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Abstract

Dairy cows suffer from hypocalcaemia in the days around calving, which may result in a condition generally known as milk fever. Calcium metabolism sharply shifts at the start of lactation, because Ca needs suddenly become much greater than at the end of gestation. Calcium metabolism is able to adapt to different physiological situations, but adaptation requires several days to be effective, resulting in this transient hypocalcaemia. A way to prevent milk fever is to induce adaptation of Ca metabolism weeks before calving by reducing dietary availability of Ca, to prepare Ca metabolism for calving. Rice bran contains a very low level of Ca and a high level of phytic acid, which is a well-know dietary antagonist of Ca in monogastric species. Preventing the ruminal degradation of phytic acid, rice bran can reduce the nutritional availability of dietary Ca in cows. In this thesis, fat coating and formaldehyde treatment proved effective to protect phytic acid in rice bran from ruminal degradation. Formaldehyde treatment was chosen as the preferred method, because it had no detrimental effects on voluntary feed intake. Feeding rumen-protected rice bran reduced dietary Ca availability, thereby inducing the adaptation of Ca metabolism. Furthermore, the product, fed before calving to multiparous cows, improved calcaemia for the first three days after calving. Rumen-protected rice bran, fed in the last weeks of gestation, could represent a practical dietary strategy to prevent milk fever.

Table of contents

CHAPTER 1

General introduction

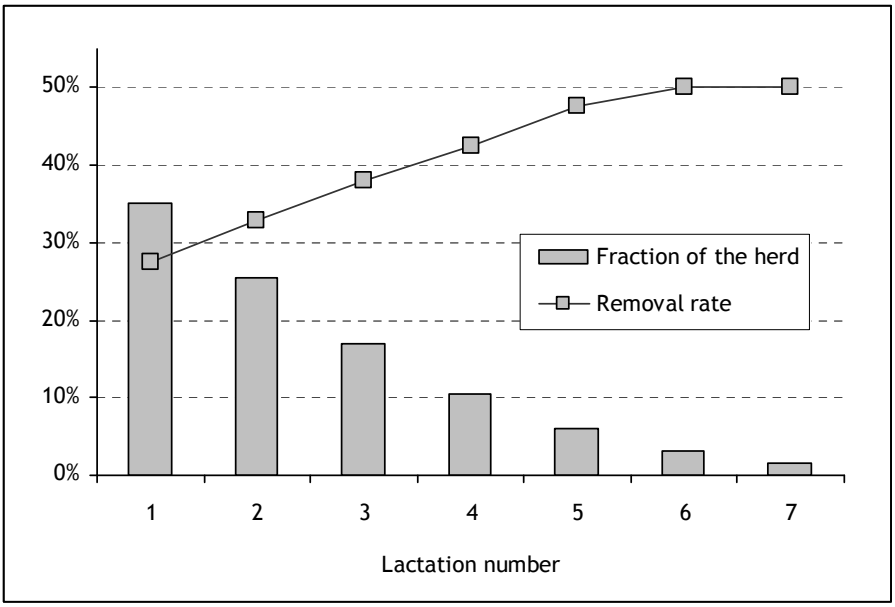
The personal connection that often takes place between dairy cows and their human keepers is a unique case in animal production. Cows have a longer life span than other farm animals, and they are producers and not the final product, because they yield milk and calves. Cattle naturally develop social structures among themselves, which was a differentiating characteristic in their early election for domestication (Diamond, 2002), and dairy breeds have been strongly selected for tameness, because they have to be daily in close contact with humans for milking. All these factors have made cow welfare traditionally important to farmers from long before any ethical code of practices was introduced into animal production. A recent survey has shown that the majority of farmers recognise their cows individually, and consider them intelligent beings capable of experiencing emotions (Bertenshaw and Rowlinson, 2009). This may be a major reason for many farmers to keep health and welfare as a priority, but it is also of outstanding importance for dairy production as an industry.

Animal health and welfare are also highly related to farm profitability. When the costs generated by a cow and the value of the milk produced are integrated along its life, the two values converge sometime during the second lactation. This implies that first and second lactation cows can be considered in terms of profit "replacement stock". Hence in the current productive scenario, keeping older cows in the herd is a prerequisite for profitability.

At the end of this decade, the raw material markets and an increasing sensibility for environmental impact of economic activities have created greater awareness of the exhaustibility of natural resources. Costs are ultimately the measurable expression of the resources used, and economic profitability may indirectly reflect the efficiency by which these resources are turned into milk. Excessive replacement stock represents a waste of resources that increases the environmental footprint of milk production, therefore replacement rate should be kept as low as possible to minimise pollution (Tamminga, 2003).

Dairy herds present a pyramidal age structure. The distribution of animals by parity from 1 through 7 in dairy herds of the United States in the last 25 years is described in figure 1. First and second lactation animals constitute more than 60% of the animals. This is the consequence of a combined rate of removal and mortality of 29.3% per year (USDA, 2008). The odds to leave the herd are not equal for all animals but they increase linearly as the animals get older. Improving longevity has been a priority for years; however, cows still leave the herds at a similar rate as 10 years ago (USDA, 2008).

Figure 1. Age structure of the herd, expressed as percentage of animals of each parity, and evolution of removal rate as lactation number increases.



Data from Hare et al. (2006)

Modern dairy cows have a production potential that greatly exceeds the original purpose of lactation, which is feeding the offspring. Cows were part of the first group of domesticated species about 10,000 years ago (Diamond, 2002), and dairy breeds have existed for centuries. Nevertheless, in the last decades intensification of dairy production brought cows into even closer symbiosis with humans, which required the adaptation of genotypes through selection. Massive milk production

has been obtained by genetic improvement, but the physiological robustness required for it is lagging behind, resulting in a high culling rate. Darwin (1859) early defined evolution under domestication as the cumulative result of two means of selection. *Methodical selection* being rapid, while *unconscious selection* being slower. Modern genetic improvement has increased milk production fast, and now culling rate is a powerful means of *unconscious selection* driven by the insufficient physiological robustness of these cows. If Darwin's intuition proves true, these means of selection will not bring a solution to the problem in the short term.

The mismatch between milk yield and physiological robustness is most dramatic when cows get older and reach maximum production. They require efficient physiological adaptation of their energy and mineral metabolism at the onset of lactation, which is a critical shift in the production cycle. The failure to adapt energy metabolism at calving results in ketosis. In a similar way, the failure to adapt Ca homeostasis results in milk fever. Dairy nutritionists aim to facilitate this adaptation with the purpose of preventing health disorders, which should result in improved animal welfare, improved economic profitability, and a reduced environmental footprint of milk production.

Milk fever: Definition, aetiology, and implications

Milk fever is the common name for the syndrome associated with hypocalcaemia that multiparous cows suffer from in the hours around parturition. Most cows have some degree of hypocalcaemia at calving; therefore it is difficult to define milk fever as a discrete parameter. Although it is generally accepted that cows which require treatment constitute clinical cases of milk fever, this remains a subjective judgement. Periparturient hypocalcaemia has an imprecise definition, but in turn it is a continuous parameter that can be measured objectively analysing Ca.

The United States Department of Agriculture (USDA) has the largest survey on intensive dairy production. In 2006, 84% of the farms reported milk fever incidence, and declared a fraction of affected animals of 4.9%, having decreased from 5.9% since 1996 (USDA, 2008). Considering that heifers are not susceptible to milk fever (Moore et al., 2000), recalculating this data results in clinical milk fever incidence in multiparous cows of 7.5%. Jordan and Fourdraine (1993) describe a maximum incidence in their survey of 44%, and Lean et al. (2006) describe experiments averaging 21% incidence, with a few herds exceeding 80%. This

enormous variability between herds exposes the real dimension of the problem, while offering an opportunity to control the disease by different means.

Variation of milk fever incidence is explained by several factors. Intrinsic to the cow are breed and age. Jerseys are known to be more susceptible to milk fever. This may be explained by their greater milk production potential per unit of metabolic body size, and by the greater Ca content of their milk. As cows get older, their susceptibility to milk fever increases by 9% per lactation (DeGaris and Lean, 2008). Increased milk production with age partially explains this factor, but the decrease in bone turnover rate could be the main cause of the effect of age on milk fever. It has been suggested that age decreases the presence of calcitriol receptors. This was shown in rats (Horst, 1990) and in cows (Horst et al., 1994). However, Liesegang et al. (2008) could not confirm this in cows. In fact, old cows do not experience hypocalcaemia during the peak of lactation, which demonstrates their Ca homeostatic competence when Ca demand is at its highest. More likely, the greater susceptibility to milk fever of older cows than young cows is explained by the greater contrast in Ca balance before and after calving. Older cows have a very low Ca requirement in the dry period, as compared with their Ca intake. Contrarily, after calving they need large amounts of Ca for their high milk yield. Instead, younger cows have a smaller Ca surplus before calving, and a smaller deficit after calving, suffering a less pronounced shift in Ca homeostasis.

The most important dietary factors which may explain milk fever incidence are Dietary Cation-Anion Difference (DCAD) and Ca intake. Systemic acid-base balance affects Ca homeostasis and it is strongly influenced by DCAD. The prophylactic value of acidifying blood and urine through dietary modulation of DCAD has been extensively documented. However, despite that the effect of DCAD on milk fever incidence has been described as linear (Lean et al., 2006), it seems that only negative DCAD values will reduce milk fever incidence consistently. This would coincide with a response of urinary pH and urinary Ca to DCAD. The response of urinary pH to DCAD is curvilinear and the pH decreases only if DCAD gets into the negative range (Roche et al., 2003).

Dietary Ca will affect milk fever incidence in a quadratic way with a maximum incidence at 11.6 g of dietary Ca per kg DM intake (Oetzel, 1991) or 13.5 g of dietary Ca per kg DM (Lean et al., 2006). Apparently, low dietary Ca induces an adaptation in the intestinal absorption of Ca before calving. This adaptation

prevents milk fever (Green et al., 1981, Kichura et al., 1982, Shappell et al., 1987). On the other hand, a high level of Ca intake around and after parturition can increase passive Ca absorption feed and this may compensate for the losses of Ca from blood (Ca clearance) into milk.

Clinical hypocalcaemia is relatively easy to treat by Ca borogluconate injection. In this way, milk fever seldom results in mortality. However, hypocalcaemia at calving is a predisposing factor for other production diseases. Curtis et al. (1985) describes a several-fold increase in the odds ratios for dystocia, retained placenta and clinical mastitis, and a 24-fold chance for ketosis. Indirect effects of milk fever on early lactation disorders are associated with low dry matter intake (DMI), because hypocalcaemia has proven to negatively affect feeding behaviour (Hansen et al., 2003). Milk fever has therefore a major indirect impact on culling rate because it strongly affects general heath status in early lactation. Furthermore, milk fever has a clear direct effect on animal welfare.

Calcium homeostasis

Evolution has provided elaborate mechanisms for physiological control of Ca. At cellular level, the concentration of ionic Ca in the cytoplasm is kept several-fold below that of the external environment, through means of membrane channels and complexation with proteins. With the development of higher forms of life, accurate control of Ca in the macro-organisms remained a high priority. Animals benefit from precise homeostasis of the blood Ca pool, because positive or negative fluctuations can have fatal consequences. At animal level, hormonal signals direct the Ca control systems in the different tissues. Among these, trans-epithelial transport in the kidneys and the intestine are the most important. This is done to preserve vital functions, such as signalling and muscle contraction, while at the same time managing Ca reserves and structural function in the bones.

Monitoring of blood Ca takes place in the parathyroid gland by the Ca^{2+} sensing receptor (CaR) (Suzuki et al., 2008). The parathyroid will respond to a decrease in blood Ca level with the release of parathormone (PTH). This hormone modulates renal Ca resorption, starts bone Ca mobilisation and induces the activation of calcitriol (1, 25-dihydroxyvitamin D) in the kidney. The latter will induce the activation of Ca absorption in the intestine, and will sustain bone Ca mobilisation, if gastrointestinal absorption alone can not correct the Ca deficit. Positive

deviations of blood calcium can only be naturally caused by excessive dietary intake through passive absorption, which represents a moderate inflow that can be effectively corrected by modulation of renal reabsorption of Ca. The most usual challenge to Ca homeostasis is an increased clearance rate of Ca from the blood, as for example, at the start of the lactation or by hypercalciuria induced by metabolic acidosis. Corrective mechanisms to this increased Ca clearance are increased renal reabsorption, increased intestinal absorption and bone mobilisation. Renal reabsorption responds within hours to PTH secretion (Schonewille et al., 1999) but it presents quantitative limitations because basal renal Ca excretion is small. Intestinal reaction is mediated by calcitriol and offers a greater resource of Ca allowing for a very efficient absorption of dietary Ca and reabsorption of endogenously secreted Ca. However, this may take longer than a day to become effective (Armbrecht et al., 1998). Bone mobilisation of Ca offers a large pool capable of Ca to sustain homeostasis, but it is only sustained in time when gastrointestinal input is insufficient (Erben, 2001).

Dairy cows undergo severe adaptations in their Ca metabolism during the productive cycle. At lactation peak, even a very efficient active absorption of dietary Ca is insufficient to cover the high Ca requirements of lactation. Therefore also Ca from the bone is mobilised. At a certain moment in lactation, dietary Ca matches demand for milk yield, but a high intestinal absorption must be maintained for several months to replenish the Ca mobilised from bone tissue. In late lactation and the dry period, passive intestinal Ca absorption is sufficient to compensate blood clearance from faecal and urinary excretions and foetal needs. In a natural scenario, placental Ca transfer before calving is similar to Ca clearance into milk at the start of lactation (Ramberg et al., 1984). In beef cows this is the case, but not in the modern dairy cow, in which Ca yield in milk exceeds greatly the needs of a new-born calf. Consequently calving represents a great challenge for the adaptation of Ca homeostasis.

Dietary prophylaxis of milk fever

For many decades, dairy nutritionists have searched for a dietary strategy to prevent milk fever. The preference for dietary prevention may be related to the ease of application. Moreover, nutrient imbalances tend to be understood as nutritional problems, although as already discussed, milk fever has to be

understood as a failure of physiological adaptation, rather than a case of inadequate nutrient supply. In the mid-last century, dietary strategies such as vitamin D supplementation and changes in dietary Ca/P ratios were proposed for prevention of mik fever; see review by Boda and Cole (1956). There is also a long history of exploration of low Ca diets and reduction of cation-anion difference to prevent milk fever.

Vitamin D, and its hydroxylated forms in positions 1 or 25, and calcitriol in oral or injected applications have been extensively tested for milk fever prevention. These products were promising initially, but were eventually discarded because effective doses were too close to toxicity, and because preventive or inductive effects of the treatments seem to be related to time of exposure before calving (Littledike and Horst, 1982). This makes dietary application unfeasible.

Once milk fever was understood as a failure of the adaptation of Ca homeostasis, low Ca diets were proposed with the intention of challenging Ca homeostasis and anticipating the necessary adaptation to the event of calving. This strategy was proven effective in numerous studies (Goings et al., 1974, Wiggers et al., 1975, Yarrington et al., 1977, Green et al., 1981, Kichura et al., 1982, Shappell et al., 1987). The efficacy of this strategy has been reviewed to be near 100%, when daily Ca intake is kept below 20 g per day (Thilsing-Hansen et al., 2002b). This approach brought valuable insight to the aetiology of the disorder, but never represented a feasible dietary means for prevention. Formulating a dry cow ration with less than 1.5 g of dietary Ca per kg DM is incompatible with the main nutritional targets of these rations. Green forages exceed by several-fold that Ca level. Hence their inclusion would need to be limited, and this would result in an energy density that would exceed recommendations and in the difficulty to cover the needs for effective fibre.

The most widespread and successful dietary prevention of milk fever until now is the modification of DCAD in order to induce a moderate state of metabolic acidosis. This dietary factor can be obtained by calculation of the added cation equivalents of Na and K and the subtraction of the anion equivalents of Cl and S. The level of DCAD is expressed in meq per kg DM. The abundant data supporting the preventive value of this strategy indicates that milk fever incidence is reduced when DCAD ranges between -100 to -200 meq per kg DM. Most diets would naturally have a DCAD value between +100 and +400 meq/kg DM. In order to reach the

target, the diet requires the inclusion of mineral salts containing S or Cl, without Na or K, the so-called anionic salts. Despite the well-documented positive effect of this strategy, prevention of milk fever by reducing DCAD is seldom total. In addition to this, the application of this strategy has practical difficulties and important drawbacks.

As already discussed above, prevention potential seems to relate in a non-linear way with DCAD, requiring negative values of dietary DCAD for providing protection. This implies that in productive scenarios with forages high in K, which are typical to high fertilisation in intensive forage production, the need for anionic salts can be very high. However, mineral salts cause palatability problems (Oetzel and Barmore, 1993) and S content in the ration should be kept below 4 g/kg DM (NRC, 2001). Thus practical application of anionic salts in the diet is a challenge. Also, DCAD reduction is associated to DMI depression, which can be detrimental to postpartum health (Bertics et al., 1992).

The mode of action of the preventive effect of low DCAD is still under discussion. It has been suggested that metabolic alkalosis reduces renal responsiveness to PTH (Horst et al., 1994), which would be reversed by metabolic acidification. However, positive DCAD is not unique to the periparturient period. In fact it is recommended for lactation diets (Hu and Murphy, 2004). When DCAD is low enough, it can increase apparent Ca absorption in cows (Schonewille et al., 1994), as consequence of the effect of DCAD on urinary Ca excretion. Urinary Ca clearance from blood must therefore be compensated in the first instance by intestinal absorption, because reduction of endogenous intestinal Ca is not among the control mechanisms of Ca homeostasis. It seems plausible that lowering DCAD, similar to lowering dietary calcium, anticipates the adaptation of Ca homeostasis by activating intestinal absorption, and if this would result insufficient to compensate urinary loses, by bone resorption (Schonewille et al., 1994).

In the last decade, the principle of low Ca to prevent milk fever has been reinvented with dietary interventions to reduce Ca availability, without necessarily modifying Ca intake. Zeolite clays have been extensively studied consistently demonstrating their ability to prevent milk fever (Thilsing-Hansen et al., 2002a, Enemark et al., 2003a, Enemark et al., 2003b, Thilsing-Hansen et al., 2003, Katsoulos et al., 2005, Thilsing et al., 2007, Grabherr et al., 2008a, Grabherr et al., 2008b). The effectiveness seems similar to that of the synthetic low Ca diets.

The mode of action of zeolites is still under discussion. The initial hypothesis was that intestinal binding of Ca would challenge Ca absorption; however, recently it has been proposed that perhaps the induction of hypophosphatemia by the product may play an active role in milk fever prevention (Pallesen et al., 2008).

Zeolites have demonstrated great potential for dietary prophylaxis of milk fever. However, a mayor drawback of this application is DMI depression. Initial data did not report the large effect on DMI that was later observed (Grabherr et al., 2008a). Feeding management in these studies can be understood as restricted feeding because leftover feed was minimal (Thilsing-Hansen et al., 2002a, Pallesen et al., 2008). However, it must be mentioned that effect on DMI depends on feeding dose, and that a compromise between effectiveness and DMI depression has been proposed to be at 23 g/kg DM (Grabherr et al., 2008b).

A different approach for reducing Ca availability was proposed by Wilson (2001, 2003). In his case, unsaturated fat was used to improve Ca homeostasis with apparent success. Dietary fat has been considered a dietary antagonist of Ca (Palmquist et al., 1986), but the magnitude of this effect is questionable in ruminants because Ca fat complexes will dissociate in the duodenum (Doreau and Ferlay, 1994). An additional drawback to this approach is also that feeding fat before calving has a negative effect on DMI (Douglas et al., 2004).

It would be very desirable to benefit from lowering dietary availability of Ca without inducing DMI depression. Among the natural components known to reduce Ca availability, phytic acid is abundantly available in rice bran. This feed has no known negative effects on DMI, and has an adequate feed value for ruminants. However, phytic acid is rumen-degradable (Clark Jr et al., 1986); therefore the simple dietary inclusion of rice bran would not represent a viable alternative to zeolites.

Phytic acid as dietary antagonist of calcium availability

Myoinositol hexaphosphate (phytic acid) is the most common form of P in plants, accounting for approximately 85% of total P. It is commonly referred to with the salt name (phytate) and less frequently with the Ca-Mg salt name, phytin. Phytic acid in feeds is often reported as phytate P, in order to easily compare it with total P. In this way, it provides an estimation of nutritionally available P. Conversion from phytate P to phytic acid is rather simple, provided that phytic acid contains

28.2% P. Yet another nomenclature exists to present the degree of phosphorilation of inositol. Inositol phosphate (IP) followed by a number from 1 to 6 indicates the number of phosphate molecules attached to the inositol ring. Less relevant to the present document is the more elaborate nomenclature available to define the different isomers of the incomplete phosphorilated IP forms.

Inositol phosphates play a role in the cell physiology of animals and plants, but the main nutritional relevance for animal nutrition is the impact of phytic acid in feeds on P availability in monogastric species. Furthermore, phytic acid is taken into account for its indirect detrimental effects on the availability of other essential minerals, caused by complexation of these minerals by phytate in the gastrointestinal tract.

Complexation of Ca by phytic acid has been described as nutritionally relevant effect in monogastric animals. Potentially phytic acid can bind 6 moles of Ca per mol, but this is conditioned by the concentration of other chemical species in the solution and pH. Integrity of phytic acid also affects its binding power. Only inositol 5 and inositol 6 phosphate seem to reduce Ca availability (Lonnerdal et al., 1989), which is of special relevance in ruminant animals that can dephosphorilate phytic acid during ruminal digestion. Another factor is the competition by other elements for which phytic acid may have higher affinity as trace metals (Crea et al. 2008), but this is of minor importance given their naturally low presence in the diets. In a process of such complexity, only the maximum binding potential can be estimated; 4 kg of rice bran containing 240 g of phytic acid could bind 88 g of Ca, an amount which is in line with the gross Ca intake of a dry cow.

Phytic acid from rice bran has been used to reduce dietary Ca availability in the prevention of renal calculi in humans (Ohkawa et al., 1984, Ebisuno et al., 1991). Among the most common cereal brans, rice bran contains the highest phytic acid levels and it also presents the greatest in vitro binding potential (20g Ca/kg bran) (Siener et al., 2001). Rice bran was tested with other brans for its ability to reduce urinary Ca in humans (Jahnen et al., 1992). This study confirmed that rice bran is the most effective in reducing intestinal Ca availability.

Unfortunately, the effects of rice bran on dietary Ca availability can not simply be extrapolated from monogastric animals into ruminants. Microbial phytase in the rumen is able to extensively degrade dietary phytic acid (Morse et al., 1992). In

fact, ruminal degradation of phytic acid has been studied extensively in recent decades with the aim to calibrate the need for supplemental P in ruminants, and in this way minimise environmental impact. These studies demonstrated that ruminal degradation of phytic acid is associated to that of protein, and that feed treatments used to promote higher rumen escapes of protein result in lower digestibility values for P. Formaldehyde treatment reduces phytic acid breakdown (Park et al., 1999, Bravo et al., 2002) as well as heat treatment (Konishi et al., 1999). Another factor that affects ruminal degradation of phytic acid is dietary Ca. Sansinena (1999) demonstrated that high dietary Ca reduces the rumen escape of phytic acid of rice bran, suggesting that phytic acid can to a certain extent complex with Ca in the rumen. If the effect of rumen-protection methods for protein on escape of phytic acid proves true also for rice bran, this cereal by-product may represent a feasible dietary ingredient to reduce Ca availability in ruminants.

Objective of this thesis, general hypothesis

The present thesis will explore the potential application of rumen-protected rice bran in the dietary induction of the adaptation of Ca metabolism around calving, in order to prevent milk fever. The effectiveness of rumen-protection of rice bran to promote rumen escape of phytic acid will be studied. Also, the potential for rice bran to induce adaptive changes in Ca homeostasis will be analysed together with any potential effects from dietary rumen escape phytic acid on mineral metabolism, which could be detrimental to animal health. Ultimately, the prophylactic effect of rumen-protected rice bran against milk fever will be tested in multiparous dairy cows at calving. The general hypothesis of this thesis is that rumen-protected rice bran, fed for some weeks before calving, could improve Ca homeostasis in multiparous dairy cows around calving.

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CHAPTER 2

Urinary calcium excretion in non-lactating dairy cows in relation to intake of fat-coated rice bran

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Summary

At calving, many older cows fail to compensate the sudden demand of calcium by an adequate activation of intestinal absorption. This results in a variable degree of hypocalcaemia. Reducing intestinal availability of calcium during the close up period can prevent milk fever. Fat-coated rice bran (FCRB) was investigated for its potential to reduce Ca availability in pre-calving cows. FCRB was incubated in situ to estimate ruminal degradation of dry matter and phytic acid. Also, seven dry multiparous dairy cows were used for a feeding trial in 3 periods of about one week each: P1: adaptation P2: feeding of 2 kg of FCRB per day and P3: withdrawal of FCRB. Feed intake was recorded and daily urine samples were analysed for pH, Ca and creatinine. The bypass fraction of phytic acid (passage rate 5%/h) was 30%. FCRB depressed dry matter intake in P2, resulting in a lower Ca intake. In P2 urine pH and calcium excretion were lower. Daily calcium excretion decreased after introduction of FCRB, peaked after withdrawal and dropped two days later.

Changes in urinary Ca excretion indicate that FCRB affected Ca homeostasis in dry multiparous dairy cows.

Introduction

Milk fever is one of the most relevant metabolic disorders in high producing dairy cows, with an incidence frequency averaging 7.2% per herd. The frequency within herd can be as high as 44% (Jordan et al., 1993). Milk fever is the clinical manifestation of various degrees of hypocalcaemia that cows develop around calving, when the nutritional needs for calcium suddenly increase from a low requirement in the dry period to a high requirement due to lactation. In the Netherlands, a commonly used nutritional strategy to prevent milk fever is to modify the dietary cation anion balance by feeding anionic salts. Unfortunately, intensively grown forages with high manure fertilisation contain high levels of K (Schonewille et al., 1997). Under these conditions, the adjustment of the dietary cation anion balance requires high doses of anionic salts, which can cause a reduction in dry matter intake (Moore et al., 2000).

Thilsing-Hansen et al. (2002 a) reviewed the different existing preventive strategies and concluded that feeding low calcium diets before calving seems to be the most effective way to prevent milk fever. Unfortunately, considering the high calcium contents in most forage types, in practice, it is not easy to formulate low calcium diets for the close-up period.

Recently, several research groups have been looking at possibilities to alter calcium digestibility by feeding calcium antagonists with the purpose of reproducing the effect of low calcium diets with practical forage based diets. Enemark et al. (2003 a, b) and Thilsing-Hansen et al. (2001, 2002 b and 2003) described positive results feeding zeolites. Wilson (2001 a, b) also reports preventive effects in ewes feeding soy oil. Also, Katsoulos et al. (2005) described the prevention of parturient paresis by feeding clinoptilolite clay, a natural zeolite that binds calcium. Thus, there seems to be potential for impairing calcium absorption through dietary interactions. However, there are yet unsolved drawbacks to these strategies. Unsaturated fat has negative effects on rumen function, and zeolites considerably increase the ash fraction of the diet and interfere with magnesium and phosphorus nutrition (Thilsing-Hansen et al. 2002 b). Despite the described disadvantages of zeolites in the prevention of milk fever, the European Food Safety Authority considers them effective and safe for cows (EFSA, 2007).

Dietary fibre can decrease calcium digestibility. Jahnen et al. (1992) in humans studied the effect of different cereal brans on calcium availability. They found that several cereal brans, especially rice bran, reduced excretion of calcium in urine. This indicates reduced intestinal calcium availability. Siener et al. (2001) confirmed in vitro that rice bran is very effective as calcium binding fibre source. It is thought that phytic acid is the most relevant component for calcium binding in rice bran. Ebisuno et al. (1991) described the preventive properties of rice bran on the prevention of kidney stone formation in humans through its negative effect on calcium absorption.

The difficulty in applying this principle in ruminants is the degradability of brans and their components in the rumen, where the microflora can degrade phytic acid (Clark et al., 1986). There are, however, different techniques available to make feed constituents rumen bypass. Rumen-protected rice bran would have the advantage of being a natural feedstuff which, in its defatted version, should not impair ruminal digestion or increase the ash content of the diet. Additionally, it should not impair magnesium absorption which mainly takes place in the forestomach, and the bypass fraction of phytic acid should act as an antagonist further in the gastrointestinal tract.

The aim of this trial was to investigate whether fat-coated rice bran could affect urinary calcium excretion in dry multiparous dairy cows, indicating a change in intestinal availability of calcium.

Materials and methods

Product manufacture

Full-fat stabilised rice bran in a fine powder form was used in a rumen-protected form. The product was sprayed with hydrogenated palm fat in a mixer. This treatment provided a solid fat-coating to the bran particles which should represent a barrier to prevent ruminal degradation. The product was manufactured in three batches and the composition is described in Table 1.

Table 1. Composition of the batches of fat-coated rice bran product

		Batch 1	Batch 2	Batch 3
Fat	%	65	48	55
Rice bran	%	35	52	45
DM	%	95.49	95.10	95.26
Ash	% DM	2.98	4.43	3.83
CP	% DM	5.40	8.02	6.94
EE	% DM	72.63	59.34	64.81
Ca	g/kg DM	0.15	0.22	0.19
P	g/kg DM	5.92	8.80	7.62
Na	g/kg DM	0.03	0.04	0.04
K	g/kg DM	5.86	8.70	7.53
Cl	g/kg DM	0.32	0.47	0.41
S	g/kg DM	0.67	0.99	0.86
Phytic acid	%DM	2.01	2.99	2.59

In situ evaluation of the rumen-protected product

The fat-coated rice bran was evaluated in situ (Ørskov and Mc Donald, 1979) to obtain an estimate of the ruminal degradability of dry matter and phytic acid. Three lactating fistulated cows were used for the incubations. Sixty nine bags were filled with approximately 5.5 g of fat-coated rice bran. Incubation was done following the all out principle in which bags are introduced at different times (0, 2, 4, 8, 16, 24, 48 and 168 hours) and removed all at once. The number of bags was different in each incubation period in order to obtain enough sample for analyses. Bags assigned to each incubation period were distributed evenly among the three cows. After incubation, the bags were submerged in water with ice, lightly rinsed and machine washed with a cold wash program. Then bags were freeze dried, weighed and pooled for determination of dry matter and phytic acid analyses. The data obtained were regressed to an exponential equation with the program NLREG, version 5.4 (Sherrod, 1992) to determine the rate of degradation. The undegradable fraction was assumed to be the fraction left over after 168 hours, and the soluble fraction to be the 0 hour machine washable fraction.

In vivo evaluation of rumen-protected rice bran on urinary Ca excretion

Seven pregnant multiparous cows were used to test the effect of fat-coated rice bran on calcium metabolism. The animals were not lactating and their parity number ranged from 2 to 5. The cows were used for the experiment in their 5^{th}, 4^{th} and 3^{rd} week before their expected calving date. Data from cows that calved less than seven days after the end of the test period were excluded from the trial, to avoid interferences with the event of calving.

The experiment was divided in three periods of six, seven and seven days, respectively. Rumen-protected rice bran was fed only in the second period. The first period provided reference data for each cow to be its own control, and the third period was meant to illustrate any possible carry over effect of the product after withdrawal. In this way, the effect of the product on urinary calcium could be studied as the difference between periods 1 and 2, and the carry over effect as the difference between periods 1 and 3.

Urine samples were taken every day except on Saturdays and Sundays. The animals started the experiment on a fixed weekday to make the missing data from the weekends coincide in the same trial days for all animals.

The cows were transferred to the stable and tied three days before the beginning of their test period, in order to adapt to housing and diet. During period 1, the animals were fed the basic dry cow diet (Table 2). In period 2, they received two kg of the rumen-protected rice bran on the top of the basic diet. The three batches of fat-coated rice bran were used sequentially as the trial went on, and differences in composition between batches were accounted for in the calculation of nutrient intakes. At the end of period 2, the rumen-protected rice bran was removed from the diet and during period 3 the cows received the basic diet again. The amount of feed offered and leftovers were recorded daily for all animals.

Each working day from 8:30 am to 16:30 the cows were visited every two hours to obtain urine samples through manual stimulation of the vulva. Urine pH was determined for each sample with a digital pH meter, and frozen in 10 ml tubes at -20^{o}C.

Table 2. Composition of the daily diet ± standard deviation

Data		Period 1	Period 2	Period 3
DMI	kg DM	14.4 ± 2.9	11.2 ± 3.8	13.1 ± 2.4
Grass silage	kg DM	5.4 ± 1.2	3.4 ± 1.5	4.9 ± 1.0
Corn silage	kg DM	7.8 ± 1.7	4.9 ± 2.2	7.1 ± 1.3
Hay	kg DM	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
Concentrate	kg DM	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0
FCRB supplement	kg DM	---	1.9 ± 0.2	---

Urinary calcium was analysed with a photometric colour test (Ca-oCPO complex, Olympus Diagnostica GmbH, Hamburg, Germany) and urinary creatinine with a kinetic colour test (Jaffé method, Olympus Diagnostica GmbH). The forages and compound feed were analysed for DM, Ash, CP, CF, Starch, Sugars, NDF, ADF and calcium content.

Statistical analysis

For each urine sample the calcium creatinine ratio (mmol/l/g/l) was calculated and for each day the mean ratio was calculated and also linearly corrected for calcium intake. Data were transformed logarithmically to obtain a normal distribution. The resulting dataset was analysed by the mixed model procedure of SAS® (version 8.02; SAS Inst., Inc., Cary, NC, USA). Observations were considered as daily repeated measures on the subject cow, for which a First Order Autoregressive covariance structure was assigned. The model included day, treatment, animal and the interaction day x treatment. Weekends were considered as missing data to comply with the requirement of equal spaced observations. A multiple comparison test of LSMEANS was adjusted with the Tukey-Kramer method for the three different periods and for each two consecutive days throughout the trial.

Results

In situ evaluation of fat-coated rice bran

The in situ ruminal degradation curves of DM and phytic acid are shown in Figure 1. The rate of ruminal degradation of phytic acid was three times higher than that of DM in the fat-coated rice bran (Table 3). Passage rates of 4% and 5%/h were assumed to calculate the bypass fractions, estimated with the model of NRC 2001, which was validated by Seo et al., (2006). For these passage rates the bypass fractions of phytic acid were 26.3% and 30.3% respectively. This represents a duodenal delivery of 12 to 14 g of phytic acid per day.

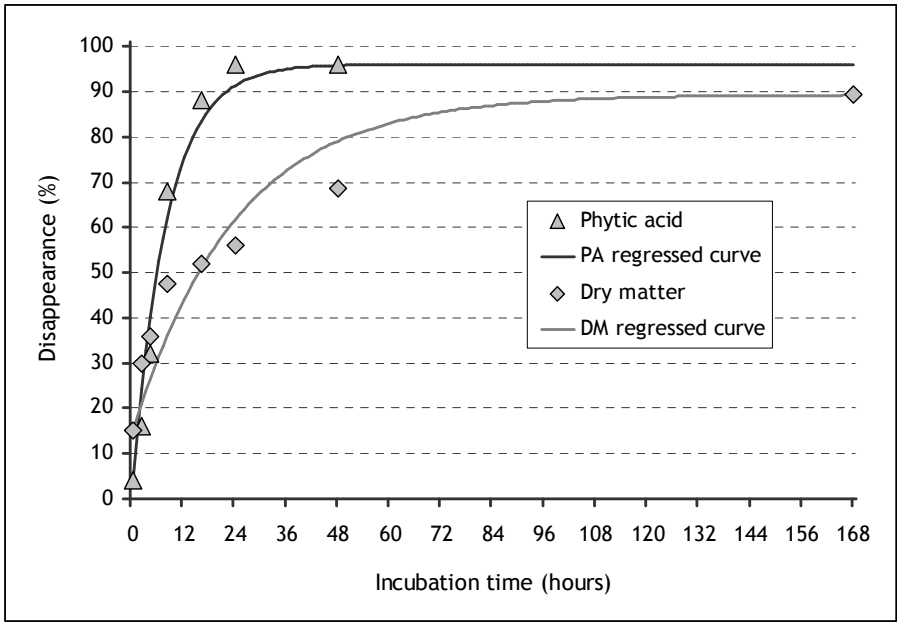

Figure 1. *In sacco* degradation of dry matter and phytic acid of fat-coated rice bran

Table 3. Degradation parameters of DM and phytic acid and estimates of rumen bypass fractions

	Washable fraction (%)	Undegr. fraction (%)	Degr. fraction (%)	Degr. rate kd (/h)	Bypass fraction(%)	
					kp=0.04/h	kp=0.05/h
DM	15.2	10.7	74.1	0.041	47.3	51.4
Phytic acid	4.0	4.0	92.0	0.125	26.3	30.3

Calcium intake

The cows showed a very sharp decrease in dry matter intake from 14.4± 2.7 to 11.3± 3.7 kg when the product was introduced in period 2 (Table 2), creating differences in all nutrient intakes (Table 4). This reduced feed intake resulted in significant differences ($p<0.01$) in calcium intake during that period compared with period 1 and period 3 (Table 5). Daily calcium intake decreased at the beginning of period 2 with a significant change between the last day of period 1 and the first day of period 2 (Figure 2).

Table 4. Daily nutrient intakes ± standard deviation

Data		Period 1	Period 2	Period 3
Ca	g	41.0 ± 9.0	26.4 ± 10.9	37.0 ± 7.5
P	g	42.0 ± 9.8	43.1 ± 12.9	39.3 ± 7.1
Na	g	8.4 ± 1.7	5.9 ± 2.2	7.7 ± 1.4
K	g	277.3 ± 62.1	191.4 ± 77.0	252.8 ± 49.1
Cl	g	37.1 ± 8.0	24.1 ± 10.0	33.6 ± 6.6
S	g	18.7 ± 3.9	14.8 ± 4.9	16.9 ± 3.3
DCAB	meq/kg DM	363.4 ± 21.0	307.5 ± 32.5	364.2 ± 5.2
Supplemental phytic acid	g	---	47.7 ± 9.3	---

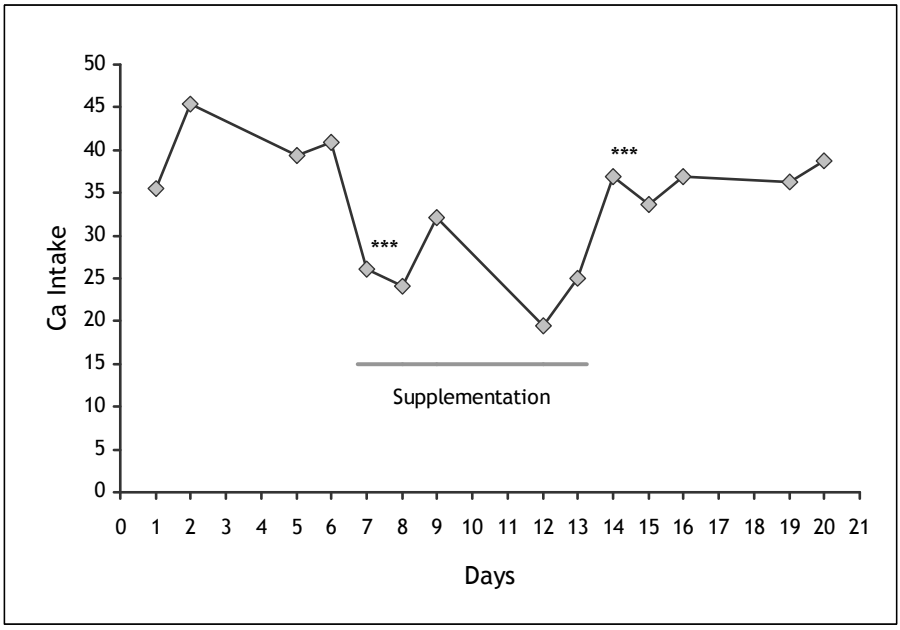

Figure 2. Evolution of Ca intake through the trial

*** Lsmeans are for that day significantly different from the day before $p<0.01$

Urine pH

Urine pH was high (around 8.5) throughout the trial for all animals (Table 5). During period 2 urine pH was one tenth of a point lower than in the other two periods ($p<0.05$).

Calcium excretion

Differences in calcium excretion among periods are given in Table 5. Urinary calcium creatinine ratio while feeding the fat-coated rice bran was significantly lower ($p<0.01$) compared with periods 1 and 3. Calcium creatinine ratio divided by daily calcium intake was lower in period 2 compared to period 1 ($p<0.05$) and in period 3, this ratio was higher than in both period 1 ($p<0.05$) and period 2 ($p<0.01$).

Regarding the mean daily calcium-creatinine ratio measurements, a sudden decrease in calcium excretion occurred at the start of the supplementation phase (Figure 3). Calcium excretion on day 7 was significantly lower ($p<0.01$) compared to

that of day 6. After linear correction for calcium intake the difference is much smaller and not statistically significant between day 6 and 7.

The most distinctive characteristic of the calcium excretion curve is the sharp increase after withdrawal of the product. On the first and the third day after the change from period 2 to period 3, calcium excretion shows significant differences ($p < 0.01$) when compared with the preceding day, increasing right after product withdrawal and then dropping again on the third day (Figure 3). Daily Ca excretion rate corrected for differences in daily calcium intake showed the same differences at product withdrawal as described for Ca creatinine ratio (Figure 4).

Table 5. Differences among LS means for calcium balance indicators

	1st period	2nd period	3rd period
DMI	a 13.97	bB 11.02	A 13.04
Ca intake (g/day)	a 39.86	b 25.64	a 36.73
Urine pH	A 8.50	B 8.42	A 8.51
Urine Ca/creatinine	a 0.3467	b 0.1084	a 0.6504
Urine Ca/creat./Ca intake x 100	A 0.8925	bB 0.4722	cC 1.8527

Differences in capital letters indicate p<0.05, differences in lower case letters indicates p<0.01

Discussion

The fat-coating applied to the rice bran in this trial was done with the purpose of testing the physiological principle of manipulating calcium homeostasis with rumen-protected rice bran, before searching for a more adequate rumen-protection method. Fat-coating has several known drawbacks: The dilution of phytic acid content, and the final high fat content which is inadequate for pre-calving diets because of its negative effect on dry matter intake (Douglas et al., 2004). The fat-coated product showed ruminal degradation characteristics that should deliver about 7g of phytic acid per kg of product to the duodenum. Also one kg of product contained on average 650 g of fat, which could have had an effect on calcium availability. It has been suggested that dietary fats can form insoluble calcium soaps in the rumen (Palmquist et al., 1986; Wilson, 2004). On the other hand, it has also been described that calcium soaps will dissociate completely in

the duodenum (Doreau et al., 1994) and that fat supplementation up to 5% does not impair calcium digestibility (Zinn et al., 1996).

The decrease of feed intake in period 2 concomitantly reduced calcium intake. Therefore, direct effects on urinary calcium excretion caused by the supplement can not be separated from indirect effects through the difference in calcium intake. However, calcium excretion is not proportional to calcium intake, because urine is not the major excretory route for calcium. Therefore, linear correction of calcium excretion by calcium intakes does not represent an appropriate way to separate the two effects. Regardless of these considerations, we have used a linear correction of the calcium creatinine ratio with total calcium intake as a parameter to evaluate calcium excretion relative to calcium intake.

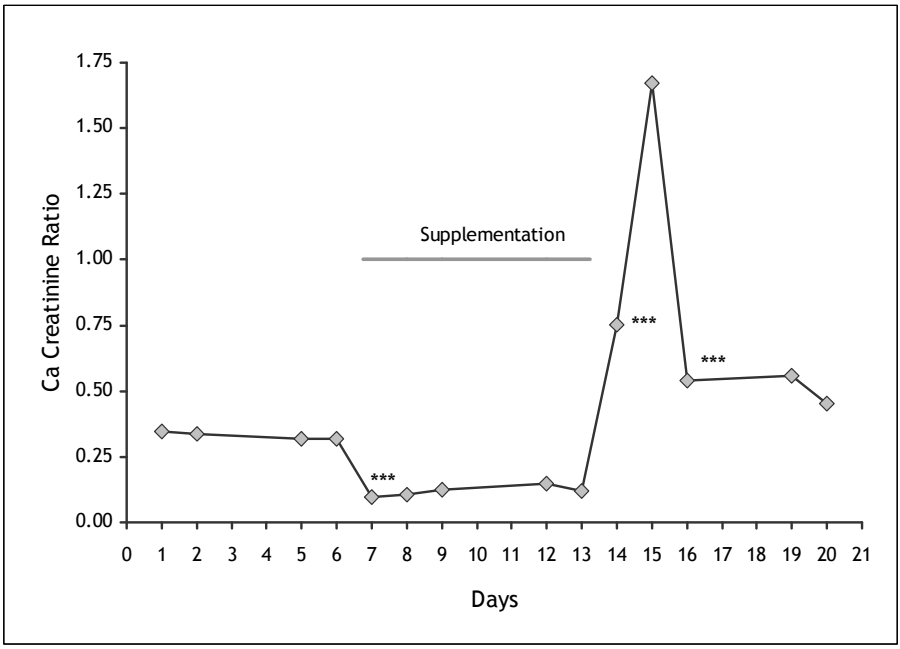

Figure 3. Daily evolution of the urine calcium creatinine ratio

*** Lsmeans are for that day significantly different from the day before $p<0.01$

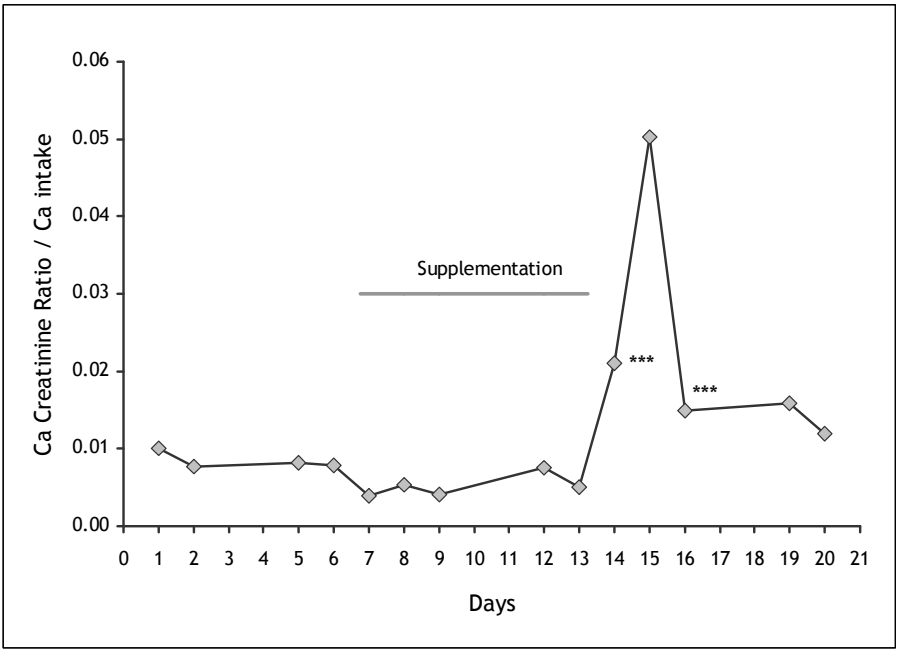

Figure 4. Daily evolution of the urine Ca creatinine ratio corrected for Ca intake
***Lsmeans are for that day significantly different from the day before $p<0.01$

Urine pH was throughout the trial very high, although the supplement slightly reduced it. This was in agreement with the positive dietary cation anion balance (DCAB) of the control diet, and the small reduction observed in period 2 (Table 4). Both urine pH and DCAB were very different from the recommendations set to trigger calcium metabolism in close up diets (Horst et al., 1997), because this was beyond the scope of the study. The milk fever prevention effects of DCAB and low calcium have been shown to be independent from each other in a meta-analysis (Lean et al., 2006).

Calcium excretion decreased in the supplementation period in a sudden way coinciding with product introduction. It can be concluded that in period 2 the animals experienced a difference in calcium availability as compared to period 1, expressed by the activation of urinary calcium resorption. Parathormone release reduces urinary excretion of calcium to minimum (Schröder and Breves, 2006). Unfortunately, parathormone was not measured in blood; however this rapid

change in urinary calcium can indirectly indicate that this hormone reacted to the lower calcium intake, or to the hypothetical lower calcium availability or to a combination of both.

At the end of period 2, the withdrawal of the coated fibre caused a clear peak in calcium excretion in the first two days, although only the increase from the last day of period 2 to the first day of period 3 was statistically significant. On the third day of period 3, calcium excretion showed a significant drop again. This indicates that after withdrawal, calcium homeostasis needs to readjust to the new intestinal availability of calcium. A similar effect in calcium excretion after calcium antagonist withdrawal is described by Enemark et al. (2003 a).

The evolution of calcium excretion could indirectly reflect that the supplementation period affected calcium homeostasis. A plausible explanation to the observations would be that parathormone would have in first instance activated renal reabsorption of calcium, and subsequently have increased intestinal absorption via the activation of 1-25 dihydroxyvitamin D. At product withdrawal, down regulation of parathormone would have inactivated renal calcium resorption in a rather short term, while the action of 1-25 dihydroxyvitamin D at intestinal level would have been maintained for two more days.

Conclusion

Fat-coated rice bran supplied about one third of the original content of phytic acid to the duodenum. However, supplementation of 2 kg of fat-coated rice bran to non lactating dairy cows had a strong negative effect on dry matter intake.

Feeding fat-coated rice bran influenced calcium homeostasis. However, it is not clear whether the observed effect was caused by a depressed calcium intake or by a reduced calcium availability created by phytic acid or a combination of both.

In order to test the effect of rumen bypass phytic acid from rice bran, an alternative protection method is necessary. Further research with different protection techniques is needed to elucidate if protecting rice bran from ruminal degradation is a feasible way to reduce calcium availability in the close up period, with the ultimate goal of stimulating calcium homeostasis and preventing milk fever.

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CHAPTER 3

In situ ruminal degradation of phytic acid in formaldehyde-treated rice bran

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Abstract

Rice bran has a very high content of phytic acid (IP6), which is a nutritional antagonist of Ca. Microbial phytase degrades IP6, but ruminal degradation of nutrients can be reduced by formaldehyde treatment. Milk fever in dairy cows can be prevented by reducing available dietary Ca to stimulate Ca homeostasis. In the present study, effects of formaldehyde treatment (FT) on ruminal degradation of IP6 in rice bran were investigated. Two samples of full-fat rice bran were treated with 4 levels of formaldehyde (i.e., 0, 1000, 2500 and 5000 ppm fresh weight) and ruminally incubated in situ for 0, 4, 8, 16, 32, 64 and 336 hours in 3 rumen-fistulated lactating dairy cows. Dry matter (DM) disappearance was determined, residues were analysed for P and, for one of the products, also for inositol phosphate (IP) forms. Degradation parameters were calculated for DM, P, total IP, and IP6. The in situ washable fraction (W), undegradable fraction (U) and degradable fraction (D) were measured and the rate of degradation (kd) was calculated by exponential regression to the equation: Y(t) = U + D*EXP(-kd*t). HPLC analyses confirmed that most P in the original sample and residues was phytate, mostly IP6. DM and P degraded differently in the two rice brans and FT reduced degradability, lowering W and increasing D and kd. The calculated rumen escape (kp=0.05/h) for P increased from 0.082 at 0 ppm to 0.136, 0.284 and 0.398 at 1000, 2500 and 5000 ppm of FT respectively. Degradation of total IP forms and IP6 corresponded with P disappearance. Formaldehyde treatment reduced W in total IP and IP6, proportionally increasing D, while U was 0 in both. Kd decreased with FT in total IP and IP6, which decreased from 0.309/h at 0 ppm to 0.217, 0.116 and 0.071/h as FT level increased. The calculated rumen escape (kp=0.05/h) of IP6 were 0.079, 0.126, 0.229 and 0.318 for the increasing FT levels. Formaldehyde treatment reduced rumen degradability of IP6 in rice bran. One kg of formaldehyde treated rice bran could bind 7 g of dietary Ca post ruminally, making it a potentially feasible tool to decrease intestinal Ca availability to aid in the prevention of milk fever.

Introduction

Rice bran is an inexpensive by-product of rice milling that is commonly used as an animal feed. The practical limitation for its use in foods or feeds is the susceptibility of its fat to rancidity (Goffman and Bergman, 2003). In recent years many studies have suggested a beneficial health effect provided by the antioxidant and cholesterol reducing compounds in rice bran (Chen and Bergman, 2005). Among these beneficial effects of rice bran, its ability to reduce Ca availability has been proposed for prevention of kidney stones in humans (Ohkawa et al., 1984). This property is related to its high phytic acid content, which is a strong antagonist to Ca absorption.

Phytate is the most prominent storage form of P in grains and legume seeds. It consists of the sugar molecule myo-inositol and up to six attached phosphate groups. The form with six phosphate groups is called phytic acid, or myo-inositol hexaphosphate (IP6), and is the most common in feeds.

Dietary actions to reduce Ca availability of dairy cows close to calving have been shown to prevent parturient hypocalcaemia (Thilsing-Hansen et al., 2002). However, use of rice bran for this purpose is limited because dietary IP6 is degraded to a large extent in the rumen (Morse et al., 1992), thereby nullifying its antagonistc effects to Ca absorption. To be able to use rice bran as a Ca antagonist for prevention of milk fever, ruminal degradation of IP6 must be prevented.

Ruminal degradation of IP6 seems to be connected to that of protein. Techniques to make proteins higher in rumen escape characteristics, such as formaldehyde treatment, appeared to also reduce IP6 degradation (Park et al., 1999; Bravo et al., 2002). Moreover, heat treatment of soybean and rapeseed meal diminished ruminal degradation of IP6 (Konishi et al., 1999).

Formaldehyde treatment is simple and inexpensive. However, its use is constrained by legal and practical restrictions because it is a potential toxin. Formaldehyde has known carcinogenic effects, but it is also a naturally occurring substance in foods and animals can metabolize it to some extent without adverse effects (Owens et al., 1990). In a typical ruminal protection application, transfer of formaldehyde into animal products is negligible, and final product levels are within the range of natural occurrence of formaldehyde in foods, which means it is not a direct food

safety hazard (Gulati et al., 2005). However, formaldehyde treatment of feedstuffs represents a hazard in the manufacturing process, and precautions need to be taken to guarantee labour safety.

The present study was undertaken to evaluate effects of formaldehyde treatment of rice bran on the ruminal in situ disappearance of dry matter (DM), P and the different phytate forms.

Materials and methods

Description of the two rice bran samples

Two rice bran samples were obtained from feed factories in Europe. Rice bran 'A' was obtained from Nanta Zaragoza (Zaragoza, Spain) and rice bran 'B' from Hendrix Haeck (Ingelmunster, Belgium). Both samples were analysed for DM (European Commission, 1971a), ash (European Commission, 1971b), ether extract (EE) (European Commission, 1998), crude protein (CP) (European Commission, 1993), starch (NEN 3574/C1, 1979), and sugars (European Commission, 1971a). Neutral detergent fibre (NDF) was analysed with alpha amylase (Van Soest et al., 1991), but without sodium sulphite, and is expressed without residual ash. Acid detergent fibre (ADF) was also analysed according to Van Soest et al. (1991) and expressed exclusive of residual ash. Lignin was analysed using sulphuric acid according to Robertson and Van Soest (1981). Calcium was analysed by acid digestion with hydrochloric acid and later determination with an atomic absorption spectrophotometer (Perkin Elmer AAnalyst 800). Phosphorus was analysed by spectrophotometry (AOAC, 2005; 4.8.14). Oxalic acid was analysed according to Canale et al. (1984). Phytic acid content was analysed by a colorimetric method (AOAC, 2005; 965.17) based on reaction of vanadomolybdate on inorganic phosphate produced by action of 6-phytase on phytic-containing substrate. Additionally, inositol phosphate (IP) fractions of the original rice bran samples were analysed by HPLC as described in Muzquiz et al. (1999) using a PRP-1 column (150 x 4.1 mm, 5µm) at 45ºC, with a refractive index detector and 10 µL injection volume.

Formaldehyde treatment

The two rice bran samples were treated with formaldehyde in a similar way to that by Subuh et al. (1994) and Park et al. (1999). Four subsamples of three kilograms of

each product were each sprayed with 600 mL of various dilutions of formalin (370 g/L formaldehyde) in water to obtain four levels of formaldehyde in the product being: 0, 1000, 2500 and 5000 ppm fresh weight. The subsamples were again divided into three portions of one kg, which were placed in large transparent plastic bags. The spraying device was fitted to the bag with rubber bands to prevent volatilisation losses of formaldehyde. The rice bran inside the bag was spread in a thin layer and the solutions were finely sprayed over it. The product was then mixed and spread again to repeat the process. After 200 mL had been sprayed into each bag, the device was removed and the bags were closed air tight. The powder was then vigorously shaken for five minutes and left in storage for three days at room temperature (i.e., 18˚C to 22˚C). After that time, the three portions of each subsample were pooled, thoroughly mixed and spread in trays and left to dry in open air at room temperature. One week later, the samples were again placed in plastic bags and DM was determined. This procedure led to eight products for ruminal in situ degradation studies, originating from two rice brans, and four formaldehyde treatment levels (i.e., 0, 1000, 2500 and 5000 ppm).

In situ procedure

Three Holstein cows fitted with a 10 cm diameter rumen fistula (Bar-Diamond, Parma ID, USA) were used for the ruminal in situ incubation (Ørskov and Mc Donald, 1979). The cows were housed in a free stall barn and chosen to have a milk production of at least 15 litres throughout the study.

A total of 1224 nylon bags, each measuring 10 x 20 cm and with a 40 µm pore size (Nybolt PA37/24, Kabel Zaandam BV, Zaandam, The Netherlands), were filled with approximately 5 g DM of sample. Each of the 8 products was incubated in the three cows in an incubation scheme of 4, 8, 16, 32, 64 and 336 hours. Bags were introduced at different times and extracted at once, rinsed in cold tap water, washed in a washing machine (Brandt AA-Class, Brandt Industries, Rueil Malmaison, France) tap water temperature, wool wash program without centrifuging) and dried at 70 ˚C for 48 hours. The number of bags per cow and incubation period was adjusted to provide a minimum residue of 10 grams per product and time point. A maximum of 32 bags was incubated at the same time in each cow, which made four consecutive incubation periods necessary. Prior to analysis, nylon bag residues were pooled over incubation periods and within cow and time points. For each of

the 8 samples of treated rice bran this procedure gives individual residues for each incubation time point and within each individual cow. The washable fraction (i.e., 0 hour incubation) was determined by machine washing the samples in tap temperature water.

Both products and their residues were analysed for DM and P. In addition, residues of 'A' were also analysed for IP forms as described above. Disappearance of DM, P, total IP and IP6 were calculated relative to the initial content of these components.

Calculations and statistical analysis

The analytical determinations on the original products were processed through analysis of variance. Duplicate analyses of IP forms done on the original rice bran 'A' and the formaldehyde-treated samples, were statistically processed to contrast the difference between treated and untreated samples and contrast linear and quadratic effects of formaldehyde dose on IP profile.

Residue data from the 8 products incubated in the three cows were used to fit 24 exponential degradation curves. The washable fraction (W) was defined as disappearance at time=0, and the undegradable fraction (U) was assumed to be the residue after 336 incubation hours. The degradable fraction (D) was calculated from the washable fraction (W) and the U fraction as: D = 100 – W – U. (Ørskov and McDonald, 1979).

The degradation rate (kd) was determined by non-linear regression fitting an exponential curve with the residues of different time points with the NLIN procedure of SAS (1999) as: Y = U + D*EXP(-kd*time) where U was the undegradable fraction and D is the degradable fraction. These parameters were used to calculate ruminal escape fractions (i.e; RE g/kg), defined as the fraction of the original matter that would escape ruminal disappearance for a fixed passage rate (Kp). Calculation was done through the equation: RE = U+ D* kp/(kp+kd) (Ørskov and McDonald, 1979), where kp was the fractional passage rate expressed per hour. Rumen escape fractions were calculated with kp of 0.05/h, which was chosen from an estimate of passage rate calculated with the feed intake of a close-up ration with the NRC model (2001), which was validated by Seo et al. (2006).

Degradation characteristics of DM and P were statistically analysed by the MIXED procedure in SAS (1999) with the model: Y = original product + formaldehyde level + original product X formaldehyde level + cow. Degradation characteristics of total IP and IP6 were analysed by the MIXED procedure for the model: Y = formaldehyde level + cow. In both cases, the method was REML NOBOUND and DDFM option was KENWARDROGER, given the structure of the data as suggested by Spilke et al. (2005). Least squares means were compared between formaldehyde treatment levels and products. Degradation parameters were tested for linear and quadratic polynomials against formaldehyde level. Inositol phosphate forms of less than 6 phosphate groups were not analysed with exponential degradation curves because they are not only subject to disappearance as they lose phosphate groups but they also appear as higher forms lose phosphate groups.

Results

Product characteristics

The two rice bran samples used in the study were full-fat rice bran products and were similar in composition (Table 1). The main difference was the higher sugar content and the lower starch content of rice bran 'A'. Calcium was much higher in rice bran 'B' as compared with rice bran 'A' which had a much higher IP6 content than rice bran 'B' as reflected indirectly by the higher P content. HPLC determination confirmed the higher IP6 content of rice bran 'A', although the difference was much smaller. IP6 was 11 g/kg lower in product 'B', but IP5 was 3 g/kg higher, therefore total IP was only 8 g/kg lower (Table 1).

The formaldehyde treatments applied to the rice brans affected the IP profile (Table 2). Treatment with water numerically decreased phytate content compared to the original product. Formaldehyde-treated samples also showed reduced phytate contents but this effect was reduced as formaldehyde dose increased.

Dry matter and P degradation

The washable DM fraction differed between the rice bran products, but for both products it was clearly reduced by formaldehyde treatment (Table 3). This effect became smaller as formaldehyde dose increased, and seemed to be independent of rice bran source. The U fractions of the two rice bran products differed and had small changes with formaldehyde treatment. Dry matter kd differed between

sources and was strongly diminished with increasing formaldehyde treatment. This reduction was significantly correlated with formaldehyde dose in rice bran 'B' but not in rice bran 'A'.

Washable P was reduced to half by 1000 ppm of formaldehyde treatment and decreased even further with higher doses (Table 4). The washable P fraction differed between sources, and the effect of formaldehyde was greater for rice bran 'A'. The undegradable P fraction was very small and was affected by neither treatment nor product. Degradation rate of P differed between sources and was clearly influenced by formaldehyde treatment. In both products, formaldehyde reduced kd. The same applied to the calculated rumen escape fractions of P. Both rice bran 'A' and 'B', treated at 5000 ppm formaldehyde, resulted in an escape of undegraded P of about 7 g/kg of original product, more than one third of the original content of rice bran 'A' and nearly half that of rice bran 'B'.

Table 1. Chemical composition of the two original rice brans obtained from different locations (g/kg DM)

	Rice Bran 'A'	Rice Bran 'B'	SEM	P
DM	899	911	0.5	<0.01
CP	169	150	<0.1	<0.01
EE	220	188	1.8	<0.01
Ash	89	123	0.4	<0.01
aNDF	213	196	4.6	0.04
ADF	86	72	0.8	0.06
Lignin	34	29	4.3	0.48
Starch	246	334	0.8	<0.01
Sugar	82	41	1.2	<0.01
Ca	0.54	24.85	0.672	<0.01
P	21.02	16.84	0.965	0.09
Phytic acid*	56	33	0.4	<0.01
Oxalic acid	13	10	0.3	<0.01
Total IP	69.10	61.61	0.212	<0.01
IP6	64.21	53.39	0.202	<0.01
IP5	2.76	5.93	0.072	<0.01
IP4	1.16	1.38	0.061	0.12
IP3	0.97	0.91	0.014	0.10

**Colorimetric method*

Table 2. Inositol phosphate (IP) fractions in rice bran 'A' before and after treatment with different levels of formaldehyde (g/kg DM)

	Formaldehyde (ppm fresh weight)						P		
	raw	0	1000	2500	5000	SEM	raw vs. 0	Lin.	Quad.
Total IP	69.13	55.85	59.65	63.64	67.44	0.147	<0.01	<0.01	<0.01
IP6	64.24	49.93	55.17	58.83	62.29	0.132	<0.01	<0.01	<0.01
IP5	2.76	3.51	2.64	2.91	3.15	0.035	<0.01	0.05	0.05
IP4	1.16	1.74	1.09	1.16	1.24	0.021	<0.01	0.02	0.03
IP3	0.97	0.67	0.75	0.74	0.76	0.003	<0.01	0.06	0.14

Inositol phosphates degradation

HPLC analyses on the incubation residues of rice bran 'A' illustrate the disappearance of IP forms from the product for the different formaldehyde treatments (Table 5). The W fraction of total IP of the untreated rice bran is quite high (384 g/kg), but formaldehyde linearly reduced this fraction to 190 g/kg at 5000 ppm. The U fraction of total IP6 was 0 at all formaldehyde doses. Degradation rate was reduced progressively by increasing doses of formaldehyde treatment. The rumen escape of total IP increased as formaldehyde dose increased. For the 5000 ppm dose, it was calculated to be 0.332 for a 0.05/h passage rate. Rice bran 'A' treated with formaldehyde at 1000, 2500 or 5000 ppm could deliver 8.4, 15.1 or 21.3 g, respectively, of undegraded total IP per kg of original product to the duodenum, as compared to 5.5 g in the untreated product.

Phytic acid was the most common form of phytate in the original product. Its degradation pattern (Table 6) was similar to that described for total IP in Table 5. Washable fraction of IP6 was higher than that of total IP, and was similarly affected by formaldehyde treatment. There was no U fraction for IP6, as it was not found in the 336 hour residues. The kd of IP6 was strongly reduced by formaldehyde treatment from 0.309/h in the original product to only 0.071/h with 5000 ppm formaldehyde. Estimated rumen escape fractions increased with formaldehyde treatment and were quantitatively equivalent to those for total IP.

The profile of IP forms in product 'A' through incubation time is illustrated in Figure 1. At time 0, washing reduced IP6 and caused a slight increase of IP5. Ruminal incubation extensively degraded IP6 during the first 4 hours and affected IP5, IP4 and IP3 in a similar proportional way. Formaldehyde treatment prevented IP6 degradation, as well as degradation of IP5, IP4 and IP3.

Discussion

The composition of the two rice bran products used in the experiment differed substantially in Ca, P and IP6 content. The difference in P content between the two products agrees with the large variation described for P in rice bran by NRC (2001). They describe 18 g/kg DM as an average with a standard deviation of 4 g/kg. This variation in P directly reflects the variability of IP6 content because most P in rice bran is in the IP6 form (Ravindran et al., 1994). Rice bran 'B' had 25 g/kg Ca compared with a level of only 0.5 g/kg in rice bran 'A'. This is most likely related to use of Ca carbonate as abrasion agent in the milling technique for rice bran 'B', which is one of the possible ways to polish rice (Marshall and Wadsworth, 1994). If rice bran is to be used as a Ca absorption antagonist, sources produced with Ca carbonate should be avoided.

Analyses of the IP profile confirmed that IP6 was the main form of phytate in these two rice bran products. Despite differences in total IP content, the proportions of the different forms of IP were similar, except for a slightly higher IP5 fraction in rice bran 'B'. It is remarkable that products of different origin and produced apparently with different methods, had similar profiles of IP forms even if total IP level was different.

Total IP and IP6 content were reduced by spraying of formaldehyde solutions. Water treatment without formaldehyde (i.e., 0 ppm) had the largest reduction in total IP and IP6. The presence of formaldehyde in the solution reduced this effect as dose increased. Also, incubation for 0 hours by machine washing only showed that plain water can wash away a substantial fraction of phytate from the product. The solubilised phytate may have separated from the sample in the treatment process and remained behind in the plastic bag. Formaldehyde, in contrast, reduced phytate solubility, which would explain why phytate losses were much smaller with increasing formaldehyde dose. IP5 and IP3 losses presented a weaker correlation with formaldehyde treatment than total IP and IP6.

Table 3. In situ ruminal degradation characteristics of DM from rice bran A and B treated with different levels of formaldehyde

	Formaldehyde level (ppm fresh weight)									P						
	Rice bran 'A'				Rice bran 'B'					Rice bran			Rice bran 'A'		Rice bran 'B'	
	0	1000	2500	5000	0	1000	2500	5000	SEM	Lin.	Quad.	RB x Level	Lin.	Quad.	Lin.	Quad.
Washable DM (g/kg)	309	195	127	97	194	125	62	57	17.4	<0.01	<0.01	0.23	--	--	--	--
Degradable DM (g/kg)	590	708	791	802	730	801	862	863	18.7	<0.01	<0.01	0.21	--	--	--	--
Undegradable DM (g/kg)	101	97	82	101	77	74	77	80	4.4	0.04	0.02	0.17	--	--	--	--
Kd of DM (/h)	0.07	0.09	0.05	0.04	0.07	0.05	0.04	0.03	0.002	--	--	<0.01	0.36	0.94	<0.01	<0.01
Rumen escape DM (Kp=0.05/h)	0.35	0.36	0.49	0.57	0.39	0.48	0.58	0.63	0.008	--	--	<0.01	<0.01	0.35	<0.01	<0.01

Table 4. In situ ruminal degradation characteristics of P from rice bran 'A' and 'B' treated with different levels of formaldehyde

	Formaldehyde level (ppm fresh weight)								SEM	RB x Level	P			
	Rice bran 'A'				Rice bran 'B'						Rice bran 'A'		Rice bran 'B'	
	0	1000	2500	5000	0	1000	2500	5000			Lin.	Quad.	Lin.	Quad.
Washable P (g/kg)	470	201	83	73	318	147	105	62	16.8	<0.01	<0.01	<0.01	<0.01	0.02
Degradable P (g/kg)	524	794	912	923	677	848	890	933	16.8	<0.01	<0.01	<0.01	<0.01	0.02
Undegradable P (g/kg)a	6	5	5	5	5	5	5	5	0.2	0.91	--	--	--	--
Kd of P (/h)	0.30	0.26	0.11	0.07	0.21	0.10	0.06	0.05	0.012	<0.01	<0.01	0.06	<0.01	<0.01
Rumen escape fraction P (Kp=0.05/h)	0.08	0.14	0.28	0.40	0.14	0.30	0.42	0.48	0.006	<0.01	<0.01	0.05	<0.01	<0.01
Rumen escape P (Kp=0.05/h) (g/kg rice bran)	1.5	2.4	5.0	7.0	2.1	4.4	6.3	7.1	0.10	<0.01	<0.01	0.05	<0.01	<0.01

a -- effect of RB Linear P=0.03; Quadratic P=0.16.

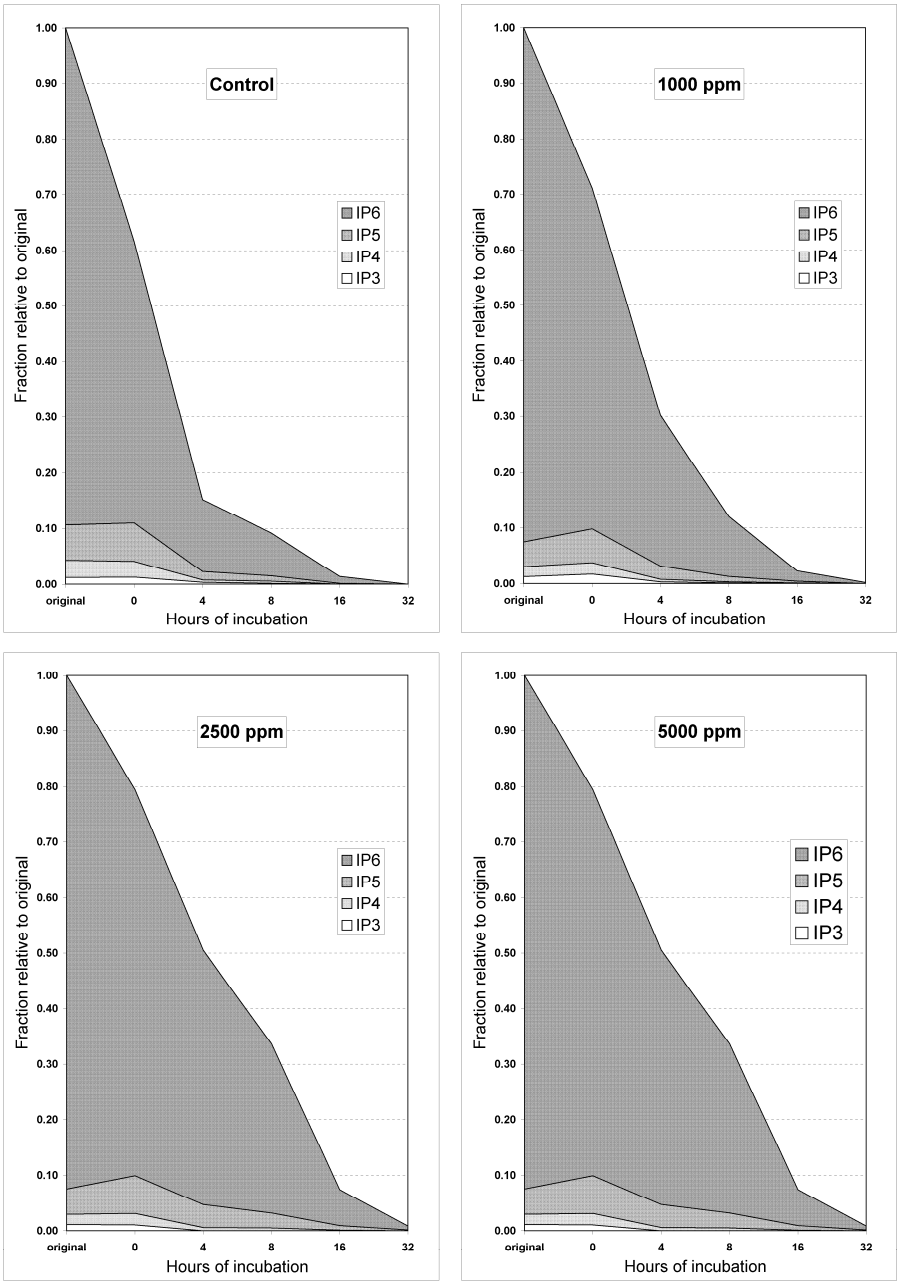
Table 5. In situ ruminal degradation characteristics of total inositol phosphate (IP) forms from rice bran A treated with different levels of formaldehyde

	Formaldehyde level (ppm)				SEM	P	
	0	1000	2500	5000		Linear	Quad.
Washable Total IP (g/kg)	384	289	206	190	36.5	<0.01	0.08
Degradable Total IP (g/kg)	616	711	794	811	36.5	0.02	0.08
Undegradable Total IP (g/kg)	0	0	0	0	--	--	--
Kd Total IP (/h)	0.32	0.23	0.12	0.07	0.002	<0.01	<0.01
Rumen escape Total IP (Kp=0.05/h)	0.09	0.13	0.24	0.33	0.057	<0.01	0.05
Rumen escape Total IP (Kp=0.05/h) (g/kg rice bran)	5.5	8.4	15.1	21.3	0.37	<0.01	0.05

Table 6. In situ ruminal degradation characteristics of phytic acid (IP6) from rice bran A treated with different levels of formaldehyde (g/kg)

	Formaldehyde level (ppm)				SEM	P	
	0	1000	2500	5000		Linear	Quad.
Washable IP6 (g/kg)	433	337	248	230	32.9	<0.01	0.05
Degradable IP6 (g/kg)	567	663	752	770	32.9	<0.01	0.05
Undegradable IP6 (g/kg)	0	0	0	0	--	--	--
Kd IP6 (/h)	0.31	0.22	0.12	0.07	<0.001	<0.01	<0.01
Rumen escape IP6 (Kp=0.05/h)	0.08	0.13	0.23	0.32	0.061	<0.01	0.04
Rumen escape IP6 (Kp=0.05/h) (g/kg rice bran)	4.5	7.1	12.9	18.0	0.34	<0.01	0.04

Figure 1. Evolution of inositol phosphate fractions through incubation time



The effect of formaldehyde treatment on degradation of DM was especially clear for the W fraction and kd. Increasing doses of formaldehyde progressively decreased the W fraction to one third for the highest level compared to control. The W fraction of DM of untreated rice bran 'A' was 309 g/kg, in agreement with the results of DePeters et al. (1997), who determined an average of 328 g/kg in three rice bran samples. Product 'B' had a lower W fraction, which could be related to its higher starch and lower sugar contents. The ruminal D fraction increased with formaldehyde treatment as a result of the reduction of the W fraction. The effect of formaldehyde treatment on the U fraction was quantitatively negligible. Formaldehyde treatment had a very marked effect in reducing the ruminal kd, consistent with observations in other raw materials (Bravo et al., 2002; Wulf and Südekum, 2005).

The W fraction of P was higher than that of DM. This indicates that the DM which leaves the bag after a short time of incubation contained relatively more P than the raw material. This was also found by Bravo et al. (2002). Formaldehyde treatment with 1000 ppm reduced the W fraction to about half in both rice brans. Treatment with higher formaldehyde levels further reduced the W fractions of the bran samples, although the difference between 2500 and 5000 ppm was smaller.

The reduction in degradability caused by formaldehyde treatment seemed to be stronger for P than for total DM. Rumen escape fractions of undegraded P increased with the level of formaldehyde treatment, but clear differences in rumen escape P between the two rice bran products occurred, probably related to differences in P content. However, compensation by the different W fractions and kd resulted in similar amounts of undegraded P per kg of original product for the 5000 ppm treatment.

Ruminal disappearance of IP in product A was affected by treatment in the same way as was for P disappearance. The W fraction was strongly reduced by formaldehyde treatment. Phytates were not detected in the undegradable residue, which indicates that the nature of the P in the residue is not phytic. The kd of total IP was strongly reduced by formaldehyde treatment similar to that of total P. Degradation characteristics of the individual IP forms revealed that IP6 remained the most common IP form throughout the process of ruminal digestion, as

illustrated in Figure 1. The evolution of the IP forms in the residues showed that formaldehyde treatment decreases ruminal degradation of all IP forms in an equivalent manner, because none of the IP forms accumulated in the residues or degraded away faster than others.

The reduction in IP degradation by formaldehyde yielded a substantially different estimate of rumen escape of total IP for each treatment level. Total IP escape increased to as much as 0.332 of the total at 5000 ppm formaldehyde. Expressed as grams per kilogram of original rice bran, total IP increased from 5.5 g for the control (water treatment) to 21.3 g for 5000 ppm formaldehyde. Considering a Ca phytate binding molar ratio of 6 to 1, the 21 g of phytate supplied by rice bran 'A' treated with 5000 ppm formaldehyde could potentially bind about 7 g of daily dietary Ca. This estimate accounts only for the fraction that remains within the nylon bag residues. Phytate, however, could bind Ca in the rumen before it is dephosphorilated by ruminal phytase, but our experimental approach did not provide insight into any process beyond nylon bag disappearance. Sansinena (1999) described that IP6 degradation in the rumen is strongly reduced by dietary Ca, and their results suggest that IP6 binds Ca already in the rumen, thereby preventing the action of ruminal phytase. Therefore, it could be expected that not only the rumen escape fraction of phytate may reduce Ca availability and that that the actual potentially bound Ca exceeds the above estimate of 7 g/kg product. The effect of formaldehyde-treated rice bran on Ca availability could be relevant in activating Ca homeostasis in dry cows (Chapter 2).

Degradation characteristics of IP show that, through the process of ruminal disappearance, IP6 remains as the most common IP form (Figure 1). The effect of formaldehyde treatment on disappearance of IP6 was similar to that for total IP. However, washing with water seemed to decrease the fraction of IP6 in the samples at time=0. The evolution of the IP forms of the residues showed that formaldehyde treatment decreases ruminal degradation of all forms in an equivalent manner because none of the IP forms accumulated in the residues or degraded faster than others.

Conclusions

Formaldehyde treatment reduced DM and phytate degradation in two rice bran samples. In both samples, IP6 was the most prominent form of phytate. For product 'A', where the different phytate forms were analysed, IP6 remained the most prominent form as the product was degraded in the rumen. Formaldehyde-treated rice bran could supply enough rumen escape IP6 to potentially bind post-ruminally 7 g of dietary Ca /kg product. By reducing Ca availability, formaldehyde-treated rice bran could be a means to activate Ca homeostasis in dry cows. However, in vivo data are necessary to confirm this antagonistic effect on Ca absorption, as well as to study its potential role in prevention of milk fever.

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CHAPTER 4

Effect of feeding rumen-protected rice bran on mineral status of non-lactating dairy heifers

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Summary

Adapting Ca homeostasis of dairy cows before calving can prevent milk fever. Rice bran, treated with formaldehyde to prevent ruminal degradation of phytic acid, was fed to heifers to study its effect on Ca homeostasis. For 3 weeks 18 heifers were supplemented 3 kg of 2 feeds: placebo (PF) and Rice Bran (RBF), defining 3 treatments: control (CRT), low dose (LD) and high dose (HD). In week 1 and 3 all animals received 3 kg of PF and in week 2: CRT received 3kg of PF, LD 1.5 kg of PF and 1.5 kg of RBF and HD 3 kg of RBF. Treatments did not affect DMI. Feed intakes and growth rates indicated that all heifers had Ca intakes below their nutritional requirements. Serum Ca, urinary Ca, calcitriol or hydroxyproline remained unaffected. Urinary Ca was consistently low indicating high renal Ca resorption, which is indicative of insufficient Ca supply. RBF influenced P, Mg and Zn intakes and serum and urine presence. Most heifers already presented an up-regulated Ca metabolism, being inadequate to study adaptive changes in Ca homeostasis of multiparous dry cows. This metabolic difference can be explanatory to the very low susceptibility of heifers to milk fever, further supporting the induction of homeostatic adaptation before calving to prevent milk fever. RBF did not reduce DMI, and was not detrimental to P, Mg or Zn status.

Introduction

Calcium homeostasis of the modern dairy cow deserves most attention in its adaptation at the event of calving. The cow turns from a situation where dietary Ca exceeds the maintenance requirements, into a sudden increase of demand brought by the start of lactation. To meet these demands, the animal needs to increase intestinal Ca metabolism and reduce urinary Ca excretion. These metabolic adaptations constitute a reversal of the direction of Ca homeostasis. Failure to do this rapidly enough may result in different degrees of hypocalcaemia, which is commonly referred to as milk fever.

Adaptation of the control mechanisms of Ca homeostasis several weeks before calving has proven to be an effective way to prevent milk fever. Low Ca diets (Green et al. 1981) and feeding antagonists of Ca availability such as zeolites (Thilsing-Hansen et al. 2002) have shown to effectively reduce hypocalcaemia at calving. The principle of these strategies is to initiate active intestinal Ca absorption to break the quiescence of this mechanism weeks before the sudden increase of Ca needs.

The use of rumen-protected rice bran to reduce Ca availability has been proposed as an alternative to low Ca diets in the prophylaxis of milk fever (Chapter 2). Rice bran naturally contains has a low Ca content, 0.39±0.01 g/kg DM (Farrell, 1994) and is very rich in phytate, approximately 65.3±9.6 g/kg DM (calculated from phytate P reported by Selle et al., 2003, assuming 28.2%P in phytate and 86%DM). The low Ca content in rice bran reduces Ca intake, and the phytic acid will precipitate Ca, either in the rumen or, for phytic acid that escapes the rumen, further in the gastrointestinal tract, making Ca unavailable for absorption.

Normally dietary phytic acid is extensively broken down in the rumen by the action of ruminal phytase (Morse et al., 1992). However, various studies have shown that it is possible to significantly increase the rumen escape fraction of phytic acid in rice bran by techniques such as fat coating (Chapter 2) and formaldehyde treatment (Chapter 3).

Phytic acid in rice bran can reduce Ca availability, but not only, also can Zn availability be affected especially in combination with Ca (Fordyce et al., 1987). The purpose of this trial was to test the effects of ruminally-protected phytic acid,

therefore assessing the effect of formaldehyde-treated rice bran on Zn status was necessary. Furthermore, phytic acid can chemically complex Mg (Crea et al., 2008), although the nutritional effect on this nutrient seems to be negligible (Coudray et al., 2003). Rice bran feeding implies also an increased dietary supply of P, thus it was necessary to study its effects on P status.

Heifers before calving could be used as a model for the non-lactating dairy cow. Replacement heifers are easily available non-lactating animals in dairy herds, in contrast with multiparous dry cows, which are only available for few weeks per year. Nevertheless, heifers present substantial differences in their Ca metabolism with multiparous cows, including their Ca requirements for growth and their apparent lack of susceptibility to clinical milk fever.

The present trial intended to study the stimulation of Ca homeostasis by feeding formaldehyde-treated rice bran, and to explore whether heifers are suitable animals to study this process. Furthermore, this trial studied the effects of rumen-protected rice bran on voluntary DMI, and P, Mg and Zn status of dairy heifers.

Materials and methods

The protocol of the study was approved by the Ethical Committee of Animal Experiments (DEC) of the University of Nijmegen (Nijmegen, the Netherlands).

Eighteen pregnant heifers were selected from the replacement stock of Kempenshof (the experimental station of Nutreco) in Boxmeer, the Netherlands. The replacement heifers were chosen among those confirmed pregnant, but those close to calving were discarded in order to avoid any interference with Ca metabolism at the start of lactation. All animals were older than 14 months and younger than 2 years, and were less than 260 days pregnant. One of the heifers was found to not have been pregnant during the trial, but data of this animal were included in the statistical analyses.

The heifers were blocked in 6 blocks of 3, according to their weight and month of gestation, and randomised in 3 dietary treatments. The experiment was carried out in 3 periods of 3 weeks each. In each period, 6 heifers were used. During these 3 weeks intakes were recorded and urine and blood samples were collected daily. Heifers were housed in a tie stall and were fed individually. The diet consisted of a forage mixture fed ad libitum and 3 kg of concentrates. The forage mix consisted

of grass silage, corn silage and hay. The concentrates consisted of 2 different pelleted compound feeds according to the experimental design and treatment assignment.

Two different batches of full-fat rice bran were obtained from a feed plant in Belgium (Hendrix Haeck, Ingelmunster, Belgium) and were mixed in equal amounts. This mixture of the 2 products was brought into a mixer with formalin (370g/litre formaldehyde) to obtain a final formaldehyde content of 3000 ppm in fresh product. After mixing, the rice bran was kept enclosed air-tight for 72 hours. Thereafter, the formaldehyde-treated product was pelleted into a compound feed (RBF), which contained the rice bran at a level of 85%. A separate compound feed was formulated to match the macro nutrient profile of the rice bran feed, without containing rice bran, to be used as placebo feed (PF).

In the first and third week, all animals received 3 kg of PF/day. The second week, the heifers assigned to control (CRT) received 3 kg of PF/day, those assigned to a low dose of rice bran (LD) received 1.5 kg of RBF/day and 1.5 kg of PF/day, and those assigned to a high dose of rice bran (HD) received 3 kg of RBF/day.

Every day the amount of forage offered to each animal was recorded. Compound feed was offered separately from forage and was consumed in total. Forage was always fed in excess to ensure voluntary intake, and the amount of left-over forage was weighed daily and sampled for DM analyses.

Samples and analyses

Blood samples were taken from the jugular vein with vacuum syringes every morning before feeding. Serum was separated by centrifugation and stored at -20˚C. Urine samples were taken every morning after feeding. Samples were obtained from midstream at spontaneous urination or induced by stimulation of the perineum. Immediately after sampling, urine pH was measured with an electronic pH meter, which was calibrated daily and samples were stored at -20˚C.

Forages were sampled from the silage clamps and hay bails by taking different subsamples that were pooled to create a representative sample. Compound feeds were sampled upon arrival to the farm. These feed samples were analysed for dry matter content (DM), ash, ether extract (EE), crude protein (CP), and sugars (European Commission, 2009). Starch was analysed according to NEN 3574/C1

(1979). Neutral detergent fibre (NDF) was analysed with alpha amylase (Van Soest et al., 1991), but without sodium sulphite, and is expressed without residual ash. Acid detergent fibre (ADF) was also analysed according to Van Soest et al. (1991) and expressed without residual ash. Lignin was analysed using sulphuric acid according to Robertson and Van Soest (1981). Calcium, Mg, Na, and K, as well as the trace metals zinc, copper, manganese and iron were analysed by calcination at 500˚C, hydrochloric acid digestion, and subsequent determination with an atomic absorption spectrophotometer (Perkin Elmer AAnalyst 800, Norwalk, CT, USA). Phosphorus was analysed by spectrophotometry (AOAC, 2005; 4.8.14). Chloride was analysed according to the official method number 969.10 (AOAC, 2005) and S content was determined with a dual range sulphur carbon analyser (Leco SC-144DR, Leco Corporation, St Joseph, MI, USA). Dietary cation-anion difference was calculated with Na, K, Cl and S by simple sum of cation and subtraction of anion equivalents as in Block (1984). Oxalic acid was analysed according to Canale et al. (1984). Phytic acid was analysed by colorimetric method number 965.17 (AOAC, 2005) based on reaction of vanadomolybdate on inorganic phosphate produced by action of 6-phytase on phytic-containing substrate. Inositol phosphate (IP) fractions of the rice bran samples were analysed by HPLC (Muzquiz et al. 1999) using a PRP-1 column (150 x 4.1 mm, 5μm) at 45ºC, with a refractive index detector and 10 μL injection volume.

Serum and urinary Ca were analysed with a photometric colour test (Ca-oCPO complex). Serum and urinary Mg analyses were performed by photometric colour test, and P with a photometric UV test. Creatinine in serum and urine was analysed with a kinetic colour test (Jaffé method). All of these were done with equipment from Olympus Diagnostica GmbH (Hamburg, Germany). Serum 1,25 dihydroxycholecalciferol was measured by radioimmunoassay RIA using IDS (Immunodiagnostic System Limited, Boldon, UK), and urine hydroxyproline with a HPLC assay (Bio-Rad Labs, Hercules, CA, USA). Serum Zn was determined by colorimetric method (Randox Zn-2341, Randox Laboratories Ltd, Antrim, UK). All serum and urine analyses were done at Synlab.vet (Augsburg, Germany).

Statistical analysis

The distribution of the observations was checked for normality and presence of outliers. Daily DM, Ca, P and Mg intakes and daily dietary DCAD, as well as serum

values of Ca, P, Mg and Zn values and urine pH were statistically analysed as such. Urine Ca, P and Mg values (mmol/l) were expressed as a ratio with urinary creatinine content (g/l). The fractional excretion (FE) of Ca, P and Mg was calculated as 100 multiplied by the urine mineral / urine creatinine ratio divided by the serum mineral / serum creatinine ratio. Urinary mineral / creatinine ratios as well as fractional mineral excretions, 1,25 dihydroxycholecalciferol and hydroxyproline data required logarithmic transformation to obtain a normal distribution.

Daily measures of dietary intakes, urine and blood parameters were processed as daily repeated measures on the subject animal with the MIXED procedure of SAS (1999), for which an autoregressive covariance structure was assigned. The model included block, period and the interaction between week and treatment as factors. Least squares means were calculated for the week treatment combinations, and differences between the means were contrasted between treatments across weeks and between weeks across treatments.

Additionally, the Ca / creatinine ratio was analysed with the MIXED procedure of SAS (1999), in a model that included animal and animal x week interaction. Least squares means were calculated for the individual animals and animal week x combinations, and differences between the weeks for the individual animals were compared.

Results

The two rice bran products used for the manufacture of RBF were both full-fat and had a low Ca content (Table 1). As expected, P content was high and colorimetric analyses of phytic acid resulted in around 60 g/kg in both products. HPLC determination of the inositol phosphate forms showed that the majority of phytate in rice bran was phytic acid. The HPLC analyses resulted in lower values for phytic acid than estimated with the colorimetric method, especially for product A, for which this difference was more than 18 g/kg DM.

Table 1. Composition of the two rice bran products used in the experimental feed

	Rice bran A	Rice bran B
DM	905.0	918.0
CP	149.2	176.5
Fat	181.0	312.0
Sugar	40.0	8.0
Starch	325.0	124.0
Ca	0.4	1.0
P	17.9	23.5
Phytic (IP6)*	59.7	63.2
Oxalic	3.8	1.2
Total IP	46.00 ± 0.02	69.93 ± 0.70
IP6	40.98 ± 0.03	61.55 ± 0.67
IP5	3.19 ± 0.03	6.24 ± 0.01
IP4	1.03 ± 0.00	1.36 ± 0.00
IP3	0.80 ± 0.02	0.77 ± 0.01

*Expressed as g/kg DM. *Colorimetric*

The forage mix contained 3.0 g/kg DM Ca and had a positive DCAD value of 34 meq/kg DM (Table 2). The supplemental feeds were formulated to have similar macro nutrient content based on table values for the raw materials (Table 2). The later analyses showed differences in CP between PF and RBF. Crude protein in the PF was 40 g/kg DM lower and fat (EE) was 30 g/kg DM lower than for RBF. As expected, Ca and P and phytic acid also differed between the feeds. Calcium was 3 g/kg DM lower in the rice bran feed and P was about 12 g/kg DM higher than in the placebo feed, as well as phytic acid, which was 32 g/kg DM higher in the rice bran feed. DCAD balance was not much different although it was 15 meq /kg DM lower in the rice bran feed.

Table 3 gives intakes by week and by treatment after analysis of variance. Dry matter intake was similar between weeks and between treatments. Calcium, P, Mg, Zn and DCAD intakes showed significant differences between diets. Intake of nutrients was not different between treatments in the first and last week, but it was different in week 2, and between weeks in the two rice bran treatments. Differences between the means reflected the differences of the nutrient profile of the supplemental feeds.

between weeks for the HD treatment. Serum Ca was numerically lower in the second week of the HD treatment and increased after withdrawal of the supplemental feed. Serum P was different between treatments in week 2 and the differences remained in week 3. Serum P increased with rice bran supplementation and decreased after withdrawal (HD treatment). Serum Mg was affected by treatment in week 2 expressing differences between weeks in the rice bran treatments. With LD and HD rice bran in the diet, Mg in serum increased and decreased after withdrawal. Serum Zn was slightly higher in week 2 and 3 than in week 1 in heifers receiving LD and HD supplement. Contents of 1,25 dihydroxycholecalciferol were similar in all weeks and treatments.

Results of analysis of variance of serum values are presented in table 4. Serum Ca showed no differences among treatments in any of the weeks, but showed a trend

Urine data are given in table 5. Urinary Ca and FE of Ca was not different with diet or week. Urinary excretion of P, just like its FE, was different between weeks in the HD treatment, increasing in week 2 and decreasing in week 3. In week 3, P excretion was lower in association with increasing rice bran levels. Urinary Mg and Mg FE and was not different between weeks in any of the diets, but in week 2, heifers on the HD treatment had a higher urinary Mg than heifers receiving CTR or LD treatment. Urinary pH was independent from treatment or week. Hydroxyproline content in urine differed between animals on different treatments in week 1 and 2, and between weeks for the HD treatment.

Discussion

The two rice brans used in this experiment (table 1) were both full-fat. These products typically contain more than 200 g fat /kg DM. Rice bran A compared with rice bran B contained a much greater fraction of starch and sugars, indicating that the process of separation of the bran was more efficient in product B, providing more fat, crude protein, P and phytic acid, which are higher in the bran fraction as compared to the endosperm (Resurrection et al., 1979). Both products had a very low Ca content. Occasionally, rice bran can contain high levels of Ca (24 g/kg DM), because Ca carbonate can be used as an abrasion agent in the production process (Marshall and Wadsworth, 1994). Low Ca rice bran limits dietary Ca availability by diluting the total Ca content in the diet, as well as by providing phytic acid. HPLC analyses of the inositol phosphate profile of the brans indicated that the majority

of phytate in the products was IP6 and IP5. This is important because two or more dephosphorilations of phytic acid eliminate its negative effect on Ca availability (Lonnerdal et al. 1989).

Table 2. Composition of the feeds

Composition in % fresh weight	**Forage mix**	**Placebo**	**Rice bran**
Wheat		38.1	7.9
Full fat rice bran A		--	42.5
Full fat rice bran B		--	42.5
Wheat bran		30.0	--
Linseed		4.0	--
Palm oil		12.0	--
Soybean meal 44%		12.8	4.0
Molasses		3.0	3.0
Mineral premix		0.1	0.1
Grass silage	33.4		
Corn silage	65.0		
Hay	1.5		
Nutrient contents expressed in DM			
DM	322	894	885
CP	127	133	173
Ash	67	50	72
CF	35	162	192
Starch	205	352	281
Sugars	10	41	23
NDF	415	229	279
ADF	227	100	85
ADL	15	21	31
Ca	3.0	4.3	1.5
P	3.3	4.1	15.9
Mg	1.5	2.6	8.1
Na	0.1	1.0	0.3
K	11.6	11.2	11.5
Cl	5.2	2.3	0.9
S	1.9	1.9	2.5
DCAD[a]	34	145	128
Fe[b]	176.0	483	198
Mn[b]	58.4	65	132
Cu[b]	3.5	16	23
Zn[b]	43.0	69	159
Oxalic	nd	1.5	9.4
Phytic*	nd	10	42

*g/kg DM. [a]meq/kg DM. [b]mg/kg DM. *Colorimetric. nd. Not determined*

The heifers consumed about 8 kg of DM per day and the dietary treatments had no effect on DMI (table 3). Formaldehyde-treated full-fat rice bran was not rejected by the animals, and was consumed in similar amounts as the PF. Additionally, the supplemental feeds did not create any difference in voluntary forage consumption in contrast with fat coating, which depressed voluntary DMI, when used to prevent ruminal degradation of phytic acid in rice bran (Chapter 2). Mineral intakes were different for the different diets as it would be expected from the differences in the supplemental feeds. However, P, Mg and Zn intakes were in all cases above NRC requirements (National Research Council, 2001), whereas Ca intake was below requirements. Several factors contributed to this nutritional situation among which the fraction of corn silage in the forage mix and the high growth rates were the most relevant.

Serum Ca and 1,25 dihydroxycholecalciferol and urinary Ca excretion were unaffected by the diet. Feeding formaldehyde-treated rice bran did not induce a change in Ca metabolism and homeostasis. Fat coated rice bran in previous studies reduced urinary Ca (Chapter 2) but it also reduced DMI. This reduction in urinary Ca could be caused by the lower Ca intake, created by the lower DMI and by the low Ca content of rice bran, or by the negative effect of phytic acid on Ca availability, or by a combination of both. In that study Ca/creatinine ratio was reduced from 0.35 to 0.11 by product supplementation. In the current study, Ca/creatinine ratio was already very low (near 0.10) before rice bran supplementation in week 1, and it remained in that range throughout the whole test period (Table 5). Urinary regulation can not reflect changes in Ca availability if Ca metabolism is already up-regulated. Heifers in this study received insufficient Ca in their diet to cover their demand for growth. Therefore, the low dietary Ca supply and in addition a hypothetical reduction of Ca availability by phytic acid did not alter Ca metabolism any further.

The present data show that in contrast with multiparous dairy cows, pregnant heifers have an already adapted Ca metabolism before calving. Their minimised urinary Ca excretion indirectly indicates that dietary supply is insufficient to compensate blood Ca clearance by means of passive Ca absorption, which is the original purpose of feeding rumen-protected rice bran. Dairy heifers are naturally not susceptible to milk fever. The confirmation that their Ca metabolism is already

adapted to greater Ca demands is explanatory to the aetiology of milk fever and further supports the value of induction to homeostatic adaptation in its prevention.

Table 3. LSMeans of the intakes contrasted by week and treatment

	week			
	1	2	3	Pr > F
DMI kg				
Control	7.77	8.07	8.06	0.48
Low Dose	7.91	7.97	7.94	0.96
High Dose	8.60	8.37	8.45	0.66
Pr > F	0.07	0.47	0.34	
Ca intake g				
Control	26.73	27.62	27.59	0.48
Low Dose	27.13	23.57	27.22	<0.01
High Dose	29.19	21.05	28.76	<0.01
Pr > F	0.07	<0.01	0.34	
P intake g				
Control	27.95	28.95	28.91	0.48
Low Dose	28.40	44.35	28.50	<0.01
High Dose	30.70	61.26	30.22	<0.01
Pr > F	0.07	<0.01	0.34	
Mg intake g				
Control	14.42	14.86	14.84	0.48
Low Dose	14.62	22.04	14.67	<0.01
High Dose	15.63	29.87	15.42	<0.01
Pr > F	0.07	<0.01	0.34	
Zn intake mg				
Control	385.2	398.1	397.6	0.48
Low Dose	391.0	498.9	392.3	<0.01
High Dose	420.6	620.3	414.5	<0.01
Pr > F	0.07	<0.01	0.34	
Dietary DCAD meq/kg				
Control	72.5	70.9	70.9	0.43
Low Dose	71.3	68.4	71.7	0.01
High Dose	68.4	64.0	69.7	<0.01
Pr > F	0.04	<0.01	0.43	

Rice bran contains a high level of P. About one third of phytic acid is elemental P, and a considerable part of this P is digested as phosphate resulting from the process of ruminal dephosphorylation of phytate. A high level of rice bran supplementation increased dietary supply of P and generated a response in serum concentration and especially in urinary excretion of P. Both parameters increased in the second week, and then decreased below the initial value after withdrawal.

This indicates a homeostatic reaction to the increased supply, which was sustained after product withdrawal. Phosphorus is less tightly regulated than Ca and its absorption is directly related to dietary supply. In ruminants, saliva and not urine represent the main source of P excretion (Horst, 1986). Saliva P content is directly related to blood P (Valk et al. 2002). On the other hand, urinary excretion also represents a relevant route for excretion of excess P when serum concentration of P is high (Wu et al. 2000). In the present study, serum P rose with the increase of dietary P. It can be assumed that salivary P also was increased. Urinary P increases with extra P absorption but it may have reacted faster and with greater intensity to dietary withdrawal than salivary excretion. This may explain the lower serum and urinary values after withdrawal as compared to the initial level.

Table 4. LSMeans of the blood parameters contrasted by week and treatment

	week			
	1	2	3	Pr > F
Serum Ca (mmol/l)				
Control	2.40	2.48	2.48	0.28
Low Dose	2.46	2.43	2.51	0.31
High Dose	2.50	2.47	2.60	0.06
Pr > F	0.26	0.62	0.12	
Serum P (mmol/l)				
Control	2.07	1.90	1.92	0.21
Low Dose	2.18	2.32	2.13	0.08
High Dose	2.06	2.21	1.80	<0.01
Pr > F	0.54	<0.01	<0.01	
Serum Mg (mmol/l)				
Control	1.03	1.00	1.02	0.60
Low Dose	1.02	1.06	0.98	0.01
High Dose	1.06	1.12	1.00	<0.01
Pr > F	0.42	<0.01	0.37	
Serum Zn (mmol/l)				
Control	7.98	7.96	8.00	0.56
Low Dose	8.03	7.99	8.02	0.70
High Dose	8.09	8.02	8.03	0.20
Pr > F	0.50	<0.01	0.03	
1,25 dihydroxycholecalciferol * (µg/l)				
Control	39.04	21.93	19.24	0.14
Low Dose	24.89	25.89	23.22	0.93
High Dose	23.37	37.09	25.76	0.30
Pr > F	0.40	0.18	0.66	

**LSMeans were calculated with a log transformation to fit a normal distribution.*

Table 5. LSMeans of the urinary parameters contrasted by week and treatment

	week			
	1	2	3	Pr > F
Urinary Ca/Creatinine*				
Control	0.09	0.12	0.11	0.38
Low Dose	0.10	0.10	0.12	0.72
High Dose	0.09	0.09	0.14	0.16
Pr > F	0.89	0.49	0.75	
FE Ca*				
Cnt	3.43	4.89	4.41	0.38
Low Dose	3.35	3.86	4.31	0.65
High Dose	3.04	3.29	4.85	0.19
Pr > F	0.90	0.38	0.90	
Urinary P/Creatinine*				
Control	0.77	0.25	0.51	0.11
Low Dose	0.42	0.28	0.19	0.41
High Dose	0.28	0.54	0.10	0.01
Pr > F	0.32	0.21	0.02	
FE P*				
Control	31.43	13.95	30.23	0.22
Low Dose	19.23	12.39	9.17	0.47
High Dose	13.85	25.39	5.30	0.02
Pr > F	0.48	0.27	0.01	
Urinary Mg/Creatinine*				
Control	8.12	10.28	9.50	0.17
Low Dose	11.09	10.89	11.44	0.90
High Dose	10.67	13.26	12.59	0.21
Pr > F	0.08	0.04	0.07	
FE Mg*				
Control	931.54	1088.68	986.51	0.45
Low Dose	1048.82	1008.79	1312.20	0.07
High Dose	979.72	1147.89	1257.48	0.19
Pr > F	0.74	0.47	0.06	
Urinary pH				
Control	7.98	7.96	8.00	0.56
Low Dose	8.03	7.99	8.02	0.70
High Dose	8.09	8.02	8.03	0.20
Pr > F	0.06	0.40	0.81	
Hydroxiproline/Creatinine*				
Control	47.39	43.86	41.73	0.47
Low Dose	65.28	54.82	52.74	0.12
High Dose	69.02	57.28	50.18	0.02
Pr > F	<0.01	<0.01	0.05	

**LSMeans were calculated with a log transformation to fit a normal distribution.*

Magnesium content in rice bran is very high as compared to common ruminant feeds and its effect on Mg intake in this trial was clear. Fibrous feeds contain phytic acid and this has been associated with impaired availability of divalent elements such as Ca and Zn, however this effect seems to be less evident for Mg (Coudray et al. 2003). The response of blood and urine Mg to rice bran reflected an increase in Mg supply. Therefore any detrimental effects of rice bran on Mg status can be excluded. Factors which affect Mg availability such as concentrate to forage ratio or K supply, did not differ between the treatments. Hence differences in serum Mg can be attributed to this increased Mg intake, which was even two-fold in the HD treatment. Blood Mg is not hormonally regulated to a constant level, but it is strongly affected by Ca regulation (Fontenot et al. 1989). In the current study, no differences were observed for Ca homeostasis indicators between treatments, so most likely the effects observed on Mg are due to differences in Mg supply.

Zinc absorption is clearly affected by phytic acid intake (Lonnerdal, 2000). The purpose of this trial was to test the effect of ruminally protected phytic acid on Ca availability. Therefore assessing the effect of formaldehyde-treated rice bran on Zn status is necessary. Rice bran is very rich in Zn, and RBF supplied a daily intake which was 50% greater in the HD treatment than in CRT. Absorption is the main regulatory system for Zn in cattle (Miller, 1975), just as in other species. This explains the small differences in serum Zn observed among treatments. Zinc levels were higher in the rice bran treatments than in CRT, and these differences remained after product withdrawal. Under these conditions, feeding rumen-protected rice bran represented a positive factor for Zn status. The greater Zn supply was able to compensate any degree of nutritional antagonism of phytic acid.

Conclusion

The pregnant heifers used in this trial already had an up-regulated Ca metabolism, so they are not a good model for the transition dairy cow. Apparently, growth with its extra needs for Ca had already activated Ca metabolism to increase Ca retention, and thus urinary excretion of Ca was already minimised, which invalidates the value of this parameter as an indicator of changes in intestinal availability of Ca. This trial therefore provided no evidence for any response to formaldehyde-treated rice bran on Ca metabolism in these growing animals. Based on this experiment, the hypothesis that the product reduces dietary availability of

Ca can neither be proven nor disproven. Pregnant heifers, in contrast with multiparous cows, have an already adapted Ca metabolism before calving, and they are naturally not susceptible to milk fever, which supports the preventive value of induction to homeostatic adaptation in the prevention of milk fever. Formaldehyde-treated rice bran had effects on P, Mg, and Zn status, but those could not be considered detrimental to the health of the animal.

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CHAPTER 5

Effect of feeding rumen-protected rice bran on Ca homeostasis of non-lactating multiparous cows

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Summary

Milk fever in dairy cows can be prevented by activating Ca homeostasis before calving. Homeostatic adaptation can be achieved by reducing dietary Ca availability. Formaldehyde-treated rice bran was studied to supply rumen-protected phytic acid to reduce Ca availability. Twelve multiparous dry cows were used in a 3X3 Latin square change-over design with 5 day periods to test 3 dietary treatments. Diets consisted of a forage mix (maize silage, grass silage and hay), being 77% of ration dry matter, supplemented with 3 concentrates: Control (no formaldehyde-treated rice bran), T1 (100% formaldehyde-treated rice bran) and T2 (99.5% formaldehyde-treated rice bran with 0.6% Ca carbonate, to equal Ca content of Control). Dietary treatments did not affect urine pH (8.14, 8.13 and 8.11 for Control, T1 and T2, respectively) or dry matter intake (13.9, 13.7 and 13.8 kg for Control, T1 and T2, respectively). Including formaldehyde-treated rice bran in the diet resulted in lower urinary Ca/creatinine ratio (0.970, 0.457 and 0.618 for Control, T1 and T2, respectively). This parameter peaked for 2 days after withdrawal of T1 and T2 at introduction of Control. Peak was greatest after T1 and was not observed in transitions between rice bran treatments. This is understood as indirect evidence for activation of intestinal Ca absorption during formaldehyde-treated rice bran feeding, because renal adaptations to changes in blood Ca clearance are immediate and intestinal adaptations delay 2 days. It was concluded that including formaldehyde-treated rice bran in rations before calving may represent a dietary strategy to prevent milk fever without reducing dry matter intake.

Introduction

Milk fever is one of the most relevant metabolic disorders in dairy cattle. The high milk production of modern dairy cows requires a rapid adaptation of Ca homeostasis at calving. Especially multiparous cows can not cope with this challenge and will frequently suffer from various degrees of hypocalcaemia. Preventive strategies for this disease involve inducing this adaption some weeks before calving. Low Ca diets have been proven effective (Green et al., 1981, Kichura et al., 1982, Shappell et al., 1987), although difficult to implement in practice. In the last decade, a reduction of dietary Ca availability through different means has been proposed (Wilson, 2001), and specially the use of zeolites has been sufficiently tested with consistent successful results (Thilsing-Hansen and Jorgensen, 2001, Thilsing-Hansen et al., 2002, Katsoulos et al., 2005). Unfortunately, zeolites will negatively affect dry matter intake (DMI) (Grabherr et al., 2008), which represents a risk factor for other metabolic diseases (Grabherr et al., 2009).

Rice bran has been proposed as an inhibitor of Ca availability (Chapter 2). Preliminary assessment of full fat rice bran, coated with hydrogenated fat to prevent ruminal degradation, showed homeostatic adaptation of Ca homeostasis in the form of changes in urinary Ca, but also presented a reduction of DMI. In addition, formaldehyde treatment of rice bran has been studied successfully as a means to prevent ruminal degradation of phytic acid (Chapter 3). Formaldehyde-treated rice bran was tested in vivo, demonstrating no inhibition of DMI and no significant detrimental effects on P, Mg or Zn status in heifers (Chapter 4). Fat extracted rice bran, produced without the use of Ca carbonate, has been identified as the preferred phytic acid source to inhibit Ca availability in dry cows. Formaldehyde treatment is a rumen protection alternative that does not negatively affect feed value or DMI. This product has an adequate nutrient profile that allows a high inclusion rate in pre-calving dairy rations and it could affect Ca availability in two ways. First, its low Ca content reduces total Ca intake and second, its high phytic acid content can reduce Ca availability.

The present experiment was done to study if formaldehyde-treated rice bran can activate Ca homeostasis in multiparous dry cows. An additional aim was to

determine if such an effect is caused by the low content of Ca in rice bran, by the negative effect of phytic acid on dietary Ca availability, or by both.

Materials and methods

Twelve multiparous Holstein dairy cows from the experimental dairy farm of Nutreco in Boxmeer, the Netherlands, were used for this experiment (Table 1). The animals were in the last two months of gestation and were dried off at least 7 days before the test. All cows had completed at least one lactation, and the test period ended at least 2 weeks before the expected calving date. The cows were assigned to blocks of 3 cows according to parity. A total of 4 blocks were composed. Two blocks at a time, cows were tested in 2 phases of 15 days. Each phase consisted of 3 feeding periods of 5 days, in which a 3x3 Latin square change-over design was organised to test 3 diets. The 2 phases had an opposite sequence of treatments, to obtain the complementary combinations of consecutive treatments. One week before the test, 6 cows at a time were brought to the stable, where they were tied in cubicles fitted with individual drinking bowls and separate feeding space. In this week, cows were fed the control ration in an adaptation period in which dry matter intake (DMI) was measured to estimate the amount of feed needed to guarantee ad libitum feeding. A forage mixture was used mixed with treatment specific concentrates to constitute the total mixed rations (TMR). The forage mixture consisted of maize silage, grass silage and hay and represented 77% DM of the ration.

In the test period, cows were provided with 110% of their observed voluntary feed intake measured in the adaptation period. Dry matter (DM) of the forage mixture and the individual left over feed was determined daily to measure individual DMI.

Fat extracted rice bran was imported from Vietnam. In the sourcing process, Ca content was checked to avoid Ca rich sources from milling processes based on Ca carbonate abrasion. The product was brought into a mixer with formalin (370 g/L formaldehyde) to provide a treatment level of 3000 ppm formaldehyde in fresh weight. The mixture was left in an air tight container for 72 hours to enable a crosslink reaction of formaldehyde with protein. After 72 hrs the mixture was put in bags and brought to the farm. A compound feed was formulated to match the macro nutrient profile of the rice bran using common feeds, and was produced in the meal form to be used as control concentrate (Table 2). Three treatment diets

were fed in this experiment. The treatment was defined by the choice of concentrate feed used in the TMR: control (C) composed of a placebo compound feed, treatment 1 (T1), feeding formaldehyde-treated rice bran, and treatment 2 (T2), feeding formaldehyde-treated rice bran and Ca carbonate to provide an equal Ca content as the control ration.

Samples and analyses

Forages samples were taken from the silage clamps with a drill from three evenly separated locations at the top of each clamp. Also three samples were taken at random from the hay bails. Samples were pooled by forage clamp and by hay batch for analyses.

Samples of the control and rice bran concentrates were also taken upon arrival to the farm. Forage and concentrate samples were analysed for DM, ash, ether extract (EE), crude protein (CP), sugars (European Commission, 2009) and starch (NEN3574/C1, 1979). Neutral detergent fibre (NDF) was determined with alpha amylase (Van Soest et al., 1991) without sodium sulphite and expressed without residual ash. Acid detergent fibre (ADF) was determined as in Van Soest et al. (1991) and expressed without residual ash. Lignin content was measured with sulphuric acid according to Robertson and Van Soest (1981). Calcium, Mg, Na, and K were analysed by calcination at 500˚C, digested with hydrochloric acid and later measurement by atomic absorption spectrophotometry (Perkin Elmer AAnalyst 800). Phosphorus was analysed by spectrophotometry (AOAC; 4.8.14, 2005), and Cl was analysed following the AOAC (AOAC; 969.10, 2005). Sulphur was analysed by dual range sulphur carbon analysis (Leco SC-144DR, Leco Corporation, St Joseph, MI, USA). Oxalic acid content was determined as in Canale et al. (1984). Phytic acid was analysed by colorimetry with the AOAC method number 965.17 (AOAC, 2005), which consists in the reaction of vanadomolybdate with inorganic phosphate generated by catalysis with phytase. Analyses of Na, K, Cl and S were used to calculate dietary cation anion difference (DCAD), by adding cation equivalents and subtracting anion equivalents, in the way calculated by Block (1984).

Urine samples were obtained from spontaneous urination, or by induction through manual stimulation, from midstream micturition with a clean plastic cup. Within minutes, pH was measured with an electronic pH meter that was calibrated daily, and subsamples were taken and preserved frozen at -18˚C for later analyses.

Table 1. Description of the cows and diet assignment in the Latin-Square Change-Over design

Cow num.	Completed lactations	Years of age	Days to calving	Phase	Block	Diets		
						Period 1	Period 2	Period 3
460	4	6.1	30	1	1	T2	C	T1
467	4	6.0	34	1	1	T1	T2	C
484	4	5.6	55	1	1	C	T1	T2
529	2	4.0	54	1	2	T1	T2	C
533	2	3.9	31	1	2	C	T1	T2
572	1	3.1	40	1	2	T2	C	T1
525	2	4.3	55	2	3	C	T2	T1
538	2	3.9	44	2	3	T2	T1	C
575	1	3.0	31	2	3	T1	C	T2
287	10	13.0	45	2	4	T1	C	T2
380	6	8.5	50	2	4	T2	T1	C
573	3	5.2	58	2	4	C	T2	T1

Urinary Ca was measured by photometry (Ca-oCPO complex) whereas creatinine in urine was analysed with a kinetic colour test (Jaffé method). Both Ca and creatinine were measured using an Olympus analyser (Olympus Diagnostica GmbH, Hamburg, Germany). Calcium/creatinine ratio was calculated to correct for variation in urine volume.

Statistical Analysis

Analysis of variance was done on the daily observations of DMI, Ca intake, dietary DCAD, urinary pH, and Ca/creatinine ratio. In order to obtain a normal distribution, a logarithmic transformation of the Ca / creatinine ratio was required. Differences between the diets were contrasted for these parameters with the MIXED procedure of SAS (version 9.1.3; SAS Inst., Inc., Cary, NC, USA), using the daily observations as repeated measures on the subject cow. The model included diet, block, period and the diet of the preceding period as factor, considering the diet before period 1 as control. An autoregressive covariance structure was assigned, and differences were contrasted with the Tukey-Kramer method.

Ca excretion was also analysed with the interaction of day in the period and the diet as additional factors. Least square means for the 5 days in the period for the 3 diets were calculated and differences between the diets in each day were contrasted. In addition to this also Ca excretion during feeding of the control diet was calculated depending on diet given in the preceding period. Differences in Ca excretion during control feeding after these preceding diets were calculated. Dietary transitions between treatments from period 1 into period 2 and from period 2 into period 3 were analysed to check for effects of the 3 preceding diets.

Results

The cows used in the experiment had completed at least one lactation, and were older than 3 years. In the middle of the testing period all cows were at least 30 days before the expected calving date (Table 1).

Nutrient analyses of the forage mixture and concentrates used in the experiment are reported in Table 2. Overall, the macro nutrient profile of the 3 supplemental feeds was similar. Only the starch and sugar contents were higher in the rice bran feeds. Control and rice bran concentrates present clear differences in the mineral contents, except for the rice bran with extra Ca added, which was meant to have the same Ca content as the control concentrate. Phytic acid in the rice bran concentrates was 6 times higher than in control. Oxalic acid in the rice bran feeds was 0.6 of that in the control.

The calculated nutrient composition of the TMR was very similar for all non-mineral nutrients (Table 3). Calcium content of T1 TMR was 0.6 g/kg DM lower than that of both C and T2. Phosphorus content of the rice bran TMR more than doubled that of the control TMR, and the phytic acid content of the rice bran TMR was 7 times that of the control TMR. Magnesium content was also greater as a consequence of the rice bran inclusion, 3.7 g/kg DM as compared to 2.0 g/kg DM in the control diet. All other mineral contents were similar, and this was reflected in the similar DCAD.

Data on the dietary and urinary parameters and their statistics are presented in Table 4. Dry matter intake remained unchanged between the diets, and there was always some leftover feed throughout the experiment. As expected, Ca intake was lowest in T1 and equal for C and T2. Rice bran total mixed rations had a somewhat higher DCAD than the control TMR, but this difference was quantitatively small.

Urinary pH was not different amongst treatments. Urinary Ca excretion was lower in the rice bran treatments as compared with the control. Treatment 1 tended to have lower Ca excretion than T2.

Table 2. Raw material and chemical composition of forage base, control and rice bran feeds.

	Forage	Control	Rice bran	Rice bran +Ca
Grass silage	32.3	--	--	--
Corn silage	65.0	--	--	--
Hay	2.7	--	--	--
Corn	--	30.0	--	--
Wheat	--	23.0	--	--
Beet pulp	--	9.0	--	--
Soy hulls	--	25.0	--	--
Soybean meal	--	13.0	--	--
Defatted rice bran	--	--	100.0	99.4
CaCO3	--	--	--	0.6
% fresh weight				
DM	384	885	895	896
CP	121	171	165	164
Ash	69	40	102	108
CF	41	32	34	33
Starch	196	302	384	382
Sugars	21	38	63	62
NDF	427	272	295	293
ADF	240	166	103	102
ADL	21	7	44	43
Ca	3.2	3.3	0.7	3.3
P	3.3	3.3	22.0	21.9
Mg	2.0	1.9	9.7	9.7
Na	1.1	0.1	0.1	0.1
K	21.4	10.7	18.5	18.4
Cl	4.9	0.6	0.6	0.6
S	2.0	1.8	2.7	2.7
DCAD	332	150	294	292
Oxalic	nd	13.6	8.6	8.5
Phytic acid	nd	8.9	61.5	61.0
g/kg DM				

nd (not determined)

Table 3. Composition of the total mixed rations

	Control	Treatment 1	Treatment 2
DM	440	441	441
Concentrate	224	228	227
Forage	776	772	773
CP	132	131	131
Ash	62	76	77
CF	39	39	39
Starch	220	239	238
Sugars	25	31	30
NDF	392	397	397
ADF	223	209	209
ADL	18	26	26
Ca	3.2	2.6	3.2
P	3.3	7.5	7.5
Mg	2.0	3.7	3.7
Na	0.9	0.8	0.8
K	19.0	20.7	20.7
Cl	4.0	4.0	4.0
S	1.9	2.1	2.1
DCAD	291	324	323
Oxalic acid*	3.0	2.0	1.9
Phytic acid*	2.0	14.0	13.8
g/kg DM			

**Only from concentrates*

The patterns of Ca excretion in time from diet introduction to the next dietary change are plotted in Figure 1. Calcium excretion showed differences between the 5 consecutive days of the period for the different diets. Differences in Ca excretion between the diets occurred only in the first 2 days. In these two days, Ca excretion was higher for C compared to T1 and T2 treatments. From day 3 in control fed periods Ca excretion was similar to that observed for T1 and T2 throughout the 5 days. In order to explain the higher Ca excretion in the first days of the C diet, the observations of this diet were analysed separately divided according to the diet fed before. One curve defined with observations of C treatment in the first period, and two more curves defined with observation made during C treatment, in the second of third period, separated according to if cows received T1 or T2 before (Figure 2). The first 2 days, animals on C that consumed T1 before had higher Ca excretion ($P<0.05$) than cows for which the diet fed before was T2 or C. Between these two groups, animals on C after T2 also had the highest Ca excretion, but this difference was not statistically significant. Animals fed C treatment in the first period

excreted similar amounts of Ca throughout the 5 days. Ca excretion for C treatment was high for two days when T1 was the preceding diet.

Analysis of the transitions between diets showed that withdrawal of C and introduction of T1 or T2 presented no difference in urinary Ca output, and that both withdrawing of T1 or T2 diets into C created a significant urinary Ca spike (Figure 3). This spike was of greater magnitude after T1 than after T2 withdrawal. Comparing the different diets as the change into a specific diet (Figure 3) shows that the spike at the start of C after T1 was significantly greater than that after T2. The start of T1 or T2 presented no differences regardless of the preceding diet.

Table 4. Least square means of the dietary intakes and urinary parameters

	C	T1	T2	SEM
DMI (kg)	13.9 a	13.7 a	13.8 a	0.28
Ca Intake (g/day)	44.7 a	36.1 b	44.5 a	0.85
Dietary DCAD (meq/kg DM)	291 a	324 b	323 b	0.21
Urinary pH	8.14 a	8.13 a	8.11 a	0.02
Ca/creatinine (mmol/l/g/l)	0.970 a	0.457 b	0.618 b	*1.186

*Different letters indicate differences at P<0.05. * SEM is reported in log scales, because confidence interval is not symmetric from the mean*

Discussion

Rice bran feeding with or without added Ca had no detrimental effect on voluntary DMI. This is of especial importance for the adequacy of this raw material for pre-calving diets. Reduction of DCAD, being the most common approach to prevent milk fever, linearly decreases DMI (Charbonneau et al., 2006). Also previous attempts to reduce Ca availability in the prevention of milk fever have shown negative effects on DMI. Fat-coated full-fat rice bran reduced DMI by as much as 3 kg per day (Chapter 2). Working with zeolites, Grabherr et al. (2008) observed a severe reduction in DMI of 48%. In a later study,

Grabherr et al. (2009) described that zeolite clays at 43 g/kg DM significantly reduced DMI by 4.0 kg/day. However, in the same study, zeolites at 23g/kg DM did not affect DMI and proved to be effective against hypocalcaemia. Other studies done with zeolites indicate reductions of DMI that were smaller in magnitude (Thilsing-Hansen et al., 2002), or not different with control (Pallesen et al., 2008).

However, these studies report feed residues smaller than 1% in the control group, which to our understanding is not indicative of free access to feed. Pair feeding below voluntary feed intake may be very valuable to separate the indirect effects caused by differences in nutrient intakes from the direct effects of the product, but does not allow for determination of effects on voluntary feed intake.

Urinary pH remained high and unchanged with all diets. In situations of high positive DCAD and alkaline urine, changes in urine pH are small when DCAD decreases (Roche et al., 2003). In fact, McNeill et al. (2002) observed that urine pH is unlikely to respond before DCAD is reduced below 200 meq/kg DM.

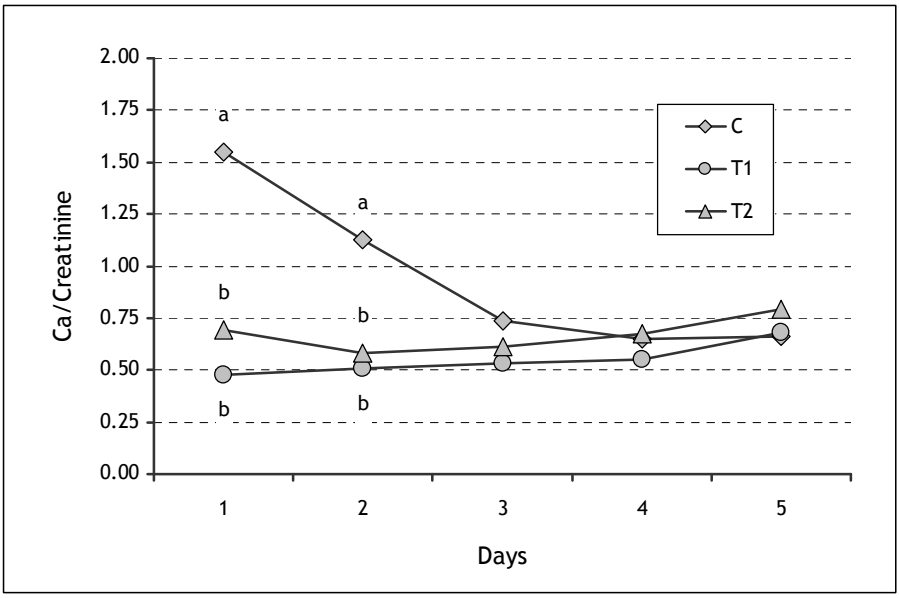

Figure 1. Evolution of urinary Ca excretion in the 5 days of feeding for the control (C), rice bran (T1) and rice bran with Ca (T2) diets. (Different letters indicate differences at P<0.05)

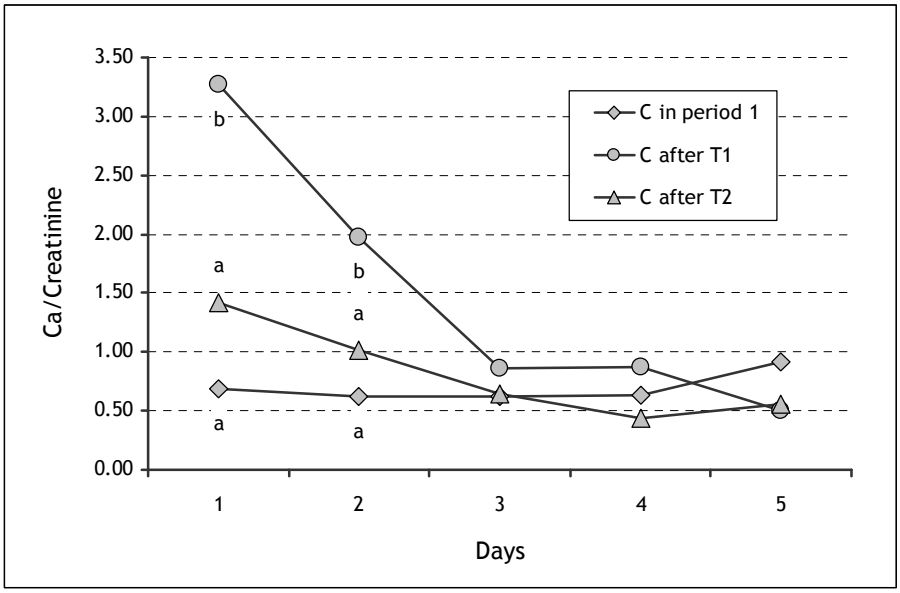

Figure 2. Urinary Ca / creatinine ratio duting control diets fed in period 1, vs control diets fed in periods 2 and 3 after having been fed either rice bran (T1) or rice bran with Ca (T2) in the preceding period. Different letters indicate differences at P<0.05

Urinary pH and DCAD here described are much higher than the range recommended for the prevention of milk fever. Our study uses urinary Ca excretion as an indicator for Ca homeostasis. This parameter is highly correlated (R2=0.99) in a curvilinear manner with DCAD (Roche et al., 2003), but in the range observed in the present study, the effect on Ca excretion should be negligible. The DCAD difference in this experiment does not explain the differences in Ca excretion or should effectively affect Ca homeostasis, so it should not constitute a confounding factor in the interpretation of Ca excretion.

Ca/creatinine ratio in urine was clearly affected by rice bran feeding. Excretion was greater at C feeding as compared with T1 and T2. Calcium supplementation of rice bran resulted in a somewhat (non significant) greater urinary excretion of Ca. Although urine is not a major excretory route for Ca in the ruminant, reduced urinary Ca excretion indicates reduced Ca availability (Chapter 2). Moreover renal reabsorption of Ca increases as response to PTH (Schröder and Breves, 2007).

However, daily Ca excretion patterns in the feeding periods show higher Ca excretion in C in the first two days after the introduction of control diet, and it is reduced again from the third day onwards (Figure 1). The only factor that could explain this pattern is the proximity to the preceding diet. In fact, as observations in C are divided by the preceding diet (Figure 2), those high Ca values are explained as a spike in Ca excretion at withdrawal of T1 diet. This pattern of urinary Ca excretion has been described before as a reaction to the withdrawal of the dietary element that was limiting intestinal Ca availability (Enemark et al., 2003, Chapter 2). Urinary Ca is considered to function as controlling signal of the positive errors of Ca homeostasis (Ramberg et al., 1984). Renal Ca clearance responds to PTH signals within a short time. When Ca entrance into the blood exceeds total Ca clearance, urinary excretion of Ca increases to prevent a rise in blood Ca. This adaptation can be as fast as within 3 hours (Schonewille et al., 1999).

Transcellular Ca absorption in the duodenum is required in situations of low intestinal availability of Ca. This process is mostly vitamin D dependent (Bronner, 2003). Regulation includes Ca uptake by the enterocyte from the intestinal lumen, intracellular Ca buffering and transport by calbindin, and later extrusion to the basolateral side. Maximum transcellular Ca absorption takes place through the mature enterocytes at the villus tips, but activation by calcitriol seems to take place earlier during differentiation at the crypts (Walters and Weiser, 1987). Centeno et al. (2004) described limited presence of vitamin D receptors (VDR) in mature enterocytes, supporting the idea of that activation of enterocytes for this absorptive function could take place at earlier development stages of enterocytes. Calbindin competence of enterocytes is acquired at the basal villus, and reaches a maximum 24 hours after calcitriol signal is received (Smith, 1993). Programming of intestinal Ca at early differentiation stages justifies a delayed intestinal response to calcitriol presence or absence. The peak in urinary Ca excretion observed at rice bran withdrawal indirectly indicates that the product activated transcellular Ca absorption during supplementation.

Delayed intestinal response to calcitriol, explained by the differentiation process of enterocytes, represents a plausible explanation for the adaptive failure in the aetiology of milk fever. Intestinal adaptation may be the limiting controlling mechanism to compensate for increased Ca needs. Reduction of Ca urinary losses

occurs within a shorter time span, but it is quantitatively insufficient to compensate Ca clearance for milk production (Schonewille et al., 1999). On the other hand, effective bone resorption has proven to be delayed for several days (Green et al., 1981), because although responsiveness of osteoclasts to PTH is relatively immediate, recruitment of new osteoclasts is delayed for 2 days (Erben, 2001).

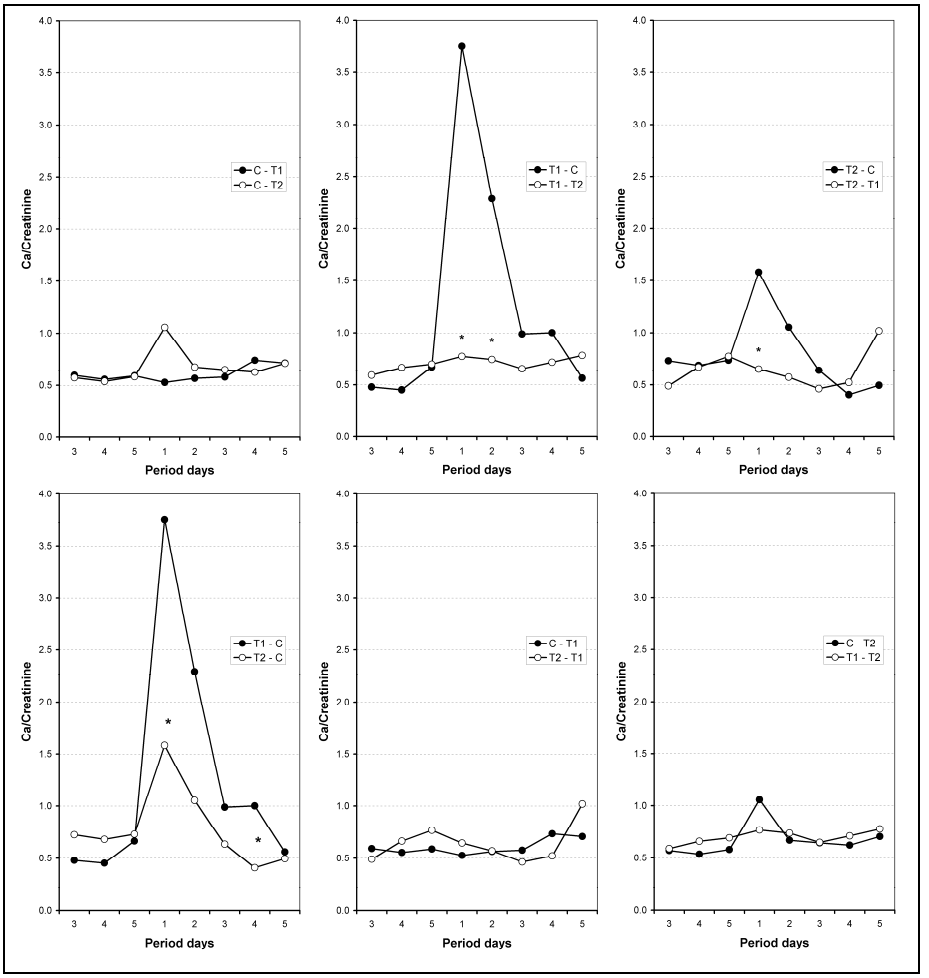

*Figure 3. Evolution of urinary Ca/creatinine ratio at diet withdrawal contrasted between the diets fed in the precedent and in the following period. * Indicates differences with a P<0.05*

Hypocalcaemia takes place normally in a similar time frame as the delay for intestinal adaptation for active Ca absorption and bone mobilisation. Limitation of Ca availability for several days before calving should be able to anticipate adaptation of Ca homeostasis. Whether gastrointestinal absorption alone or also bone mobilisation will become available to compensate Ca clearance without a time delay should depend on the degree of the reduction of dietary Ca availability. If enhanced dietary absorption compensates Ca deficit, bone mobilisation will stop when PTH signal would cease with the recovery of Ca balance, but if such limited availability is severe enough and extended in time, bone mobilisation should stay active to cooperate with intestinal absorption to sustain Ca homeostasis in the challenge of calving.

Conclusion

Formaldehyde-treated rice bran affected Ca homeostasis in non lactating multiparous dairy cows. This effect persisted when the low Ca content of the product was compensated with supplemental Ca, although intensity of the effect was reduced. Rumen-protected rice bran added to a close-up dairy ration at 23% DM induced changes in urinary excretion of Ca that are indicative of reduced dietary availability of Ca, and an induction of an adaptation of Ca absorption. Activation of paracellular Ca absorption mechanisms from days before calving could prevent that the delay of this adaptation results in hypocalcaemia. Feeding rumen-protected rice bran could represent a strategy to prevent milk fever.

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CHAPTER 6

Effect of rumen-protected rice bran on calcium homeostasis of multiparous dairy cows at calving

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Summary

Multiparous dairy cows can have different degrees of hypocalcaemia around calving, which can lead to milk fever. At parturition, there is a steep increase in Ca needs, which combined with a delayed homeostatic adaptation, results in a transient state of hypocalcaemia. Anticipating homeostatic adaptation through lowering dietary Ca availability can prevent milk fever. Rice bran, treated to increase its rumen escape fraction of phytic acid, can reduce dietary availability of Ca. A total of 113 multiparous cows calved during 3 periods of 3 weeks, in which the close-up group was fed first a control diet, then a diet containing 140 g/kg DM of rumen-protected rice bran, and as last the control diet again. The animals joined the close-up group 3 weeks before the expected calving date. Blood samples were taken weekly before parturition, at calving, 6 and 12 hours after calving, and 3 and 28 days in lactation, further urine samples were taken once, right after calving. Serum was analyzed for Ca, Mg, and P, and urine was analyzed for Ca, Mg, P and creatinine. Analysis of variance of the repeated measures on the cows was done to compare effects of the diets, including contrasts for days of exposure to the rice bran diet, and days after diet withdrawal. Rice bran feeding produced a transient decrease of serum Ca during feeding, indicating a challenge to Ca homeostasis. Rice bran also reduced serum P, and withdrawal reduced serum Mg, indirectly indicating a hormonal reaction to the diet. At calving, the treatment improved the nadir of serum Ca, although did not change the odds of receiving a Ca infusion. Serum Ca after calving recovered faster after rice bran feeding, and blood Ca level was higher at calving and for at least 72 hours. Improved calcaemia after calving indicates that anticipation of homeostatic adaptation by feeding protected rice bran was effective in improving homeostatic competence. Magnesium status was little affected by the diet. Serum P was better sustained in cows that gave birth during rice bran feeding. The decrease of blood P levels at calving was less profound, and recovery to pre-calving levels was already 6 hours after parturition. Rumen-protected rice bran reduced dietary availability of Ca before calving and induced adaptation of Ca homeostasis in multiparous dairy cows, which improved Ca and P homeostasis at calving.

Introduction

Ca homeostasis has a high priority to every animal because high positive or negative fluctuations of blood Ca can result in death. Consequently, natural selection has led to accurate physiological mechanisms to monitor blood Ca, that respond to fluctuations by means of modulation of urinary excretion, enhancement of gastrointestinal absorption and control of bone turnover (DeGaris and Lean, 2008, El-Samad et al., 2002, Goff, 2008).

Modern dairy cows produce quantities of milk that by far exceed the amount required to feed their progeny. This surplus of milk is a distinctive feature of dairy cows from naturally evolved mammals and has physiological implications for which these man-selected genotypes are not yet adequately adapted. Their ancestors, and still nowadays beef cows, supplied Ca to their foetus before birth in comparable amounts to that provided in milk right after calving (Ramberg et al., 1984). However, in high producing dairy cows, Ca needs follow no longer a continuous function at calving and create an unequalled challenge for Ca homeostasis. Multiparous dairy cows are suffering from this challenge for homeostatic control of Ca at a moment - before parturition - when net supply has exceeded daily requirement for several months, and consequently gastrointestinal absorption and bone mobilization have been down-regulated. The delay in up-regulation of intestinal absorption, combined with reduced feed intake at calving can explain hypocalcaemia in dairy cows around parturition. This disease is specific to dairy cattle breeds and is known as milk fever.

Limiting Ca availability in the weeks before calving has been proposed as means to activate the dormant homeostatic mechanisms, and prepare the animals preventing in this way milk fever. Reducing Ca intake was proposed already decades ago (Goings et al., 1974, Kichura et al., 1982, Shappell et al., 1987, Wiggers et al., 1975, Yarrington et al., 1977). More recently it has been successfully attempted to reduce intestinal availability by dietary means (Enemark et al., 2003a, Enemark et al., 2003b, Thilsing-Hansen et al., 2002, Thilsing-Hansen et al., 2003, Thilsing et al., 2007).

Phytic acid is a well studied dietary antagonist of Ca absorption in monogastric animals (Lonnerdal et al., 1989). Rice bran is the common feed with the highest content of phytic acid, and the protection of this component from ruminal

degradation has been demonstrated by different means as fat coating (Chapter 2) and formaldehyde treatment (Chapter 3). Formaldehyde treatment is a technological aid to increase rumen escape of feed components that does not affect feed intake. Although inhalation of formaldehyde is a health threat, its use for treatment of feeds is considered to be of no concern for food safety as residual levels are similar as natural formaldehyde contents in feeds and foods (Gulati et al., 2005).

Formaldehyde-treated rice bran has proven to affect Ca homeostasis of dry cows (Chapter 3) and hence the purpose of this trial is to evaluate the prophylactic value of formaldehyde-treated rice bran included in the diet against periparturient hypocalcaemia.

Materials and methods

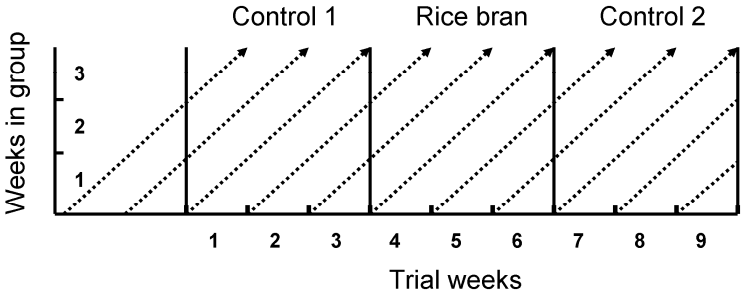
This trial was approved by the ethical committee of the Thueringer Landesanstalt fuer Landwirtschaft and took place at a commercial dairy farm in Thuringia, Germany. The herd consisted of approximately 1100 Holstein dairy cows, excluding replacement stock. The close-up group included cows and heifers from 3 weeks before expected calving date until calving. Affected by calving pattern, the size of this group varied between 60 and 100 animals. At calving the cows were brought into a separate calving pen, and hours after calving they were transferred to an early lactation group.

Before the start of the trial, the close-up group was fed a total mixed ration (TMR) that included Ca chloride to reduce the dietary cation-anion difference (DCAD). For the trial 3 kg of concentrates of the TMR including all supplemental minerals were replaced by 3 kg of experimental feeds. Two different feeds: control (C) and rice bran (RB) defined the treatments. Feed formulas and nutrient compositions are described in Table 1. Rice bran feed contained 800 g/kg fresh weight of fat-extracted rice bran, treated with formaldehyde at 3000 ppm fresh weight. A placebo feed was formulated with common feedstuffs to match the macronutrient profile of the RB feed.

The dietary treatments were applied to ration of the close-up group in 3 periods of 3 weeks, first control (C1), then RB and then back to control (C2). Each diet was fed for 3 weeks to the cows in the close-up group and then the diet was switched

(Figure 1). Because cows joined the group according to their expected calving date and left the group after parturition, the number of days of exposure to the RB treatment varied between 1 and 21 days.

Figure 1. Scheme of cow flow through the close-up group and exposure to the diets (each week a new group of about 10 to 15 cows entered). The arrows indicate the expected exposure to the diets by the cows



Samples and analyses

The original rice bran, the 2 experimental feeds and pools of samples of the 3 TMR rations taken during feeding at the farm were analyzed for DM , ash, ether extract (EE), crude protein (CP), sugars (European Commission, 2009) and starch (NEN 3574/C1, 1979). Neutral detergent fiber (NDF) was determined with alpha amylase (Van Soest et al., 1991) without sodium sulphite and expressed without residual ash. Calcium, Mg, Na, and K were determined by calcination at 500˚C, digested with hydrochloric acid and later measurement by atomic absorption spectrophotometry (Perkin Elmer AAnalyst 800). Phosphorus was analysed by spectrophotometry (AOAC, 2005; 4.8.14). Chloride was analyzed following the AOAC method number 969.10 (AOAC, 2005) and S was analyzed by dual range sulphur carbon analysis (Leco SC-144DR, Leco Corporation, St Joseph, MI, USA). Oxalic acid content was determined as in Canale et al. (1984). Phytic acid was analyzed by colorimetry with the AOAC method number 965.17 (AOAC, 2005), which consists on vanadomolybdate reaction with inorganic phosphate generated by phytase catalysis. Analyses of Na, K, Cl and S were used to calculate DCAD, by

adding Na and K equivalents and subtracting Cl and S equivalents, as calculated by Block (1984).

Every week a blood sample was taken from all multiparous cows in the close-up group. Blood samples were also taken right after and at 6 and 12 hours after calving. Urine samples were taken right after calving. Additional blood samples were taken in the early lactation period at 3 and 28 days in lactation.

Urine samples were analyzed for Ca, Mg, P and creatinine. Blood samples were analyzed for total Ca, Mg and P. Ca and Mg in blood and urine were determined by atomic absorption spectrophotometry (Thermo Scientific, Oberhausen – Germany). P in blood and urine were measured according to the standard procedure using an autoanalyser (Cobas Mira Plus, Roche Diagnostic Systems, D-79639 Grenzach-Wyhlen – Germany).

Calculation of days in treatment

Cows joined the close-up group 3 weeks before their expected calving date. Gestation length has a standard deviation of 6.3 days according to Jamrozik et al. (2005), therefore cows spent a variable number of days in the group. Subsequently, diets were fed to the group for 3 weeks; hence cows were exposed to the diets for a variable number of days ranging between 1 and 21. A scheme of the distribution of treatments over the periods in the trial is displayed in Figure 1.

Previous experiments have demonstrated that rice bran feeding has effects on Ca metabolism depending on time of exposure and on time after withdrawal (Chapter 2). In order to study the effect of the time of exposure to the product, for each pre-calving sampling of the rice bran feeding period the number of days of rice bran consumption was determined, and also if the sample was taken during C2, the number of days after withdrawal of rice bran was calculated.

The effect of time of product exposure on post-calving observations was investigated by calculating the total number of days of rice bran received by the cows before calving. When rice bran was fed until the moment of calving (all cows in RB group), the number of days was considered a separate variable. Conversely, if calving took place after rice bran feeding ended (treatment switched back to control), the number of days of before calving but after withdrawal was also considered as a separate time variable.

Table 1. Raw material composition and chemical analysis of the original rice bran and the experimental feeds

	Rice bran	Rice bran feed	Placebo
Formaldehyde-treated rice bran	100.00	80.00	--
Soybean meal 44%	--	9.78	--
Maize	--	--	20.25
Wheat gluten	--	--	6.52
Palm kernel expeller	--	--	6.51
Rapeseed meal	--	--	28.00
Beet pulp	--	--	8.00
Soy hulls	--	--	20.50
Urea	--	1.25	1.25
Cane molasses	--	3.50	3.50
Vinasses	--	3.50	3.50
Salt	--	0.35	0.35
MgO_2	--	0.54	0.54
Mineral vitamin premix	--	1.08	1.08
% fresh weight			
DM	895	858	890
Ash	102	107	72
CF	34	28	42
CP	165	245	230
Starch	384	284	140
Sugars	63	82	74
NDF	295	234	336
Ca	0.7	3.6	6.0
P	22.0	18.2	5.6
Mg	9.7	11.9	7.4
Na	0.9	2.7	2.9
K	18.5	19.1	12.5
Cl	6.1	5.6	5.1
S	2.7	4.4	4.8
DCAB*	172	172	2
Oxalic	8.6	5.9	3.0
Phytic acid	61.5	42.0	9.1
g/kg DM, * meq/kg DM			

Statistical analysis

Before analysis of variance, the distribution of the different observations was checked for normality, and those presenting positive or negative skewness were mathematically transformed to fit a bell curve. Blood Ca, Mg and P in the pre-

calving period presented a normal distribution after the elimination of samples from the 3 days before calving, but post-calving Ca and P analyses presented negative skewness and required transformation with the inverse functions "Ca' = 1 / (4 - Ca)" and "P' = 1 / (4.5 - P)". Post-calving blood Mg instead presented negative skewness and was transformed with "Mg' = 1 / (Mg + 1)". Urinary excretion of Ca, P and Mg after calving were expressed as a fraction of creatinine and also transformed as the inverse of the value added to one to make all values greater than one. Further, binomial observations such as receiving infusion or not, retained placenta and culling in the first 8 weeks of lactation were processed under logit transformation and were analyzed with PROC GENMOD of SAS.

Analysis of variance was done on blood and urine observations with the MIXED procedure of SAS considering observations as repeated measures on the subject cow. Post-calving samples were assigned a banded (Toeplitz) covariance structure because samples were not equally spaced in time. Weekly samples from the pre-calving period were computed with an autoregressive (AR1) covariance structure with random effect cow, because they were equally spaced in time.

Analysis of variance of pre-calving observations included diet and parity. Days on rice bran feeding and days after withdrawal were included for estimating linear and quadratic contrast. Least square means by diet were calculated including only the factors that were significant at $P < 0.10$ in the model.

Analysis of variance of post-calving observations included parity, diet at calving, time point after calving, and the interaction of diet at calving and time point. Additionally, per cow the average of the last 2 pre-calving determinations of that parameter was included as a co-variable, and linear and quadratic contrasts were tested for total days of rice bran consumption, rice bran consumption ending at calving, and days between rice bran withdrawal and calving. Least square means by diet at calving were calculated using a model with only the factors that presented a $P < 0.10$.

Incidence of production diseases, metabolic indicators and production after calving were analyzed including diet at calving, parity, production in the previous lactation and linear and quadratic contrast were tested for total days of rice bran consumption, rice bran consumption ending at calving, and days between rice bran

withdrawal and calving. Least square means by diet at calving were calculated including in the model the factors that were significant at $P < 0.10$.

Table 2. Raw material composition and analyses of the TMR diets fed in the three periods

	Control 1	Rice bran	Control 2
Grass hay	3.0	3.0	3.0
Grass silage	4.0	4.0	4.0
Corn silage	12.0	12.0	12.0
Pressed beetpulp	2.6	2.6	2.6
Total corn cob silage	1.6	1.6	1.6
Rice Bran feed	--	3.0	--
Placebo feed	3.0	--	3.0
Kg fresh weight over a daily ration of 26.2kg fresh weight (14.6 kg DM)			
Ash	74	73	75
CF	35	39	42
CP	132	143	133
Starch	145	177	168
Sugars	25	28	26
NDF	433	400	403
Ca	4.7	4.2	4.8
P	3.6	5.7	3.6
Mg	2.3	3.3	2.5
Na	3.6	2.1	3.1
K	15.9	17.1	14.6
Cl	7.6	6.3	7.3
S	2.6	2.4	2.3
DCAB	191	200	163
g/kg DM, * meq/kg DM			

Results

Chemical analysis of original rice bran confirmed the expected very low Ca, low fat and high phytic acid content (Table 1). The placebo feed prepared for the control diet had a comparable macro nutrient composition as compared with the rice bran experimental feed, which contained 80% rice bran. An exception to this rule was

starch content, for which the rice bran feed had higher contents, and NDF, which was higher in the control feed. Further differences resulted from the nutrient profile of rice bran; phytic acid in rice bran feed was four fold that of placebo, resulting in a greater P content. Calcium was also 2.6 g/kg DM lower in the final experimental feed as compared to the control (Table 1).

The differences in composition of the TMR diets were small except for those in mineral profiles, which are created by the treatments (Table 2). The diet during the rice bran period contained 0.5 g/kg DM less Ca and 2.1 g/kg DM more P compared to the control feed. This reflects the high amounts of phytic acid supplied by rice bran (Table 2). The calculated inclusion of rice bran in the final experimental diet was 140 g/kg DM.

The pyramidal age structure of the multiparous animals used for the experiment is described in Table 3. One third of the cows were about to calve for the second time and almost another 30% were approaching their third calving. Cows having completed more than 4 lactations were the exception, being less than 10% of the total. The distribution between the dietary periods was not completely balanced in numbers and parities. Somewhat more cows calved during rice bran feeding than during control periods and these cows were older. In addition one third of these had completed more than 4 lactations.

The effect of the diet on mineral status in the blood before calving is displayed in Table 4. Rice bran feeding significantly reduced blood Ca, and this reduction was dependent on the number of days of exposure to the diet before sampling. Blood Mg was reduced by the withdrawal of rice bran from the diet, and was affected by the time elapsed from the withdrawal. Phosphorus was reduced by the inclusion of rice bran and the effect was sustained in time after withdrawal.

Table 3. Parity, previous milk production and distribution in the three calving periods of the cows in the trial

Completed lactations	Average age	Milk yield in previous lactation (kg)	Parity structure of the total trial and animals calving in the feeding periods (%)			
			Trial	Control 1	Rice bran	Control 2
1	3.3	7833	33.6	41.9	28.9	32.4
2	4.5	9495	27.4	16.1	31.1	32.4
3	5.3	9272	16.8	25.8	13.3	14.2
4	6.5	10044	12.4	9.7	11.1	16.5
5	7.6	9431	7.1	6.5	8.9	5.4
6	8.5	9182	1.8	0.0	4.4	0.0
7	9.4	8280	0.9	0.0	2.2	0.0
			100%	100%	100%	100%
Total animals	4.8	8956	113	31	45	37

The incidence of blood Ca infusions, retained placenta, culling in the first 8 weeks, and blood Ca Nadir is presented in Table 5. The odds ratio for cows to receive a Ca infusion after calving was affected by parity and by milk production level in the preceding lactation, but appeared to be independent from the dietary treatments. Instead, the nadir of blood Ca for the animals was significantly higher for cows calving during rice bran supplementation. Variation of Ca nadir was explained by dietary treatment, and also by days of exposure to the treatment, and more clearly when rice bran treatment was maintained until calving. Additionally, just like for the odds to get a Ca infusion, parity and production level were explanatory to the lowest Ca level observed in each cow. Risk for retained placenta and culling in early lactation were calculated for the different calving periods, but neither diet, parity nor production level was explanatory for these factors.

Average blood Ca level after calving was higher in cows calving during the period of rice bran feeding and in cows which were switched back to the control in the third feeding period (Table 6). Calcaemia after calving was influenced by days of exposure to the product, parity and sampling time. These results were very similar

when the samples taken after a Ca infusion were not excluded from the calculation. Blood Ca drop at calving was less severe for animals consuming rice bran until calving, and immediately started a trend of recovery, whereas control cows stayed at minimum values for hours after calving (Figure 2). Three days after calving, treated cows still had significantly better Ca status than the controls.

Blood Mg seemed to be mostly dependent on sampling time after calving and on the total exposure time to rice bran diet in days. Blood Mg was not clearly influenced by calving period and was independent from parity (Table 6). Magnesium level increased at calving and then declined to return to the original range 3 days after calving (Figure 3).

Blood P was clearly higher in cows that calved during rice bran supplementation, and was somewhat dependent on the number of days of dietary treatment before calving. In this case, parity was an important factor, but also sampling time and its interaction with diet at calving (Table 6). Blood P decreased much less in animals that were exposed to rice bran until calving as compared with the controls, and already 6 hours after calving they had recovered values in the range of those observed before calving (Figure 4). Differences with controls were sustained until day 3 after calving and were not evident anymore 28 days after calving.

Urinary excretion of Ca, Mg and P, in the hours after calving, were affected by the close-up diet (Table 7). Calcium excretion was much greater when cows calved during the second control period, and was explained by the time between product withdrawal and calving. Urinary excretion of Mg was unaffected by diet at calving, and only parity was explanatory to its variation. Phosphorus excretion was much greater when rice bran was fed before calving, although unaffected by the number of days of exposure tot the diet.

Table 4. Blood parameters during feeding period, by diet, parity and days of RB feeding or days after product withdrawal (mmol/l)

	Diet at sampling				Diet	Parity	Days in RB diet		Days after withdrawal RB	
	Control 1	Rice bran	Control 2	SEM	P<	P<	P<		P<	
							Lin.	Quad.	Lin.	Quad.
Ca blood	2.45a	2.29b	2.45a	0.018	<0.01	<0.01	<0.01	<0.01	ns	ns
Mg blood	0.87a	0.89a	0.80b	0.015	<0.01	0.02	0.08	ns	<0.01	0.02
P blood	1.95a	1.81b	1.71b	0.035	<0.01	<0.01	<0.01	<0.01	ns	ns

Difference in letters indicates P<0.05

Table 5. Incidence of production diseases and metabolic indicators in early lactation

	Diet at calving				Diet at calving	Parity	Production last lactation (305days)	Total days RB		Days RB (before calving)		Days between withdrawal RB and calving	
								P<		P<		P<	
	Control 1	Rice bran	Control 2	SEM*	P<	P<	P<	Lin.	Quad.	Lin.	Quad.	Lin.	Quad.
Ca infusion at calving	0.44	0.36	0.35	0.381	ns	<0.01	0.01	ns	ns	ns	ns	ns	ns
Nadir blood Ca (mmol/l)	1.68a	1.88b	1.71a	0.008	<0.01	<0.01	<0.01	0.10	0.07	<0.01	<0.01	ns	ns
Retained placenta	0.10	0.13	0.11	0.521	ns	ns	ns	ns	ns	ns	ns	ns	ns
Culling in first 8 weeks	0.10	0.07	0.08	0.602	ns	ns	ns	ns	ns	ns	ns	ns	ns

Difference in letters indicates P<0.05

** SEM is reported in the different transformed scales, because confidence intervals are not symmetric from the mean.*

Table 6. Blood parameters after calving as affected by diet at calving, sampling time, parity and time of exposure to rice bran in the close up period (mmol/l)

	Diet at calving				Base level	Diet	Time	Diet x Time	Parity	Total days RB		Days RB (before calving)		Days between withdrawal RB and calving	
	Control 1	Rice bran	Control 2	SEM*	P<	P<	P<	P<	P<	P<		P<		P<	
										Lin.	Quad.	Lin.	Quad.	Lin.	Quad.
Ca	2.05a	2.18b	2.13b	0.0079	ns	0.01	<0.01	0.08	<0.01	ns	0.09	<0.01	<0.01	ns	ns
Ca[1]	2.08a	2.20b	2.15b	0.0066	ns	<0.01	<0.01	0.18	<0.01	0.09	0.05	<0.01	<0.01	ns	ns
Mg	0.93a	0.94ab	0.98b	0.0046	<0.01	0.08	<0.01	ns	ns	0.03	0.01	ns	ns	ns	ns
P	1.34a	1.69b	1.29a	0.0063	0.04	<0.01	<0.01	<0.01	0.02	ns	ns	0.04	0.06	ns	ns

Difference in letters indicates P<0.05. Ca[1] are blood Ca measurements including those obtained 24 hours after Ca infusion.

** SEM is reported in the different transformed scales, because confidence intervals are not symmetric from the mean.*

Table 7. Urinary content of Ca, Mg and P after calving relative to creatinine (Ca/creat.)(mmol/l/mmol/l)

	Diet at calving				Diet	Parity	Total days RB		Days RB (before calving)		Days after withdrawal RB	
							P<		P<		P<	
	Control 1	Rice bran	Control 2	SEM*	P<	P<	Lin.	Quad.	Lin.	Quad.	Lin.	Quad.
Ca / creat.	1.37a	1.19a	2.37b	0.033	0.01	ns	ns	ns	ns	ns	0.05	0.05
Mg / creat.	8.08	6.52	9.52	0.190	ns	0.05	ns	ns	ns	ns	ns	ns
P / creat.	3.28a	4.73b	3.42a	0.011	<0.01	0.06	ns	ns	ns	ns	ns	ns

Difference in letters indicates P<0.05.

** SEM is reported in the different transformed scales, because confidence intervals are not symmetric from the mean.*

Discussion

Milk fever is caused by the delay to adapt Ca homeostasis to the new Ca balance between blood clearance and blood input. Three physiological mechanisms can be modulated to respond to changes in Ca clearance, these are: Renal reabsorption, bone turnover and gastrointestinal absorption. The kidneys are naturally fast reacting to PTH signals, and it is efficient for correcting positive serum Ca fluctuations (Ramberg et al., 1984), but quantitatively of little value to prevent hypocalcaemia (Schonewille et al., 1999).

Bone is an extensive resource of Ca, and its mobilization is induced by PTH in close coordination with calcitriol (Horst et al., 2005). Bone tissue mobilization is only sustained in time with the simultaneous stimuli of both hormones, therefore if Ca absorption for the gastrointestinal tract is able to compensate blood Ca clearance, PTH signal would disappear and bone mobilization would cease. This mechanism defines a priority for gastrointestinal Ca above bone Ca to compensate for Ca clearance. Activating existing osteoclasts can take place within hours. However, the increase of osteoclasts numbers takes about 2 days (Erben, 2001). This time delay is caused by the process of cell differentiation, and coincides with the time of PTH stimulation for bone mobilization in pregnant cows (Goff et al., 1986).

Active transcellular Ca uptake from the gut in ruminants takes place not only in the intestine, but also in the rumen (Dua et al., 1994). This mechanism is induced by calcitriol and presents many similarities with renal reabsorption (Hoenderop et al., 2005). A very relevant difference in regulation of these mechanisms of transepithelial Ca transport is time of response. As in the case of bone tissue, cell differentiation seems to play a role in the regulation of absorption (Walters and Weiser, 1987). Maximum Ca transfer across these epithelia reaches a maximum after 24-48 hours, coinciding with the typical time for crypt cells to migrate to the tips (Kumar, 1986). Anticipating the signal to induce adaptation is the only way to prevent that the lag time of adaptation coincides with the sudden increase in Ca clearance.

Low Ca intake is one measure to activate the regulatory response of Ca homeostasis and the current study simulated this approach by preventing Ca absorption with rice bran, which acts on Ca availability in two ways: diluting Ca content in the diet with its very low Ca content, and affecting the nutritional

accessibility of Ca by gastro-intestinal precipitation with phytic acid (Chapter 2). The process of rice milling concentrates P, Mg, K, Zn and Fe in the by-product (Resurrection et al., 1979). Nearly all phytic acid remains in the outer layers during polishing, while the gradient between the fractions is less marked for Na and it is moderate for Ca and K (Kennedy and Schelstraete, 1975). Therefore Ca content in rice bran is in general below 1 g/kg DM. Consequently, the rice bran used for feed manufacture in this trial had a very low Ca content as compared with the other feed ingredients (Table 1). The inclusion of rice bran in the ration created a difference in Ca intake of 0.5 g/kg DM (Table 2), which for a DMI of 14 kg causes a reduction of 7 g/d, representing a decrease in Ca intake of about 10%.

The rice bran used in this trial contained a high amount of phytic acid (Table 1), which is the main Ca binding component of rice bran. It accounts for more than 80% of its Ca binding capacity (Siener et al., 2001). The 61.5 g of phytic acid per kg DM possesses a theoretical binding potential of 22 g of Ca, for a 6 to 1, Ca to phytic acid molar ratio. In the ration, the inclusion of approximately 140 g/kg DM rice bran represents a potential binding in the total ration of 3 g of Ca per kg DM. Previous work by our group has shown that formaldehyde treatment of rice bran leads to a rumen escape fraction of phytic acid of nearly 30% (Chapter 3), which for the present rice bran inclusion results in a post-ruminal binding capacity of near 1 g of intestinally available Ca per kg DMI.

Natural variation in the distribution of the calving events caused that 45 cows calved during rice bran supplementation, as compared to the control periods in which 31 and 37 cows calved. In addition to that, cows calving during that period were older (Table 3). Parity increases the risk of milk fever by 9% per lactation (DeGaris and Lean, 2008), so it is logical to expect that cows calving during rice bran feeding period were substantially more susceptible to hypocalcaemia than the controls.

Cows in the close-up group showed lower blood Ca during rice bran supplementation (Table 4). Blood Ca during rice bran feeding was strongly related to the number of days between product introduction and the date of sampling, because the decrease in blood Ca after rice bran introduction was corrected by Ca homeostasis after a few days. This effect is in agreement with the similar drop and later recovery consistently observed at the introduction of low Ca diets (Goings et al., 1974, Green et al., 1981, Kichura et al., 1982, Shappell et al., 1987). In

contrast, this pattern is not observed at the introduction of zeolites in the diet (Grabherr et al., 2009, Pallesen et al., 2008, Thilsing-Hansen et al., 2002).

The odds to receive a Ca infusion at calving were not affected by the dietary treatments, although parity and production level were clearly explanatory of this parameter (Table 5). This means that under these conditions, rice bran did not affect the external perception of milk fever by farm staff. In contrast, rice bran had a clear effect on the magnitude of hypocalcaemia measured in the cows. Also the number of days of rice bran feeding affected the preventive value of the treatment. The effectiveness was also associated to feeding until the calving date, because the number of days of feeding before calving was more explanatory of calcaemia at calving than the total number of days of rice bran consumption. Rice bran induced an adaptation of Ca homeostasis that was useful to sustain serum Ca at calving, confirming the main hypothesis of the experiment. This effect is further confirmed by the average serum Ca (Table 6) and its evolution after calving (Figure 2). Average blood Ca was higher for animals that consumed rice bran, especially those that stayed on the diet until calving, regardless of the consideration or exclusion of samples taken 24 hours after an infusion with Ca. The consistency between the two sets of Ca observations is of practical relevance for the design of milk fever trials, when the inevitable intervention to save the most severe milk fever cases, can be a confounding factor, either if data is used or excluded from the analyses. The evolution of serum Ca after calving is explanatory of the effect of the treatment. Not only calcaemia at calving is higher for the treated animals, but also recovery starts immediately whereas control animals exhibited serum Ca at minimum values for hours after calving. Speed of recovery of Ca may be more indicative of dietary induction of homeostatic adaptation than the extent of hypocalcaemia.

The immediate start of the recovery of calcaemia in treated cows (Figure 2) demonstrates that homeostatic mechanisms are capable to compensate Ca clearance without requiring a lag time for adaptation to take place. However, it is possible that only gastrointestinal absorption is prepared for increased absorption, which can be the case if increased absorptive efficiency made bone mobilization redundant. In the present experiment, hypocalcaemia still takes place and the recovery after calving is relatively slow as compared with the preventive effect of low Ca diets and zeolites.

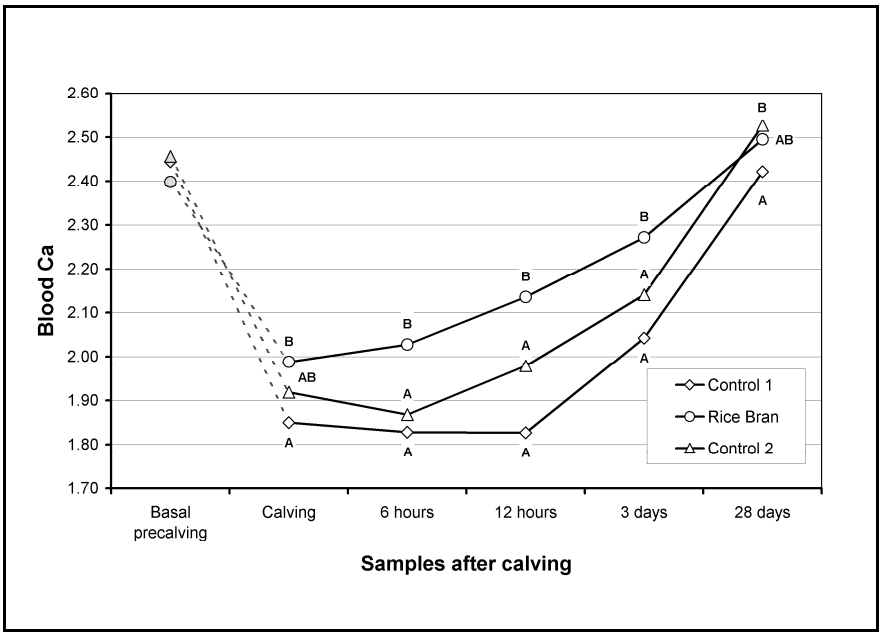


Figure 2. Effect of diet at calving on the time course of blood Ca after calving (mmol/l)

The moderate preventive value of the treatment could be explained by a limited adaptation of bone tissue, leaving gastrointestinal Ca as the only effective means for compensation. Considering that feed intake is normally reduced in the hours around calving, gastrointestinal Ca content might have been limiting to effectively avoid hypocalcaemia. Rice bran dose was relatively low for the Ca content of the diet, as compared to previous experiments (Chapter 2) and the concept of induction of homeostatic adaptation before calving is dose dependent in the case of zeolites (Grabherr et al., 2009). Additionally, the time of exposure to the treatment was variable between 1 and 21 days, which may have further reduced the average degree of induction to adaptation of the treated animals.

Calcium intake at calving could be a major factor for the efficacy of previously adapted gastrointestinal absorption to compensate for Ca clearance. Early studies done with zeolites included an oral supplementation at calving of 90 g of Ca (Thilsing-Hansen and Jorgensen, 2001, Thilsing-Hansen et al., 2002), which may explain in part the total hypocalcaemia alleviation demonstrated in these studies. On the other hand, despite the exclusion of this practice in other trials, the

product maintained a high preventive efficacy (Grabherr et al., 2009, Pallesen et al., 2008). Urinary Ca after calving was higher for the animals calving after product withdrawal, and was conditioned by the number of days after withdrawal. This effect of Ca temporarily increasing after withdrawal, was observed in previous experiments (Chapter 2), and its understood to be caused by a disturbance in PTH signal when active gastrointestinal absorption stays up-regulated for about 2 days, and the kidneys correct the temporary positive fluctuation in calcaemia.

Blood Mg is not as tightly regulated as Ca, but it is affected by the homeostatic system of Ca (Fontenot et al., 1989). In the present experiment, rice bran feeding had no apparent effect on Mg status, although there was a clear reduction caused by the withdrawal of the product. Urinary excretion of Mg is modulated by PTH (Deetz et al., 1982) in parallel with Ca excretion. Rice bran withdrawal has shown to produce peaks of urinary Ca excretion for about 2 days, caused by a ceased PTH signal while intestinal Ca absorption is maintained active (Chapter 2).

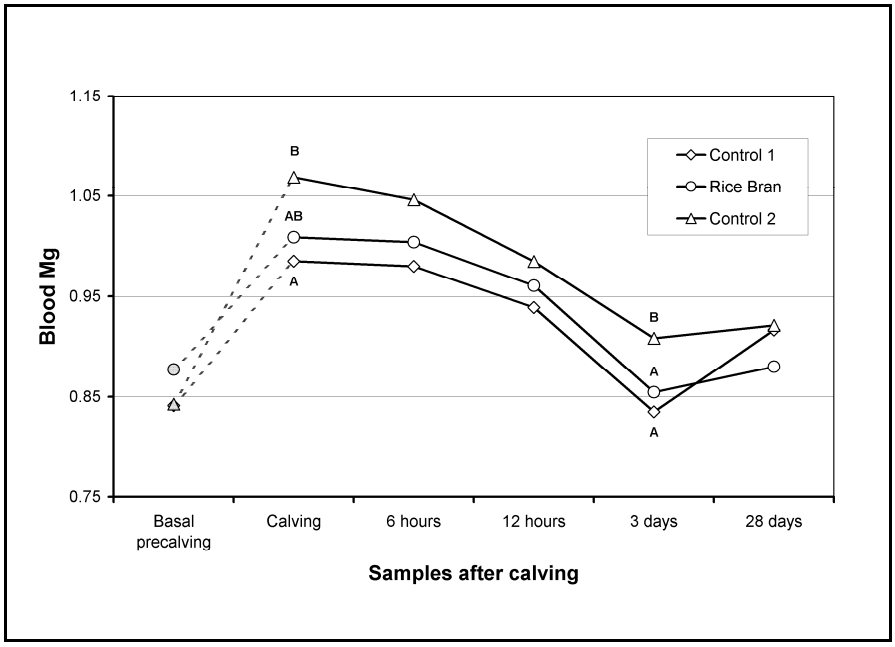


Figure 3. Effect of diet at calving on the time course of blood Mg after calving (mmol/l)

Serum Mg presented a transient decrease after rice bran withdrawal, possibly as consequence of the ceased PTH signal at recovery of calcaemia, which may have induced a greater urinary Mg excretion. The increase of serum Mg at calving and the hours after observed in Figure 3 is typical of periparturient cows with an adequate Mg status (Goff, 2006), and reflects PTH secretion in a situation of hypocalcaemia, which should increase renal reabsorption of Mg. However, this effect on Mg regulation was not affected by the diet (Table 7). Rumen-protected rice bran has shown to increase serum Mg in heifers in previous experiments (Chapter 4). Rice bran is a rich source of Mg, and phytic acid does not clearly affect the Mg availability (Coudray et al., 2003). Feeding of rumen-protected rice bran is not a threat to Mg status because of these factors, together with the fact that the main site of absorption for Mg in the ruminant is the forestomach (Martens, 1983).

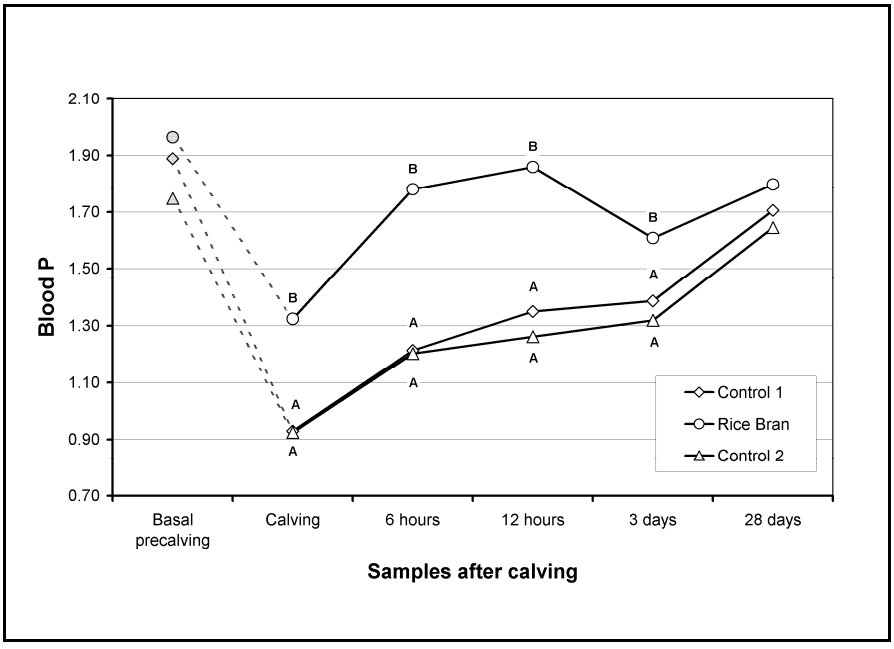


Figure 4. Effect of diet at calving on the time course of blood P after calving (mmol/l)

Serum P was reduced by rice bran feeding despite the high P content in this diet (Table 4). In contrast to Ca, P absorption is mostly directly related to intake, since its regulation is controlled by salivary excretion and to a lesser extent by urinary excretion (Horst, 1986). Under specific conditions, serum P correlates positively with P intake (Lopez et al., 2004), although this relation is not consistently observed (Peterson et al., 2005). Lower serum P during high P intake may be explained by indirect effects of the reduction in Ca availability. PTH reduces serum P by its effect on salivary excretion (Horst, 1986) and by increasing its urinary excretion (Goff et al., 1986). This effect of PTH lowering serum P has also been observed during low Ca diets for the prevention of milk fever (Green et al., 1981, Shappell et al., 1987). A much more severe hypophosphatemic effect is described for zeolites, for which there seems to be an additional direct effect on P availability (Thilsing et al., 2006).

Serum P decreases at calving with the combined effect of P drain into colostrum (Kume and Tanabe, 1993) and the effect of PTH on increased urinary and salivary P excretion (Goff et al., 1986). However, serum P was clearly higher in the cows that consumed rice bran at calving, and this effect was independent from the number of days of treatment before calving (Table 6). Calcitriol stimulates P absorption (Breves and Schröder, 1991), and in this way, serum Ca and P are simultaneously brought back to normal levels. Rice bran fed before calving created wide differences in the evolution of P concentration after calving (Figure 4). Not only was the value reached at calving higher, but also recovery to pre-calving levels was immediate. Because this effect is strictly related to rice bran fed the day before calving and independent from time of exposure to the product, most likely the difference is caused by a much greater presence of P in the gastrointestinal tract in the treatment group, and not by any form of P homeostatic competence acquired before calving. Urinary P is higher for cows that calved during treatment, and this excretion was also independent of days on product or days after withdrawal, further supporting that the abundance of P in the diet is the main explanation for better recovery of serum P and greater urinary excretion mediated by PTH (Table 7).

Conclusion

Feeding rumen-protected rice bran, at an inclusion of 140 g/kg DM, and with a phytic acid content of 61.5 g/kg DM, induced changes in Ca homeostasis. These can be explained by a reduced gastrointestinal availability of dietary Ca. It is understood that Ca availability was reduced below the threshold of covering basal requirements by means of paracellular passive absorption, inducing an adaptation of Ca homeostasis. Anticipation of this adaptation before calving could explain the observed improved Ca homeostatic competence at calving. Serum Ca in the hours after calving was improved by rice bran fed before calving; however the magnitude of the improvement was moderate. The present trial confirmed the hypothesis that rumen-protected rice bran can be used to anticipate homeostatic adaptation and prevent hypocalcaemia, but it is necessary to study whether a higher intake of protected rice bran could provide complete prevention of hypocalcaemia, as described for low Ca diets.

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CHAPTER 7

General discussion

Milk fever is a unique disease because hypocalcaemia is a rare condition in animals. Calcium homeostasis is a robust system that seldom fails under natural conditions. Dairy cows in the time around calving, and only then, represent the exception to this rule. Even in early lactation, when Ca metabolism is strongly challenged by high milk yields, Ca homeostasis proves to be robust enough. The cause for hypocalcaemia around calving is to be found in the delayed reaction of adaptive mechanisms of Ca homeostasis. In dairy breeds these mechanisms fail to compensate the sharp increase in Ca withdrawal from blood, caused by the greater Ca needs of early lactation as compared to those of late gestation. In order to understand the aetiology of milk fever, it is necessary to understand these adaptive mechanisms in their ability to respond to natural challenges, such as calving, or dietary induced challenges such as that proposed in this thesis. The reduction of dietary availability of Ca through rice bran feeding is meant to challenge Ca homeostasis, anticipating adaptation and therefore preparing the cow for the challenge of calving.

Adaptive mechanisms of Ca homeostasis

Animal nutrition often considers nutrient absorption as a constant competence, while instead it is constantly changing due to internal signals from the body and external stimuli from the diet. Many examples of these adaptations are well known (see review by Ferraris and Diamond, 1989). Calcium homeostasis can be described as a system with controlled signals, disturbing signals and controlling signals (Ramberg et al., 1984). A very interesting approach that already at that time pointed out that the cause of milk fever could be a delay in the adaptive mechanisms (controlling signals) that are responsible to compensate for sudden changes in Ca clearance from the blood. These adaptive mechanisms involve renal reabsorption, intestinal absorption and bone turnover. Now these mechanisms are much better understood, thanks to new molecular techniques that have become available in recent years. Renal reabsorption and intestinal absorption are both

tightly regulated processes of transepithelial Ca transport. Bone turnover is a tissue modification process regulated in such a way that it can anabolically or catabolically respond to aid Ca homeostasis, but also with respect for the structural function of bone.

Transepithelial transport processes

Passive non-saturable Ca diffusion takes place through the tight junctions of epithelia when Ca gradients allow for this paracellular transport. This mechanism allows for limited regulation by means of modification of epithelial permeability (Pérez et al., 2008). In transepithelial transport processes that require regulation competence, the leading mechanism of transfer is a transcellular saturable transport. Calcium re-absorption in the kidney and active intestinal absorption are transcellular processes that are coordinated by the hormonal Ca homeostatic system, and that can operate against the Ca concentration gradient.

Transcellular epithelial transport of Ca consists of three steps: facilitated entry into the epithelial cell, intracellular diffusion mediated by a binding protein, and active extrusion of Ca into the next extracellular compartment (Hoenderop et al., 2005). Such an elaborate system allows for efficient and controlled Ca transport, while complying with the need to keep free intracellular Ca at a minimum.

Calcium enters the epithelial cells through two highly specific Ca channels; these are TRPV5 (formerly known as ECaC1 or CaT2) and TRPV6 (formerly known as ECaC2 or CaT1). Calcium entry, although it is a passive transfer, is believed to represent the limiting and the key regulatory step of the process, strongly regulated by calcitriol (Bouillon et al., 2003) and extracellular Ca concentration (Nilius et al., 2002).

Once inside the epithelial cell, Ca needs to diffuse across the cytoplasm. Luminal Ca concentration is kept extremely low to protect normal cell function, and at that concentration simple diffusion can not quantitatively provide for the needs of Ca transfer of the intestine or kidney. Two cytosolic Ca binding proteins Calbindin-D9k and Calbindin-D28k have been described (Hoenderop et al., 2005). These proteins are calcitriol dependent, and are responsible for ionic Ca buffering in the cell and facilitating intracellular diffusion in transepithelial transport (Bronner, 2003). This step can be limiting for the process because when there is a lack of Calbindin proteins Ca transport can not be effective (Bronner, 2003).

The final step in transcellular Ca transport is extrusion out of the cell. This process is ATP mediated by two active Ca transporters; the Ca ATPase protein, also called plasma membrane Ca ATPase (PMCA), and the Na+/Ca2+ exchanger (NCX) (Bouillon et al., 2003). This step is also understood to be as well regulated by calcitriol (Hoenderop et al., 2005), but is less likely to be rate-limiting (Bronner, 2003), which reduces its importance in the regulation of the total transport process.

As many characteristics as transepithelial transport of Ca in the intestine and the kidney share, there are important differences in the regulation mechanisms that establish their different functions in Ca homeostasis, and also other differences based on the distinct nature of these tissues.

There are molecular differences between renal and intestinal transcellular Ca transport. The main Ca entry channel in the intestine is TRPV6 (Suzuki et al., 2008), whereas TRPV5 is the only entry channel in the kidney (Khanal and Nemere, 2008). Also, in mammals the predominant intracellular Ca binding protein in the kidney is Calbindin-D28k while in the intestine this role is played by Calbindin-D9k (Bouillon et al., 2003). It is understood that PTH has a predominant role in controlling renal Ca reabsorption, and that calcitriol plays the equivalent role in the intestinal system (Schröder and Breves, 2007). However, Vitamin D Receptors (VDR) are present in both tissues (Liesegang et al., 2008) and regulation has proven to have greater complexity, because both PTH and calcitriol can directly affect both renal and intestinal transport processes (Hoenderop et al., 2005). The differences between tissues are in the specific regulation of the different steps according to not only to hormonal actions, but also physiological situation and Ca presence in the compartments. Armbrecht et al. (1998) describes age dependent differences in Calbindin induction by calcitriol in speed of response and intensity between Calbindin-D28k in the kidney and Calbindin-D9k in the intestine. Also the structural differences between TRPV5 and TRPV6 result in different inactivation kinetics in their down-regulation as induced by Ca (Nilius et al., 2002), demonstrating further divergences between renal and intestinal Ca transport.

A specific characteristic of TRPV5 is its pH sensitivity, inhibiting its action to half during conditions of metabolic acidosis (Suzuki et al., 2008). This inhibition results in hypercalciuria, a common condition in cattle that are fed anionic salts (Schonewille et al., 1994, Roche et al., 2003). TRPV5 failure has been studied with TRPV5 gene knock-out mice. These animals combine the expected hypercalciuria

with hyperactivation of intestinal Ca absorption, combining high circulating calcitriol levels and increased TRPV6 expression (Suzuki et al., 2008).

Prevention of milk fever with anionic salts is well substantiated experimentally, although its mode of action has remained unclear. It has been proposed that bone mobilisation caused by metabolic acidosis would increase urinary Ca, creating a condition of increased intestinal absorption derived from increased calcitriol levels (DeGaris and Lean, 2008), but higher calcitriol levels are not justified in a situation of urinary correction of too high levels of blood Ca. Earlier it was proposed that lowering systemic pH would allow for functionality of calcitriol receptors that would have lost receptivity with metabolic alkalosis (Horst et al., 1994), however PTH responsiveness is adequate in other physiological stages with high systemic pH, as for example during lactation. TRPV5 inactivity in acidic conditions, combined with the counter-reaction of intestinal TRPV6, represents a plausible explanation for the mode of action of the prevention of milk fever by lowering DCAD.

A major difference between renal and intestinal epithelial tissues is the cell differentiation process of enterocyte maturation through migration from the crypts to the villus tips (Figure 1). Intestinal epithelium cells have a short live span of around 4 days only whereas that of the renal cells is about 160 days (Norman et al., 1981). The constant enterocyte turnover may allow for irreversible genetic regulation of the cells, on the contrary, the life span of renal cells is too long to maintain a fixed regulatory configuration. It has been suggested that enterocytes acquire Ca transport competence in early stages of differentiation, and express that ability in the mature form (Walters and Weiser, 1987), which is supported by the fact that maximum Ca transport is reached after 24-48 hours. This delay coincides with that required for migration of crypt cells to the villus tips (Kumar, 1986). Villus tips respond to calcitriol with Ca uptake and villus base cells do not, while Calbindin response is more evident in base cells than in cells from villus tips (Bikle et al., 1984). Also, VDR presence is greater in crypt cells as compared with tip cells, suggesting that calcitriol induces differentiation to produce cells that are more capable to express calcitriol dependent genes for active calcium absorption in the mature stage (Centeno et al., 2004). The main implication of this different adaptation to calcitriol action between renal and intestinal tissue is its effect on the time lag to acquire Ca transport competence, and the time for which this competence is maintained when the calcitriol stimulus ceases.

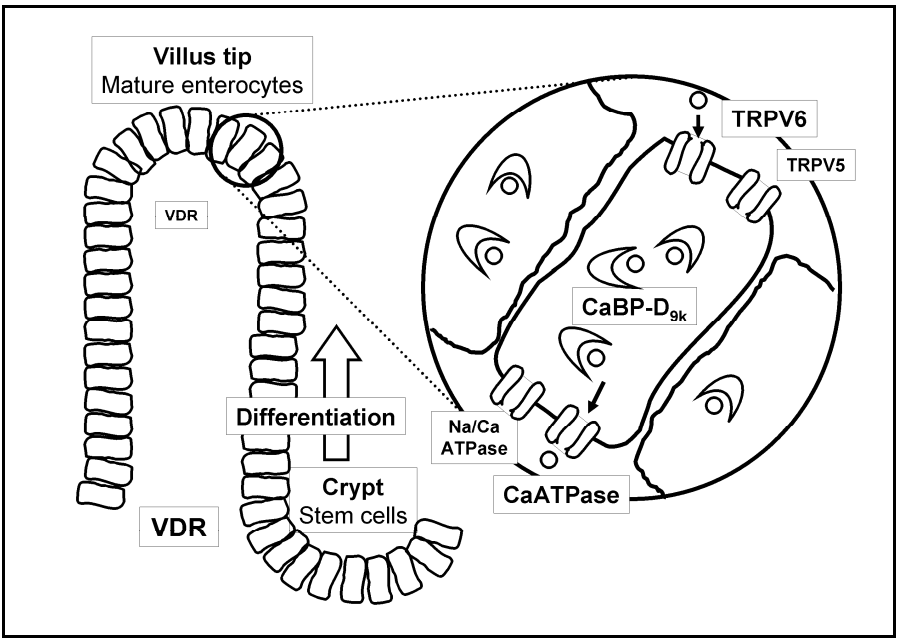

Figure 1. Regulation of gastrointestinal Ca absorption. Schematic representation of gastrointestinal transepithelial transport mechanism. VDR: Vitamin D receptor; TRPV5/6: Ca entry channels; CaBP-D_{9k}: Intestinal Calbindin; CaATPase, Na^{+}/Ca^{2+}: Ca transporters.

Calbindin synthesis peaks 10 hours after calcitriol induction, and its presence in intestinal tissue reaches a maximum at 20 hours and this level is held up to 48 hours after induction (Norman et al., 1981). A similar pattern of Calbindin response to calcitriol has been explained by changes in the mechanisms of control of Calbindin synthesis during enterocyte differentiation, because Calbindin mRNA expresses maximally in basal villus enterocytes (Smith, 1993). In contrast, TRPV6 is only expressed in villi tips (Suzuki et al., 2008), further supporting the hypothesis of the delay in positive or negative adaptation to be caused by enterocyte differentiation (Figure 1). Rapid adaptation of renal reabsorption and delayed adaptation of intestinal absorption explain the increases in urinary Ca excretion after rice bran withdrawal observed in chapters 2, 4 and 5 of this thesis. Urinary

excretion of Ca is an effective means to correct positive disturbances in Ca homeostasis (Ramberg et al., 1984). At product withdrawal, the negative signal for Ca regulation disappears but gastrointestinal absorption remains up-regulated for about 2 days. This surplus of Ca is corrected by the down-regulation of renal reabsorption, which also ceases when intestinal absorption is inactivated again.

Tissue remodeling

Another important adaptive mechanism of Ca homeostatic control is bone remodeling. Osseous tissue requires Ca to maintain its structural function; therefore it can be a factor for blood Ca clearance. On the other hand, bone Ca represents quantitatively a tremendous resource to sustain blood calcium. Bone remodeling is a continuous process that results from the anabolic actions of osteoblasts and the catabolic actions of osteoclasts. The prevalence of the activity of either of these cell types results in net bone calcification or resorption. The control of this process presents great complexity, because it involves not only direct hormonal regulation of the activity of these cells, but also because the differentiation and maturation of these two cell lineages from stem cells is as well regulated (Pivonka et al., 2008).

Bone remodeling is controlled by local and systemic regulation (Hadjidakis and Androullakis, 2006) and it is through systemic hormonal control that it is integrated within Ca homeostasis. The most important hormone affecting bone remodelling is PTH, but its action is tightly coordinated with calcitriol (Parfitt, 1976). Parathyroid hormone induces bone mobilisation when its presence is sustained in time, although pulsatory administration has shown to have anabolic effects on bone (Lemaire et al., 2004). Calcitriol also presents opposite effects on bone depending on time exposure. Contrarily to PTH, calcitriol is catabolic in acute applications but suppresses resorption in continuous administrations (Erben, 2001), and it is mostly understood as a direct or indirect stimulator of bone formation.

In fact, the actions of these hormones on bone metabolism can only be understood together. Parathyroid hormone initiates bone resorption to compensate for decreases in blood Ca, but at the same time it induces the synthesis of calcitriol, which in turn activates intestinal Ca absorption. If blood Ca is normalised by intestinal input, PTH ceases, consequently inducing renal excretion of the surplus. In a situation of sufficient dietary Ca, the half-life of PTH being around 4 minutes

(Bieglmayer et al., 2002), as compared to that of calcitriol, which is several hours, would create a pulsatory PTH release and continuous action of calcitriol, therefore inducing bone formation. If instead intestinal absorption does not suffice, PTH signal is sustained and bone resorption continues. Through this coupling, bone resorption only takes place when intestinal absorption is insufficient for Ca homeostasis.

Transient regulatory effects of PTH and calcitriol are explained by their effects on activity and by the recruitment of bone remodeling cells through stimulation of their differentiation. Osteoclasts are inhibited through direct hormonal action, but their activation is mediated indirectly through osteoblasts (Greenfield et al., 1999). Osteoblasts have specific receptors for PTH and calcitriol, and act on the pre-osteoclasts inducing their differentiation into active osteoclasts. Activation of existing osteoclasts takes place within 6 hours, but increases in osteoclasts numbers delay for 2 days. In case of sufficient Ca, this process is reversed by a decrease in osteoclasts numbers by day 7, although bone mobilisation is sustained in lack of sufficient dietary Ca (Erben, 2001).

There are many practical implications for the transition cow associated to the nature of the regulation in bone remodeling. First of all, it seems that adaptation, just like in the case of the intestine, is not able to react fast enough to a sudden change in blood Ca clearance. Also in this case, the involvement of a cell differentiation process delays adaptation for about 2 days, coinciding with the time term in which the cow suffers from hypocalcaemia. A second implication is that bone remodelling comes only into effect if adaptation of intestinal absorption does not suffice to compensate Ca clearance. A last conclusion would be that pharmacological induction of adaptation by injections of vitamin D metabolites can turn into effects opposite to those intended. If bone adaptation is reversed one week after application with sufficient dietary Ca, calving challenge may occur when bone resorption is inhibited. This provides an explanation for some of the problems observed in the exploration of the use of vitamin D applications in the prevention of milk fever (Littledike and Horst, 1982, Horst et al., 1997b).

Conclusion on adaptive mechanisms

Intestinal absorption and bone resorption present a delay in adaptation of about 1 or two days. This time frame coincides with the period around calving in which

cows suffer from hypocalcaemia. Renal adaptation is much faster, but because urinary Ca excretion is very small, reabsorption of Ca in the kidney is insufficient to cope with the homeostatic challenge.

Induction of homeostatic adaptation before calving should trigger gastrointestinal absorption, which seems to be the first limiting mechanism in the line of reactions against hypocalcaemia. Only if the challenge is beyond the capacity of intestinal Ca absorption, bone remodeling will be adapted to represent a means to compensate Ca clearance from blood.

Mechanistic analysis of homeostatic adaptation

In the dry period, dairy cows do not produce milk and thus have a low metabolic expenditure of Ca as compared with their supply of gastrointestinally available Ca. Passive paracellular absorption from the gut is sufficient to cover their basal metabolic needs, and the surplus either goes into bone deposition if necessary, or is excreted into urine. As already discussed, anticipating homeostatic adaptation can prevent that the sudden increase of Ca demand at calving results in hypocalcaemia during the inevitable lag time of adaptation.

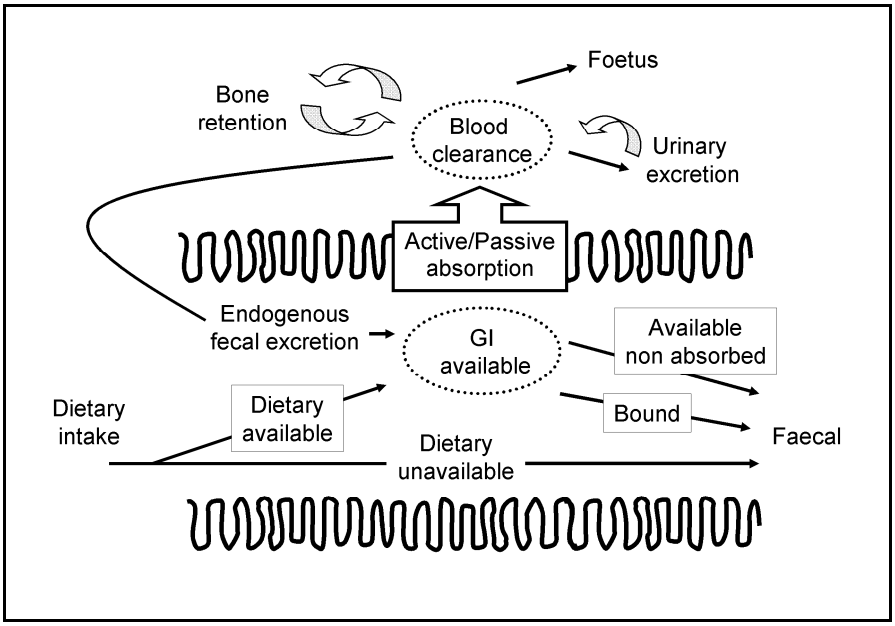

Figure 2. Diagram of digestive and physiological fate of Ca in pre-calving dairy cows

Adaptation can be anticipated either by increasing Ca clearance from blood or by reducing intestinally available Ca. Any of these two actions can make passive Ca absorption insufficient to compensate metabolic needs, triggering homeostatic adaptation. In order to understand the effectiveness of milk fever prevention strategies, it is important to quantify their effects on Ca clearance and on gastrointestinally (GI) available Ca. A scheme of the factors affecting these is described in Figure 2.

Estimation of blood Ca clearance

The factors defining blood Ca clearance are: Endogenous faecal output, urinary excretion, net bone deposition, foetal requirements and lactation requirements.

The endogenous faecal output is the sum of the Ca excreted into the gastrointestinal tract with saliva, bile, gastric and pancreatic juices and intestinal secretions. It is a function of body weight and feed intake (Braithwaite, 1982), and represents almost the total of the maintenance requirements. Endogenous faecal clearance is mostly independent from Ca intake (Lengemann, 1965, Braithwaite, 1982). In the present mechanistic analysis, it will be assumed to be 6 g/day in multiparous dry cows and 5.5 g/day in dry heifers based on values from the literature (Comar et al., 1953, Visek et al., 1953, Martz et al., 1999).

Urinary excretion is a small fraction of maintenance requirements. As already described, it can increase or be minimised during the transient times for intestinal and bone adaptation, but in general reabsorption is as high as 99% (Ramberg et al., 1984). Therefore urinary losses are as low as 0.4 g/day (Schonewille et al., 1994) and 0.8 g/day (Roche et al., 2006). An exception to this rule is during metabolic acidosis, when failure of TRPV5 provokes an increase. Urinary Ca excretion increases in a curvilinear fashion as systemic pH decreases and can be as high as 6 g/day (Schonewille et al., 1994) and 5.3 g/day (Roche et al., 2006). The relation between dietary DCAD and urinary Ca has been described by Roche et al. (2003). In order to translate urinary Ca excretion values expressed as a fraction of creatinine excretion, it is necessary to assume a constant daily creatinine excretion for the dry cow. This is defined as 17.5 g/day in the calculations as estimated from literature references (Erb et al., 1977, Valadares et al., 1999). From this assumption, Ca excretion in chapters 2, 4 and 5 of this thesis can be estimated to be between 0.3 and 0.5 g/day as basal excretion, and about 0.08 g/day when

minimised in up-regulated Ca resorption. In this mechanistic analysis, urinary Ca excretion will be estimated as a function of DCAD with the equation from Roche et al. (2003), which predicts an excretion of about 0.5 g/day in high DCAD and about 7 g/day for low DCAD. Despite that homeostatic adaptation can bring urinary excretion down to 0.1 g/day, this mechanism is not included in the model. The purpose of these estimates is to predict if homeostatic adaptation takes place under certain conditions. Therefore it is not possible to include this urinary effect in the calculation. Besides, quantitatively renal adaptation has little effect on total Ca clearance.

Net bone deposition of Ca depends on the long term Ca balance of the cow. At the end of the dry period, if the lactation diet provided Ca to recommendations, multiparous cows should already have replenished the bone reserves mobilised in early lactation. Heifers instead are still growing and their increase in body mass is a relevant factor for Ca clearance from blood. The National Research Council of the United States proposes a factorial approach to derive net Ca requirements in which growth needs are a function of daily weight gain, body weight and mature weight gain (NRC, 2001). According to this model, a heifer weighing 700 kg, aiming for a mature weight of 750 kg and growing before calving at 800 g/day would have a net Ca requirement for growth of 8 g/day.

The intrinsic cause of milk fever is the discontinuity of Ca yield to the calf between foetal and lactation needs. The factorial model of NRC (2001) estimates foetal requirements in the last days of pregnancy to be around 7 g/day. This contrasts with Ca export in the first colostrums, which in the dairy cow represents about 23 g (Horst et al., 1997b).

Gastrointestinal (GI) available pool

The GI available pool is the Ca transported through the whole GI tract that can potentially be absorbed. Calcium absorption is a regulated process, which clearly differentiates between availability and absorption. The pool is constituted by the endogenous faecal excretions, plus the fraction of dietary Ca that becomes available during the digestion process. In this pool we exclude the fraction of these two that may become unavailable, e.g. by precipitation with other dietary components.

Dietary Ca intake is the result of total feed intake and Ca content of the feeds, including eventual supplemental mineral Ca. Voluntary feed intake is a very important factor affecting Ca intake in the transition cow, because it is greatly affected by the characteristics of the diet, the health status of the animal and the event of calving. Ca content in feeds is highly variable. Roughly, in legume forages it can range from 12 to 15 g/kg DM, while grasses are in the range of 4 to 8 g/kg DM, and corn silages are mostly within 2 to 4 g/kg DM. Among the concentrates, most grains are poor sources of Ca being mostly under 1 g/kg DM and protein concentrates range from 3 to 6 g/kg DM. Depending on ration formulation, final Ca content is very variable, although there is a clear association between effective fibre and Ca content, which makes it unfeasible to formulate low Ca diets for dry cows, while complying with their requirements for effective fibre.

A similar correlation also exists between Ca content and Ca availability, although in this case it is an inverse correlation. NRC (2001) assigns an availability coefficient of 0.6 for Ca in concentrates and corn silages, and 0.3 for Ca in other forages. This negative correlation greatly reduces the variability of available Ca in the rations. For the present analysis, rations below 2 g/kg DM Ca will be calculated with an availability of 0.6, and rations above 8 g/kg DM Ca with an availability of 0.3. Availability of intermediate Ca contents will be calculated with values between 0.3 and 0.6 determined by a linear function.

Ca availability is not simply an intrinsic property of the feeds. Digestive processes can result in the formation of chemical Ca species that are not susceptible to intestinal absorption. Endogenous faecal Ca can also be subject to availability loss. Precipitation with phytate is the means for induction of adaptation studied in this thesis. The present analysis aims to evaluate the effect of Ca precipitation in the gastrointestinal tract on the GI available Ca pool.

Ca clearance/GI available Ca ratio as predictor of homeostatic adaptation

Predicting the threshold in which GI Ca availability and physiological requirements induce homeostatic adaptation is not simple. The greatest challenge is that both Ca clearance and intestinal availability do not present constant rates in time, and choosing the day as a time unit for the assessment may be inadequate. Clearance rates for colostrums production may be greater at a given moment than as a daily average, and also intestinal availability will depend very much on transit speed through the gastrointestinal tract, and the residence times of chime in the different compartments (Bronner, 2003). Despite this, it can be assumed that induction of adaptation before calving will be directly related to Ca clearance and inversely related to intestinal available Ca.

A representative set of dietary and physiological pre-calving scenarios are evaluated with the proposed model and displayed in Table 1. This includes the high and low range of dietary Ca fed to multiparous cows, and an average diet fed to heifers. Also, three milk fever prevention strategies are evaluated: (1) a low Ca diet, low enough for milk fever prevention, (2) a low DCAD diet, and (3) a hypothetical Ca diet in which, available Ca is reduced by 15 g through feeding a dietary agent. At first sight, the calculations show that multiparous cows require the absorption of a smaller fraction of the GI available Ca regardless of dietary Ca within a natural range. Heifers instead are more in range with the situation estimated for the milk fever prevention strategies, which is in line with the fact that heifers are considered not to be susceptible to clinical manifestations of hypocalcaemia. The ratio between Ca clearance and GI available Ca seems to be indicative for sufficient adaptation of Ca homeostasis before calving. Within the assumptions of the proposed model, it seems that when more than 70% of the intestinally available Ca needs to be absorbed before calving, milk fever is effectively prevented, and that even at lower rates, as in the case of the low DCAB diet, a relatively good degree of prevention can be achieved.

Table 1. Estimated daily blood Ca clearance and gastrointestinal available Ca pool in different pre-calving scenarios: multiparous dairy cows fed two levels of Ca, and heifers, multiparous cows fed a low DCAD diet and multiparous cows fed a Ca binder, all fed an average Ca level.

	Mid-Low Ca	Mid-High Ca	Heifer	Low Ca diet	Low DCAD	Ca binder
Endogenous faecal excretion (g)	6.0	6.0	5.5	6.0	6.0	6.0
Urinary excretion (g)	0.7	0.7	0.7	0.7	7.2	0.7
Bone deposition (g)	--	--	8.0	--	--	--
Foetal needs (g)	7.0	7.0	7.0	7.0	7.0	7.0
Total blood Ca clearance (BCC) (g)	13.7	13.7	21.2	13.7	20.2	13.7
DMI (kg)	14.0	14.0	12.0	14.0	13.0	14.0
Dietary Ca (g/kg DM[1])	2.5	5.5	4.0	1.5	4.0	4.0
DCAD[2] (meq/kg DM)	300	300	300	300	-150	300
Ca Intake (g)	35.0	77.0	48.0	21.0	52.0	56.0
Availability	0.58	0.43	0.50	0.60	0.50	0.50
Available Ca intake (g)	20.1	32.7	24.0	12.6	26.0	28.0
Gastrointestinal precipitation (g)	--	--	--	--	--	15.0
Total GI available pool (GIAP) (g)	26.1	38.7	29.5	18.6	32.0	19.0
Ratio BCC/GIAP	0.53	0.35	0.72	0.74	0.63	0.72

[1] DM: Dry matter. [2] DCAD: Dietary Cation Anion Balance

The effect of parity and dietary Ca the calculated ratio of Ca clearance to GI available Ca is further explored in figure 3. The model clearly explains the different susceptibilities to milk fever of heifers and cows in terms of induction to homeostatic adaptation. It predicts that heifers always require absorbing more than 60% of intestinal available Ca even at high dietary Ca, while instead older cows only require absorbing such fraction at very low amounts of dietary Ca. It is also remarkable, how the curve gains slope when dietary level reaches 1.5 g/kg DM (approx. 20 g/day), the dietary intake considered to effectively prevent milk fever (Thilsing-Hansen et al., 2002b). These estimates explain the absence of homeostatic changes in heifers observed in chapter 4 of this thesis. Ca homeostasis of heifers is adapted to their situation of great Ca clearance as compared to their intestinally available Ca, therefore a further reduction of the GI available pool only represents a change in magnitude of their Ca deficit, and it does not further induce a major adaptation of their homeostatic competence.

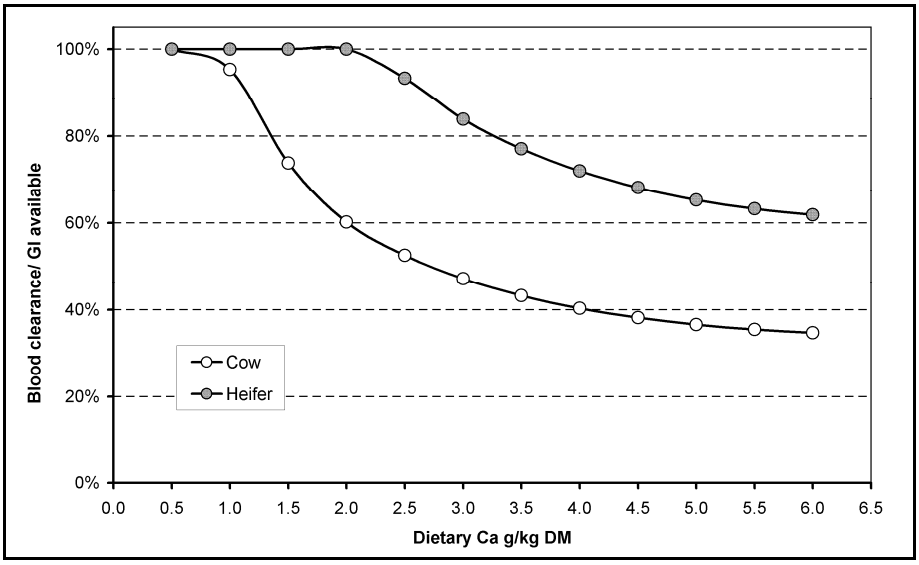

Figure 3. Predicted effect of dietary Ca (g/kg DM) on the fraction of the gastrointestinally available Ca required for physiological purposes in heifers and multiparous cows

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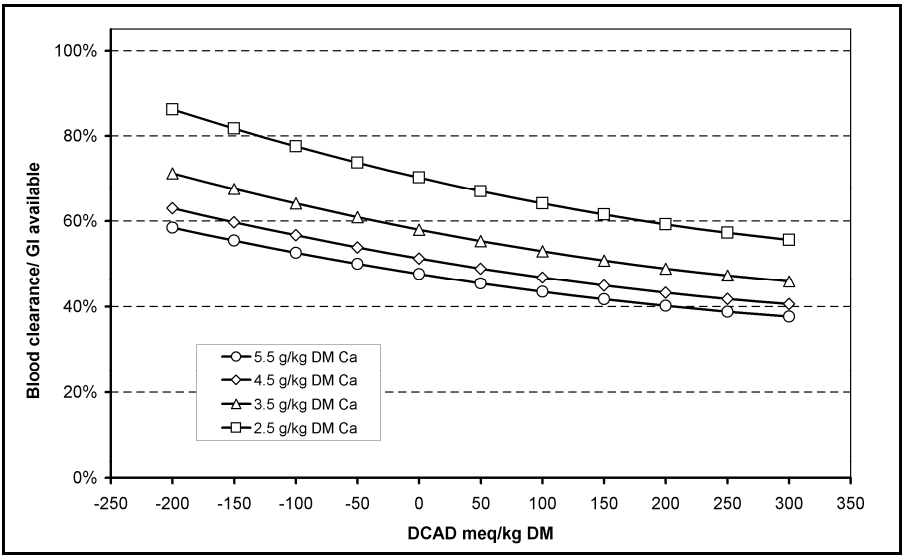

Figure 4. Predicted effect of DCAB on the fraction of available Ca required for physiological purposes at different levels of dietary Ca

The model is also used to study the effect of DCAD on urinary Ca excretion and the fraction of intestinal available Ca that is absorbed at different dietary levels (Figure 4). Reduction of DCAD increases the fraction absorbed. At the recommended DCAD levels between -200 and -100 meq/kg DM, blood Ca clearance / GI available Ca ratios reach levels in the range of those calculated for heifers for most dietary Ca levels. This is supportive to the milk fever prevention value of this strategy, and explains its mode of action through the induction of homeostatic adaptation.

The effect of dietary Ca on the effectiveness of DCAD has been controversial. While some authors proposed that increasing dietary Ca may have preventive value in low DCAD diets (Horst et al., 1997a), others suggest that the quadratic effect of dietary Ca on milk fever incidence is independent from DCAD level (Lean et al., 2006). The present mechanistic simulation (Figure 4) predicts a preventive value of lowering Ca even at low DCAD, and that this effect would not be linear but curvilinear, suggesting a smaller stimulation of adaptation to changes of dietary Ca in the higher range than in the lower range. This would correspond with the left slope of

the quadratic relation empirically determined by Lean et al. (2006) and earlier by Oetzel (1991). Because the present model only studies milk fever prevention through induction of homeostatic adaptation, it is not suitable to describe the preventive effect of high levels of dietary Ca, which mode of action is most likely explained by sufficient paracellular intestinal absorption that compensates Ca clearance at calving.

The model proposed here can be used to quantify the amount of Ca that needs to be made unavailable in order to induce homeostatic adaptation (Figure 5). Obviously, higher dietary Ca levels require more intestinal precipitation to induce adaptation. The model, however, also shows that at high dietary Ca, the preventive efficiency of precipitation decreases. At higher Ca intake a greater fraction of the intake needs to be made unavailable to induce adaptation. Roughly, at intakes of 2.5 g/kg DM precipitating 15% of the intake could be sufficient, while at intakes of 5.5 g/kg DM as much as 25% of dietary Ca would need to be sequestrated.

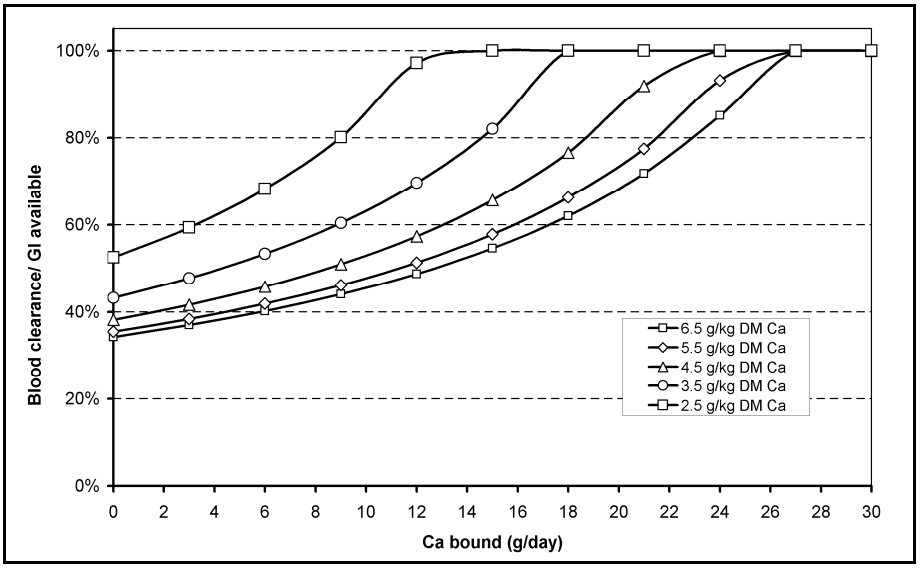

Figure 5. Predicted effect of Ca binding on the fraction of available Ca required for physiological purposes at different levels of dietary Ca

Efficiency of rumen-protected rice bran to reduce GI available Ca and induce homeostatic adaptation

Rumen-protected rice bran reduces GI available Ca in two ways. The very low Ca content of this feed (<1 g/kg DM) results in a reduced Ca intake because the feeds displaced by its inclusion would almost surely contain more Ca. Additionally, phytic acid in rice bran reduces the availability of the intestinally available Ca from the ration.

The dilution of dietary Ca content by rice bran inclusion was shown in chapter 5 of this thesis. Formulation of a placebo feed to simulate the nutrient profile of rice bran using common feeds resulted in a feed containing 2.6 g Ca/kg DM more than in rice bran. This means that in an application of rice bran of 25% (DM) inclusion in the ration, it would already subtract around 10 g from the daily gross Ca intake.

The formation of complexes between phytic acid and Ca is a well studied process although it is not simple to predict. Complexation of up to 6 moles of Ca per mol of phytic acid depends on the pH and their total concentration and ratio. It also depends on whether the phytic acid remains intact or is degraded into lower phytate forms. In addition, it depends on the presence of other complexing elements (Crea et al., 2008). Therefore, the effect of feeding rumen-protected rice bran on Ca digestion is extremely difficult to predict. Assuming the maximum integrity of phytic acid and maximum binding capacity this would mean that 2.5 kg of rice bran containing 150 g of phytic acid (60g/kg) could bind as much as 55 g of Ca (1:6 phytic acid:Ca molar ratio). This amount of Ca would be the typical total gross Ca intake of a dry cow. The effect of ruminal phytase and the evolution of pH in the gastro intestinal tract should result in a much smaller fraction of Ca being affected by rice bran.

Ruminal destruction of phytic acid is thought to be the greatest factor which reduces the efficiency of Ca binding by dietary rice bran. It is known that dephosphorylation beyond the loss of a second phosphate group of phytic acid destroys its Ca binding ability (Lonnerdal et al., 1989). In chapter 3 of this thesis, the process of dephosphorylation of rice bran in the rumen is described. A major finding of that study was that in the catalytic process intermediate inositol phosphate forms do not become more abundant than in the original product, implying that once the first phosphate is lost, the rest are removed at least as fast

as the first one. Also, it was described that ruminal degradation was strongly linked to that of protein, because formaldehyde protection, which is based on cross-links of lysine residues (Broderick and Lane, 1978), proved effective in reducing ruminal degradability of phytic acid. Phytic acid seems to degrade rather rapidly once it is released from the protein matrix. In this thesis the rumen escape fraction of phytic acid is considered to be the main fraction of phytic acid that acts on Ca availability. However, it is important to consider that phytic acid released into the rumen during degradation of rice bran may also influence Ca availability. Ca is known to impair efficiency of phytase enzymes in monogastric animals (Lei et al., 1994, Qian et al., 1996, Sebastian et al., 1996) and also dietary Ca has proven to have a strong negative effect on ruminal degradation of phytic acid from rice bran (Sansinena, 1999). Therefore, some degree of Ca binding must take place at ruminal level, although given the uncertainty of this process, only the effect of rumen escape phytic acid is considered for the quantification of the intended reduction of Ca availability.

In chapter 3, formaldehyde-treated rice bran was estimated to potentially bind 7 g of available Ca per kg, based on the rumen escape of phytic acid. The untreated product would have approximately 4 times less binding potential compared to the treated product. Considering an application of 25% (DM) inclusion in the diet and a DM intake for dry cows of 13-14 kg/day, a total of about 24 g of available Ca are made unavailable. Going back to Figure 5, such binding potential seems sufficient for induction of adaptation in rations within the common range of Ca content. Additionally, as already discussed, such rice bran inclusion would displace about 10 g of gross dietary Ca from the diet. This represents between 4 to 5 g of available Ca. Therefore, it can be expected that rumen-protected rice bran fed at 25% (DM) of a dry cow ration would induce homeostatic adaptation of gastrointestinal Ca absorption.

Chapter 5 of this thesis demonstrated homeostatic responses to feeding rumen-protected rice bran, with and without Ca supplementation, to compensate Ca dilution caused by the low Ca content of rice bran. In that experiment, product inclusion in the ration was 23% (DM), and Ca content of the reference diet was 3.2 g/kg DM. These results demonstrate that homeostatic response is caused by nutritional effects of rice bran and its components, and not solely by the reduction of Ca intake. Nevertheless, a greater response was observed for rice bran without

supplemented Ca, which underlines the importance of the combination of both modes of action.

In the experiment of chapter 6, rice bran inclusion was 14% (DM), much lower than in previous trials, and the ration contained much more Ca, between 4 and 6 g/kg DM. Still, under these less favourable conditions, rumen-protected rice bran induced homeostatic adaptation.

Efficacy of rumen-protected rice bran to prevent milk fever

Induction of adaptation of Ca homeostasis acts on the most apparent direct cause of hypocalcaemia, the delay in response of Ca gastrointestinal absorption and Ca mobilisation from the bone. However, training the homeostatic system, while being necessary, may not be sufficient to solve a problem that has its cause in more than one factor.

At calving, the cow may compensate Ca clearance into colostrums by bone mobilisation or by active gastrointestinal absorption. Bone reserves are quantitatively sufficient to compensate Ca drain into milk, but only if intestinal Ca is insufficient, because intestinal Ca uptake can induce the PTH signal to cease, stopping bone catalysis. Therefore, depending on the magnitude of inhibition of gastrointestinal Ca availability before calving, two scenarios are possible. If gastrointestinal absorption is insufficient to sustain calcaemia, bone mobilisation is maintained. In such scenario, at calving, Ca homeostasis can rely on the vast resource of bone Ca. Instead, if active gastrointestinal absorption suffices to maintain blood Ca before calving, bone mobilisation will remain inactive, and the only ready mechanism to obtain Ca would be gastrointestinal absorption. In this last scenario, Ca intake in the hours around calving would be of critical importance. Even if gastrointestinal Ca absorption is well prepared, the amount of Ca in the GI tract can be limiting to compensate Ca clearance, and hypocalcaemia would still take place.

Cows reduce their feed intake as much as 30 to 35% as they approach calving (Grummer, 1995). Therefore reducing their Ca intake proportionally, and limiting the total amount of Ca that can be absorbed from the gastrointestinal tract. Although, Ca absorption in the ruminant is not restricted to a single region of the tract (Schröder and Breves, 2007), transit and residence times in the different

compartments may further limit the accessibility of this Ca resource for the animal. If in addition to this, the cow has been consuming until calving a product that limits Ca availability, thus it could be questioned if gastrointestinal absorption can represent a sufficient means to compensate for Ca clearance. For this reason, some of the trials done with zeolites included an oral Ca supplementation at calving of 90 g Ca (Thilsing-Hansen and Jorgensen, 2001, Thilsing-Hansen et al., 2002a), and this may be explanatory for the very high preventive value demonstrated by the treatment in those trials. On the other hand, other trials testing zeolites have also shown similar prevention of hypocalcaemia without oral supplementation at calving (Pallesen et al., 2008, Grabherr et al., 2009).

The study described in chapter 6 presents a significant alleviation of hypocalcaemia at calving associated with rice bran feeding. However, the differences observed are smaller than those described for zeolites in various studies (Thilsing-Hansen and Jorgensen, 2001, Thilsing-Hansen et al., 2002a, Pallesen et al., 2008, Grabherr et al., 2009). There are three main reasons that could explain this lower degree of prevention: The intrinsic differences between products, a difference in magnitude of the reduction in Ca availability, and differences in the experimental design.

Both zeolites and rumen-protected rice bran are presupposed to have the same mode of action; however the products are very different. Zeolites and phytic acid both have Ca binding properties, but also present many nutritional differences. Rice bran supplies a large quantity of dietary P and is a relatively rich source of Mg, which demonstrated to positively influence the presence of these elements in blood in chapter 4. Instead, feeding zeolites reduces blood levels of P and Mg (Thilsing-Hansen et al., 2002a), and when fed in combination with additional P, the milk fever preventive effect seems to be reduced (Pallesen et al., 2008).

The effect of Ca binding in the prevention of milk fever has proven to be dose dependent (Grabherr et al., 2009), which opens the possibility for the observed difference between rice bran and zeolites to be caused by the magnitude of the nutritional challenge. In chapter 6, rice bran dose was relatively low for the level of dietary Ca, and also time of exposure was variable from 0 to 21 days, while in most zeolites trials the aim was to feed for several weeks before calving. An additional factor affecting the magnitude of the nutritional challenge is DMI. While rice bran has shown no effect on DMI, zeolites proved to reduce DMI (Thilsing-Hansen et al., 2002a, Grabherr et al., 2008), which magnifies their effect on Ca

availability by reducing Ca intake. However, this could not have been the case in the trials where feed intake was kept similar between treatments and control (Pallesen et al., 2008).

Rumen-protected rice bran has proven to induce homeostatic adaptation in non-lactating dairy cows (chapters 2, 4, 5), and has proven to improve calcaemia after calving (chapter 6). However, further research experience is needed to evaluate its effectiveness to prevent milk fever under practical conditions. It is necessary to test the concept feeding the product for 2-3 weeks before calving, taking the dietary treatment until the moment of calving. It is also necessary to experimentally calibrate the response to different doses of rumen-protected rice bran, in terms of milk fever incidence and calcaemia post-partum. Further, it would be necessary to compare the preventive efficacy of this strategy as compared to the traditional DCAD concept.

Feasibility to use rumen-protected rice bran for milk fever prevention

Any new nutritional application should be suitable to be inserted in current animal husbandry practices. Ease of practical implementation can be as important as effectiveness. In fact they are very much interrelated in defining the final acceptation into the production system. Highly effective applications may justify some practical drawbacks, and very easy to implement solutions may be used even if the nutritional effectiveness is moderate. The here presented rice bran concept intends to overcome the practical limitations of formulating very low Ca diets, and to resolve the drawbacks of zeolites and anionic salts in terms of DMI depression. Therefore it is necessary to critically evaluate the practical implications of feeding rumen-protected rice bran in pre-calving diets.

Nutritional implications

Transition feeding schemes are fully focussed on preventing production diseases such as milk fever and ketosis. Milk fever is a predisposing factor for ketosis (Curtis et al., 1985), and as discussed in the introduction of this thesis, milk fever prevention is crucial as a means for reducing the incidence of energy related disorders in early lactation. Feed intake decrease during the days before calving is a major predisposing factor for metabolic problems. Fatty liver after calving is

highly correlated with feed intake before calving (Grummer, 1993). Therefore, milk fever prevention strategies need to sustain feed intake in order to achieve its final goal, which is to preserve health in early lactation. Fat-coated full-fat rice bran used in the first experiment of this thesis caused a DMI depression that would make its use unfeasible for practical nutrition. Later trials showed that formaldehyde-treated full-fat rice bran (chapter 4) and formaldehyde-treated defatted rice bran (chapter 5) presented no differences in dry matter intake compared to controls.

Another nutritional factor relevant for using rice bran is its nutritional value. Rice bran is a common feedstuff in dairy rations there where it is available, and among by-products it is easily included in diets that are least cost formulated (Grasser et al., 1995). This is indicative of an adequate nutrient profile of rice bran for dairy cow diets. This may not be true for the full-fat version of rice bran, which contains about 20% of highly unsaturated oil. This fat content does not only represent a difficulty in formulation of ruminant diets, but also represents a major risk for the feed to turn rancid (Goffman and Bergman, 2003). The use of defatted rice bran permits inclusion in the diet to reach effective doses, and fat extraction has the additional advantage of proportionally increasing phytic content of the feed. Unsaturated fat is inadequate in ruminant diets because of its effects on ruminal fermentation (Palmquist and Jenkins, 1980), therefore total inclusion needs to be limited. However, there are indications that rice bran fat, in moderate quantities, can induce positive effects on reproductive performance, as has been observed in Brahman animals (De Fries et al., 1998, Webb et al., 2001).

Another factor to keep under consideration about the nutrient profile of rice bran is Ca content. Some rice brans are rich in Ca because they are produced through abrasion of the rice with Ca carbonate (Marshall and Wadsworth, 1994). These sources are obviously less suitable to limit dietary Ca availability.

Rice bran prevents hypocalcaemia by means that are innocuous to acid-base balance. Inducing metabolic acidosis in pre-calving diets is justified by the benefits obtained in Ca homeostasis at calving, but it is a major physiological challenge for the animals that has additional metabolic consequences such as impaired insulin response (Bigner et al., 1996), which may not be convenient during the challenge to energy metabolism that takes place around calving.

A potential drawback of rice bran is that it could affect availability of other minerals in the diet. Phytic acid does not only have nutritional effects on Ca, but also on Zn availability, especially in combination with Ca (Fordyce et al., 1987). Nevertheless, the unchanged Zn blood levels during rice bran feeding, as presented in chapter 4, indicate that if such effect takes place, it is overruled by the greater Zn supply associated with the high Zn content in rice bran. Furthermore, mineral inhibition on Mg as divalent cation should not be expected from phytic acid (Coudray et al., 2003), as shown in chapter 4.

Non-nutritional factors affecting practical suitability of the concept

Nutritional value is objectively important, but product perception can be a subjective factor to take into account for practical applicability. Rice bran has a positive image in human nutrition because several health promoting effects have been attributed to the different components that it contains (Abdul-Hamid and Luan, 2000, Jariwalla, 2001). In animal nutrition, these properties are shadowed by the susceptibility of the full-fat form to become rancid. Another major factor affecting product perception is formaldehyde treatment, which is recognised as a potentially dangerous product. This is true when it comes to handling of the product in the treatment process, where it represents a hazard by inhalation. However, formaldehyde is also a naturally occurring substance (Owen et al., 1990), and the typical treatment of ruminant feeds does not represent a food safety hazard (Gulati et al., 2005). In any case, as perception is not necessarily based on facts, if formaldehyde treatment would be a limitation, other methods for increasing the rumen escape fraction of protein should be suitable to protect phytic acid (Konishi et al., 1999).

Nutritional applications need to fit in the reality of farm management. In most practical conditions, it is not possible to establish a differentiated pre-calving diet for heifers. Overton and Waldron (2004) reviewed nutritional management in the transition period, underlining the fact that modern dairy herds unfortunately include a large fraction of heifers, which undergo pre-calving ration with multiparous cows. Consequently, it is very important to evaluate if this diet is detrimental to these animals, as they will not benefit from milk fever prevention. Chapter 4 showed that heifers were not negatively affected by rice bran, either in their mineral status or DMI. On the contrary, low DCAD diets currently used in milk

fever prevention, when fed to heifers have a negative effect on their energy balance, and do not improve their Ca homeostasis (Moore et al., 2000).

The combination of rice bran with oral applications of Ca at calving needs to be studied. If this would prove to be synergetic to the here proposed induction of homeostatic adaptation, such additional application would imply greater labour input at calving. In that case, the preferred form of application would be Ca suspensions for voluntary consumption. Calcium drenches can produce lesions in the rumen (Thilsing-Hansen et al., 2002b), and in practice are not easy to apply and are detrimental to animal welfare.

Under the current understanding of the effectiveness of the proposed preventive approach, and considering the most relevant nutritional and practical constrains, it seems that rumen-protected rice bran could be used in dairy operations as a means to prevent milk fever.

Conclusions and recommendations

Observations from the experiments and their interpretation result in the following conclusions:

✓ Rumen-protected rice bran reduces dietary availability of Ca by dilution of dietary Ca and by gastrointestinal precipitation of Ca. Lower dietary Ca availability enhances renal Ca reabsorption and activates gastrointestinal absorption. Upon cessation of the restriction on availability, gastrointestinal down-regulation delays two days, time in which the excessive absorption is excreted in urine, shown by the two day peak of urinary Ca after withdrawal of the rice bran product. This observed delayed homeostatic adaptation may be related to the differentiation process of gastrointestinal epithelium, and is understood to be the underlying cause of hypocalcaemia in the days around calving. Therefore, rumen-protected rice bran can be used to stimulate homeostatic adaptation before calving, avoiding this delay at parturition.

✓ A rumen protection method is necessary in order to use dietary phytic acid as Ca antagonist. Fat coating and formaldehyde treatment have proven effective in promoting rumen escape of phytic acid. Fat coating is not appropriate because it depresses DMI, but formaldehyde treatment is innocuous to DMI and provides rice bran with a post-ruminal binding potential of 7 g of available Ca per kg of product.

✓ Cows before their first parturition are not susceptible to dietary induction of homeostatic adaptation because their Ca metabolism is already up-regulated. These animals still have significant growth requirements for Ca, which makes passive gastrointestinal absorption insufficient to cover their needs, in contrast with multiparous cows. This difference explains the resistance of heifers to hypocalcaemia, and supports the value of induction of homeostatic adaptation as an effective preventive strategy.

✓ Rumen-protected rice bran supplies phytic acid post-ruminally, and significantly increases dietary P. Beyond the intentional nutritional antagonism with Ca, the nutritional status of other minerals such as Mg and Zn could potentially be compromised. However, high content of Zn and Mg in rice bran seem to compensate any such effects, and experimentally it was shown that the product has no apparent detrimental effects on mineral status.

✓ Homeostatic responses were observed without induction of metabolic acidosis, feeding diets with positive DCAD. The here proposed preventive feeding strategy is considered to be independent of dietary acid-base modulation, although this strategy is also considered to be based on the induction of homeostatic adaptation. Rice bran reduces dietary availability to induce adaptation, while dietary induction of metabolic acidosis would produce a failure of the main Ca channel in renal epithelium (TRPV5), increasing urinary excretion, which induces adaptation as reaction to a greater blood Ca clearance. In different ways, they affect the ratio between blood Ca clearance and gastrointestinal available Ca, which can be considered a good predictor of induction of homeostatic adaptation.

✓ Rumen-protected rice bran, fed before and until the day of calving, improves periparturient calcaemia. Animals exposed to rumen-protected rice bran, at an inclusion of 14% on DM basis, and for a variable number of days before calving, had a moderate but significant improvement in blood Ca in the first 3 days after calving. The hypocalcaemia prevention was dependent on days of exposure, and it is expected to be rice bran dose dependent. Rumen-protected rice bran can be a dietary means for milk fever prevention in dairy cows under practical feeding and management conditions.

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SUMMARY

Cow health and welfare are important, not only in an ethical context of dairy production, but they also have great implications in the profitability and environmental impact of the production system. In the last decades, dairy production has rapidly evolved towards high milk yields and feed efficiency in the animal. The downside of this progress has been an increase in culling rates. Today, dairy production faces the challenge to increase cow longevity in order to improve economic and nutrient efficiencies of the farm system.

Milk fever is one of the most relevant diseases in dairy production. At calving, multiparous dairy cows develop hypocalcaemia because they are not able to adapt Ca homeostasis fast enough in the transition between gestation and lactation. Milk fever is important as a life threatening condition for the cow and it is also a predisposing factor for other production diseases, which amplify its effects on culling rate.

The difference between Ca needs of gestation and lactation create a sudden shift in the rate at which Ca is exported from blood into milk. Animals normally respond with physiological adaptations to fluctuations in blood Ca clearance. However, in this case urinary adaptation is insufficient and intestinal and bone adaptations are not fast enough to avoid hypocalcaemia. After a few days, however, when adaptation is complete, Ca homeostasis is effectively maintained despite the still growing Ca demand as milk production increases.

Dietary prevention of milk fever has focused on anticipating the challenge to prepare homeostatic competence before calving, because delayed homeostatic adaptation is understood to be the main cause of milk fever. The proposed approaches to adapt the homeostatic system include: (1) dietary induction of metabolic acidosis to create a state of hypercalciuria, (2) reduction of dietary Ca, and (3) reducing Ca availability by dietary means. Metabolic acidosis, the first approach mentioned, is induced by the reduction of the dietary cation anion difference, by means of feeding anionic salts. This is the most common dietary prevention method. However, prevention is seldom absolute and it presents practical difficulties in its application, especially with forages high in potassium.

The second approach, reducing dietary Ca, is very effective, but the low target Ca intakes are difficult to reach in practical ration formulation, which is why it has not been implemented in practice. For the third and last approach, reducing Ca availability rather than dietary levels, in the last decade, zeolite clays have been proposed as dietary antagonist of Ca. Efficacy of milk fever prevention seems to be very high, but this application is associated with dry matter intake depression.

Dietary phytic acid binds to Ca during the digestive process, making Ca unavailable for absorption. This is a well-known effect in monogastric farm animals and humans. In contrast, in ruminants phytic acid is extensively degraded in the rumen by the microbial enzyme phytase; therefore, in ruminants effects of phytic acid on Ca availability are mostly negligible. However, because phytic acid is integrated in the protein matrix of feeds its ruminal destruction is connected with ruminal degradability of protein. In fact, treatments used to enhance the rumen escape fraction of protein in feedstuffs, have a negative effect on the digestibility of phytate phosphorous.

Among feedstuffs, rice bran has the highest phytic acid content. Rice bran is the by-product of rice polishing. Full-fat rice bran contains too much unsaturated fat to be used in ruminant rations. Nevertheless, after oil extraction, defatted rice bran has an adequate nutrient profile for pre-calving dairy rations.

Defatted rice bran, treated to reduce phytic acid degradability was proposed as a dietary means to reduce Ca availability. It was hypothesised that rumen-protected rice bran could be fed to anticipate homeostatic adaptation of Ca metabolism and provide greater homeostatic competence at calving thereby preventing milk fever.

Five trials were carried out to study this hypothesis in its different aspects: In the first trial, fat-coated rice bran was produced, and its effects on Ca homeostasis were tested in vivo in multiparous dry cows. In the next trial, formaldehyde treatment of rice bran was studied in situ as a rumen protection method for phytic acid. In a third experiment, formaldehyde-treated rice bran was fed to growing dairy heifers to evaluate its effects on Ca homeostasis in vivo. In the fourth trial, formaldehyde-treated defatted rice bran, fed with or without supplemental dietary Ca, was tested in multiparous dry cows before calving. In the fifth and last trial, formaldehyde-treated defatted rice bran was fed to multiparous dry cows, to study its effects on Ca homeostasis around calving.

Feeding fat-coated rice bran affects Ca homeostasis but reduces dry matter intake

Fat-coated rice bran was manufactured by spraying melted hydrogenated fat over rice bran in a mixer. The product was later incubated in situ, to estimate ruminal degradation of dry matter and phytic acid. The rumen escape fraction of phytic acid was 0.3 (passage rate 0.05/h). In addition to this, the product was fed to seven dry multiparous dairy cows for a week, and feed intakes and urinary Ca were monitored during feeding, and the weeks before and after. Fat-coated rice bran caused a substantial reduction of dry matter intake, which implied also a much lower Ca intake. Urinary pH and calcium excretion also decreased during feeding. Daily Ca excretion decreased the day after product introduction and peaked for two days after withdrawal. The pattern observed in urinary Ca excretion following fat-coated rice bran feeding, indicated an influence on Ca homeostasis in dry multiparous dairy cows. However, dry matter intake depression showed that fat coating, although effective at reducing rumen degradability of phytic acid, is not a suitable treatment for feeds to be fed before calving.

Formaldehyde treatment substantially reduces ruminal degradation of phytic acid

Formaldehyde treatment was explored as a method to promote the rumen escape of phytic acid in rice bran. Two samples of full-fat rice bran were treated with 4 levels of formaldehyde and ruminally incubated in situ in 3 rumen-fistulated cows. Dry matter disappearance was determined, and residues were analysed for P and phytate, including individual inositol phosphate forms. Ruminal degradation parameters were calculated for dry matter, P, total phytate, and phytic acid. Most P in the original sample and residues was phytate P, mainly phytic acid. Formaldehyde treatment reduced degradability, increasing the estimated rumen escape (passage rate 0.05/h) of P from 0.08 in the control to 0.40 at the highest formaldehyde dose. Ruminal degradation of total phytate and phytic acid behaved similarly to P degradation. Formaldehyde treatment reduced the rate of degradation of phytic acid from 0.31/h in the control to 0.07/h at the highest formaldehyde treatment level. Similarly, rumen escape (passage rate 0.05/h) of phytic acid was 0.08 without treatment and 0.32 for the highest dose of formaldehyde. Based on the level of rumen protection and assumptions of the Ca binding capacity of phytic acid, one kg of formaldehyde-treated rice bran could

deliver enough phytic acid post ruminally to bind 7 g of dietary Ca. These results indicated that it could be feasible to decrease intestinal Ca availability with formaldehyde-treated rice bran in dairy cows.

Dairy heifers have an already adapted Ca metabolism

Formaldehyde-treated rice bran was fed to heifers to study effects on Ca homeostasis. Two levels of dietary rice bran were tested against a control. Rice bran did not affect DMI. Calcium intakes and rate of growth were indicative of an insufficient Ca supply in relation to requirements, in agreement with a consistently low urinary Ca observed throughout the treatments. Serum Ca, urinary Ca, calcitriol and hydroxyproline remained unaffected. Rice bran influenced P, Mg and Zn intakes and serum and urine presence, but effects can not be interpreted as detrimental to the nutritional status. Most heifers had an already adapted Ca metabolism, because in contrast with dry multiparous cows, their Ca supply is relatively low compared to their needs. Multiparous dry cows are required to study dietary activation of Ca metabolism.

Rumen-protected rice bran induces adaptation of Ca homeostasis by reducing dietary Ca and its gastrointestinal availability

Formaldehyde-treated defatted rice bran was tested in vivo to study its effects on Ca homeostasis, separating the effects of its naturally low Ca content and its effect on Ca availability. To this end, not only rumen-protected rice bran alone but also rumen-protected rice bran plus supplemental Ca were compared with a control treatment. Treatments did not affect urine pH or dry matter intake. Urinary Ca excretion peaked for 2 days after withdrawal of the rice bran treatments. The peak was greatest after feeding rice bran alone. This reaction is interpreted as indirect evidence for the activation of intestinal Ca absorption during rice bran feeding, because urinary Ca can be rapidly modulated to correct blood Ca balance, whereas intestinal adaptations delay two days. From this study it was concluded that formaldehyde-treated rice bran is able to adapt Ca regulation by reducing dietary Ca and its availability. Therefore it could be a suitable product to prevent milk fever without reducing dry matter intake.

Rumen-protected rice bran improves homeostatic competence of multiparous dairy cows at calving

Formaldehyde-treated defatted rice bran was fed before calving to study its effect on Ca homeostasis at calving. The trial included 113 multiparous cows, which calved during 3 feeding periods of 3 weeks. In the first period, a control diet was fed, then 140 g/kg DM of rumen-protected rice bran was included in the diet, after which the group returned to the control diet. Blood was monitored weekly before calving, at calving, 6 and 12 hours after calving, and 3 and 28 days in lactation. Urine samples were taken right after calving. Rice bran reduced serum Ca during feeding, indicating a challenge to Ca homeostasis. At calving, the nadir of serum Ca was raised by rice bran feeding, but odds of receiving a Ca infusion were comparable to the control. Serum Ca at calving was higher and the difference was maintained for at least 72 hours after calving. This is indicative of improved homeostatic competence provided by rumen-protected rice bran.

General discussion

Hypocalcaemia is an uncommon condition in animals. Calcium homeostasis is a robust system because blood Ca is a critical priority for the animal. Homeostatic control includes monitoring and correcting mechanisms. Positive or negative fluctuations in blood Ca can be corrected by modulation of urinary excretion, adaptation of gastrointestinal absorption and remodelling of bone tissue. Each of these mechanisms is controlled by specific signals and has its strengths and limitations. These specific characteristics are intrinsic to the nature of each process, either transepithelial transport or tissue remodelling, but also are associated with their specific function within the greater homeostatic system.

Renal reabsorption and gastrointestinal absorption are both tightly regulated transepithelial transport processes that share many structural and regulatory characteristics. However, major differences exist. Renal reabsorption is mainly controlled by parathormone, and its major function is to correct positive fluctuations by increasing excretion, which makes it the short term controlling system. Gastrointestinal absorption is mainly controlled by calcitriol, and its main function is to correct supply to the system, modulating absorption and faecal losses to adjust midterm balance. The major regulatory difference between the two seems to be that while renal reabsorption adapts to changes within hours,

gastrointestinal absorption maintains its configuration for days before full adaptation. The evolutionary cause for this difference may be that negative Ca fluctuations are almost always associated with slow physiological changes, while positive fluctuations are more likely to be sudden in time. There are indications that delayed adaptation of gastrointestinal absorption is related to cell the relatively slow differentiation processes of the gastrointestinal epithelium.

Bone remodelling is controlled by parathormone and calcitriol. In this way, bone Ca reserves and structural function of Ca in bone, are prioritised. Substantial mobilisation takes place only when gastrointestinal absorption is insufficient to sustain blood Ca. Although a fraction of bone Ca can be relatively quickly mobilised, effective Ca mobilisation involves a delay of days, because increases in osteoclasts also involves a process of cell differentiation from stem cells.

The transition between gestation and lactation in dairy cows represents an uncommonly sudden increase in blood Ca clearance. Delayed gastrointestinal and bone adaptation is understood as the main cause of milk fever, therefore the key to dietary prevention should be anticipating homeostatic adaptation either by reducing the supply of available Ca or by increasing Ca clearance days before calving.

Mechanistic analysis of the induction of homeostatic adaptation

A simplified model was proposed to quantify the effects of physiological status and diet on Ca homeostasis to predict homeostatic adaptation for the prevention of milk fever. The ratio between blood Ca clearance and gastrointestinally available Ca (BCC/GIA) was calculated assuming that adaptation can be induced either by reducing available Ca or by increasing its physiological demand. Multiparous dairy cows fed on typical pre-calving diets were estimated to have a much lower BCC/GIA ratio than heifers, or multiparous cows fed very low Ca diets or a low Dietary Cation Anion Difference (DCAD) diet.

Heifers, because of their Ca requirements for growth have high BCC/GIA ratios at a broad range of dietary Ca levels, whereas multiparous cows had much lower BCC/GIA ratios except when fed extremely low dietary Ca levels. Cows fed low enough DCAD diets have high urinary losses of Ca, because metabolic acidosis

inhibits the functionality of the renal Ca channel (TRPV5). This results in high BCC/GIA ratios, which are in the range of those found in heifers.

Because BCC/GIA ratios before calving seem explanatory to milk fever prevention, the model was used to determine the desirable reduction of gastrointestinally available Ca in order to achieve similar BCC/GIA ratios known to prevent milk fever. The surplus of gastrointestinally available Ca would then need to be made unavailable by phytic acid. Gastrointestinal precipitation of 24 g of Ca would be sufficient to induce adaptation of Ca absorption for any common dietary Ca level. Theoretically, this binding could be achieved by dietary inclusion of rice bran at a level of 25% DM.

Practical implications of milk fever prevention with rice bran

The search for an alternative strategy for milk fever prevention is justified by the practical limitations of the current approaches. If rumen-protected rice bran is to be used in practical dairy feeding, the application should be compatible with the nutritional and practical constrains of the production system.

Defatted rice bran presents an adequate nutritional ingredient to be used in dairy rations. The low fat content makes it less susceptible to become rancid, and its nutrient profile allows for relatively high inclusion rates without conflicts with the main feed formulation boundaries. Furthermore, formaldehyde-treated rice bran does not impair dry matter intake, in contrast with zeolite clays and low DCAD rations. Reduced voluntary feed intake before calving can influence the incidence of metabolic disorders in early lactation. Rice bran does not affect acid base balance, which avoids the negative effects of the metabolic acidosis induced by reduced DCAD. Additionally, feeding rumen-protected rice bran does not involve any known detrimental effects on other mineral nutrients. Given the current understanding of the concept, rice bran may be used in practical pre-calving diets for dairy with the purpose of preventing milk fever.

Conclusions

✓ Rumen-protected rice bran reduces dietary availability of Ca by dilution of dietary Ca and gastrointestinal precipitation of Ca. Lower urinary Ca excretion indicates lower Ca availability, and the two day peak of urinary Ca after withdrawal is indicative of the unavoidable delay in the adaptation of gastrointestinal absorption, which may be explained by the differentiation process of gastrointestinal epithelium.

✓ A rumen protection method is necessary to use dietary phytic acid as Ca antagonist. Fat coating and formaldehyde treatment are effective to promote rumen escape of phytic acid. However, fat coating is not a valid method because it reduces dry matter intake.

✓ Pregnant heifers are not susceptible to dietary induction of homeostatic adaptation because their Ca metabolism is already up-regulated. This is explanatory of the resistance of heifers to hypocalcaemia, and further supports the preventive value of induction of homeostatic adaptation before calving.

✓ Rumen-protected rice bran supplies phytic acid post-ruminally, and significantly increases dietary P. Nevertheless, no negative effects on Zn or Mg status have been observed.

✓ Feeding rumen-protected rice bran and reducing dietary DCAD share the objective to induce homeostatic adaptation. Rice bran reduces dietary available Ca, whereas lower DCAD diets increase urinary Ca excretion by inhibiting the main Ca channel in renal epithelium (TRPV5). Both approaches increase the ratio between blood Ca clearance and gastrointestinal available Ca, which can be considered a predictor for homeostatic adaptation.

✓ Rumen-protected rice bran fed before and until calving produced a substantial improvement in blood Ca during the 3 days after calving.

✓ Rumen-protected rice bran can be a dietary means for milk fever prevention in dairy cows under practical feeding and farm management conditions.

SAMENVATTING

Het welzijn en de gezondheid van melkvee zijn niet alleen van belang vanuit ethisch perspectief, maar hebben ook een grote invloed op de economische en ecologische duurzaamheid van het productiesysteem. De laatste decennia heeft de melkveehouderij zich sterk ontwikkeld richting dieren met een hoge melkproductie en voerefficiëntie. De keerzijde hiervan is dat dit gepaard ging met een toename van het vervangingspercentage van melkvee. Op het moment staat de melkveesector voor de uitdaging de levensduur van melkkoeien te verlengen om zo de economische en ecologische efficiëntie van de melkproductie te verhogen.

Melkziekte is één van de belangrijkste ziektes in de melkveehouderij. Rondom het afkalven kunnen oudere koeien hypocalcemie ontwikkelen omdat het regulatiemechanisme van het Ca-niveau in het bloed zich niet snel genoeg kan aanpassen aan het verschil in Ca-behoefte tussen droogstand en lactatie. Melkziekte is van belang vanwege de levensbedreigende aard van de ziekte en is bovendien een predispositie voor andere productieziekten.

Het verschil in Ca-behoefte tussen de droogstand en lactatie veroorzaakt een plotselinge verandering in de snelheid waarmee Ca uit het bloed verdwijnt. In normale omstandigheden reguleert het dier fluctuaties in het Ca-gehalte in het bloed door een fysiologische aanpassing van Ca-uitscheiding in de urine. Echter, in dit geval is aanpassing van Ca-uitscheiding in urine onvoldoende en is de adaptatie van darmabsorptie of botresorptie niet snel genoeg om hypocalcemie te voorkomen. Wanneer na een paar dagen deze adaptatieprocessen volledig op gang gekomen zijn, wordt de bloed Ca-homeostase effectief gereguleerd, ondanks de nog steeds sterk stijgende Ca-behoefte door de stijgende melkproductie.

Melkziektepreventie middels het rantsoen richt zich vooral op het voorbereiden van de homeostatische regulatiemechanismen van bloed Ca. Dit aangezien de vertraagde homeostatische adaptatie van de regulatie van het bloed Ca gezien wordt als de belangrijkste oorzaak van melkziekte. De voorgestelde benaderingen om het homeostatische systeem te adapteren zijn: (1) Inductie van metabole

acidose via het rantsoen, waardoor hypercalciuria veroorzaakt wordt, (2) verlaging van het Ca-gehalte in het rantsoen en (3) een reductie van de Ca-beschikbaarheid door middel van het rantsoen. Metabole acidose, de eerste benadering, wordt bereikt door een verlaging van het kation-anion verschil, door middel van anionische zouten. Dit is de meest gangbare preventiemethode. Echter, de preventie is zelden volledig en de toepassing is soms moeilijk, vooral in combinatie met ruwvoeders met een hoog kaliumgehalte. De tweede benadering, reductie van het Ca-gehalte in het droogstandsvoer, is zeer effectief, echter de gewenste lage Ca-opname is rantsoentechnisch moeilijk te bereiken, waardoor het vrijwel niet toegpast wordt in de praktijk. Voor de derde en laatste benadering, reductie van de Ca-beschikbaarheid, is het gebruik van zeolieten (kleimineralen) voorgesteld, als antagonist van Ca-absorptie. De effectiviteit van melkziektepreventie lijkt erg groot, maar de toepassing gaat gepaard met een depressie van de drogestofopname.

Fytinezuur bindt Ca in het maagdarmkanaal, waardoor het onbeschikbaar wordt voor absorptie. Dit is een bekend effect in monogastrische landbouwhuisdieren en mensen. Bij herkauwers wordt fytinezuur in de pens afgebroken door microbieel fytase, daarom is het effect van fytinezuur op Ca-beschikbaarheid in herkauwers veelal verwaarloosbaar. Fytinezuur is geïntegreerd in de eiwitmatrix van voeders en de afbraak in de pens is gekoppeld aan de pensafbreekbaarheid van eiwit. Behandelingen gericht op het verhogen van de pensbestendigheid van eiwit, hebben inderdaad een negatief effect op de verteerbaarheid van het fosfor in fytaat.

Van de reguliere voedermiddelen heeft rijstevoermeel het hoogste gehalte fytinezuur. Rijstevoermeel is het bijproduct van het polijsten van rijst. Volvet-rijstevoermeel bevat te veel onverzadigde vetzuren om in rantsoenen voor herkauwers te gebruiken. Echter, na vetextractie ontstaat ontvet rijstevoerschroot, dat een zeer geschikte samenstelling voor droogstandsrantsoenen heeft.

Ontvet rijstevoerschroot, behandeld om de afbraak van fytinezuur te reduceren, is een product dat mogelijk de beschikbaarheid van Ca kan verminderen. De hypothese van deze studie is dat pensbestendig rijstevoerschroot gevoerd kan worden om de homeostatische regulatie van het Ca-metabolisme te adapteren, om zo melkziekte te voorkomen.

Vijf experimenten zijn uitgevoerd om de verschillende aspecten van deze hypothese te testen. In het eerste experiment is vetgecoat rijstevoermeel geproduceerd en het effect hiervan op de Ca-homeostase in vivo getest bij multipare droogstaande koeien. In het volgende experiment is de effectiviteit van formaldehydebehandeling van rijstevoermeel, als methode het fytinezuur te beschermen tegen pensafbraak, in situ onderzocht. In het derde experiment is formaldehydebehandeld rijstevoermeel gevoerd aan groeiend jongvee om het effect op de Ca-homeostase in vivo te onderzoeken. In het vierde experiment is formaldehydebehandeld rijsteschroot met of zonder extra Ca gevoerd en is het effect op de Ca-homeostase getest bij multipare droogstaande koeien voor afkalven. In het vijfde en laatste experiment is formaldehydebehandeld rijstevoerschroot aan multipare droogstaande koeien gevoerd om het effect op de Ca-homeostase bij afkalven te onderzoeken.

Vetgecoat rijstevoermeel beïnvloedt Ca-homeostase, maar verlaagt drogestofopname

Vetgecoat rijstevoermeel is gemaakt door rijstevoermeel in een mixer met gesmolten gehydrogeneerd vet te besproeien. Dit product is in situ geïncubeerd om de pensafbraak van droge stof en fytinezuur te bepalen. De pensbestendige fractie van fytinezuur was 0.3 (passagesnelheid 0.05/h). Aanvullend hierop is het product één week aan zeven droogstaande mulipare koeien gevoerd. Gedurende deze week en in de week ervoor en erna werd de voeropname geregistreerd en urine verzameld. Vetgecoat rijstevoermeel veroorzaakte een substantiële verlaging van de drogestofopname, hetgeen ook een veel lagere Ca-opname inhield. Urine pH en Ca-uitscheiding waren lager gedurende de week waarin rijstevoermeel gevoerd werd. De Ca-uitscheiding verminderde de dag na introductie van het product en vertoonde een piek gedurende de twee dagen nadat het product niet meer gevoerd werd. Het waargenomen patroon in Ca-uitscheiding in de urine na het voeren van vetgecoat rijstevoermeel impliceert een effect op de Ca-homeostase van droogstaande multipare melkkoeien. Echter, de reductie van de drogestofopname laat zien dat vetcoating, ondanks de effectieve reductie van de pensafbraak van fytinezuur, niet geschikt is als behandeling van voeders die voor het afkalven gevoerd worden.

Formaldehydebehandeling verlaagt de pensafbraak van fytinezuur

Formaldehydebehandeling is onderzocht als methode de pensbestendigheid van fytinezuur in rijstevoermeel te verhogen. Twee monsters volvet rijstevoermeel zijn behandeld met vier concentraties formaldehyde en zijn in situ geïncubeerd in de pens van drie gefistuleerde koeien. De verdwijning van droge stof is bepaald en de residuen zijn geanalyseerd op fosfor (P) en fytaat, inclusief fytinezuur en de andere verschillende inositolfosfaatvormen. Pensafbraakparameters zijn berekend voor droge stof, P, totaal fytaat en fytinezuur. Het meeste P in de oorspronkelijke monsters was fytaat-P, vooral in de vorm van fytinezuur. Formaldehydebehandeling verlaagde de pensafbraak, waardoor de berekende fractie pensbestendige P (passagesnelheid 0.05/h) steeg van 0.08 in de controle naar 0.4 bij de hoogste dosis formaldehyde. Pensafbraak van totaal fytaat en fytinezuur gedroeg zich vergelijkbaar met de P-afbraak. Formaldehydebehandeling verlaagde de afbraaksnelheid van fytinezuur van 0.31/h in de controle tot 0.07/h bij het hoogste niveau van formaldehydebehandeling. Zo was ook de pensbestendigheid (passagesnelheid 0.05/h) van fytinezuur 0.08 zonder behandeling en 0.32 bij de hoogste dosis formaldehyde. Op basis van de pensbestendigheid en aannames over het Ca-bindend vermogen van fytinezuur kan een kg formaldehydebehandeld rijstevoermeel genoeg fytinezuur beschermen om na de pens 7 gram Ca te binden. Deze resultaten geven aan dat het mogelijk kan zijn de Ca-beschikbaarheid op darmniveau te verlagen met formaldehyde behandelde rijstvoermeel.

Vaarzen zijn geen geschikt model om de Ca-homeostase van melkvee te onderzoeken

Formaldehydebehandeld rijstevoermeel is aan vaarzen gevoerd om het effect op Ca-homeostase te bestuderen. Twee niveaus van rijstevoermeel zijn getest ten opzichte van een controlebehandeling. Rijstevoermeel had geen effect op drogestofopname. Calciumopname en de groeisnelheid van de dieren duidden op een onvoldoende Ca-voorziening ten opzichte van de behoefte. Dit was in overeenstemming met de consistent lage Ca-uitscheiding in de urine. Calcium in serum, in urine, calcitriol en hydroxyproline bleven gelijk. Echter, vier dieren reageerden met een verlaagd Ca-gehalte in de urine gedurende het voeren van het product, of met een stijging van het Ca-gehalte in de urine nadat het voeren gestopt was. Rijstevoermeel beïnvloedde de opname en uitscheiding in de urine van P, Mg en Zn, echter deze effecten kunnen niet als nadelig voor de

voedingsstatus gezien worden. De meeste vaarzen hadden een reeds geadapteerd Ca-metabolisme, aangezien hun Ca-voorziening beperkt was ten opzichte van de behoefte, in tegenstelling tot droogstaande multipare koeien. Om activatie van het Ca-metabolisme met rantsoenmaatregelen te testen zijn multipare droogstande koeien vereist.

Pensbestendig rijstevoerschroot induceert adaptatie van de Ca-homeostase

Formaldehydeontvet rijstevoerschroot is in vivo getest om het effect op de Ca-homeostase te bestuderen, waarbij de effecten van het lage Ca-gehalte in rijstevoerschroot en het effect op Ca-beschikbaarheid gescheiden werden. Daarvoor is niet alleen pensbestendig rijstevoerschroot, maar ook pensbestendig rijstevoerschroot met Ca-supplementatie vergeleken met een controlebehandeling. De behandelingen hadden geen effect op de pH van urine of op drogestofopname. Ca-uitscheiding in de urine vertoonde een piek gedurende twee dagen na stoppen van de rijstevoerschrootbehandelingen. De piek was het hoogst na het voeren van alleen rijstevoerschroot. Deze reactie wordt geïnterpreteerd als indirect bewijs voor de activatie van de Ca-absorptie in de darm gedurende het voeren van rijstevoerschroot. Dit omdat Ca in de urine snel aangepast wordt om de Ca-balans in het bloed te corrigeren, terwijl adaptatie van de darm twee dagen duurt. De conclusie uit deze studie is dat formaldehydebehandeld rijstevoerschroot de Ca-regulatie kan adapteren door zowel een verlaagd Ca-gehalte als een verlaging van de Ca-beschikbaarheid. Formaldehydebehandeld rijstevoerschroot is daarom mogelijk een geschikt product om melkziekte te voorkomen zonder verlaging van de drogestofopname.

Pensbestendig rijstevoerschroot verbetert Ca-regulatie van multipare melkkoeien bij afkalven

Formaldehydebehandeld ontvet rijstevoerschroot is voor het afkalven gevoerd om het effect op de Ca-homeostase bij het afkalven te bestuderen. Het experiment omvatte 113 multipare koeien die kalfden gedurende drie voerperioden van drie weken. In de eerste periode werd een controlevoer gevoerd, vervolgens werd in de tweede periode 140 g/kg droge stof pensbestendig rijstevoerschroot aan het rantsoen toegevoegd, waarna in de derde periode wederom het controlevoer gevoerd werd. Bij afkalven, 6 en 12 uur na kalven en 3 en 28 dagen in lactatie werden bloedmonsters genomen. Urinemonsters werden direct na het kalven

genomen. Gedurende het voeren van rijstevoerschroot was het serum Ca verlaagd, hetgeen een beïnvloeding van de Ca-homeostase aangeeft. De nadir van serum Ca na afkalven was verhoogd door het voeren van rijstevoerschroot, echter de kans op het ontvangen van een Ca-infuus was vergelijkbaar met de controlebehandeling. Het Ca-gehalte in het serum bij afkalven was hoger en dit verschil bleef aanwezig gedurende 72 uur na afkalven. Dit duidt op een verbeterde homeostatische competentie, veroorzaakt door pensbestendig rijstevoerschroot.

Algehele discussie

Hypocalcemie in dieren is een ongebruikelijke aandoening. De regulatie van de Ca-homeostase is robuust, aangezien het Ca-niveau in het bloed een prioriteit is voor het dier. Homeostase bevat controle- en correctiemechanismen. Positieve of negatieve afwijkingen in het Ca-niveau in het bloed kunnen gecorrigeerd worden door veranderingen in de uitscheiding in de urine, adaptatie van de absorptie in de darm en door aanpassing van beenweefsel. Elk van deze mechanismen wordt gestuurd door specifieke signalen en heeft zijn sterke kanten en beperkingen. De specifieke eigenschappen van deze drie mechanismen zijn gebaseerd op twee processen, transepitheel transport of aanpassing van beenweefsel, maar zijn ook gekoppeld aan hun specieke functie in het gehele homeostatische systeem.

Ca-absorptie in de nieren en de darm worden beide gereguleerd door transepithele transportprocessen met vergelijkbare structurele aspecten en regulatiemechanismen. Er zijn echter grote verschillen. Absorptie in de nieren wordt vooral gereguleerd door het parathyroid hormoon en de belangrijkste functie is het corrigeren van positieve afwijkingen, door de uitscheiding te verhogen. Hierdoor is het een korte-termijn regulatiemechanisme. Absorptie in de darm wordt vooral gereguleerd door calcitriol, waarvan de functie vooral is de Ca-aanvoer naar het systeem te corrigeren. De Ca-absorptie en feacale uitscheiding worden zo gemoduleerd om de middellange termijn te reguleren. Het grootste regulatoire verschil tussen beide systemen lijkt te zijn dat terwijl reabsorptie in de nier binnen enkele uren op veranderingen reageert, darmabsorptie meerdere dagen nodig heeft voor volledige adaptatie. De evolutionaire achtergrond voor dit verschil kan zijn dat negatieve Ca-fluctuaties vrijwel altijd geassocieerd zijn met langzame fysiologische veranderingen, terwijl positieve fluctuaties veel abrupter kunnen zijn. Additioneel zijn er indicaties dat de vertraagde adaptatie van de

darmabsorptie verband houdt met de relatief langzame celdifferentiatieprocessen van het darmepitheel.
Aanpassing van het beenweefsel wordt gereguleerd door parathyroidhormoon en calcitriol. Hierbij zijn de Ca-reserves en de structurele functie van het bot prioriteit. Substantiële mobilisatie van botweefsel vindt alleen plaats wanneer absorptie uit de darm onvoldoende is om het Ca in bloed op niveau te houden. Alhoewel een fractie van het Ca in bot relatief snel gemobiliseerd kan worden, heeft een effectieve Ca-mobilisatie een vertraging van enkele dagen, aangezien de toename van osteoclasten ook een proces van celdifferentiatie vanuit stamcellen betreft.

De overgang van droogstand naar lactatie in melkvee veroorzaakt een ongebruikelijke, tijdelijke toename in opname van Ca uit het bloed. Vertraagde darmabsorptie en botweefselmobilisatie worden gezien als de belangrijkste oorzaken van melkziekte. De sleutel voor preventie door voedingsmaatregelen zou gezocht moeten worden in het voorbereiden van de homeostatische regulering reeds dagen voor afkalven. Dit kan gedaan worden door het aanbod van beschikbaar Ca te verminderen, óf door de uitscheiding van Ca te verhogen.

Mechanistische analyse van de inductie van homeostatische regulatie

Er is een simpel model gemaakt om de effecten van het fysiologische stadium en het rantsoen op Ca-homeostase te kwantificeren om hiermee de homeostatische aanpassing ter preventie van melkziekte te voorspellen. De verhouding tussen Ca-opname uit het bloed en beschikbaar Ca in de darm (BCC/GIA: Blood Ca Clearance/GastroIntestinal Available Ca) is berekend met de aanname dat adaptatie wordt geïnduceerd door de Ca-beschikbaarheid te reduceren of de fysiologische behoefte te verhogen. Multipare melkkoeien die gevoerd worden op een typisch droogstandsrantsoen hebben een veel lagere geschatte BCC/GIA verhouding dan groeiend jongvee, multipare koeien gevoerd op een laag Ca-rantsoen, of koeien met een rantsoen met een laag kation-anion verschil.

Jongvee heeft vanwege de hoge Ca-behoefte voor groei een hoge BCC/GIA verhouding bij een brede range van Ca in het rantsoen, terwijl multipare koeien veel lagere BCC/GIA verhoudingen hebben, behalve voor extreem lage Ca rantsoenen. Koeien gevoerd met een rantsoen met een laag genoeg kation-anion verschil hebben een hoge uitscheiding van Ca in de urine, omdat metabole acidose

de werking van de Ca-resorptiekanalen in de nier vermindert. Dit resulteert in een hoge BCC/GIA verhouding, welke in de buurt ligt van die van jongvee.

Aangezien de BCC/GIA-verhouding voor afkalven een verklaring lijkt te zijn voor de preventie van melkziekte, is het model gebruikt om de gewenste reductie van de Ca-beschikbaarheid in de darm te berekenen, waarbij een BCC/GIA-verhouding ontstaat waarvan bekend is dat deze melkziekte voorkomt. Het overschot aan beschikbare Ca in de darm moet dan door fytinezuur onbeschikbaar gemaakt worden. Precipitatie van 24 gram Ca in de darm zou voldoende moeten zijn om adaptatie van Ca-absorptie te induceren bij elk gebruikelijk niveau aan Ca in het rantsoen. Het is theoretisch mogelijk deze hoeveelheid te binden met een droogstandsrantsoen waarin 25% rijstevoerschroot op basis van droge stof is opgenomen.

Praktische implicaties van melkziektepreventie met rijstevoerschroot

Het zoeken naar een alternatieve preventieve strategie voor melkziekte is gerechtvaardigd door de praktische limitaties van de huidige methoden. Voor praktische toepassing van pensbestendig rijstevoerschroot dient deze toepassing te passen binnen de nutritionele en praktische grenzen van het productiesysteem.

Nutritioneel is rijstevoerschroot een geschikt ingrediënt voor melkveerantsoenen. Het lage vetgehalte zorgt dat het minder gevoelig is voor oxidatie en de nutritionele samenstelling laat, binnen de belangrijkste grenzen van de voersamenstelling, een hoge opname in het rantsoen toe. Verder heeft formaldehydebehandeld rijstevoerschroot geen negatief effect op de drogestofopname, dit in tegenstelling tot zeolieten en rantsoenen met een laag kation-anion verschil. Dit is relevant aangezien een verminderde drogestofopname voor het afkalven de prevalentie van metabole stoornissen in het begin van de lactatie kan verhogen. Rijstevoerschroot heeft geen invloed op het metabole zuur-base evenwicht, waardoor het negatieve effect van metabole acidose, zoals veroorzaakt door een laag kation-anion verschil, voorkomen wordt. Aanvullend heeft pensbestendig rijstevoerschroot geen bekende negatieve effecten op andere mineralen. Met de huidige kennis van dit concept kan rijstevoerschroot toegepast worden in praktische droogstandsrantsoenen voor melkvee voor de preventie van melkziekte.

Conclusies

✓ Pensbestendig rijstevoerschroot/rijstevoermeel verlaagt de beschikbaarheid van Ca door verdunning van Ca in het rantsoen en binding van Ca in het maagdarmkanaal. Een lagere uitscheiding van Ca in de urine weerspiegelt een lagere Ca-beschikbaarheid. De tweedaagse piek in uitscheiding van Ca in de urine, na het stoppen van het voeren van rijstevoerschroot, is een weerspiegeling van de vertraagde adaptatie van darmabsorptie, die verklaard kan worden door het proces van differentiatie van het darmepitheel.

✓ Bescherming tegen fermentatie in de pens is noodzakelijk om fytinezuur in rijstevoerschroot als Ca-absorptie-antagonist te gebruiken. Vetcoating en formaldehydebehandeling zijn effectief in het verhogen van de pensbestendigheid van fytinezuur. Echter, vetcoating is geen geschikte methode aangezien het de drogestofopname vermindert.

✓ Drachtig jongvee is niet gevoelig voor inductie van homeostatische adaptatie via het rantsoen, omdat het Ca-metabolisme reeds geactiveerd is. Dit verklaart de lage prevalentie van hypocalcemie bij jongvee en is een verdere onderbouwing voor de preventieve waarde van de inductie van homeostatische adaptatie voor afkalven.

✓ Pensbestendig rijstevoerschroot zorgt ervoor dat fytinezuur onaangetast de pens passeert en verhoogt het fosforgehalte in het rantsoen. Er zijn geen negatieve effecten op zink of magnesium status waargenomen.

✓ Het voeren van pensbestendig rijstevoerschroot en het verlagen van het kation-anion verschil richten zich beide op het induceren van homeostatische adaptatie. Rijstevoerschroot verlaagt beschikbaar Ca terwijl rantsoenen met een laag kation-anion verschil uitscheiding van Ca in de urine verhogen, door inhibitie van de belangrijkste Ca-resorptiekanalen in het nierepitheel. Beide toepassingen verhogen de ratio tussen verwijdering van Ca uit bloed en het beschikbare Ca in de darm. Dit kan gezien worden als een voorspelling van homeostatische adaptatie.

✓ Het voeren van pensbestendig rijstevoerschroot voor en tot aan afkalven veroorzaakt een substantiële verbetering in het Ca-gehalte in bloed gedurende de drie dagen na afkalven.

✓ Pensbestendig rijstevoerschroot is een mogelijke rantsoentoepassing voor de preventie van melkziekte onder praktijkomstandigheden.

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Quiero dedicar esta tesis a mis padres, para quien nuestra formación fue siempre su principal prioridad. Espero seguir vuestro ejemplo con nuestros hijos.

Biography

Javier Martín-Tereso López was born in Madrid on May 7th, 1974. He lived and attended school in the city, but spent most weekends and holidays in the countryside near the mountains of Toledo. Since very young, his greatest interest was biology. In secondary school, he developed an interest for other sciences, which led him to consider studying engineering. At the age of 16, he studied for one year in the USA, having the opportunity to live on a farm in Ohio, his first international experience and first contact with agriculture. Two years later, he brought together his interest for biology and engineering by joining the School of Agricultural Engineering of the Universidad Politécnica de Madrid. Throughout his university years, he combined his studies with rugby, backpacking, teaching science to secondary school students, and teaching Spanish in Minnesota during the summer holidays. One school year, he studied with an EU grant at the Faculty of Agriculture in Ancona, Italy. There, he learnt Italian and became interested in aquaculture. After his return to Madrid, he participated in aquaculture research at the university. In 2001, he graduated as an Agricultural Engineer specialising in Animal Production, and joined the Nutreco Ruminant Research Centre in the Netherlands as a researcher, a position he held for 8 years. During this time, he had two main research areas: First, control of ruminal acidosis, including ad libitum compound-feed systems for beef and dairy animals, and alternatives to ionophores; and second, mineral nutrition, covering modelling, trace element regulation, environmental impact, and milk fever prevention. This last project resulted in the present thesis. Since 2009, he works at the Nutreco Ingredient Research Centre as Senior Project Manager. His current research target is mineral and vitamin nutrition of all farm species.

Javier lives in Nijmegen, the Netherlands. He shares his life with Lara. Together they have two children, Miguel and Ana.

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Training and Supervision Plan

	year	credits*
Basic Package		
WIAS Introduction Course	2007	1.5
Course on philosophy of science and/or ethics	2007	1.5
Scientific Exposure		
Mineralenvoorziening van rundvee, schapen en geiten, Lelystad, the Netherlands.	2005	0.3
Production Diseases of the Transition Cow, Dublin, Ireland.	2006	1.0
WIAS Science Day (2006, 2007). Wageningen, the Netherlands.	2006-07	0.6
Chinese Conference of Animal Nutrition, Zhuhai, China.	2006	1.2
OTEANE, Geneva, Switzerland.	2007	1.0
DIGAL Delicias, Chihuahua, Mexico.	2007	0.9
1^{era} Reunión Int. de Sistemas de Producción de Forrajes y Leche, Jalisco, Mexico.	2007	0.9
International Conference on Production Diseases 2007, Leipzig, Germany.	2007	1.0
Congreso Latinoamericano Nutrición Animal (CLANA), Cancun, Mexico.	2008	1.2
Presentations		
Chinese Conference of Animal Nutrition, Zhuhai, China. (Invited-Oral)	2006	1.0
DIGAL Chihuahua, Mexico. (Invited-Oral)	2007	1.0
1er R. Int. Sist. de Producción de Forrajes y Leche, Jalisco, Mexico. (Invited-Oral)	2007	1.0
International Conference on Production Diseases 2007, Leipzig, Germany. (Oral)	2007	1.0
Congreso Latinoamericano Nutrición Animal (CLANA), Cancun, Mexico. (Invited-Oral)	2008	1.0
In-Depth Studies		
WIAS advanced statistics Design of animal experiments	2006	1.0
Eco-physiology of the gastrointestinal tract	2007	1.5
Speciation and bioavailability	2007	1.0
Statistics for the animal sciences	2007	1.5
Bioreactor design and operation	2008	1.2
Professional Skills Support Courses		
Culture and Change Programme	2005	0.6
Techniques for Scientific Writing	2006	1.2
WGS Course Presentation Skills	2006	1.0
PhD Competence assessment or Job assessment	2006	0.3
Research Skills Training		
Preparing own PhD research proposal		6.0
Supervising theses		
Michiel Derks (Wageningen University)	2005	2.0
Carmelo Distefano (Università di Bologna)	2007	2.0
Luis Calderón (Universidad Politécnica de Madrid)	2007	2.0
Ron van Puijenbroek (Wageningen University)	2009	2.0
Membership of boards and committees		
Gebruikerscommissie: Biobeschikbaarheid van sporenelementen (Universiteit Gent)	2007-09	1.0
Education and Training Total		**40.4**

* one ECTS (European Credit Transfer System) credit equals a study load of approximately 28 hours

Colophon

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On the cover:

Anna 376 from Kempenshof, the Nutreco experimental farm, better known as cow 287. At the time of print, she was 14 years old, had gone through 11 lactations and was pregnant once again. She had taken part in many trials, of which three are described in this book. She had also yielded 124,000 kg of milk with 4.1% fat and 3.0% protein.

Increasing the longevity of dairy cows is possible, desirable and necessary for a profitable and environmentally sustainable dairy production.