(DMI\ \left(\frac{kg}{d}\right) x1.0g\ P\right) + \left(milk\ yield\ \left(\frac{kg}{d}\right) x1.0g\ P\right)}{Total\ utilization} x100$$

Zinc

Function and sources of Zn

Dietary Zn is required in many living processes including protein synthesis and energy metabolism. Zn occurs as an essential part of many metalloenzymes and as activator for some metalloenzyme complexes (Rose, 1983). The physiological functions of Zn are numerous: it plays a part in the metabolism of DNA, RNA, carbohydrates, lipids and energy (Miller, 1983). Zn is required for the structural and functional integrity of over 2000 transcription factors. As a consequence, almost every signaling and metabolic pathway depends on one or more zinc-requiring proteins (Suttle, 2010). Furthermore, Zn plays a crucial role in maintaining cell membrane structure and function. In some species of animals, including swine and ruminants, supplemental Zn is essential for the hampering of deficiency effects such as disease in animals which are fed commonly used diets. It is unknown, at what point ruminants or humans suffer from a marginal deficiency. This uncertainty partially results from the lack of definitive biochemical measures for determining Zn status of individual animals. Zn is regarded as relatively non-toxic and as such its toxicity does not pose a major problem.

The provision of adequate dietary supplies of Zn can be a matter of life or death. Information about the Zn content of feeds is summarized in **Table 1**.

Table 1: Mean Zn-concentrations found in common livestock forages and feeds in the UK (MAFF, 1990) and elsewhere (Suttle, 2010)

Roughages	Zn (mg/kg DM)	Concentrates	Zn (mg/kg DM)	By-products	Zn (mg/kg DM)
Straw	14	Barley	33	Wheat feed	104
Grass		Maize	19	Rice bran	77
Fresh	36	Oats	26	Brewers´grain	73
Dried	33	Wheat	26	Distillers´grain	55
Grass		Maize gluten	80	Rapeseed meal	82
Hay	21	Cassava meal	12	Meat and bone meal	130
Dried	28	Linseed meal	66	Feather meal	152
Kale	27	Soybean meal	49		
Maize silage	45	Sorghum	14		
		Field beans	38		

The Zn content of forages and cereal grains varies a little among plant species, but is greatly influenced by the soil Zn status (Suttle, 2010).

Zn digestion, absorption and metabolism

In contrast to other essential trace elements such as selenium, cobalt, iodine and copper, animals require relatively large amounts of Zn. The estimated requirement for cattle is 40 ppm in diet DM. This provides a margin of safety for varying conditions and many factors influence the actual amount needed by the animal (Miller, 1979). Likewise, higher dietary content of Ca and P can increase the Zn needs of animals (Miller, 1971, Underwood, 1977).

In most animal´s tissues and milk, Zn content is not greatly affected by dietary intake, though there are exceptions. If a large amount of Zn is fed, the content in liver, kidney and some other vital organs could be influenced (Miller et al., 1970, Stake et al., 1975, Rose, 1983).

The major route of homeostatic Zn control bases on variable absorption (Rose, 1983). In animals Zn is almost exclusively bound to other substances such as proteinaceous or skeletal material. The remaining part is distributed in the other tissues of the animal (Miller, 1971, Underwood, 1977, Miller, 1979, Rose, 1983). Even though there is variability among different tissues, this variation is much less pronounced than for other trace elements. The Zn-content is low in body fat, either in the animal body or the milk. Most of the milk Zn is

located in association with casein, whey or with lactose. Furthermore it occurs in milk fat globule membrane and in lipoprotein membranes (Parkash and Jenness, 1969, Matrone, 1970, Miller, 1971, Miller and Neathery, 1980). In a few tissues such as liver, hair and bones the decline is smaller. Commonly used dietary Zn-concentration, generally have relatively minor influence on the Zn-content of most body tissues. Certain features of Zn metabolism are necessary to understand if the nutritional value of feeds, in their role as Zn sources, is to be accurately measured.

Zn is marginally excreted by the kidney (Suttle, 2010). The major route of endogenous Zn excretion happens predominantly via pancreatic secretions and faeces. The largest part comes from the direct Zn transfer through the intestinal wall with one-quarter or less from pancreatic juice. Likewise, some is lost through the sweat and in hair and sloughing skin (Stake et al., 1974). With little regulation of excretion, Zn retention is closely related to Zn absorption. Zn is absorbed from the small intestine, principally in the duodenum. Substantial and definitive evidence indicates that the absorption takes place throughout the length of small intestine with approximately equal amounts absorbed per unit of intestinal length in cattle (Hampton et al., 1976). This process accords to need through an active saturable process at normal dietary Zn concentrations. Sheep and cattle can absorb with a maximal efficiency of 0.75 (Suttle, 2010). Zn absorption can be visualized as a two-step process in which Zn is taken up by the mucosa cells of intestine and subsequently transferred to plasma. This latter step is slower than the initial mucosal uptake (Pate et al., 1970).

As a consequence of Zn deficiency, ruminants get clinical symptoms located at the skin. In the case of a minor deficiency, the feed intake of calves, bulls and cows decreases. Therefore the performance such as physical growth rate of calves, daily live weight gain of bulls and milk performance of dairy cows decline. In contrast a higher deficiency becomes apparent by the appearance of skin disorders. This clinical picture emerges over the entire skin surface of calves, whereas bulls and cows show symptoms only on the back and croup areas (Dirksen et al., 2006).

Zn requirement

The GfE-recommendations estimate a Zn-requirement of 50 mg Zn/kg DM feed for dairy cows (GfE, 2001) and 40 mg Zn/kg DM feed for fattening bulls with a live weight over 175 kg (GfE, 1995). Normally the milk of cows contains 3-5 mg Zn/L. With a concentration

between 30 and 40 mg Zn/kg, dried skimmed milk and buttermilk are appropriate sources of Zn (Suttle, 2010).

Phytase

Sources and function of phytase

Phytase is widespread in nature, occurring in plants, microorganisms and in some animals (Dvorakova, 1998). Ruminants are able to digest phytate P because rumen microorganisms synthesize the phytase enzyme. Phytase breaks the phosphate groups from the inositol in order to make P available for absorption in the small intestine (Reid and Franklin, 1947; Raun et al., 1956; Nelson et al., 1976; Morse et al., 1992). As already Raun et al. (1956) found out, the rumen microorganisms produce the enzyme phytase, which is capable of hydrolyzing phytate present in plant feedstuffs and that factors like pH, time of incubation, concentration of substrate, or concentration of active cells which alter fermentation rate, also alter the activity or the amounts of phytase produced. The duration of incubation also increases the amount of phytate P hydrolyzed (Raun et al., 1956).

The enzyme and its activity are frequently present in the plant kingdom. It appears in some cereal grains such as barley, rye and wheat. Furthermore, phytase activity is found in peas, beans, soybeans, maize, rice, white mustard, potato, radish, lettuce, spinach, grass and others (Dvorakova, 1998). This activity is mainly associated with the aleurone layer (34%) in cereals (Gabard and Jones, 1986). Therefore, in wheat kernels, phytase is distributed through the endosperm (34%) and scutellum (15%) (Peers, 1953), however in barley it contained around the protein bodies of the aleurone layer (Tronier et al., 1971). Phytase activity in corn is only little or to nonexistend (Eeckhout and De Paepe, 1994). Dvorakova (1998) summarized previous studies and considered that a rapid increase of phytase activity can be found in plant seeds during their germination. The determination of phytase activity in the feedstuff follows the procedure of Engelen et al. (1994). Phytase activity is determined in rumen samples and expressed in FTU adapted from the definition of Engelen et al. (1994), while one FTU is the amount of enzymes that liberates 1µmol of inorganic P per minute from an excess of Na phytate at pH 5.5 and 37°C. **Table 2** summarizes the phytate P content and phytase activities of the most common feed ingredients. Wheat and wheat-by products show the highest phytase activity, while in corn and oilseed meals activity is much lower. There are differences between the ingredients depending on the treatment, such as pelleting or milling. Phytase

activity is not related to total P content or phytate-P content in feedstuff (Eeckhout and De Paepe, 1994).

Table 2: Phytate P content and phytase activity of some common feed ingredients

Ingredient	Phytate P[1,2] (%)	Phytate P[1,2] (% of total P)	Phytase activity [2] (FTU*)
Cereals and by-products			
Corn	0.19-0.28	68-72	15
Corn silage	0.30	43	12
Corn gluten feed	0.47	54	48
Wheat	0.27	69	1193
Wheat bran	0.92	71	2957
Wheat gluten feed	0.56	71	25
Sorghum	0.19-0.27	66-70	24
Barley	0.27	64	582
Oats	0.21-.29	59-67	42
Oilseed meals			
Soybean extracted	0.32-0.39	53-60	8-40
Canola meal	0.70	59	16
Sunflower meal	0.89	77	60
Groundnut meal	0.48	80	3
Cottonseed meal	0.84	70	-
Roots and tubers			
Beet Pulp	0	0	3

[1] Data adapted from Ravindran (1996) and (Ravindran et al., 1994, Ravindran et al., 1995)

[2] Data from Eeckhout and De Paepe (1994). One unit is defined as that amount of phytase which liberates inorganic P from a 5.1 mM Na-phytate solution at a rate of 1µmol min^{-1} at pH 5.5 and 37°C.

[*] FTU: one FTU is the amount of enzymes that liberates 1µmol of inorganic P per minute from an excess of Na phytate at pH 5.5 and 37°C (Engelen et al., 1994)

In the rumen, phytase is synthesized by ruminal microflora. *Selenomonas ruminantium* is one of the most important and functionally diverse bacteria present in the rumen. This micro-organism influences the phytase activity positively (Raun et al., 1956, Caldwell and Bryant, 1966, Yanke et al., 1998). Moreover, Yanke et al. (1998) investigated, that high phytase activity is mostly associated with bacteria whereas low enzymatic activity occurs in connection with protozoa, feed particle, and fungal fraction of rumen fluid.

Additionally, Yanke et al. (1998) have represented a higher phytase activity if the level of phytate in feed increases. Furthermore it is determined that almost the complete dietary P to

ruminants is splitted up by microbial built phytase in the intestinal tract (Reid and Franklin, 1947; Raun et al., 1956; Nelson et al., 1976; Morse et al., 1992). For example bacterial starch fermentation is associated with the highest ruminal activity of phytase (Eeckhout and De Paepe, 1994). Consequently, phytate has to be consumed by the bacteria to hydrolyze myo-inositol hexakisphosphate. Phytin hydrolysis might also be caused by intrinsic phytase contained to the diet (Eeckhout and De Paepe, 1994).

The enzyme phytase plays an extraordinary role in the reduction of P excretion by excrements. Due to its ability of splitting phosphate from the inositol ring, phytase makes P more available for absorption in the small intestine (Guyton et al., 2003). Kincaid et al. (2005) tested the effects of grain source and exogenous phytase supplementation on the digestibility of P and concluded that exogenous phytase might influence the faecal P excretion of dairy cows. According to current knowledge, there is reliable method to identify the quantitative influence of plant phytase on ruminal phytate hydrolysis (Kincaid et al., 2005). The P application of inorganic and organic origin by ruminal bacteria in a semi-continuous culture demonstrated that the outflow has a higher content of inorganic P than organic (Godoy and Meschy, 2001). Consequently, the natural ruminal phytase activity does not decompose the complete dietary phytate (Godoy and Meschy, 1999).

Using in vitro ruminal techniques Morse et al. (1992) and Brask-Pedersen et al. (2011) found that the effect of exogenous phytase is closely related to the composition of the feedstuff, the pH-value, the kind of phytase and incubation time. Brask-Pedersen et al. (2011) found out that the supply of exogenous phytase in vitro can influence the P-absorption positively. Otherwise, there have been no *in vitro* studies to investigate whether exogenous phytase can influence the Zn-absorption. Experiments in a semi-continuous culture system by Godoy and Meschy (1999) with inorganic and organic P compounds suggest that in some situations the ruminal phytase activity does not hydrolyze all dietary phytate.

The time of feed incubation in the gastrointestinal tract gets shorter in high performance fattening bulls and lactating cows, as feed intake increases with improving performance. Consequently, on the one hand a decelerated passage rate increases, whilst on the other hand a faster passage rate decreases incubation time and P and Zn-hydrolyses.

3. Scope of the thesis

Of all essential dietary mineral elements for dairy animals, P represents the greatest potential risk if excess is released into the environment contaminating surface waters and causing eutrophication.

Therefore the aim of this work was to examine the effects of exogenous phytase, P and Zn and their effects on P- and Zn-status of ruminants. Although ruminants generally have the ability to use phytin-bound phosphorus through ruminal hydrolysis, different studies have shown that the addition of exogenous phytase into the diet leads to a reduced faecal phosphorus excretion. In addition, the apparent P-digestibility and the inorganic phosphorus-concentration (Pi) in serum were increased at the same time. However, not only phosphorus is bound to phytin, but also trace minerals such as zinc and copper are known to be released by the action of exogenously added phytase in monogastric animals. The occurrence of such effects is not yet known in ruminants. Additional phytase might mediated by the liberation of these trace elements, may increase their bioavailability.

Therefore the following hypotheses should be investigated in the present thesis:

1. Supplementation of phytase to ruminant diets improves the P-digestibility.
2. By ruminants faecal P-excretion (g/d) is linear to P-intake with the feed (g/d).
3. Different P-intake with feed affect the P-digestibility and P-balance of dairy cows and bulls.
4. The effect of exogenous phytase, P and Zn supplementation has an influence on performance and P- and Zn levels in liver, testes, bones and faeces.
5. Supplemented phytase has no influence on the ruminal fermentation parameters and the nutrient duodenal fluxes, the P-balance and P-digestibility.

For investigation of these topics three experiments were conducted. 24 German Holstein dairy cows, were used to investigate the effect of P or phytase supplementation on the P-balance (Paper I), 48 German Holstein bulls were used to investigate the effect of P-, Zn- or phytase-supplementation on the P- and Zn-metabolism of fattening bulls (Paper II), and nine pluriparous fistulated dairy cows were used to determine the effects of an exogenous phytase is added to the diet in the rumen (Paper III).

4. Paper I

Effect of exogenous phytase on the phosphorus balance of lactating cows fed a corn based diet

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ABSTRACT

The present study investigated the effect of P or phytase supplementation on the P-balance of dairy cows. 24 lactating German Holstein cows were used for a 5-weeks feeding trial and were allocated to three dietary treatments, P+MIN, P-MIN and P+PHY. All cows received a total mixed ration (TMR) composed of 63% corn silage and 37% concentrate on a dry matter (DM) basis for *ad libitum* intake. The concentrate of the P+MIN group was supplemented with dicalcium phosphate and represents the control group. The concentrate of the P-MIN group was unsupplemented and the concentrate of the P+PHY group was supplemented with an exogenous phytase (0.1 g/kg DM in the TMR; 50 000 FTU/g). The P concentration in the TMR of the P+MIN, P-MIN and P+PHY groups were 3.98, 3.46 and 3.26 g P/kg DM, respectively.

Dry matter intake (DMI) and milk yield were recorded daily. In the last two weeks samples of milk, urine and blood were collected. Samples of faeces were collected to determine the P-balances by using the acid insoluble ash (AIA) marker technique.

No differences in P-concentration of milk, urine and faeces were observed between the treatments. The P-digestibility of Group P+MIN and P-MIN was 60 and 56%, respectively. These values were not different compared to the P-digestibility of 57% in the P+PHY-group. The P-balance in the P+MIN group (26 g/d) was higher compared to the P-MIN (16g/d) and P+PHY (17g/d) treatment. Overall, phytase supplementation had no effect on P-digestibility and P-balance of dairy cows in this trial.

Keywords: phytase, ruminants, P-digestibility, P-balance

INTRODUCTION

In the past, dairy cows were often fed diets containing P levels markedly higher than the recommendations for P supply. The most common explanation for this oversupply is the perception that high-P diets improve reproductive performance. In addition, the recommendations for adequate P supply differ from nation to nation (GfE, 2001, NRC, 2001, INRA, 2002, CVB, 2005, Schlegel, 2011). Consequential the proportion of excreted P, which is not used to meet the requirements of the cow, increases. Moreover, natural P sources used as mineral feedstuffs become more and more limited in the future (Rodehutscord, 2008). Therefore, it remains a challenge for animal nutrition to reduce the dietary P supply while

meeting the requirement at the same time. One way to increase the P-absorption and to reduce faecal P is the supply of exogenous phytase to the diets (Knowlton et al., 2007). The enzyme phytase has a relevant impact in the reduction of P-excretion by excrements from monogastrics. Due to its ability to cleave phosphate from its binding to the inositol ring, phytase supply more P for absorption in the small intestine (Guyton et al., 2003). In ruminants, phytase is secreted intracellular by ruminal bacteria (Yanke et al., 1998) and phytate hydrolysis also occurs in the lower gastrointestinal tract (small intestine with duodenum, jejunum, ileum) of ruminants. Thus the total tract hydrolysis of phytate is nearly complete (Brask-Pedersen et al., 2013). However, for P to be absorbed from the small intestine, the phytate hydrolysis must occur in the rumen. Using *in vitro* ruminal techniques Morse et al. (1992) and Brask-Pedersen et al. (2011) found out that the effect of exogenous phytase is closely related to the composition of the feedstuff, the pH-value level, the kind of phytase and the time of incubation. Additionally, Brask-Pedersen et al. (2011) observed that the supply of exogenous phytase *in vitro* can influence the P-utilization positively. These results are sustained by Garikipati (2004), who figured out a positive effect of the influence of exogenous phytase in dairy cows. However, data regarding the intake of P and the use of exogenous phytase are inadequate. Kincaid et al. (2005) tested the effects of grain source and exogenous phytase supplementation on the digestibility of P and concluded that exogenous phytase could have an influence on the faecal P-excretion of dairy cows. Although the main part of phytase activity in the rumen is of bacterial origin (Yanke et al., 1998), phytin hydrolysis might also be caused by intrinsic phytase contained in the diet, whereby however only some cereals and their by-products show phytase activities of more than 100 units/kg (Eeckhout and De Paepe, 1994). The phytase activity in corn is below the detection limit (Zimmermann et al., 2002). According to current knowledge, there is no certain way to identify the quantitative influence of plant phytase on ruminal phytate hydrolysis (Kincaid and Rodehutscord, 2005). Experiments in a semi-continuous culture system by Godoy and Meschy (1999) with P of inorganic and organic origin suggest that in some situations the ruminal phytase activity does not hydrolyze all dietary phytate. Time of incubation of feed in the gastro intestinal tract is getting shorter in high lactating cows, because the feed intake increases with the increasing performance. Based on this, the passage rate increases and with this the time of P-hydrolyses gets even lower (Garikipati, 2004).

Overall, these observed effects of phytase could influence the P-balance of dairy cows and previous studies (Kincaid et al., 2005, Knowlton et al., 2007) comprise no complete P-

balances. In addition the interaction between inorganic phosphorus (P_i) in serum and P-excretion via urine and milk remained unconsidered. Due to the limited quantity of studies to the supply of exogenous phytase *in vivo*, the aim of the present study was to determine a P-balance of lactating cows fed a corn based diet with a commonly used P-concentration, to ascertain further indicators for the effects of exogenous phytase and to study the impact on the concentration of P in milk, urine, faeces and blood.

MATERIAL AND METHODS

Animals, treatments and experimental design

The experiment was carried out with 24 lactating pluriparous German Holstein cows at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Federal Research Institute for Animal Health, in Braunschweig, Germany. At the beginning of the trial, the cows had an average milk yield of 26.8 ± 1.9 kg/d and a mean body weight of 648 ± 64 kg. The mean lactation day was 196 ± 26 and the animals were, on average, in their 2.8 ± 1.0 lactation. The animals were randomly assigned to one of three feeding groups, with eight cows each, based on milk yield, body weight, days in lactation and the number of lactation. The cows were kept in a free stall barn with a slatted floor and cubicles.

The animals were fed *ad libitum* with corn silage and concentrate as a TMR, with 37% concentrate and 63% corn silage (on a DM basis). The concentrates were added in pelleted form to the corn silage and mixed in a feed mixer just before feeding. The diets were intended to cover the demand of energy and protein according to the recommendations of the Society of Nutrition Physiology (GfE, 2001).

Three experimental diets were fed. Mineral P is supplied to the P+MIN diet. The diet represents the commonly used P-concentration for dairy cows with a milk yield of approximately 30 kg/d and a feed intake of about 20 kg DM/d. The TMR of group P-MIN included the native P of the feedstuffs without P-supplementation. The animals of group P+PHY got the same concentrate as group P-MIN e.g. **Table 1**, but supplemented with an experimental phytase (0.1 g/kg DM in the TMR). The phytase had an activity of min. 50 000 FTU/g according to the manufacturer´s specifications (Experimental Phytase, manufacturer DSM-Nutritional Products Ltd, Basel, Switzerland). Determination of phytase activity in the

feedstuff followed the procedure of Engelen et al. (1994). Phytase activity was determined in rumen samples and expressed in FTU adapted from the definition of Engelen et al. (1994).

Table 1. Composition of the concentrates used during the trial

	Concentrate 1 (%)	Concentrate 2 (%)	Concentrate 3 (%)
Corn	40.0	40.0	40.0
Soybean meal	35.0	35.0	35.0
Dried sugar beet pulp	19.7	20.0	20.0
Calcium carbonate	0.7	1.3	1.3
Urea	1.5	1.5	1.5
Sodium chloride	0.2	0.2	0.2
Mineral premix*	2.0	2.0	-
Mineral premix with phytase†	-	-	2.0
Dicalcium phosphate	0.9	-	-

* Composition (per kg): 200g calcium, 120g sodium, 40g magnesium, 1 000 000 IU vitamin A (E672), 100 000 IU vitamin D3 (E671), 1500 mg vitamin E (alpha Tocopherolacetat), 5400 mg Mangan (Mangan (II)sulfat, Monohydrat E5), 6000 mg Zinc (Zincoxide E6), 1000 mg copper (copper sulfate pentahydrate E4), 100 mg iod (calcium jodate, waterfree E2), 40 mg selenium (Sodium Selenate E8), 25 mg cobalt (cobalt sulfate, monohydrate, E3)

† Composition (per kg): see premix [1] added with 14.8 g phytase (phytase activity amounted to 50 000 FTU**/g)

**FTU: one FTU is the amount of enzymes that liberates 1µmol of inorganic P per minute from an excess of Na phytate at pH 5.5 and 37°C (Engelen et al., 1994)

Measurements and sampling procedure

Individual feed and water intake were recorded continuously by an automatic feeding system (manufacturer Insentec B.V., Marknesse, The Netherlands). The first three weeks of the trial were conducted as an adaptation period, followed by a two- week sampling period.

During the sampling period, representative samples of the diet were collected daily. After collection, the samples were stored at -18°C and pooled to one sample.

Cows were milked twice daily at 05:30 am and 03:30 pm in a milking parlour (manufacturer Lemmer-Fullwood, Lohmar, Germany). The milk yield was recorded automatically. Twice a week milk samples were taken during morning and afternoon milking to determine fat, protein, lactose, urea and somatic cell count (SCC). The samples were conserved with bronopol and stored at 8°C until analysis. Individual body weight was determined twice daily after milking.

Blood samples were collected to prove that only cows in good metabolic condition were included in the study. The blood samples were taken for analysis of clinical blood parameters at day 0 of the adaptation period and at the end of the trial. Additionally, samples of blood, urine, faeces and milk were taken for P- and Ca-analysis from every cow on day 1, 3, 5, 8, 10 and 12 during the last two weeks of the experiment. That means that during the sampling period, blood was collected six times in serum tubes from a *vena jugularis externa*. Approximately one hour after sampling, serum was separated by centrifugation at 3000 x g for 30 minutes at 15°C. The serum was filled into tubes and stored at -18°C until analysis of clinical blood parameters.

Analyses

Samples of feedstuff and faeces were analyzed for DM, ash, crude protein (CP), crude fibre (CF) and ether extract (EE) according to the methods of the Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA, 1997). Feedstuff samples were dried at 60°C for 72 hours and ground through a 1-mm screen. Analysis of acid and neutral detergent fibre (ADF resp. 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 Ca and P. Faeces samples were taken during the last two experimental weeks and freeze dried for the determination of nutrients and acid AIA.

Ca and P in TMR, milk, urine and faeces were analyzed by an optical emissions spectrometer with inductively coupled plasma (ICP-OES) according to VDLUFA (1997).

The phytase content of the concentrates is determined via the phytase activity and expressed as units (U)/kg feed. The analytical method is based on the method of the draft of the Comite Europeen de Normalisation (CEN) standard (12) which is published as ISO 30 024 (Gizzi et al., 2008).

The milk samples were analyzed for fat, protein, lactose, urea and SCC with a Fourier transform infrared spectroscopy and flow cytometric measurement system (Milkoscan FT 6000 combined with a Fossomatic 5000, Foss Electric, Hillerød, Denmark).

Measurements of AIA are suited to predict the digestibility in ruminants (Sunvold and Cochran, 1991). The AIA in feed and faeces was analyzed with the 4N HCl-method. This is an adapted form of the method described by Wünsche et al. (1984) and McCarthy et al. (1974). A total of 2 to 5 g of freeze dried faeces or feed were ashed. The ignition of the

samples lasted 5 hours at a temperature of 550°C. The ashes were boiled for 15 minutes with 4N HCl and the residues were filtered with an ashless filter paper. After drying the filters with the residues, they were ashed again to obtain the amount of AIA. The nutrient concentration in faeces samples was analyzed in the same way as in the feed. The concentration of P, Ca and different clinical chemical parameters like total protein, aspartate aminotransferase (AST), gamma glutamyltransferase (GGT), glutamate dehydrogenase (GLDH), cholesterine, glucose, free fatty acids (FFA), ß-hxdroxybutyrate, urea and creatinine were analyzed in blood serum samples. The analysis of the clinical chemical parameters was carried out using a Cobas Mira Plus Chemistry Analyzer (Hoffmann-La Roche Ltd., Basel, Switzerland).

Inositol-P (IP-6/5/4/3/2/1) was analyzed in all samples using high-performance ion chromatography according to the method of Brejnholt et al. (2011). The majority of total P was present as phytate P. Phytate P was determined by a standard `ferric chloride precipitation`-method (Selle et al., 2003).

Calculations

The apparent total tract digestibility (AD) of organic matter (OM), CF, EE, P and Ca was estimated by AIA as a marker occurring naturally in the diet. The results of AIA and OM, CF, EE, P and Ca analysis from the different diets and faeces were used to calculate the AD of OM, CF, EE as well as Ca and P as follows:

$$AD\ [\%] = ([(N_{diet}/AIA_{diet})-(N_{faeces}/AIA_{faeces})]/N_{diet}/AIA_{diet}) * 100,$$

where N_{diet} is the nutrient concentration (OM, CF, EE, P, Ca) in g/kg DM and AIA_{diet} is the AIA concentration in g/kg DM in the feed. N_{faeces} is the nutrient concentration (OM, CF, CL, P, Ca in g/kg DM) and AIA_{faeces} the AIA concentration (g/kg DM) in faeces.

The ME content was calculated according to GfE (2001) by using the digestibilities from the AIA method:

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

Where CP is crude protein, DEE is digestible ether extract; DCF digestible crude fibre and DOM digestible OM.

P-excretion with urine was calculated as:

P-excretion in urine [g/d] = P-concentration in urine [g/mL]*40 [mL/kg/d] *BW [kg].

According to Kienzle (1991) it is assumed that the mean urine excretion of dairy cows is 40 mL/kg body weight (BW) per day.

P-balance was calculated with the following equitation:

Balance [g/d] = P-intake [g/d]- faecal P [g/d]- urinary P [g/d]- milk P [g/d].

The phytase activity for the corn silage is calculated according to tabulated values of Eeckhout and De Paepe (1994). This results in a phytase activity for corn silage of 12 FTU/kg DM.

Statistical analysis

The statistical analysis was carried out with the SAS-software package Version 9.1.3 (SAS Institute, Cary, NC, USA 2004).

The procedure "MIXED" with a compound symmetry covariance was used to analyse the data. The treatment group was assumed to be the fixed effect. The fact that each cow was used for frequent measurements was considered using a "REPEATED" statement for the individual animal effect. 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 means (LS means) ± standard error of the mean (SE). Effects are graded as significant with $P < 0.05$.

RESULTS

Chemical composition of the feedstuffs

The mean nutrient and energy content of the diets is shown in e.g. **Table 2**. All three experimental groups received a TMR with an average energy content of 11.4 MJ ME/kg DM and 7.0 MJ NEL/kg DM. P-concentrations in the TMR were 3.98, 3.46, and 3.26 g/kg DM in groups P+MIN, P-MIN and P+PHY, respectively. The concentration of phytate P in total P of the different diets is listed in e.g. **Table 2**. The proportion of phytate P was between 58.5 and 69.9% of the total P-concentration in the diets.

Table 2. Chemical composition of the total mixed rations (TMR) in the experimental groups (g/kg DM)

	TMR 1 P+MIN[1]	TMR 2 P-MIN[2]	TMR 3 P+PHY[3]
	(g/kg DM)		
Organic matter	947	943	945
Crude protein	137	151	152
Ether extract	24	35	28
Crude fibre	152	158	153
ADF	164	178	172
NDF	346	376	358
P	3.98	3.46	3.26
Ca	5.79	5.54	5.10
	(mg/g DM)		
IP-6[4]	1.87	1.92	1.84
IP-5[4]	0.44	0.45	0.42
IP-4[4]	0.02	0.03	0.02
IP-1/2/3[4]	†	†	†
	(MJ/kg DM)		
ME[5]	11.49	11.21	11.46
NEL	7.05	6.82	7.02

† out of level of quantification, while the lowest standard (0.5-3.2mg/g DM) is defined as the `level of quantification`

[1] diet with supplemented mineral P

[2] diet with native P content

[3] diet with native P content, added with phytase

[4] Inositol Phosphorus

[5] calculated according to GfE (2001) by using the digestibility's from the AIA-Method

The concentrates used in the diets of group P+MIN and P-MIN (concentrate 1 and concentrate 2) showed no phytase activity, while concentrate 3 (P+PHY group) showed a phytase activity of 5859±15 FTU/kg.

General performance

The feed intake of the dairy cows during the sampling period is presented in e.g. **Table 3**. TMR intake amounted on average 21.6 kg DM/d for all groups. No differences were observed in TMR intake across the treatments. The mean P concentration of the concentrate was 5.95, 4.49 and 4.37 g/kg DM in group P+MIN, P-MIN and P+PHY, respectively. Group P+PHY showed the highest CP intake (3.35 kg/d) and P-MIN the highest intake of EE, ADF and NDF.

Table 3. Mean feed and nutrient intakes during the sampling period (LS means ± standard error)

	Group P+MIN[1]	Group P-MIN[2]	Group P+PHY[3]	*P*-value
Animals/group	8	8	8	
TMR (kg DM/d)	21.3 ± 1.03	21.3 ± 1.03	22.1 ± 1.03	0.413
Crude protein (kg/d)	2.92 ± 0.07[a]	3.21 ± 0.07[b]	3.35 ± 0.07[b]	<0.001
Ether extract (kg/d)	0.51 ± 0.01[a]	0.75 ± 0.01[c]	0.62 ± 0.01[b]	<0.001
ADF (kg/d)	3.50 ± 0.08[a]	3.79 ± 0.08[b]	3.80 ± 0.08[b]	0.012
NDF (kg/d)	7.38 ± 0.17[a]	8.00 ± 0.17[b]	7.80 ± 0.17[b]	0.021

[a, b, c] Different letters in one row show significant differences (P<0.05)

[1] diet with supplemented mineral P

[2] diet with native P content

[3] diet with native P content, added with phytase

The milk performance data showed no differences between the treatments e.g. **Table 4**. Average milk yield amounted to 26.8±1.9 kg/d, fat corrected milk (FCM) to 27.4±1.9 kg/d. The mean milk fat content was 4.15±0.23 %, the protein content 3.46±0.1 % and the lactose content 4.72±0.1 %. The averaged SCC was 175±55 tsd/ml and the urea concentration 264±12 ppm.

Table 4. Average milk yield and milk composition as well as milk P- and Ca-concentration during the sampling period (LS means ± standard error)

	P+MIN[1]	P-MIN[2]	P+PHY[3]	*P*-value
Animals/group	8	8	8	
Milk yield (kg/d)	27.1 ± 1.9	26.8 ± 1.9	26.6 ± 1.9	0.984
Fat Corrected Milk (kg/d)	27.5 ± 1.9	27.4 ± 1.9	27.3 ± 1.9	0.997
Fat content (%)	4.08 ± 0.23	4.22 ± 0.23	4.15 ± 0.23	0.914
Protein content (%)	3.39 ± 0.083	3.44 ± 0.082	3.54 ± 0.082	0.436
Lactose content (%)	4.64 ± 0.088	4.70 ± 0.088	4.81 ± 0.088	0.398
Somatic cell count (tsd/ml)	146 ± 55	278 ± 54	100 ±55	0.079
Urea (ppm)	245 ± 12	275 ± 12	272 ± 12	0.171
Phosphorus (g /kg DM)	0.90±0.03	0.92±0.03	0.91±0.03	0.885
Calcium (g/kg DM)	1.2±0.03	1.2±0.03	1.1±0.03	0.794

[1] diet with supplemented mineral P

[2] diet with native P content

[3] diet with native P content, added with phytase

The P-concentration in milk was on average 7.1±0.2 g P/kg DM and did not differ between groups. The Ca-concentration was on average 9.0±0.1 g/kg DM.

Digestibility and balances of P and Ca

The mean daily P-and Ca-intake is listed in e.g. **Table 5**. P-intake differed between the groups without P-supplementation and the P-supplemented group and was 74 and 72 g P/d for group P-MIN and P+PHY compared with 85 g P/d for group P+MIN. The ratio between Ca- and P-intake was 1.6:1 for group P-MIN, 1.5:1 for group P+MIN and 1.6:1 for group P+PHY. The ratio differed significantly ($P<0.001$), whereby the animals fed the highest amount of P (Group P+MIN) showed the lowest value.

There was no influence of treatment on the excretion of P and Ca with milk, faeces and urine during the sampling period e.g. **Table 5**.

Table 5. Mean P- and Ca-intakes and excretion with milk, faeces and urine as well as P- and Ca-balance during the sampling period (LS means ± standard error)

	P+MIN[1]	P-MIN[2]	P+PHY[3]	*P*-value
Animals/group	8	8	8	
Intake (g/d)				
Phosphorus	84.9 ± 4[a]	73.7 ± 4[b]	71.8 ± 4[b]	0.039
Calcium	123.5 ± 6	117.9 ± 6	112.6 ± 6	0.414
Excretion with faeces (g/d)				
Phosphorus	34.5 ± 3	32.4 ± 3	31.0 ± 3	0.636
Calcium	64.3 ± 6	78.8 ± 6	74.9 ± 6	0.221
Excretion with urine (g/d)				
Phosphorus	0.57 ± 0.1	0.60 ± 0.1	0.36 ± 0.1	0.196
Calcium	0.57 ± 0.1	0.29 ± 0.1	0.49 ± 0.1	0.186
Excretion with milk (g/d)				
Phosphorus	24.3 ± 2	24.5 ± 2	24.0 ± 2	0.971
Calcium	31.3 ± 2	31.0 ± 2	30.0 ± 2	0.907
Balance (g/d)				
Phosphorus	26.4 ± 2[a]	16.2 ± 2[b]	16.5 ± 2[b]	0.004
Calcium	27.3 ± 3[a]	7.8 ± 3[b]	7.2 ± 3[b]	<0.001
Total tract digestibility (%)				
Phosphorus	59.8 ± 2.4	56.1 ± 2.4	56.8 ± 2.4	0.519
Calcium	48.6 ± 2.8[a]	33.9 ± 2.9[b]	33.5 ± 2.8[b]	0.001

[a, b] Different letters in one row show significant differences (P<0.05)

[1] diet with supplemented mineral P

P-concentration in faeces for the different groups was averagely 6.7±0.6 g/kg DM. Ca-concentration in faeces of group P+MIN was lower (13.6±1 g/kg DM; P = 0.019) compared to both other groups (15.1 and 15.4 g/kg DM for P-MIN and P+PHY). Total tract apparent digestibility of P and Ca estimated by the AIA method is shown in e.g. **Table 5**. The P-

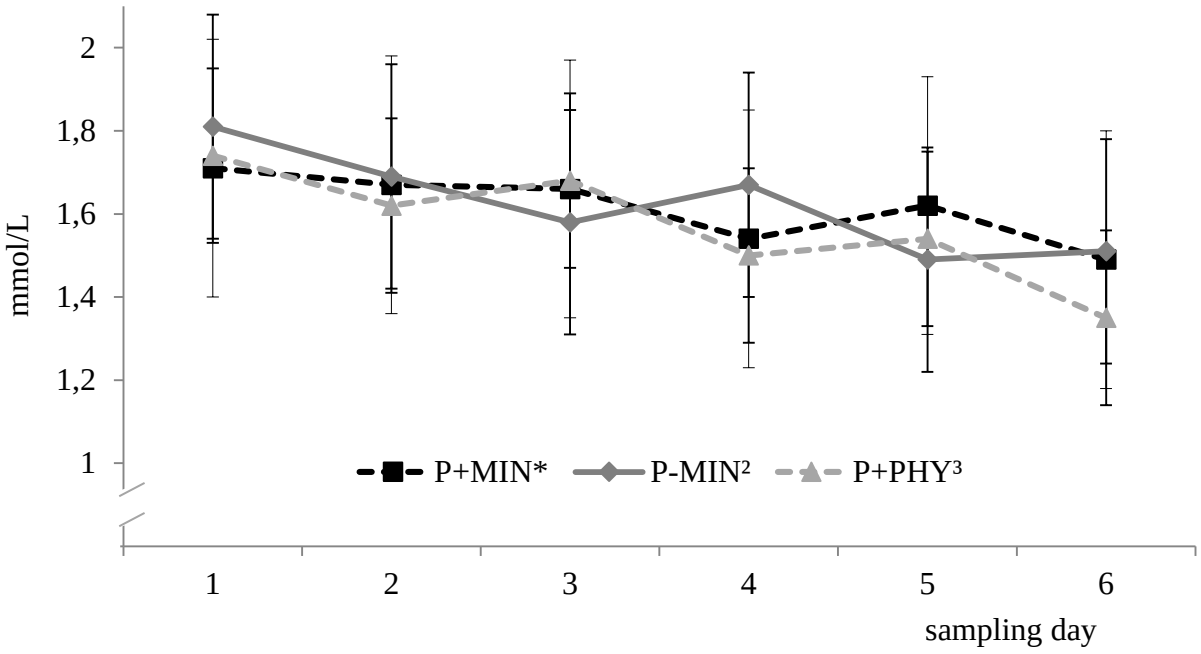
digestibility coefficients (on average 57.6 %) did not differ among treatments (P=0.519). In contrast, the digestibility of Ca was higher for P+MIN (P=0.001) and showed no differences between P-MIN and P+PHY.

There was no influence of treatment on the excretion of P and Ca with milk, faeces and urine during the sampling period. The P+MIN group retained more P and Ca with the consequence that nearly 80% of the additional P in group P+MIN were retained.

Blood Analysis

The course of the Ca and P-concentrations in blood serum during the sampling period did not show differences between the treatments.

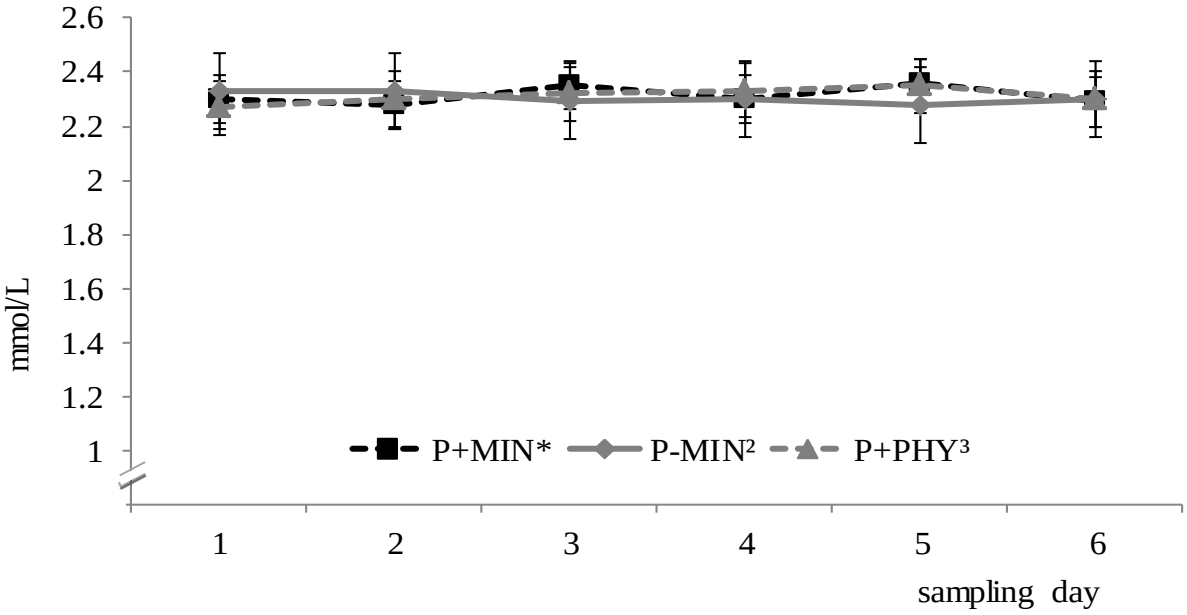
There was a slight decrease in serum P-concentration during the sampling period e.g. **Figure 1**. All feeding groups started with a higher P-concentration in blood serum than they finished with at sampling day 6. Group P-MIN had a P-concentration of 1.8 mmol/L at sampling day 1, while it closed at sampling day 6 at a concentration of 1.5 mmol/L. Both other groups started with 1.7 mmol/L, the group P+MIN ended with the same concentration as the control group P-MIN, while the phytase group P+PHY signs up the lowest concentration with 1.35 mmol/L.



* diet with supplemented mineral P [2] diet with native P content [3] diet with native P content, added with phytase

Figure 1. P-concentration (mmol/L) in blood during the sampling period (n=8)

The concentration of Ca in blood serum is constant in all groups e.g. **Figure 2**.



* diet with supplemented mineral P [2] diet with native P content [3] diet with native P content, added with phytase

Figure 2. Ca-concentration (mmol/L) in blood serum during the sampling period (n=8)

Results of blood serum analysis for total protein (71.4-78.4 g/L), AST (65.4-78.9 IU/L), GGT (28.5-33.1 IU/L), GLDH (16.1-33.1 IU/L), cholesterine (4.3-4.5 mmol/L), FFA (105-166 µmol/L) and ß-hydroxybutyrate (BHB) (0.3-0.6 mmol/L) from the beginning and the end of the experiment are within the reference values given by Kraft and Dürr (2005). The values of glucose (3.7-3.9 mmol/L), Urea (3.2-5.9 mmol/L) and creatinine (µmol/L) are near the reference values. No treatment effects were observed in the blood parameters at the beginning of the adaptation period and at the end of the sampling period.

DISCUSSION

P-requirements for milk synthesis represent the largest proportion of the total P amount required by lactating dairy cows. If the milk performance rises, an increasing amount of P must be fed, but a P-concentration higher than 4 g/kg DM is, even at high performance levels, not necessary (Brintrup et al., 1993, Wu et al., 2000, Valk et al., 2002). The P-concentration of the P+MIN diet was calculated to represent the commonly used diet for a dairy cow with a milk yield of about 30 kg/d. Therefore mineral P was supplied to this diet. The TMR of group P-MIN (3.46 g P/kg DM) was intended to include the native P of the feedstuffs, only. Thus this group has a 20 percent lower P-concentration than the diet of the

P+MIN group (3.98 g P/kg DM). The animals of group P+PHY received a concentrate similar to group P-MIN (3.26 g P/kg DM), but supplemented with an experimental phytase.

The feed ingredients including corn silage, corn, soybean meal and dried sugar beet pulp contributed to the comparatively low dietary P as compared with other diets commonly used for dairy cows. The P-concentration of the corn silage was on average 2.72 g/kg DM and the mean P-concentration of the concentrate was 4.49 g/kg DM. With 59-70 percent phytate P (IP-6/5/4/3/2/1) in total P, the part of phytate P is higher than half of the total P in the diets and should be available for hydrolysis catalyzed by phytase.

Milk yield was unaffected by the dietary treatments. A similar result was reported by Kincaid et al. (2005) and by Yang et al. (1997) who found no differences in milk yield, when the P-content of TMR was 0.45%.

Commonly, a P-concentration in milk of about 0.9 g/kg milk is indicated in the literature (Brintrup et al., 1993, Knowlton and Herbein, 2002, Pfeffer et al., 2005). Nevertheless, there are variations between experiments with values ranging from 0.7 to 1.1 g/kg milk (Pfeffer and Hristov, 2005). Reference values for P in milk (g/kg) of lactating Holstein cows over the complete lactation are between 0.85-0.94 g P/kg milk (Brintrup et al., 1993, Wu et al., 2000, Valk et al., 2002) and for early- and mid-lactation between 0.68 and 0.89 g P/kg milk (Knowlton et al., 2001, Knowlton and Herbein, 2002). In the present trial the values ranged between 0.88 and 0.91 g P/kg milk and 1.09 and 1.12 g Ca/kg milk. They are similar to the mean P-and Ca-concentration of 0.9 and 1.1 g/kg milk given by Pfeffer et al. (2005). The present results show and Pfeffer and Hristov (2005) concluded that the P-intake has no influence on the P-excretion with milk. It should be still recognized, that the current study contradicts experiments conducted by Coates and Ternouth (1992) with cattle.

With regard to the P-recommendations, it is conspicuous that the excretion of P with milk is assumed between 0.9 and 2.0 g P per kg milk (GfE, 2001, NRC, 2001, INRA, 2002, Beyer et al., 2004, CVB, 2005, Schlegel, 2011). Bearing these recommendations in mind, the values out of the present study are just below values given by the GfE (2001) even though the P-intake was comparatively high. This confirms the statement that P-intake with feed has no influence on P-concentration in milk and supports the previous studies of Wu et al. (2001). Considering the actual study, it would be possible to decrease the value for P-concentration in milk to 0.9 g per kg milk, like other recommendations already did (NRC, 2001, INRA, 2002). Moreover the actual results shows that the presumption of 2 g P per kg milk (Beyer et al.,

2004) is overestimated. The reduction of P-concentration in milk in the recommendations of Schlegel (2011), CVB (2005), Beyer et al. (2004) and GfE (2001) from 2 resp. 1 g to 0.9 g P per kg milk could help to reduce the faecal P-concentrations without it would be harmful for the health of the cows.

Overfeeding of dietary P is common in the diets for dairy cows. P is often fed to dairy cattle 20 to 40 percent in excess of published requirements (Sink et al., 2000, Knowlton et al., 2004). P fed in excess of the requirements is excreted and a reduction of overfeeding could be a powerful tool to reduce the P-concentration in faeces (Knowlton et al., 2004). Pfeffer and Hristov (2005) observed correlations between faecal P-excretion and DMI indicating that per kg DMI 0.88 g P was inevitable lost in faeces of goats. Similar to Pfeffer and Hristov (2005) other authors found the direct line between P-intake and P-excretion in dairy cows (Morse et al., 1992, Wu et al., 2000, Knowlton et al., 2001, Wu et al., 2001, Knowlton and Herbein, 2002, Knowlton et al., 2004). In the current study P-excretion with faeces was similar to a study conducted by Coates and Ternouth (1992). In this study, faecal P-excretion was unaffected by either the dietary P-intake or the added exogenous phytase fed to cows. Dvorakova (1998) determined the pH-value, temperature, moisture and time of incubation in the rumen and ileum as relevant factors affecting the digestibility of P. Consequently, caused by the high feed intakes in high-producing dairy cows, ruminal passage-rates are rapid, which limits the time for hydrolysis in the rumen (Garikipati, 2004). In the current study, this assumption cannot be proved, thus there was no lower P-excretion with faeces of the cows fed the phytase supplementation.

The total tract apparent digestibility of P and Ca is described differently in the literature. While Wu et al. (2000) presents interactions between the P-intake and P-digestibility, Silva Filho et al. (1992) determined missing interactions between these parameters. In accordance with Silva Filho et al. (1992) who studied the effect of dietary P-concentrations on P-metabolism in *Bos indicus*, in the current study no effect of P-intake on P-digestibility was observed. In contrast, the Ca-digestibility which showed a significantly influenced by P-intake. The group with the highest P-intake (P+MIN) was able to digest about 15 percentage points more Ca compared with the other groups.

P and Ca retention for all dietary treatments was positive. However, the P+MIN group showed a higher P- (P=0.004) and Ca-balance (P<0.001) compared with both other groups. This means that more P and Ca were retained in the body of the animals than in the P-MIN and P+PHY groups. In the current study, a higher mineral P intake enabled the cows to retain

more P and Ca. Valk et al. (2002) found comparable results for lactating dairy cows even at a lower P-intake level. P and Ca could be stored in bones, teeth, soft tissues and blood. Because there were no differences in the P-concentration in blood e.g. Figure 1, it can be assumed that bones or soft tissues would have shown differences between the groups.

When comparing the retention of the P, it can be noticed that the group P+MIN had the highest P-retention. This higher value can be explained by the used P-supplement. The group P+MIN received inorganic P supplements while the diets of both other groups contained only native P. The comparison between the group P-MIN and P+MIN showed that there is a difference of 11g/d in the P-intake and of 10 g/d in the P-balance. 91% of the supplemented P in group P+MIN were utilized. The comparison of group P+PHY and P+MIN showed that the intake of the groups differed about 13g/d and the balance about 9 g/d. Consequently 69% of the supplemented P in group P+MIN were utilized.

The concentrations of P and Ca in serum were not affected either by the exogenous phytase or by the different P-intake. According to literature data Hadzimusic (2011) summarized that the reference values of P in blood of cows are between between 1.4 and 2.5 mmol/L (Merck, 2008). The range of reference values for Ca in blood serum of lactating cows is between 2.1 to 3.1 mmol/L (Jovanovic et al., 1997, Radostits et al., 2000, Kraft and Dürr, 2005, Kaneko, 2008, Merck, 2008, Hadzimusic, 2011). The values of Ca and P in this experiment are below or respectively near the minimum level of the reference values e.g. **Figures 1 and 2**.

Morse et al. (1992), Knowlton et al. (2001) and Wu et al. (2001) investigated that there is a relationship between the intake and output of minerals. The present analytic values for P- and Ca-balance support this statement that the P-intake has an influence on the P-balance. The desired positive effect of phytase with regard to a better P-digestibility did not occur. In accordance with Kiarie and Nyachoti (2010) and under the conditions examined the supply of phytase could not reduce the excretion of non-digestible P. Brask-Pedersen et al. (2013) figured out, that a higher supply of exogenous phytase increases the ruminal degradation of inositol P_6 (IP_6). The supply of phytase increases the ruminal phytase activity. The phytase concentration in the diets of the current study are nearly similar to the high concentrated group of the study by Brask-Pedersen et al. (2013). However the actual study shows no phytase activity in the rumen. Against this back ground, the question arises if a higher amount of phytase supplementation could have resulted in differences between the treatments. In previous studies ruminal and total-tract degradations of IP_6 were higher when exogenous phytase was added to the TMR. Degradation of IP_6 occurred mainly in the rumen as the

content of IP_6 was lower in the duodenum samples in animals fed phytase (Brask-Pedersen et al., 2013). Bearing this in mind, it could be advantageous to examine the pH-value and temperature in the rumen to prove that the phytase can work efficiently. Dvorakova (1998) determined that the rated range 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 phytase used in their study was 5 to 5.5. The lower pH-optimum of the phytase can constitute a reason that in the current study the phytase has no influence on the P-retention, in so far that the pH-value of the rumen of a dairy cow is higher than 5.5. Under conditions as presented here, it makes more sense to determine the lower limits for the P-supply of the different ruminants and different performances than to use the tested phytase in this quantity. Furthermore research is needed to determine the effect of incubation time, passage rate, pH-value and phytase activity in the rumen and duodenal chyme.

CONCLUSION

Dietary phytase supplementation in dairy cows fed different amounts of P showed no effect on P-excretion with faeces and urine as well as the P-balance. The results of the experiment indicate that the addition of the tested phytase had no influence on the P-excretion in milk. The reduction of P concentration in milk in the recommendations from 2 resp. 1g to 0.09g per kg milk could help to reduce the faecal P-concentration without it would be harmful for the health of the cows.

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REFERENCES

Beyer, M., Chudy, A., Hoffmann, L., Jentsch, W., Laube, W., Nehring, K., & Schiemann, R., (2004). *Rostocker Futterbewertungssystem*. Rostock, Germany: Jentsch, W., A. Chudy and M. Beyer.p.392.

Brask-Pedersen, D. N., Glitso, L. V., Skov, L. K., Lund, P., & Sehested, J. (2011). Effect of exogenous phytase on feed inositol phosphate hydrolysis in an in vitro rumen fluid buffer system. *Journal of Dairy Science.* 94(2). 951-959.10.3168/jds.2010-3504.

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. *Journal of Dairy Science.* 96(3). 1691-1700.DOI 10.3168/jds.2011-5278.

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. *Journal of the Science of Food and Agriculture.* 91(8). 1398-1405.Doi 10.1002/Jsfa.4324.

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. *Journal of Animal Physiology and Animal Nutrition-Zeitschrift Fur Tierphysiologie Tierernahrung Und Futtermittelkunde.* 69(1). 29-36.

Coates, D. B., & Ternouth, J. H. (1992). Phosphorus Kinetics of Cattle Grazing Tropical Pastures and Implications for the Estimation of Their Phosphorus Requirements. *Journal of Agricultural Science.* 119. 401-409.

CVB. (2005). Centraal Veevoederbureau. Voedernormen landbouwhuisdieren en voederwaarde veevoeders. .

Dvorakova, J. (1998). Phytase: Sources, preparation and exploitation. *Folia Microbiologica.* 43(4). 323-338.

Eeckhout, W., & De Paepe, M. (1994). Total Phosphorus, Phytate-Phosphorus and Phytase Activity in Plant Feedstuffs. *Animal Feed Science and Technology.* 47(1-2). 19-29.Doi 10.1016/0377-8401(94)90156-2.

Engelen, A. J., Vanderheeft, F. C., Randsdorp, P. H. G., & Smit, E. L. C. (1994). Simple and Rapid-Determination of Phytase Activity. *Journal of Aoac International.* 77(3). 760-764.

Garikipati, D. (2004). Effect of exogenous phytase addition to diets on phytate phosphorus digestibility in dairy cows. Masterthesis, Master of Science In Animal Science, Washington State University.

GfE, (2001). *Gesellschaft für Ernährungsphysiologie. Empfehlungen zur Energie- und Nährstoffversorgung der Milchkühe und Aufzuchtrinder*.

Gizzi, G., Thyregod, P., von Holst, C., Bertin, G., Vogel, K., Faurschou-Isaksen, M., Betz, R., Murphy, R., & Andersen, B. B. (2008). Determination of phytase activity in feed: Interlaboratory study. *Journal of Aoac International.* 91(2). 259-267.

Godoy, S., & Meschy, F. (1999). Utilisation of phytic P by rumen bacteria in a semi-continuous culture system (rusitec). *South African Journal of Science.* 29. 148-149.

Guyton, A. D., McKinney, J. M., & Knowlton, K. F. (2003). The effect of steam-flaked or dry ground corn and supplemental phytic acid on phosphorus partitioning and ruminal phytase activity in lactating cows. *Journal of Dairy Science.* 86(12). 3972-3982.

Hadzimusic, N. J. K. (2011). Values of Calcium, Phosphorus and Magnesium Concentrations in Blood Plasma of Cows in Dependence on the Reproductive Cycle and Season. *Journal of the Faculty of Veterinary Medicine, Istanbul University* 38. 1-8.

INRA, (2002). *Institut National de la Recherche Agronomique. Tables de l´alimentation des ruminants* (Vol. 2): Institut National de la Recherche Agronomique.

Jovanovic, J. M., Rajic, I., Pesterac, V., Crcev, D., & Cokrevski, S. (1997). Parametri krvi visoko steonih I tek oteljenih krava hranjenih obrocima razlicitog sastava. *Veterinarski Glasnik.* 51. 231-244.

Kaneko, J. J., (2008). *Carbohydrate Metabolism and Its Diseases* (Vol. 6.th edition). New York: Academic Press.p.64..

Kiarie, E., & Nyachoti, C. M., (2010). *Bioavailibilty of Calcium and Phosphorus in Feedstuffs for Farm animals*. United Kingdom: CABI publishing, Oxfordshire.p.76-93.

Kienzle, E., (1991). *Ernährung und Urolithiasis bei Haussäugetieren* (Vol. 19).p.157-200.

Kincaid, R. L., Garikipati, D. K., Nennich, T. D., & Harrison, J. H. (2005). Effect of grain source and exogenous phytase on phosphorus digestibility in dairy cows. *Journal of Dairy Science.* 88(8). 2893-2902.

Kincaid, R. L., & Rodehutscord, M., (2005). *Phosphorus Metabolism in the rumen*. United Kingdom: CABI Publishing, Cambridge.p.189-191..

Knowlton, K. F., & Herbein, J. H. (2002). Phosphorus partitioning during early lactation in dairy cows fed diets varying in phosphorus content. *Journal of Dairy Science.* 85(5). 1227-1236.DOI 10.3168/jds.S0022-0302(02)74186-6.

Knowlton, K. F., Herbein, J. H., Meister-Weisbarth, M. A., & Wark, W. A. (2001). Nitrogen and phosphorus partitioning in lactating Holstein cows fed different sources of dietary protein and phosphorus. *Journal of Dairy Science.* 84(5). 1210-1217.

Knowlton, K. F., Radcliffe, J. S., Novak, C. L., & Emmerson, D. A. (2004). Animal management to reduce phosphorus losses to the environment. *Journal of Animal Science.* 82. 173-195.

Knowlton, K. F., Taylor, M. S., Hill, S. R., Cobb, C., & Wilson, K. F. (2007). Manure nutrient excretion by lactating cows fed exogenous phytase and cellulase. *Journal of Dairy Science.* 90(9). 4356-4360.Doi 10.3168/Jds.2006-879.

Kraft, W., & Dürr, U. M., (2005). *Klinische Labordiagnostik in der Tiermedizin*. Stuttgart/New York.

McCarthy, J. F., Aherne, F. X., & Okai, D. B. (1974). Use of Hcl Insoluble Ash as an Index Material for Determining Apparent Digestibility with Pigs. *Canadian Journal of Animal Science.* 54(1). 107-109.

Merck. (2008). In Co., M. (Ed.), *Merck veterinary manual* (Vol. 8th ed.). Inc Whitehause Station, NJ, USA..

Morse, D., Head, H. H., & Wilcox, C. J. (1992). Disappearance of Phosphorus in Phytate from Concentrates Invitro and from Rations Fed to Lactating Dairy-Cows. *Journal of Dairy Science.* 75(7). 1979-1986.

NRC. (2001). National Research Council, Nutrient requirements of dairy cattle. 7th revised edition ed. National Academy of Science, Washington D.C. .

Pfeffer, E., Beede, D. K., & Valk, H., (2005). *Phosphorus Metabolism in Ruminants and Requirements of Cattle*. United Kingdom: CABI publishing, Cambridge.p.195-231..

Pfeffer, E., & Hristov, A. N., (2005). *Nitrogen and P nutrition of cattle, reducing the environmental Impact of cattle operations*. United Kingdom: CABI Publishing, Cambridge.p.208-219..

Radostits, O. M., Blood, D. C., & Gay, C. C., (2000). *A textbook of the diseases of cattle, sheep, goats and horses* (Vol. 8th edition). London.

Rodehutscord, M. (2008). Approaches for saving limited phosphate resources. *Archiv Fur Tierzucht-Archives of Animal Breeding.* 51. 39-48.

Schlegel, P., ALP. (2011). *Phosphorempfehlung für die Milchkuh*. Paper presented at the ALP Tagung 2011. .

Selle, P. H., Walker, A. R., & Bryden, W. L. (2003). Total and phytate-phosphorus contents and phytase activity of Australian-sourced feed ingredients for pigs and poultry. *Australian Journal of Experimental Agriculture.* 43(5). 475-479.Doi 10.1071/Ea02155.

Silva Filho, J. C., Lopes, H. O. S., Meirelles, C. F., Vitti, D. M. S. S., & Abdalla, A. L. (1992). Absorcao real de fosforo do fosfato bicalcico, fosfato monoamonio, superfosfato triplo e do fosfato de ureia em bovinos. . *Pesquisa Agropecuaria Brasileira.* 35. 1861-1865.

Sink, S. E., Knowlton, K. F., & Herbein, J. H. (2000). Economic and environmental implications of overfeeding phosphorus on Virginia Dairy Farms. *Journal of Animal Science.* 78 (Suppl.2)(4).

Sunvold, G. D., & Cochran, R. C. (1991). Technical Note - Evaluation of Acid Detergent Lignin, Alkaline Peroxide Lignin, Acid Insoluble Ash, and Indigestible Acid Detergent Fiber as Internal Markers for Prediction of Alfalfa, Bromegrass, and Prairie Hay Digestibility by Beef Steers. *Journal of Animal Science.* 69(12). 4951-4955.

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.

Journal of Dairy Science. 85(10). 2642-2649.DOI 10.3168/jds.S0022-0302(02)74349-X.

VDLUFA. (1997). Die chemische Untersuchung von Futtermitteln. Ergänzungslieferungen von 1983, 1988, 1992, 1997, 2004, 2006, 2007. *VDLUFA-Methodenbuch.* 3.

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. *Journal of Dairy Science.* 84(7). 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. *Journal of Dairy Science.* 83(5). 1028-1041.

Wünsche, J., Borgmann, E., Henning, U., Kreienbring, F., & Bock, H. D. (1984). Application of the HCl-insoluble ash as an indicator for the determination of nutrient including amino-acid digestibility at the end of the small intestine and the whole digestive-tract of pigs. *Archive of Animal Nutrition.* 34. 817-831.

Yang, W. Z., Beauchemin, K. A., Farr, B. I., & Rode, L. M. (1997). Comparison of barley, hull-less barley, and corn in the concentrate of dairy cows. *Journal of Dairy Science.* 80(11). 2885-2895.

Yanke, L. J., Bae, H. D., Selinger, L. B., & Cheng, K. J. (1998). Phytase activity of anaerobic ruminal bacteria. *Microbiology-Uk.* 144. 1565-1573.

Zimmermann, B., Lantzsch, H. J., Mosenthin, R., Schoner, F. J., Biesalski, H. K., & Drochner, W. (2002). Comparative evaluation of the efficacy of cereal and microbial phytases in growing pigs fed diets with marginal phosphorus supply. *Journal of the Science of Food and Agriculture.* 82(11). 1298-1304.Doi 10.1002/Jsfa.1190.

5. Paper II

Effect of exogenous phytase on the P- and Zn-metabolism of fattening bulls fed a corn silage based diet

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ABSTRACT

This study investigated the effects of exogenous phytase, different dietary phosphorus (P) and zinc (Zn) levels on the P- and Zn-metabolism of fattening bulls.

48 German Holstein bulls (average initial live weight 312 ± 49 kg) were used for a feeding trial and allocated to four dietary treatments, P/Zn, P+MIN/Zn, P/Zn+PHY and P/Zn+MIN. All bulls received a diet of 80 % corn silage and 20% concentrate on a dry matter (DM) basis. The corn silage intake was for ad libitum and the concentrate intake restricted.

The concentrate of P+MIN/Zn was supplemented with dicalcium phosphate. The concentrate of the P/Zn+PHY group was supplemented with an exogenous phytase (0.1 g/kg DM in the diet, 50 000 FTU/g) and the concentrate of the P/Zn+MIN group was supplemented with Zn. The P- and Zn-concentration in the diet of the P/Zn-, P+MIN/Zn-, P/Zn+PHY- and P/Zn+MIN-groups were 2.41, 2.99, 2.48 and 2.41 g P/kg DM and 33.2, 33.6, 34.0 and 38.8 mg Zn/kg DM, respectively.

No differences in P- and Zn-concentration of faeces, liver, testes and performance were observed between the treatments. In the *Os metacarpale*, P- and phytase supplementation resulted in a slightly higher P-concentration, while the Zn addition led to the lowest value (P=0.062). Overall, it becomes clear that the microbial phytase of the rumen is sufficient enough to make the indigestible phytate-P and the hardly digestible Zn digestible for ruminants. The supplemented dietary phytase has no influence on the bioavailability of the mineral P and the trace element Zn.

Keywords: Phytase, Ruminants, P-metabolism, Zn-metabolism

INTRODUCTION

Phytase releases phosphorus (P) from inositol phosphate (InsP) by hydrolysis (Suttle, 2010). P from phytate is suggested to be highly available to ruminants because of the microbial phytase activity in the rumen (Clark et al., 1986, Morse et al., 1992). However, ruminal P-excretion with faeces seems to be linear to P-concentration in the diet of ruminants (Call et al., 1987, Suttle, 2010). Although ruminants generally have the ability to use phytin-bound phosphorus through ruminal hydrolysis, different studies showed that the dietary supplementation of exogenous phytase leads to reduced faecal excretion and an increased P-concentration in bones. However, not only P is bound to phytin but also trace elements such

as zinc (Zn) are known to be released by the action of exogenously added phytase in monogastric animals (Garikipati, 2004). If such effects occur in ruminants is not known. Additionally phytase mediated release of these trace elements might increase their bioavailability.

Zn deficiency in the animals may occur due to different reasons. The first one is the primary deficiency because of an inadequate content in the diet of the animal. The secondary deficiency is caused by other elements that might hamper the Zn-absorption (Adeola et al., 1995). Because of this in the actual study it has to be taken into account that metal cations, such as Zn form insoluble complexes with phytate, which decrease the enzymatic rate of hydrolysis by phytase (Garikipati, 2004). P-supplementation might result in Zn-deficiency symptoms as high concentrations of phytate in diets may lead to insoluble complexes of phytic-acid and Zn which are poorly available (Kirchgessner et al., 1994). Kornegay (2001) also described the influence of phytase on the Zn bioavailability in pig, poultry and fish diets and emphasized that Zn is closely connected to the phytate complex. Another important aspect of Zn in the feeding is that Zn is indispensable for the fertility of cattle (Van Laar and Jongbloed, 2010). A study with rats showed that Zn is important for the spermatogenesis and that testes exhibit a high concentration of Zn. Feeding rats with a low Zn diet resulted in lower weight of testes, epididymis and dorsal prostate (Millar et al., 1958). Not only rats were affected by Zn-deficiency, but also male goats showed reductions in testicular size and loss of libido (Neathery et al., 1973). Previous studies (Millar et al., 1958, Barney et al., 1968, Neathery et al., 1973) comprise investigations in rats and goats but not in bulls.

Thus one aim of the present experiment was to elucidate the effects of exogenous phytase, Zn and P on P- and Zn-status of growing bulls. Therefore it is appropriate to examine the P- and Zn-levels in liver, testes and bones to consider this when the results are evaluated. A further important point is to investigate the effect on the P- and Zn-concentration of faeces of the bulls. Do the animals differ in their P- and Zn-concentration if reduced or higher amounts of minerals are fed and if there is an exogenous phytase in the feed?

For this reason, a P- and Zn-reduced diet should be compared to a full supplied diet and a P- and Zn-reduced diet added with phytase. Feeding these four different treatments, it becomes possible to figure out if a supplemented enzyme is able to compensate a deficiency by splitting up more indigestible P-and Zn-complexes than the rumen microbes do, and to figure out if phytase supplementation in combination with a reduced level of P and Zn-intake has effects on the performance and the P and Zn-concentration in faeces and bones.

MATERIAL AND METHODS

Animals and experimental design

The experiment with 48 German Holstein bulls 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 animals were randomly assigned based on age and body weight to one of four experimental groups of 12 animals each. At the beginning of the trial, the bulls had a mean body weight of 312 ± 49 kg. The bulls were housed in a thermally non-isolated stable and were kept in pens (8 animals per pen, each pen two animals per group) on slatted floor. The body weight was measured weekly with cattle weighing scales.

The experimental design included different P- and Zn-concentrations and phytase supplementation in the feed. The intended supply of P and Zn is related to the recommendations given by the GfE (1995). The feeding group P/Zn, P/Zn+PHY and P/Zn+MIN achieved 80%, P+MIN/Zn achieved 100% of the intended P-concentration given by GfE (1995). Feeding group P/Zn, P+MIN/Zn and P/Zn+PHY got 80%, P/Zn+MIN 100% of the intended Zn-concentration given by GfE (1995). The diet of the experimental group P/Zn+PHY received additionally 0.1 g experimental phytase per kg DM.

The experimental diets differed in the concentration of P, Zn and phytase e.g. **Table 1**. The P/Zn-group received a basal diet with a P-concentration of approximately 2.4 g/kg dry matter (DM) and a Zn-concentration of approximately 32 mg/kg DM to cover approximately 80% of the present P and Zn recommendations (GfE, 1995) without phytase supplementation. The P+MIN/Zn-group was fed the same diet added with P covering the P-demand of about 2.97 g/kg DM. The P/Zn+PHY-group obtained the control diet added with an experimental phytase. According to the manufacturer´s specifications the phytase had an activity of minimum 50.000 FYT/g. The quantity of the enzyme added to the concentrate amounted to 100 g/t DM. The P/Zn+MIN-group received the control diet with Zn-supplementation according to the recommendation (40 mg/kg DM) (GfE, 1995).

Table 1. Experimental design and realized concentrations

	P/Zn[1]	P+MIN/Zn[2]	P/Zn+PHY[3]	P/Zn+MIN[4]
Intended concentration				
P (g/kg DM)	2.4	2.97	2.3	2.4
Zn (mg/kg DM)	32	32	32	40
Phytase (g/kg DM)	-	-	0.1*	-
Intended supply related to the recommendations given by the GFE (1995)				
P (%)	80	100	80	80
Zn (%)	80	80	80	100
Realised concentration				
P (g/kg DM)	2.41	2.99	2.48	2.41
Zn (mg/kg DM)	33.2	33.6	34.0	38.8
Phytase (g/kg DM)	-	-	0.1*	-
Realised percentage related to the recommendations given by the GFE (1995)				
P (%)	79	100	86	82
Zn (%)	83	84	85	97

* The phytase had an activity of min. 50 000 FYT/g according to the manufacturer´s specifications.

FYT: one FYT is the amount of enzymes that liberates 1µmol of inorganic P per minute from an excess of Na phytate at pH 5.5 and 37°C

[1] diet with native P and Zn content

[2] diet with supplemental mineral P and native Zn content

[3] diet with native P and Zn content, added with phytase

[4] diet with native P content and supplemental mineral Zn

The components of the concentrates used during the trial are shown in e.g. **Table 2**. Changes in mineral content were achieved by changes in the mineral premix in the concentrates.

Table 2. Components, mean nutrient, fibre and energy of the concentrates and corn silage of the experimental diets

	Corn silage	C-P/Zn[1]	C-P+MIN/Zn[2]	C-P/Zn+PHY[3]	C-P/Zn+MIN[4]
Components of the concentrates (g/kg DM)					
Wheat gluten		100	100	100	100
Corn		300	300	300	300

Dried sugar beet pulp	483.7	483.7	483.7	483.7
Calcium carbonate	36	16	36	36
Dicalcium phosphate	-	20	-	-
Soybean oil	12	12	12	12
Urea	30	30	30	30
Premix Control*	8.3	-	-	-
Premix with P†	-	8.3	-	-
Premix with phytase ‡	-	-	8.3	-
Premix with Zn§	-	-	-	8.3
Mineral premix [a] (without P and Zn)	30	30	30	30

Nutrient, fibre and energy content of the concentrates and corn silage (g/kg DM)

Organic matter	961	949	950	949	949
Crude ash	39	51	50	51	51
Crude protein	82	118	115	119	117
Ether extract	38	33	34	33	34
Crude fibre	184	166	168	166	167
ADF	208	190	192	190	191
NDF	403	372	374	371	372
P	2.33	2.85	5.59	2.93	2.76
Zn	0.02	0.13	0.14	0.14	0.18
IP-6[5]	#	0.67	0.47	0.67	0.68
IP-5[5]	#	0.02	0.05	0.03	0.04
IP-1/2/3/4[5]	#	#	#	#	#
ME (MJ/kg DM)	10.7	11.9	11.9	11.9	11.9

[1] concentrate with native P and Zn content

[2] concentrate with supplemental mineral P and native Zn content

[3] concentrate with native P and Zn content, added with phytase

[4] concentrate with native P content and supplemental mineral Zn

[5] Inositol Phosphate

*/† 98.76 % Corn and 1.24 % Zinc sulphate monohydrate (35% Zinc in Zinc sulphate monohydrate)

‡ 93.97 % Corn, 1.23 % Zinc sulphate monohydrate and 4.8 %, Experimental phytase (phytase activity amounted to 50 000 FYT/g)

§ 97.6 % Corn and 2.4 % Zinc sulphate monohydrate

out of detection limit

[a] Composition (per kg): 560.000 IU vitamin A (E672), 70.000 IU vitamin D3 (E671), 1.050 mg vitamin E (alpha tocopherolacetat), 3.000 mg manganese (manganese (II) sulphate, monohydrate E5, 700 mg copper, 50 mg iodine (calcium jodate, water-free E2), 25 mg cobalt (cobalt sulphate, monohydrate, E3), 30 mg selenium (sodium selenate E8)

The animals were fed with restricted amounts of concentrate (2 kg/d) and corn silage for *ad libitum* intake.

The concentrate was provided via feeding stations (Type AWS HF 2ST, manufacturer: Insentec, Marknesse, The Netherlands). Water was available for *ad libitum* intake during the whole experiment. Because corn silage and concentrates were fed separately the added phytase was not exposed to moisture and was therefore assumed to be inactive prior to feeding.

The diets were intended to cover the energy and protein requirements according to the recommendations of the Society of Nutrition Physiology (GfE 1995).

Measurements and Sampling Procedure

Individual silage and water intake were recorded continuously by an automatic feeding system (RIC, manufacturer Insentec B.V., Marknesse, The Netherlands). The body weight was measured weekly with cattle weighing scales. During the experiment, representative samples of the diets were collected regularly. Silage and concentrate samples were collected twice a week and weekly, respectively. After collecting, the samples were stored at -18°C and pooled over a 4 weeks period.

Additionally, once during the experiment, samples of faeces were taken from every fourth bull of every treatment group for P-, Zn- and acid insoluble ash-analysis.

The bulls were slaughtered at an average live weight of 578 kg at the slaughtering house of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute, FLI, Braunschweig. At the day of slaughter samples of *Os metacarpale,* liver and testes were taken and stored frozen at -20°C until further analysis.

Analyses

Feedstuff samples were dried at 60°C for 72 hours and ground to pass a 1-mm screen. The samples of *Os metacarpale*, testes and liver were freeze dried for the determination of P

and Zn. After freeze drying, the samples were ground in mortars and after that passed through a spiral screw mincer.

Samples of feedstuff and faeces were analyzed according to the methods of the Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA 1997). Analysis of acid and neutral detergent fibre (ADF resp. NDF) was conducted following methods of VDLUFA (1997). P and Zn in feedstuff, bones, testes liver and faeces were analyzed by an optical emissions spectrometer with inductive coupled plasma (ICP-OES) according to VDLUFA (1997).

The digestibility of Zn and P was estimated by acid insoluble ash (AIA) as marker (Sunvold and Cochran, 1991). AIA in feed and faeces was analyzed with an adapted 4N HCl-method based on the method described by Wünsche et al (1984) and McCarthy et al. (1974). A total of 2 to 5 g of freeze dried faeces or feed were ashed. The ignition of the samples lasted 5 hours at a temperature of 550°C. The ashes were boiled for 15 minutes with 4N HCl and the residues were filtered through an ashless filter paper. After drying the filters with the residues, they were ashed again to obtain the amount of AIA. Inositol-P was analysed in all feedstuff samples using high-performance ion chromatography according to the method of Brejnholt et al. (2011).

Calculations

The apparent total tract digestibility (AD) of P and Zn was estimated by acid insoluble ash as a marker occurring naturally in the diet as follows:

$$AD\ [\%] = ([(M_{diet}/AIA_{diet})-(M_{faeces}/AIA_{faeces})]/M_{diet}/AIA_{diet}) * 100,$$

where M_{diet} is the mineral content (P, Zn) in g/kg DM and AIA_{diet} is the AIA content in g/kg DM in the feed. M_{faeces} is the mineral content (P, Zn) in g/kg DM and AIA_{faeces} the AIA content (g/kg DM) in faeces.

The energy content of the diets was calculated based on table values given by the DLG (1997).

The phytase activity for the corn silage is calculated according to tabulated values of Eeckhout and De Paepe (1994). This results in a phytase activity for corn silage of 12 FYT/kg DM.

Empty Body weight is defined as the body weight without the content of the rumen, intestine, gall and urinary bladder. Dressing percentage was calculated as quotient of warm carcass weight and body weight.

Statistical analysis

The statistical analysis was carried out with the SAS-software package Version 9.1.3 using the MIXED-procedure (SAS Institute, Cary, NC, USA 2004).

Treatment group was assumed to be the fixed effect. The fact that each bull was used for frequent measurements was considered using a "REPEATED" statement for the individual animal effect. 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 in form of least square means (LS means) and standard error (SE) of the mean. Effects are considered as significant with a $P< 0.05$.

RESULTS

Chemical composition of the feedstuffs

The chemical composition of the concentrates is shown in e.g. **Table 1**. As intended the P and Zn-concentration of the groups manifested variations.

The proportion of phytate-P (IP-6/5/4/3/2/1) of the total P of the concentrates amounted to 24%, 24% and 26% phytate-P in the P/Zn-, P/Zn+PHY- and P/Zn+MIN-group. Because of the supplementation of inorganic P in the P+MIN/Zn-group, the percentage of phytate-P was lower and amounted to 9%.

The concentrated feed of group P/Zn, P+MIN/Zn and P/Zn+MIN showed no detectable phytase activity, while the feed of the P/Zn+PHY group showed a phytase activity of 5859±15 FYT/kg.

Performance

Corn silage intake amounted on average to 6.65 kg DM/d. No differences were observed in corn silage intake across the treatments. The mean concentrate intake was 1.75 ± 0.03 kg DM/d and ranged from 1.72 to 1.78 kg DM/d e.g. Table 3. As intended the mean P-intake was significantly higher in the P+MIN/Zn-group with 26.2 g/d and the mean Zn-intake was significantly higher in the P/Zn+MIN-group with 0.32 g/d, as compared to the other groups.

Table 3. Mean feed and nutrient intakes during the experimental period (LS-means, SE)

	P/Zn[1]	P+MIN/Zn[2]	P/Zn+PHY[3]	P/Zn+MIN[4]	*SE	*P*-value
Animals/group	12	12	12	12		

DM intake (kg/d)	8.1	8.8	8.2	8.3	0.27	0.072
Corn silage (kg DM/d)	6.4	7.1	6.5	6.6	0.32	0.442
Concentrate (kg DM/d)	1.74	1.74	1.78	1.72	0.02	0.060
Crude protein (kg/d)	0.95	1.01	0.98	0.97	0.03	0.421
Ether extract (kg/d)	0.27	0.29	0.28	0.28	0.01	0.449
ADF (kg/d)	1.53	1.68	1.57	1.58	0.07	0.432
NDF (kg/d)	3.00	3.28	3.05	3.08	0.13	0.423
P (g/d)	19.6[b]	26.2[a]	20.3[b]	20.1[b]	0.65	<0.001
Zn (mg/d)	270[a]	294[b]	280[ab]	321[c]	6.5	<0.001
ME intake (MJ/d)	89.1	93.7	90.6	89.9	3.4	0.791

[a,b,c] Different letters in one row show significant differences (P<0.05)

[*] Standard Error

[1] diet with native P and Zn content

[2] diet with supplemental mineral P and native Zn content

[3] diet with native P and Zn content, added with phytase

[4] diet with native P content and supplemental mineral Zn

The average live weight gain did not differ between the treatments e.g. **Table 4**. The average live weight gain during the whole experiment was 1360±84 g/d. In the P/Zn- and P/Zn+PHY-group the daily live weight gain was numerically higher at the beginning of the experiment, while the bulls of the P+MIN/Zn- and P/Zn+MIN-group had a numerically higher daily live weight gain from day 101 until the end of the experiment. The average live weight gain of all groups was for the period from day 0-100 1381±64 g/d. It was 1345±107 g/d for day 101 to the end of trial. Mean feed per gain ratio (kg DM/kg LWG) was 6.03±0.15 kg DM/kg LWG, until day 101 and 6.78±0.24 kg DM/kg LWG from day 101 until the end. For the whole period mean feed per gain was 6.30±0.19 kg DM/kg LWG. Overall means for energy intake per gain were 66±2 MJ ME/kg LWG for day 1-100, 74±3 MJ ME/kg LWG for day 100-end and for the whole period 69±2 MJ ME/kg LWG.

Table 4. Live weight gain (LWG) and feed efficiency subjected to the feeding groups (n=12) (LS-means, SE)

	P/Zn[1]	P+MIN/Zn[2]	P/Zn+PHY[3]	P/Zn+MIN[4]	[*]SE	P-value
Animals/group	12	12	12	12		

Daily live weight gain (g/d)						
day 1-100	1455	1413	1321	1334	165	0.824
day 101-end	1284	1474	1234	1387	154	0.417
Total period	1379	1438	1275	1348	83	0.265
Feed per gain (kg DM/kg LWG)						
day 1-100	5.87	5.93	6.20	6.11	0.5	0.914
day 101-end	6.74	6.70	7.11	6.55	0.6	0.840
Total period	6.14	6.25	6.57	6.23	0.3	0.611
Energy intake per gain (MJ ME/kg LWG)						
day 1-100	64.8	63.4	68.3	66.3	5.7	0.844
day 101-end	74.4	71.7	78.3	71.2	6.9	0.713
Total period	67.8	66.9	72.4	67.7	3.5	0.376

* Standard Error

[1] diet with native P and Zn content

[2] diet with supplemental mineral P and native Zn content

[3] diet with native P and Zn content, added with phytase

[4] diet with native P content and supplemental mineral Zn

Twenty four out of the 48 bulls were slaughtered. Six bulls out of every feeding group were sampled. Mean body weight of the slaughtered bulls was of 581±8 kg, while the dressing percentage amounted to 56±1 %. Considering the treatments, body weight, empty body weight, retroperitoneal fat and dressing did not show any significant differences e.g. **Table 5**.

Table 5. Body weights of the bulls at slaughter and carcass characteristics (n=6) (LS-means, SE)

	P/Zn[1]	P+MIN/Zn[2]	P/Zn+PHY[3]	P/Zn+MIN[4]	*SE	*P*-value
Animals/group	6	6	6	6		
Body weight (kg)	577	584	577	584	24	0.984
EBW† (kg)	514	525	516	519	22	0.959
Fat of pelvic cavity and kidneys (g)	2365	2052	2371	2087	238	0.391
Dressing‡ (%)	55.5	56.4	55.6	55.9	0.90	0.760

* Standard Error

† Empty Body weight is defined as the body weight less the content of the rumen, intestine, gall and urinary bladder

‡ Dressing is calculated as carcass weight as percentage of body weight at slaughter

[1] diet with native P and Zn content

[2] diet with supplemental mineral P and native Zn content

[3] diet with native P and Zn content, added with phytase

[4] diet with native P content and supplemental mineral Zn

The weight of the spleen tended to be lower in the P/Zn- and P/Zn+PHY-group than in the P/Zn+MIN-group and the weight of the heart tended to be lower in the P/Zn+PHY- as compared to the P/Zn+MIN-group e.g. **Table 6**. The treatments had no influence on the weight of lung, liver, kidneys, testes, pancreas, prostate and thyroid gland.

Table 6. Weight of different organs at slaughter (g per 100 kg LWG) depending on the diet (n=6) (LS-means, SE)

	P/Zn[1]	P+MIN/Zn[2]	P/Zn+PHY[3]	P/Zn+MIN[4]	*SE	*P*-value
Animals/group	6	6	6	6		
Heart	420	425	406	392	9	0.082
Liver	1357	1360	1348	1314	30	0.689
Kidneys	225	202	222	202	9	0.138
Testes	174	169	176	175	10	0.950
Lung	650	663	620	743	38	0.156
Spleen	190	195	180	216	9	0.062
Pancreas	55	70	74	73	7	0.228
Prostate	59	70	58	70	10	0.746
Thyroidgland	6	7	6	6	1	0.855

[a, b] Different letters in one row show significant differences (P<0.05)

[*] Standard Error

[1] diet with native P and Zn content

[2] diet with supplemental mineral P and native Zn content

[3] diet with native P and Zn content, added with phytase

[4] diet with native P content and supplemental mineral Zn

P and Zn in faeces

There were no significant differences between the treatments, but the P+MIN/Zn-group showed the numerically highest P-concentration e.g. **Table 7**.

All four groups together showed a mean P-concentration of faeces of 4.6±0.9 g/kg DM. The averaged Zn-concentration of the faeces samples was 102±8 mg/kg DM.

P and Zn-digestibility

The digestibility of P and Zn showed no significant differences between the treatments e.g. **Table 7**. All four groups together showed a mean P digestibility of 48 percent and a mean Zn digestibility of 16.3 percent.

Table 7. P- and Zn- concentration in faeces (g/kg DM), excretion with faeces (g/d) and -digestibility depending on the diet (n=3) (LS means, SE)

	P/Zn[1]	P+MIN/Zn[2]	P/Zn+PHY[3]	P/Zn+MIN[4]	*SE	*P*-value
Concentration (g/kg DM)						
P	4.1	5.6	3.9	4.5	0.60	0.218
Zn	0.1	0.1	0.1	0.1	0.01	0.452
Excretion (g/d)						
P	9.0	11.9	10.6	10.2	1.77	0.718
Zn	0.04	0.04	0.04	0.08	0.04	0.786
Digestibility (%)						
P	49.0	47.7	48.9	50.2	6	0.994
Zn	14.1	12.3	13.9	24.8	13	0.889

* Standard Error

[1] diet with native P and Zn content

[2] diet with supplemental mineral P and native Zn content

[3] diet with native P and Zn content, added with phytase

[4] diet with native P content and supplemental mineral Zn

Analysis of organs

There were no differences in P- and Zn-concentration in liver and testes e.g. **Table 8**. In *Os metacarpale* the P-concentration tended to be influenced by the treatments (*P*=0.062). The animals of the P+MIN/Zn-group showed the highest P-concentration in bones (95.9 g/kg DM) while the P/Zn+MIN-group had the numerically lowest value (90.0 g/kg DM) e.g. **Table 8**.

Table 8. P- and Zn-concentration of liver, testes and *Os metacarpale* (n=6) (LS-means and SE)

	P/Zn[1]	P+MIN/Zn[2]	P/Zn+PHY[3]	P/Zn+MIN[4]	*SE	*P*-value
Animals/group	6	6	6	6		

Liver						
P (g/kg DM)	10.7	10.5	10.3	10.1	0.3	0.181
Zn (mg/kg DM)	104	97	103	93	6	0.247
Testes						
P (g/kg DM)	6.1	5.5	6.2	5.4	0.9	0.756
Zn (mg/kg DM)	41.5	36.7	39.9	35.4	5	0.641
Os metacarpale						
P (g/kg DM)	92.6	95.9	95.1	90.0	2	0.062
Zn (mg/kg DM)	57.9	60.4	57.3	58.0	3	0.657

* Standard Error

[1] diet with native P and Zn content

[2] diet with supplemental mineral P and native Zn content

[3] diet with native P and Zn content, added with phytase

[4] diet with native P content and supplemental mineral Zn

DISCUSSION

The P- and Zn-reduced diet should be compared to P or Zn supplemented diets and a P- and Zn-reduced diet added with phytase. Feeding these four different diets, it should be possible to figure out if the supplemented enzyme is able to compensate the deficiency by splitting up more indigestible P- and Zn-complexes than the rumen microbes do. The intended P-concentrations in the diets of group P/Zn, P+MIN/Zn, P/Zn+PHY and P/Zn+MIN as well as the desired difference of 20% between the groups with or without P- and Zn-supplementation were almost achieved. The feed ingredients including corn silage, corn, wheat gluten and dried sugar beet pulp contributed to the comparatively low dietary P-concentration. The P-concentration of the corn silage was on average 2.33 g/kg DM and the mean P-concentration of the unsupplemented concentrates was 2.85 g/kg DM. With 24 to 26 % phytate P (sum of InsP-6/5/4/3/2/1) of total P of concentrates of the unsupplemented groups, the current study supports the data of Maenz (2001). Maenz (2001) investigated the occurrence of phytic acid in plants and found that cereals and grain legumes that are commonly used as feed ingredients all have similar phytate levels. The content of phytate P in the concentrate of the P-supplemented group was only 9%. This can be explained with the supplementation of inorganic P in the P+MIN/Zn-group, while the P content of the other groups is native.

The requirement of Zn is met when the diet contains 40 mg Zn/kg dry matter intake (DMI) for bulls (GfE, 1995). The P- and Zn-reduction of group P/Zn+PHY to 86 respectively 85% related to the recommendations given by the GfE (1995) should be compensated by the effect of the supplemented exogenous phytase. Brask-Pedersen et al. (2011) and Garikipati (2004) found, that exogenous phytase can influence the availability of P positively. As Zn is probably the most vulnerable mineral to phytate complexation (Kornegay, 2001) it seems possible that exogenous phytase could decrease Zn-excretion with faeces, too.

But in the actual study, there are no differences visible between the P/Zn- and P/Zn+PHY-group. Phytase showed neither an effect on the P and Zn-excretion with faeces nor on the P and Zn digestibility of the animals.

Geisert et al. (2010) investigated the relationship between P requirement and excretion of finishing beef cattle fed different concentrations of P. Geisert et al. (2010) did not find differences in the DMI depending on P-intake. In contrast, in the present study there was a tendency towards a higher feed intake of the P+MIN/Zn group (P=0.072). The DMI of this group was 0.5 to 0.7 kg per day higher compared with the other groups. Like in the study of Geisert et al. (2010) there were no differences in DMI depending on P-intake. The observation that feed intake was not affected by Zn-treatment is in agreement with Khan (1978) and Mandal et al. (2007), where feed intake of growing calves and bulls was unaffected by an increase of dietary Zn-concentration of 2.5 mg Zn/kg DM. Supplementations of Zn methionine to a diet containing more than 25 mg Zn/kg DM did not affect feed intake in ewes (Salama Ahmed, 2003), goats (Puchala et al., 1999), growing lambs (Droke et al., 1998), beef steers (Duff et al., 2000) and bulls (Mandal et al., 2007). Mandal et al. (2007) investigated diets for growing bulls with a Zn-concentration of 32 and 35 mg Zn/kg DM, respectively. That complies with 80% and 87.5% of the recommendations given by the GfE (1995). The different Zn-concentrations did not affect the intake and balance of minerals like Ca and P in bulls. In accordance with Mandal et al. (2007) in the present study no effect of Zn-treatment on P-intake, P-excretion and P-digestibility was detected, while Zn-supplementation to feed results as desired in higher Zn-intake in group P/Zn+MIN. Contrary to Mandal et al. (2007), the current study showed no influence of Zn-supplementation on Zn-excretion with faeces and the Zn-digestibility.

Fattening bulls excrete the main part with faeces and only 1% of total P- excretion with urine (Klosch et al., 1994, Vitti et al., 2000). Klosch et al. (1994) investigated that the faecal P-excretion of fattening bulls is between 0.71 and 1.10 g/kg DMI. Calculating the faecal P-

excretion of the animals during the current study considering these assumptions, the P-excretion should amount from 5.75 to 9.7 g/d. In the actual study the analyzed P-excretion with faeces ranges from 9.0 g to 11.9 g/d. Consequential the faecal P-excretion was between 1.0 and 1.6 g/kg DMI. While Geisert et al. (2010) observed a significant effect of P-intake on the faecal P-excretion in a study with steers, the P+MIN/Zn-group of the actual study showed a tendency towards a higher P-excretion compared with the other groups (P=0.085). Both results indicate that faecal losses were related to P-intake. This was also confirmed by studies with dairy cattle (Braithwaite, 1985, Scott and Buchan, 1985, Ternouth, 1989, Khorasani et al., 1997, Valk et al., 2002).

To get an impression of the P retention in body, the P-balance was calculated. The P-excretion with urine was assumed to be 1% of total P-excretion and consequently amounts to 0.1 g/d. Taking the P-excretion with faeces e.g. **Table 7** and the calculated P-excretion with urine into account, P-balances are as follows: 10.4, 14.2, 9.6 and 9.8 g/d for groups P/Zn, P+MIN/Zn, P/Zn+PHY and P/Zn+MIN. Winter et al. (2013) investigated the P-balance of lactating cows fed comparable diets. The study resulted in P-balances between 16.2 and 26.4 g/d. The difference between the studies can be explained with the gender and the age of the animals.

Compared to the P-excretion, the Zn-excretion showed a similar pattern. While Mandal et al. (2007) found significantly higher Zn-excretion with faeces and urine of Zn-supplemented groups, in the actual experiment with fattening bulls, these results could not be confirmed.

The skeleton is the main P-storage (Fernandez, 1995, Pfeffer et al., 2005). 80% of whole body P is stored in bones, while the remaining part is contained in tissues and fluids (Kirchgessner et al., 1994). Taking this fact and the P-balance in the actual study into account, a quantity of 8.3, 11.4, 7.7 and 7.8 g P/d is stored in the bones (groups P/Zn, P+MIN/Zn, P/Zn+PHY and P/Zn+MIN). The P-concentration of bones is different, depending on the particular type of bone. Results of investigations by Williams et al. (1991) indicate that chemical and physical properties of bovine bones are sensitive to dietary P. Erickson et al. (2002) studied the effects of dietary P-concentration on quantity and route of P-excretion and P-concentration in the bones of cattle feed finishing diets over 180 days. They concluded like Shupe et al. (1988), Ternouth (1989) and Geisert et al. (2010) that the metacarpal bone of cattle could be an indicator of mineral status. The ash of *Os metacarpale* showed an average P content of 17.3%. In the actual study, the phytase as well as the P-supplementation to a diet low in P tended to increase the P-concentration in *Os metacarpale* (P=0.062). Therefore, it can be

ascertained that there seems to be a potential of the tested phytase to replace mineral P-supplementation under P deficient conditions.

An interesting aspect of the present study is the liver with its Zn binding protein, metallothioneins (MTs). MTs have the ability of releasing Zn, when necessary (Tapiero and Tew, 2003). For this reason the question arises if the Zn-concentration in the liver increases linear to the Zn-supplementation of the diet. Cao et al. (2000) analyzed the Zn-concentration of the liver of growing lambs depending on the dietary Zn-intake and ascertained that the Zn-concentration of the liver increased linearly with the Zn-concentration of the diet. Wright and Spears (2004) studied the effect of different Zn sources and different amounts of supplementation on the Zn-concentration in the liver and bones of calves. They found that the Zn-concentration in the liver and bones rose linearly with the Zn-intake. In contrast to these results, no relation between the dietary Zn-intake and Zn-concentration in the liver and bones were observed in the present study. An explanation for the differences between calves and fattening bulls could be a more effective homeostatic control mechanism of older animals which is responsible for the regulation of the Zn content of liver tissue (Kincaid et al., 1997).

In the actual study, the P- and Zn-concentration of the testes was not influenced by the dietary treatment. These results were in line with the studies of Wright et al. (2004) and Cao et al. (2000) who found similar effects. There was no effect of dietary treatment on the weight of testes, too. Foote et al. (1977) observed a positive relation between the number of sperms and the size of scrotum. Amstutz (1979) detected a positive relation between the size of scrotum and the motility of sperms in ejaculate of bulls. Furthermore density of ejaculate is sensitive to external parameters like genetic and feed. In addition there is evidence that a Zn supplementation could have a positive influence on the fertility of bulls.

The logical effect of this study is that the microbial phytase of the rumen is sufficient enough to make the indigestible phytate-P and the hardly digestible Zn digestible for ruminants.

CONCLUSIONS

In the present study a supplementation of phytase has no influence on the feed intake, live weight gain, feed efficiency, slaughter characteristics and P and Zn digestibility. Only the P-concentration of *Os metacarpale* tended to be increased for animals fed a P deficient diet supplemented with phytase. Under the conditions of the present study the microbial phytase in

the rumen appears to be sufficient to make the phytate bound P and the hardly digestible Zn available for the animals.

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REFERENCES

Adeola, O., Lawrence, B. V., Sutton, A. L., & Cline, T. R. (1995). Phytase-Induced Changes in Mineral Utilization in Zinc-Supplemented Diets for Pigs. *Journal of Animal Science.* 73(11). 3384-3391.

Amstutz, H. E. (1979). Bull Infertility. *Modern Veterinary Practice.* 60(1). 52-53.

Barney, G. H., Orgebinc.Mc, & Macapinl.Mp. (1968). Genesis of Esophageal Parakeratosis and Histologic Changes in Testes of Zinc-Deficient Rat and Their Reversal by Zinc Repletion. *Journal of Nutrition.* 95(4). 526.

Braithwaite, G. D. (1985). Endogenous Fecal Loss of Phosphorus in Growing Lambs and the Calculation of Phosphorus Requirements. *Journal of Agricultural Science.* 105(Aug). 67-72.

Brask-Pedersen, D. N., Glitso, L. V., Skov, L. K., Lund, P., & Sehested, J. (2011). Effect of exogenous phytase on feed inositol phosphate hydrolysis in an in vitro rumen fluid buffer system. *Journal of Dairy Science.* 94(2). 951-959.10.3168/jds.2010-3504.

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. *Journal of the Science of Food and Agriculture.* 91(8). 1398-1405.Doi 10.1002/Jsfa.4324.

Call, J. W., Butcher, J. E., Shupe, J. L., Lamb, R. C., Boman, R. L., & Olson, A. E. (1987). Clinical Effects of Low Dietary Phosphorus Concentrations in Feed Given to Lactating Dairy-Cows. *American Journal of Veterinary Research.* 48(1). 133-136.

Cao, J., Henry, P. R., Guo, R., Holwerda, R. K., Toth, J. P., Littell, R. C., Miles, R. D., & Ammerman, C. B. (2000). Chemical characteristics and relative bioavailability of supplemental organic zinc sources for poultry and ruminants. *Journal of Animal Science.* 78(8). 2039-2054.

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. *Journal of Dairy Science.* 69(12). 3151-3155.

DLG, D. U. H., (1997). *DLG Futterwerttabellen Wiederkäuer (Feed value tables for ruminants)* (Vol. 7th). Frankfurt am Main, Germany: DLG-Verlag.

Droke, E. A., Gengelbach, G. P., & Spears, J. W. (1998). Influence of level and source (inorganic vs organic) of zinc supplementation on immune function in growing lambs. *Asian-Australasian Journal of Animal Sciences.* 11(2). 139-144.

Duff, G. C., Walker, D. A., Malcolm-Callis, K. J., Wiseman, M. W., & Hallford, D. M. (2000). Effects of preshipping vs arrival medication with tilmicosin phosphate and feeding chlortetracycline on health and performance of newly received beef cattle. *Journal of Animal Science.* 78(2). 267-274.

Eeckhout, W., & De Paepe, M. (1994). Total Phosphorus, Phytate-Phosphorus and Phytase Activity in Plant Feedstuffs. *Animal Feed Science and Technology.* 47(1-2). 19-29.Doi 10.1016/0377-8401(94)90156-2.

Erickson, G.E., T.J. Klopfenstein, M.W.Orth, D. Brink, and K.M.nWhittet.2002. Phosphorus requirementsof finishing steer calves. *Journal of Animal Science,* In press

Fernandez, J. A. (1995). Calcium and Phosphorus-Metabolism in Growing Pigs .2. Simultaneous Radio-Calcium and Radio-Phosphorus Kinetics. *Livestock Production Science.* 41(3). 243-254.

Foote, R. H., Seidel, G. E., Hahn, J., Berndtson, W. E., & Coulter, G. H. (1977). Seminal Quality, Spermatozoal Output, and Testicular Changes in Growing Holstein Bulls. *Journal of Dairy Science.* 60(1). 85-88.

Garikipati, D. (2004). Effect of exogenous phytase addition to diets on phytate phosphorus digestibility in dairy cows. Masterthesis- Master of Science in Animal Science, Washington State University.

Geisert, B. G., Erickson, G. E., Klopfenstein, T. J., Macken, C. N., Luebbe, M. K., & MacDonald, J. C. (2010). Phosphorus requirement and excretion of finishing beef cattle fed different concentrations of phosphorus. *Journal of Animal Science.* 88(7). 2393-2402.10.2527/jas.2008-1435.

GfE, G. f. E., (1995). *Empfehlungen zur Energie- und Nährstoffversorgung der Mastrinder* (Vol. 6).

Khan, S. A. (1978). *Interaction of cooper and zinc and its influence on the metabolism of major nutrients in growing calves.* . Ph.D., Nuslim University, Aligarh, India.

Khorasani, G. R., Janzen, R. A., McGill, W. B., & Kennelly, J. J. (1997). Site and extent of mineral absorption in lactating cows fed whole-crop cereal grain silage or alfalfa silage. *Journal of Animal Science.* 75(1). 239-248.

Kincaid, R. L., Chew, B. P., & Cronrath, J. D. (1997). Zinc oxide and amino acids as sources of dietary zinc for calves: Effects on uptake and immunity. *Journal of Dairy Science.* 80(7). 1381-1388.

Kirchgessner, M., Heindl, U., & Schwarz, F. J. (1994). Content and Deposition of Trace-Elements in Various Tissues and in the Empty Body of Growing German Simmental Bulls. *Journal of Animal Physiology and Animal Nutrition-Zeitschrift Fur Tierphysiologie Tierernahrung Und Futtermittelkunde.* 72(4-5). 260-271.

Klosch, M., Ranft, S., Richter, G. H., & Flachowsky, G. (1994). Effect of Phosphorus Repletion after P-Poor Feeding on Feed Intake, Live Weight Gain and Selected Parameters of Metabolism in Fattening Bulls. *Deficient and Excessive Levels of Macroelements and Trace Elements in Nutrition*. 680-685.

Kornegay, E. T., (2001). *Digestion of Phosphorus and other Nutrients: the Role of Phytases and Factors Influencing their Activity*. United Kingdom: CABI Publishing p.252..

Maenz, D. D., (2001). *Enzymatic Characteristics of Phytase as they Relate to their Use in Animal Feeds* (Vol. 1). UK: CABI Publishing.p.61-84.

Mandal, G. P., Dass, R. S., Isore, D. P., Garg, A. K., & Ram, G. C. (2007). Effect of zinc supplementation from two sources on growth, nutrient utilization and immune response in male crossbred cattle (Bos indicus X Bos taurus) bulls. *Animal Feed Science and Technology.* 138(1). 1-12.DOI 10.1016/j.anifeedsci.2006.09.014.

McCarthy, J. F., Aherne, F. X., & Okai, D. B. (1974). Use of Hcl Insoluble Ash as an Index Material for Determining Apparent Digestibility with Pigs. *Canadian Journal of Animal Science.* 54(1). 107-109.

Millar, M. J., Fischer, M. I., Elcoate, P. V., & Mawson, C. A. (1958). The Effects of Dietary Zinc Deficiency on the Reproductive System of Male Rats. *Canadian Journal of Biochemistry and Physiology.* 36(6). 557.

Morse, D., Head, H. H., & Wilcox, C. J. (1992). Disappearance of Phosphorus in Phytate from Concentrates Invitro and from Rations Fed to Lactating Dairy-Cows. *Journal of Dairy Science.* 75(7). 1979-1986.

Neathery, M. W., Miller, W. J., Blackmon, D. M., Pate, F. M., & Gentry, R. P. (1973). Effects of Long-Term Zinc Deficiency on Feed Utilization, Reproductive Characteristics, and Hair Growth in Sexually Mature Male Goat. *Journal of Dairy Science.* 56(1). 98-105.

Pfeffer, E., Beede, D. K., & Valk, H., (2005). *Phosphorus Metabolism in Ruminants and Requirements of Cattle*. United Kingdom: CABI publishing, Cambridge.p.195-231.

Puchala, R., Sahlu, T., & Davis, J. J. (1999). Effects of zinc-methionine on performance of Angora goats. *Small Ruminant Research.* 33(1). 1-8.Doi 10.1016/S0921-4488(98)00194-1.

Salama Ahmed, A. K., Cajat, G., Albanell, E., Snch, X., Casals, R. (2003). Effects of dietary supplements of zinc-methionine on milk production, udder helth and zinc metabolism in dairy goats. *Journal of Dairy Research.* 70. 9-17.

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

Scott, D., & Buchan, W. (1985). The Effects of Feeding Either Roughage or Concentrate Diets on Salivary Phosphorus Secretion, Net Intestinal Phosphorus Absorption and Urinary Phosphorus Excretion in the Sheep. *Quarterly Journal of Experimental Physiology and Cognate Medical Sciences.* 70(3). 365-375.

Shupe, J.L., J.E. Butcher, J.W. Call, A.E. Olson, and J.T. Blake.1988. Clinical signs and bone changes associated with phosphorus deficiency in beef cattle. *Am. J.Vet Res.* 49:19129-16136.

Sunvold, G. D., & Cochran, R. C. (1991). Technical Note - Evaluation of Acid Detergent Lignin, Alkaline Peroxide Lignin, Acid Insoluble Ash, and Indigestible Acid Detergent Fiber as Internal Markers for Prediction of Alfalfa, Bromegrass, and Prairie Hay Digestibility by Beef Steers. *Journal of Animal Science.* 69(12). 4951-4955.

Suttle, N. F. (2010). Minerals in livestock production. *Asian-Australasian Journal of Animal Sciences.* 13. 1-9.

Tapiero, H., & Tew, K. D. (2003). Trace elements in human physiology and pathology: zinc and metallothioneins. *Biomedicine & Pharmacotherapy.* 57(9). 399-411.Doi 10.1016/S0753-3322(03)00081-7.

Ternouth, J. H. (1989). Endogenous Losses of Phosphorus by Sheep. *Journal of Agricultural Science.* 113. 291-297.

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. *Journal of Dairy Science.* 85(10). 2642-2649.DOI 10.3168/jds.S0022-0302(02)74349-X.

Van Laar, H., & Jongbloed, A. W. (2010). Effect of zinc and copper on health and fertility in cattle *Wageningen UR Livestock Research, Partner in livestock innovations*. Wageningen UR: Livestock Research.

VDLUFA. (1997). Die chemische Untersuchung von Futtermitteln. Ergänzungslieferungen von 1983, 1988, 1992, 1997, 2004, 2006, 2007. *VDLUFA-Methodenbuch.* 3.

Vitti, D. M. S. S., Kebreab, E., Lopes, J. B., Abdalla, A. L., De Carvalho, F. F. R., De Resende, K. T., Crompton, L. A., & France, J. (2000). A kinetic model of phosphorus metabolism in growing goats. *Journal of Animal Science.* 78(10). 2706-2712.

Williams, S. N., Lawrence, L. A., Mcdowell, L. R., Wilkinson, N. S., & Warnick, A. C. (1991). Phosphorus concentrations in blood, milk, feces, bone and selected fluids and tissues of growing heifers as affected by dietary phosphorus. *Livestock Research for rural Development.* 3(2).

Winter, L., Meyer, U., Spolders, M., Hüther, L., Lebzien, P., & Dänicke, S. (2013). Effect of exogenous phytase on the phosphorus balance of lactating cows fed a corn based diet. *Journal of Biology and Life Science.* 4(2). 232-250.DOI: 10.5296/jbls.v4i2.3697.

Wright, C. L., & Spears, J. W. (2004). Effect of zinc source and dietary level on zinc metabolism in Holstein calves. *Journal of Dairy Science.* 87(4). 1085-1091.

Wünsche, J., Borgmann, E., Henning, U., Kreienbring, F., & Bock, H. D. (1984). Application of the HCl-insoluble ash as an indicator for the determination of nutrient including amino-acid digestibility at the end of the small intestine and the whole digestive-tract of pigs. *Archive of Animal Nutrition.* 34. 817-831.

6. Paper III

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 (VFA) were not different between the treatments. The HP-group shows a higher P-flow at the duodenum than both 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.

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

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.

MATERIAL 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 DM/d as given by the 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 (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**.

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

Variable	Corn Silage	Concentrate HP[§]	Concentrate LP[^]	Concentrate 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				
Organic matter	955	915	917	924
Crude Protein	83	258	254	256
Ether extract	32	28	27	26
Acid detergent fibre	263	112	118	120
Neutral detergent fibre	501	265	269	280
Minerals				
P	2.74	4.89	1.88	1.96
$InsP_6$	-	0.57	0.39	0.53

[°] Composition (per kg): 200 g calcium, 120 g sodium, 40 g magnesium, 1,000,000 U vitamin A (E672), 100,000 U vitamin D3 (E671), 1500 mg vitamin E (alpha tocopherolacetate), 5.4 g manganese (manganese (II)sulphate, monohydrate E5), 6 g zinc (zincoxide E6), 1 g copper (copper sulphate pentahydrate E4), 100 mg iod (calcium jodate, waterfree E2), 40 mg selenium (Sodium Selenate E8), 25 mg cobalt (cobalt sulphate, monohydrate, E3)

[#]Composition (per kg): 0.952 kg corn grain added with 0.048 kg experimental phytase

[§]Concentrate added with dicalcium phosphate

[^]Concentrate without supplementation

[$]Concentrate without P-supplementation, but added with an exogenous phytase

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 German Society of Nutrition Physiology (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 handmixed 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 am and 5:15 pm into the rumen beginning ten days before the beginning of 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 am; 11:45 am, 5:45 pm and 11:45 pm.

Analysis

Samples of feedstuffs, refusals, duodenal chyme and faeces were analyzed according to the methods of the Association of German Agricultural Analytic and Research Institute (VDLUFA, 1997). Samples were dried at 60°C for 72 hours and ground through a 1-mm screen. Analysis of acid (ADF) and neutral 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 units (FTU)/kg feed (Engelen et al., 1994). Phosphorus in diet, milk, urine and faeces was analyzed 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 analyzed 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 analyzed 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, 3400 Hillerød, Denmark).

Volatile fatty acids (VFA) in rumen fluid were analysed using a gas chromatograph (HP5890II, Hewlett Packard, 71034 Böblingen, Germany) equipped with an automatic injector (HP7673 II, Hewlett Packard, 71034 Böblingen, Germany), a flame ionization detector and an integrator (HP 3396 II). For sample preparation samples were centrifuged at 40,000 g at 4 °C. 1 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 (NPGS) and two percent ortho-phosphoricacid (Analyt, 79379 Mühlheim, Germany) was used for separation of VFA. Flow rates of the flame ionization detector combustion gases hydrogen and synthetic air were 30 and 420 ml/min, respectively. N 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 analyzed according to DIN 38406-E5-2 (Anonymous, 1998). The following analyses were carried out in the freeze dried and ground duodenal samples. The DM and ash contents of duodenal chyme were analyzed 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, GBC Scientific Equipment Pty Ltd, 3195 Victoria, Australia) 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 analyzed by the same methods as the feedstuff.

Calculations

The metabolizable 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), digestible organic matter (DOM) was obtained from the digestion trial.

GE [MJ] = 0.0239 x g CP+0.0398 x g EE+ 0.0201 x g CF+0.0175 x 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 x *q*) x ME (MJ), where *q* is ME/GE.

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

FCM [kg/d] = ((% milk fat x 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 dry matter 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 utilizable 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 x 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 x kg DMF) x 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 x microbial N [kg/d].

Total tract digestibility was calculated with the following equations:

Total tract digestibility [%] =

((nutrient intake [g/d] – nutrient in feaces [g/d])/ nutrient intake [g/d]) x 100

The ME of the diets was calculated using the results from the sampling period and resulted in 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 NEL (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 Institute, 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 means (LS-means) ± standard error (SE). Effects are graded as significant with $P < 0.05$, a trend was considered if $P < 0.10$ and $P \geq 0.05$.

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 requires according to the GfE recommendations 3.3 g P/kg DM in the diet. This content is equal to the analysed content in the HP diet. In the LP and the LP+PHY-group 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.

Table 2. Nutrient intakes by the fistulated cows during the sampling period (LS-means, ±SE)

	Experimental diets			
	HP°	LP#	LP+PHY§	*P*-value
Animals/group	7	5	5	
Intakes, kg/d				
Dry matter	13.6±0.15	13.3±0.18	13.6±0.18	0.414
Organic matter	12.7±0.13	12.6±0.15	12.8±0.15	0.709
Crude protein	1.8±0.17	1.8±0.20	1.8±0.20	0.602
Ether extract	0.4±0.01	0.4±0.01	0.4±0.01	0.335
Nitrogen	0.3±0.003	0.3±0.003	0.3±0.003	0.602
Acid detergent fibre	3.0±0.05	3.0±0.06	3.1±0.06	0.337
Neutral detergent fibre	6.0±0.09	5.8±0.11	6.1±0.11	0.318

°diet added with dicalcium phosphate

#diet without P and phytase supplementation

§diet without P-supplementation, but added with an exogenous phytase

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 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 between the three treatments. The acetic acid to propionic acid ratio was about 2.2 in the study and did not differ between the treatments.

Table 3. Effects of supplemental P and phytase on rumen fermentation parameters in dairy cows (LS-means, ±SE)

	Experimental diets			
	HP°	LP#	LP+PHY§	*P*-value
Animals/group	7	5	5	
pH-value	6.7±0.1	6.5±0.1	6.7±0.1	0.242
NH_3-N^ (mg/100g)	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

°diet added with dicalcium phosphate

#diet without P and phytase supplementation

§diet without P-supplementation, added with an exogenous phytase

^ Ammonia Nitrogen

$ Volatile Fatty Acids

Treatments had no effect on the amount of fermented organic matter and the portion of organic matter 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)**.

Table 4. Effects of supplemental P and phytase on nutrient and P-flows at the duodenum and amount of fermented organic matter in the rumen (LS-means, ±SE)

	Experimental diets			
	HP°	LP#	LP+PHY§	*P*-value
Animals/group	7	5	5	
Organic matter				
(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
Neutral detergent fibre				
(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
Acid detergent fibre				
(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
Fermented organic matter				
(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

[a,b] Different letters in one row show significant differences (P<0.05)

[°]diet added with dicalcium phosphate

[#]diet without P and phytase supplementation

[§]diet without P-supplementation, but added with an exogenous phytase

The supplementation of feed with P and phytase had no effects on nitrogen flow at the duodenum, the rumen degradable and undegradable protein and the microbial protein synthesis **(Table 5)**.

Table 5. Effects of supplemental P and phytase on nitrogen flow at the duodenum as well as microbial protein synthesis and feed protein degradation in the rumen (LS-means, SE)

	Experimental diets			
	HP[°]	LP[#]	LP+PHY[§]	*P*-value
Animals/group	7	5	5	
Nitrogen (g/d)	228±10	223±12	219±11	0.775
Non-ammonia nitrogen (g/d)	216±8.9	212±10.9	210±10.1	0.855
Microbial crude protein				
(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 crude protein)	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
Utilizable crude protein (g/d)	1177±51.7	1142±62.5	1127±58.3	0.679

[°]diet added with dicalcium phosphate

[#]diet without P and phytase supplementation

[§]diet without P-supplementation, but added with an exogenous phytase

[^]ruminal nitrogen balance

There were no differences between 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 between 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)**.

Table 6. Effects of supplemental P and phytase on nutrient and P concentration of faeces, P- and N-concentration of urine in dairy cows as well as apparent total tract digestibility (LS-means, SE)

	Experimental diets			
	HP[°]	LP[#]	LP+PHY[§]	*P*-value
Animals/group	7	5	5	
Faeces (g/kg DM)				
Organic matter	899±4.0	888±4.8	901±4.8	0.097
Crude protein	132±2.4	130±2.8	135±2.8	0.280
Ether extract	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 (g/kg DM)				
P	0.10±0.03	0.02±0.03	0.04±0.03	0.180
N	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 (%)				
Organic matter	69±1.4	68±1.6	68±1.6	0.589
Ether extract	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

[°]diet added with dicalcium phosphate

[#]diet without P and phytase supplementation

[§]diet without P-supplementation, but added with an exogenous phytase

Milk yield amounted to 20.6 ± 0.2 kg/d on average. No differences were observed in milk yield and milk composition between the different treatments **(Table 7)**. The mean P concentration in milk was 0.90 g P/kg milk and did not differ between the groups. The SCC showed no differences between the groups.

Table 7. Effects of supplemental P and phytase on milk production and composition in dairy cows (LS-means, SE)

	Experimental diets			
	HP°	LP#	LP+PHY§	*P*-value
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				
Fat (g/d)	750±51.7	746±61.1	774±55.8	0.931
Protein (g/d)	556±35.9	570±42.1	559±38.4	0.949
Lactose(g/d)	978±80.1	975±95.6	988±86.5	0.993
Urea (ppm)	115±19.0	132±21.6	117±20.0	0.696

°diet added with dicalcium phosphate

#diet without P and phytase supplementation

§diet without P-supplementation, but added with an exogenous phytase

As intended by the experimental design, the P-intake differed between the groups. Group LP and LP+PHY had nearly the same intake (33.5 resp. 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 of the other groups ($P=0.057$) **(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 ($P=0.014$). The 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%.

Table 8. Mean P-intake and P secretion with milk, P secretion with faeces, urine as well as P-balance of the dairy cows during the sampling period (LS-means, SE)

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

[a, b] Different letters in one row show significant differences ($P<0.05$)

°diet added with dicalcium phosphate

#diet without P and phytase supplementation

§diet without P-supplementation, but added with an exogenous phytase

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 increases 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 indicated that the supply of phytase increases the ruminal phytase activity. The phytase concentration in the diets of the current study were similar to the highly supplemented 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 indicated that $InsP_6$ degradation and absorption of released P in the duodenum as a result of ruminal phytase activity was 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 was 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 were 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 Schöder 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 was 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 (GfE, 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 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 between 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 utilized. The desired positive effect of phytase with regard to a higher P-digestibility and P-balance did not occur.

CONCLUSION

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.

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REFERENCES

Anonymous, 1998 DIN 38406-E5-2. Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung [German unitymethods for water, sewage and sludge analysis]. Berlin, Germany: Beuth Verlag GmbH.

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. Anim. Nutr. 46:39-48.

Brask-Pedersen DN, Glitso LV, Skov LK, Lund P and 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 Agric. 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. Anim. Nutr. 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. Technol. 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. Anim. Nutr. 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 Agricultural Experiment Station Bulletin 308, pp 401-438.

GfE, 2001. Empfehlungen zur Energie- und Nährstoffversorgung der Milchkühe und Aufzuchtrinder, DLG Verlag ed., 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. and Rodehutscord M. 2005 Phosphorus metabolism in the rumen. In Nitrogen and Phosphorus Nutrition of Cattle, Eds E Pfeffer and A Hristov, Wallingford, UK: CABI Publishing, 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. Technol. 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 *Enzymes in Farm Animal Nutrition*, Eds MR Bedford and GG Partridge, Wallingford, UK: CABI Publishing, 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 Nitrogen and Phosphorus Nutrition of Cattle, Eds E. Pfeffer and A. Hristov, Wallingford, UK: CABI Publishing, 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 Institute, 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. Anim. Nutr.50:34-35.

Suttle, N.F., 2010. Mineral Nutrition of Livestock. (4th Edition). Wallingford, UK: CABI Publishing.

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). Vol III. Die chemische Untersuchung von Futtermitteln. VDLUFA-Verlag ed., 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.

7. General Discussion

The aim of the present study was to investigate the effects of exogenous phytase and different dietary P-levels on the P-excretion with faeces. Furthermore the effect on the P-concentration of duodenal chyme, urine, milk, and the P-balance of dairy cows and fattening bulls, as well as the effects on P-concentration of bone, liver and testes of fattening bulls should be investigated. The core of this study was to identify, if it is possible to decrease P-excretion through faeces to the environment by using exogenous phytase and to conclude whether there are differences between the three experiments.

P is essential for the metabolism (Puggaard et al., 2011) and microbial phytase makes P available for the ruminal metabolism. Taking into account, that the functionality of phytase is sensitive to many parameters (Winter et al., 2013), the present study investigates lactating cows (**Experiment 1**) as well as fattening bulls (**Experiment 2**). To figure out the effects of phytase on ruminal parameters, a study with ruminal- and duodenal-fistulated cows (**Experiment 3**) was also carried out.

Although cows and fattening bulls are of the same species, there are differences between their P-metabolism processes, mainly due to the fact that cows secrete large amounts of P with the milk while bulls are growing. Another important aspect is the age of the animals. While the pluriparous lactating cows are in their averagely 2.8 respectively 3.6 lactation and fully grown, the fattening bulls are still in the state of growth. Their live weight increased from, on average, 312 kg to 581 kg during the study. There are two methods for calculating P-recommendation for the experimental animals. The net requirement for the lactating cows takes the inevitable losses (g/d), the P secretion with milk (g/d), intrauterine deposition (g/d), accretion during growth (g/d) and the digestibility (%) into account, while the requirement for fattening bulls is calculated with inevitable losses (g/d), accretion during growth (g/d) and digestibility (%) (GfE, 1995, GfE, 2001). That implies that the daily quantity of P fed to the bulls is less, with an average 21.5 g/d being fed (Paper II) than the average 77 g/d was fed to the dairy cows (Paper I) and of average 37.7 g/d fed to the fistulated dairy cows (Paper III). The higher number of P-intake of cows in Experiment 1 compared to Experiment 3 is based on the higher feed intake and higher milk yield of the animals of Experiment 1. In Experiment 3 the cows were fed restricted amounts while the cows of Experiment 1 had free access to feed. In previous studies no effect of P on the DMI was measured (Geisert et al., 2010). The current studies support this result. However the study made with fattening bulls shows a

different trend. DMI tended to be higher in the P+MIN/Zn-group compared to the other treatments (*P*=0.072). The intake of this group and this tendency of higher feed intake supports previous studies made by Ternouth (1990) who maintains that a P-depletion can lead to anorexia. Though in comparison to the DMI of Experiment 1 and 3, the 500-700 g higher feed intake of the fattening bulls (Experiment 2) in group P+MIN/Zn should not be overestimated, because it makes only a marginal part. Not only DMI tended to be higher in the study with fattening bulls, but concentrate intake of the phytase supplemented group also (*P*=0.060). The cows of Experiment 1 even show a significant difference between the treatments (*P*=0.007). The phytase supplemented group had a higher concentrate intake than both other groups. These results are contrary to Kincaid et al. (2005) where DMI was not affected by exogenous phytase. Kincaid and Harrison (2002) discovered that generally, differences occur only when the cereal grains constitute 50% of the whole diet. This 50% of cereal grains are not in the composition of the diet used in the current study.

The phytate P concentration in the diets is different between the experiments. While the phytate P content is similar in all groups for corn silage, there are differences in the concentrates. Corn, soya and dried sugar beet pulp are the main components of the concentrate fed to the animals of Experiment 1, while wheat replaces soya in the concentrated fed to the animals of Experiment 2 and 3. In fact, the amount of phytate P in extracted soybean is lower than in wheat, though the cows of Experiment 1 eat 35 % soya instead of 10% wheat with their concentrates. The additional amount is reduced in the quantity of dried sugar beet pulp (20% instead of 48%). As dried sugar beet pulp has no measureable phytate P concentration, the cows of Experiment 1 had a better phytate P care with concentrates than the animals of Experiment 2 and 3 even the total P content is similar (Eeckhout and De Paepe, 1994).

The three different experiments have the purpose out of discovering nearly the whole P balance of cows and bulls. Experiment 1 served to investigate mainly the P- and phytase-supplementation on performance, P-balance and P-concentration in milk, while the samples of liver, testes and bones of the bulls (Experiment 2) help to investigate the effect on the P-concentration of organs and the Experiment 3 represent the effect of the different treatments on P-flow at the duodenum and rumen parameters. In addition, all three trials give a general view on the influence of P- and phytase-supplementation of cattle.

Differences between the milk yield of Experiment 1 and 3 can be explained with the experimental design. While the cows of Experiment 1 have a milk yield of, on average, 26.8 kg/d on lactation day 197, the fistulated cows from Experiment 3 show only a milk yield of 20.6 kg/d on lactation day 96. It must be taken into account, that the cows from Experiment 1 had free access to feed, whilst the animals of Experiment 3 were fed a restrictive ration. In a recent study Brask-Pedersen et al. (2013) and Brintrup et al. (1993) have shown again, that milk yield and composition were unaffected by dietary P supply and that intake of various nutrients was not affected by treatment. Primarily, the content of P in milk is a function of milk protein percentage and is not affected by dietary P level (Forar et al., 1982, NRC, 2001). This statement can be supported with the results of the current studies. P-excretion with milk is the same for the experimental groups, and between the experiments. The studies show a P-excretion with milk of 0.9 g/kg. This value for P-secretion to milk is 0.1g/kg lower than the recommendations stated by the GfE (2001), CVB (2005) and Schlegel (2011). In contrast it supports the calculations of NRC (2001) and INRA (2002) who calculate the P-requirement with 0.9 g P/kg milk.

Experiment 2 allows study into the storage of P in some organs such as the liver and testes and in the bones of cattle. There were no differences in the P contents of liver and testes between the treatments. Contrary to this *Os metacarpale* tended to have a lower P-concentration in the Zn supplemented group, but dietary P-concentration did not affect the P-concentration in *Os metacarpale*. This result is supported by data by Geisert et al. (2010) who did not find a difference in skeletal maturity in yearling steers fed different dietary P-concentrations. The total P-content of the metacarpal bone did not differ with an overall average P concentration of 17.1% of total ash and there was no difference in the weight of P in the individual bones as percentage of hot carcass weight (HCW) (Geisert et al., 2010). Furthermore Salazar et al. (2013) investigated the P bone metabolism of cows during lactation and found out, that cows mobilized P from the bone during late dry stage and early lactation, and restored P in late lactation and that this pattern of dynamics did not differ when varying amounts of P were fed. The P-content in bones of the current study (Experiment 2) is approximately 9.0 to 9.5% P of the bone and comparable with data of Salazar et al. (2013) who found a bone P-content between 9.8 and 11.8%. The slight difference can be explained by the fact that the data are from lactating cows and not from fattening bulls.

Another important aspect is to answer the question whether phytase has an influence on the ruminal fermentation parameters and the nutrient duodenal fluxes. According to

manufacturer´s specification, the highest efficiency of the experimental phytase is at a pH of 5.5. Therefore it was appropriate to examine the pH-value of the rumen in Experiment 3. However the results of rumen pH-value go not along with literature. While Geisert et al. (2010) found out a main effect of dietary treatment on ruminal pH, with averaged pH between 5.7 to 5.9, there was no effect in the current study for this parameter. In contrast, time affected rumen pH, which decreased with time after feeding from averaged 7.1 (0 minutes after feeding) to 6.2 (300 minutes after feeding). Cattle consumed a meal when fresh feed was offered and thus increased the starch load and fermentable feed in the rumen, leading to a decrease in ruminal pH (Fulton et al., 1979). The higher pH-value of the rumen than the pH-optimum for the supplemented phytase can be an explanation for the missing phytase effect in the current studies. The difference between the actual study and Geisert et al. (2010) could be explained by the gender and age of the animal. While Experiment 3 worked with fistulated cows Geisert et al. (2010) investigated in five ruminally fistulated steers with an initial BW of 386 kg. Another important difference is that Geisert et al. (2010) fed his steers mainly with brewers grain.

The second important aspect of Experiment 3 is the sampling of duodenal chyme of the fistulated cows. It makes it possible to compare if the exogenous phytase has an effect on the daily duodenal nutrient flows of ruminants.

All three experiments are comparable for the component phytase, because all animals of the P(/Zn)+PHY-resp. LP+PHY-group received the same phytase concentration per kg DMI, independent of their age or gender. Brask-Pedersen et al. (2013) realized that an increasing supplementation of phytase is directly linked with a decreasing IP 6-concentration in feed. Furthermore they investigated, that the daily duodenal flows of IP 6 were all lower with exogenous phytase, and the flows were also lower with a higher dose than with a lower one (Brask-Pedersen et al., 2013). In the current study the phytase supplementation did not affect duodenal P-flow. Instead of this the varying P-concentration in feed resulted in differences between the P+MIN- resp. HP- and both other groups. All together, the duodenal P-flow of the current study is lower than the values of Brask-Pedersen et al. (2013). It must be taken into account, that the cows of the current study had a lower DMI and consequential a lower P-intake per day than the cows used in the study by Brask-Pedersen et al. (2013).

Klop et al. (2013) found a relationship between P intake and P excretion in faeces, this was in line with general expectations. Furthermore other authors support this assumption, that the

faecal P-excretion increases linearly with an increasing amount of P-intake (Spiekers et al., 1993). Spiekers et al. (1993) studied the influence of DMI on faecal P-losses in dairy cows rations which were low in P and discovered, that there is a relation between the inevitable losses and the DMI. By comparing all three experiments with each other, the study supports the statement of Spiekers et al. (1993) and of Klop et al. (2013). The tendency for a higher P-concentration in faeces of the animals of Experiment 1 compared to both other experiments can be explained by the higher P intake in Experiment 1 **(Table 3)**. This assumption is supported by a comparison of Experiment 1 and the study of Spiekers et al. (1993). The faecal P excretion in Experiment 1 is, on average, 33 g/d at a mean feed P intake of 77 g/d. This is higher than the values in the study of Spiekers et al. (1993) who realized that a mean faecal P concentration of 20 respectively 13 g/d at a feed P intake of 37 respectively 22 g/d.

Table 3. Comparison of the faecal P excretion and P-balance between the trials with dairy cows (Experiment 1), fattening bulls (Experiment 2) and fistulated cows (Experiment 3)

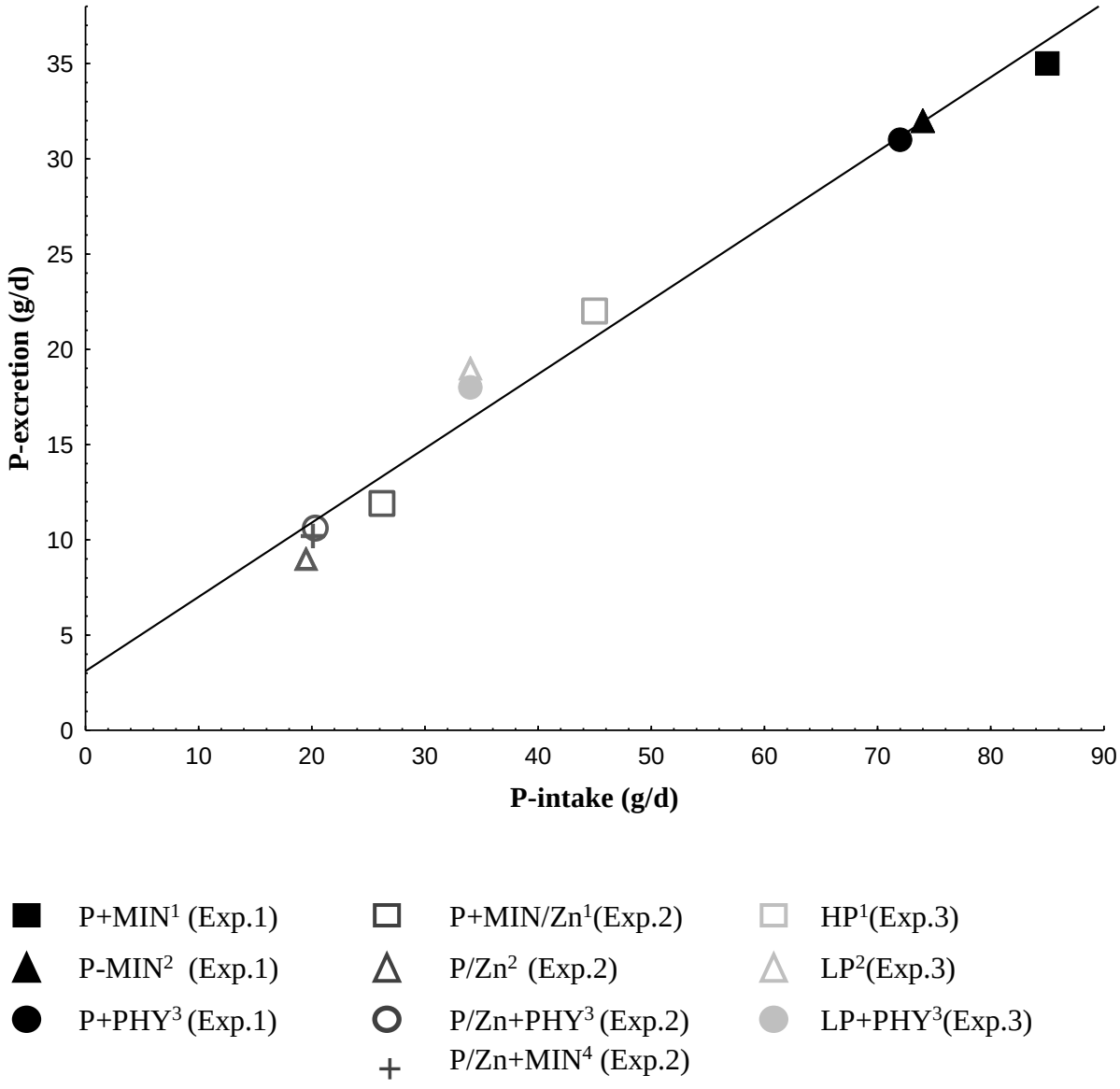
Group	P-Intake (g/d)	Faecal P (g/d)	Faecal P/P-Intake (g/g)	P-Balance (g/d)
Experiment 1				
P+MIN[1]	85	35	0.41	26.4
P-MIN[2]	74	32	0.43	16.2
P+PHY[3]	72	31	0.43	16.5
Experiment 2				
P/Zn[2]	20	9	0.45	10.4
P+MIN/Zn[1]	26	12	0.46	14.2
P/Zn+PHY[3]	20	11	0.55	9.6
P/Zn+MIN[4]	20	10	0.50	9.8
Experiment 3				
HP[1]	45	22	0.49	2.6
LP[2]	34	19	0.56	-3.2
LP+PHY[3]	34	18	0.53	-3.0

[1] diet with supplemented mineral P
[2] diet with native P content
[3] diet with native P content, added with phytase
[4] diet with supplemented mineral Zn

Klop et al. (2013) investigated the ratio of P-excretion to P intake (g/g) with 0.60 g/g, that means that 60% of the P-intake with feed are excreted with faeces. This value cannot be supported with the values of the current study **(Table 3)**. The actual studies showed with 41-43% (Experiment 1), 48-50% (Experiment 2) and 49-56% (Experiment 3) lower values than previous studies, even if the P-intake is similar (Klop et al., 2013). By comparing all three experiments with each other it is apparent that the ratio of faecal P to P-intake ranges between 0.41 and 0.56 g/g. In all three experiments the P+MIN- resp. HP- group has the numerically lowest ratio, but the differences are so marginal that its effect should not be overstretched.

In Experiment 1 and 2 urinary losses are only calculated, while they are measured in Experiment 3. Nevertheless inevitable urinary losses are generally thought to be comparatively small (Spiekers et al., 1993). In the actual study, the amount of P excreted in urine was low, varied slightly between cows and bulls for all diets, but was only once significantly higher for the P-supplemented group. Cows of Experiment 1 excreted on average 0.51 g P/d in urine. The bulls (Experiment 2) were calculated with 1% of P-excretion, and in the urine of the cows of Experiment 3 on average 0.90 g P/d were excreted. While Spiekers et al. (1993) studied lactating cows, Geisert et al. (2010) was concentrated on young steers. The steers were comparable to the bulls of the current study and excreted urinary P, without significant differences (averagely 2.1 g P/d). The urinary P concentration is sensitive to changes in dietary P-content and Geisert et al. (2010) determined that a higher the dietary P-concentration resulted in a higher P excretion with urine of steers. The values for P-excretion with urine were only calculated for the bulls (Experiment 2), whereas the values of the cows of Experiment 3 were measured. They ranged between 0.1 and 2. g P/d. Morse et al. (1992) showed that when lactating Holstein cows were fed diets containing small or medium amounts of P, the quantity of P excreted in urine was small (1.4 g/d) and did not differ significantly. Compared to Experiment 1 and 3 these values are still higher than the values of the current study, but it has to be taken into account, that the P-intake per day ranged between 60 and 112 g/d. These higher amounts of P-intake could explain the higher P-excretions in urine. Furthermore exogenous phytase had no influence on P-concentration in urine in the Experiments. These results were in accordance to a study of Brask-Pedersen et al. (2013) who did not find an effect of exogenous phytase on urinary P-excretion either. However it should be borne in mind that P-excretion with urine only is a marginal part of the P metabolism and that the kidney is not a major excretory route (Morse et al., 1992).

Figure 4 represents an overview of all three Experiments in relation to P-intake and P-excretion of the animals. The animals were fed varying concentrations of dietary P and each point represents the averaged P-intake and excretion of the animals of each treatment. Excretion (g/d) is calculated with faecal P-excretion (g/d) in addition to urinal P-excretion (g/d) plus P-excretion with milk (g/d). P-excretion is linear to P-intake in all three experimental groups.



[1] diet with supplemented mineral P
[2] diet with native P content
[3] diet with native P content, added with phytase
[4] diet with supplemented mineral Zn

Figure 4. Relationship between P-intake and P-excretion (with milk, urine and faeces)

for ruminants fed varying amounts of dietary P in Exp. 1-3. Each point represents P-intake and excretion by animals (P-intake:P-excretion: y = 3,1116 + 0,3896*x; r = 0,9892; p=0,00000; r^2 = 0,9785)

The special feature of the current study is that it is possible to get P-balances for all three experiments. In this way enables to compare the P-metabolism of dairy cows and fattening bulls fed similar dietary P-concentration while on the other hand the balances can prove the missing phytase effect.

Comparing the P-balances of this study, all differences of the P-balance were equal between the three experiments. In all three experiments the P supplemented group has the highest P-balance, independently of gender, age or lactation period. In Experiment 1 and 2 the P-balances are positive while in Experiment 3 they are like intended in two groups negative. Highest value was in group HP (2.6 g/d) compared to group LP (-3.2 g/d) and LP+PHY (-3.0 g/d). The results show that P-intake with feed has an influence on P-balance. P-balance is calculated as:

P-intake (g/d) - P-excretion^{milk} (g/d) - P-excretion^{faeces} (g/d) - P-excretion^{urine} (g/d).

Salazar et al. (2013) support this statement. The study investigated in body P mobilization and deposition during lactation in dairy cows fed varying P-concentrations. The milk yield was averaging 34.6 kg/d and the dietary P fed to the cows was between 72 and 88 g P/d. That is nearly similar to P-intake of dairy cows in Experiment 1 with 72-85 g P/d. It is striking, that P-balance values of Salazar et al. (2013) are lower than the values of Experiment 1. Although the proportion between the treatments is similar, nevertheless P-balances of Salazar et al. (2013) range from -3.9 to 1.1 g/d while they are between 16.2 and 26.4 g/d in Experiment 1. The differences can be explained by the P-excretion with faeces. The animals in the study of Salazar et al. (2013) excreted 46 to 54 g P/d. This is 15 to 19 g P/d compared to Experiment 1 more. Moreover, the P-excretion with milk is higher in the study of Salazar et al. (2013) than in the current one. This is based on the slightly lower milk yield of Experiment 1. Altogether the milk yield is responsible for the differences in P-balance between these two studies. While Salazar et al. (2013) calculates the averaged P-balance for the whole lactation period, the current study only takes weeks 28 to 33 into account. Although Salazar et al. (2013) investigated that over the entire experiment, including the late dry period and subsequent lactation, P balance changed little for all groups, suggesting that, by end of lactation, all groups had re-stored most of what had been mobilized earlier, the study is split into lactating

periods. Salazar et al. (2013) realized, that in weeks -4 to -1 the P-balance was negative, while it became positive from weeks 1 to 5. In weeks 6 to 13 it decreases again and increases from weeks 19 to 42. These many sampling times were not possible in the Experiment 1 and Experiment 3 caused by the short experimental time period. The difference between the values of Experiment 1 and 3 can be explained by the different day of lactation, the different amount of feed intake and with this the different amount of dietary P-intake. While the cows of Experiment 3 are, on average, on lactation day 96, the cows of Experiment 1 are on day 197. Calculating the P-balance of the three experiments as part of P-intake with feed, there are differences to see. The part of P-balance on P-intake of Experiment 1 was between 22-31%, of Experiment 2 49-52% and for Experiment 3 it ranges between -9 and 6%. Comparing these values, the bulls retained more P in the body than the cows. The young age of the bulls could be the reason for this. They need the dietary P for growing processes. The P-balance of Experiment 1 and 2 shows that the intended P-depletion with feed of the animals did not happened as intended. The animals of Experiment 1 and 2 did not suffer P-deficiencies. Due to this fact, it was not possible to investigate the influence of the supplemented phytase like intended. For the models, the requirement for absorbed P was factorially derived by summing estimates of true requirements for maintenance, growth, pregnancy and lactation, divided by the total utilization for P from the diet. The total utilization in the denominator of the factorial equitation potentially has more influence on the final computed dietary requirement than any of the single or combined requirement values for absorbed P. The smaller the total utilization, the greater will be the calculated dietary requirement (NRC, 2001). The recommended values for the total utilization differ between scientific societies (GfE, 1986, GfE, 1995, GfE, 2001, NRC, 2001, INRA, 2002, CVB, 2005). **Table 4** compares the P-requirements for the different diets calculated with different total utilization.

Table 4: P-supply for the animals of Experiment 1, 2 and 3 calculated with different total utilization for P

Group	P-supply (g/d) calculated with total utilization of							
	90 %	80%	75%	70%*	65%	60%#	55%	50%
Experiment 1								
P+MIN[1]	54	61	65	69	74	81	88	97
P-MIN[2]	43	48	51	55	59	64	70	77
P+PHY[3]	43	49	52	56	60	65	71	78
Experiment 2								
P/Zn[1]	15	17	18	20	21	23	25	28
P+MIN/Zn[2]	20	23	24	26	28	30	33	37
P/Zn+PHY[3]	15	17	18	19	20	22	24	27
P/Zn+MIN[4]	15	17	18	20	21	23	25	28
Experiment 3								
HP[1]	38	43	46	49	53	57	62	68
LP[2]	31	34	37	39	42	46	50	55
LP+PHY[3]	30	34	36	39	42	45	50	55

[1] diet with supplemented mineral P
[2] diet with native P content
[3] diet with native P content added with phytase
[4] diet with supplemented mineral Zn
* absorption coefficient according to GfE (2001)
# absorption coefficient according to GfE (1986)

The aim of all three Experiments was to use feed components with a low P-availability. As known corn and corn silage has a low total P-content, but a high percentage of phytate P (Eeckhout and De Paepe, 1994). Based on this the main feed component of the total diets in Experiment 1 was corn silage (63%) and in the concentrates corn (40%). Although ruminants generally have the ability to use phytin-bound phosphorus through ruminal hydrolysis, the P-availability of the feeding component corn is low. The low P-availability of corn led to following calculation for total utilization: the assumed total utilization of Experiment 1 was calculated with 55%. The diet was composed to have a low P-concentration, but afterwards it got clear that calculating the diets with a total P-utilization of 55% makes it impossible to get a P-depletion of 20% into the feed. Based on the experience of Experiment 1 the total P-utilization was calculated with 70% for Experiment 2 and 3. The realized P-intake is

conforming to the intended. In experiment 2 the P/Zn-group received a basal diet with a P-concentration of approximately 2.4 g/kg dry matter (DM) to cover approximately 80% of the present P recommendations (GfE, 1995) without phytase supplementation. The P+MIN/Zn-group was fed the same diet added with P covering the P-demand of about 2.97 g/kg DM. The P/Zn+PHY-group obtained the control diet added with an experimental phytase. However despite the compliance of the P-concentration in the 80%-groups, no P-depletion was investigated. The P-balance of the 80%-groups was positive and therefore not only P-depletion but also a phytase effect was not evidenced in Experiment 2.

In contrast to Experiment 1 and 2 the P-balance of Experiment 3 showed clearly that the P-intake of the LP- and LP+PHY-group was too low for their lactation state and performance. The cows had to set free P from bones and tissues to cover the P demand. However, there was no influence of exogenous phytase on the P-balance of the fistulated cows to see, either. Due to the fact, that the fistulated animals of group LP and LP+PHY were undersupplied, an effect was expected. The missing effect of the exogenous phytase could be attributed by the relatively small amount of dry matter intake. As known, the total utilization of P was calculated similar to Experiment 2 with 70%, but fistulated cows were fed restricted amounts of feed. This way it was possible to feed them into depletion. The low amount of feed leads to a lower passage rate of the rumen, then usual. Flachowsky et al. (2009) investigated, that DMI has an effect on the passage rate of the digesta through the rumen. That means that a higher DMI increase the passage rate, while at the same time the digestibility of nutrients decreases. It is possible that there is a relationship between feeding sequences and microbial protein synthesis. Some authors (Kolver et al., 1998, Vaughan et al., 2002) indirectly indicate that feeding sequence could affect microbes synthesis, too. The lower passage rate conducts, that the microbes have more time to synthesis P in the rumen, than normally. Consequential, the P-depletion of the feed could be compensated by the longer incubation time. This could be one reason, to explain the missing phytase effect, even there is a P-depletion in the diet of the two feeding groups of Experiment 3.

P-digestibility of all three experiments falls between 44 and 59.8%. Literature is not uniform for this aspect. Investigations of Wu and Satter (2000), Knowlton and Herbein (2002) and Wu et al. (2003) reported that apparent P digestibility decreases when P was fed in excess of the requirement. In contrast to these investigations, the current three studies cannot support these facts. In the actual studies, the P digestibility is independent of the treatments. These results confirm the investigations of Salazar et al. (2013) who discovered that the digestibility did not

differ among treatments over lactation or during any of the collection times during lactation. However P-digestibilities obtained in the actual study are not consistent with literature values that have ranged from 24 to 44% (Wu et al., 2000, Wu et al., 2003, Wu, 2005).

8. Conclusion

The result of the present study confirms that supplemented phytase had neither an effect on DMI, P-balance, P-excretion with milk and faeces, performance nor on the duodenal flow and total tract digestibility of P of cows and bulls supplied with P slightly below the recommendations.

The first experiment investigated dietary phytase supplementation in dairy cows fed different amounts of P and showed no effect on P-excretion with faeces and urine as well as the P-balance. The results of the experiment indicate that the addition of the tested phytase had no influence on the P-secretion in milk. The reduction of P concentration in milk in the recommendations from 2 resp. 1g to 0.9g per kg milk could help to reduce the faecal P-concentration as without it, it would be harmful for the health of the cows.
The study with fattening bulls ascertains that a supplementation of phytase did not affect the P- and Zn-digestibility of fattening bulls. Only the P-concentration of faeces and bones tended to be influenced by treatments. The P-concentration tended to be affected by the higher P-intake, but not by the phytase supplementation.
The result of the third investigation, shows that the supplemented phytase had no effect on duodenal flow and total tract digestibility of P of cows supplied with P slightly below the recommendations. The absence of phytase effects on P excretion with milk, urine and feces resulted in an unaffected P balance. On the contrary, the supplementation with mineral P lead to an increased duodenal P flow, no higher P excretion with milk and a slightly higher excretion with faeces. However, questions remain as to whether a higher supplementation level of the exogenous phytase is more suitable or whether another exogenous phytase with a higher pH-optimum would be able to work more efficiently. Altering the composition of the tested diet might also lead to other results such as an improved ruminal fermentation or an increased digestibility of P.

Overall, it can be concluded, that the tested phytase has no effects on the P-digestion of dairy cows and fattening bulls even if the time of ruminal incubation decreases, while time of passage rate increases in high performing animals. A reason for this kind of result can be that

in Experiment 1 a too low total utilization was assumed and that therefore the phytase was not able to work. In Experiment 2 to P-concentrations in feed were as intended, but the bulls were fed *ad libitum.* This results in a positive P-balance and not as intended in a P-depletion. Because of the missing P-depletion the exogenous phytase was not able to influence the P-excretion with faeces.

In Experiment 3 the animals were fed as intended into a P-depletion, but phytase did not affect P-excretion with faeces and the P-balance, either. The three trials show that the conclusion of this thesis is that the microbial phytase of the rumen is sufficient enough to make the indigestible phytate-P digestible for ruminants. Therefore a supplementation of exogenous phytase of this range is not necessary and an exogenous phytase cannot help to reduce P-excretion with faeces.

9. Summary

P plays an important role in the metabolism and health of lactating cows and fattening bulls. P is an essential mineral for the metabolism with diverse functions in the body. Thus P is a necessary constituent of bones and teeth and plays a part in cell membrane structure, energy transfer and in structure of DNA. To be available for the metabolism, the phytate P has to be split up. The enzyme phytase breaks the phosphate groups from the inositol ring in order to make P available for absorption in the small intestine. Phytase is widespread in nature, occurring in plants, microorganisms and in some animals. Ruminants are able to digest phytate P because rumen microorganisms synthesize the enzyme phytase. The enzyme and its activity are frequently present in the plant kingdom. It appears in some cereal grains such as barley, rye and wheat. Furthermore, phytase activity is found in peas, beans, soybeans, maize and other feed plants. The effect of phytase as a P-splitting feeding supplement is known in the feeding of poultry and swine. Therefore in the current study the question arises if is it possible to compensate a lower P-concentration in the feed by the supplementation of phytase. Another question comes up as to wether the phytase has an effect on the accommodation with trace elements. Zn for example often forms a heavy soluble complex with P. In contrast to other trace elements animals require relatively large amounts of Zn. Likewise, increasing dietary P or the supplementation of phytase could have an influence on the Zn needs of the animals.

To investigate the effect of exogenous phytase on the P- and Zn-metabolism of ruminants, three studies were done at the Friedrich-Loeffler-Institute in Braunschweig: a study with dairy cows (Experiment 1), a study with fattening bulls (Experiment 2) and at least a study with fistulated cows (Experiment 3). The cows and fattening bulls of the trial were German-Holstein bred. The experimental design of all three studies included different P-concentrations and phytase supplementations of the feed. In the study with fattening bulls it gets completed with varying Zn-concentrations between the feeding groups. The similar experimental designs of the three studies make a comparison between all the investigated parameters possible.

24 lactating pluriparous cows were used for Experiment 1. The trial lasted 5 weeks. The cows were randomly assigned to one of the three feeding groups (P+MIN, P-MIN, P+PHY) with eight cows each. The diets of group P+MIN and P-MIN showed no phytase activity, while the diet of group P+PHY was supplemented with an exogenous phytase. The feed of group P+MIN was supplemented with dicalcium phosphate, while the TMR of group P-MIN and P+PHY included only the native P content of the feed components. The cows were fed *ad libitum* with corn silage and concentrate as a TMR with 37% concentrate and 63% corn silage (on a DM basis). Cows were milked twice daily and milk yield recorded automatically. Samples of blood, faeces, urine and milk were taken for P-analyses for every cow in regular intervals. Analysing the results of the study no differences were observed in TMR intake across the treatments. There were no differences between the treatments for P-excretion with milk, faeces and urine during the experimental period, either. In contrast, the P-balance of group P+MIN is higher, compared to the other two groups. The higher dietary P-intake is the reason for this increase. The supplementation of phytase had neither influence on the P-excretion with faeces and urine, nor on the P-digestibility and the P-concentration in milk and blood.

In order to assess the different treatments on the performance and organ weights of fattening bulls, a trial with 48 fattening bulls was carried out. The bulls were allocated to four dietary treatments (P/Zn, P+MIN/Zn, P/Zn+PHY and P/Zn+MIN). The P+MIN group got a higher P-concentration with feed as the other groups. Animals of the P+PHY group achieved an exogenous phytase and P/Zn+MIN a higher Zn-concentration in feed. All bulls received a diet of 80% corn silage and 20% concentrate on a dry matter (DM) basis. The corn silage intake was for ad libitum and the concentrate intake restricted. 24 of the 48 bulls were slaughtered. Six bulls from every feeding group were sampled. Considering the treatments, body weight, empty body weight, organ weight, retroperitoneal fat and dressing did not show any

differences. P- and Zn-excretion with faeces, P- and Zn-digestibility and P- and Zn-concentration of liver and testes did not show differences. In contrast, the P-concentration of the bone *Os metacarpale* tended to be lower in the P/Zn+MIN group (*P*=0.062).

Experiment 3 was based on a balance study by using fistulated dairy cows. Nine lactating, ruminal and duodenal fistulated cows were used for three periods, consisting of three weeks of adaption period and three weeks of sample collection. Six cows in the first and third periods, as well as five cows in the second period, were assigned to one of three dietary treatments: HP (diet supplemented with dicalcium phosphate), LP (native P-content) or LP+PHY (diet supplemented with an exogenous phytase). Samples of milk, urine and faeces were taken to calculate the P-balance. Furthermore samples of ruminal fluid were taken to examine parameters such as pH-value SCFA and NH_3-N. Duodenal chyme was collected for the investigation of duodenal P-flow and blood samples were taken. The results of the trial with fistulated cows showed, that there were no differences in nutrient intake between the treatments and the P- and phytase supplementation did not affect the pH-value and NH_3-N concentration of ruminal fluid. The concentration of total SCFA were not affected by the P-supplementation. The nutrient flow at the duodenum was not influenced by the feeding. However, the P-flow was affected. The HP group had a 9 respectively 15% higher P-flow than both other groups. P-excretion with faeces and urine tended to be higher in the HP group, the P-concentration in milk did not differ between the groups (*P*=0.984). That implies that the P-balance was different between the groups. P-balance of group HP was positive, the balance of the other groups was negative. These differences showed the intended slight shortage of the LP- and LP+PHY-groups, but it also showed that the phytase supplementation was not able to compensate the deficit of the LP+PHY-group. These results affirm that the P-digestibility of ruminants can be influenced by the P-intake with feed, but not by a supply of exogenous phytase.

Comparing all three studies, considering the fact that the same parameters were investigated in the same species but with different gender and age, it seems that the supplementation of P has an influence on the P-metabolism, whereas the supplementation of phytase did not show any effects on the investigated parameters. It can be concluded from this, that ruminants are able to digest P with the rumen microbes, even if the performance, the feed intake and with this the passage rate increases, while the time of incubation decreases.

10. Zusammenfassung

Der Mineralstoff P spielt eine wichtige Rolle in der Ernährung und dem Stoffwechsel von laktierenden Kühen und Mastbullen. P hat einen Einfluss auf den Stoffwechsel und auf viele biologische Funktionen. Unter anderem dient P als Baustein für den Aufbau von Knochen und Zähnen. Außerdem stellt P einen essentiellen Bestandteil von Zellmembranstrukturen, ATP und des Puffersystems von Blut und anderen Körperflüssigkeiten dar. Um dem Körper P aus dem Futter verfügbar zu machen, muss das Element aus Pflanzenphytaten freigesetzt werden. Während bei Wiederkäuern größtenteils der Pansen mit den Pansenmikroben diese Freisetzung veranlasst, sind Monogastrier auf das Phytat spaltende Enzym Phytase als Futterzusatz angewiesen. Phytase ist in der Natur weit verbreitet. Unter anderem ist Phytase in einigen Pflanzen, Mikroorganismen und wenigen Tieren zu finden. Getreidesorten wie Roggen, Gerste und Weizen weisen relativ hohe Phytaseaktivitäten auf, aber auch Erbsen, Bohnen, Soja, Mais, Gras und einige andere Futterpflanzen enthalten Phytase. Daher stellt sich in den aktuellen Versuchen die Frage, ob es möglich ist durch eine Phytasesupplementation bei gleichzeitig leicht reduzierter P-Fütterung das P Defizit im Futter auszugleichen. Weiter soll abgeleitet werden, ob die Phytase sich auch auf die Spurenelementversorgung auswirkt. Beispielsweise bildet P häufig mit Zn einen schwerlöslichen Komplex. Zn ist essentiell und im Gegensatz zu den anderen Spurenelementen ist der Bedarf relativ hoch. Daher ist es wichtig mögliche Effekte des Phytaseeinsatzes auf die Zn-Verwertbarkeit in Betracht zu ziehen.

Um die Auswirkung der exogenen Phytase auf den P- und Zn-Stoffwechsel zu untersuchen wurden drei Versuche auf der Versuchsstation des Friedrich-Loeffler-Instituts in Braunschweig durchgeführt: ein Versuch mit Milchkühen (Experiment 1), ein Schlachtversuch mit Mastbullen (Experiment 2) und ein Fistelkuhversuch (Experiment 3). Für die Versuche standen laktierende Kühe und wachsende Bullen der Rasse Deutsch-Holstein zur Verfügung. Das Design der drei Versuche beinhaltet verschiedene P-Konzentrationen und Phytasesupplementationen im Futter. Im Bullenversuch wird es um verschiedene Zn-Konzentrationen im Futter ergänzt. Das ähnliche Versuchsdesign ermöglicht einen Vergleich der einzelnen Parameter zwischen den Versuchen. Es kann der Einfluss der Behandlungen auf die P-Aufnahme mit dem Futter, die P-Ausscheidung mit Kot, Harn und bei den Kuhversuchen mit der Milch, sowie die P-Bilanz untersucht werden. Ergänzt werden diese Standarddaten durch die Auswirkung auf Leistungsparameter und Organgewichte der

Mastbullen, sowie durch die Pansenfermentationsparameter und Darmsaftuntersuchungen der Fistelkühe.

Im Experiment 1 wurden 24 laktierende mehrkalbige Milchkühe in einem fünfwöchigen Versuch in drei verschiedenen Futtergruppen (P+MIN, P-Min, P+PHY) gefüttert. Jeweils acht Kühe waren in einer Futtergruppe und erhielten unterschiedliche P- und Phytasemengen. Die höhere P-Konzentration der P+MIN Gruppe ist auf die Gabe von Dicalciumphosphat zurückzuführen, während der P-Gehalt in den anderen beiden Gruppen nativ ist. Das Futter wurde den Kühen *ad libitum* als TMR, bestehend aus 37% Kraftfutter und 63% Maissilage vorgelegt. Die Milchleistung der Kühe wurde zweimal täglich beim Melken erfasst. In regelmäßigen Abständen wurden Blut-, Milch- und Kotproben zur Bestimmung des P-Gehaltes genommen. Während der Versuchsperiode gab es keine Unterschiede in der P-Ausscheidung mit Milch, Kot und Harn. Die P-Bilanz war im Vergleich zu den beiden anderen Gruppen in der P+MIN Gruppe höher. Diese Erhöhung resultierte aus der höheren P-Aufnahme mit dem Futter. Die Phytasezulage hatte weder Auswirkung auf die P-Konzentration im Kot und Harn, noch auf die Verdaulichkeit und die P-Konzentration in der Milch. Auch im Blut zeigten sich keine Unterschiede zwischen den Gruppen.

Um die Auswirkung der unterschiedlichen Behandlungen auf die Organgewichte und Leistungsparameter zu untersuchen wurde das Experiment 2 mit 48 Mastbullen durchgeführt. Jeweils 12 Tiere waren in vier Futtergruppen (P/Zn, P+MIN/Zn, P/Zn+PHY und P/Zn+MIN) und erhielten unterschiedliche P-, Zn- und Phytasemengen. Die höhere P-Konzentration der P+MIN/Zn-Gruppe wurde durch die Gabe von Calciumphosphat erreicht, während der P-Gehalt der anderen Gruppen nativ war. Die P/Zn+MIN-Gruppe hat einen nativen P-Gehalt und es wurde Zn-supplementiert. In Gruppe P/Zn+PHY wurde der Ration exogene Phytase zugesetzt. Insgesamt 24 Tiere der 48 wurden mit einer durchschnittlichen Lebendmasse von 581±8 kg geschlachtet. Es wurden sechs Bullen aus jeder Futtergruppe beprobt. Die verschiedenen Behandlungen zeigten keinen Effekt auf die Schlachtausbeute, die Körper-, und Leerkörpermasse und die Masse des retroperitonealen Fettdepot. Auch bei den Organmassen, der P und Zn Verdaulichkeit und Exkretion mit dem Kot gab es keine Einflüsse des Futters. Bei der P- und Zn-Analyse der Organe waren in der Leber und dem Hoden keine Differenzen zu erkennen. Die Beprobung des Knochen *Os metacarpale* in der P/Zn+MIN-Gruppe wies dagegen eine tendenziell niedrigere P-Konzentration auf als die anderen Gruppen.

Grundlage des dritten Versuches war ein Bilanzversuch mit neun duodenal- und pansenfistulierten Milchkühen. Auch hier war Ziel des Versuches den Einfluß einer exogenen Phytase bei verschiedenen P Konzentrationen im Futter auf die P-Ausscheidung mit Kot, Milch und Harn, die P-Verdaulichkeit und auf die Pansenfermentations Parameter zu untersuchen. Der Versuch wurde in drei Versuchsperioden durchgeführt die jeweils drei Wochen Anfütterungszeit und drei Wochen Probenentnahme beinhalteten. Die Tiere wurden wie in Versuch 1 in drei Futtergruppen (HP, LP, LP+PHY) gefüttert und erhielten unterschiedliche P- und Phytasemengen. Das Futter wurde den Kühen restriktiv vorgelegt. Es wurden eine Totalsammlung von Harn und Kot, die Entnahme von Pansensaft-, Darmsaft-, Milch- und Blutproben durchgeführt. Die Milchleistung der Tiere wurde täglich dokumentiert. Der Zusatz von P und Phytase zeigte keine Auswirkungen auf den pH-Wert und die NH_3-N Konzentration im Pansensaft. Der Nährstofffluss im Dünndarm wurde nicht durch die Fütterung beeinflusst. Der P-Fluss hingegen schon. Die HP-Gruppe zeigte einen 9 bzw. 15% höheren P-Fluss als die beiden Gruppen mit dem niedrigeren P-Gehalt im Futter. Die Analyse der P-Ausscheidung mit Kot, Harn und Milch ermöglichte auch in diesem Versuch das Erstellen einer P-Bilanz. Während die HP-Gruppe eine positive P-Bilanz (2.6 g/Tag) hatte, war sie für die beiden anderen Gruppen negativ (-3.2 und -3.0 g/Tag). Diese Unterschiede zeigten, dass wie gewünscht eine leichte Unterversorgung der LP- und LP+PHY-Gruppen erfolgte, dass die Phytase aber die Mangelsituation der Gruppe LP+PHY nicht ausgleichen konnte. Dieses Ergebnis bestätigt, dass in dieser Studie die P-Verdaulichkeit bei Wiederkäuern zwar durch die Menge an gefütterten P aber nicht durch den Zusatz einer exogenen Phytase beeinflusst werden konnte.

Bei der Betrachtung der drei Versuche mit den gleichen Parametern aber verschieden geschlechtlichen und unterschiedlich alten Tieren scheint es letztlich, dass die P-Zulage zum Futter Auswirkungen auf den P-Stoffwechsel haben kann. Die Zugabe von exogener Phytase zeigt dagegen keine Wirkung auf die verschiedenen untersuchten Parameter. Daraus kann geschlussfolgert werden, dass Rinder auch in Zeiten von immer steigenden Leistungen und den damit einhergehenden höheren Futtermengen, niedrigeren Inkubationszeiten im Pansen und einer höheren Passagerate, immer noch in der Lage sind mit den panseneigenen Mikroben das Element P zu verstoffwechseln.

11. References

Bailey, C. B. and C. C. Balch. 1960. Some Aspects of the Secretion of Parotid Saliva in the Bovine. J Anim Sci. 19(3):961-961.

Bannink, A., L. Sebek, and J. Dijkstra. 2010. Efficiency of Phosphorus and Calcium Utilization in Dairy Cattle and Implications for the Environment.151-172.

Bertoni, G. W., M.J.; Savage, G.P.; Armstrong, D.G. 1976. The movements of minerals in the digestive tract of dry and lactating Jersey cows 1. Net movements of Ca, P, Mg, Na, K and Cl. 2:107-118.

Bortolussi, G., J. H. Ternouth, and N. P. McMeniman. 1996. Dietary nitrogen and phosphorus depletion in cattle and their effects on liveweight gain, blood metabolite concentrations and phosphorus kinetics. J Agr Sci. 126:493-501.

Boxebeld, A., L. Gueguen, G. Hannequart, and M. Durand. 1983. Utilization of Phosphorus and Calcium and Minimal Maintenance Requirement for Phosphorus in Growing Sheep Fed a Low-Phosphorus Diet. Reprod Nutr Dev. 23(6):1043-1053.

Boyd, L. O´Dell, R. A. S. 1997. Handbook of nutritionally essential mineral elements. Vol. 2. Marcel Dekker, New York, United States of America.

Braithwaite, G. D. 1984. Changes in Phosphorus-Metabolism of Sheep in Response to the Increased Demands for P-Associated with an Intravenous-Infusion of Calcium. J Agr Sci. 102(Feb):135-139.

Braithwaite, G. D. 1985. Endogenous Fecal Loss of Phosphorus in Growing Lambs and the Calculation of Phosphorus Requirements. J Agr Sci. 105(Aug):67-72.

Brask-Pedersen, D. N., L. V. Glitso, L. K. Skov, P. Lund, and J. Sehested. 2011. Effect of exogenous phytase on feed inositol phosphate hydrolysis in an in vitro rumen fluid buffer system. J Dairy Sci. 94(2):951-959.

Brask-Pedersen, D. N., L. V. Glitso, L. K. Skov, P. Lund, and J. Sehested. 2013. Effect of exogenous phytase on degradation of inositol phosphate in dairy cows. J Dairy Sci. 96(3):1691-1700.

Breves, G. and B. Schröder. 1991. Comparative aspects of gastrointestinal phosphorus metabolism. Nutr Res Rev Vol 105:125-140.

Brintrup, R., T. Mooren, U. Meyer, H. Spiekers, and E. Pfeffer. 1993. Effects of 2 Levels of Phosphorus Intake on Performance and Fecal Phosphorus Excretion of Dairy-Cows. J. Anim. Physiology and Anim. Nutrition. 69(1):29-36.

Caldwell, D. R. and M. P. Bryant. 1966. Medium without Rumen Fluid for Nonselective Enumeration and Isolation of Rumen Bacteria. Appl Microbiol. 14(5):794-&.

Call, J. W., J. E. Butcher, J. L. Shupe, R. C. Lamb, R. L. Boman, and A. E. Olson. 1987. Clinical Effects of Low Dietary Phosphorus Concentrations in Feed Given to Lactating Dairy-Cows. Am J Vet Res. 48(1):133-136.

Challa, J., G. D. Braithwaite, and M. S. Dhanoa. 1989. Phosphorus Homoeostasis in Growing Calves. J Agr Sci. 112:217-226.

Cheryan, M. 1980. Phytic Acid Interactions in Food Systems. Crc Cr Rev Food Sci. 13(4):297-335.

Clark, W. D., J. E. Wohlt, R. L. Gilbreath, and P. K. Zajac. 1986. Phytate Phosphorus Intake and Disappearance in the Gastrointestinal-Tract of High Producing Dairy-Cows. J Dairy Sci. 69(12):3151-3155.

CVB. 2005. CVB Table Booklet Feeding of Ruminents. Centraal Veevoederbureau. Voedernormen landbouwhuisdieren en voederwaarde veevoeders. Lelystad, the Netherlands.

Dirksen, G., H. D. Gründer, and M. Stöber. 2006. Innere Medizin und Chirurgie des Rindes. Vol. 5. Paul Parey Verlag, MVS Medizinverlage Stuttgart GmbH, Stuttgart.

Durand, M. and R. Kawashima. 1980. Influence of minerals in rumen microbial digestion. In: Y. Ruckebusch and P. Thivend (eds.) Digestive Physiology and Metabolism in Ruminants. AVI Publ Co. Westport, CT.

Dvorakova, J. 1998. Phytase: Sources, preparation and exploitation. Folia Microbiol. 43(4):323-338.

Eeckhout, W. and M. De Paepe. 1994. Total Phosphorus, Phytate-Phosphorus and Phytase Activity in Plant Feedstuffs. Anim Feed Sci Tech. 47(1-2):19-29.

Ekelund, A. 2003. Phosphorus and the dairy cow-influence of intake level, source and stage of lactation on apparent digestibility and bone turnover. PhD.Thesis.SLU, Kungsängen Research Centre, Uppsala.

Engelen, A. J., F. C. Vanderheeft, P. H. G. Randsdorp, and E. L. C. Smit. 1994. Simple and Rapid-Determination of Phytase Activity. J. AOAC Int. 77(3):760-764.

Flachowsky, G., P. Lebzien, and U. Meyer. 2009. Deduction of energy and nutrient requirements for high yielding cows. Züchtungskunde. 81(6):429-441.

Flachowsky, G. and M. Rodehutscord. 2008. Steigerung der P-Effizienz in der Tierproduktion. in Braunschweiger Nährstofftage 2008. Institut für Tierernährung, Braunschweig.

Forar, F. L., R. L. Kincaid, R. L. Preston, and J. K. Hillers. 1982. Variation of Inorganic Phosphorus in Blood-Plasma and Milk of Lactating Cows. J Dairy Sci. 65(5):760-763.

Frolich, W., T. Drakenberg, and N. G. Asp. 1986. Enzymatic Degradation of Phytate (Myoinositol Hexaphosphate) in Whole Grain Flour Suspension and Dough - a Comparison between P-31 Nmr-Spectroscopy and a Ferric Ion Method. J Cereal Sci. 4(4):325-334.

Fulton, W. R., T. J. Klopfenstein, and R. A. Britton. 1979. Adaptation to High Concentrate Diets by Beef-Cattle .1. Adaptation to Corn and Wheat Diets. J Anim Sci. 49(3):775-784.

Gabard, K. A. and R. L. Jones. 1986. Localization of Phytase and Acid-Phosphatase Isoenzymes in Aleurone Layers of Barley. Physiol Plantarum. 67(2):182-192.

Geisert, B. G., G. E. Erickson, T. J. Klopfenstein, C. N. Macken, M. K. Luebbe, and J. C. MacDonald. 2010. Phosphorus requirement and excretion of finishing beef cattle fed different concentrations of phosphorus. J. Anim. Sci. 88(7):2393-2402.

GfE 2001. Gesellschaft für Ernährungsphysiologie. Empfehlungen zur Energie- und Nährstoffversorgung der Milchkühe und Aufzuchtrinder.

GfE 1995. Empfehlungen zur Energie- und Nährstoffversorgung der Mastrinder. Vol. 6. Energie- und Nährstoffbedarf landwirtschaftlicher Nutztiere.

GfE 1986. Energie- und Nährstoffbedarf landwirtschaftlicher Nutztiere, Milchkühe und Aufzuchtrinder. Vol. 3. DLG Verlag, Frankfurt am Main.

Godoy, S. and F. Meschy. 1999. Utilisation of phytic P by rumen bacteria in a semi-continuous culture system (rusitec). S. Afr. J. Sci. 29:148-149.

Godoy, S. and F. Meschy. 2001. Utilisation of phytate phosphorus by rumen bacteria in a semi-continuous culture system (Rusitec) in lactating goats fed on different forage to concentrate ratios. Reprod. Nutr. Dev. 41(3):259-265.

Grace, N. D., M. J. Ulyatt, and J. C. Macrae. 1974. Quantitative Digestion of Fresh Herbage by Sheep .3. Movement of Mg, Ca, P, K and Na in Digestive-Tract. J Agr Sci. 82(Apr):321-330.

Guyton, A. D. 2002. Starch digestion and Phosphorus Excretion in lactating Dairy Cows Page 86 in Faculty of the Virginia Polytechnic Institute and State University. Faculty of the Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Guyton, A. D., J. M. McKinney, and K. F. Knowlton. 2003. The effect of steam-flaked or dry ground corn and supplemental phytic acid on phosphorus partitioning and ruminal phytase activity in lactating cows. J Dairy Sci. 86(12):3972-3982.

Hampton, D. L., W. J. Miller, D. M. Blackmon, R. P. Gentry, M. W. Neathery, J. W. Lassiter, R. L. Kincaid, and P. E. Stake. 1976. Zinc Absorption from Small-Intestine in Young Calves. J Dairy Sci. 59(4):712-715.

Hill, S. R., K. F. Knowlton, E. Kebreab, J. France, and M. D. Hanigan. 2008. A Model of Phosphorus Digestion and Metabolism in the Lactating Dairy Cow. J. Dairy Sci. . 91:2021–2032.

Horst, R. L. 1986. Regulation of Calcium and Phosphorus Homeostasis in the Dairy-Cow. J Dairy Sci. 69(2):604-616.

INRA. 2002. Institut National de la Recherche Agronomique. Tables de l´alimentation des ruminants. Vol. 2. Institut National de la Recherche Agronomique.

Johnson, L. F. and M. E. Tete. 1969. Structure of ´phytic acids´. 47:67-73.

Jongbloed, A. W., P. A. Kemme, and Z. Mroz. 1993. The role of phytases in pig production. Pages 173-180 in Proc. Enzymes in Animal nutrition-Proceedings of the 1st Symposium, Switzerland.

Karn, J. F. 2001. Phosphorus nutrition of grazing cattle: a review. Anim Feed Sci Tech. 89(3-4):133-153.

Khorasani, G. R. and D. G. Armstrong. 1992. Calcium, Phosphorus, and Magnesium Absorption and Secretion in the Bovine Digestive-Tract as Influenced by Dietary Concentration of These Elements. Livest Prod Sci. 31(3-4):271-286.

Kincaid, R. and J. Harrison. 2002. Feeding barley to dairy cattle may have advantages. 74(2):33-40.

Kincaid, R. L., D. K. Garikipati, T. D. Nennich, and J. H. Harrison. 2005. Effect of grain source and exogenous phytase on phosphorus digestibility in dairy cows. J Dairy Sci. 88(8):2893-2902.

Klop, G., J. L. Ellis, A. Bannink, E. Kebreab, J. France, and J. Dijkstra. 2013. Meta-analysis of factors that affect the utilization efficiency of phosphorus in lactating dairy cows. J Dairy Sci. 96(6):3936-3949.

Knowlton, K. F. and J. H. Herbein. 2002. Phosphorus partitioning during early lactation in dairy cows fed diets varying in phosphorus content. J. Dairy Sci. 85(5):1227-1236.

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

Kolb, E. and H. Gürtler. 1971. Ernährungsphysiologie der landwirtschaftlichen Nutztiere. VEB Gustav Fischer Verlag, Jena, German Democratic Republic.

Kolver, E., L. D. Muller, G. A. Varga, and T. J. Cassidy. 1998. Synchronization of ruminal degradation of supplemental carbohydrate with pasture nitrogen in lactating dairy cows. J Dairy Sci. 81(7):2017-2028.

Lim, P. E. and M. E. Tate. 1973. Phytases .2. Properties of Phytase Fractions F1 and F2 from Wheat Bran and Myoinositol Phosphates Produced by Fraction F2. Biochim Biophys Acta. 302(2):316-328.

Lott, J. N. A. 1984. Accumulation of seed reserves of phosphorus and other minerals. In: D.R. Murray (ed.) Seed physiology No.1: 139-166. Academic Press, New York.

Maenz, D. D. 2001. Enzymatic Characteristics of Phytase as they Relate to their Use in Animal Feeds. Vol. 1. Enzymes in Farm Animal Nutrition. CABI Publishing, UK.

MAFF. 1990. UK Tables of Nutritive Value and Chemical Composition of Foodstuffs. Rowett Research Services. Aberdeen, UK.

Martz, F. A., A. T. Belo, M. F. Weiss, R. L. Belyea, and J. P. Goff. 1990. True Absorption of Calcium and Phosphorus from Alfalfa and Corn-Silage When Fed to Lactating Cows. J Dairy Sci. 73(5):1288-1295.

Matrone, G. 1970. Biochemistry and mechanismen of action of trace elements. Pages 171-175 in 8th Int. Congress Nutr., Prague.

Matsui, T. 2002. Relationship between mineral availabilities and dietary phytate in animals. Anim Sci J 73: 21-28.

McCance, R.A., and E.M. Widdowsen. 1935. Phytic acid in human nutrition. Biochem J 29: 42694-42699.

McCleary, B. V. 2001. Analysis of Feed Enzymes. Vol. 1. Enzymes in Farm Animal Nutrition. CABI Publishing, Finnfeeds, Marlborough, Wiltshire, UK.

McDowell, L.R. 1992. Minerals in Animals and Human Nutrition. Academic Press, San Diego, CA.

Miller, W. J. 1971. Zinc metabolism in farm animals. Mineral Studies with Isotopes in Domestic Animals Joint FAO/IAEA Division of Atomic Energy in Food and Agriculture, Vienna.

Miller, W. J. 1979. Dairy Cattle Feeding and Nutrition. Academic Press, New York.

Miller, W. J. 1983. Zinc in animal and human health. Pages 182-191 in Trace elements in health. Vol. 1. J. Rose, ed. Butterworth & Co, Cambridge, UK.

Miller, W. J., D. M. Blackmon, R. P. Gentry, and F. M. Pate. 1970. Effects of high but nontoxic levels of zinc in practical diets on Zn and Zn metabolism in Holstein calves. 100:893-902.

Miller, W. J. and M. W. Neathery. 1980. Manifestations of zinc abnormalities in animals. Vol. 2, Health Effects. Zinc in the Environment. John Wiley & Sons, New York, Chichester, Brisbane, Toronto.

Morris, E. R. and R. Ellis. 1976. Isolation of mono ferric phytate from wheat bran and its biological value as an iron source to the rat. J Nutr 106:753-760.

Morse, D., H. H. Head, and C. J. Wilcox. 1992. Disappearance of Phosphorus in Phytate from Concentrates Invitro and from Rations Fed to Lactating Dairy-Cows. J Dairy Sci. 75(7):1979-1986.

Moustgaa, J. 1972. Nutritive Influences Upon Reproduction. J Reprod Med. 8(1):1-6.

Nelson, T. S. 1967. The utilization of phytate phosphorus by poltry-a review. Poult Sci 46: 862-871.

Nelson, T. S., L. B. Daniels, J. R. Hall, and L. G. Shields. 1976. Hydrolysis of Natural Phytate Phosphorus in Digestive-Tract of Calves. J Anim Sci. 42(6):1509-1512.

Nikolic, J. A., M. Jovanovic, R. Andric, and J. Krsmanovic. 1978. Influence of Mineral Element Concentration on Protein-Synthesis by Rumen Microorganisms Invitro .2. Zinc. Acta Vet-Beograd. 28(2):57-66.

NRC. 1996. National Research Council, Nutrient requirements of dairy cattle. National. Academy of Science, Washington D.C.

NRC. 2001. National Research Council, Nutrient requirements of dairy cattle. 7th revised edition ed. National Academy of Science, Washington D.C.

O´Dell, B. L., A. R. de Boland and A. R. Koirtyohann. 1972. Distribution of phytate and nutritionally important elements among the morphological components of cereal grains. J Agric Food Chem 20: 718-721.

Parkash, S. and R. Jenness. 1969. Status of zinc in cow´s milk. Journal of Dairy Science 50:127-134.

Pate, F. M., W. J. Miller, D. M. Blackmon, and R. P. Gentry. 1970. Early Tissue Zn-65 Distribution after Duodenal Dosing in Calves Fed Zinc-Deficient and Control Diets. P Soc Exp Biol Med. 135(3):653-658.

Peers, F.G. 1953. The phytase of wheat. Biochem J 53: 102-110.

Pfeffer, E., D. K. Beede, and H. Valk. 2005. Phosphorus Metabolism in Ruminants and Requirements of Cattle. Nitrogen and Phosphorus Nutrition of Cattle. CABI publishing, Cambridge, United Kingdom.

Pfeffer, E. and A. N. Hristov. 2005. Nitrogen and P nutrition of cattle, reducing the environmental Impact of cattle operations. CABI Publishing, Cambridge, United Kingdom.

Pfeffer, E., A. Thompson, and Armstron.Dg. 1970. Studies on Intestinal Digestion in Sheep .3. Net Movement of Certain Inorganic Elements in Digestive Tract on Rations Containing Different Proportions of Hay and Rolled Barley. Brit J Nutr. 24(1):197-201.

Pointillart, A. A. Fourdin and N. Fontaine. 1987. Importance of cereal phytase activity for phytate phosphorus utilization by growing pigs fed diets containing triticale or corn. J Nutr 117: 907-913.

Pointillart, A. A. 1991. Enhancement of phosphorus utilization in growing pigs fed phytate diets by using rye bran. J Anim Sci 69: 1109-1115.

Puggaard, L., N. B. Kristensen, and J. Sehested. 2011. Effect of decreasing dietary phosphorus supply on net recycling of inorganic phosphate in lactating dairy cows. J Dairy Sci. 94(3):1420-1429.

Raun, A., E. Cheng, and W. Burroughs. 1956. Phytate Phosphorus Hydrolysis and Availability to Rumen Microorganisms. J Agr Food Chem. 4(10):869-871.

Ravindran, V. 1996. Occurence of phytic acid in plant feed ingredients. Phytase in Animal Nutrition and Waste Management. BASF Corporation, Mount Olive, New Jersey.

Ravindran, V., W. L. Bryden, and E. T. Kornegay. 1995. Phytates: occurence, bioavailability and implications in poultry nutrition. 6:125-143.

Ravindran, V., G. Ravindran, and S. Sivalogan. 1994. Total and Phytate Phosphorus Contents of Various Foods and Feedstuffs of Plant-Origin. Food Chem. 50(2):133-136.

Reid, R. L., M.C. Franklin. 1947. The utilization of phytate phosphorus by sheep. 23:136-140.

Reinhardt, T. A., R. L. Horst, and J. P. Goff. 1988. Calcium, Phosphorus, and Magnesium Homeostasis in Ruminants. Vet Clin N Am-Food A. 4(2):331-350.

Rodehutscord, M. 2008. Approaches for saving limited phosphate resources. Arch Tierzucht. 51:39-48.

Rose, J. 1983. Trace Elements in health. Vol. 1. A review of current isssues. Butterworth & Co Ltd. , UK.

Salazar, J. A. E., J. D. Ferguson, D. B. Beegle, D. W. Remsburg, and Z. Wu. 2013. Body phosphorus mobilization and deposition during lactation in dairy cows. J Anim Physiol an N. 97(3):502-514.

Satter, L.D., T.J. Klopfenstei and G.E. Erickson. 2002. The role of nutrition in reducing nutrient output from ruminants. J Anim Sci 80 (Suppl): E143-E156.

Schlegel, P., ALP. 2011. Phosphorempfehlung für die Milchkuh. in ALP Tagung 2011. Department federal de l´economie DFE Station de recherche Agroscope Liebefeld-Posieux ALP.

Spiekers, H., R. Brintrup, M. Balmelli, and E. Pfeffer. 1993. Influence of Dry-Matter Intake on Fecal Phosphorus Losses in Dairy-Cows Fed Rations Low in Phosphorus. J Anim Physiol an N. 69(1):37-43.

Stake, P. E., W. J. Miller, D. M. Blackmon, R. P. Gentry, and M. W. Neathery. 1974. Role of Pancreas in Endogenous Zinc Excretion in Bovine. J Nutr. 104(10):1279-1284.

Stake, P. E., W. J. Miller, R. P. Gentry, and M. W. Neathery. 1975. Zinc Metabolic Adaptations in Calves Fed a High but Nontoxic Zinc Level for Varying Time Periods. J Anim Sci. 40(1):132-137.

Suttle, N. F. 2010. Minerals in livestock production. Asian Austral J Anim. 13:1-9.

Ternouth, J. H. 1990. Phosphorus and Beef-Production in Northern Australia .3. Phosphorus in Cattle - a Review. Trop Grasslands. 24(3):159-169.

Ternouth, J. H. 1997. Phosphorus metabolism in ruminant animals. CABI Publishing. Oxfordshire. 167-177.

Ternouth, J. H., H. M. S. Davies, J. T. B. Milton, M. W. Simpsonmorgan, and N. E. Sands. 1985. Phosphorus-Metabolism in Ruminants .1. Techniques for Phosphorus Depletion. Aust J Agr Res. 36(4):637-645.

Tronier, B., R.L. Ory and K.W. Henningsen. 1971. Characterization of the fine structure and proteins from barley protein bodies. Phytochem 10: 1207-1311.

Underwood, E. J. 1977. Trace Elements in Human and Animal Nutrition 4. 4th ed. Academic Press. New York. P118

Valk, H., L. B. J. Sebek, and A. C. Beynen. 2002. Influence of phosphorus intake on excretion and blood plasma and saliva concentrations of phosphorus in dairy cows. J Dairy Sci. 85(10):2642-2649.

Vaughan, J. M., J. A. Bertrand, T. C. Jenkins, and B. W. Pinkerton. 2002. Effects of feeding time on nitrogen capture by lactating dairy cows grazing rye pasture. J Dairy Sci. 85(5):1267-1272.

Vitti, D. M. S. S., E. Kebreab, J. B. Lopes, A. L. Abdalla, F. F. R. De Carvalho, K. T. De Resende, L. A. Crompton, and J. France. 2000. A kinetic model of phosphorus metabolism in growing goats. J Anim Sci. 78(10):2706-2712.

Vohra, A. and T. Satyanarayana. 2003. Phytases: Microbial sources, production, purification, and potential biotechnological applications. Crit Rev Biotechnol. 23(1):29-60.

Wasserman, R. H. and A. N. Taylor. 1976. Gastrointestinal absorption of calcium and phosphorus. . Vol. VII, Parathyroid Gland. Handbook of Physiology. No. 7: Endocrinology. G. D. Aubach, Washington, D.C.

Winter, L., U. Meyer, M. Spolders, L. Hüther, P. Lebzien, and S. Dänicke. 2013. Effect of exogenous phytase on the phosphorus balance of lactating cows fed a corn based diet. 4(2):232-250.

Wu, Z. 2005. Utilization of phosphorus in lactating cows fed varying amounts of phosphorus and sources of fiber. J Dairy Sci. 88:222-223.

Wu, Z. and L. D. Satter. 2000. Milk production, reproductive performance of dairy cows fed two concentrations of phosphorus for two years. J Dairy Sci. 83(5):1052-1063.

Wu, Z., L. D. Satter, A. J. Blohowiak, R. H. Stauffacher, and J. H. Wilson. 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(7):1738-1748.

Wu, Z., L. D. Satter, and R. Sojo. 2000. Milk production, reproductive performance, and fecal excretion of phosphorus by dairy cows fed three amounts of phosphorus. J Dairy Sci. 83(5):1028-1041.

Wu, Z., S. K. Tallam, V. A. Ishler, and D. D. Archibald. 2003. Utilization of phosphorus in lactating cows fed varying amounts of phosphorus and forage. J Dairy Sci. 86(10):3300-3308.

Wylie, M. J., J. P. Fontenot, and L. W. Greene. 1985. Absorption of Magnesium and Other Macrominerals in Sheep Infused with Potassium in Different Parts of the Digestive-Tract. J Anim Sci. 61(5):1219-1229.

Yanke, L. J., H. D. Bae, L. B. Selinger, and K. J. Cheng. 1998. Phytase activity of anaerobic ruminal bacteria. Microbiol-Uk. 144:1565-1573.

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation: “Effects of addition of phytase, phosphorus and zinc to ruminant diets on rumen metabolism, digestion and performance” selbstständig und nur unter der Verwendung der angegeben Literatur und Hilfsmittel angefertigt habe. Die Arbeit lag bisher in gleicher oder ähnlicher Form keiner Prüfungsbehörde vor.

Halle/Saale, den

Laura Schulte-Ebbert, geb. Winter

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