Feeding Practices and Potential Risk Factors for Laminitis in Dairy Cows in Thailand

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Feeding Practices and Potential Risk Factors for Laminitis in Dairy Cows in Thailand

Voederpraktijken en potentiële risicofactoren voor hoefbevangenheid bij melkkoeien in Thailand

(met een samenvatting in het Nederlands)

Proefschrift

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Contents

This thesis is dedicated to my late mother, Pulsawat Pilachai,
with all loving memories

Chapter 1

General Introduction

1. Dairy industry in Thailand

The dairy industry in Thailand was initially started by Indian immigrants approximately 100 years ago. At this time, fresh milk consumption in Thailand was very limited due to a lack of understanding as to its benefits. The small demand for dairy products was met by imported products from overseas. During the 1950s, milk processing industries were established by the Thai government to develop and promote milk production, but the rate of consumption remained low, primarily due to the higher cost of locally produced milk products compared to their imported counterparts. During the 1960s, the Thai and Danish governments cooperated to set up the Thai-Danish Dairy Farm in Muak Lek, Saraburi Province. Both governments provided training facilities and other services to a dairy cow colony nearby, which supplied raw milk to a small plant for pasteurization. In 1971, the Thai government established the Dairy Farming Promotion Organization of Thailand (DPO) to support the Thai dairy industry. At the same time, the Nong Pho Dairy Cooperative was established with the aim to distribute ultra heat treated (UHT) milk for human consumption. The DPO was highly instrumental in implementing the government policy to develop the dairy industry in Thailand. Currently, most dairy milk is produced by small to medium scale farms averaging 10 to 20 milking cows. In 2012, there were 316,130 dairy cows and 20,624 dairy farms mostly located in the central area of Thailand (Department of Livestock Development, 2012). The average milk yield per cow is approximately 4,000 kg in 324 days containing 3.8% fat, 3.2% of protein and 12.2% of total solids (Aiumlamai, 2009).

An adequate nutrition of dairy cows is a prerequisite for the efficient production of milk. In association with low milk yields, poor reproduction and a suboptimal health status, improper nutrition is a major problem encountered in the Thai dairy industry and can be characterized by a poor quality of the rations and a general lack of knowledge regarding feeding management by the farmers (Aiumlamai, 2009).

2. Lameness and laminitis

Lameness is one of the most serious health problems facing dairy cows worldwide (Greenough et al., 2007). The prevalence of lameness of dairy cows ranges from 2 to 55% (Table 1) throughout the world depending on area, and has dramatically increased in herds over the past 20 years (Vermunt, 2007). Apart from the fact that lameness is considered to be a crucial welfare issue, lameness has also a significant economic impact due to a loss in milk

Table 1. Prevalence of lameness in dairy cows.

Reference	Farm characteristics or Region	Prevalence (%)
Thailand		
Poolket et al. (2000)	Western areas	54.5
Pratumchai (2005)	Farms with 50 to 100 cows per farm	4.7 – 13.0
Thapsitsarinya (2007)	Smallholder farms in Western areas	7.7
Arunvipas et al. (2011)	Smallholder farms in Western areas	21.0
Other countries		
Clarkson et al. (1996)	Wales (summer time)	18.6
	Wales (winter time)	25.0
Hernandez et al. (2002)	USA, Florida	31.0
Whay et al. (2003)	England	22.1
Somers et al. (2003)	The Netherlands	30.0
Huxley et al. (2004)	England (organic herds)	24.0
Sogstad et al. (2005)	Norway	2.0
Haskell et al. (2006)	England (zero-grazing herds)	39.0
Bach et al. (2007)	Spain (milking robot)	28.7
Barker et al. (2010)	England and Wales	36.8

production (Warnick et al., 2001; Green et al., 2002; Amory et al., 2008), reduction in fertility (Hernandez et al., 2001; Garbarino et al., 2004; Huxley, 2004) and hence an increased risk of culling (Enting et al., 1997; Booth et al., 2004). It has been estimated that the economic losses associated with lameness vary from €104 to €374 per clinical case of lameness (Table 2). The variation in the estimated economical loss associated with lameness can be explained by factors such as herd size, general management strategies, milk yield and definition of lameness (Enting et al., 1997).

Table 2. Estimated economic loss due to dairy cattle lameness.

Reference	Estimated economical loss (€)				
	DD[1]	WLD[2]	IDD[3]	SU[4]	Per lame cow/yr
Enting et al. (1997)	-	-	-	-	104
Kossaibati and Esslemont (1997)	259	-	141	459	-
Ettema and Østergaard (2006)	-	-	-	-	192
Willshire and Bell (2009)	85	336	173	581	362
Archer et al. (2010)	84	370	170	587	374

[1]DD=digital dermatitis, [2]WLD=white line disease, [3]IDD=interdigital disease, [4]SU=sole ulcer.

Laminitis can be defined (Mülling and Lischer, 2002) as a diffuse aseptic inflammation of the dermis of the claw (*Pododermatitis aseptica diffusa*) and is considered to be an important cause of lameness (Vermunt, 1992; Nocek, 1997). It is well documented that laminitis-related claw lesions including hemorrhage of the sole and the white line along with sole ulcers, are considered to be the most important causes of lameness in dairy cows (Vermunt and Greenough, 1994; Clarkson et al., 1996; Greenough et al., 2007). Furthermore, claw horn defects are usually observed in cows suffering from laminitis (Leach et al., 1997; Knott et al., 2007; Archer et al., 2010). Many predisposing factors are associated with the occurrence of laminitis including farm management, housing, genetics, breeding, and nutrition (Vermunt and Greenough, 1996; Ossent and Lischer, 1998). The latter is considered to be an important factor in the occurrence of laminitis, although the underlying mechanisms by which the characteristics of ration and/or feeding management contribute to laminitis have not yet been settled. However, it has been suggested that the condition of rumen acidosis is one of the most important factors in the etiology of bovine laminitis (Nocek, 1997; Ossent and Lischer, 1998; Owens et at., 1998, Greenough et al., 2007). Although the precise relationship between rumen acidosis and laminitis is hitherto unknown, one possible mechanism may be that toxic compounds such as lipopolysaccharide (LPS), histamine or other toxic components are released from the rumen into the circulation and may directly or indirectly trigger a vascular disturbance in the corium of the bovine claw, thereby, contributing to the development of laminitis (Nocek, 1997; Müller and Lischer, 2002). However, it is not yet clear how these substances are involved and whether they trigger laminitis or are just metabolites released during laminitis. As such, the relationships between

rumen acidosis and toxic compounds warrant further investigation in relation to the etiology of laminitis.

3. Thai dairy nutrition

Computerized optimization of dairy rations is hardly practiced in Thailand and due to the practice of manual, on farm mixing of the feedstuffs, a balanced nutrient intake can be questioned and nutrient intake may vary from day to day. Roughage supply to dairy farms in Thailand typically depends on the availability of agricultural waste and by-products instead of grass or its derivates such as silage and hay, due to the fact that farmers own a limited amount of land. During the dry season, roughages such as corn stover, soybean straw, soybean pods, sugarcane tops, bagasse and most importantly rice straw are used. Generally, the use of fresh grass as a source of roughage is, in practice, restricted to the rainy season. In quantitative terms, the feeding of grasses can be considered less important, most likely because its supply is based on a cut and carry system; i.e. grasses are manually collected from publically available lands and fed to the cows the same day. Grasses such as Ruzzi grass (*Brachiaria ruziziensis*), Para grass (*Brachiaria mutica*) Napier grass (*Pennisetum purpureum*) and Pangola (*Digitaria eriantha*) are among the species most commonly used.

In general, the digestibility of the roughages used to formulate dairy rations in Thailand, is low and thus the use of concentrates is necessary to ensure dairy productivity (Wanapat, 2000). In practice, the amount of concentrate offered to the cows is calculated on the basis of milk production. A commonly applied rule is that 1 kg of concentrate is supplied for each 2 kg of milk produced (Wanapat, 2000; Sruamsiri, 2007). Usually, concentrates are fed in a non-pelleted form and the ingredients are manually mixed, although some regional differences exist. For example, in the provinces west of Bangkok (amongst others Kanchanaburi and Ratchaburi), the use of pelleted concentrates is more common. Feedstuffs used as concentrates are mainly agro-industrial and industrial by-products such as cassava chips, cassava pulp, soybean hull, pineapple waste, tomato waste, potato waste, molasses, distiller's grain waste, sweet corn peel and baby corn waste. As previously mentioned, dairy ration formulation in Thailand depends on the availability of the for mentioned feedstuffs.

In the north-east of Thailand, dairy rations usually contain 50 to 70% cassava chips, and rice straw as the sole source of roughage (Wanapat, 2003). Consequently, such conditions may increase the risk of rumen acidosis due to the high content of rapidly fermentable carbohydrates. It has been shown that rations rich in rapidly fermentable

carbohydrates, low in fiber or high in rumen degradable protein, are associated with the occurrence of laminitis (Weaver, 1971; Manson and Leaver, 1988; Greenough et al., 1990). The cause and effect relationships between dietary, practical feeding, rumen acidosis and the occurrence of (sub) clinical laminitis under typical Thai conditions have not been extensively documented. Both from an economic and animal welfare perspective, studying the potential risk factors related to laminitis are important in order to reduce the occurrence of laminitis in the Thai dairy industry.

4. Aim of the thesis

The objective of the research described in this thesis was to investigate some potential risk factors in relation to laminitis in dairy cows under typical Thai feeding conditions. Four experimental objectives were identified for this research, with the anticipation that the outcome of the research can assist the Thai dairy industry to find effective prevention strategies for laminitis.

5. Outline of the thesis

Selected nutritional risk factors related to the occurrence of subclinical laminitis (SCL) in smallholder dairy farms in Thailand are investigated in **Chapter 2**.

Most Thai dairy farm rations typically contain a high level of rapidly fermentable carbohydrates in combination with a low level of crude protein (CP), which could be a major factor affecting rumen fermentation and milk production. For this reason, protein supplementation to improve milk production may result in recurrent low rumen pH values accompanied by high histamine levels in the rumen fluid, a condition associated with laminitis in dairy cows (Nocek, 1997). In order to evaluate the effect of a high CP content in the ration on rumen pH and histamine concentration of dairy cows, CP was supplemented in two different forms: formaldehyde-treated or -untreated soybean meal (**Chapter 3**).

Rumen acidosis was investigated to test the relationships between the proportion of dietary cassava meal and rumen characteristics associated with the etiology of laminitis, i.e., rumen concentrations of LPS and histamine **(Chapter 4)**.

Specific phyllosilicates have the ability to bind LPS in aqueous solutions. Hydrate sodium calcium aluminosilicate (HSCAS), a phyllosilicate, is commonly used to prevent aflatoxin toxicity but it’s the efficacy to remove free LPS from rumen fluid is not known.

Therefore, the possible preventative action of HSCAS on free LPS levels in rumen fluid was investigated in **Chapter 5**.

Finally, the results of these studies are summarized and discussed in **Chapter 6** and the main conclusions are provided in the final chapter of this thesis (**Chapter 7**).

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Chapter 2

Diet factors and subclinical laminitis score in lactating cows of smallholder dairy farms in Thailand

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Abstract

The objective of this study was to evaluate the importance of dietary crude protein (CP) content, dietary neutral detergent fiber (NDF) content and feeding regime as well as other factors related to management and demographics on the occurrence of (subclinical) laminitis under practical Thai feeding conditions. Hemorrhage of the white line and the sole, sole ulcer and white-line fissure of all four claws of milking cows (*n*=119) on 25 farms (selected based on the occurrence of lameness) were macroscopically assessed to calculate the prevalence of subclinical laminitis (SCL) on each farm. Data were collected on farm characteristics, feed and feeding management, floor type and hoof care. Dry matter intake was assessed on each farm and feed ingredients collected and analyzed for dry matter (DM), CP and NDF. No significant differences were found for farm characteristics such as herd size, number of milking cows, parity and body condition scoring between farms with a low (<25%) or a high prevalence (> 25%) of SCL. Percentages of DM and CP content of the rations did not differ, whereas mean NDF content in the ration was significantly higher in the low compared to the high prevalence farms. Multiple regression analysis of the data showed that a ration low in NDF content and/or in combination with the separate feeding of roughage and concentrate was associated with a high SCL prevalence. The results suggest that mixing concentrate with a substantial part of the roughage is an important strategy to prevent SCL in smallholder dairy farms under Thai feeding conditions. In addition, the dietary NDF content but not the dietary CP level is associated with SCL prevalence in dairy cows under Thai feeding conditions.

Keywords: subclinical laminitis; nutritional risk factor; dairy cow

1. Introduction

Lameness is a major health problem in the dairy industry worldwide (Greenough et al., 2007) including Thailand. An important cause of lameness is subclinical laminitis (SCL), which is characterized by claw lesions such as hemorrhages of the sole and the white line, and sole ulcers (Greenough and Vermunt, 1991). Subclinical laminitis is associated with low milk production, poor health and reduced reproductive performance (Nordlund et al., 2004), which negatively affect the economical return of dairy cows. Many predisposing factors are associated with the occurrence of (subclinical) laminitis in dairy cattle including farm management, housing, genetics, breeding, and nutrition (Vermunt and Greenough, 1996; Ossent and Lischer, 1998). The latter is considered to be an important factor (Nocek, 1997), although the underlying mechanisms by which nutrition contributes to the occurrence of laminitis are not yet fully understood.

Several reports have indicated that ration characteristics and/or feeding management are involved in the etiology of laminitis (Vermunt and Greenough, 1994; Nocek, 1997; Greenough et al., 2007). It has been shown by Weaver (1971) that the feeding of supplemental barley was associated with the occurrence of laminitis. Furthermore, work by Manson and Leaver (1988a) and Greenough et al. (1990) indicates that the dietary roughage to concentrate ratio also contributes to the development of laminitis as well as the intake of large amounts of concentrates; i.e. 11 kg per day (Manson and Leaver, 1988a). These results may be interpreted as effects of the dietary content of neutral detergent fiber (NDF) on the etiology of laminitis (Stone, 2004). Apart from the dietary NDF content, the level of rumen degradable protein also has been reported to be important in the etiology of laminitis (Manson and Leaver, 1988b).

Dairy production in Thailand is characterized by small-scale operations managing 20 to 50 dairy cows (Aiumlamai, 2009; Koonawootrittriron et al., 2009) and common Thai feeding practices of dairy cows are not extensively documented. Generally, cows are fed poor quality forages particularly rice straw, supplemented with non-pelleted concentrates. Cassava chips are widely available in Thailand and may be incorporated up to 70% in dairy rations (Aiumlamai, 2009; Wanapat, 2003) resulting in low dietary NDF contents. However, it is unknown whether the typical Thai feeding conditions predispose cows to laminitis. The aim of the present study was to evaluate the importance of dietary CP content, dietary NDF content and feeding regime as well as other factors related to management and demographics on the occurrence of (subclinical) laminitis under practical Thai feeding conditions.

2. Materials and methods

2.1. Farm selection

This study was conducted on farms located in the Saraburi and Khon Kaen provinces of Thailand, two of the top ten dairy farming areas in Thailand. The use of farms from two different provinces increased the variation in feeding management because mixed feeding of roughage and concentrate is more common in the Khon Kaen province, while farmers in Saraburi province traditionally feed concentrate and roughage separately. From each province a list of all farms under veterinary control, was provided by local veterinarians. Then, the farms on this list were divided into two groups; i.e. farms without and with reported incidences of lameness during the last six months preceding the current study. From each group of farms, individual farms were randomly selected as described by Thrusfield (1997). It was anticipated that the selection of farms with and without previous reports on lameness would increase the variation in the prevalence of SCL so as to enhance the interpretation of the data. All cows that participated in the study were a cross of Holstein-Friesian (HF) × Brahman (86.4% HF). At all farms, cows were housed in a loose-housing system as typical in these provinces and none of the farms used formaldehyde and/or copper sulphate as a prophylactic agent for hoof problems.

2.2. Cow selection

Subclinical laminitis was assessed by investigation of claw lesions in dairy cattle in the selected farms. On each farm, the sample size to detect SCL was calculated by assuming a power of 80% and SCL prevalence of 50% (Thapsitsarinya, 2007), with a confidence interval of 95%. Therefore, approximately 20% of the lactating cows on each farm were randomly selected to estimate SCL prevalence. Cows in milk on farms were randomly selected from a list containing cow numbers, which was the sole selection criterion. In total, 119 lactating cows were evaluated originating from 12 smallholder dairy farms in Saraburi and 13 in the Khon Kaen province, representing 21.1% of all lactating cows on the selected farms. Toes length of all four limbs in each of the selected cows was measured along the dorsal border using a Vernier caliper. Claw lesions were assessed by trimming the hooves using a hoof-trimming machine (Robert Bosch GmbH, Bosch®, Stuttgart, Germany). The same person throughout the study then evaluated claw lesions of all four limbs on the basis of macroscopic examination. Hemorrhage of the white line and the sole, sole ulcer and white-line fissure were scored using the 4-point scoring as 0=not present, 1=mild, 2=moderate or 3=severe

according to the method described by Sogstad et al. (2005). By definition, any appearance of the white line hemorrhage, sole hemorrhage or white line fissure on one of the claws was considered as a clinical case of SCL with a score ≥2. Prevalence of SCL was calculated as a proportion of the affected cows relative to the number of selected cows on each farm. The data were categorized using medians and standard deviation.

2.3. Data collection

Each farm was visited once (May 2008 to Feb 2009) during which time, cows were scored and farm owners/managers were interviewed using a standardized questionnaire. Questions related to the history of the farm and incidences of SCL were confined to a period of the previous 6 months prior to the farm visit. The questionnaire was divided into four sections, including general information concerning farm management and location, feed and feeding management, floor type and hoof care. The characteristics of housing factors and feeding management were classified into two categories as yes (1) or no (0). The categories for floor type were a solid concrete floor with a partial soil or muddy area (1) and a sand or muddy floor only (0). Feeding practice was categorized as separate (SFRC, 1) or combined (CFRC, 0) feeding of roughage and concentrate. Feeding frequency was divided in two times per day (1) and over two times per day (0). The categories for supplementation of vitamins and minerals were divided as farms with (1) and without supplementation of minerals and vitamins (0). Data related to herd size, parity, stage of lactation (d in milk) and milk yield were obtained from farm records.

Total dry matter intake (DMI) of the cows was recorded on the day the farm was visited by weighing the concentrate and roughage provided by the farmer. Feed refusal collection was endeavored but no refusals were found for any of the cows on any of the farms. Body condition score (BCS) of the selected cows was determined according to the method described by Ferguson et al. (1994). Ration ingredients were collected at each farm. Samples from concentrates were taken after the mixing of the unpelleted concentrates alone (SFRC) or in combination with roughages (CFRC). Grab samples of both the mixed concentrates and roughage were taken from proportional locations of the feed through to attain approximately 2 kg (as fed) of sample. Then, the samples were dried at 103 °C for 16 h and subsequently stored in sealed plastic bags at room temperature (25 °C) until analyses. Nitrogen content was measured by the macro Kjeldahl method; with a factor of 6.25 used to convert to CP (AOAC, 1990). The NDF content was determined according to the method of Van Soest et al. (1991).

2.4. Statistical analysis

All statistical analyses were performed using SPSS version 17 for Windows. Differences between farms with regard to continuous variables were compared using Student's t-test. To detect risk factors that were related to the occurrence of SCL, multiple regression analysis was performed with floor type, breeding, parity, province and BCS as factor, the prevalence of SCL as dependent variable, and stage of lactation, milk yield, grazing frequency, feeding frequency, total DMI, dietary CP and NDF contents as predictor variables. Prior to multiple regression analysis, potential explanatory variables were assessed for their independency by means of Pearson's correlation matrix. Forward stepwise regression was performed by incorporating the variable into the model showing the highest significant, partial correlation coefficient for its relation with the prevalence of SCL. Throughout, the level of statistical significance was pre-set at $P<0.05$.

3. Results

Twelve farms had a prevalence of SCL above 25%. Subclinical laminitis prevalence was not correlated with province ($P=0.359$). Sole hemorrhage was the most frequently observed claw lesion and was higher in the farms with a previous report of lameness (Table 1). Likewise, hemorrhages of the white line and white line fissures were observed more on the farms with a report of lameness 6 months before commencement of the study (Table 1). Consequently, SCL prevalence was found to be 64.0% and 15.3% for the farms with or without a previous history of lameness, respectively. The length of the two front hooves and the two hind hooves were not different (data not shown) for farms with or without a previous lameness report ($P=0.426$ and 0.436, respectively).

The majority (72%) of the farm holders kept their cows on a solid concrete floor with a partial soil or mud area, while the remaining farms had a sand or mud floor only (data not shown). The number of lactating cows on the selected farms ranged from 14 to 38 (Table 2). The SFRC was practiced in 56% of the selected farms. The remaining farms fed roughage and a mixture of predominantly concentrates (farmer information and visual inspection) and roughage (CFRC). On all farms, both commercial and homemade concentrates were fed in a non-pelleted form and consisted of varying proportions (farmer information and visual inspection) of a wide range of feedstuffs (Table 2).

Table 1. Observed claw lesions of dairy cows on farms with or without a previous incidence of lameness six months before determination of the prevalence of subclinical laminitis in Thailand.

Parameter	Previous lameness incidence on farm (± SD)	
	No	Yes
Number of farms	13	12
No. of cows on farm	41.2±11.29	36.8±10.25
No. of lactating cows	23.1±6.85	22.1±7.83
% of lactating cows sampled	21.0	21.1
	% of total cows	
Sole hemorrhage		
Khon Kaen	13.2	54.3
Saraburi	11.3	52.1
White line hemorrhage		
Khon Kaen	3.4	39.0
Saraburi	4.6	41.5
White line fissure		
Khon Kaen	5.5	26.8
Saraburi	7.4	25.6
Prevalence of subclinical laminitis (%)		
Khon Kaen	16.2	63.3
Saraburi	14.0	64.5

Furthermore, on 80% of the farms, concentrates were offered twice a day while on 20% of the farms concentrates, including CFRC, were offered more than twice a day. Supplemental vitamins were provided to the cows on all farms. Rice straw was the main source of roughage while on 32% of the farms other forms of roughages such as fresh ruzie grass (*Brachiaria ruziziensis*), fresh napier grass (*Pennisetum purpureum*) and sugar cane leaves were supplied in various proportions (Table 2). To enhance animal welfare, outside grazing of cows was practiced on 26% of the selected farms (data not shown). The amount of fresh grass ingested during grazing was ignored because the actual grazing time was restricted to a maximum of 3 h/d and the amount of grass available for grazing (farmer information and visual inspection) was very low. Therefore, intake from fresh grass was

considered to be low for the cows on farms practicing outside grazing. Estimated DM intake of concentrates and CFRC ranged from 5.8 to 14.1 kg/d (Table 3). The NDF and CP content of the total rations (DM basis) were found to range between 349 to 653 g/kg DM and from 75 to 166 g/kg DM, respectively (Table 3).

No significant differences were found for herd size, number of milking cows, parity and BCS between the two groups (Table 4). Approximately 65% and 57% of the cows in the low and high prevalence SCL farms respectively were multiparous. A significant difference was found for days in milk for cows on the low and high prevalence SCL farms (Table 4). Therefore, the difference in stage of lactation was taken into account when data on milk production were statistically analyzed and a day in milk was used as covariate. Consequently, the difference in milk production between the low and high prevalence SCL farms was not significant (Table 4). Mean total DMI was 19.6 kg (SD 3.28) and 15.5 kg (SD 4.20) on the farms with the low and high SCL prevalence, respectively. The mean dietary DM and CP contents were similar for the low and high SCL prevalence farms; i.e. 595 (SD 120.4) and 113 (SD 28.1) g/kg DM, respectively. In contrast, mean dietary NDF contents were 35.9% higher on the farms with a low SCL prevalence; i.e. 587 (SD 51.2) and 432 (SD 68.9) g/kg DM for the low and high prevalence farms, respectively.

Table 2. Number of cows, milk yield, dry matter intake and ration information for 25 Thai small holder dairy farms with a variety of subclinical laminitis prevalence.

Farm[a]	Case report[b]	No. of cows		Laminitis prevalence (%)	Milk yield[c] (kg/d)	DMI[d] (kg/d)	Feeding method[e]	Ingredients	
		Lactating	Sampled					Concentrate	Roughage
1	no	28	6	0.0	16.7	15.1	CFRC	CC[f], cassava pulp, brewers meal, palm meal, napier grass, sugar cane leaf	Rice straw
2	no	20	4	0.0	21.3	17.1	CFRC	CC, brewers meal, palm meal, leucaena leaf meal, napier grass, sugar cane leaf	Rice straw
3	no	20	4	0.0	27.5	23.9	CFRC	CC, brewers meal, palm meal, leucaena leaf meal, corn meal, napier grass, sugar cane leaf	Rice straw, ruzie grass
4	no	14	3	0.0	22.3	14.8	SFRC	Commercial concentrate, soybean meal	Rice straw
5	no	35	7	14.3	21.7	17.7	CFRC	CC, cassava pulp, brewers meal, palm meal, molasses, pangola grass	Rice straw, pagola grass
6	no	24	5	20.0	26.6	23.6	CFRC	CC, cassava pulp, brewers meal, palm meal, molasses, napier grass, sugar cane leaf	Rice straw
7	no	24	5	20.0	25.6	17.1	CFRC	CC, cassava pulp, brewers meal, palm meal, para grass	Rice straw, para grass
8	no	20	5	20.0	15.6	21.5	SFRC	CC, cassava pulp, brewers meal, palm meal, leucaena leaf meal	Rice straw, ruzie grass

Table 2. Continued

Farm[a]	Case report[b]	No. of cows		Laminitis prevalence (%)	Milk yield[c] (kg/d)	DMI[d] (kg/d)	Feeding method[e]	Ingredients	
		Lactating	Sampled					Concentrate	Roughage
9	no	20	4	25.0	18.5	20.2	CFRC	CC, cassava pulp, brewers meal, palm meal, napier grass, sugar cane leaf	Rice straw
10	no	18	4	25.0	27.5	19.2	CFRC	CC, cassava pulp, brewers meal, palm meal, leucaena leaf meal, coconut meal, sugar cane leaf	Rice straw, ruzie grass
11	no	20	4	25.0	27.0	17.5	CFRC	CC, cassava pulp, palm meal, leucaena leaf meal, coconut meal, para grass	Rice straw, para grass
12	no	19	4	25.0	12.7	23.8	SFRC	CC, soybean meal	Rice straw, pangola grass
13	no	38	8	25.0	17.5	22.8	CFRC	CC, commercial concentrate, cassava pulp, palm meal, leucaena leaf meal, napier grass, corn silage	Rice straw, ruzie grass
14	yes	35	7	28.6	15.4	13.9	SFRC	Commercial concentrate, soybean meal	Rice straw
15	yes	15	3	33.3	13.0	10.6	SFRC	CC, cassava pulp	Rice straw
16	yes	25	5	40.0	21.8	25.1	CFRC	CC, cassava pulp, brewers meal, molasses, pangola grass	Rice straw, pangola grass
17	yes	35	7	42.9	16.5	9.2	SFRC	Commercial concentrate	Rice straw
18	yes	30	6	66.7	23.8	17.1	SFRC	CC, palm meal, leacena leaf meal	Rice straw
19	yes	15	3	66.7	15.7	14.4	SFRC	Commercial concentrate	Rice straw

Table 2. Continued

Farm[a]	Case report[b]	No. of cows		Laminitis prevalence (%)	Milk yield[c] (kg/d)	DMI[d] (kg/d)	Feeding method[e]	Ingredients	
		Lactating	Sampled					Concentrate	Roughage
20	yes	22	5	75.0	18.3	20.5	SFRC	CC, commercial concentrate, palm meal, luecaena leaf meal, coconut meal, molasses	Rice straw
21	yes	18	4	75.0	13.50	13.9	SFRC	Commercial concentrate, soybean meal	Rice straw
22	yes	16	4	75.0	17.25	16.7	SFRC	CC, cassava pulp, palm meal, luecaena leaf meal, coconut meal, molasses	Rice straw
23	yes	25	5	80.0	15.4	14.8	SFRC	CC, palm meal, luecaena leaf meal	Rice straw
24	yes	14	4	100.0	11.9	14.1	SFRC	Commercial concentrate, soybean meal	Rice straw
25	yes	15	3	100.0	9.7	15.2	SFRC	CC, cassava pulp, molasses	Rice straw

[a] Farm number 1, 2, 7, 12, 13, 14, 15, 17, 18, 23, 24, 25 were located in Saraburi province and 3, 4, 5, 6, 8, 9, 10, 11, 16, 19, 20, 21, 22 in the Khon Kaen province

[b] Lameness incidence of farms 6 months prior to commencement of the study.

[c] Mean of milk production of the selected cows.

[d] Dry matter intake.

[e] Feeding method was classified as combined (CFRC) and separated (SFRC) feeding of roughage and concentrate.

[f] The constant components consist of cassava chips, rice bran, soybean meal and mineral premix.

Table 3. Dry matter intake, neutral detergent fiber content and crude protein content of the ration concentrate and roughage fed to cows on 25 Thai small holder dairy farms with a variety of subclinical laminitis prevalence.

Farm[a]	DMI (kg/d)				NDF (g/kg DM)				CP (g/kg DM)			
	CFRC[b]	C[c]	R[d]	Total	CFRC	C	R	Total	CFRC	C	R	Total
1	8.3	-	6.8	15.1	445	-	705	562	189	-	38	121
2	8.7	-	8.4	17.1	579	-	705	641	173	-	39	107
3	14.1	-	9.8	23.9	617	-	686	645	109	-	56	87
4	-	9.3	5.5	14.8	-	596	705	637	-	194	38	136
5	10.8	-	6.9	17.7	445	-	688	540	159	-	54	118
6	16.4	-	7.2	23.6	579	-	705	617	91	-	39	75
7	10.2	-	6.9	17.1	617	-	706	653	174	-	52	125
8	-	13.5	8	21.5	-	375	683	489	-	210	56	153
9	13.3	-	6.9	20.2	550	-	705	603	131	-	38	99
10	10.3	-	8.9	19.2	495	-	706	592	119	-	52	88
11	10.3	-	7.2	17.5	410	-	706	532	194	-	51	135
12	-	9.3	14.4	23.7	-	326	689	546	-	245	54	129
13	8.7	-	14.1	22.8	423	-	674	578	115	-	71	88
14	-	10.0	3.9	13.9	-	328	705	434	-	117	39	95
15	-	7.1	3.5	10.6	-	239	705	393	-	140	39	107
16	13.8	-	11.3	25.1	528	-	689	601	93	-	56	76
17	-	5.8	3.4	9.2	-	385	705	504	-	241	38	166
18	-	12.5	4.6	17.1	-	249	705	371	-	127	37	103
19	-	8.8	5.6	14.4	-	256	705	432	-	111	39	83
20	-	12.8	7.7	20.5	-	235	705	412	-	154	37	110
21	-	9.0	4.9	13.9	-	316	705	454	-	188	37	135
22	-	10.6	6.1	16.7	-	157	705	357	-	99	38	77
23	-	8.2	6.6	14.8	-	221	705	438	-	240	38	150
24	-	8.3	5.8	14.1	-	250	705	437	-	255	39	166
25	-	9.0	6.2	15.2	-	104	705	349	-	118	39	86

[a] Farm 1-13 and 14-25 without and with lameness incidence of farms 6 months prior to commencement of the study, respectively.

[b] Combined feeding of roughage and concentrate

[c] Concentrate

[d] Roughage

Table 4. Herd and cow characteristics of 25 Thai small holder dairy farms with a low and high subclinical laminitis score.

	Subclinical laminitis score[a]			
Variable	Low (n=13)	High (n=12)	S.E.	*P*-value
Herd size	41.2	36.8	4.4	0.346
Lactating cows	23.1	22.1	2.9	0.739
Parity	3.2	2.7	0.79	0.280
Body condition scoring	2.8	2.7	0.52	0.880
Stage of lactation (d in milk)	113.5	156.5	18.19	0.027
Milk yield (kg/cow/d)	21.6	16.0	1.83	0.077

[a] low prevalence ≤25%, high prevalence >25%.

Visual inspection of the data (Fig.1), clearly show that feeding method (CFRC vs. SFRC) and the dietary NDF content are not independent. Therefore, two series of multiple regression analyses were performed. After stepwise regression, the model showing the highest percentage of explained variance in the SCL prevalence (R^2=0.631; P<0.001) only contained a constant and the dietary NDF content as the predictor variable (Fig. 1). It appeared that a SCL prevalence < 50% only occurred when the ration contained at least 480 g NDF/kg DM and a maximum SCL prevalence of 25% was, except for one herd, associated with a dietary NDF content of at least 525 g/kg DM. Furthermore, when the ration contained less than 550 g NDF/kg DM cases of laminitis where always observed at the selected farms (Fig. 1). When the dietary NDF content was replaced by feeding method as an predictor variable, feeding method contributed significantly to the explained variance of SCL prevalence (R^2=0.585; P=0.016).

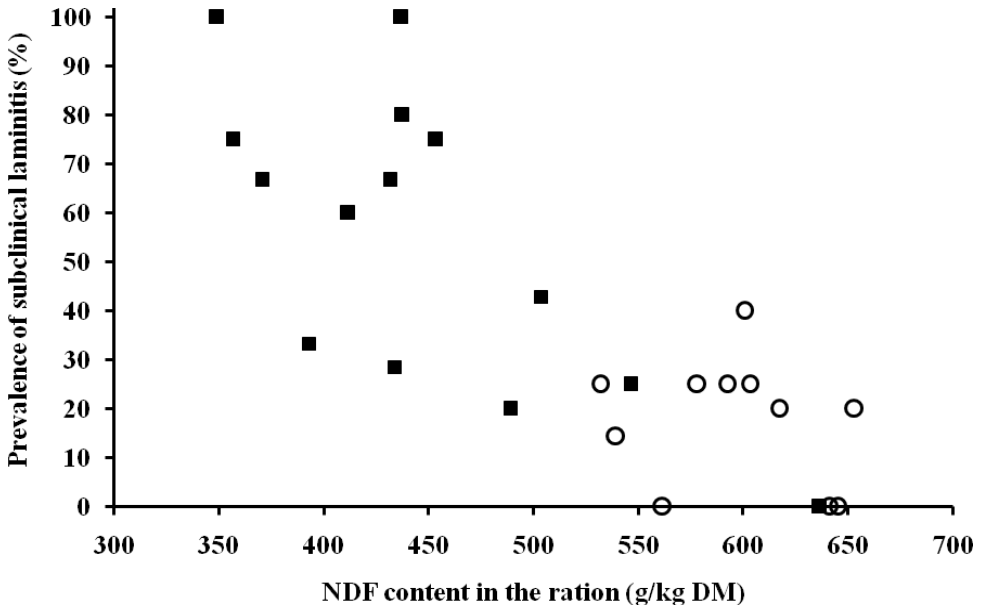

Fig. 1. The relationship between the prevalence of subclinical laminitis and the dietary content of neutral detergent fiber (NDF) in dairy cows on farms in two provinces of Thailand. (○) farms practicing combined feeding of roughage and concentrate, (■) farms practicing separate feeding of roughage and concentrate. The linear correlation coefficient and regression formula are: $R^2 = 0.631$, $y = -0.246x + 165.1$ ($P<0.001$).

4. Discussion

In the current study, SCL prevalence ranged from 0% to 100% with a mean prevalence of 39%. Sole hemorrhages (31.8%) contributed most to the SCL score followed by hemorrhages of the white line (21.4%) and white line fissure (16.0%). Bargai and Levin (1993) found a similar percentage for hemorrhages in Israel while much higher values (i.e. 62.1%) were reported for cows in Ohio, USA (Smilie et al., 1999).

The objective of the current study was to evaluate the importance of the dietary CP content, dietary NDF content and feeding regime on the occurrence of (subclinical) laminitis in Thailand. Multiple regression analysis (Fig. 1) showed that the level of NDF in the ration was associated with the SCL prevalence. The mean NDF content of the rations fed on the low SCL prevalence farms was higher ($P<0.001$) compared to that fed on the high SCL prevalence farms. The NDF content of the ration was found to be greater when feeding roughage and concentrate combined (CFRC). Furthermore, feed refusals were not detected which implies that all cows were fed restrictively. In contrast to the cows fed by the CFRC

method, cows fed by the SFRC method could only ingest roughage that was offered separately. The feeding of restrictive amounts of roughage can negatively affect the buffer capacity of the rumen content because cows cannot compensate by roughage intake (Maekawa et al., 2002). Thus, it can be speculated that the feeding of restrictive amounts of roughage in combination with SFRC has influenced the prevalence of SCL on the farms that practice SFRC.

In four out of the 25 farms, the number of milking cows ranged from 14 to 16. Therefore, only 3 cows were selected from these farms. Consequently, one case of SCL has a large impact on the SCL prevalence and significantly affecting the outcome of the regression analysis. However, when these four farms were excluded from the dataset, the dietary NDF content remained the only variable that significantly (P<0.001) explained the observed variance in the SCL prevalence. Therefore, it seems that the dietary NDF content was the most important risk factor for SCL prevalence in the current study.

The outcome of the current study strongly indicates that the feeding of low NDF rations, eventually in a separate roughage-concentrate feeding management (SFRC) is associated with a higher incidence of SCL. Both low dietary NDF contents and practicing SFRC can negatively affect rumen pH and this condition may be related to the occurrence of laminitis (Nocek, 1997). The mean NDF content of the rations fed on either the high and low SCL prevalence farms, was 596 and 454 g/kg DM, respectively. Nevertheless, these values are higher than minimum requirements of NRC (2001); i.e. >30%. The physiological background of the NRC recommendation for dietary NDF content is related to rumen function (Welch and Smith, 1969; Allen, 1997). In this respect, the differentiation of dietary NDF towards physically effective NDF (Mertens, 1997) might be more relevant. Physically effective NDF (peNDF) promotes chewing activity and secretion of saliva (Yang and Beauchemin, 2007), thereby, promoting rumen pH and preventing rumen acidosis (Krause et al., 2002; Yang and Beauchemin, 2006) and therefore laminitis (Nocek, 1997). Several studies have shown that increasing peNDF, particularly in the form of forage NDF could reduce ruminal acidosis (Oba and Allen, 1999; Krause and Oetzel, 2006; Yang and Beauchemin, 2006). However, the estimated mean intake of rice straw, a feedstuff rich in peNDF, was identical for both groups of farms in the present study (~8.5 kg DM/d). Therefore, it seems that the difference in SCL prevalence cannot be explained by a difference in intake of rice straw. Thus, it might be that the CFRC is an important strategy to prevent SCL, at least in the condition when restrictive amounts of rice straw are offered. Because CFRC contained both roughage and concentrates it increases the intake of peNDF and might

affect the fermentation rate of the fermentable organic matter. In the current study, all but one of the farmers on the high SCL prevalence farms provided their cows with concentrates and roughage separately. In these cases, concentrates were provided during milking time and roughage was supplied 1 to 3 hrs later. Furthermore, the majority of dairy farms used non-pelleted concentrates that contained a variety of rapidly fermenting ingredients such as cassava chips, cassava pulp and rice bran. Thus, it might be that in these cases rumen pH was compromised. It has been shown by Maekawa et al. (2002) that rations rich in rapidly fermentable carbohydrates and inadequate amounts of fiber contents may contribute to an increase in VFA production in the rumen leading to a decrease in rumen pH. Furthermore, various studies (Sutton et al., 1986; Yang and Varga, 1989) have demonstrated that the feeding of concentrates rich in rapidly fermentable carbohydrates twice daily negatively affects rumen pH.

In the present study, the mean CP content of the rations was 112 g/kg DM (SD 2.83), irrespective of the prevalence of SCL. This value is substantially lower than the minimum amount of CP required for maintenance and production of dairy cows recommended by the NRC (2001); i.e. 150-160 g/kg DM. However, the CP content of the rations investigated in the current study are in line with those reported by Wachirapakorn (2006) for Thai dairy rations which typically contain 100 to 120 g CP/kg DM. Furthermore, it is generally accepted that the rate of rumen fermentation is decreased when the ration contains only 100 to 120 g CP/kg DM (Satter and Slyter, 1974). Therefore, it might be of interest to supplement the ration with rumen degradable protein (RDP) to stimulate milk production. However, Garner et al. (2004) reported that the feeding of RDP resulted in higher ruminal histamine concentrations, a condition associated with the occurrence of laminitis (Nocek, 1997). The issue on dietary CP in relation to SCL under Thai feeding conditions was investigated by Pilachai et al (2012). In this study, (Pilachai et al., 2012) cows were fed high CP rations (260 g CP/kg dm); either rich in RDP (62% of total CP) or rich in rumen undegradable protein (RUP, 57.9% of total CP) and one case of laminitis occurred on each ration. However, the observed ruminal histamine concentrations were low (< 1.3 mg/l) and it was concluded that the observed cases of laminitis could not be related to the ruminal histamine concentrations (Pilachai et al., 2012) Clearly, the issue on dietary CP in relation to SCL is not yet settled.

The lack of influence of floor type on the prevalence of SCL was probably due to the fact that all the cows were housed in a loose housing type system in combination with at least partially, a sandy floor. This condition allowed the cows to walk on softer surface, which

might reduce the pressure on the claws and the incidence and severity of claw lesions (Rouha-Mülleder et al., 2009; Somers et al., 2003; Vanegas et al., 2006).

5. Conclusion

Dairy cows kept on smallholder farms in Thailand and fed a ration low in NDF content and/or in combination with restrictive feeding of roughage have a high risk of subclinical laminitis. Dietary CP level was not associated with a higher prevalence of subclinical laminitis. The results suggest that an increased proportion of forage NDF content in the concentrate diet may be an important factor in the prevention of subclinical laminitis in Thai dairy cows.

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Chapter 3

The effects of high levels of rumen degradable protein on rumen pH and histamine concentrations in dairy cows

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Abstract

An experiment was conducted to test the hypothesis that the supplementation of crude protein (CP) results in rumen acidosis and increased histamine concentrations in dairy cows. Six ruminally fistulated, non-pregnant dry cows were fed 3 experimental rations in a replicated 3 × 3 Latin square design. The CP contents in the low-CP, the high rumen undegradable protein (high-RUP) and the high rumen degradable protein (high-RDP) rations were 112, 259 and 266 g/kg DM, respectively. The cows were fed 7.7 kg DM of the concentrates and 2.7 kg DM of rice straw. High levels of RDP in the ration significantly increased the ammonia, total volatile fatty acid (VFA) and histamine concentrations in the rumen fluid. However, supplemental CP either degradable or undegradable, did not significantly affect the pH of rumen fluid. Plasma urea nitrogen concentration was higher in both high-RDP and high-RUP compared to cows fed by the low-CP ration. The rise in ruminal histamine concentrations was physiologically non-relevant, most likely because rumen pH was not affected by supplemental CP at the installed level of DM intake. Therefore, it can be concluded that the issue on supplemental CP, rumen pH and ruminal histamine concentrations has not yet settled. Further research is warranted to understand these relationships.

Keywords: rumen degradable protein; fermentation; rumen acidosis; histamine; dairy cow

1. Introduction

In Thailand, dairy cows commonly produce 3,500 kg of milk in 305 days and in large parts they are typically fed high concentrate rations (75 to 80% of total dry matter). The concentrates are usually fed in the non-pelleted form. In practice, dairy rations typically contain 50 to 70% cassava chips and rice straw as the main source of roughage for dairy cattle (Aiumlamia et al., 2003; Wanapat, 2003). Consequently, the rations are high in rapid fermentable carbohydrates and low in CP which may be a major constraint for both rumen fermentation and milk production. Theoretically, CP supplementation to high concentrate rations should be in the form of true protein to optimize microbial growth (Russell et al., 1992). However, under Thai feeding conditions, CP supplementation, if any, is usually in the form of urea because of economical reasons. Protein supplementation to rations high in rapid fermentable starch substantially increases the nitrogen supply to the rumen microbes, resulting in an increased production of VFA (Satter and Slyter, 1974; Davidson et al., 2003). Increasing levels of VFA are associated with a decline in rumen pH which enhances the risk on rumen acidosis (Goad et al., 1998; Nagaraja et al., 1998). Furthermore, it has been suggested by Garner et al. (2004) that rations high in rumen degradable protein induce increased ruminal histamine concentrations through the decarboxylation of histidine. Therefore, protein supplementation may result in recurrent low rumen pH values accompanied by high histamine levels in the rumen fluid (Irwin et al., 1979), a condition associated with laminitis in dairy cows (Nocek, 1997). Currently, it is unknown whether CP supplementation to dairy cows kept under the unique Thai feeding conditions would result in rumen acidosis and increased rumen histamine concentrations. In the present study, CP was supplemented in two different forms of either formaldehyde-treated or -untreated soybean meal. It was hypothesized that the supplementation of CP results in rumen acidosis and increases ruminal histamine concentrations. It was anticipated that the fermentation rate of the untreated soybean meal was much higher than formaldehyde treated soybean meal, which offers the possibility to distinguish between the effect of protein and rate of fermentation.

2. Materials and methods

2.1. Ethical considerations

The experimental protocol was approved by the Animal Ethics Committee of Khon Kaen University, Khon Kaen, based on the Ethics of Animal Experimentation of National Research Council of Thailand.

2.2. Animals and experimental design

Six crossbred Holstein-Friesian × Brahman, non-pregnant, dry cows with the average age of 3.8 yr (SEM = 0.19) and BW of 465 kg (SEM = 27.7) were used. They were fitted with a rumen cannula and housed on a concrete floor in individual pens (2 × 3.5 m^2) with roof and natural ventilation during the experiment. All cows were examined and appeared healthy, without clinical signs of foot pain and no swelling over the coronary band at the beginning each period of the study.

The trial had a 3 × 3 Latin square design, with two cows per treatment order and was preceded by a 14-d pre-experimental period that allowed the cows to adapt to the experimental rations. Each experimental period lasted 21 days. The animals were randomly assigned to each sequence of feeding on the three experimental rations and had free access to water.

2.3. Experimental rations

The cows were fed restricted amounts of the experimental rations (7.7 kg DM of concentrates and 2.7 kg DM of rice straw) to ensure constant intake of non-variable nutrients. The rations were offered daily in two equal portions at 08:00 and 17:00 h and feed refusals, if any, were recorded. The ingredient and chemical composition of the experimental rations are presented in Table 1.

Except for the experimental soybean meals, estimates of protein degradability of all other experimental ingredients were calculated according to NRC (2001). The protein degradability of both types of soybean meal was determined in triplicate using the method as described (Ørskov and McDonald, 1979). Briefly, the samples were incubated in the rumen for 0, 4, 8, 12, 24, 48 and 72 h. Effective degradability (ED) of DM and CP was calculated according to the equation (Ørskov and McDonald, 1979): $ED = a + bc/(k + c)$ where a = constant representing the portion of CP soluble at time 0, b = the portion of CP potentially degradable in the rumen, c = rate constant of degradation of fraction b, and k = estimated outflow rate from the rumen. Kinetics of the *in sacco* disappearance of DM and CP was estimated using the non-linear regression procedure of SAS (SAS, 1996).

2.4. Collection of samples

In order to monitor the feed during the last week of each experimental period, the experimental concentrates were sampled daily and then pooled, ground, and stored in sealed plastic bags at ambient temperature (25 ºC) until analysis.

On days 18 and 21, rumen fluid samples were taken 15 min before feeding and 1, 2, 3, 4, 5, 7 and 9 h after the morning feeding. Rumen content (200 ml) was collected from the ventral sac of the rumen using a vacuum pump (220 Volts, 1.9 Amps and 50 Hz, GAST manufacturing, Inc., U.S.A.) into a 500-ml Erlenmeyer flask. Immediately after collection, pH of ruminal fluid was recorded (Mettler-Toledo GmBh 8603, Schwerzenbach, Switzerland) and subsequently filtrated through four layers of cheesecloth. A 55-ml filtered sub-sample taken at 15 min before feeding and 1, 3, 5, 7 and 9 h after feeding was preserved by adding 5 ml of 1 M H_2SO_4 and stored at -20 ºC until analysis of volatile fatty acids (VFA) and ammonia. Another 20 ml of filtered rumen fluid sample taken at 0, 2, and 4 after feeding was mixed with 5 ml of 30% trichloroacetic acid (w/v), immediately placed on ice and centrifuged at 4ºC at 16 000 *g* for 30 min within 30 min after collection. The supernatant was stored in Eppendorf tubes at -80 °C until histamine analyses.

On d 18 and 21 of each period, blood samples were taken at 0, 2 and 4 h after feeding from the jugular vein of each cow using a 6-ml Vacutainer tube containing lithium-heparin (0804-01; Zenimed Co., Ltd.) and immediately placed on ice. Plasma was then separated within 30 min after blood collection by centrifugation at 4 ºC (1 500 *g*, 15 min). The plasma was collected and stored in Eppendorf tubes at -20 °C until analysis.

Throughout the experiment all cows were clinically examined daily for any signs related to subacute laminitis. They were observed for a claw inflammation and foot pain according to the definition of subacute laminitis described by Greenough et al. (2007) with some modifications. Briefly, the coronary band was observed and evaluated for swelling as 0 = no swelling; or 1 = a swelling and pink in color. Weight shifting was defined by a shifting of weight laterally from one leg to another in a monotonous manner as 0 = no reaction; or 1 = marked reaction.

2.5. Chemical analysis

The DM content of the experimental rations was determined by drying at 135 ºC for 3 h (AOAC, 1990). The ash content of the rations was analyzed by combustion at 550 ºC for 16 h. Nitrogen contents were determined by the macro Kjeldahl method (International Dairy Federation, 1986); a factor of 6.25 was used to convert nitrogen into CP. Ether extracts of the

rations were determined according to the AOAC (1990). The NDF and ADF content of the rations were analyzed according to the method of Van Soest et al. (1991).

Table 1. Ingredient and chemical composition of the experimental rations.

Item	Experimental rations [a]		
	Low-CP	High-CP	
		High-RUP	High-RDP
Ingredient composition, % of DM			
Constant components [b]	45.4	45.4	45.4
Cassava chips	40.7	13.9	13.9
Soybean meal	13.9	-	40.7
Formaldehyde treated soybean meal	-	40.7	-
Analysed chemical composition, % of DM			
Ash	9.5	11.0	11.3
Crude protein	11.2	25.9	26.6
RDP, as formulated [c]	7.8	9.6	16.5
RUP, as formulated [c]	3.0	15.0	9.0
NDF	47.1	37.7	37.8
ADF	24.3	27.2	27.3
Ether extract	1.6	1.8	1.8

[a] Low-CP = low crude protein (control); High-CP = high crude protein; High-RUP = high rumen un-degradable protein; High-RDP = high rumen degradable protein.

[b] The constant components consisted of (in % of ration DM) 26.1 rice straw (consisted of % DM; 13.4 Ash, 3.8 CP, 81.6 NDF, 71.4 ADF and 3.0 EE), 13.3 rice bran, 3.2 molasses, 0.8 salt, 0.8 calcium phosphate and 1.2 premix (consisted per kg DM; 6 g Zn, 8 g Mn, 10 g Fe, 1.6 g Cu, 0.10 g I, 0.02 g Co, 0.06 g Se, 10 g Mg, 2,000,000 IU vitamin A, 400,000 IU vitamin D and 3,000 IU vitamin E (TS Dairy mix®, Thailand)).

[c] Ruminally degradable protein (RDP) estimates base on NRC (2001) values for constant compositions and mean RDP values in sacco of 36.4 and 66 % of DM for formaldehyde treated soybean meal and soybean meal, respectively. Ruminally un-degradable protein (RUP) calculated as 100 minus RDP.

The H_2SO_4 preserved rumen samples were thawed and centrifuged at 10 000 *g* for 10 min and the supernatant was collected for VFA analysis using gas chromatograph (Varian 3600 Star; Varian Specialties Ltd., Ontario, Canada) fitted with a nitroterephthalic acid modified polyethylene glycol megabore column (25 m × 0.25 mm i.d. with 0.2 µm Film; CP-WAX 58 (FFAP) CB; J&W Scientific, Folsom, CA USA). Initial oven temperature was 130 ºC for 7 min and temperature was increased at the rate of 2.9 ºC/min over 7 min to a final temperature of 150 ºC. Helium, air and hydrogen flows were set to 30, 300, and 30 ml/min, respectively. Ammonia concentrations of rumen fluid were determined using the method of Bremner and Keeney (1965).

After thawing, plasma urea nitrogen (PUN) was measured according to enzyme metric method (Cobas Integra 400 plus S/N 39-6352; Roche Ltd., Switzerland).

Histamine in rumen fluid was extracted with the use of dansyl chloride (Sigma-Aldrich, Netherland Ltd.) and after derivatization procedures, 10 µl of the extract, with an internal standard of diaminohexane was injected onto a HPLC (model: RF-10AXmugiL; Shimadzu, Japan; Phenomenex Luna column, 5 µm RP-18 250 mm × 4.6 mm i.d.) according to the method of Eerola et al. (1993).

2.6. Calculations and statistical analysis

All data for each dietary treatment were checked for normal distribution using Kolmogorov-Smirnov test. Five postprandial data of rumen pH, VFA and ammonia were subjected to analysis of variance (ANOVA) with a repeated measurement model. Whereas, the mean of two postprandial values of rumen histamine (2 and 4 hours post feeding) and PUN were subjected to analysis of variance with cow, experimental period and dietary treatments as factors (SAS, 1996). When the influence of dietary treatment reached statistical significance, Fisher's *t* test was used to separate treatment means. In addition, for the data from each cow (n = 6) and for each experimental diet (n = 3), linear correlations were calculated between rumen histamine concentrations and rumen pH. The calculations were done under the assumption that the 18 sets of data could be considered independently. Significant differences between treatments were considered when $P<0.05$.

3. Results

3.1. Feed intake and body weight

Experimental rations were completely consumed throughout the experiment and no residuals were collected. Mean BW (n = 6) at the end of the experiment was significantly higher (Students paired *t* test; *P*=0.002) than pre-experimental values; i.e. 501 (SEM = 29.4) and 465 (SEM = 27.7) kg, respectively.

3.2. Rumen fermentation, histamine and plasma urea nitrogen

In contrast to the intake of supplemental rumen undegradable protein, the intake of supplemental rumen degradable protein increased the concentrations of ammonia (*P*<0.001; Fig. 2) and total VFA *P*<0.001; Fig. 3) in rumen fluid. The increase in total VFA was mainly caused by the increase in acetate concentration (Table 2). Supplemental CP either degradable or undegradable, did not significantly alter (*P*=0.141) the postprandial pH of rumen fluid (Fig. 1) and no interaction of time and experimental rations was observed.

Table 2. Effects of experimental rations on ruminal VFA in dairy cows[a].

Measure	Ration[b]	Point of time post-feeding (h)					Pooled SEM	*P* value for diet effect
		1	3	5	7	9		
Volatile fatty acids, m*M*								
Total	Low-CP	107	113	113	110	107		
	High-RUP	118	108	116	113	111	2.3	<0.001
	High-RDP	129	127	134	127	122		
Acetate	Low-CP	67	70	69	68	67		
	High-RUP	75	66	72	70	70	2.1	0.019
	High-RDP	79	78	80	78	77		
Propionate	Low-CP	20	22	21	22	20		
	High-RUP	22	20	22	22	20	1.3	0.080
	High-RDP	27	24	27	25	24		
Butyrate	Low-CP	17	17	18	16	17		
	High-RUP	17	18	18	18	17	0.3	0.002
	High-RDP	19	20	20	20	19		

[a] Values are means in 2 nonconsecutive days in each period and pooled SEM for 6 animals.

[b] Low-CP = low crude protein; High-CP = high crude protein; High-RUP = high rumen un-Degradable protein; High-RDP = high rumen degradable protein.

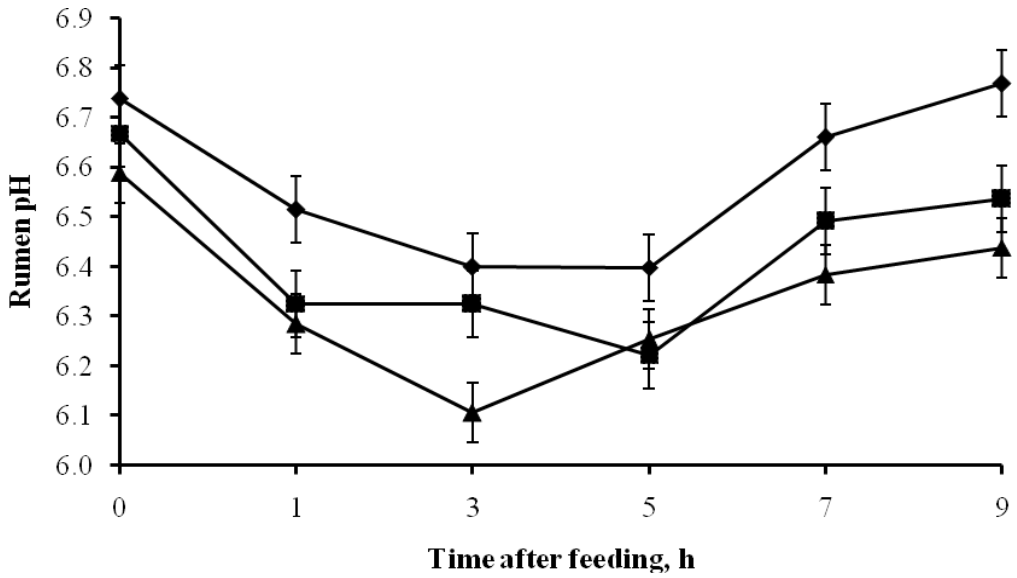

Fig. 1. Rumen pH based on point of time during a day in dairy cows fed rations with a low crude protein (Low-CP = ♦), high un-degradable protein (High-RUP = ■) and high rumen degradable protein (High-RDP = ▲). Error bars = SE.

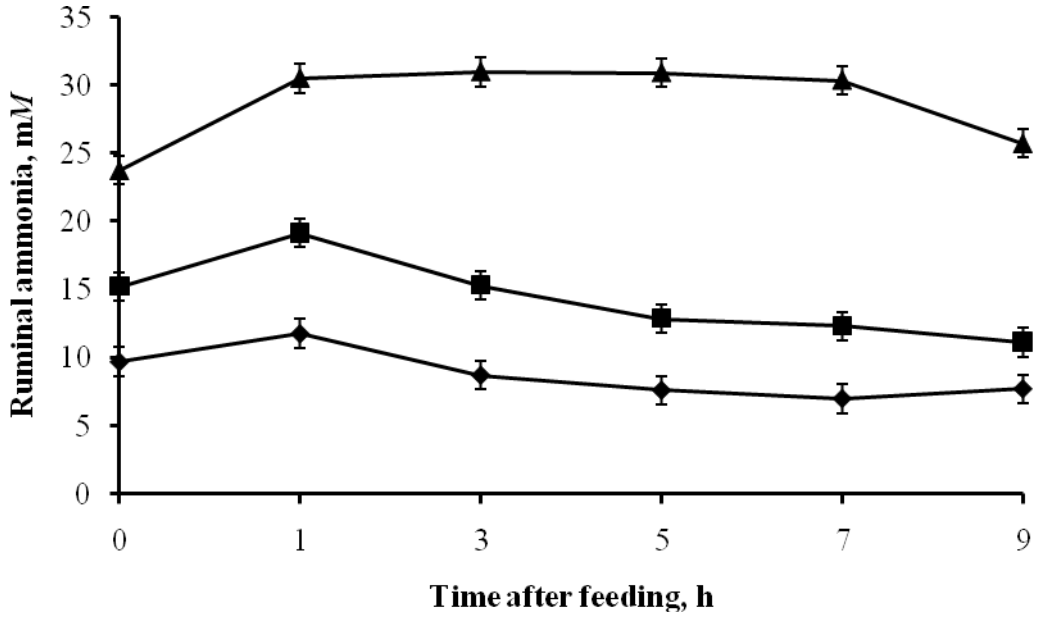

Fig. 2. Ruminal ammonia based on point of time during a day in dairy cows fed rations with a low crude protein (Low-CP = ♦), high un-degradable protein (High-RUP = ■) and high rumen degradable protein (High-RDP = ▲). Error bars = SE.

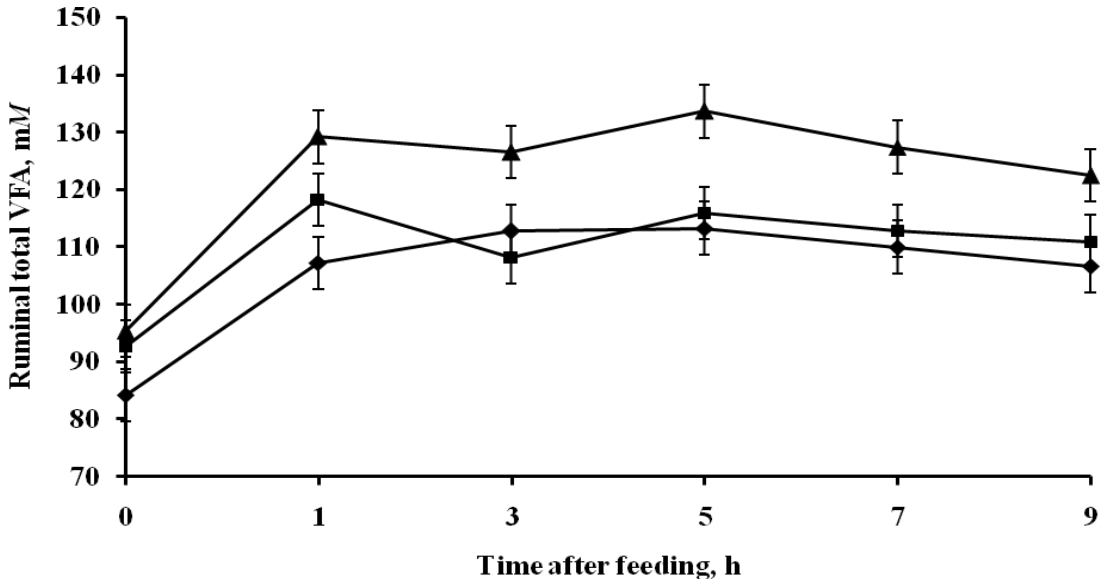

Fig. 3. Ruminal total VFA based on point of time during a day in dairy cows fed rations with a low crude protein (Low-CP = ♦), high un-degradable protein (High-RUP = ■) and high rumen degradable protein (High-RDP = ▲). Error bars indicate SEM for n = 6.

The concentrations of histamine in the ruminal fluid (Fig. 4) were significantly higher (P<0.05) after the feeding of high-RDP both in 2 and 4 hours after the morning feeding. In contrast, supplemental rumen undegradable protein did not significantly affect ruminal histamine concentrations. Furthermore, mean postprandial histamine concentrations in rumen fluid, as measured in individual cows for each experimental ration, were not significantly correlated with rumen pH (r = -0.285, P=0.251).

Plasma urea nitrogen concentrations increased significantly (P<0.001) from 1.47 mM to 4.38 mM when the high protein rations were fed. The source of protein did not significantly influence PUN values (data not shown).

3.3. Clinical sign of laminitis

During the last experimental period, there were 2 out of 6 cows displaying marked reaction of weight-shifting (score 1) and swelling over the coronary band (score 1). One cow was fed the high-RDP ration; the other cow was fed the high-RUP ration.

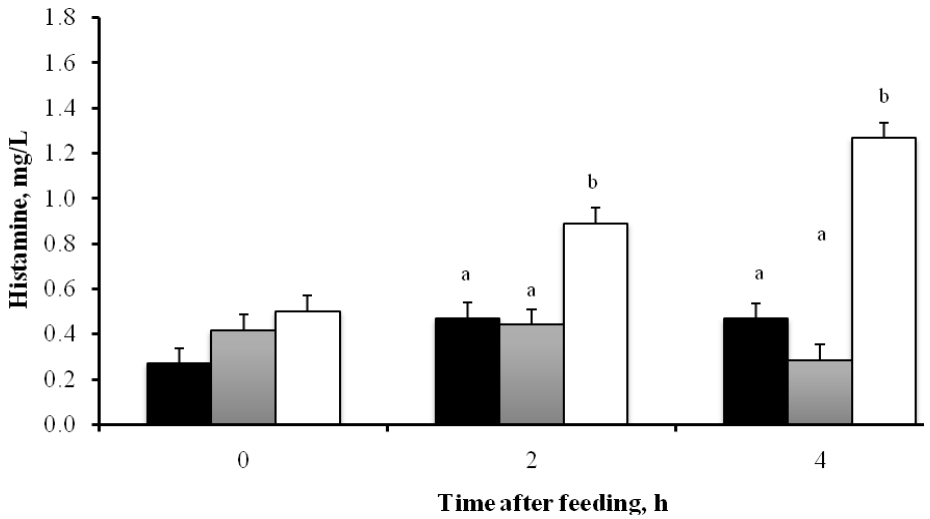

Fig. 4. Rumen histamine concentrations in dairy cows fed with control diet (black bar), high-RUP (gray bar) and high-RDP (white bar). The cross on the x-axis indicates feeding time. Error bars = SE.

[a, b] Treatments with different letters within time period differ significantly ($P < 0.05$).

4. Discussion

This study showed that supplementation of high rumen degradable CP in the form of untreated soybean meal increased the concentrations of total VFA in the rumen fluid, which is in line with the results of Arroquy et al. (2004) and Köster et al. (1996). Thus, it can be concluded that the supplementation of degradable CP had a stimulatory effect on rumen fermentation. Indeed, it has been shown by Satter and Slyter (1974), that a ration CP content lower than 15% inhibits the production of VFA. Despite the fact that rumen fermentation was enhanced after the feeding of the ration rich in degradable CP, rumen pH was not significantly affected in the current study. In general, rumen pH was significantly decreased after the ingestion of the diets in response to the increment of rumen fermentation (Nocek, 1997). However, mean postprandial rumen pH was 6.4 units, which clearly indicates that the cows did not develop rumen acidosis. The lack of effect of supplemental rumen degradable CP on rumen pH may be explained by the low level of DM intake. This explanation is corroborated by the observation of Schonewille et al. (2000) who also could not demonstrate a clear effect of readily fermentable carbohydrates on postprandial rumen pH after the intake of a ration containing 18% CP at a level of 6.5 kg DM/day. It has been shown by Robinson et al. (1986) that the level of feed intake is negatively associated with the buffering capacity of rumen fluid. Thus, it may be that the level of DM intake in the present study was too low to

effectively reduce rumen pH. Alternatively, the proportion of rice straw in the experimental rations possibly promoted chewing activity and its associated saliva secretion (Krause et al., 2002; Yang and Beauchemin, 2006) to maintain rumen pH within the physiological range. Finally, the iso-energetic replacement of cassava chips by soybean meal was associated with an increase in the fiber content of the ration. Therefore, it cannot be excluded this increase in fiber is at least partly, responsible for the lack of effect on rumen pH. On the other hand, it is well known that the digestibility of fiber from soybean meal is relatively high (CVB, 2007). Nevertheless, it is clear that supplemental CP did not result in rumen acidosis under the current feeding conditions.

Ruminal histamine concentrations were significantly higher after the feeding of the ration rich in rumen degradable CP, and somewhat higher as compared to previous reports with the values range from 0.009 to 0.3 mg/L (Ahrens, 1967; Sjaasted, 1967). However, the ruminal histamine concentrations observed in the current study are much lower than values reported by Sanford (1963) and Suber et al. (1979) who observed histamine levels ranging from 3 to 70 mg/L in rumen fluid during an acidic condition at a pH level of 4.5. It has been demonstrated that rumen pH and histamine are inversely correlated (Irwin et al. 1979; Sanford, 1963; Suber et al., 1979). In the current study, mean postprandial histamine concentrations in the rumen were not correlated with mean postprandial rumen pH values. Therefore, it might be suggested that the relatively low levels of histamine could be explained by the high level of the postprandial rumen pH, which was maintained at a level of 6.4. The formation of histamine in rumen fluid depends upon both histidine decarboxylation and recurrent low rumen pH values (Garner et al., 2004; Irwin et al., 1979; Rodwell, 1953; Schelp et al., 2001). Thus, in case that a dietary degradable CP content of 16.5% was sufficiently high to support the production of high levels of histidine, the high rumen pH prevented the actual decarboxylation of histidine, which resulted in low rumen histamine levels.

In the present study, PUN concentrations were significantly higher after the feeding of the high CP rations, irrespective of the CP degradability of the supplement. This result was relevant to previous studies (Harmeyer and Martens, 1980; Olmos Colmenero and Broderick, 2006; Reynal and Broderick, 2003). It was likely that the observed lower ruminal ammonia concentrations after the feeding of the formaldehyde-treated soybean meal was counteracted by increased deammination of absorbed protein leading to a similar N flux to the urea cycle and therefore, similar PUN values (Olmos Colmenero and Broderick, 2006).

In the current study, two cows which were fed high CP rations, developed clinical signs of laminitis. However, it seems unlikely that the occurrence of laminitis was related to

the histamine concentrations in their rumen; i.e. 0.44 and 1.27 mg/L. Indeed, MacLean (1970) suggested that the occurrence of laminitis is associated with ruminal histamine concentrations in the order of 60 mg/L or higher. Generally, the occurrence of laminitis is associated with many causes beside ruminal acidosis and already discussed in the literature such as sudden changes in the diet, enhanced endotoxin levels (due to a rise in the Gram negative bacterial in ruminal content) and pressure forces to the claw (due to enhanced weight, less time spent lying, hard concrete flooring).

Vermunt and Leach (1992) suggested that endotoxin released in the rumen fluid is also important in the etiology of laminitis. However, it has been shown by Khafipour et al. (2009) that high levels of lipopolysaccharides in rumen fluid only occur at a low rumen pH (pH < 5.6). Thus, the occurrence of laminitis in the current cannot be easily explained. Because the two cows were showing the clinical signs of laminitis at the end of our experiment, it can be speculated that there is a cumulative effect of high CP feeding on the development of laminitis. However, this notion is not corroborated by the fact that only one of the affected cows was fed the high CP ration for six weeks in a row. Clearly, this issue has not settled yet. Alternatively, it may be suggested that the clinical signs of laminitis are related to increased pressure forces to the claw because of the observed increased in BW in the course of the experiment. As far as we know, there is no indication that an increase in BW in dairy cattle is associated with the incidence of laminitis. However, Carter et al. (2009) reported that obese ponies were more sensitive to pasture-associated laminitis. Therefore, it cannot be excluded the observed cases of laminitis were at least partly, related to the increase in BW. Clearly, this issue has not been settled yet.

5. Conclusion

This study shows that the supplementation of high rumen degradable soybean meal increased rumen fermentation (more VFA) and histamine concentration, but had no effect on the pH of rumen fluid. The development of laminitis symptoms cannot be explained by the increased rumen histamine concentration. Further research is warranted to understand the relationship between high CP in the diet, rumen pH and histamine concentrations and the etiology of laminitis in dairy cows.

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Chapter 4

Starch source in high concentrate rations does not affect rumen pH, histamine and lipopolysaccharide concentrations in dairy cows

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Abstract

The replacement of ground corn by cassava meal on rumen pH, lipopolysaccharide (LPS) and histamine concentrations under typical Thai feeding conditions (high concentrate diets and rice straw as the sole source of roughage) was investigated. Four rumen-fistulated crossbred Holstein, non-pregnant, dry cows were randomly assigned to the four experimental rations in a 4 × 4 Latin square design with 21-d periods. Each period consisted of a 14-d run-in/wash out period, followed by a 7-d experimental period. During the run-in/wash out period, the cows were offered 5.4 kg DM of concentrate containing 4.6% cassava meal and rice straw was provided *ad libitum*. During the 7 days of each experimental period, cows were offered 10.5 kg DM of concentrate containing either 4.6% or 18.3% or 32.4% or 46.2% cassava meal and 1.5 kg of rice straw. Irrespective of dietary treatments, rumen total VFA and lactate concentrations were significantly affected by sampling day, which were significantly higher on day 1 versus days 3 and 7 of the experimental periods. Mean postprandial rumen pH was reduced to values <5.6 for at least 6 h on days 3 and 7 of each experimental period. However, the relationship between rumen pH, total VFA and lactate concentrations was not straight forward. Rumen LPS and histamine concentrations were not affected by either ground corn or cassava mean, but values significantly increased from day 1 to 7 of each experimental period with the values ranging from 10.3×10^3 to 40.3×10^3 EU/ml and 11.5 to 33.2 μM, respectively. Both plasma LPS and histamine concentrations were below the detection limit. It can be concluded that the amount of concentrate but not the replacement of ground corn by cassava meal, affect rumen pH, rumen fluid concentrations of total volatile fatty acids, lactate, histamine and LPS. Irrespective of the dietary treatments, the results suggested that a low rumen pH is associated with an increase in rumen histamine and LPS concentration, which is not accompanied by an increase in plasma histamine or LPS concentration.

Keywords: cassava meal, rumen pH, histamine, lipopolysaccharide, dairy cow

1. Introduction

Currently, laminitis (*Pododermatitis aseptica diffusa*) is considered as an important health problem in the dairy industry worldwide, including Thailand (Pilachai et al., 2009; Seesupa, 2010). Generally, nutrition is believed to play an important role in the occurrence of laminitis, but the underlying mechanism by which the ration or feeding management of dairy cows contributes to the etiology of laminitis remains obscure. It has been suggested that the condition of rumen acidosis is an important factor that contributes to the development of laminitis (Nocek, 1997; Cook et al., 2004; Nordlund et al., 2004; Greenough et al., 2007). The acidic conditions in the rumen are associated with release of lipopolysaccharides (LPS) (Gozho et al., 2007) likely due to the lysis of ruminal bacteria which after absorption may trigger inflammatory responses, thereby, causing vasoconstriction and subsequent development of laminitis (Nocek, 1997; Vermunt and Leach, 1992). Moreover, recurrent low rumen pH values may also result in increased ruminal histamine concentrations (Irwin et al, 1979), a condition also associated with laminitis in cattle (Nocek, 1997).

In Thailand, dairy cows are typically fed rations containing high levels of concentrate with rice straw as the sole source of roughage. The non-pelleted concentrates typically consist of a high proportion (50-70%) of cassava chips (Sommart et al., 2000; Wanapat, 2003), which contain relatively high concentrations of rapidly degradable starch (Richard et al., 1991; Chanjula et al., 2003). Consequently, such rations may predispose cows to rumen acidosis and a subsequent release of LPS (Emmanuel et al., 2008; Zebeli and Ametaj, 2009) and histamine (Irwin et al., 1979) in the rumen.

The aim of the current experiment was to gain insight into the relationships, if any, between the source of starch, rumen pH, and rumen concentration of LPS and histamine under Thai feeding conditions; i.e. high concentrate diets and rice straw as the sole source of roughage. In order to enhance proper interpretation of the data, two sources of starch were used; i.e. corn and cassava. These sources of starch were selected because it has been shown by Cone et al. (1989), that the rumen degradability of cornstarch is much lower compared to cassava starch. Furthermore, proportions of dietary fiber (acid detergent fiber, ADF) in the experimental concentrates were kept constant by replacing corn with cassava meal. It was anticipated that the replacement of corn meal by cassava meal induces rumen acidosis in dairy cows. In other words, it was anticipated that corn meal could serve as a control.

2. Materials and methods

2.1. Ethic considerations

The current experiment was approved by the Animal Ethics Committee of Khon Kaen University, Khon Kaen and conformed to the Ethics of Animal Experimentation of the National Research Council of Thailand. Prior to the execution of the experiment, it was decided to immediately terminate the experiment if any cow refused to eat and concurrently presented itself with severe diarrhea and clinical signs of metabolic acidosis. At the end of each experimental period, all cows received an intra-ruminal dose of sodium bicarbonate (50 g/cow) to facilitate recovery of rumen pH.

2.2. Animals and experimental design

Four, rumen fistulated, Holstein-Friesian-Brahman crossbred, non-pregnant, dry cows with an average age of 4.5 years (SE 0.5) and mean BW of 408 kg (SE 43) were used. Cows were housed on a concrete floor in individual pens (3×3 m^2) with natural ventilation at the Ruminant Research Unit of Udon Thani Rajabhat University, Udon Thani, Thailand. The trial design was a 4×4 Latin square with 21-d periods (Cochran and Cox, 1957). Each period consisted of a 14-d run-in/wash out period, followed by a 7-d experimental period. The animals were randomly assigned to each sequence of feeding on the four experimental rations and had free access to water. All cows were examined at the beginning of each period of the study, and they appeared healthy, without clinical signs of foot pain and no swelling over the coronary band.

2.3. Experimental rations

During the run-in/wash out period, the cows were offered 5.4 kg DM concentrate containing 4.6 % cassava meal (Table 1) and rice straw was provided *ad libitum*. During the 7 days of each experimental period, each cow was offered 10.5 kg DM of experimental concentrate and 1.5 kg of rice straw. Four experimental concentrates were formulated containing either 4.6% or 18.3% or 32.4% or 46.2% cassava meal (dry matter basis). The control concentrate contained 4.6 % cassava meal and 41.7% finely ground corn which was replaced by increasing amounts of cassava meal (Table 1). The analyzed composition of the rice straw (930 g DM/kg) was as follows (g/kg DM): crude ash, 123; crude protein, 21; neutral detergent fiber (NDF), 705; ADF, 496; ether extract, 15. The rations were offered daily in two equal portions at 08:00 and 17:00 h, and feed refusals, if present were collected.

Table 1. Ingredient and analyzed composition of the experimental concentrates[a].

	Experimental ration, % cassava meal inclusion			
	4	18	32	46
Ingredient composition, % of DM				
Soybean meal	35.2	35.2	35.2	35.2
Rice bran	17.1	17.1	17.1	17.1
Ground corn	41.7	28.0	13.9	0.0
Ground cassava chips	4.6	18.3	32.4	46.3
Premix[b]	1.4	1.4	1.4	1.4
Analyzed composition				
Dry matter, g/kg	900	890	890	900
Crude ash, g/kg DM	64	67	71	74
Crude protein, g/kg DM	211	202	195	199
NDF, g/kg DM	460	447	413	381
ADF, g/kg DM	147	145	145	147
Ether extract, g/kg DM	29	26	22	21
NSC[c], g/kg DM	236	258	299	325

[a] Concentrates were offered in a non-pelleted form.

[b] Premix, 1.4. The premix consisted of (g per kg): 10 Mg; 6 Zn; 8 Mn; 10 Fe; 1.6 Cu; (mg per kg) 100 I; 20 Co; 60 Se; 2,000,000 IU vitamin A; 400,000 IU vitamin D; 3000 IU vitamin E (TS Dairy mix®, Thailand).

[c] NSC = Non structural carbohydrates, calculated as: 100 – NDF – crude protein – ether extract – crude ash.

2.4. Collection of samples

Throughout the experiment, the concentrates and rice straw were sampled daily and pooled, ground, and stored in sealed plastic bags at an ambient temperature (25 ºC) until analysis. On days 1, 3 and 7 of each experimental period, pH of rumen contents was recorded 15 min before feeding and 9 times hourly after feeding. The pH was measured by a pH electrode (Mettler-Toledo GmBh 8603, Schwerzenbach, Switzerland), which was inserted into the ventral sac of the rumen through the cannula. On the same days, rumen fluid samples (approximately 100 ml) were taken from the ventral sac of the rumen, 15 min before feeding and 1, 3, 5, 7 and 9 h after the morning feeding. Immediately after collection, the rumen fluid

was filtered through four layers of cheesecloth and a ~55-ml filtered sub-sample was preserved (-20 ºC) by adding 5 ml of 1 M H_2SO_4 until analysis of volatile fatty acids (VFA) and lactate. Lactate was only analyzed in the sub-samples prepared 15 min before feeding and 1, 3 and 5 h post feeding. A second filtered sub-sample (10 ml) taken 15 min before feeding and 3 and 5 h post feeding, was transferred directly into a pyrogen-free centrifuge tube and centrifuged at 5000g for 30 min. Then, the supernatant was filtered by means of a disposable 0.22-µm sterile filter and subsequently heated for 30 min at 100 ºC. Thereafter, the filtered supernatant was stored in a pyrogen-free tube at -20 °C until LPS analysis. On days 1 and 7, a third filtered sub-sample (20 ml) was taken 15 min before feeding and 5 h after feeding. This sub-sample was mixed with 5 ml of 30% trichloroacetic acid (w/v), immediately placed on ice and centrifuged at 4 ºC at 15000g for 30 min within 30 min after collection. The supernatant was stored in Eppendorf tubes at -80 °C until histamine analysis.

On days 1, 3 and 7 of each experimental period, blood (20 ml) was aseptically collected 15 min before feeding and 3 and 5 h after feeding from the jugular vein into a syringe. The blood was transferred into evacuated heparinized tubes (Zenimed Co., Ltd., Bangkok, Thailand), immediately placed on ice and centrifuged at 4 ºC at 1500*g* for 15 min within 30 min after collection. Directly after centrifugation, plasma was collected and stored in two pyrogen-free tubes at -80 °C until analysis of LPS. Plasma histamine concentrations were analyzed in the samples taken 15 min before feeding and 5 h after feeding on days 1 and 7 of each experimental period.

Throughout the experiment, all cows were clinically examined for signs related to sub-acute laminitis daily which involved observations for claw inflammation and foot pain according to the definition of subacute laminitis modified from Greenough et al. (2007). Briefly, the coronary band was observed and evaluated for swelling as 0=no swelling, 1=a swelling and pink in color. Weight shifting was defined by a shifting of weight laterally from one leg to another in a monotonous manner as 0=no, 1=slight and 2=marked weight shifting. Furthermore, at 15 min before the morning feeding and at 12:00 h on days 1, 3 and 7 of each experimental period, all cows were clinically monitored, including auscultation of heart rate and rumen contraction per 5 min, measurement of rectal temperature, and observation of respiration frequency. The consistency of feces were inspected visually and graded as 1=dry, firm; 2=normal; 3=pasty, soft; 4=diarrhea, thin; or 5=watery modified from Hughes (2001).

2.5. Chemical analysis

The DM content of the concentrates and rice straw was determined by drying at 135 ºC for 3 h (AOAC, 1990). The ash content was analyzed by combustion at 550 ºC for 16 h and nitrogen contents were determined by the macro Kjeldahl method (International Dairy Federation, 1986) with a factor of 6.25 used to convert to CP. Ether extract was determined according to the procedure of the AOAC (1990). The NDF and ADF content were analyzed according to the method of Van Soest et al. (1991).

The H_2SO_4 preserved rumen samples were thawed and centrifuged at 10000*g* for 10 min and the supernatant was collected for VFA and lactate analysis. Volatile fatty acids were analyzed using gas chromatograph (Varian 3600 Star; Varian Specialties, Midland, ON, Canada) according to the method described by Pilachai et al. (2012). Lactate concentration in the rumen fluid was determined by means of HPLC (Shimadzu Class-VP, version 5.03; Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) according to the method described by Samuel et al. (1997).

Both histamine in rumen fluid and plasma were extracted with dansyl chloride (Sigma-Aldrich, Chemie B.V., Zwijndrecht, The Netherlands) and after derivatization, 10 µl of the extract containing diaminohexane as an internal standard (Eerola et al., 1993) was injected onto an HPLC (Shimadzu Class-VP, version 5.03; Shimadzu Scientific Instruments, Inc., Columbia, MD, USA; Phenomenex Luna column, 5 µm RP-18 250 mm × 4.6 mm i.d.).

Both rumen and plasma LPS concentrations were determined by a chromogenic *Limulus* amebocyte lysate assay (LAL) (QCL-1000, Lonza Group Ltd., Breda, The Netherlands) as described by Gozho et al. (2005).

2.6. Statistical analysis

Before statistical evaluation of the effect of starch source on selected indices of rumen fermentation and LPS levels in rumen fluid, mean postprandial values on each sampling day (days 1, 3 and 7 of each experimental period) were calculated. In contrast to the indices on fermentation, mean rumen LPS was calculated including the preprandial value. Rumen concentrations of free LPS were normalized by a logarithmic transformation before statistical analysis. Except for rumen histamine concentrations, all data were subjected to repeated measures analysis of variance (ANOVA) with cow, experimental period and dietary treatments as factors (SPSS® version 17). Rumen histamine concentrations were subjected to ANOVA with cow, experimental period and dietary treatments as factors. Tukey's multiple range test was used to separate treatment means. Upon statistical evaluation, rumen pH, total

VFA and lactate concentrations were significantly affected by sampling day but not by experimental treatment. Therefore, rumen pH, total VFA and lactate concentrations were pooled across dietary treatments and subjected to repeated measures ANOVA with cow and experimental period as factors. Student's paired *t* test was used to compare the differences between sampling days and times. In addition, data from each cow (n=4) and for each experimental diet (n=4) were used to derive linear correlations between rumen histamine concentrations and rumen pH on days 1 and 7. The calculations were done under the assumption that the 32 sets of data could be considered independent. Throughout, the level of statistical significance was pre-set at $P<0.05$.

3. Results

3.1. Clinical observations

Throughout the experiment, cows remained healthy; heart rate, respiration rate, rectal temperature, and rumen contraction were found to be within the normal ranges (data not shown). During the run-in period and each wash out period, all cows had normal feces consistency (score 2). During the 7 days of each experimental period, 18.8% of cows excreted watery feces (score 5), 50% of the cows had a feces score of 4 and 31.3% of the cows scored a feces consistency of 3. Neither feces scores of 1 and 2 was observed. The effect on feces consistency did not differ between the four dietary treatments.

Only on day 3 of the first experimental period, weight shifting was observed in all animals; three out of the four cows showed a marked weight shifting (score 2) and swelling over the coronary band (score 1). The remaining cow displayed slight weight shifting (score 1), and inflammation of the coronary band was not observed.

3.2. Feed intake

During the run-in and each wash out period, cows consumed all concentrates offered at all times. The intake of rice straw ranged from 4 to 5 kg DM per day during the first 13 days of each run-in/wash out period. The mean intake of rice straw on day 14 of each run-in/wash out period for all treatments (n=4) was 4.4 kg DM (SE 0.03). During the 7 days of the experimental period, mean DM intake of rice straw was 1.45 kg DM (SE 0.02). Mean DM intake of the concentrate decreased from 10.0 kg/day (SE 0.43) on day 1 to 9.3 kg/day (SE 0.48) on day 7.

3.3. Rumen pH, volatile fatty acids and lactate

Rumen pH, concentration of total and individual VFAs and lactate in the ruminal fluid were not significantly different between dietary treatments (Table 2). In contrast, mean postprandial values for rumen pH, total and individual VFAs and lactate were significantly affected by sampling day (P=0.046). Rumen pH was lower (P=0.040) on days 3 and 7 compared to the first sampling day. Mean postprandial rumen pH (n=16 per time point) was <5.6 for at least 6 h on days 3 and 7 (Fig. 1). The observed rumen total VFA and lactate concentrations were significantly higher on day 3 versus days 1 (P=0.035) and 7 (P=0.027) (Fig. 1).

3.4. Rumen LPS and histamine

Rumen LPS concentrations were not affected (P=0.448) by dietary treatment. Mean rumen LPS concentrations (n=4) across dietary treatments were similar on days 1 and 3; the mean values being 10.3×10^3 EU/ml (CV=197.3 %) and 9.9×10^3 EU/ml (CV=108.4 %), respectively. However, average values were significantly increased (P=0.016) to 40.3×10^3 EU/ml (CV=93.2 %) on the last day of each experimental period. The latter value was highly influenced by the high mean value measured 15 min before the morning meal on day 7; i.e. 76.9×10^3 EU/ml. The histamine concentrations in the rumen fluid were not affected (P=0.268) by dietary treatment, but values were significantly (P<0.001) higher on day 7 compared to day 1. Furthermore, mean rumen histamine values tended to be higher at 5 h after feeding (P=0.051) compared to the preprandial value (Fig. 2).

Table 2. Effect of experimental rations on selected indices of rumen fermentation[a] in dairy cows.

	Experimental ration, % cassava meal inclusion				Pooled SEM	P-value[b]
	4	18	32	46		
Rumen pH						
day 1	5.92	5.87	5.75	5.72		
day 3	5.53	5.42	5.55	5.44	0.08	0.269
day 7	5.61	5.51	5.48	5.46		
Total VFA, mM						
day 1	94	98	100	106		
day 3	118	123	109	115	4.47	0.677
day 7	109	109	111	115		
Acetate, mM						
day 1	61	65	67	71		
day 3	72	75	69	71	2.98	0.452
day 7	65	67	68	66		
Propionate, mM						
day 1	23	20	23	22		
day 3	26	25	23	24	1.20	0.251
day 7	27	26	28	31		
Butyrate, mM						
day 1	9	10	11	12		
day 3	16	20	13	17	2.11	0.542
day 7	12	12	14	12		
Lactate, mM						
day 1	5.8	2.8	5.1	2.5		
day 3	5.2	8.1	9.0	6.8	1.67	0.077
day 7	2.7	5.4	5.7	1.2		

[a] Selected indices of rumen fermentation are means of 10 postprandial values for rumen pH, 6 postprandial values for VFA (total and individual VFA) and 4 postprandial values for lactate.

[b] Values generated from repeated measures ANOVA. Days 1, 3 and 7 are treated as repeated measures.

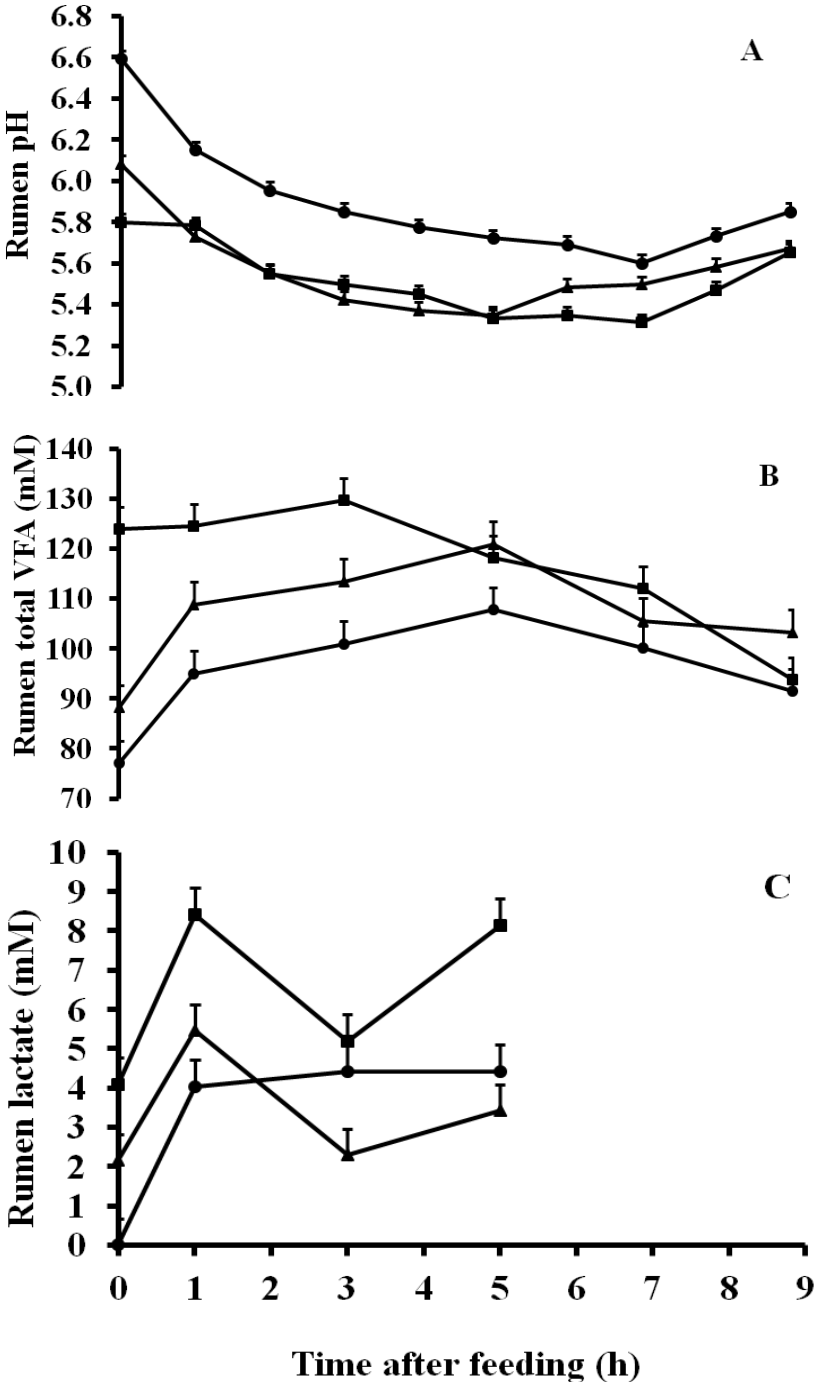

Fig. 1. Mean postprandial rumen pH values (A) and concentration of total volatile fatty acid (VFA; B) and lactate (C) in the rumen fluid of dairy cows fed the experimental rations. Data were pooled (*n*=16 per time point) across dietary treatments for day 1 (●), day 3 (■) and day 7 (▲) of each experimental period. Error bar indicate standard errors.

3.5. Plasma LPS and histamine

Both, plasma LPS and histamine concentrations were at all times below the detection limit of the assay of 0.1 EU/ml and 1 μM, respectively.

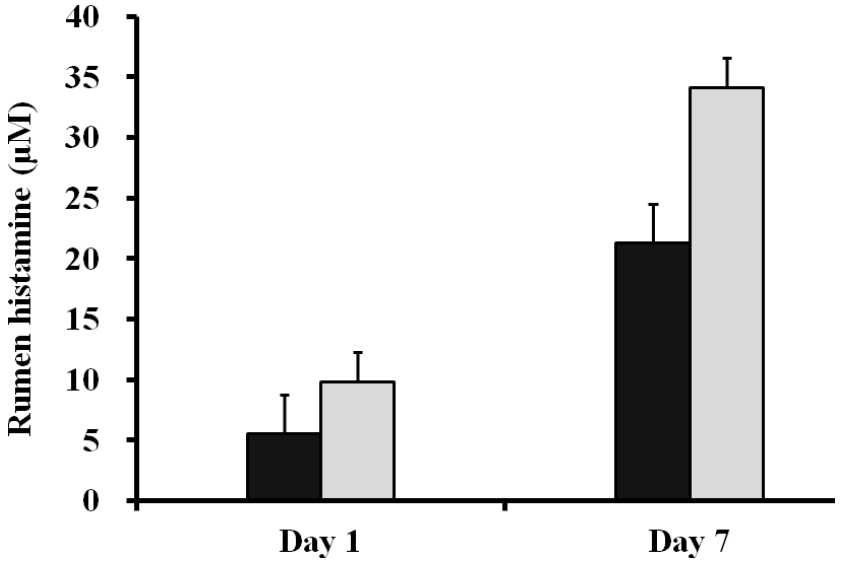

Fig. 2. Mean rumen histamine concentration before (black bar) and at 5 h after feeding (gray bar) in dairy cows fed the experimental ration on day 1 and 7. *P*-value $_{day}$<0.001; *P*-value $_{time}$=0.051. Error bar indicate standard errors (*n*=4).

4. Discussion

Interestingly, rumen pH was not affected by the source of starch in the current experiment. This outcome was somewhat unexpected because it is well known that the rumen degradability of cassava starch is much higher compared to that of corn starch (Cone et al., 1989; Richard et al., 1991; Chanjula et al., 2003). Furthermore, the degradation rate of dietary starch has a major impact on rumen pH (Owens et al., 1997; Krause et al., 2002; Gulmez and Turkmen, 2007). The lack of response of source of starch on the rumen pH may be explained by the fact that both the cassava chips and corn were the finely ground (Lykos and Varga, 1995). It has been demonstrated by Owens et al. (1997), Firkins et al. (2001) and Bengochea et al. (2005) that finely processed grain increase the ruminal degradability of starch, resulting in a decline in rumen pH, which enhances the risk of rumen acidosis.

Rumen pH was clearly depressed during each experimental period with a similar pattern for all experimental rations. It was already mentioned that postprandial rumen pH was

found to be below 5.6 for at least 6 h on days 3 and 7 (Fig. 1) indicating that the cows suffered from subacute rumen acidosis (SARA; Gozho et al., 2005). Rumen lactate concentrations remained relatively low (<9.0 mM) during the time that rumen pH <5.6. Lactic acidosis in the rumen is associated with lactate concentrations >50 mM (Dunlop, 1972; Nagaraja et al., 1985) and such high values were not observed in the current study. Rumen lactate concentrations were higher on day 3 compared to days 1 and 7 (Fig. 1). On the first day of the experimental period rumen lactate concentrations were relatively low which may be explained by the fact that the roughage to concentrate ratio was abruptly changed. It can be speculated that rumen microbial flora was not yet adapted, thereby, preventing the proliferation of lactate producing bacteria (Goad et al., 1998). Furthermore, it may be suggested that in the course of the experimental period, the growth of lactate utilizing bacteria was triggered, leading to the conversion of lactate into propionate (Nocek, 1997). This is in line with the observed propionate concentrations on days 3 and 7 which were significantly (P=0.016) higher compared to day 1 (Table 2). The observed low postprandial rumen pH values are difficult to explain by rumen lactate concentrations. The decrease in rumen pH is most likely explained by VFA concentrations in combination with the reduced forage to concentrate ratio of the ration. Consumption of the high, starch rich, concentrate diet may have reduced the chewing activity which was associated with a relative high rate of fermentation of organic matter in the rumen, thereby, reducing the buffer capacity of the rumen content leading to a decrease in rumen pH (Maekawa et al., 2002). Indeed, Allen (1997) showed that the relationship between rumen pH and the concentration of VFA is not highly significant due to a number of factors such as variation in buffering, absorption rate of VFA, water fluxes across the rumen wall, diet patterns, and fractional rates of OM degradation and passage.

During the 7 days of each experimental period, mean rumen LPS concentrations were highest on day 7 (40.3×10^3 EU/ml; CV = 93.2%; Table 3). This value is substantially lower than the mean rumen LPS values reported by Gozho et al. (2007), Emmanuel et al. (2008) and Khafipour et al. (2009b) of 128.8×10^3, 88.7×10^3 and 107.2×10^3 EU/ml, respectively. The discrepancy in rumen LPS values between the current study and those of Gozho et al. (2007), Emmanuel et al. (2008) and Khafipour et al. (2009b) are difficult to explain. It has been suggested by Andersen et al. (1994) that mean rumen pH values <5.6 are associated with lyses of gram-negative bacteria resulting in a subsequent release of LPS in the rumen fluid. Clearly, other factors than rumen pH are also important to explain the release of LPS from gram negative bacteria in rumen fluid (Nagaraja et al., 1978). The data shown in Table

3 indicate that LPS levels > 88.0 × 10^3 EU/ml, are only observed at a dry matter intake (DMI) of >18 kg/day. The underlying mechanism for a relationship between DMI and LPS is not clear, but it can be suggested that the profile of the microbial flora may provide a key in relation to dietary responses on rumen LPS (Plaizier et al. 2008). Further studies are warranted to investigate the relationship between DMI, microbial flora and LPS concentrations in the rumen.

Table 3. Dry matter intake (DMI) and rumen lipopolysaccharide (LPS) concentrations in dairy cows[a].

Reference	DMI (kg/d)	Rumen LPS concentration (×10^3 EU/ml)
Khafipour et al. (2009a)	23.4	145.6
Khafipour et al. (2009b)	19.0	107.2
Emmanuel et al. (2008)	25.2	88.7
Gozho et al. (2007)	18.3	128.8
Gozho et al. (2006)	6.7	13.6
Current study	9.3	40.3

[a] All cows had subacute rumen acidosis (rumen pH < 5.6 at least 3 h a day).

Rumen histamine concentrations were significantly increased from day 1 to day 7 of each experimental period, irrespective of dietary treatment. In the current study, the increased rumen histamine concentrations observed at day 7 are consistent with values of 64.0, 47.4 and 50.1 µM reported by Ahrens (1967), Koers et al. (1976) and Irwin et al. (1979), respectively. In contrast, Pilachai et al. (2012) reported ruminal histamine concentrations lower than observed in the current study. However, in the latter study, the pH of rumen fluid was 6.4, which is probably too high to trigger the activity of histidine decarboxylase, thereby, preventing the formation of histamine (Schelp et al., 2001). This is in line with the observation that mean rumen histamine concentrations observed on days 1 and 7, were negatively correlated with mean postprandial rumen pH values (R^2=0.335, P<0.001; Fig. 3).

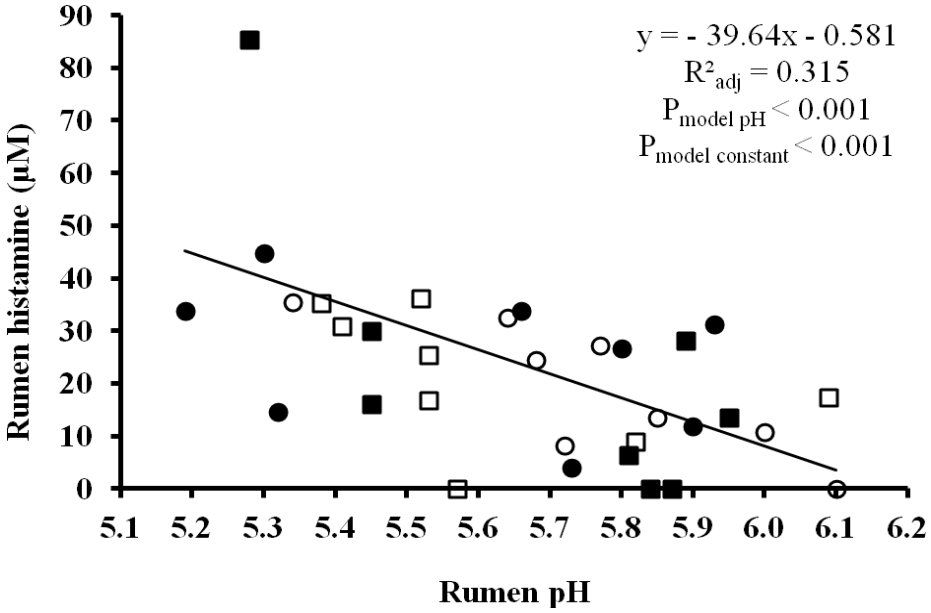

Fig. 3. Rumen histamine concentrations of individual cows fed the four experimental rations containing 4.6% (○), 18.3% (■), 32.4% (□) and 46.2% (●) of cassava meal in relation to rumen pH on days 1 and 7.

Lipopolysaccharide concentrations in plasma were below the detection limit of the assay used in the current study. It is likely that the free rumen LPS concentrations in the current study were too low to result in translocation of free LPS from the rumen content to the plasma pool. Alternatively, the translocation of free LPS from rumen to plasma not only depends on the concentration gradient of LPS but most likely also on the barrier function of epithelium (Li et al., 2012). Unfortunately, integrity of the rumen wall was not assessed in the current study. Khafipour et al. (2009b) showed that ruminal LPS concentrations of 107,152 EU/ml were associated with increases in plasma LPS concentrations (0.52 EU/ml). In contrast, rumen LPS concentrations of 128.8×10^3 and 145.6×10^3 EU/ml reported by Gozho et al. (2007) and Khafipour et al. (2009a), respectively did not increase plasma LPS concentration above the detection limit of the assay used. The ingredient composition of the rations may have interfered with LPS metabolism as plasma LPS concentrations were increased when SARA was induced in cows which were fed a grain-based ration (Khafipour et al., 2009b) instead of an alfalfa based ration (Khafipour et al, 2009a). Alternatively, it cannot be excluded that LPS was cleared by the liver, thereby, preventing the appearance of LPS in peripheral blood (Andersen, 2003). Clearly, the relationship between the rumen LPS and plasma LPS remains to be elucidated.

Histamine concentrations in plasma were <1 μM despite the elevated rumen histamine concentrations during the 7 days of each experimental period. The low plasma histamine concentration may have been the result of histamine catabolism in the rumen epithelial cells (Aschenbach et al., 2000), thereby, preventing appearance of histamine in plasma. Several studies (Ahrens, 1967; Irwin et al., 1979; Suber et al., 1979) have demonstrated that histamine disappeared rapidly from the rumen contents, and only ruminal histamine concentrations >64 μM were associated with increased plasma histamine concentrations (Ahrens, 1967). Therefore, it seems that the increased histamine concentrations in the rumen fluid in the current study where too low to affect plasma histamine concentrations.

Clinical signs of laminitis where only observed on day 3 during the first experimental period although plasma concentrations of LPS and histamine were below the detection limit. Thus, it seems unlikely that the occurrence of laminitis was related to either LPS or histamine. However, it has been suggested by Greenough et al. (2007), Danscher et al. (2011) and Penner et al. (2011) that increased inflammatory responses triggered by absorbed LPS from the rumen may play a role in the etiology of laminitis. Nocek (1997) and Vermunt and Leach (1992) speculated that mediators of inflammatory responses such as serum amyloid-A, haptoglobin and LPS-binding protein lead to the disruption of peripheral microvasculature in the corium, which contribute to the development of laminitis. It can be speculated that in the present study, absorption of LPS occurred, thereby, inducing an increased inflammatory response followed by rapid clearance of LPS by the liver (Andersen, 2003). Furthermore, sudden changes in the diet and inflammation of the rumen epithelium might also play a role in the development of laminitis (Plaizier et al., 2012). Alternatively, it may be suggested that the clinical signs of laminitis are related to an increased pressure force to the claw due to body weight, less time spent lying and hard concrete floors (Bergsten, 2003; Cook et al., 2004). The fact that clinical signs of laminitis were only observed on day 3 of the first experimental period may indicate that cows adapted to the LPS induced inflammatory response. To the authors' knowledge there are no reported studies that can support this observation and as such the cause of the clinical signs of laminitis in the current study remains unknown.

5. Conclusion

This study showed that the source of dietary starch did not affect rumen pH, concentrations of total VFA, histamine and LPS. Both ground corn and cassava meal can

induce rumen acidosis which is associated with an increased rumen LPS and histamine concentration. The relative high rumen LPS and histamine concentrations did not affect plasma LPS and histamine concentrations, probably cause by the relative low DMI in the current study. The observed cases of laminitis are difficult to explain from the measured plasma and rumen LPS and histamine concentrations. The results of the current study do not support the idea that typical Thai rations (i.e. high starch, low roughage) result in a high incidence of laminitis.

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Chapter 5

Hydrate sodium calcium aluminosilicate does not reduce rumen lipopolysaccharide concentrations in dairy cows

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Submitted

Abstract

The efficacy of hydrate sodium calcium aluminosilicate (HSCAS) to reduce the concentrations of free lipopolysaccharide (LPS) in rumen fluid of dairy cows was investigated. Six, rumen-fistulated crossbred Holstein, non-pregnant, dry cows were randomly assigned to three experimental rations in a study with a replicated 3 × 3 Latin square design with 28-d periods. During the first 20 days of each experimental period, cows were offered concentrate (5.4 kg dry matter; DM) without HSCAS and rice straw *ad libitum*. On day 21, cows were fasted for 12 h prior to commencement of a 7-d measurement period during which time cows were offered concentrate (10.5 kg DM) containing either 0, 0.5 or 1.0% HSCAS and 1.5 kg DM rice straw. Rumen fluid was collected on days 1, 3 and 7 during the measurement period for analysis of pH, LPS and volatile fatty acids (VFA). Mean postprandial rumen pH was reduced to values below 5.6 at all time. Rumen LPS concentrations were not significantly affected by the supplemental HSCAS, but significantly increased from day 1 to 7 during each measurement period with values ranging from 4,489 to 104,000 endotoxin units (EU)/mL. The increase in free LPS in the rumen fluid was related to the developed early signs of laminitis. Hydrate sodium calcium aluminosilicate supplementation did not affect (P>0.05) the pH or the concentrations of total and individual VFA in the ruminal fluid. The results of the present study show that HSCAS supplementation does not affect the concentration of free LPS in the rumen fluid and does not prevent the occurrence of early signs laminitis in dairy cows.

Keywords: hydrate sodium calcium aluminosilicate; supplementation; lipopolysaccharide; rumen; dairy cow

1. Introduction

Laminitis in dairy cows is considered to be an important health problem worldwide. A recurrent low rumen pH appears to be an important factor contributing to the development of laminitis (Nocek, 1997; Cook et al., 2004; Nordlund et al., 2004; Greenough et al., 2007). The acidic conditions in the rumen are associated with the formation of lipopolysaccharides (LPS) in rumen fluid (Andersen et al., 1994; Gozho et al., 2005, 2006, 2007; Khafipour et al., 2009a, 2009b; Pilachai et al., 2012). Transfer of LPS into the blood (Khafipour et al., 2009b) may trigger an inflammatory response (Emmanuel et al., 2008; Danscher et al., 2011), thereby, causing vasoconstriction and subsequent laminitis (Vermunt and Leach, 1992; Greenough et al., 2007). The use of agents that can irreversibly trap rumen LPS may as such be of interest to prevent laminitis in dairy cows.

Phyllosilicates are a group of minerals that are based on SiO_4 tetrahedra polymers linked to octahedral sheets. These sheets are arranged in such a way that they form parallel layers with interlayer spaces (Kloprogge et al., 1999). Due to their chemical and conformational properties, these phyllosilicates are of interest as sorbents for the use in animal production and veterinary practice (Slamova et al., 2011). Smectites are representatives of these phyllosilicates and are well known for their capacity to counteract aflatoxin toxicity (Dixon et al., 2008). Furthermore, Weese et al. (2003) and Lawler et al. (2008) demonstrated that di-tri-octahedral smectite (DTOS) effectively adsorbed *Clostridium perfringens* exotoxins. Weese et al. (2003) reported that DTOS also has the ability to bind LPS in aqueous solutions while Bysani et al. (1990) demonstrated that phyllosilicates such as kaopectate and bentonite effectively removed LPS from both water and human plasma. Interestingly, Wang et al. (2005) reported that calcium silicate hydrate with the crystalline structure of gyrolite (a phyllosilicate) has an endotoxin removal capacity of 6×10^6 EU/g. To the authors' knowledge, gyrolite is not available as a feed additive. In addition, there are no studies in the scientific literature reporting the efficacy of other phyllosilicates to remove free LPS from rumen fluid under practical feeding conditions. The aim of the current study was to test the efficacy of hydrate sodium calcium aluminosilicate (HSCAS) to reduce the concentrations of free LPS in rumen fluid of dairy cows.

2. Materials and methods

2.1. Ethical considerations

The committee of Ethics of Animal Experimentation of Khon Kean University, Khon Kean, Thailand, approved this experiment. As is generally accepted that acidic conditions in the rumen are a prerequisite to trigger the release of LPS (Gozho et al., 2007; Emmanuel et al., 2008; Khafipour et al., 2009b; Pilachai et al., 2012), cows were subjected to a feeding protocol to induce a low rumen pH (Pilachai et al., 2012). Prior to the start of the experiment, it was decided to immediately terminate the experiment if any cow refused to eat and concurrently presented itself with severe diarrhea and clinical signs of metabolic acidosis (e.g. tachycardia and tachypnea). At the end of each 28-d experimental period, all cows received an intra-ruminal dose of sodium bicarbonate (100 g/cow) to increase buffer capacity.

2.2. Animals and experimental design

Six, rumen fistulated, Holstein-Friesian × Brahman crossbred, non-pregnant, dry cows with an average age of 4.6 years (SE 0.13) and a mean body weight of 449 kg (SE 53.4) were used. Cows were housed on a concrete floor in individual pens (3 × 3 m^2) with natural ventilation at the Ruminant Research Unit of Udon Thani Rajabhat University, Udon Thani, Thailand. All cows were examined at the start of the study and found to be without clinical signs of foot pain and swelling over the coronary band.

The experimental design consisted of a replicated 3 × 3 Latin square with 28-d periods (Cochran and Cox, 1957). Each period consisted of a 21-d run-in/wash out period, followed by a 7-d measurement period. The animals were randomly assigned (within the restrictions of the Latin square design) to each sequence of feeding on three experimental rations and had free access to water.

2.3. Experimental rations

During the first 20 days of each run-in/wash out period, the cows were offered 5.4 kg DM of concentrate without HSCAS (Table 1) and rice straw was provided *ad libitum*. The analyzed composition of the rice straw (940 g DM/kg) was as follows (g/kg DM): crude ash, 123; crude protein (CP), 21; neutral detergent fiber (NDF), 705; acid detergent fiber (ADF), 496; ether extract, 15. On day 21 of each period, cows were fasted for 12 h prior to the 7-d measurement period, during which time cows were offered 10.5 kg DM concentrate (Table 1) containing 0, 0.5 or 1.0% NOVASIL plus® (purity 93% HSCAS, Trouw Nutrition Inc.,

Rotterdam, The Netherlands) and 1.5 kg DM rice straw. The levels of HSCAS in the current study are in accordance with the levels commonly applied to adsorb aflatoxin in dairy cows (Harvey et al., 1991; Kutz et al., 2009) and in line with the levels recommended for dairy cows by the producer. The rations were offered daily in two equal portions at 08:00 and 17:00 hours and feed refusals, if present, were collected.

Table 1. Ingredient and analyzed composition of the experimental concentrates[a].

	Control	Low HSCAS[b]	High HSCAS
Ingredient composition (g of DM)			
Rice bran	171	171	171
Soybean meal	349	349	349
Ground cassava chips	463	463	463
Mineral premix[c]	17	17	17
HSCAS	0	5	10
Analyzed composition (g/kg of DM)			
Dry matter (g/kg as is)	924	922	925
Crude ash	78	79	80
Crude protein	229	229	224
NDF	373	375	379
ADF	162	159	168
Ether extract	29	25	24
Non structural carbohydrates[d]	291	291	294

[a] Concentrates were offered in a non-pelleted form.

[b] Hydrate sodium calcium aluminosilicate.

[c] Premix consisted of (g per kg): 10 Mg; 6 Zn; 8 Mn; 10 Fe; 1.6 Cu; (mg per kg) 100 I; 20 Co; 60 Se; 2,000,000 IU vitamin A; 400,000 IU vitamin D; 3,000 IU vitamin E (TS Dairy mix®, Thailand).

[d] Calculated as: 100 – NDF – crude protein – ether extract – crude ash.

2.4. Collection of samples

Throughout the 7-d measurement periods, the concentrates and rice straw were sampled daily, pooled, ground, and stored in sealed plastic bags at an ambient temperature (25 ºC) until analysis. On the last day of each run-in/wash out period and on days 1, 3 and 7

of each measurement period, pH of rumen contents was recorded 15 min before feeding and 9 times hourly after feeding. The pH was measured using a pH electrode (Mettler-Toledo GmBh 8603, Schwerzenbach, Switzerland), which was inserted into the ventral sac of the rumen through the cannula. On days 1, 3 and 7 of each measurement period, rumen fluid samples (approximately 20 ml) were taken from the ventral sac of the rumen 15 min before feeding and 4, and 8 h after the morning feeding. Immediately after collection, the rumen fluid was filtered through four layers of sterile cheesecloth and a ~10-ml filtered sample was transferred directly into a pyrogen-free centrifuge tube and immediately placed on ice. Thereafter, the sample was centrifuged at 10 000 *g* for 15 min, the supernatant collected and filtered using a disposable 0.22-µm sterile filter. The filtered supernatant sample (3 ml) was stored in 2 pyrogen-free tubes at -20 °C for LPS and VFA analysis.

Throughout the experiment, 15 min before the morning feeding at 12:00 hours on the last day of each run-in/wash out period and days 1, 3 and 7 of each measurement period, all cows were clinically monitored, including auscultation of heart rate and rumen contraction per 5 min, measurement of rectal temperature, and observation of respiration frequency. The consistency of feces was inspected visually and graded as 1=dry, firm; 2=normal; 3=pasty, soft; 4=diarrhea, thinly spread; or 5 = watery modified from Hughes (2001). Furthermore, all cows were clinically examined daily for signs related to sub-acute laminitis which involved observations on claw inflammation and foot pain according to the definition of sub-acute laminitis as modified by Greenough et al. (2007). Briefly, the coronary band was observed and evaluated for swelling as 0=no swelling, 1=a swelling and pink in color. Weight shifting was defined by a shifting of weight laterally from one leg to another in a monotonous manner as 0=no, 1=slight and 2=marked weight shifting.

2.5. Chemical analysis

The DM content of the concentrates and rice straw was determined by drying at 105 ºC for 16 h (AOAC, 1990; ID 967.03). The ash content was analyzed by combustion at 550 ºC for 16 h and nitrogen content was determined by the macro Kjeldahl method (International Dairy Federation, 1986) with the factor 6.25 used to convert N into CP. Ether extract was determined according to the procedure of the AOAC (1990; ID 954.02). The NDF and ADF content were determined according to the method described by Van Soest et al. (1991).

After thawing, 0.6 ml of the filtered supernatant was acidified with 60 µl of formic acid containing 1% ethyl-butryic acid as an internal standard. The samples were stored at 4 °C until analysis. Rumen VFA concentrations were determined by gas chromatography

(7890A, Agilent Technologies SA/NV, Diegem, Belgium) equipped with a FID detector on a Nukol column (30 m × 0.25 mm × 0.25 µm, Supelco®) as described by Fievez et al. (2003). Briefly, 0.3 µl sample was injected with a split of 1:25, the injector temperature was 250 °C and column flow 0.8 ml/min (carrier gas H_2). The temperature program was 120 °C at the start of the injection, increasing 10 °C per minute until reaching 180 °C, which was kept for 3 minutes. The detection temperature was 255 °C. Free rumen LPS concentrations were determined by a chromogenic *Limulus amebocyte* lysate assay (LAL) (QCL-1000, Lonza Group Ltd., Breda, The Netherlands) as described by Gozho et al. (2005).

2.6. Statistic analysis

All statistical analyses were performed using SPSS version 17 for windows. Rumen concentrations of free LPS were normalized by a logarithmic transformation before statistical analysis. Except for fecal consistency scores and the score of weight-shifting and swelling over the coronary band, all data were subjected to repeated measures analysis of variance with cow, experimental period and dietary treatments as factors. Student's paired t-test was used to compare the mean differences of rumen LPS concentration between the sampling times. Effects of dietary treatments on consistency of feces, scores on weight-shifting and swelling over the coronary band were determined using a Chi-square test. Differences among treatments were considered significant when $P<0.05$.

3. Results

3.1. Clinical observations

Throughout the experiment, heart rate, respiration rate, rectal temperature, and rumen contractions were found to be within the normal ranges (data not shown). During the run-in/ wash out period, all cows had normal feces consistency (score 2). During the 7-d of each measurement period, 5.6% of the cows scored a feces consistency of 2, 72.2% of the cows had a feces score of 3 and 22.2% of cows had thinly spread feces (score 4). Neither feces scores of 1 and 5 were observed. The inclusion of HSCAS in the rations did not significantly affect ($P=0.112$) feces consistency.

No clinical signs in relation to sub-acute laminitis were observed during the run-in/ wash out period and on the first day of each measurement period. However, during days 3 and 7 of the measurement periods, four out of the six cows fed concentrate without HSCAS displayed marked reactions of weight shifting (score 2) and swelling over the coronary band

(score 1). Two out of the six cows fed the concentrate containing 0.5% HSCAS showed signs of laminitis. One cow showed slight reaction of weight-shifting and a swelling over the coronary band with a score of 1; the other cow scored 2 on weight-shifting and 1 on the swelling over the coronary band. Furthermore, three out of the six cows fed the concentrate containing 1.0% HSCAS observed signs of laminitis. Two cows presented scored 1 on both weight-shifting and swelling over the coronary band. The remaining cow scored 2 and 1 on weight-shifting and swelling over the coronary band, respectively. The inclusion of HSCAS in the rations did not affect (P=0.513) the clinical signs of laminitis in the current study.

3.2. Feed intake

During the first 20 days of each run-in/wash out period, the concentrate without HSCAS was completely consumed by all cows and the mean intake of rice straw was 4.7 (SE 0.05) kg DM/d. On day 21 of each run-in/wash out period, mean intakes of concentrate and rice straw were 2.8 and 2.4 kg DM/d, respectively. During the 7-d measurement period, cows consumed almost all rice straw supplied; i.e. mean intake of rice straw was 1.4 kg DM (SE 0.02) irrespective of dietary treatment. In contrast, cows only consumed 63.8 to 73.3% of the amount of concentrates supplied; overall mean intakes were 7.1 (SE 0.38), 6.7 (SE 0.43) and 7.7 (SE 0.37) kg DM/d for the control, low- and high HSCAS concentrates, respectively.

3.3. Rumen pH and volatile fatty acids

On day 21 of each run-in/wash out period, mean postprandial rumen pH was 5.9 (SE 0.03). During each measurement period, rumen pH significantly decreased (P<0.001) after the feeding of experimental rations, but differences between the dietary treatments were not observed (Table 2). During each sampling day of each measurement period, postprandial rumen pH was below 5.6 during all times, irrespective of the dietary treatment (data not shown). Supplemental HSCAS did not significantly affect (P=0.268) the concentrations of both total and individual VFA, in the ruminal fluid (Table 2). Total VFA concentrations were significantly greater (P=0.017) on days 3 and 7 *versus* day 1 (Table 2). Likewise, ruminal acetate and butyrate concentrations on days 3 and 7 were higher compared to day 1, but only the difference in ruminal butyrate concentration reached statistical significance (P=0.002). Furthermore, ruminal propionate concentrations were significantly increased at day 7 (P<0.001) on all treatments.

Table 2. Effect of varying inclusion rates of hydrate sodium calcium aluminosilicate (HSCAS) on selected indices of rumen fermentation[a] in dairy cows at day 1, 3 and 7 after inclusion.

	Experimental ration, % HSCAS inclusion			SEM	*P-value*	
	0	0.5	1.0		Day	Treatment
Rumen pH						
day 1	5.19	5.26	5.23			
day 3	4.99	5.03	4.89	0.153	0.122	0.878
day 7	5.00	5.08	5.22			
Total volatile fatty acids, mM						
day 1	88	86	84			
day 3	118	117	118	5.2	0.017	0.903
day 7	119	116	117			
Acetate, mM						
day 1	57	57	55			
day 3	63	67	63	4.1	0.069	0.908
day 7	60	58	59			
Propionate, mM						
day 1	18	17	18			
day 3	23	23	23	1.9	<0.001	0.510
day 7	35	31	35			
Butyrate, mM						
day 1	9	9	8			
day 3	24	20	24	2.2	0.002	0.935
day 7	16	18	15			

[a] Selected indices of rumen fermentation are means of 9 postprandial values for rumen pH, 2 postprandial values for volatile fatty acids (total and individual volatile fatty acids).

3.4. Rumen lipopolysaccharides

During each experimental period rumen LPS concentrations (Table 3) were not significantly affected (P=0.543) by supplemental HSCAS. However, sampling day and sampling time significantly affected rumen LPS values (P<0.05), rumen LPS values were significantly lower (P=0.003) on the first day of the measurement periods. Within sampling days, the highest rumen LPS concentrations were observed before the morning feed for all

treatments, the differences between pre- and post-feeding were significant on days 1 and 3 (P=0.026).

Table 3. Effect of varying inclusion rates of hydrate sodium calcium aluminosilicate (HSCAS) on rumen lipopolysaccharide (LPS) concentrations (EU/mL) in dairy cows at day 1, 3 and 7 after inclusion.

	Experimental ration, % HSCAS inclusion				*P-value*[a]	
	0	0.5	1.0	SEM	Hour	Treatment
Day 1						
07:45	6,755	4,774	7,133			
12:00	5,087	3,209	7,953	2,160.6	0.026	0.691
16:00	2,267	1,688	1,531			
Day 3						
07:45	94,828	15,903	97,541			
12:00	5,190	6,693	4,942	25,768.9	0.009	0.391
16:00	20,517	5,083	8,656			
Day 7						
07:45	116,621	236,199	235,168			
12:00	12,833	14,593	140,532	91,987.3	0.114	0.362
16:00	29,198	20,356	130,505			

[a] P-value of sampling day was 0.003 (SEM = 35,451.9).

4. Discussion

4.1. Rumen lipopolysaccharides

Generally, high LPS concentrations in the rumen can be found with a low rumen pH (<5.6) after the feeding of a diet high in concentrates (Gozho et al., 2007; Emmanuel et al., 2008; Khafipour et al., 2009b). In the present study, mean rumen LPS concentrations increased significantly (P<0.001) from day 1 to 7, i.e. from 4,489 (CV = 73.2%) to 104,001 EU/mL (CV = 130.0 %), respectively. The highest rumen LPS values are in line with those

reported by Gozho et al. (2007), Emmanuel et al. (2008) and Khafipour et al. (2009b), i.e. 128,825, 88,700 and 107,152 EU/mL, respectively. In contrast, the observed rumen LPS concentrations were approximately 2.5 times higher than values reported in our previous study (Pilachai et al. 2012). The difference in rumen LPS concentration between the previous and the current study may be related to the observed lower rumen pH in the current study (i.e. pH 5.6 *vs.* pH 5.1). It can be suggested that a lower rumen pH triggers a more pronounced lysis of gram-negative bacteria and its associated release of LPS in the rumen fluid. This reasoning is corroborated by Zebeli et al. (2012) who demonstrated a significant, negative relationship between mean rumen pH and the concentration of rumen LPS. In the current study, the highest rumen LPS levels were observed 15 min before the morning feeding. This is in line with the results of Khafipour et al. (2009a) and Pilachai et al. (2012) who also reported the highest rumen LPS values prior to the morning feeding. However, proper interpretation of the observed rumen LPS values is difficult because they are also affected by rumen volume and passage rate. Unfortunately, values on rumen volume and passage rate were not determined in the fore mentioned studies (Khafipour et al., 2009a; Pilachai et al., 2012).

4.2. Efficacy of HSCAS to reduce rumen lipopolysaccharides

In the present study, supplemental HSCAS did not reduce the concentration of free LPS in the rumen fluid. The lack of an effect of supplemental HSCAS on rumen LPS cannot be unequivocally explained but it may be related to the specificity of phyllosilicates to adsorb LPS or the applied dosage of HSCAS. It was suggested by Wang et al. (2005) that LPS may form cationic species due to the strong binding between the phosphate groups of LPS and Ca ions released from the interlayers of calcium silicate hydrate. The release of Ca ions from calcium silicate hydrate results in more negatively charged surface layers of the phyllosilicate which attracts the cationic LPS species. Because both calcium silicate hydrate and HSCAS are Ca-containing phyllosilicates, it was anticipated that HSCAS also has the potential to adsorb LPS. However, despite the overall resemblance between calcium silicate hydrate and HSCAS, there are also differences. For example, the compositions of the tetrahedral-octahedral sheet types are not the same (Kloprogge et al., 1999; Wang et al., 2005) which may affect the electrogenic properties of these phyllosilicates. Therefore, it may be suggested that only specific phyllosilicates have the potential to bind LPS and thus of potential veterinary relevance (Slamova et al., 2011).

The maximum intake of HSCAS in the present study was 82.4 g/d after feeding the high HSCAS concentrate. Unfortunately, reported *in vitro* values on the maximum LPS removal capacity of Ca containing phyllosilicates are not consistent and ranges from 400 EU LPS/g (Weese et al., 2003) to 6×10^6 EU LPS/g (Wang et al., 2005). Under the assumption that HSCAS can maximally adsorb 6×10^6 EU LPS/g, it can be calculated that maximally 4.9×10^8 EU of LPS could be adsorbed after the ingestion of the high HSCAS concentrate. Furthermore, assuming a rumen volume of 70 L (Schonewille et al., 2000), maximum rumen LPS concentrations that can be fully adsorbed under the current feeding conditions are estimated to be 7,000 EU LPS/mL. This value is higher than all the mean values observed at the first day of the measurement period when the concentrate without HSCAS was fed (Table 3). However, supplemental HSCAS did not exert any effect on rumen LPS and values were similar to the control (no HSCAS). Therefore, it can be concluded that HSCAS is unsuitable to adsorb LPS in rumen fluid.

4.3. Rumen pH and volatile fatty acids

This study shows that the dietary inclusion with HSCAS did not alter rumen pH and concentrations of total VFA in rumen fluid. Rumen pH was clearly depressed during the 7-d of each measurement period. Mean postprandial rumen pH was significantly decreased ($P<0.001$) from day 21 of each run-in/wash out period to days 1, 3 and 7 of each measurement period; i.e., 5.9 (SE 0.03) to 5.2 (SE 0.05), 5.0 (SE 0.06) and 5.1 (SE 0.05), respectively. The decrease in rumen pH is most likely explained by the increased VFA concentrations in the rumen fluid. Alternatively, it cannot be excluded that rumen lactate also was responsible for the low rumen pH (Goad et al., 1998). However, comparison with our previous study (Pilachai et al., 2012), rumen pH was somewhat higher but rumen lactate was probably not important in determining rumen pH. Consumption of the high, starch rich, concentrate diet may have reduced the chewing activity which was associated with a relative high rate of fermentation of organic matter in the rumen, thereby, compromising the buffer capacity of the rumen content leading to a decrease in rumen pH (Maekawa et al., 2002). Rumen propionate concentrations were higher ($P<0.001$) on day 3 and 7 compared to day 1 (Table 2). It is likely that the growth of lactate utilizing bacteria was triggered by the high grain diet, leading to the conversion of lactate into propionate (Nocek, 1997; Nagaraja and Titgemeger, 2007).

4.4. Signs of laminitis

Irrespective of the dietary treatments, 33.3% of cows displayed marked weight shifting (score 2) and swelling over the coronary band (score 1), while 11.1% of cows displayed slight weight shifting (score 1) and swelling over the coronary band (score 1). Most (83.3%) of the laminitis signs (marked weight shifting and swelling over the coronary band) were observed already at day 3 of the measurement period, whereas laminitis signs of only one cow developed at day 7. It seems likely that the developed laminitis signs were related to the increasing concentrations of free LPS in the rumen fluid. This is in line with the observed level of free rumen LPS on day 3 and 7 which were significantly (P=0.003) higher compared to day 1. Indeed, mean log LPS concentrations observed on the laminitis cows was significantly higher (P<0.001) compared to non-laminitis cows. The underlying mechanism for the relationship between LPS concentration and laminitis is not clear, but it may be speculated that the elevated rumen LPS may trigger mediators of inflammatory responses (Danscher et al., 2011), which contribute to the development of laminitis. Furthermore, sudden changes in the diet and inflammation of the rumen epithelium may also play a role in the development of laminitis (Plaizier et al., 2012). Alternatively, it cannot be excluded that the clinical signs of laminitis are related to an increased pressure force to the claw due to body weight, less time spent lying or the hard concrete floors (Bergsten, 2003; Cook et al., 2004).

5. Conclusions

Dietary supplementation of hydrate sodium calcium aluminosilicate to cows had no effect on the concentration of free LPS in the rumen fluid. Furthermore, dietary supplementation of hydrate sodium calcium aluminosilicate did not prevent the occurrence of laminitis. As such, the use of supplemental hydrate sodium calcium aluminosilicate to prevent laminitis in dairy cows does not appear to be an effective strategy to prevent laminitis.

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Chapter 6

General Discussion

1. Introduction

It is generally accepted that, at least in cattle, many predisposing factors are associated with the occurrence of laminitis (*Pododermatitis diffusa aseptica*) including farm management, housing, genetics, breeding, and nutrition (Vermunt and Greenough, 1994; Ossent and Lischer, 1998). Nutrition has been identified as a major contributing factor in relation to laminitis (Greenough et al., 1990), but the precise cause of laminitis is not yet known. The occurrence of laminitis seems to be restricted to ungulates held in captivity in which the gastro-intestinal breakdown of macro-nutrients from feed mainly occurs through the process of fermentation. To the author's knowledge, laminitis has only been reported in ungulates such as cattle (Greenough and Vermunt, 1991), horses (Coffman et al., 1970), goat (Mgasa and Mbassa, 1988), sheep (Morrow et al., 1973; Pierson and Jensen, 1975), donkeys (Walmsley, 1995, Collins et al., 2012), elk (Gray et al., 2001) and moose (Clauss et al., 2009) but not in animals that predominantly rely on small intestinal digestion of feed. Therefore, it can be considered opportune to compare studies undertaken with both cattle and horses, to discuss various nutritional aspects in relation to laminitis in these species. This chapter provides an overview of the nutritional studies on bovine and equine laminitis. Together with the results from the experiments presented in this thesis, new clues for a better understanding of the underlying mechanism(s) of laminitis in dairy cattle (and horses) are provided.

2. Clinical signs, pathology and pathophysiology of laminitis in bovine and equine

Clinical signs of bovine laminitis can be classified into the following categories: an acute and sub-acute clinical form, a subclinical form and a chronic form (Vermunt and Greenough, 1994). Acute laminitis is associated with a rapid onset of foot pain, detectable lameness and an arching of the back of the animal. Shifting weight from one foot to the other of both front and hind limp is an early clinical sign of acute laminitis development including signs of claw inflammation, e.g. warmth and increased pulsation of the coronary band (Boosman et al., 1991). Animals with subacute laminitis show moderate to severe lameness with general stiffness, weight shifting and coronary band inflammation. Hemorrhagic lesion in the sole and along with the white line, double soles and ulcers of the sole are usually attributed to subclinical laminitis (Ossent and Lischer, 1998). If left untreated, the disease can result into chronic laminitis when characteristic alteration in the shape of the claw due to a disturbed horn growth pattern can occur. The claw becomes elongated with a flattened and

broadened and concave of the dorsal wall (Vermunt and Greenough, 1994). Bovine suffering from either subclinical or chronic laminitis do not necessarily show obvious signs of lameness.

In horses, generally four stages of clinical signs are distinguished: the developmental stage, the acute stage, the sub-acute stage and the chronic stage. The latter is always characterized by the rotation and/or sinking of the distal phalanx (Herthel and Hood, 1999; Swanson, 1999). The acute stage is most precisely described and the most observed clinical signs in the acute stage of laminitis are an increased digital pulse and difficulty to move (Wylie et al., 2013). In the latter study, swelling of the coronary band was characterized as one of the least prevalent signs (<10%). This seems to be in contrast to a review concerning laminitis in geriatric horses (Hunt, 2002). Wylie et al. (2013) reported that in 57% of the cases both forefeet were suffering from the disease and that in 41% all four feet were involved.

2.1. Pathology of laminitis

In general, pathological changes of bovine laminitis are commonly present in the hind limbs of cattle. In acute laminitis, stretching of lamellae, dermal edema, hemorrhages, changes in basal cell morphology, presence of white blood cells in dermis, and signs of basement membrane detachment are seen (Nilsson, 1963; Thoefner et al., 2004, 2005). The degradation of collagen fibre systems (essential for supporting and suspending the pedal bone) and the change in the position of the third phalanx have been demonstrated in several studies (Nilsson, 1963; Thoefner et al., 2004; Danscher et al., 2010). In subclinical and chronic forms of laminitis, weakening of the horn capsule is usually observed. A dark brown red discoloration is often observed in the horn of the sole and the wall as well as a yellowish discoloration of the sole.

The anatomical differences between the hoof of horses and the claw of cattle have been described by Mülling and Lischer (2002).The equine hoof has a larger dermo-epidermal contact area due to the larger laminar region compared to the bovine claws. The claw has a digital cushion layer that can be seen as a shock absorber that improves the stability between the pedal bone and the horn capsule. The consequences of these anatomical differences in relation to laminitis have been summarized by Mülling and Lischer (2002). The horse has a highly stable connection between the hoof capsule and the third phalanx due to the large contact area with primary and secondary lamellae. During standing and running the mechanical load in the horse is transferred completely to the hoof by the connective tissue

between the hoof and the third phalanx. In cattle, the weight load is transferred on one hand by the connective tissue between the pedal bone and the claw capsule and on the other hand by the digital cushions that are positioned between the pedal bone to sole and the heel. This anatomical difference in the transfer of weight between the horse and cattle helps to understand the different consequences of laminitis. In the horse, laminitis has an impact on mainly the connection between the lamellae and the hoof while in cattle there can be additional damage to the sole and the heel due to a shift of mechanical load to these areas where a disturbed circulation can cause clinical signs of laminitis.

2.2. Pathophysiology of laminitis

Currently, it is believed that two potential processes may play an important role in the pathogenesis of bovine laminitis. First, a compromised microcirculation of the dermis, which is contributes to hypoxia and degeneration of the epidermal basal cells and to the separation of the dermal and epidermal tissues. Secondly, degradation of collagen essential for supporting and suspending the pedal bones (Mülling and Lischer, 2002). Nocek (1997) indicated that the alteration of vascular endothelium and microcirculation is the initial process of bovine laminitis. Increased blood pressure may result in edema, internal hemorrhages and expansion of the corium, thereby, causing severe pain. Generally, the pathogenesis of bovine and equine laminitis is characterized by the degeneration of the basement membrane of the lamellar regions (Vermunt and Leach, 1992; Katz and Bailey, 2012). In equine, the physiological factors involved in laminitis are inflammation and extracellular matrix degeneration, metabolic abnormalities and vascular dysfunction (Moore et al., 2004). The latter is considered the main factor that initiates the clinical signs laminitis in both bovine and equine.

Several factors involved in vascular disturbances such as endotoxin, histamine and lactate have been implicated to play an important role in the etiology of bovine laminitis (Nocek, 1997). Katz and Bailey (2012) described a broad range of factors that were related to the occurrence of clinical signs of equine laminitis and they summarized to three theoretical concepts, i.e. a traumatic, an endocrinopathic and an inflammatory theory.

The traumatic theory: poor blood flow and ischemia are the key words in this concept which is closely related to mechanical overload of one or more legs.

The endocrinopathic theory: stretching of the secondary epidermal lamellae, mitotic activity and cell proliferation are the key words in this concept which can be induced by

corticosteroid administration and is seen in animals with pituitary pars intermediate dysfunction and insulin-resistance.

The inflammatory theory: inflammatory toxins, degradation of the lamellar basement membrane, matrix metalloproteinase (MMP), leukocyte infiltration and hemodynamic changes are the key words in this concept which can be induced by an overload of the hindgut fermentation with starch or fructan or by the administration of the aqueous extract of the black walnut.

The so-called inflammatory theory for horses contains many elements that are also involved in the development of bovine laminitis. The role of a deteriorated fermentation seems to be of major importance. Vascular dysfunction in the claw/hoof is important in the initial stages of the pathogenesis of laminitis in both bovine and equine.

2.3. Conclusions

The precise etiology of both bovine and equine laminitis remains unknown. However, it is clear that its pathogenesis is multifactorial in nature. The clinical signs, etiology and pathogenesis of bovine laminitis are characteristic and different from equine laminitis due to the structural and biomechanical differences between claw and hoof, respectively. However, a deteriorated fermentation in association with a vascular dysfunction in the claw/hoof appears to be common denominators in bovine and equine laminitis.

3. Intake of readily fermentable carbohydrates, acidosis and laminitis

Unfortunately, there is no general agreement on the definition of rumen acidosis in terms of pH (Kleen et al., 2003; Krause and Oetzel, 2005; Plaizier et al., 2008) and it is somewhat beyond the scope of this overview to extensively address this issue. The reader is referred to the papers of Gozho et al. (2005), Khafipour et al. (2009b) and Li et al. (2012). In the current overview rumen acidosis is arbitrarily defined as a rumen pH < 5.6 for at least 3 consecutive hours (Gozho et al., 2005; Khafipour et al., 2009b; Li et al., 2012). Unless otherwise indicated, a cecal pH below 6.0 was considered to represent hindgut acidosis in horses (Radicke et al., 1991).

In cattle, various studies under practical feeding conditions have clearly shown that rumen acidosis can be successfully induced by dietary measures such as the feeding of high proportions of grain (Khafipour et al., 2009b), starch (Krause et al., 2006) or an decreased roughage to concentrate ratio (Gozho et al., 2006, Khafipour et al., 2009a). However, cases of

(sub) clinical laminitis were not reported in all fore mentioned studies. In contrast, in recent studies by Pilachai et al. (2012b), the feeding of high amounts of starch rich concentrates (i.e. 10.5 kg DM containing 40% starch) in combination with a low roughage intake (i.e. 1.5 kg DM) resulted in rumen acidosis and cases of subacute laminitis. In these studies, 3 out of 4 (Pilachai et al. (2012b) and 4 out of the 6 (Chapter 5) animals showed clear signs of subacute laminitis. Likewise, in horses excessive ingestion of starch and an overload of rapid fermentable carbohydrates (inulin) can induce laminitis in this species (Garner et al., 1978; Rowe et al., 1994; Pollit et al., 2003). Furthermore, oral doses of high amounts of oligofructose in both bovine and equine, appear to be highly effective in inducing laminitis (Table 1).

Taken all fore mentioned studies into account it can be speculated that cases of laminitis occur under conditions of a deteriorated fermentation. However, it is not clear whether pH is an appropriate indicator of a deteriorated fermentation. At least in bovine, the relationship between the feeding of high amounts of rapid fermentable carbohydrates and the occurrence of laminitis is not unequivocal although in all fore mentioned cases, rumen acidosis occurred. Rumen acidosis may occur when production rates of volatile fatty acids exceed the buffer capacity of the rumen fluid (Nagaraja et al., 1998). When rumen pH decreases below 5.5, a shift in the microbial population towards lactate producing bacteria occurs and this compromises the buffer capacity of the rumen fluid even more. Ultimately, rumen pH may become as low as 4.0 due to high concentrations of lactate (Owens et al., 1998).

Interestingly, the oligofructose load used by Danscher et al. (2009; Table 1) effectively induced laminitis in heifers. This was associated with a very low rumen pH. Unfortunately, rumen lactate was not measured in that study but it can be speculated that rumen lactate levels were high in those studies (Danscher et al., 2009, 2010). It was suggested by Nocek (1997), that the productions of ruminal lactate play an important role with the respect to the development of laminitis (Nocek, 1997). This speculation is corroborated by Dunlop and Hammond (1965) who found that cattle fed an overload of dietary grain, developed lactic acidosis with associated clinical signs of laminitis.

Table 1. Rumen or fecal pH and the occurrence of laminitis after an oral dose of oligofructose.

Reference	Animal	Oral dose of oligofructose (g/kg of body weight)	pH[a]	Laminitis (no. of cases / no. of animals)
Thoefner et al. (2004)	Heifer	0	7.0	0/2
		13	4.8	1/2
		17	4.7	2/2
		21	4.6	1/2
Danscher et al. (2009)	Heifer	0	7.3	0/8
		17	4.3	8/8
Danscher et al. (2010)	Heifer	0	-	0/6
		17	3.8	9/10
Van Eps and Pollit (2006)	Horse	0	7.3	0/6
		7.5	5.6	2/2
		10	5.0	6/6
		12.5	4.5	2/2
Bailey et al. (2009)	Horse	0	6.9	0/6
		10	4.6	5/6
Onishi et al. (2012)	Horse	0	-	0/8
		10	-	6/6

[a] pH of rumen or fecal fluid collected from heifer or horse, respectively.

In lambs, an overload of corn syrup induced lactic acidosis and acute laminitis (Pierson and Jensen, 1975) and Morrow et al. (1973) showed that an intraruminal administration of a high dose of lactic acid (0.35% of body weight) induced laminitis in these animals. In contrast, Brown et al. (2000) induced high rumen lactate concentrations (i.e. 48 mM) in steers by means of a steam-flaked corn based ration, but clinical cases of laminitis were not reported in this study. Furthermore, Pilachai et al. (2012b) observed that 3 out of 4 cows showed signs of subacute laminitis in association with rumen pH levels < 5.6 but rumen lactate levels were relatively low; i.e. < 9.0 mM. Therefore, the outcome of the latter two studies (Brown at al., 2000, Pilachai et al., 2012b) do not corroborate the notion that rumen lactate is important with respect to the development of laminitis. Regrettably, the equine studies listed in Table 1 do not provide further clues because indices of fermentation including lactate, were not assessed in those studies.

It can be concluded that the role of lactate in relation to the occurrence of laminitis is unclear. Nocek (1997) proposed a mechanistical model for the relationship between rumen acidosis and the occurrence of laminitis. Unfortunately, the role of lactate and/or rumen pH does not become clear from the model proposed by Nocek (1997) but it was suggested that not a low rumen pH *per se* but a systemic metabolic acidosis plays an important role in the initial phase of the development of laminitis. However, data on systemic acid base balance including the role of systemic lactate were not assessed in all but one studies mentioned. Brown et al. (2000) monitored systemic acid base balance and found that in steers suffering from lactic acidosis in the rumen, metabolic acidosis did not occur. This observation seems to be in line with the model proposed by Nocek (1997) which suggests that systemic acidosis is important in relation to the etiology of laminitis. Nevertheless, the issue on rumen lactate and systemic acid base balance should be taken into account in future studies to verify the proposed model on rumen acidosis induced laminitis of Nocek (1997) and consequently gain more insight into the pathophysiology of laminitis.

4. Lipopolysaccharides and laminitis

Lipopolysaccharides (LPS) have been implicated to play a role in the etiology of laminitis in both bovine (Nocek, 1997) and equine (Boosman et al., 1991). Lipopolysaccharides are derived from the cell membrane of gram-negative bacteria and consist typically of a hydrophobic domain known as lipophilic component lipid A, a core oligosaccharide, and a distal polysaccharide (or O-antigen). The lipid A domain is the most

biologically active portion of the LPS molecule and is considered to be responsible for the toxic proportions of LPS (Raetz and Whitfield, 2002).

4.1. Origin of LPS

In bovine, the rapid fermentation of carbohydrates can have a major impact on the composition of the microbial flora in the rumen. The feeding of high concentrate rations can induce a shift in the microbial population towards gram negative bacteria such as Bacteroidetes, Anaerophygy and Prevotella (Fernando et al., 2010). This observation is corroborated by others showing that the feeding of high grain rations can trigger the increase of lactate-producing bacteria (Russell and Rychlik, 2001) and stimulates the growth rate of *Escherichia coli* (*E. coli*) (Diez-Gonzalez et al., 1998; Khafipour et al., 2011). The proliferation of lactate-producing bacteria and *E. coli* occur with a concomitant decrease in rumen pH (Nagaraja et al., 1978) and it is generally accepted that a rumen pH < 6 cause lysis of *E. coli* which is associated with the release of LPS into the rumen fluid. This reasoning is in line with the data provided in Table 2, showing that high levels of rumen LPS are usually observed during prolonged episodes of a low rumen pH. Therefore, it can be speculated that most of the free LPS in the rumen originate from gram-negative Bacteriodetes, *E. coli* in particular.

Gram negative bacteria are generally found in various parts of the gastrointestinal (GI) tract such as the rumen, cecum and colon (Nagaraja et al., 1978). Therefore, it cannot be excluded that release of LPS also occurs in the hindgut of ruminants. Generally, most of the dietary carbohydrates are fermented in the rumen and only up to 10 % of the dietary carbohydrates are fermented in the large intestine (Owens et al., 1986; Huntington, 1997) and are primarily associated with the fiber fraction of the ration (Ørskov et al., 1970; Firkins, 1997). However, the feeding of a ration with a high proportion of grain can lead to an excessive supply of starch to the hindgut which increases the rate of fermentation of the carbohydrate fraction at that location (Reynolds, 2006). Li et al. (2012) found an increased starch content of cecal digesta when dairy cows were fed pellets based on grain instead of forage. The high starch content of the cecal digesta was associated with a 7-fold increase in cecal LPS concentration and levels up to 118.6×10^3 EU/g were found (Li et al., 2012).

Table 2. Ration characteristics, rumen pH and the concentration of lipopolysaccharide (LPS) in rumen fluid of dairy cows.

Reference	Ration composition (% of dry matter)	Rumen variable		
		pH		LPS
		Time pH<5.6 (min/d)	Mean	(10^3 EU/ml[a])
Andersen et al. (1994)	100 % hay	-	6.9	0.2
	25% hay, 75% concentrate	-	5.7	1.6
Gozho et al. (2005)	60% - 40% alfalfa, 40% - 60% wheat/barley based concentrate	98	6.3	8.2
Gozho et al. (2006)	100 % -59% hay, 0% - 41% wheat/barley based concentrate	0	6.7	6.3
	24% hay, 76% wheat/barley based concentrate	121	6.1	26.9
Gozho et al. (2007)	43% roughage, 57% concentrate	187	6.2	24.5
	32% roughage, 68% concentrate	309	6.0	128.8
Emmanuel et al. (2008)	100% basal ration (85 % roughage,15% concentrate)	-	6.8	6.5
	85% basal ration, 15% of barley based concentrate	-	6.7	7.9
	70% basal ration, 30% of barley based concentrate	-	6.7	50.2
	55% basal ration, 45% of barley based concentrate	-	6.5	88.7

Table 2. Continued.

Reference	Ration composition (% of dry matter)	Rumen variable		
		pH		LPS
		Time pH<5.6 (min/d)	Mean	(10^3 EU/ml[a])
Khafipour et al. (2009a)	50% alfalfa hay, 50% concentrate	112	6.3	42.1
	40% alfalfa hay, 60% alfalfa based pellets	558	5.8	145.6
Li et al. (2012)	70% roughage, 30% grain based concentrate	56	6.3	10.4
	30% roughage, 70% grain based concentrate	299	5.9	168.4
	36% roughage, 64% alfalfa based pellets	225	5.9	30.7
Pilachai et al. (2012b)	12.5 % rice straw, 87.5 % corn/cassava based concentrate	-	5.6	40.3
Pilachai et al. (Chapter 5)	46.5 % rice straw, 53.5 % corn/cassava based concentrate	-	5.9	4.5
	12.5 % rice straw, 87.5 % corn/cassava based concentrate	-	5.0	104.0

[a] EU = Endotoxin Units

Therefore, it can be speculated that the feeding of high amounts of starch potentially can stimulate the growth of gram negative bacteria in the hindgut and concomitantly increase release of LPS from this part of the gastro intestinal tract. It is possible that the contribution of LPS from the hindgut is relevant under Thai feeding conditions due to the fact that dairy rations may contain large amounts of starch rich cassava (Aiumlamai, 2009; Wanapat, 2003) in combination with a restricted roughage intake (Chapter 2).

In equine, the gastrointestinal tract also contains large quantities of gram negative organisms (Garner et al. 1978). It has been shown by Onishi et al. (2012) that an overload of dietary carbohydrates fed to equines resulted in increased levels of LPS in the hindgut. Likewise, Moor et al. (1979) demonstrated a carbohydrate induced increase of LPS in the cecum with a subsequent development of acute laminitis. Recently, Bailey et al. (2009) demonstrated that in five out of the six horses that were exposed to an oligofructose overload, significant concentrations of LPS could be detected in blood plasma. Unfortunately, not indices of hindgut fermentation nor LPS concentrations were measured in this study (Bailey et al., 2009). Thus, it can be concluded that most of LPS found in the digestive tracts of both bovine and equine originate from gram negative bacteria and that high levels of LPS can be induced by excessive digestion of carbohydrate in rumen and large intestine.

4.2. Absorption of LPS

Khafipour et al. (2009b) demonstrated that an increase in free rumen LPS from 28.2×10^3 to 107.2×10^3 EU/ml was associated with increased plasma LPS levels peaking at 0.81 EU/ml. In contrast, an increase in plasma LPS concentrations could not be demonstrated in various other studies (Gozho et al., 2007; Khafipour et al., 2009a; Pilachai et al., 2012b, Li et al., 2012) despite the fact that high levels of rumen LPS were observed. Thus, it seems likely that the translocation of free LPS from rumen to plasma not only depends on the concentration gradient of LPS but most likely also on the barrier function of epithelium (Li et al., 2012). The ruminal mucosa exhibits a low permeability towards LPS under physiological conditions (Mani et al., 2012). However, Zebeli et al. (2012) suggested that in the condition of rumen acidosis, the barrier function of the rumen epithelium may be compromised in such a way that transport of LPS across the rumen wall can occur. This reasoning is in line with the outcome of an *in-vitro* study by Emmanuel et al. (2007) who demonstrated that under acidic conditions (pH=4.5), LPS transport occurred across the rumen wall. The precise mechanism of ruminal LPS absorption is not yet known but it was suggested by Mani et al.

(2012) that paracellular transport through nonspecific tight junctions is involved in the process of LPS absorption by the rumen epithelium.

Next to absorption through the rumen wall, LPS absorption can also occur in the large intestine (Khafipour et al., 2009b; Li et al., 2012) and these investigators suggested that absorption of LPS from the hindgut is quantitatively more important than that from the rumen. For obvious reasons, absorption of LPS in equines most likely occurs in the distal part of the gastrointestinal tract. The underlying mechanism of LPS absorption from the hindgut is unknown but it has been speculated that it resembles the process of LPS absorption in the rumen, both in bovine (Khafipour et al., 2009b; Li et al., 2012) and equine (Werners et al., 2005).

4.3. Plasma LPS

Currently, the relationship between LPS levels in the rumen and plasma is not exactly clear and is not straightforward. It was already mentioned that LPS can also be absorbed from the hindgut which provides at least a partial explanation for the poor relationship between rumen- and plasma LPS. Furthermore, the liver plays an important role in the clearance of LPS that enters portal blood which prevents the accumulation of LPS in peripheral blood (Andersen, 2003; Satoh et al., 2008). Nevertheless, Khafipour et al. (2009b) reported high levels of rumen LPS and elevated plasma LPS levels when rumen acidosis was induced by means of grain feeding. In contrast, induction of rumen acidosis by means of a reduction in the effective fiber content of the ration (i.e. alfalfa pellets; Khafipour et al., 2009a) or a combination of a low effective fiber content together with a high dietary starch content (Chapter 5) resulted in high rumen LPS values but responses in plasma LPS were not detected. The differential responses of plasma LPS to high ruminal LPS concentrations are difficult to explain but it may be that the extent of LPS release in the rumen not only depends on rumen pH but also on the profile of the microbial flora (Russel, 1976; Plaizier et al. 2008). It was already mentioned that LPS originates from gram negative bacteria but it can be speculated that the proliferation of gram negative bacteria not only depends on pH but also on the substrate available for ruminal fermentation. Furthermore, it cannot be excluded that also the level of dry matter intake is important (Pilachai et al., 2012b) with respect to the interrelationship between composition of the ration, rumen LPS and the plasma LPS concentration.

Lipopolysaccharide has been found in the blood circulation of horse suffering with gastrointestinal tract diseases including hindgut acidosis (Sprouse et al., 1987). Recently,

Bailey et al. (2009) detected in five out of the six horses LPS concentrations of 0.019 EU/ml in plasma after oligofructose overload, thereby, triggering platelet activation. Such a condition can contribute to the development of laminitis. Sprouse et al (1987) showed increased plasma LPS levels in horses with a carbohydrate induced laminitis. It can be concluded that the relationship between ration and plasma LPS is complex and has not been settled yet, both in bovine and equine.

4.4. Putative role of LPS in the etiology of laminitis

Several reports in relation to laminitis in equine (Moore et al. 1979; Hunt et al. 1986; Weiss et al. 1996; Rodgerson et al. 2001) and bovine (Vermunt and Greenough, 1994; Nocek, 1997; Ossent and Lischer, 1998) suggest that LPS is one of the relevant factors in the development of the disease. Currently, the role of LPS in the pathophysiology of laminitis is not yet known but it seems unlikely that LPS itself causes laminitis. Several equine studies have shown that infusions of LPS do not induce clinical signs of laminitis (Duncan et al., 1985; Hunt et al., 1990; MacKay et al., 1991; Toth et al., 2009; Tadros and Frank, 2012). Furthermore, local infusions of high doses of *E. coli* LPS (10 – 75 µg) in a ligated lower limb of dairy cattle also did not result in clinical signs of laminitis (Boosman et al., 1991). Therefore, it can be concluded that LPS can only have an indirect effect in relation to the etiology of laminitis.

Under the condition of experimentally induced rumen acidosis (Ametaj et al., 2009; Dionissopoulos et al., 2012; Emmanuel et al., 2008; Khafipour et al., 2009b) it has been shown that high levels of rumen LPS were accompanied by increased levels of LPS-binding protein (LBP; Li et al., 2012) and acute phase proteins in bovine plasma (Dancher et al., 2011; Gozho et al., 2005, 2006, 2007). Thus, it appears that LPS can trigger an immune response when it enters the circulation (Alexander and Rietschel, 2001). It has been shown by Bannerman et al. (2003, 2004) that an intra-mammary challenge with LPS was followed by a rapid (within 24 h) increase in circulating levels of LBP and cluster of differentiation molecule 14 (CD14). Activation of CD14 may ultimately result in the activation of macrophages in liver Kuppfer cells and consequently the release of different pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8, tumor-necrosis factor (TNF)-alpha (Sweet and Hume, 1996; Zebeli et al., 2012). It was suggested by Nocek (1997) that these pro-inflammatory cytokines may play a role in the etiology of laminitis in dairy cattle. Interestingly, Raffetto and Khalil (2008) reported that LPS and its associated pro-inflammatory cytokines, increase the activity of MMPs while others (Frears et al., 1996;

Rajagopalan et al., 1996; Okamoto et al., 2001) reported an increase in MMP activity and a decreased activity of tissue inhibitors of metalloproteinases (TIMPs). An imbalance between the activity of MMP and that of TIMP may cause pathological changes in the vessel wall and ultimately to a vascular dysfunction (Lalu et al., 2006). It was already mentioned that a vascular dysfunction in the claw/hoof is important in the initial stages of the pathogenesis of bovine and equine laminitis. Therefore, a LPS induced impairment of the remodeling of the wall of blood vessels might be one of the underlying mechanisms to explain laminitis. Alternatively, Bailey et al. (2009) suggested that LPS may cause an indirect activation of circulating platelets and a subsequent release of inflammatory mediators and vasoactive compounds such as thromboxanes and 5-hydroxytryptamine, respectively. Alterations of vascular circulation also have been implicated as a key factor in the etiology of laminitis in horses (Weiss et al., 1996) and in cattle (Nocek, 1997). Apart from 5-hydroxytryptamine, histamine a potent vasodilator, also is indicated to be important in relation to the pathogenesis of laminitis (see below).

Evidence exists that LPS generated from the gastrointestinal tract can induce a systemic inflammatory response, but the relationship between a LPS induced inflammatory response and the occurrence of laminitis is not straightforward. Several authors (Ametaj et al., 2009; Emmanual et al., 2008; Khafipour et al., 2009b; Li et al., 2012) reported a significant increase in LPB and Ametaj et al. (2009), Gohzo et al. (2005, 2006, 2007) and Khafipour et al. (2009b) have reported a significant LPS induced increase in serum amyloid-A (SAA) and Heptoglobin (Hp). However, clinical cases of (sub)clinical laminitis in dairy cows were not reported. Danscher et al. (2011) reported that all heifers (n=10) that participated in their study, developed laminitis which was associated with mean SAA concentrations of 325 mg/L and mean Hp concentrations of 4,226 mg/L. In contrast, Gohzo et al. (2007) and Khafipour et al. (2009) reported higher SAA levels but laminitis did not occur. Bailey et al. (2009), van Eps and Pollit (2006) and Visser and Pollit (2011) reported that almost 100% cases of laminitis occurred in horses after an oligofructose overload in association with several indices of inflammatory responses such as TNFα, thromboxane A2 (Bailey et al., 2009), plasma fibrinogen (Van Eps and Pollit, 2006) and gene expression of IL-6 (Visser and Pollit, 2011). Clearly, a cause and effect relationship between the different indices of inflammatory response and the occurrence of laminitis cannot be deducted from the fore mentioned studies.

4.5. Conclusions

To date, it is believed that LPS may contribute indirectly to the development of laminitis by the activation of an inflammatory response. The activation of such a response of contributes to hemodynamic disturbances of the claw/hoof in both bovine and equine. However, it is clear that further studies are needed to elucidate the role of a LPS induced inflammatory response in the pathophysiology of laminitis.

6. Histamine and laminitis

For a long time, there has been a belief that histamine might be involved in the pathogenesis of equine (Chavance, 1946; Rautschka et al., 1991) and bovine (Nilsson, 1963; Koers et al., 1976; Irwin et al., 1979; Nocek, 1997; Nagaraja and Titgemeyer, 2007) laminitis. Histamine is a potent vasodilator and as such can potentially influence the hemodynamics in the claw or hoof and thus contribute to the development of laminitis (Takahashi et al., 1981; Manson and Leaver, 1988).

Histamine is produced by *Allisonella histaminiformans* and these bacteria are present in the rumen as well as in the cecum of horses (Garner et al., 2002). The production of histamine involves the decarboxylation of the amino acid histidine (Rodwell, 1953; Irwin et al., 1979; Schelp et al., 2001; Garner et al., 2004). Therefore, high levels of histamine can only be produced after the ingestion of appropriate amounts of protein. However, Pilachai et al. (2012a) could not demonstrate a relevant rise in ruminal histamine concentrations after the feeding of a ration high in crude protein, i.e. 26% on the basis of dry matter. In this study, rumen pH was > 6.1 and this condition may have prevented the formation of histamine. It is known that the formation of histamine in rumen fluid depends on both histidine decarboxylation and recurrent low rumen pH values (Rodwell, 1953; Sanford, 1963; Irwin et al., 1979; Suber et al., 1979; Schelp et al., 2001; Garner et al., 2004; Pilachai et al., 2012b). Therefore, histamine can be considered as a potential factor to explain the proposed relationship between rumen acidosis and laminitis (Nocek, 1997). It has been suggested by MacLean (1970) that laminitis only occurs in association with ruminal histamine concentrations in the order of 60 mg/L or higher. Furthermore, Rautschka et al. (1991) reported that plasma histamine ranged from 73.4 to 194.5 mg/L in horses with laminitis. However, whether in the increased levels of histamine in rumen (MacLean, 1970) or plasma (Rautschka et al., 1991) caused laminitis cannot be elucidated from those studies but it was shown by Takahashi et al. (1981) that an injection of histamine did not induce any clinical

signs of laminitis in horses. Furthermore, Ammann and Almasy (1950) did not find any difference in plasma histamine levels in healthy horses and those suffering from laminitis. Finally, Pilachai et al. (2012a) observed that two out of the six cows developed clinical signs of laminitis while rumen histamine levels remained very low (< 1.3 mg/L).

6.1. Conclusion

On the basis of theoretical considerations histamine seems to be of interest in relation to the etiology of laminitis. However, this notion is not supported by the provided experimental data. Therefore, the mechanism if any, by which histamine contributes to the development of laminitis remains unclear.

7. Advanced glycation endproducts and laminitis

In equines, the role of obesity and insulin resistance are nowadays well known factors that are closely related to laminitis (Jeffcott et al., 1986; Treiber et al., 2005, 2006). The role of insulin was experimentally confirmed by De Laat et al. (2010) who were able to induce laminitis in clinically normal horses by prolonged infusions of insulin and glucose to maintain physiological levels of plasma glucose. With respect to the underlying mechanism of insulin induced laminitis, De Laat et al. (2012) suggested that the formation of advanced glycation endproducts (AGEs) might have played a role.

In cattle, the role of AGEs and insulin resistance in development of laminitis has not been studied. It has been shown by Gearhart et al. (1990) and Geelen and Wensing (2006) that insulin resistance is often seen in early lactating cows, which may be exacerbated by high intakes of rapidly fermentable carbohydrates or starch (Holtenius et al., 2003). Interestingly, high prevalence of laminitis lesions, i.e. hemorrhage of the sole and white line are often observed during early lactation (Leach et al., 1997; Livesey et al., 1998; Webster, 2001; Tarlton et al., 2002). Although no study has been conducted, it can be argued that the occurrence of laminitis during early lactation may be related to insulin resistance and the formation of AGEs.

Advanced glycation end products are derived from glucose through intermediates such as glyoxal, 3-deoxyglucosone and methylglyoxal (Shinohara et al. 1998) and it has been postulated by Thornalley (1990) that methylglyoxal is a major source of intracellular and plasma AGEs. It has been shown by Shinohara et al. (1998) that under *in-vitro* conditions, the enzymes glyoxalase I and II act in concert to convert methylglyoxal into D-lactate, thereby,

preventing the formation of AGEs. In bovine and equines suffering from a systemic acidosis induced by the feeding of high amounts of fermentable carbohydrates and subsequent acidosis of either the rumen or cecum, bovine plasma levels of D- lactate may increase up to 25 mmol/L (Moller et al., 1997). Unfortunately, to the author's knowledge, it is not yet known whether the end product D-lactate exerts a negative feedback on the activity of glyoxalase I but this notion may be of interest in relation to the development of laminitis.

Apart from the potential that AGEs may be derived from methylglyoxal that originate from the animals intermediary metabolism, methylglyoxal can also be formed during the anaerobic fermentation of rapidly fermentable carbohydrates (Cooper and Anderson, 1970, Ackerman et al., 1974). It was already mentioned that methylglyoxal is converted to D-lactate under physiological conditions. Therefore, it can be speculated that under practical feeding conditions of cows and horses, the fermentation of rapidly fermentable carbohydrates results in the accumulation of both D-lactate and methylglyoxal. Methylglyoxal is toxic to cells (Együd and Szent-Györgyi, 1966) which ultimately results in the lysis of bacteria (Ackerman et al., 1974) and the subsequent release of lipopolysaccharides which are implicated in the etiology of laminitis in both bovine (Nocek, 1997) and equine (Boosman et al., 1991). Alternatively, methylglyoxal may be absorbed by the rumen epithelium and subsequently trigger the formation of AGEs. However, data on absorption of methylglyoxal across the epithelium of the gastro-intestinal tract of bovine and equine is, to the author's knowledge, not available.

Apart from the fact that AGEs can be metabolically generated, they can also be present in feedstuffs, for instance in heated hay. When hay becomes heated beyond 38° C. so called Maillard reactions can occur which involve the binding of plant sugars with amino acids, especially lysine (McDonald et al., 2011). Currently, it is not exactly clear whether the fore mentioned Maillard products can be absorbed by cows or horses but it was shown by Negrean et al. (2007) that the intake of an AGE-enriched meal induced a significant raise in serum methylglyoxal in humans. However, it is yet not known whether this observation can be extrapolated to bovines or equines.

The mechanism by which AGEs may play role in the development of laminitis is currently not clear but it is known that AGEs can bind to its receptor (RAGE, Bucciarelli et al., 2002) which results in amongst others, the release of pro-inflammatory cytokines, oxidative stress and overexpression of some proteins in the extra-cellular matrix such as collagen. Therefore, it seems that at least theoretically, AGEs might be important to study in relation to laminitis.

In all bovine studies mentioned in the current chapter, methylglyoxal nor other related compounds were measured. However, further studies on the relationship between AGEs and the development of laminitis might yield new clues as to nutrition related aspects of laminitis.

8. Concluding remarks

It can be concluded that laminitis in bovines and equines is associated with vascular dysfunction in the claw/hoof. Furthermore, it seems clear that the occurrence of laminitis in both species is associated with a deteriorated fermentation. Many studies were successful to induce laminitis using different approaches, but could not unravel the underlying mechanism of laminitis. In clinical cases of laminitis, the relationship between laminitis and practical feeding conditions is difficult to understand. In the last decade, the role of insulin and AGEs in the etiology of equine laminitis has drawn serious attention. It can be suggested that this notion can also be applied in the study on bovine laminitis. The progress in the understanding of laminitis seems to be hampered by the disciplinary approach when the etiology of laminitis is studied. For instance, when the effect of deteriorated rumen/cecal fermentation on the occurrence of laminitis is studied, the role of lactate, systemic acid base balance and insulin/AGEs are not taken into account. Therefore, an interdisciplinary approach and cooperation between bovine and equine scientists might be instrumental in the research on laminitis.

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Chapter 7

General Conclusions

In this chapter, the main conclusions of the thesis are listed and an overall conclusion in relation to the occurrence of laminitis in Thailand and practical feeding conditions is provided.

1. Diet factors and subclinical laminitis score in lactating cows of smallholder dairy farms in Thailand

The observational study described in Chapter 2 shows a wide range in the prevalence of subclinical laminitis (SCL) in 25 smallholder dairy farms in Thailand. Sole hemorrhages contributed most to the SCL score followed by hemorrhages of the white line and white line fissure. The importance of the dietary crude protein (CP) content, dietary neutral detergent fiber (NDF) content and feeding regime as well as other factors related to management and demographics on the occurrence of SCL under practical Thai feeding conditions were investigated. It appeared that Thai dairy rations typically contain a variety of agricultural waste and by-products such as cassava chips, cassava pulp, rice bran, and rice straw as the main source of roughage. Thai dairy rations are generally low in NDF content, roughage is not always provided *ad libitum* and the CP content is usually low. It was shown that dairy cows kept in smallholder farms in Thailand and fed a ration low in NDF content and/or in combination with restrictive feeding of roughage have a high risk of SCL. Dietary CP level was not associated with a higher prevalence of subclinical laminitis. It was suggested that an increased proportion of forage NDF content of the ration may be an important factor in the prevention of SCL in Thai dairy cows.

2. The effects of high levels of rumen degradable protein on rumen pH and histamine concentrations in dairy cows

It was unknown whether CP supplementation to dairy cows kept under the unique Thai feeding conditions would result in rumen acidosis and increased rumen histamine concentrations. The hypothesis tested was that the supplementation of two different forms of dietary rumen undegradable or degradable protein (formaldehyde-treated or -untreated soybean meal) results in rumen acidosis and increased histamine concentrations in dairy cows (Chapter 3). It was shown that high levels of rumen degradable protein in the ration significantly increased the ammonia, total volatile fatty acid (VFA) and histamine concentrations in the rumen fluid. However, supplemental CP either degradable or

undegradable, did not significantly affect the pH of rumen fluid probably due to the relatively low level of dry matter intake (DMI). The rise in ruminal histamine concentrations was physiologically non-relevant, most likely because rumen pH was not affected by supplemental CP at the provided level of DMI. Thus, rations containing about 25% CP did not result in rumen acidosis under the feeding conditions described in Chapter 3. Two cows which were fed high CP rations, developed clinical signs of laminitis but the clinical signs of laminitis could not be explained by the ruminal histamine concentrations.

3. Starch source in high concentrate rations does not affect rumen pH, histamine and lipopolysaccharide concentrations in dairy cows

It is commonly accepted that corn starch *versus* cassava starch is slowly degraded by rumen bacteria. Therefore, it could be suggested that the replacement of corn meal by cassava meal induces rumen acidosis, thereby, increasing the concentrations of lipopolysaccharide (LPS) and histamine in the rumen fluid. However, it appeared that the source of dietary starch did not affect rumen pH, concentrations of total VFA, lactate, histamine and LPS (Chapter 4). The lack of response of source of starch on the rumen pH may be explained by the fact that both the cassava chips and corn were finely ground. The feeding of concentrates, either rich in corn or cassava meal, induced rumen acidosis which was associated with increased rumen LPS and histamine concentrations. The relative high rumen LPS and histamine concentrations did however not affect plasma LPS and histamine concentrations. Therefore, the observed cases of laminitis were difficult to explain. It was suggested that ruminal LPS concentrations were not sufficiently high to induce an inflammatory response probably because of the relative low level of DMI.

4. Hydrate sodium calcium aluminosilicate does not reduce rumen lipopolysaccharide concentrations in dairy cows

Lipopolysaccharide is considered one of the potential risk factors in the development of laminitis in dairy cows. Therefore, the use of agents to bind rumen LPS may be of interest to prevent laminitis. Phyllosilicates such as hydrate sodium calcium aluminosilicate (HSCAS) have the capacity to bind several biological toxins and are used as sorbents in veterinary practice. On the basis of the fact that several other phyllosilicates can bind LPS, it was hypothesized that HSCAS can also adsorb LPS. However, it appeared (Chapter 5) that

supplemental HSCAS did not reduce the concentration of free LPS in the rumen fluid. The lack of effect of supplemental HSCAS on rumen LPS could not be unequivocally explained but it was probably related to the specificity of phyllosilicates to adsorb LPS. As such, the use of supplemental HSCAS to prevent laminitis in dairy cows is not an effective strategy to prevent laminitis

5. Under Thai feeding conditions, rumen acidosis is more important than LPS and histamine in relation to laminitis

It was already mentioned that there is general acceptance of the idea that rumen acidosis, LPS and histamine play a role in the development of laminitis in dairy cattle. However, the relevancy of these risk factors in relation to the occurrence of laminitis under practical feeding conditions in Thailand is not clear. In Thailand, dairy rations are generally low in physically effective NDF because roughage is not always provided *ad libitum* (Chapter 2). Furthermore, cassava which is rich in rapid fermentable carbohydrates, is an important concentrate in practice and the CP content of Thai dairy rations is usually low (< 150 g/kg dry matter; Chapter 2). When these practical feeding conditions are taken into account, it may be suggested that dairy cattle are prone to the condition of rumen acidosis. Therefore, rumen acidosis appears to be relevant in the light of the observed high incidence of laminitis in Thailand (General introduction). This reasoning is in line with the outcome of the observational study described in Chapter 2, showing that the prevalence of (sub) clinical laminitis and the dietary NDF content were negatively related. Under the condition of rumen acidosis, both histamine and LPS can be produced and both factors are implicated to play a role in the etiology of laminitis.

Under Thai feeding conditions, histamine is most likely not important to explain the occurrence of laminitis because it was shown that the prevalence of (sub) clinical laminitis was not related with the dietary CP content (Chapter 2). This observation is corroborated by Pilachai et al. (2012a,b) who showed that under controlled feeding conditions, rumen histamine levels remained too low to induce laminitis when the rations contained at least 200 g of CP/kg dry matter (Pilachai et al., 2012a,b). Therefore, the observed cases of (sub) clinical laminitis could not be explained by the relative low, rumen histamine concentrations (Pilachai et al., 2012a,b).

Rumen LPS also was monitored after the feeding of high concentrate rations (Pilachai et al., 2012b, Chapter 5). In both studies rumen acidosis was successfully induced but

Pilachai et al. (2012b) could not demonstrate a physiologically relevant rise of LPS values in neither rumen nor plasma. The observed cases of (sub) clinical laminitis could not be explained by the observed rumen LPS values. It was suggested that the lack of effect on rumen LPS was due to the relative low level of DMI. In contrast, the results presented in Chapter 5 showed much higher levels of rumen LPS despite the fact that DMI was similar to the previous study (Pilachai et al., 2012b). The differential response in rumen LPS between the two fore mentioned studies is difficult to explain. Nevertheless, observed cases of laminitis were related to the observed high rumen LPS values. Therefore, this study provides some evidences that rumen LPS and the occurrence of laminitis are directly or indirectly, related. Unfortunately, plasma LPS nor indices of inflammation were measured in that study (Chapter 5). Clearly, the issue on LPS remains debatable and the differential responses in rumen LPS between the two studies of Pilachai et al. (2012b, Chapter 5) do not allow a clear conclusion with respect to the relevancy of LPS under Thai feeding conditions.

Currently, there appears to be no agents available that can effectively adsorb LPS under practical feeding conditions. This implicates, that the prevention of rumen acidosis appears to be the most effective strategy to prevent the occurrence of nutrition related laminitis. Consequently, Thai dairy farmers should raise the content of physically effective NDF of the dairy ration. From a practical viewpoint, it is recommended that Thai dairy farmers should supply roughage (i.e. rice straw) *ad libitum* and/or mix appropriate amounts of roughage with concentrates.

Summary

Laminitis is considered an important health problem facing the Thai dairy industry. Although the etiology of laminitis is multifactorial, nutrition is considered as an important risk factor. The objective of this thesis was to investigate some potential risk factors in relation to laminitis under typical Thai feeding conditions. Common Thai feeding practices are not extensively documented but cows are generally fed with low quality roughages, such as rice straw, supplemented with non-pelleted concentrates. Cassava chips are widely used and incorporated up to 70% in dairy rations. Consequently, Thai dairy rations may contain high levels of rapid fermentable carbohydrates in combinations with low levels of neutral detergent fibre (NDF) and crude protein (CP). Therefore, the importance of dietary CP, NDF and feeding regime as well as other factors related to management on the occurrence of (subclinical) laminitis (SCL) were evaluated under practical Thai feeding conditions. Data were collected from 25 Thai dairy farms and it was found that a low dietary NDF content was associated with a high SCL prevalence (>25%). Unfortunately, this result was confounded by feeding regime (separate feeding *versus* mixed feeding of concentrates and roughages) but the results suggested that mixing concentrates with a substantial part of the roughage is an important strategy to prevent SCL under Thai feeding conditions.

Thai dairy rations typically contain high levels of rapid fermentable carbohydrates and low levels of CP, which may be a major constraint for both rumen fermentation and milk production. Therefore, protein supplementation of typical Thai dairy rations can be considered opportune for economical reasons. However, protein supplementation of rations rich in rapid fermentable carbohydrates, theoretically enhance the risk on rumen acidosis and may induce high ruminal histamine concentrations. The condition of a recurrent low rumen pH in combination with increased levels of ruminal histamine is associated with laminitis in dairy cows. Therefore, a controlled feeding trial was designed to test the idea that CP supplementation results in rumen acidosis and high ruminal histamine concentrations. It was found that supplemental CP, either degradable or undegradable, did not induce rumen acidosis but significantly increased histamine concentrations in the rumen fluid. However, the rise in ruminal histamine concentrations was considered physiologically unimportant and not related to the observed cases of SCL in that study. The lack of effect of supplemental CP on rumen pH was most likely due to the relative low level of dry matter intake.

Next to histamine, lipopolysaccharides (LPS) also are implicated in the etiology of laminitis. Lipopolysaccharides are released during rumen acidosis due to the lysis of ruminal bacteria. The high content of rapid fermentable carbohydrates, in the form of cassava chips, in Thai dairy rations may predispose the cows to rumen acidosis and a subsequent release of LPS. Therefore, an experiment was designed to gain insight between the source of starch, rumen pH, and rumen concentrations of histamine and LPS under Thai feeding conditions. In this study two sources of starch were used; cassava and corn. It was anticipated that the replacement of corn meal by cassava meal induced rumen acidosis. However, it appeared that the source of starch did not affect rumen pH. Both ground corn and cassava induced rumen acidosis which was associated with increased levels of rumen histamine and LPS. However, both histamine and LPS were not detected in plasma. Cases of SCL were only observed in the first out of the four experimental periods. Thus, it was difficult to relate these cases of laminitis with the values of LPS and histamine in rumen fluid.

From a practical viewpoint, agents that can irreversibly trap LPS are of interest because of their potential effect to prevent laminitis. Due to their chemical and conformational structure, phyllosillicates have the potential to trap LPS. The idea that a phyllosillicate, in the form of hydrate sodium calcium aluminosilicate (HSCAS), can adsorb LPS, was tested in cows suffering from experimentally induced rumen acidosis. However, it appeared that HSCAS had no effect on the concentration of free LPS in rumen fluid. Therefore, the use of supplemental HSCAS does not appear to be an effective strategy to prevent laminitis. To date, the precise etiology of laminitis remains obscure but a deteriorated fermentation in association with a vascular dysfunction in the claw, appears to be relevant to trigger laminitis. Histamine appears to be not relevant to explain the high prevalence of laminitis in Thailand because dietary CP levels are too low in practice. The relevance of LPS in relation to the high prevalence of laminitis in Thailand can be disputed and the underlying mechanism by which LPS influences the occurrence of laminitis remaines unclear.

In conclusion, the prevention of rumen acidosis appears to be the most effective strategy to prevent laminitis. Thus, it is recommended that Thai farmers should supply roughage *ad libitum* and/or mix appropriate amounts with concentrates. Finally, insulin and advanced glycation endproducts (AGEs) are implicated in relation to the etiology of equine laminitis but this concept is not yet studied in ruminant. Therefore, the relationship between insulin, AGEs and the occurrence of laminitis should be investigated in bovine because this might yield new clues on the nutrition related aspects of laminitis.

Samenvatting

Laminitis (klauwbevangenheid) wordt gezien als een belangrijk gezondheids-probleem van melkvee in Thailand. Hoewel bij het ontstaan van laminitis meerde factoren van belang zijn, wordt de voeding als een belangrijke risicofactor gezien. Het doel van dit proefschrift was na te gaan in hoeverre een aantal van deze risicofactoren van belang zijn voor het ontstaan van laminitis onder praktijkomstandigheden in Thailand. De samenstelling van Thaise melkveerantsoenen is niet goed gedocumenteerd, maar in het algemeen worden de koeien gevoerd met een lage kwaliteit ruwvoer zoals rijststro, aangevuld met niet-gepelleteerde krachtvoeders. Vooral cassave wordt veel gebruikt en het aandeel cassave in Thaise melkveerantsoenen bedraagt soms wel 70% van de droge stof. Hierdoor bevatten deze rantsoenen een hoog gehalte aan snel fermenteerbare koolhydraten, een laag gehalte aan celwand koolhydraten en een laag eiwitgehalte. Dit vormde de aanleiding om te onderzoeken in hoeverre de variatie in eiwitgehalte, celwand koolhydraten en voermanagement gerelateerd is aan de prevalentie van laminitis bij melkkoeien onder Thaise condities. In dit onderzoek zijn van 25 Thaise melkveehouderijen gegevens verzameld en het bleek dat het voeren van rantsoenen met een laag gehalte aan celwand koolhydraten gerelateerd was aan een hoge prevalentie van laminitis (>25%). Dit resultaat was niet eenduidig te interpreteren omdat de bedrijven waar rantsoenen gevoerd werden met een laag gehalte aan celwand koolhydraten, ook het ruwvoer en het krachtvoer gescheiden aan de koeien voerden. Toch lijkt het gemengd voeren van krachtvoer met een substantieel aandeel ruwvoer een belangrijke strategie om laminitis te voorkomen.

Thaise melkveerantsoenen met een hoog gehalte aan snel fermenteerbare koolhydraten in combinatie met een laag eiwitgehalte zorgen er voor dat de pensfermentatie niet optimaal verloopt en dat de melkproductie achter blijft. Verhoging van het eiwitgehalte van Thaise melkveerantsoenen lijkt een gepaste oplossing vanuit een economisch perspectief. Echter, een hoog eiwitgehalte in combinatie met een hoog aandeel snel fermenteerbare koolhydraten, kan leiden tot een te snelle pensfermentatie waardoor er pensverzuring kan optreden. Onder dergelijke condities kan er ook vorming van histamine in de pens plaats vinden. De combinatie van pensverzuring en hoge histamine concentraties wordt geassocieerd met laminitis bij melkkoeien. Daarom is in een tweede proef onderzocht in hoeverre een verhoging van het eiwitgehalte leidt tot pensverzuring en verhoogde histamine concentraties in de pens. Uit de resultaten bleek dat bij de rantsoenen met een hoog

eiwitgehalte geen pensverzuring ontstond, maar dat de histamine concentratie in de pens wel significant verhoogd werd. De verhoogde histamine concentraties hadden geen fysiologische betekenis en konden niet gerelateerd worden aan het optreden van subklinische laminitis in deze studie. Het feit dat bij de rantsoenen met het hoge eiwitgehalte geen pensverzuring ontstond kan waarschijnlijk verklaard worden door het relatief lage voerniveau in deze proef.

Naast histamine, worden ook lipopolysacchariden vaak genoemd als risicofactor voor het ontstaan van laminitis bij melkkoeien. Lipopolysacchariden zijn onderdeel van de celmembranen van pensbacterieën en komen vrij bij pensverzuring. Vanwege het hoge gehalte aan snel fermenteerbare koolhydraten (cassavezetmeel) in Thaise melkveerantsoenen is de kans op pensverzuring verhoogd met als potentieel gevolg hoge concentraties aan lipopolysacchariden in de pensvloeistof. In een derde experiment is daarom de relatie tussen zetmeelbron, pens pH en de concentraties aan histamine en lipopolysacchariden onderzocht. In deze studie zijn maiszetmeel en cassavezetmeel als zetmeelbron gebruikt. De verwachting was dat vervanging van maiszetmeel door cassavezetmeel pensverzuring tot gevolg zou hebben, maar dit werd niet door de resultaten bevestigd. Zowel op rantsoenen met maiszetmeel als die met cassavezetmeel werd pensverzuring en verhoogde concentraties aan histamine en lipopolysacchariden in de pensvloeistof waargenomen. Echter, deze stoffen konden niet in het bloed worden aangetoond. Alleen in de eerste van de vier experimentele perioden werden gevallen van subklinische laminitis waargenomen. Het bleek niet mogelijk deze gevallen van laminitis te relateren aan de histamine- en lipopolysaccharide concentraties in de pensvloeistof.

Om laminitis te voorkomen zijn stoffen die lipopolysacchariden irreversibel kunnen binden vanuit een praktisch oogpunt potentieel interessant. Op basis van hun chemische en ruimtelijke eigenschappen zijn phyllosilicaten (bodemmineralen) potentieel in staat om lipopolysaccariden te binden. Deze mogelijkheid werd onderzocht in een vierde studie bij melkkoeien waarbij pensverzuring was geïnduceerd. Tijdens deze studie werd een gehydrateerde vorm van natrium-calcium-aluminiumsilicaat (HSCAS) getest. Het bleek dat HCAS geen effect had op de concentratie van vrije lipopolysacchariden in pensvloeistof en dus praktisch niet van belang is om laminitis te voorkomen.

De exacte ontstaanswijze van laminitis is nog steeds niet bekend, maar een combinatie van een verstoorde pensfermentatie en verminderde doorbloeding in de klauw lijken belangrijk te zijn. De hoge prevalentie van laminitis in Thailand lijkt niet verklaard te kunnen worden door histamine vanwege de te lage eiwitgehalten in Thaise melkveerantsoenen. Het

belang van lipopolysacchariden in relatie tot de hoge prevalantie van laminitis in Thailand is twijfelachtig en het onderliggende mechanisme is niet duidelijk.

Geconcludeerd kan worden dat de preventie van pensverzuring de meest effectieve strategie is om laminitis te voorkomen bij Thaise melkkoeien. Voor de dagelijkse praktijk van de Thaise melkveehouders betekent dit dat zij hun melkkoeien onbeperkt ruwvoer aan moeten bieden en/of voldoende ruwvoer door het krachtvoer moeten mengen. Uit de literatuur blijkt dat bij paarden zowel insuline als versuikerde eiwitten (zogenaamde advanced glycation endproducts of AGEs) een rol lijken te spelen bij het ontstaan van laminitis (hoefbevangenheid). Bij herkauwers is hierover nog niets bekend maar onderzoek naar de rol van insuline en AGEs bij laminitis bij melkvee zou een nieuw inzicht op kunnen leveren met betrekking tot de voedings-gerelateerde oorzaak van laminitis.

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Curriculum vitae

Rittichai Pilachai was born on the 25th July 1970 in Khon Kaen, Thailand. He obtained his Doctorate of Veterinary Medicine from Chulalongkorn University, Thailand in 1995. During 1995-1998, he worked as a bovine practitioner at the Dairy Promotion Organization of Thailand. Since November 1998, he has been a lecturer at the Faculty of Science and Technology, Udon Thani Rajabhat University. In 2005, he obtained his Master of Science (Theriogenology) degree from the Faculty of Veterinary Medicine, Khon Kaen University, Thailand. One year later, he was promoted to assistant professor at the Faculty of Technology, Udon Thani Rajabhat University. In 2008, he was selected to participate in the Asia-Link Programme (Phase III) in the project entitled "The conversion of local feeds into human food by the ruminant". During this three-year EU funded programme, he started research that focused on feeding practices and potential risk factors for laminitis in dairy cows in Thailand. His position of assistant professor and his academic work in dairy cows will be continued at the Faculty of Technology, Udon Thani Rajabhat University.

List of Publications

Peer-reviewed journals

1. **Pilachai, R.**, Schonewille, J.Th., Thamrongyoswittayakul, C., Aiumlamai, S., Wachirapakorn, C., Everts, H., Hendriks, W.H., 2013. Diet factors and subclinical laminitis score in milking cows of smallholder dairy farms in Thailand. Livest. Sci. 155,197-204.
2. **Pilachai, R.**, Schonewille, J.Th., Thamrongyoswittayakul, C., Aiumlamai, S., Wachirapakorn, C., Everts, H., Hendriks, W.H., 2012. Starch source in high concentrate rations does not affect rumen pH, histamine and lipopolysaccharide concentrations in dairy cows. Livest. Sci.150, 135-142.
3. **Pilachai, R.**, Schonewille, J.Th., Thamrongyoswittayakul, C., Aiumlamai, S., Wachirapakorn, C., Everts, H., Hendriks, W.H., 2012. The effects of high levels of rumen degradable protein on rumen pH and histamine concentrations in dairy cows. J. Anim. Physiol. Anim. Nutr. 96, 206-213.
4. **Pilachai, R.**, Aiumlamai, S., Wongsrikeao, W., and Sirisatien, S. 2006. Effect of GnRH or hCG administration on the 5^{th} day-post insemination regarding serum progesterone concentration and conception rate in lactating cows during hot season. KKU. Vet. J. 16(1): 61-70.
5. **Pilachai, R.**, Aiumlamai, S., Wongsrikeao, W., and Sirisatien, S. 2004. A study on post-insemination administration of GnRH or hCG on serum progesterone concentration and conception rates in dairy heifers during hot season. J. Thai Vet. Med. Assoc. 55(2): 43-54.

Contributions to conferences and symposia

1. **Pilachai, R.**, S. Budpraserd, S. Chumnanbungkae and N. Parakun. 2011. Effect of substituting ground corn with cassava starch on rumen pH and blood parameters in dairy cows. The Third International Conference on Sustainable Animal Agriculture for Developing Countries, Nakhon Ratchasima, Thailand. (Oral presentation)

2. **Pilachai, R.**, Schonewille, J.Th., Thamrongyoswittayakul, C., Aiumlamai, S., Wachirapakorn, C., Everts, H., Mullaert, M., and Hendriks, W.H. 2011. In vitro efficacy of adsorbents in trapping lipopolysaccharides from rumen fluid of dairy cows. International Conference on Global Issues Influencing Human and Animal Health for ASEAN; One Health Concept, 2011, KhonKaen, Thailand. (Oral presentation)
3. **Pilachai, R.**, Schonewille, J.Th., Chaiyotwittayakun, A., Aiumlamai, S.,Wachirapakorn, C., Everts, H. and Hendriks, W.H.. 2010. The Effect of Variable Proportions Rapid Degradable Carbohydrate on Rumen pH and Endotoxin Concentrations in Dairy Cows. 13th Association of Institutions for Tropical Veterinary Medicine (AITVM) Conference 2010, Bangkok, Thailand. (Oral presentation)
4. **Pilachai, R.**, Schonewille, J.Th., Chaiyotwittayakun, A., Aiumlamai, S., Wachirapakorn, C., Everts, H., Hendriks, W.H. 2010. Replacement of cassava chips by corn meal does not alter rumen pH and rumen lipopolysaccharides in dairy cows fed high concentrate diets. International Conference on Production diseases in Farm Animals (ICPD) 2010, Ghent, Belgium. (Poster presentation)
5. **Pilachai, R.,** Schonewille, J.Th., Chaiyotwittayakun, A., Aiumlamai, S., Wachirapakorn, C., Everts, H., and Hendriks, W.H. 2009. The Effects of High Levels of Rumen Degradable Protein on Rumen Fermentation and Rumen Histamine Concentrations in Dairy Cows. XIth International Symposium on Ruminant Physiology – Clermont Ferrand, France. 6-9 September 2009. (Poster presentation)
6. **Pilachai, R.**, Schonewille, J.Th., Chaiyotwittayakun, A., Aiumlamai, S., Wachirapakorn, C., Seesupa, S., Everts, H., Hendriks, W.H., 2009. Feeding pattern risk factors for subclinical laminitis of milking cows in small holder dairy farm, Thailand. The 35th Veterinary Medicine and Livestock Development Annual Conference. Procongress Co. Ltd., Bangkok, Thailand, pp. 577-581. (Poster presentation)
7. **Pilachai, R.** and Parinyasutinun, U. 2001. Synchronization of estrus and fertility rate in dairy cow by luprostiol injection via vulvosubmucosal. KKU. Animal Agricultural Seminar for 2001, 26-27 January 2001. Faculty of Agriculture, KhonKaen University. pp. 260-269. (Oral presentation)