DEVELOPMENT OF ALTERNATE MARKERS FOR SUBACUTE RUMINAL ACIDOSIS (SARA)

By

Naveen Gakhar

A Thesis

Submitted to Faculty of Graduate Studies

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FACULTY OF GRADUATE STUDIES

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Of

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ABBREVIATIONS

BCS	Body condition score
CDC	Canadian Dairy Commission
CP	Crude protein
D1	Diet1
D2	Diet2
D3	Diet3
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
EU	Endotoxin unit
F:C	Forage-to concentrate ratio
IVDMD	In vitro dry matter digestibility
LAL	Limulus amoebocyte lysate
LPS	Lipopolysaccharide
MCH	Mean corpuscular hematocrit
MCHC	Mean corpuscular hematocrit
MCP	Microbial crude protein
MCV	Mean corpuscular volume
mM	millimolar
mOsm	milliosmoles
NABE	Net acid base excretion
NRC	National Research Council
PD	Purine derivative

peNDF	Physically effective neutral detergent fibre
RBC	Red blood cell
S1	Artificial saliva1
S2	Artificial saliva2
S3	Artificial saliva3
SARA	Subacute ruminal acidosis
SMLR	Stepwise multiple linear regression
TMR	Total mixed ration
VFA	Volatile fatty acid
VLDL	Very low density lipoprotein
WBC	White blood cells

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ABSTRACT

An *in vivo* and an *in vitro* experiment were conducted to study the effects of Subacute ruminal acidosis (SARA) induction on parameters that could be used for the diagnosis of SARA. The objectives of the first experiment conducted *in vivo* were to determine the relationships between rumen pH and selected biochemical markers in milk, urine, feces, blood that might be suitable for diagnosis of SARA and to compare the above relationships between the "grain-pellet SARA challenge" and "alfalfa-pellet SARA challenge". In the second experiment, the objectives of were a) to use a Rusitec in vitro continuous culture system to simulate SARA by decreasing the buffering capacity of saliva following alteration in its composition along with reducing the forage: concentrate (F:C) ratio in the diets and b) to study the effects of varying artificial saliva composition and dietary forage-to-concentrate ratios on rumen pH, free lipopolysaccharide (LPS), total coliform numbers, in vitro dry matter digestibility (IVDMD) and protozoa. In the first experiment, replacing 26% of the control total mixed ration (TMR) with alfalfa pellets in the "alfalfa-pellet SARA challenge" resulted in an increase of average duration of rumen pH < 5.6 from 43.0 to 234.3 min/d. Replacing 12% of control TMR with grain pellets in the "grain-pellet SARA challenge" resulted in an increase of average duration of rumen pH < 5.6 from 72.1 to 349.8 min/d. The "alfalfa-pellet SARA challenge" increased ($P<0.05$) milk protein (3.05 vs 3.12 %), total water intake per day (106.1 vs 116.8 L), urine pH (8.12 vs 8.24), net acid base excretion (NABE) (127.6 vs 182.2 mol/L), blood sodium (136.0 vs 137.1 mmol/L), blood PCV (0.26 vs 0.27 L/L), RBC (5.57 vs 5.76 X 10^{12} /L) and hemoglobin (97.6 vs 101.5 g/L), while milk yield, milk fat, dry matter intake (DMI), blood pH, fecal pH, microbial protein synthesis, blood lactate

remained unaffected. The "grain-pellet SARA challenge" increased ($P<0.05$) milk protein (3.06 vs 3.23), blood sodium (135.8 vs 137.0 mmol/L), blood lactate (0.80 vs 1.14 mmol/L) but decreased milk yield (22.6 vs 20.1 kg/d), and did not affect the DMI, blood pH, fecal pH, urine pH, NABE, microbial protein synthesis. Multivariate discriminant analysis indicated that the best indicator of the "grain-pellet SARA challenge" was blood glucose, blood CO_2, milk yield, milk protein % and blood sodium while in the "alfalfa-pellet SARA challenge" it was urine pH. The results of the stepwise multiple linear regression (SMLR) indicated that milk fat, milk protein and sodium were the best indicators of rumen pH.

In the second experiment, both artificial saliva and the diets were supplied at three levels for induction of SARA in Rusitec. The three levels in artificial saliva were strong (S1), medium (S2) and weak buffer (S3) based on the composition and buffering capacity of the artificial saliva. The dietary levels differed in F:C ratios with F:C ratio being 80:20, 50:50 and 20:80 for diet 1 (D1), diet 2 (D2) and diet 3 (D3) respectively. The lowest pH at 6 h after feeding and the highest dry matter digestibility was obtained at S3-D3 (weak buffer, 80% concentrate) treatment combinations. The average pH observed under this combination of diet and buffer was 5.86. Induction of SARA did not affect the concentrations of lipopolysaccharide and the number of coliform bacteria. However, SARA induction reduced the average daily pH (7.10 vs 6.85 and 5.86) and IVDMD of diets (54.54 vs 50.37 and 48.42 %) from S1 to S2 and S3. The saliva with the weakest buffering (S3) recorded lowest average daily pH with F:C ratio of 20:80. The S3 saliva also recorded the lowest IVDMD and protozoa per ml amongst all the saliva used.

In conclusion milk fat, milk protein and blood sodium appeared to be the best predictors of rumen pH. Dissimilarity in the response of urine pH, NABE, water intake per day and certain blood parameters to SARA induction in experiment 1 by both methods, further complicates the development of alternate markers for this disease. The results from experiment 2 warrant further research to determine effects of greater magnitude of decreased pH on free LPS, coliform numbers, protozoa and microbial fermentation. The detailed analysis of global rumen fluid microbiota in an in vitro model of SARA, therefore, remains to be investigated.

FOREWORD

This thesis is written in manuscript style, with each manuscript having its own abstract, introduction, materials and methods, results and discussion sections. There is a general introduction and review of the literature prior to the manuscripts, which are followed by the general discussion and conclusions, and list of references sections.

1 GENERAL INTRODUCTION

Subacute ruminal acidosis (SARA) is a common metabolic disorder in cattle that is characterized by daily episodes of low rumen pH. Various authors have defined SARA as a digestive disorder caused by ingestion of diets rich in rapidly fermentable carbohydrates and lacking physically effective neutral detergent fibre (peNDF) required for adequate rumen buffering, leading to daily episodes of low rumen pH (Kleen et al., 2003; Plaizier et al., 2008). There is a disagreement in the literature regarding exact threshold pH to be used for the presence of SARA. Threshold rumen pH values of 5.5 (Hibbard et al., 1995), 5.6 (Cooper and Klopfenstein, 1996), 5.8 (Beauchemin et al., 2001; Nordlund et al., 1995) and 6.0 (Krehbiel et al., 1995) have been used to define SARA.

This disease results in annual economic losses amounting to $500 million to $1 billion in US dairy industry (Enemark, 2008). These losses are due to reduced milk production, depression in milk fat, increased veterinary costs and high involuntary cullings due to lameness (Nordlund et al., 1995; Nocek, 1997; Stone, 1999; Kleen et al., 2003). Mild diarrhea, ruminal stasis, unexplained abscesses, dehydration, abdominal discomfort and excessive salivation are signs that can accompany SARA (Owens et al., 1998). These signs are not specific to SARA, but they can aid in the diagnosis of the disorder. Subacute ruminal acidosis also has a negative effect on the digestibility of fibre, rumen health and rumen microbial populations (Plaizier et al., 2001; Krajcarski-Hunt et al., 2002). The disease can affect 20 % of the lactating cows between 1-120 days of milk within a herd (Oetzel et al., 1999). Further, it is assumed that the depression of rumen pH in SARA is due to the accumulation of volatile fatty acids (VFA's) rather than lactate as

the concentration of lactate are maintained below 10 m*M* (Burrin and Britton, 1986; Goad et al., 1998).

Recording of feed intake, milk production in a herd and rumen pH measurements form part of the diagnostic process of SARA (Nordlund et al., 1995; Enemark et al., 2002; Nordlund, 2002). Currently, measurement of the pH of rumen fluid is the only diagnostic tool used for the confirmation of SARA. Rumenocentesis and stomach tubing are the two methods that can be used for rumen fluid collection under field conditions. The disadvantage associated with the rumenocentesis method is that it is invasive, and can result in abscesses at the site of puncture (Hollberg, 1984; Aceto et al., 2000). The samples collected by stomach tube are often contaminated with saliva and, as a result do not reflect the true rumen pH (Nordlund and Garrett, 1994; Oetzel and Nordlund, 1998).

The literature presented in this thesis outlines the etiology of SARA, its diagnosis and the effects of SARA in dairy cows. The objective of the first part of the study presented herein was to induce SARA in lactating dairy cows by feeding of diets having inadequate forage particle size and also by increasing the starch content of diets. The relationships between rumen pH and selected biochemical markers in milk, urine, feces, blood that might be suitable for prediction of SARA were determined. In the second part of this study, SARA was simulated in a "Rusitec" *in vitro* continuous culture system by varying composition of the artificial saliva in order to alter rumen buffering and by altering forage-to-concentrate ratios of the diets. The effects of the SARA induction on free liopolysaccharide (LPS), total number of coliform, protozoa, in vitro dry matter digestibility (IVDMD), ammonia nitrogen and VFA's were determined.

It was expected that these studies will aid in developing alternate tools for the diagnosis of SARA that can be used on farm.

2.0 LITERATURE REVIEW

2.1 General introduction on acidosis and definition of SARA

Improved genetic potential of cows accompanied by improved management practices such as better nutrition and health care have lead to increased milk production of dairy cows across the world. The average milk production of Canadian dairy cows has increased from 7,412 kg of milk per cow to 9,538 kg of milk per cow from 1990 to 2007 (CDC, 2003). In order to sustain high levels of production, the high yielding dairy cows require more energy and protein rich diets as compared to low yielding dairy cows. These diets are typically low in physically effective neutral detergent fibre (peNDF) and high in starch content, and, are very rapidly fermentable in the rumen. Cows not adapted to these diets become susceptible to rumen acidosis. This is commonly seen in transition cows, when they are switched from a fibre-based diet to a concentrate-based diet in order to meet high energy demands due to lactation. This change of diet requires a transition period of its own in order to allow the rumen microbes and epithelium to adapt to the increased intake of concentrate (Owens and Goetsch, 1988). Rumen acidosis results in wide array of physiological and biochemical stresses due to the accumulation of ruminal organic acids and endotoxins from excessive ruminal fermentation (Britton and Stock, 1986; Stock, 2000).

Rumen acidosis has been broadly classified into two general forms i.e. acute and subacute. Acute acidosis is more lethal and less commonly seen (Owens et al., 1998). Feeding of diets rich in readily fermentable carbohydrates e.g. grains, fodder beets or other feeds containing high levels of available starch, sucrose, fructose, lactose or glucose

predispose cows to rumen acidosis (Simensen and Konggaard, 1970; Dunlop, 1972). These feedstuffs get readily fermented in rumen, leading to accumulation of the lactic acid driving the rumen pH below 5.0 and promoting the growth of acid-resistant lactogenic bacterial species like *Streptococcus bovis* and *Lactobacillus acidophilus* (Van Soest, 1994). In acute rumen acidosis the lactic acid concentration increases to more than 5.0 m*M* (Underwood, 1992; Oetzel, 2000) and can rise up to 300 m*M* (Hyldgaard and Simensen, 1966). Fermentation of these readily fermentable carbohydrates adversely impacts the rumen microbial population by production of VFA's over and above the levels required for the maintenance of a normal rumen pH. At a rumen pH below 5.0, lactogenic bacteria dominate resulting in the accumulation of D- and L isomer of lactic acid in the rumen with D-isomer being the dominant (Dunlop and Hammond, 1965). This further leads to increased osmolality of ruminal contents which interferes with VFA absorption and exacerbates the acid accumulation (Huber, 1976; Owens et al., 1998). Anorexia, tachycardia, diarrhea, lethargy, ruminal stasis, recumbancy and death are the clinical signs associated with acute rumen acidosis.

The second form of acidosis is subacute ruminal acidosis (SARA). Various authors have defined SARA as a digestive disorder caused by excessive consumption of rapidly fermentable carbohydrates which results in low rumen buffering that is characterized by daily episodes of low rumen pH between 5.2 and 6.0 (Britton and Stock, 1986; Krehbiel et al., 1995 a, b, c; Cooper and Klopfenstein, 1996; Beauchemin et al., 2001; Cumby et al., 2001; Koenig et al., 2003). In the literature, this form of acidosis was first mentioned by Dirksen (1965). Subacute ruminal acidosis is more prevalent, but less

lethal than acute acidosis (Britton and Stock., 1986). Garrett et al. (1997) carried out a survey on 15 dairy farms of Wisconsin and reported an incidence rate for SARA as 19% in early lactation cows and 26% in mid-lactation cows. After their ingestion, the readily fermentable carbohydrates in the rumen undergo microbial digestion converting the carbohydrates to VFA's, CO_2, lactate and methane. The VFA's are absorbed across the ruminal wall and buffered in the blood (Owens et al., 1998). In normal physiological conditions, a balance exists between production of VFA and their absorption and neutralization. With the increase in microbial action, the rate of production of VFA exceeds that of absorption, and, hence VFA's accumulate in the rumen resulting in rumen pH depression. The most abundant VFA's are acetic acid, propionic acid and butyric acid. Source of carbohydrates, pre-treatment of feeds such as steam flaking, rolling or popping guides the concentration of these VFA's in the rumen fluid (Owens et al., 1998). Generally, fermentation of the starch in the rumen results in the production of propionic acid while cellulose decomposition results in acetic acid and fermentation of soluble carbohydrates results in formation of butyric acid (Van Soest, 1994). When dietary starch content is increased, the ratio is between acetic acid, propionic acid and butyric acid, is shifted towards propionic acid (Van Soest, 1994). In SARA, the level of lactic acid does not increase beyond 5-10 mmol per litre (Hibbard et al., 1995). The low pH encountered in SARA is due to the build up of VFA's rather than accumulation of lactic acid as encountered in acute rumen acidosis (Underwood, 1992; Oetzel, 2000).

Symptoms associated with SARA are diffuse and inconsistent (Nordlund et al., 1995). As a result, SARA can be difficult to diagnose and often goes undetected. Clinical signs associated with SARA include erratic feed intake, body condition losses, milk fat

depression, unexplained abscesses, and, diarrhea (Nordlund et al., 1995; Nocek, 1997; Kleen et al., 2003). Nocek (1997) stated that these changes are also associated with poor bunk management, and, hence are of limited value for diagnosis of SARA. Transition cows from the dry to the lactating stage, cows having high dry matter intakes, cows subjected to higher variability in ration and meal patterns are at the risk of SARA (Stone, 2004).

2.2 SARA and production economics

There are many direct and indirect economic losses associated with SARA. Direct losses include acute death, treatment costs, and indirect losses include wastage of feed, delay in marketing of animals, reduced feed efficiency and loss of milk production (Underwood, 1992). According to recent estimates, SARA results in annual economic losses amounting to $500 million to $1 billion in the US dairy industry through milk and reduced longevity (Enemark, 2008). Stone (1999) estimated losses occurring due to SARA at $ 1.12 per day per cow in a herd of 500 cows where at least one-third of cows had rumen pH below 5.6 and over 50% of the cows with rumen pH below 5.8.

2.3 Clinical signs associated with SARA

Various clinical signs are associated with SARA. Some of these signs are overt and can easily be detected, whereas others are inconsistent (Kleen et al., 2003). Animals affected with SARA are anorectic, dehydrated, have erratic or reduced appetite and show unexplained abscesses (Underwood, 1992; Britton and Stock, 1986). Mild diarrhea, ruminal stasis, reduced milk production, milk fat depression, abdominal discomfort,

excess salivation are the signs that accompany SARA (Owens et al., 1998). In prolonged cases of SARA, rumenitis, laminitis, fall in body condition score (BCS) and epistaxis may occur (Owens et al., 1998; Oetzel, 2000). Slight dehydration, compensatory metabolic acidosis, hypercalcuria, hyperphosphatemia, hypokalemia and hypercalcemia may also occur (Furll, 1994). Signs associated with SARA are often varied and ambiguous, which complicates the definitive diagnosis of this disease. Also, signs associated with SARA are not as distinct as they are in acute rumen acidosis. Additionally, clinical signs may appear after several weeks of the onset of this disease. Clinical signs observed in SARA are related to the herd thus the diagnosis of SARA should not be solely based on the examination of single animal (Enemark et al., 2004). Early lactation cows maintained on concentrate-based diets show considerable drop in rumen pH, rumen tympany, loose manure, stupor, reduced appetite, decreased rumen motility, decreased rumination time and drop in milk yield (Eddy, 1992)

2.4 Rumen pH

Threshold rumen pH values of 5.5 (Hibbard et al., 1995), 5.6 (Cooper and Klopfenstein, 1996), 5.8 (Beauchemin et al., 2001; Nordlund et al., 1995) and 6.0 (Krehbiel., 1995) have been used in the literature to define SARA. As already mentioned the drop in rumen pH during SARA is due to the increase in total VFA, rather than increase in lactate, as there is very small concentration (<5 mM) of lactate during SARA (Britton and Stock, 1986; Owens et al., 1998; Ghorbani et al., 2002). So, while determining decline in rumen pH during SARA total organic acid content and not just lactate should be considered (Slyter, 1976; Goad et al., 1998; Owens et al., 1998).

Effects of SARA are noticeable when the rumen pH is depressed below 5.6 for atleast 3 h in a day (Keunen et al., 2002). Depression of rumen pH below optimal rumen pH of 6.3 (de Veth and Kolver, 2001) for a brief period of time is unlikely to affect the health of cows. If the rumen pH is depressed below 6.0 for less than 4 h rumen fermentation is negatively affected in terms of VFA production, apparent dry matter digestibility and neutral detergent fibre digestibility (Calsamiglia et al., 2002). Feed factors such as type of concentrate, grain processing and forage type play an important role in predisposing the cattle to low rumen pH. Feeding highly fermentable carbohydrates to cows leads to the production of VFA's beyond the absorptive capacity of the ruminal papillae, thus resulting in the accumulation of these VFA's, and, subsequently to a fall in rumen pH (Dirksen et al., 1985; Nordlund et al., 1995; Kleen et al., 2003). This decline in rumen pH can be prevented by step-wise introduction of concentrate in the diet of animals spread over a period of several weeks. Since SARA is more common in transition cows or early lactation cows step-wise introduction of the concentrate in the diet of these animals, allows the ruminal papillae to increase in the size and density, thus enhancing the absorptive surface of rumen and preventing depression in rumen pH below optimal levels either due to accumulation of VFA or lactic acid (Kleen et al., 2003). Moreover, it helps in the maintenance of balance between lactic-acid producing bacteria and lactic-acid utilizing bacteria (Kleen et al., 2003).

Rumen pH is considered to be of utmost importance while diagnosing SARA (Enemark et al., 2004). Depression of the rumen pH below 5.6 can lead to reduced dry matter intake and hence, maintaining rumen pH above 5.6 is very important (Stock,

2000). A herd can be declared SARA positive by measuring ruminal pH in a subsample of cows (Garrett et al., 1999). If one-third of the cows in a herd experience rumen pH below 5.6, then herd is declared SARA positive, and, if the rumen pH falls between 5.6-5.8, then SARA is a marginal problem in the herd (Nordlund et al., 1995). There are different opinions regarding the number of cows to be sampled for diagnosing SARA as a herd problem. Garett et al. (1999) suggested that in a sample of 12 cows at least 3 out of those 12 cows must have rumen pH below 5.5. Nordlund et al. (1995) suggested that if 30% of 10 or more sampled cows are below 5.50, the group is classified as experiencing ruminal acidosis. They recommended that 10 or more cows should be sampled from any group in which acidosis is suspected. It is important to avoid making a herd diagnosis based upon a few samples.

Diets low in peNDF lead to insufficient rumen buffering and inadequate saliva production (Mertens, 1997; Kleen et al., 2003; Stone, 2004). Mertens (1997) highlighted the importance of having adequate peNDF in the diet. The National Research Council (NRC, 2001) recommends having a minimum of 25% DM as NDF with 75% of it coming from forage component in the diet. Diets low in peNDF are often associated with SARA due to reduced rumination, low saliva secretion and rumen buffering, reduced acetate to propionate ratio and milk fat depression (Mertens, 1997).

2.5 Methods of rumen fluid collection

Currently three methods are used for the collection of rumen fluid, with each method having its own advantages and disadvantages.

a. Rumen canula: - In this approach, rumen canula is surgically placed in the rumen and rumen fluid samples are collected. This approach provides an accurate result of rumen pH and multiple samples can be collected in a day allowing accurate monitoring of rumen pH. However, the disadvantage associated with this method is that it is impractical to canulate an entire herd, and, hence cannot be carried on commercial dairy farm (Garrett et al., 1999; Oetzel, 2000).

b. Stomach tube: - Various stomach tubes have been used to collect rumen fluid (Dirksen and Smith, 1987; Geishauser, 1993). Large volumes of samples can be collected using this approach but the samples are often contaminated with saliva (Dirksen and Smith, 1987) and therefore their pH do not reflect true rumen pH (Nordlund and Garett, 1994; Oetzel and Nordlund, 1998). There is considerable variation in pH among different locations in the rumen (Bryant, 1964; Lane et al., 1968). In order to have representative pH of rumen composite sample from different sacs of rumen i.e. cranial dorsal, central, cranial ventral and caudal ventral of rumen should be collected (Bryant, 1964). However, it is difficult to collect such samples under field conditions. The oral probe used for collection of rumen fluid can also cause damage to the esophagus.

c. Rumenocentesis: - Samples can be collected from caudoventral part of rumen using this technique without performing any surgeries or contamination concerns. But the disadvantage associated with this method is that it is quite invasive, and can result in abscesses at the site of puncture (Hollberg, 1984; Aceto et al., 2000).

All methods can be used for the collection of rumen fluid samples at specific times of day but pH measurement in these samples may not represent true picture of rumen pH due the fact that there is a large diurnal variation in this pH (Plaizier et al., 2008).

2.6 Volatile fatty acids

Dietary carbohydrates are fermented to produce VFA's which are absorbed across the ruminal wall. Build up of VFA's results in depression of rumen pH, as the two follow a negative correlation. Also, the lowest pH coincides with the highest concentration of total VFA indicating a major role of total VFA in decreasing rumen pH (Hoover and Miller, 1992).

Excessive intake of readily fermentable carbohydrates leads to production of VFA's, which is more than the required level to maintain normal rumen pH. This results in a drop in rumen pH leading to SARA. In the absence of SARA, when there is no excessive consumption of readily fermentable carbohydrates, the carbohydrates are digested and there is production of pyruvate in the rumen which is further converted to VFA's supporting microbial growth (Owens et al., 1998) and there is no accumulation of VFA's. However, excess consumption leads to accumulation of VFA's because of the insufficient absorption of the VFA's in the rumen (Owens et al., 1998). If VFA production in rumen is either increased or steady, the inhibition of VFA absorption will lead to accumulation of VFA and drop in rumen pH accompanied by increased influx of bicarbonate from the blood stream into the rumen due to decreased exchange with ionized VFA's (Owens et al., 1998). Between rumen pH of 5.0-6.0, the drop in pH is attributed to increased VFA and when the rumen pH is below 5.0, lactic acid is the primary acid responsible for

depression in pH (Owens et al., 1998). According to various authors, the maximum depression in rumen pH is observed 4-8 h (Horn et al., 1979), 10 h (Krause and Oetzel., 2005) and 15 h (Keunen et al., 2002) after feeding. When there is excessive consumption of readily fermentable carbohydrates and the rumen pH drops, the proportion of VFA's produced in rumen also shifts. The concentration of acetate in rumen decreases, and, that of propionate increases and level of butyric acid remains relatively constant (Erfle et al., 1982; Calsamiglia et al., 2002).

2.7 SARA and rumen microbial populations

Under normal physiological conditions, rumen contains a population of protozoa, fungi, gram-positive and gram-negative bacteria (Underwood, 1992). Feeding of diets rich in fibre shifts the rumen microbial population towards cellulolytic bacteria and bacteria species such as *Ruminococcus albus, Bacteroides fibrisolvens* dominate, thereby limiting the activity of starch hydrolyzing bacteria. These fibre digesting bacteria are capable of digesting cellulose and hemicelluloses. Feeding diets high in readily fermentable carbohydrates initially increase all bacterial populations in the rumen because of the substrate availability (Nocek, 1997). Diets having more concentrate enhance the digestion of starch by promoting the activity of amylolytic and saccharolytic bacteria. Maintenance of high levels of readily fermentable carbohydrates promotes the activity of *Streptococcus bovis.* The number of *Streptococcus bovis* can increase from 10^7 to 10^9 per ml within 6 h of carbohydrate feeding along with its increased activity resulting in rapid conversion of starch to lactate and other organic acids (Van Soest, 1994). The end products of microbial fermentation of these carbohydrates are VFA's and lactic acid,

carbon dioxide and methane (Owens et al., 1998). Lactic acid producers include *Ruminococcus albus, Ruminococcus flaveciens, Lactobacillus aciodphillus* and some strains of *Selenomonas ruminantium* (Slyter, 1976). During SARA, when the rumen pH is between 5.2 and 5.6 the lactic acid producers like *Streptococcus bovis* and lactic acid utilizers like *Megasphera elsdenii* exist in equilibrium thereby preventing lactic acid accumulation. Therefore, the depressed rumen pH during SARA is due to insufficient rumen buffering and increased total VFA rather than increased lactate. Gradual increase of concentrate in the diet spread over several weeks of time helps in increasing the number of lactate acid utilizing bacteria in rumen such as *Selemonas ruminantium, Megasphera elsdenii* as the bacteria have more time to adapt to the concentrate diet as compared to the abrupt increase of the concentrate . These organisms may not necessarily utilize lactate when other alternative substrates are present. For example, *Selenomonas ruminantium* utilizes glucose instead of lactate. *Megasphera elsdenii* plays an important role in preventing acid build up in rumen as it is even active at a low pH (4.8). It is not clear if gram positive or gram negative bacteria predominate at the pH levels between 5.2 and 5.6. In general, rumen acidity results in reduction in cellulolytic bacteria and a shift in bacterial population so that gram positive bacteria predominate (Goad et al., 1998) even though the gram negative bacterial population may also increase at this pH (Nagaraja et al., 1978a).

2.8 Diagnosis of SARA

Various diagnostic tools and measurement of several parameters have been suggested for the diagnosis of SARA. They can be performed either on individual animals or on a herd basis.

2.8.1 Dry matter intake

Cows suffering from SARA exhibit erratic or cyclical feed intake, and, this is one of a primary indicator for the diagnosis of SARA (Stock and Britton, 1993; Cooper et al., 1999). Cyclic feeding pattern has also been suggested as a marker for SARA, as it reflects bouts of acidosis (Fulton et al., 1979a). Several studies have shown a reduced feed intake during periods of SARA (Olson et al., 1991; Brown et al., 2000). The reduced feed intake can be attributed to the drastic fall in rumen pH and increased osmolality of the rumen fluid. The appetite is regained after the establishment of normal rumen conditions (Fulton et al., 1979a, b). It is possible to detect this pattern in herds with automated feed dispensers where feed intake of individual cow is registered.

Fluctuations in feed intake have been observed when cattle were adapted to diets containing corn and wheat 35, 55, 75 and 90% of DM (Fulton et al., 1979a). Fluctuations in intake were observed at each inclusion rate of wheat in diet. Cattle reduced their feed intake for a few days after consumption of these diets in order to counter acidotic conditions (rumen pH < 5.6) and then again overate on attaining normal conditions in the rumen thus depicting cyclical feed intake (Fulton et al., 1979a). In contrast, animals consuming the corn diet showed same cyclical feeding pattern as observed with wheat only at 90% levels (Fulton et al., 1979a). Daily intake variation was more pronounced in both corn and wheat based diets at the 90% inclusion rate. In another experiment, feeding a 90% coarsely rolled wheat diet to the steers and maintaining the rumen pH above 5.6 by intra-ruminal infusion of sodium hydroxide resulted in increased dry matter intake thus

confirming that low pH reduced feed intake (Fulton et al., 1979b). This indicates that low pH depresses voluntary feed intake in cattle.

Feed intake patterns of dairy cattle are either governed by physical fill or chemostats (energy sensing mechanism in brain) or a combination of the two (Britton and Stock, 1986). If cows are fed high forage diets, physical fill or combination of physical fill and chemostatic factors guides the feed intake patterns (Stock and Britton, 1993). When the cows are fed diets containing higher proportion of grains the feed intake patterns are governed primarily by chemostatic factors coordinated in the brain (Stock and Britton, 1993). The ruminal microbial population needs to adapt accordingly to the substrate offered. This change of substrate to grains reduces the rumen pH and cattle adjust their daily intake patterns often resulting in reduced feed intake in an attempt to increase rumen pH (Stock and Britton, 1993; Phy and Provenza, 1998).

The large amounts of VFA's in the rumen, rumen hypomotility, histamine and the increased levels of gram-negative bacterial endotoxin also called lipopolysaccharide (LPS) in the rumen encountered during SARA also contributes to the reduction in the feed intake (Slyter, 1976; Owens et al., 1998; Huber, 1976; Kleen et al., 2003; Andersen, 2000). Lactate has also been suggested as a strong inhibitor of feed intake (Britton and Stock, 1986).

Subacute ruminal acdiosis has also been associated with the increased levels of dissolved minerals and organic acids in the rumen, that increase the osmolality of the rumen contents (Carter and Grovum, 1990). The increased osmolality stimulates the sensors located in the reticulo-ruminal wall which in turn cause reduction in feed intake

through negative feedback on the brain (Carter and Grovum, 1990). Differences in fiber content, particle size, ensiled forages, starch fermentability among diets, and, interaction among metabolic factors such as plasma hormones and endotoxin tolerance (Hurley, 1995) can cause differences in DMI among animals. Positive correlations between decreased DMI and low rumen pH have already been found (Fulton et al., 1995 a, b) but in some studies the day to day variation in ruminal pH was not accompanied by changes in DMI (Ghorbani et al., 2002).

2.8.2 Milk fat depression

One of the many signs associated with SARA is milk fat depression. Many dietary and metabolic factors affect the milk fat (Griinari et al., 1998). These include stage of lactation, breed of cow, forage particle size, physically effective fibre and the proportion of concentrates in the ration (Grummer, 1991). In ruminants, 50% of the fatty acids present in milk originate from de novo synthesis and the remainder from the uptake of long chain fatty acids from the diets (Griinari et al., 1998; Ensminger, 1993). Milk fat is also influenced by ratio of 'acetate to propionate' in the rumen fluid as there is a positive correlation between acetate to propionate ratio and milk fat (Grant et al., 1990; Sutton et al., 1986). Also, acetate is an important precursor for milk fat. When highly fermentable carbohydrates are fed, propionate production increases more than that of acetate, resulting in milk fat depression.

The increase in propionate can affect the milk fat levels by three possible mechanisms.

a. Increased glucose synthesis with more propionate entering gluconeogenesis. As glucose is an important precursor for lactose, hence more glucose leads to higher

milk yield without corresponding increase in milk fat, thus resulting in reduction of overall milk fat content.

b. Increased propionate levels in the rumen also leads to lower levels of vitamin B_{12} in the rumen and lesser amount of propionate is converted to succinyl CoA. This results in increased levels of intermediary metabolite methylmalonyl CoA in blood inhibiting fat synthesis (Van Soest, 1994).

c. Stimulation of insulin secretion and subsequent decrease in tissue lipolysis resulting in low levels of very low density lipoprotein (VLDL) in liver whuch are of importance in milk fat synthesis. Increased levels of circulating insulin encountered during feeding of high concentrate diets are suggested to have a role in decreasing milk fat (Griinari et al., 1998). Insulin leads to shortage of lipogenic precursors by preferential chanelling of nutrients such as acetate, β-hydroxybutyrate and long chain fatty acids (Bauman and Griinari, 2001). Diets rich in concentrate cause reduction of biohydrogenation of polyunsaturated fatty acids in the rumen. This incomplete biohydrogenation results in milk fat decline (Griinari and Bauman, 1999; Loor et al., 2004). The low milk fat associated with these diets is due to increase in trans-10 C18:1 rather than an increase in total trans-C18:1 (Bauman and Griinari, 2001).

The importance of roughage (peNDF) in maintaining the milk fat content has also been well evinced in the literature (Sutton, 1989; Mertens, 1997). Milk fat depression accompanies depression of rumen pH encountered in SARA, and can be used as an indicator for its diagnosis. Milk fat percentage seems to be a good indicator of fermentation conditions in rumen on individual basis and should be performed at least

once per week to be of any diagnostic importance (Erdman, 1988). According to Stone (1999) early lactation cows experiencing SARA mobilize body fat to counter the falling BCS and may show higher or normal milk fat levels. Table 2.1 has been adapted from Enemark et al. (2002) and it contains several biochemical markers in the milk that have been linked to SARA.

Table 2.1 Changes occurring in biochemical markers in milk due to SARA

Marker	Normal	Pathogenesis	Changes due to SARA
Fat-protein-quotient (FPK)	1 – 1.5	Decreased intraruminal acetate: propionate-ratio and subsequent decrease in milk fat content	< 1
Soxleth-Henkel-figure (SH)	6.4 – 6.8	Elimination of H^+ via the udder	> 8.0
Lactose	4.4 – 5.2 %	Increased intraruminal propionate formation → increased blood glucose	↑
Cl, Na, K	Cl: 25 – 31mmol/l Na: 20 – 26 mmol/l K: 30 – 40 mmol/l	?	Cl ↑ Na ↑ K ↓
Milk-urea-nitrogen (MUN)	3.0 – 5.0 mmol/l	Energy (carbohydrate) content of ration in favor of protein → reduced ruminal NH_3 –formation → reduced hepatic urea formation	< 3.0 mmol/l

Source - Enemark et al., (2002)

2.8.3 Diarrhea

According to Nocek, 1997 unexplained diarrhea in a herd signals the presence of SARA but Nordlund et al. (2004) concluded that its presence has limited use for the

diagnosis of SARA. Using fecal consistency as a tool for the diagnosis of SARA is matter of controversy. Cows fed diets containing insufficient peNDF content had feces with liquid consistency (Ireland-Perry and Stalling, 1993) although rumen pH was not monitored in this study, hence it is difficult to postulate that this would be true under rumen conditions observed in SARA. Appearance of feces can yield important information about SARA. Cows affected with SARA have brighter, yellowish (Kleen et al., 2003) and foamy feces (Nordlund et al., 2004). Foamy feces is an indicator of extensive hind gut fermentation (Nordlund et al., 2004) and can be a reason for diarrhea as fecal consistency is determined by the movement of water into the digestive tract when digesta becomes hypertonic to plasma as a result of SARA (Huber, 1976). Nordlund et al., (1995) reported herds experiencing SARA have loose feces containing substantial amounts of undigested feed. Presence of intermittent diarrhea along with undigested particles in the feces indicate inadequate digestion along with fast passage rate of feed (Enemark, 2002)

2.8.4 Abomasal displacement

Subacute ruminal acidosis is also treated as an etiological factor for abomasal displacement (Markusfeld, 1987; Olson, 1991). One of the possible reasons could be the increased back and forth movement of rumen derived gases such as VFA's, CO_2 and CH_4 between the forestomachs and the abomasum resulting in abomasal dilatation, atony and subsequent dislocation. High peNDF content in the diet is the most important factor for preventing abomasal displacement (Shaver, 1997), because functional fibre mat in the rumen plays an important in the gradual absorption and production of VFA in rumen

(Olson, 1991). Also, diets lacking adequate peNDF and rich in grain component reduce the muscle tone of abomasums predisposing the animal to abomasal displacement. Feed induced acidosis has been shown to cause abomasal ulceration in goats (Aslan et al., 1995).

2.8.5 Clinical examination parameters

Feeding energy rich ration significantly affects the rumen motility, pulse and respiration in beef cattle (Leedle et al., 1995). It is not clear that how these clinical parameters can be used for routine surveillance of SARA because of considerable variation within and between herds.

2.8.6 Blood and its metabolites

Blood pH of 7.35 or below signals the presence of systemic acidosis (Owens et al., 1998). There are studies stating that changes in acid base status of blood are minor during SARA (Goad et al., 1998; Brown et al., 2000). Blood gas parameters were not notably affected in chronic cases of metabolic acidosis (Lachmann and Siebert, 1980). Increased haemoconcentration accompanied by systemic dehydration due to efflux of water from extracellular compartment into the rumen (Slyter, 1976) results in swelling of ruminal papillae and damage to ruminal mucosa (Owens et al., 1998). Increased rumen osmolality or hypertonicity reduces the frequency of ruminal contractions by stimulation of receptors on the wall of reticulo-rumen along with decrease in the saliva production (Carter and Grovum, 1990). Increased osmolarity also results in elevated levels of blood CO_2, with corresponding reduction in blood bicarbonate levels (Huntington and Britton,

1979; Burrin and Britton, 1986; Krehbiel et al., 1995; Owens et al., 1998; Brown et al., 2000).

Subacute ruminal acidosis leads to increased osmolality of rumen contents higher than that of blood resulting in net inflow of body fluids in to the blood (Hoover and Miller, 1992). Osmolality in the rumen ranges between 240 to 265 mOsm/L and 280 to 300 mOsm/L when forage and concentrate based diets are fed respectively (Owens et al., 1998). Crichlow and Chaplin (1985) found a direct correlation between high osmolality and lactate levels. Bennink et al. (1978) stated that high osmolality reduces fermentation of cellulose and glucose. Osmolality levels higher than 400 mOsm / L inhibit microbial enzyme activity (Bergen, 1972). Increased osmolality damages the ruminal epithelium along with reduction in concentration of plasma or serum minerals especially Ca, and blood lactate dehydrogenase which is required to mobilize lactate (Owens et al., 1998; Ghorbani et al., 2002). Accumulation of lactate in the rumen increase the chances of metabolic acidosis as it causes increased lactate in plasma and hence lowered blood pH i.e. below 7.35 (Burrin and Britton, 1986; Krehbiel et al., 1995; Owens et al., 1998; Brown et al., 2000). Lactic acid accumulation reduces aerobic metabolism resulting from exhaustion of compensatory mechanism (Burrin and Britton, 1986).

In addition to lactate, the levels of histamine, tryptamine and tyramine are also increased during SARA, thus causing changes in the clinical picture of blood after entry into blood (Koers et al., 1976; Dirksen, 1985). Osmolality levels higher than 300 mOsm have been suggested to reduce feed intake because of the stimulation of osmopreceptors in rumen (Owens et al., 1998; Carter and Grovum, 1990). Rumen acidosis also results in hyperphosphatemia and hypercalcemia (Furll, 1994). High rumen osmolarity is also

associated reduced starch digestion due to inhibiton of bacteria. This results in low gut motility and stagnant ruminal contents and accumulation of acid due to reduced clearing of the acid from the rumen (Slyter, 1976; Huber, 1976).

2.8.7 Laminitis

Laminitis is one of the most frequent signs associated with SARA. There are studies indicating the connection between starch content in diet and laminitis (Mortensen, 1993; Nocek, 1997). Diets that have excessive levels of readily fermentable carbohydrates are attributed to cause nutrition mediated laminitis in cows (Manson and Leaver, 1988; Kelly and Leaver, 1988). It is believed that low rumen pH encountered in SARA causes lysis of gram negative bacteria resulting in increased levels of free LPS in rumen and blood, and increased levels of the vasoactive agents (histamine, LPS) leads to damage in the capillaries of the corium of hoof resulting in reduced blood supply, inflammation and necrosis of corium (Nocek, 1997). Losses occurring due to the laminitis involve increased involuntary cullings, decreased milk production, increased treatment costs and decreased fertility. Laminitis resulting as a consequence of repeated bouts of SARA can lead to lameness. An annual incidence rate of 10% or more for laminitis and an increase in number of cases of laminitis is the first and typical sign raising suspicion for presence of SARA in dairy herds (Nordlund et al., 1995). Subacute ruminal acidosis leads to higher rate of involuntary cullings and inexplicable deaths in herd (Nordlund et al., 1995; Garett et al., 1997).

2.8.8 Urine and fecal parameters

The capacity of the lungs in ruminant is small and it plays a very minor role in regulation of acid-base balance (Enemark et al., 2004). On the other hand, the elimination of acid via kidneys is more important. Rumen pH and urine pH have been reported to have a positive correlation (Furll, 1984), but several other conditions can result in aciduria (Markusfeld, 1987). Estimation of NABE in urine by titration has been reported to be more accurate than urine pH determination as acidotic conditions result in increased amounts of inorganic phosphate excreted in urine acting as a buffer (Kutas, 1965; Furll, 1994). According to a survey carried out in six Danish dairy herds by Enemark et al. (2004), mid-lactation cows (>30 DIM) had significantly ($p=0.01$) lower fecal pH (6.13) as compared to the early-lactation cows (<30 DIM, pH=6.50). The overall correlation between fecal pH and rumen pH samples collected by rumenocentesis was not significant ($p=0.65$), indicating rumen pH is not reflected in fecal pH. The diagnostic value of fecal pH in acute ruminal acidosis is much better (Kovac et al., 1986; Aslan et al., 1995)

2.9 Implications of SARA

SARA has been responsible as etiological factor for a number of diseases (Dirksen, 1985; Nordlund et al., 1995; Nocek, 1997). Possible implications of SARA and probable causes are discussed below:-

2.9.1 Rumenitis

Rumenitis is one of the most frequent sequelle to SARA. Increased production of VFA's especially propionate and butyrate, along with temporary changes in ruminal lactate and also increased osmolality of ruminal contents can be the causative factors for

rumenitis (Dirksen, 1985; Krehbiel et al., 1995). Also parakeratosis resulting from acidotic conditions may affect VFA absorption in the long run (Krehbiel et al., 1995). Rumenitis is often accompanied by mucosal injury which may serve as entry for pyogenic bacteria such as *Fusobacterium necrophorum* along with its colonization. Embolic spread of these bacteria can result in formation of abscesses in liver (rumenitis liver abscess complex) or they may metastasize to pulmonary circulation via posterior vena cava causing rupture of minor pulmonary arteries into bronchii. Clinically this leads to epistaxis (bleeding from nostrils) and/or haemoptysis (bleeding from mouth).

2.9.2 Metabolic acidosis

Dunlop (1972) stated that lactate, particularly D-lactate, leads to uncompensated metabolic acidosis found in cases of acute rumen acidosis, but the role of lactate in SARA is not that clear. It is also not evident the extent to which rumen pH is reflected in metabolic acidosis. The depth of fall in rumen pH and duration of episodes below pH 5.5 controls the extent of metabolic acidosis (Nocek, 1997). Lactate accumulation has not been reported in SARA (Hibbard et al., 1995). Hence, it is not yet determined if lactate has any influence on metabolic acidosis (Holtershinken et al., 1997). Amongst all VFA's, acetic acid reaches into the peripheral circulation as such whereas butyric acid is converted to β-hydroxybutyric acid in the rumen wall and propionic acid is converted to glucose in the liver (Owens et al., 1998). Owens et al. (1998) also speculated that compromised cell function in the rumen wall and in the liver results in high VFA concentration in peripheral blood, which causes metabolic acidosis.

2.9.3 Mastitis

One of the important consequences of SARA is mastitis. Mastitis due to increase in bacterial endotoxins during SARA has been reported in the literature (Verheiden et al., 1981; Hoeben et al., 2000). The principles of rumen hypomotility involving toxaemia in cases of coliform mastitis have been documented (Verheiden et al., 1981; Hoeben et al., 2000).

2.9.4 Impact of SARA on digestibility parameters

Neutral detergent fibre (NDF) digestibility in the rumen declines as the rumen pH falls below 6.2 (Grant, 1994). This can be attributed to altered rumen dynamics, increased rumen osmolality and VFA concentration during SARA. During SARA cows experience repeated bouts of low pH, which inhibits cellulolytic bacteria and impair the rumen microbes (Shi and Weimer, 1992). Studies have been conducted in vivo/ and in vitro to observe the effect of low pH on fibre digestibility. Calsamiglia et al. (2002) using dual control fermenters reported that artificial rumens maintained at pH of 5.7 had lower (39.2%) true digestibility as compared to the ones subjected to pH of 6.4 (46.3%). The results obtained from in vitro trials should be used with caution, due to the fact that rumen microbes can adapt to the changed conditions and passage rate or volume of contents in rumen can also change. Cardozo et al. (2000) reported digestibility values of 37% and 12% at rumen pH levels of 6.4 and 4.9 respectively when animals are fed diet containing F:C ratio of 60:40. The average NDF digestibility of mixed hay decreased significantly by 5.4 % at 24 h and 8.6 % at 48 h, when SARA was induced as compared to the control group (Plaizier et al., 2001). Similarly, Krajcarski-Hunt et al. (2002)

reported significant ($p<0.05$) reductions by 6.9 % and 14.4 % in situ degradability of grass hay at 24 h and 48 h. It is therefore quite evident that SARA inhibits the digestibility of fibre in the rumen.

2.10 Summary

At present, there is no agreement regarding the threshold pH to be used for SARA. Threshold rumen pH values of 5.5 (Hibbard et al., 1995), 5.6 (Cooper and Klopfenstein, 1996), 5.8 h (Beauchemin et al., 2001; Nordlund et al., 1995) and 6.0 (Krehbiel., 1995) have been used in the literature to define SARA.

Rumen microbial populations undergo changes during SARA that affects fermentation in the rumen. Activity of cellulolytic bacteria is decreased and that of amylolytic or saccharolytic bacteria is increased, when cattle experience daily episodes of low rumen pH during SARA. These changes result in decreased fibre digestion. As a consequence, there is increased production of propionate and decreased production of acetate and shifting of the rumen fermentation towards propionate (Krajcarski-Hunt et al., 2002; Plaizier et al., 2001). The changes associated with these altered rumen fermentation can be reversed if SARA is diagnosed and rectified.

The altered rumen microbial population results in milk fat depression by incomplete biohydrogenation of unsaturated fatty acids in the diet (Bauman and Girinari, 2001). Signs associated with SARA are varied and ambiguous, and, may appear several weeks after the onset of the disease. Animals experiencing SARA show unexplained episodes of diarrhea, erratic feed intake, abscesses and sudden death (Nordlund et al., 1995). In the short term, SARA causes parakeratosis of the rumen epithelium which subsequently leads

to rumenitis. Pyogenic bacteria such as *Fusobacterium necrophorum* can invade different body tissues from rumen. In the long term, SARA affected animals show laminits followed by lameness (Nocek, 1997).

Diets containing insufficient peNDF or excessive readily fermentable carbohydrates predispose the cows to the risk of SARA (Mertens, 1997; Kleen et al., 2003). Insufficient peNDF results in reduced rumen buffering and saliva production that is required to neutralize low rumen pH resulting from VFA production through microbial fermentation. There is limited data in the literature regarding incidence of SARA (Garett et al., 1997) because of the absence of suitable diagnostic tests or specific signs associated with this disease. Currently, the diagnosis of SARA relies on measurement of rumen pH from rumen fluid samples collected by stomach tube, rumenocentesis or rumen canula with each method associated with its own advantages and disadvantages (Garett et al., 1999; Dirksen and Smith, 1987; Aceto et al., 2000).

It is believed that SARA causes changes in the several biochemical markers in rumen fluid, urine, feces, blood, milk and also it causes changes in microbial protein synthesis, acid-base status of blood, dry matter intake and drinking behavior. It is important to investigate the changes occurring in these parameters, in order to have better understanding of the pathogenesis and etiology of this disease. In this way, alternate markers can be developed which can assist in the diagnosis of SARA and also help in minimizing the negative effects of SARA.

2.11 HYPOTHESES

1. Subacute ruminal acidosis decreases rumen, fecal, urine and blood pH. Subacute ruminal acidosis also decreases milk production, dry matter intake and affects blood parameters, drinking behavior and microbial protein synthesis, and that these changes can be used for the diagnosis of SARA.
2. Reducing pH in a "Rusitec" *in vitro* continuous culture system by varying artificial saliva composition and forage-to-concentrate (F:C) ratios of diets increases the free LPS in the rumen fluid.

2.12 OBJECTIVES

1. To induce SARA by two different methods a) by increasing dietary starch in the diet by adding wheat barley pellets to the diet ("grain-pellet SARA challenge") and by reducing particle size of the diet by adding alfalfa pellets to the diet ("alfalfa-pellet SARA challenge").

2. To determine the relationships between rumen pH and selected biochemical markers in milk, urine, feces, blood that might be suitable for prediction of SARA.

3. To compare the above relationships between the "grain-pellet SARA challenge" and the "alfalfa-pellet SARA challenge".

4. To induce SARA in a Rusitec continuous fermenter by altering the dietary F:C ratio and saliva composition.

5. To determine the effects of diet F:C ratio and saliva composition on the rumen pH, free LPS concentration, rumen fermentation characteristics, protozoa numbers and coliform bacterial count.

Manuscript I: Development of Alternate Markers for Subacute Ruminal Acidosis (SARA)

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3.0 Development of alternate markers for Subacute Ruminal Acidosis (SARA)

3.1 Abstract

Subacute ruminal acidosis (SARA) was induced in Holstein dairy cows by feeding wheat barley pellets and alfalfa pellets in order to study the relationships between rumen pH and selected biochemical markers in milk, urine, feces, blood that might be suitable for prediction of SARA. Replacing 26% of the control TMR with alfalfa pellets in the "alfalfa-pellet SARA challenge" resulted in an increase of average duration of rumen pH < 5.6 from 43.0 to 234.3 min/d. Replacing 12% of control TMR with grain pellets in the "grain-pellet SARA challenge" resulted in an increase of average duration of rumen pH < 5.6 from 72.1 to 349.8 min/d respectively. The "alfalfa-pellet SARA challenge" increased ($P<0.05$) milk protein (3.05 vs 3.12 %), total water intake per day (106.1 vs 116.8 L), urine pH (8.12 vs 8.24), net acid base excretion (127.6 vs 182.2 mol/L), blood sodium (136.0 vs 137.1 mmol/L), blood PCV, RBC and hemoglobin while milk yield, milk fat, DMI, blood pH, fecal pH, microbial protein synthesis, blood lactate remained unaffected. The "grain-pellet SARA challenge" increased ($P<0.05$) milk protein (3.06 vs 3.23), blood sodium (135.8 vs 137.0 mmol/L), blood lactate (0.80 vs 1.14 mmol/L) but decreased milk yield (22.6 vs 20.1 kg/d) without affecting the DMI, blood pH, fecal pH, urine pH, NABE, microbial protein synthesis. Multivariate discriminant analysis indicated that the best indicator of the "grain-pellet SARA challenge" was blood glucose, blood CO_2, milk yield, milk protein % and blood sodium while in "alfalfa-pellet SARA challenge" it was urine pH. The results of the stepwise multiple linear regression indicated that milk fat, milk protein and sodium were the best indicators of rumen pH. Dissimilarity in response

of urine pH, NABE, water intake per day and certain blood parameters to SARA induction by both methods, further complicates the development of alternate markers for this disease.

3.2 Introduction

Subacute ruminal acidosis (SARA) is an important and a common metabolic disease of high yielding dairy cows (Nordlund et al., 1995; Garett et al., 1997). This disease, in addition to reducing overall farm profitability, affects fermentation in the rumen, animal welfare, milk production and also animal health by diarrhea, laminitis, erratic feed intake (Stone, 1999; Kleen et al., 2003; Morgante et al., 2007; Plaizier et al., 2008). Milk production losses occurring due to SARA are estimated at US $1.12 per day (Stone, 1999).

There is inconsistency in the literature regarding the definition of SARA. Some researchers have defined SARA as daily episodes of rumen pH between 5.2-5.6 (Cooper and Klopfenstein, 1996). Beauchemin et al. (2003) recommended having a rumen pH of 5.8 as the threshold for SARA. Garett et al. (1999) used a threshold pH of 5.5 with the rumen fluid samples collected by rumenocentesis.

Currently, the diagnosis of this disease relies on the collection of rumen fluid for pH measurement. Several methods are available for the collection of rumen fluid (Dirksen and Smith, 1987). One of the methods is to collect rumen fluid via rumen canula surgically placed in the rumen. This is not a practical method, and cannot be used in dairy herds. An alternative method is to measure pH from rumen fluid collected via a stomach tube. The rumen fluid collected by this method can be contaminated by saliva, leading to inaccurate measurements of rumen pH (Dirksen and Smith, 1987; Nordlund and Garrett, 1994; Oetzel and Nordlund, 1998). Duffield et al. (2004) reported that samples collected using an oral-ruminal probe on an average had rumen pH higher by 0.34 pH units than

the rumen fluid samples collected by rumenocentesis. Rumen fluid sample pH may also vary according to the difference in locations of the fluid collection and also due to the diurnal variation in rumen pH (Bryant, 1964; Lane et al., 1968; Duffield et al., 2004; Enemark et al., 2004). Another approach followed for rumen pH measurement is collection of rumen fluid by rumenocentesis. This method requires the preparation of site as for surgery, and may require administration of local anesthesia (Duffield et al., 2004). Rumenocentesis may result in formation of abscess at the puncture site and, also, reduction in milk yield (Aceto et al., 2000).

In absence of specific diagnostic tests and the lacunas associated with all the methods used for measurement of rumen pH, the diagnosis of this disease currently relies on secondary clinical signs which may result in under-diagnosis of SARA (Enemark et al., 2004). So, there is a need to develop some alternate methods for the diagnosis of SARA. We hypothesized that SARA causes changes in rumen, fecal, urine and blood pH, and that it also causes changes in milk production, blood parameters, dry matter intake, drinking behavior and microbial protein synthesis, and that these changes can be used for the diagnosis of SARA. The objectives of the study were to induce SARA by two different methods a) by increasing dietary starch by adding wheat barley pellets to the diet ("grain-pellet SARA challenge") and by reducing particle size of the diet by adding alfalfa pellets to the diet ("alfalfa-pellet SARA challenge"). b) To determine the relationships between rumen pH and selected biochemical markers in milk, urine, feces, blood that might be suitable for the prediction of SARA. c) To compare the above relationships between the "grain-pellet SARA challenge" and the "alfalfa-pellet SARA

challenge". We assume that method of inducing SARA does not affect these relationships between the two forms of SARA.

3.3 Material and methods

3.3.1 *Animals, Diets and Experimental procedure*

Eight lactating Holstein cows, comprising of four rumen canulated and four non-rumen canulated cows, were used in a crossover design with two 6 wk periods. Cows were with an average body weight of 638±74 (mean ± SD) kg and an average milk yield of 26.9±8.8 kg at the start of the experiment. Cows were housed in individual tie-stalls in Glenlea Dairy Research station at the University of Manitoba (Winnipeg, MB, Canada) in accordance with the guidelines of Canadian Council on Animal Care (CCAC, 1993). Animals were randomly allocated in two groups comprising of two cannulated and two non-cannulated cows each. During wk 1 and wk 2 all cows received a total mixed ration (TMR) *ad libitum* with a forage to concentrate ratio (F:C) of 58:42 (Table 3.1) at 0900 h in the morning. In this study, rumen pH depression below 5.6 for at least 3 h/d was used as the working definition of SARA (Gozho et al., 2006). During wk 3, 4, 5 of each period SARA, was induced in either group by feeding an alfalfa-pellet TMR or by feeding a grain-pellet TMR (Table 3.1). Data obtained from wk 2 of each period was considered as control. In the "alfalfa-pellet SARA challenge", 26% of control TMR was replaced with alfalfa pellets, and in the case of the "grain-pellet SARA challenge", 12% of control

Table 3.1 Ingredients, nutrient composition and forage to concentrate ratio of control TMR, alfalfa pellet diet used for SARA induction and grain pellet diet used for SARA induction

Item	Control TMR (Wk2)	Alfalfa pellet TMR (Wk 4)	Grain pellet TMR (Wk 4)
Ingredients, % of DM			
Alfalfa silage (%)	34.0	8.0	30.0
Faba bean silage (%)	7.0	7.0	6.0
Corn silage (%)	17.0	17.0	15.0
Energy supplement	33.0	34.0	30.0
Protein supplement	4.0	4.0	3.0
Soybean (%)	5.0	4.0	4.0
Wheat barley pellets (%)	-	-	12.0
Alfalfa pellets (%)	-	26.0	-
Forage: Concentrate (%)	58:42	58:42	51:49
Nutrient Composition			
DM, %	48.4	63.3	51.8
CP, % of DM	19.5	18.1	18.7
Insoluble protein,% of DM	10.8	13.7	12.4
Soluble protein, % of DM	8.7	4.4	6.3
Available insoluble protein, % of DM	9.7	12.6	11.1
Available protein, % of DM	18.4	17.0	17.4
NDF, % of DM	30.1	31.5	27.2
ADF, % of DM	17.9	21.5	17.6
NSC, % of DM	35.7	36.5	40.6
Total fat, % of DM	6.49	5.53	5.04
Ash, % of DM	8.21	8.29	8.47
Ca, % of DM	1.28	1.22	1.27
P, % of DM	0.45	0.45	0.52
K, % of DM	1.59	1.73	1.72
Mg, % of DM	0.33	0.35	0.34
Na, % of DM	0.34	0.36	0.35

TMR was replaced with wheat barley pellets. These changes resulted in unchanged F:C ratio of 58:42 in case of alfalfa-pellet TMR and 51:49 in case of grain-pellet TMR (Table 3.1). Grain pellets contained 50% ground wheat and 50% ground barley (Table 3.3). Cows had unlimited access to fresh water throughout the experiment. Data collected in wk 4 of each period were considered representative of SARA. Samples of diet, and dietary ingredients were collected and analyzed (Table 3.1, 3.2, 3.3) by wet chemistry as described by Bhandari et al., (2007).

3.3.2 *Rumen pH measurement*

Rumen pH was monitored continuously in wk 2 and 4 of both experimental periods in the four rumen cannulated cows using indwelling pH probes as described by Gozho et al. (2006). Rumen pH was measured at 1 min intervals and data were sent to a data logger for storage and used for subsequent analysis. The pH data were summarized as average pH, time spent below 6.0, time spent below 5.6, area (time x pH) spent below pH 6.0 and area spent below 5.6 for each 24 h periods as described by Gozho et al. (2006).

Rumen fluid was also collected from all non-cannulated cows at 0900 h and 1500 h on second and fourth day of wk 2 and 4 of both periods using a stomach tube (ST) (Geishauser, 1993). The first 200 mL of collected rumen fluid was discarded and the subsequent 50 mL of the rumen fluid was kept for pH measurement. pH was measured using an Accumet Basic 15 pH meter and an Accumet gel filled, polymer-body combination pH electrode (Fisher Scientific, Fairlawn, NJ), calibrated with pH 4.0 and pH 7.0 buffer solutions (Fisher Scientific, Fairlawn, NJ).

Table 3.2 Ingredient composition of energy and protein supplement (% of DM)

Ingredient	Energy supplement	Protein supplement
Rolled barley	54.0	-
Lurosil salt[1]	0.2	-
Protein pellet[2]	1.8	-
Dairy supplement[3]	40.0	-
Vegetable oil	4.0	-
Dried distillers grain	-	42.0
Fish meal	-	7.0
Canola meal	-	22.5
Soybean meal	-	20.0
Beet molasses	-	3.2
Niacin	-	0.3
Sodium carbonate	-	5.0

[1] Calcium propionate

[2] Protein pellets contain 46.1 % soybean meal, 2.6 % wheat shorts, 40 % meal, 5 % oat hulls, 0.3 % pellet binder, 1 % cane molasses, and 5 % corn gluten meal.

[3] Dairy supplement contains 0.13% vitamin A, D, and E premix (Vit A, 16800 IU/kg; Vit D, 2215 IU/kg; Vit E, 75 IU/kg, DM basis), 0.13 % trace mineral premix, 2.6 % soybean meal, 0.06 % selenium, 39.1 % wheat shorts, 5 % distillers grain, 17.5 % canola meal, 15 % ground wheat, 1.7 % dicalcium phosphate, 1.6 % salt, 2 % Dynamate (Mosaic company, Plymouth, MN), 0.3 % pellet binder, 1 % cane molasses, 3.7 % calcium carbonate, and 10% corn gluten meal.

Table 3.3 Nutrient composition of alfalfa pellets and grain (wheat barley) pellets

Items	Alfalfa pellets	Grain (wheat barley) pellets
CP, % of DM	18.7	12.4
NDF, % of DM	38.9	19.6
ADF, % of DM	31	5.8
NSC, % of DM	30.1	61.3
Total fat, % of DM	2.38	2.24
Ash, % of DM	9.86	4.46
Ca, % of DM	1.54	0.63
P, % of DM	0.22	0.69
K, % of DM	2.53	0.56
Mg, % of DM	0.38	0.17
Na, % of DM	0.09	0.1

The rumen pH was also measured in rumen fluid collected by rumenocentesis (RCT). Rumenocentesis was performed once in all the cows during wk 5 of both periods using technique described by Nordlund and Garrett (1994). An area measuring 10 cm X 10 cm was prepared on the left flanc, 20 cm caudal to the last costae and on the level of the top of the knee joint similarly to a surgical procedure. A 12-14 gauge needle, length 14-16 cm attached to 30 mL syringe was inserted gently at the site of aspiration and rumen fluid was aspirated. The pH of rumen samples was measured immediately after sampling using a pH meter as described earlier. Rumen fluid samples (RC) used for analysis were also collected from all the four sacs of rumen i.e cranial dorsal, central, cranial ventral and caudal ventral of rumen cannulated cows, 15 min before feeding, and at 2, 4, 6, 8 hours after feeding on second and fourth day of wk 2 and 4 of both periods. Approximately 150 mL of rumen fluid was collected from each sac of the rumen. The rumen fluid samples collected from each sac were mixed thoroughly and pH was measured immediately. Rumen contents collected from both ventral sacs were strained through four layers of sterile cheese cloth. Approximately 5 mL of this strained sample was stored at -20°C until analyzed for VFA's and ammonia. Rumen pH comparison was carried out between the rumen fluid samples collected by the three methods i.e. rumenocentesis, stomach tube and rumen canula. All these samples were collected at the same time of day during the wk 5 of each period.

3.3.3 *Blood sampling and analysis*

Two blood samples (10 mL each) were collected at 0900 h and 1500 h on second and fourth day of wk 2 and 4 of both experimental periods by coccygeal venipuncture

into plain and sodium heparin coated vacutainer tubes (Becton Dickson, Franklin Lakes, NJ). Blood samples were analyzed for pH, pCO_2, pO_2, Na^+, K^+, Cl^-, Ca^{2+}, glucose, lactate, blood, urea using blood gas analyzer (Nova Biomedical Corporation, Waltham, MA).

An automated hematology analyzer (Cell-Dyn 3500 system, Abbott Laboratories, Abbott Park, IL, USA) was used for enumeration of white blood cells (WBC), red blood cells (RBC), measuring hemoglobin, mean corpuscular volume (MCV), mean corpuscular hematocrit (MCH), mean corpuscular hematocrit concentration (MCHC) and packed cell volume (PCV) in the whole blood. Copper concentration was measured in the serum samples (Varian AA240FS fast sequential AAS, Varian Inc., CA, USA). Fibrinogen levels were measured in the plasma (Leica TS 400. Leica Microsystems Inc., ON, Canada). All these analyses were conducted at the Manitoba Veterinary Services Laboratory, Winnipeg, MB.

3.3.4 *Milk sampling and analysis*

Cows were milked twice daily in their stall at approximately 0400 h and 1600 h. Milk yield was recorded using Tru Test regulation meters (Westfalia surge, Mississauga, ON). Milk samples from four consecutive milkings on day 3 and 4 of wk 2 and 4 were collected into 50 mL vials in each collection period and were preserved with 2-bromo-2-nitropropane-1,3 diol. Milk samples were stored at 4°C until analyzed at the laboratory of the Dairy Farmers of Manitoba (Winnipeg, MB) by near infrared analyzed for milk fat, milk protein and lactose content using Milk-O-Scan 303AB (Foss Electric, Hillerod, Denmark).

3.3.5 *Urine samples*

Approximately 50 mL sample of midstream urine was collected from each cow at 0900 h and 1500 h on the second and fourth day of wk 2 and 4 of either period. Urine samples from each cow were mixed thoroughly and pH was measured in duplicate immediately using a pH meter as described earlier. Urine samples were stored in plastic tubes at -20°C before they were analyzed for renal NABE (Net Acid Base Excretion) and purine derivatives (PD). Renal NABE (appendix I) was assessed using the method originally described by Kutas (1965) and modified by Enemark and Jorgensen (2003). PD excretion was estimated using creatinine as an index of total urinary output (Chen et al., 1995). Estimations for creatinine, allantoin and uric acid were carried out by colorimetric methods (IAEA, 1997). In order to allow comparison between different body weight animals, the ratio of purine derivatives to creatinine (PD: C) (mmol: mmol) concentrations in spot urine samples was corrected for metabolic weight ($W^{0.75}$, kg) of each cow, as daily creatinine excretion in urine appears as a function of the metabolic weight (Chen et al., 1995).

$$\text{Corrected PD: C} = \frac{\text{PD concentration}}{\text{Creatinine concentration}} \times W^{0.75}$$

Urinary creatinine was used as a marker to estimate urine volume (Chen et al., 1995), while urinary PD allantoin and uric acid were used as markers for estimation of MCP production.

3.3.6 *Fecal samples*

Fecal samples were collected from all cows at 0900 h and 1500 h on second and fourth day of wk 2 and 4 of both periods. Samples were collected per rectally after restraining the cows and cleansing of the perineal area by fecal grab method. A 10 g of sample was mixed with 5 mL of distilled water and shaken by hand to make the consistency of feces even. Fecal pH was immediately measured from the samples as described earlier.

3.3.7 *Water intake and drinking behavior*

Water consumption was determined for each cow by continuous measurement of the flow of water through each water bowl using Elster flow meters model C700 (ABB Water meters Inc., Ocala, FL). Water flows during 1 min periods were recorded by a data logger and used for subsequent statistical analysis. Drinking bouts were defined as the combination of drinking events that were apart by less than 4 min (Dado and Allen, 1993).

3.3.8 *Dry matter intake*

The amount of TMR offered was recorded on second and fourth day of wk 2 and 4 in each period for all the cows. Diet samples were pooled for each of the collection weeks in either period. Samples of all dietary ingredients including wheat barley pellets and alfalfa pellets and forages were also collected on second and fourth day of wk 2 and 4 of either period. The DM content of the pooled diet samples, dietary ingredients and forages were determined by drying at 60°C for 48 h. Dried feed samples were ground using a Wiley

mill through a 1 mm screen (Thomas-Wiley, Philadelphia, PA) and stored at -20°C until analyzed.

3.3.9 *VFA's, Ammonia nitrogen*

Frozen rumen fluid samples were thawed at room temperature and 0.5 mL of 25% meta-phosphoric acid solution was added to 2.5 mL of rumen fluid. The tubes were vortexed and placed in a −20°C freezer for 17 h. 0.2 mL of the 25% sodium hydroxide and 0.32 mL of 0.3 M oxalic acid were added to the thawed samples. Samples were centrifuged for 20 min at 3000 rpm. Approximately 2 mL of supernatant was decanted and placed into the autosampler device for analysis. Concentrations of VFA were determined by gas chromatography (Clarius 500, Perkin Elmer) using a 2.0-m packed glass column (21179-U, Supelco, Oakville, Ontario, Canada). The injector and detector temperatures were set at 200°C, with initial and final column temperatures set at 175°C and 200°C respectively. The run time was 21.25 min followed by a 2-min thermal stabilization period. Ammonia nitrogen concentration of rumen fluid samples was determined using a colorimetric technique as described by Bhandari et al. (2007). This assay is based on the principle that in an alkaline solution (pH 10.4-11.5) ammonium ions react with hypochlorite to form monochloramine. In the presence of phenol and an excess of hypochlorite, the monochloramine will form a blue coloured compound, indophenol, when nitroprusside is used as catalyst. The concentration of ammonium is determined spectrophotometrically at 630 mn (Novozamsky et al., 1974).

3.3.10 *Veterinary examination*

All cows were examined for heart rate, respiration rate, rumen motility and rectal temperature on second day of wk 2 and 4 of either period of the experiment (Mosby, 1996).

3.3.11 *Statistical analysis*

Data collected from wk 2 and 4 of each period were analyzed as a cross over design with repeated measures using proc mixed of SAS (2004). Trends were discussed at $P \leq 0.10$. Multivariate discriminant analysis (Sann et al., 2005) was conducted using JMP IN (Version 5.1; SAS Institute Inc., Cary, NC) to examine possible relationships among models of SARA induction and milk yield, milk fat, milk protein, urine pH, glucose, blood CO_2, sodium, PCV and lactate as diagnostic markers. Further, stepwise multiple linear stepwise regression (SMLR) was employed (SAS, 2004) to help determine which of the independent variables amongst blood CO_2, urine pH, blood sodium, blood glucose, blood lactate, WBC, PCV, milk yield, milk fat % and milk protein % could be used to best predict the dependent variable rumen pH. Rumen pH used for SMLR was measured across both forms of SARA challenge.

3.4 Results

The chemical and physical compositions of the diets, alfalfa pellets and grain pellets are given in Table 3.1, 3.2 and 3.3. Replacing 26% of control TMR by alfalfa pellets increased the DM content in SARA diet (48.4 vs 63.3%), ADF (17.9 vs 21.5% of DM), NDF (30.1 vs 31.5% of DM), NSC (35.7 vs 36.5% of DM), potassium (1.59 vs 1.73% of DM) but decreased the CP content (19.5 vs 18.1% of DM) without affecting the F:C ratio. Replacing 12% of control diet by grain pellets increased the DM content (48.4

vs 51.8%), NSC (35.7 vs 40.6% of DM), potassium (1.59 vs 1.72% of DM) but decreased the CP content (19.5 vs 18.7% of DM), NDF content (30.1 vs 27.2% of DM) and did not alter the ADF content (17.9 vs 17.6 % of DM) while changing the F:C ratio from 58:42 to 51:49. The chemical composition of alfalfa pellets and wheat barley pellets is given in Table 3.3.

Week 4 was considered as SARA wk as the duration of rumen pH below 5.6 was maximum in wk 4 for both the "alfalfa-pellet SARA challenge" and the "grain-pellet SARA challenge". The "alfalfa-pellet SARA challenge", decreased ($P<0.05$) average daily rumen pH from 6.0 to 5.8 (Table 3.4), increased ($P<0.05$) the duration with rumen pH below 6.0 from 386.1 min/d to 861.1 min/d and the duration of rumen pH below 5.6 from 43.0 min/d to 234.3 min/d. The area (time x pH) below pH 6.0 increased ($P<0.05$) from 83.0 to 241.7, the area below pH 5.6 remained unaffected by the "alfalfa-pellet SARA challenge". The "grain-pellet SARA challenge", decreased ($P<0.05$) average daily rumen pH from 6.0 to 5.7 (table 3.4) and increased ($P<0.05$) the duration with pH below 6.0 from 514.8 min/d to 889.3 min/d and, increased ($P<0.05$) the duration of rumen pH below 5.6 from 72.1 min/d to 349.8 min/d. The area (time x pH) below pH 6.0 increased ($P<0.05$) from 115.3 to 307.1 and the area below pH 5.6 tended to increase ($P=0.10$) from 14.8 to 46.6 in the "grain-pellet SARA challenge".

3.4.1 *Dry matter intake, milk production*

Table 3.5 presents the summary of dry matter intake and milk production parameters. Dry matter intake remained unaffected either by the "alfalfa-pellet SARA challenge" or the "grain-pellet SARA challenge". The "alfalfa-pellet SARA challenge" did not affect milk

Table 3.4 Rumen pH variables of cows fed the control TMR or alfalfa pellet TMR or grain pellet TMR[1]

	Treatment						Effect, *P* –value		
	Alfalfa pellets			Grain pellets					
Items	Control (Wk2)	SARA (Wk 4)	SEM	Control (Wk2)	SARA (Wk 4)	SEM	Alfalfa-pellet SARA challenge vs Control	Grain-pellet SARA challenge vs Control	Interaction
Average pH	6.0	5.8	0.06	6.0	5.7	0.06	0.02	<0.01	0.47
Time <5.6, min/d	43.0	234.3	63.60	72.1	349.8	63.86	0.04	<0.01	0.37
Time <6.0, min/d	386.1	861.1	97.95	514.8	889.3	98.64	0.01	<0.01	0.53
Area <5.6, min X pH/d	2.73	33.32	12.25	14.85	46.64	15.50	0.13	0.08	0.74
Area <6.0, min X pH/d	83.07	241.77	45.10	115.38	307.17	45.35	<0.01	0.01	0.63

[1]Diets were a TMR only (control) or control TMR replaced by 26% alfalfa pellets or control TMR replaced by 12% wheat barley pellets (grain pellets).

Table 3.5 Dry matter intake and milk parameters for cows fed the control TMR or alfalfa pellet TMR or grain pellet TMR[1]

	Treatment						Effect, *P* –value		
	Alfalfa pellets			Grain pellets					
Items	Control (Wk2)	SARA (Wk 4)	SEM	Control (Wk2)	SARA (Wk 4)	SEM	Alfalfa-pellet SARA challenge vs Control	Grain-pellet SARA challenge vs. Control	Interaction
DMI (dry matter intake), kg/d	24.70	26.07	2.16	23.69	22.72	2.16	0.11	0.25	0.06
Milk yield , kg/d	23.1	22.4	1.07	22.6	20.1	1.07	0.50	0.02	0.22
Milk fat, %	3.38	3.36	0.21	3.38	3.35	0.21	0.91	0.91	0.95
Milk fat yield, kg/d	0.75	0.71	0.07	0.75	0.63	0.07	0.25	<0.01	0.09
Milk protein, %	3.05	3.12	0.01	3.06	3.23	0.01	0.04	<0.01	0.03
Milk protein yield, kg/d	0.68	0.67	0.05	0.68	0.63	0.05	0.80	0.18	0.43
Milk fat : milk protein	1.10	1.07	0.06	1.10	1.06	0.06	0.69	0.32	0.55
Lactose, %	5.77	5.73	0.05	5.74	5.74	0.05	0.25	0.86	0.22

[1]Diets were a TMR only (control) or control TMR replaced by 26% alfalfa pellets or control TMR replaced by 12% wheat barley pellets (grain pellets).

fat percentage, milk fat yield, milk protein yield, milk yield, lactose but increased (P<0.05) milk protein percentage (3.05 vs 3.12). The "grain-pellet SARA challenge" did not change milk fat and lactose but decreased (P<0.05) milk yield (22.6 vs 20.1 kg/d), milk fat yield (0.75 vs 0.63, kg/d), milk protein yield (0.18 vs 0.13, kg/d) and increased (P<0.05) milk protein percentage (3.06 vs 3.23). The fat: protein ratio declined from 1.10 to 1.07 in the "alfalfa-pellet SARA challenge" and from 1.10 to 1.03 in the "grain-pellet SARA challenge".

3.4.2 VFA's, ammonia nitrogen

The results for VFA analysis are summarized in Table 3.6. The "alfalfa-pellet SARA challenge" did not affect daily averages of acetate, butyrate, propionate, valerate but decreased (P<0.05) isobutyrate (1.11 vs 0.85 mM) and isovalerate (0.91 vs 0.70 mM). The "grain-pellet SARA challenge" did not affect acetate, butyrate, isobutyrate, valerate isovalerate but increased (P<0.05) propionate (19.85 vs 26.61 mM). In case of the "alfalfa-pellet SARA challenge", the acetate: propionate ratio and total VFA remained unaffected. However, in the "grain-pellet SARA challenge", the acetate: propionate ratio declined (2.77 vs 2.16, P<0.05) without any affect on total VFA. The "alfalfa-pellet SARA challenge" and the "grain-pellet SARA challenge" (Table 3.6) did not affect the level of ammonia nitrogen.

3.4.3 *Water intake and drinking behavior*

Table 3.7 summarizes the results for the water intake and the drinking behavior analysis. The "alfalfa-pellet SARA challenge" decreased (P<0.05) mean interval between bouts

Table 3.6 Volatile fatty acids and ammonia nitrogen in rumen fluid measured for cows fed the control TMR or alfalfa pellet TMR or grain pellet TMR[1]

	Treatment						Effect, *P* –value		
	Alfalfa pellets			Grain pellets					
Items	Control (Wk2)	SARA (Wk 4)	SEM	Control (Wk2)	SARA (Wk 4)	SEM	Alfalfa-pellet SARA challenge vs. Control	Grain-pellet SARA challenge vs. Control	Interaction
Acetate , m*M*	56.62	54.05	4.06	55.41	54.03	4.09	0.57	0.78	0.50
Propionate, m*M*	20.44	21.79	2.08	19.85	26.61	2.04	0.61	0.02	0.13
Acetate : Propionate	2.86	2.54	0.18	2.77	2.16	0.17	0.21	0.02	0.31
Butyrate, m*M*	14.87	14.26	0.93	14.55	13.52	0.88	0.63	0.47	0.84
Isobutyrate, m*M*	1.11	0.85	0.09	1.08	0.99	0.09	0.01	0.45	0.06
Valerate , m*M*	2.16	2.52	0.25	2.01	2.48	0.26	0.24	0.16	0.93
Isovalerate, m*M*	0.91	0.70	0.07	0.91	0.95	0.07	0.01	0.65	0.02
Total VFA, m*M*	95.90	94.12	6.17	93.64	98.38	6.23	0.80	0.78	0.32
Lactate, m*M*	0.011	0.014	0.009	0.002	0.004	0.009	0.75	0.81	0.70
Ammonia nitrogen, mg/dl	11.96	9.66	1.28	11.89	9.88	1.28	0.13	0.22	0.74

[1]Diets were a TMR only (control) or control TMR replaced by 26% alfalfa pellets or control TMR replaced by 12% wheat barley pellets (grain pellets).

Table 3.7 Drinking behavior parameters for cows fed the control TMR or alfalfa pellet TMR or grain pellet TMR[1]

	Treatment						Effect, *P* –value		
	Alfalfa pellets			Grain pellets					
Items	Control (Wk2)	SARA (Wk 4)	SEM	Control (Wk2)	SARA (Wk 4)	SEM	Alfalfa-pellet SARA challenge vs. Control	Grain-pellet SARA challenge vs. Control	Interaction
Mean interval between bouts , min	47.2	41.6	5.18	43.4	46.8	5.18	0.02	0.14	0.02
Drinking Rate , L / min	1.35	1.18	0.13	1.30	1.22	0.13	0.01	0.26	0.39
Duration of bout , min	2.74	3.08	0.21	2.66	2.95	0.21	<0.01	0.01	0.05
Total number of daily bouts	32.7	36.3	3.82	36.20	33.7	3.82	0.09	0.11	0.02
Total daily consumption, L	106.1	116.8	8.04	106.3	103.4	8.04	0.01	0.47	<0.01
Total duration drinking / day, min	91.4	113.3	15.43	96.9	98.7	15.43	<0.01	0.79	0.12
Mean consumption /bout/day, L	3.68	3.59	0.44	3.36	3.48	0.44	0.66	0.56	0.55

[1]Diets were a TMR only (control) or control TMR replaced by 26% alfalfa pellets or control TMR replaced by 12% wheat barley pellets (grain pellets).

(47.2 vs 41.6 min), drinking rate (1.35 vs 1.18 L/min), increased ($P<0.05$) the duration of bout (2.74 vs 3.08 min), total daily consumption of water (106.1 vs 116.8 L), total time spent drinking per day (91.4 vs 113.3 min/d), and tended to increase ($P=0.09$) the total number of daily bouts (32.7 vs 36.3) without affecting mean consumption of water per day per bout. The "grain-pellet SARA challenge" did not affect mean interval between bouts, drinking rate, total number of daily bouts, total daily consumption of water, total time spent drinking per day, mean consumption per day per bout but increased ($P<0.05$) the duration of bouts (2.66 vs 2.95 min).

3.4.4 *Fecal pH, urine pH and renal NABE*

The "alfalfa-pellet SARA challenge" and the "grain-pellet SARA challenge" did not change the fecal pH (Table 3.8). Urine pH and renal NABE (Net Acid Base Excretion) increased ($P<0.05$) from 8.12 to 8.24 and 127.6 to 182.2 mol/L respectively in case of the alfalfa- pellet induced SARA (Table 3.8). However, urine pH and NABE values did not change in case of the "grain-pellet SARA challenge" (Table 3.8).

3.4.5 *Microbial protein synthesis*

The "alfalfa-pellet SARA challenge" did not change the production of MCP (Table 3.9) as estimated by purine derivative concentration index (PDC index) for uric acid and allantoin. Also, the PDC index for allantoin remained unchanged but the PDC index for uric acid increased (0.14 vs 0.17, $P<0.05$) in case of the "alfalfa-pellet SARA challenge". The "grain-pellet SARA challenge" also did not change the production of MCP as estimated by PDC index for uric acid and allantoin. However, the "grain-pellet SARA

Table 3.8 Fecal pH, Urine pH and NABE (Net acid base excretion) in urine of cows fed the control TMR or alfalfa pellet TMR or grain pellet TMR

	Treatment						Effect, *P* –value		
	Alfalfa pellets			Grain pellets					
Items	Control (Wk2)	SARA (Wk 4)	SEM	Control (Wk2)	SARA (Wk 4)	SEM	Alfalfa-pellet SARA challenge vs. Control	Grain-pellet SARA challenge vs. Control	Interaction
Blood pH	7.45	7.46	<0.01	7.46	7.46	<0.01	0.90	0.65	0.80
Fecal pH	6.59	6.58	0.06	6.62	6.63	0.06	0.81	0.86	0.75
Urine pH	8.12	8.24	0.03	8.15	8.14	0.03	<0.01	0.89	0.01
NABE[2], mol/L	127.6	182.2	15.5	137.5	137.1	15.5	<0.01	0.97	0.03

[1]Diets were a TMR only (control) or control TMR replaced by 26% alfalfa pellets or control TMR replaced by 12% wheat barley pellets (grain pellets).
[2] NABE = Net acid base excretion

Table 3.9 Purine derivative concentration (PDC) index in urine of cows fed the control TMR or alfalfa pellet TMR or grain pellet TMR[1]

	Treatment						Effect, *P* –value		
	Alfalfa pellets			Grain pellets					
Items	Control (Wk2)	SARA (Wk 4)	SEM	Control (Wk2)	SARA (Wk 4)	SEM	Alfalfa-pellet SARA challenge vs. Control	Grain-pellet SARA challenge vs. Control	Interaction
PDC index (uric acid + allantoin)	0.62	0.62	0.04	0.65	0.60	0.04	0.86	0.21	0.27
PDC index (uric acid)	0.14	0.17	0.01	0.15	0.16	0.01	0.03	0.51	0.33
PDC index (allantoin)	0.47	0.45	0.03	0.49	0.43	0.03	0.59	0.08	0.40
Creatinine, mmol/L	1.27	1.29	0.14	1.25	1.34	0.14	0.77	0.25	0.50

[1]Diets were a TMR only (control) or control TMR replaced by 26% alfalfa pellets or control TMR replaced by 12% wheat barley pellets (grain pellets).

challenge" tended to decrease the PDC index for allantoin (0.49 vs 0.43, P=0.08) but the PDC index for uric acid did not show any change.

3.4.6 *Clinical parameters*

Neither the "alfalfa-pellet SARA challenge" nor the "grain-pellet SARA challenge", affected the heart rate, rectal temperature, respiration rate and ruminal motility (Table 3.10).

3.4.7 *Blood parameters*

The "alfalfa-pellet SARA challenge" did not change pCO_2 and pO_2 levels in blood whereas the "grain-pellet SARA challenge" increased ($P<0.05$) pCO_2 level (34.6 vs 38.3 mm of Hg) and did not affect pO_2 levels in blood (Table 3.11). Both the "alfalfa-pellet SARA challenge" and the "grain-pellet SARA challenge" tended to increase ($P<0.10$) sodium concentration in blood (136.0 vs 137.1mmol/L) and (135.8 vs 137.0 mmol/L) respectively. The "alfalfa-pellet SARA challenge" and the "grain-pellet SARA challenge" did not affect the chloride concentration in blood. Blood glucose increased ($P<0.05$) in the "alfalfa-pellet SARA challenge" from 3.4 to 3.9 mmol/L and the "grain-pellet SARA challenge" from 3.5 vs 4.1mmol/L. The "alfalfa-pellet SARA challenge" did not affect lactate level in blood. However, there was increase ($P<0.05$) in concentration of blood lactate in the "grain-pellet SARA challenge" (0.80 vs 1.14 mmol/L). The "alfalfa-pellet SARA challenge" increased ($P<0.05$) PCV (0.26 vs 0.27 L/L), RBC (5.57 vs 5.76 X 10^{12}/L) and hemoglobin level (97.6 vs 101.5 g/L). The "grain-pellet SARA challenge" did not affect PCV, RBC and hemoglobin. The "alfalfa-pellet

Table 3.10 Veterinary parameters observed for cows fed the control TMR or alfalfa pellet TMR or grain pellet TMR[1]

	Treatment						Effect, *P* –value		
	Alfalfa pellets			Grain pellets					
Items	Control (Wk2)	SARA (Wk 4)	SEM	Control (Wk2)	SARA (Wk 4)	SEM	Alfalfa-pellet SARA challenge vs. Control	Grain-pellet SARA challenge vs. Control	Interaction
Heart rate, beats / min	83.2	82.5	3.68	83.5	90.2	3.68	0.86	0.13	0.18
Rectal temperature, °C	38.6	38.5	0.14	38.6	38.7	0.14	0.49	0.36	0.33
Respiration rate, per min	40.2	40	3.40	40	46.5	3.46	0.85	0.12	0.87
Rumen motility, per min	1.5	1.8	0.10	1.5	1.7	0.10	0.15	0.14	0.25

[1]Diets were a TMR only (control) or control TMR replaced by 26% alfalfa pellets or control TMR replaced by 12% wheat barley pellets (grain pellets).

Table 3.11 Blood chemistry for cows fed the control TMR or alfalfa pellet TMR or grain pellet TMR[1]

	Treatment						Effect, *P* –value		
	Alfalfa pellets			Grain pellets					
Items	Control (Wk2)	SARA (Wk 4)	SEM	Control (Wk2)	SARA (Wk 4)	SEM	Alfalfa-pellet SARA challenge vs. Control	Grain-pellet SARA challenge vs. Control	Interaction
pCO_2, mm of Hg	35.9	37.1	0.90	34.6	38.3	0.90	0.20	0.01	0.15
pO_2, mm of Hg	49.7	53.6	5.97	59.3	51.8	6.02	0.57	0.27	0.24
Sodium, mmol/L	136.0	137.1	0.50	135.8	137.0	0.50	0.08	0.05	0.90
Potassium, mmol/L	4.2	4.3	0.08	4.1	4.2	0.08	0.20	0.68	0.58
Chloride, mmol/L	104.3	104.2	0.84	104.2	103.6	0.84	0.85	0.31	0.58
Glucose, mmol/L	3.4	3.9	0.20	3.5	4.1	0.20	<0.01	<0.01	0.65
Calcium, mmol/L	1.8	1.1	0.02	1.1	1.2	0.02	0.61	0.11	0.37
Lactate, mmol/L	1.18	1.13	0.10	0.80	1.14	0.10	0.74	0.02	0.11
Urea , mmol/L	6.0	5.6	0.32	5.8	5.5	0.32	0.09	0.18	0.84
Copper, mmol/L	0.76	0.72	0.06	0.80	0.80	0.06	0.34	0.93	0.50
WBC[2], X 10^9/L	9.7	10.9	1.66	9.9	10.7	1.66	0.09	0.30	0.69
PCV[3] , L/L	0.26	0.27	0.01	0.26	0.26	0.01	0.05	0.26	0.62
RBC[4]X 10^{12}/L	5.57	5.76	0.14	5.51	5.63	0.14	0.04	0.24	0.60
Hemoglobin , g/L	97.6	101.5	3.66	96.5	98.6	3.67	0.01	0.18	0.48
MCV[5], fL	48.0	48.1	1.43	47.9	47.8	1.43	0.87	0.42	0.54
MCH[6], pg	17.5	17.6	0.51	17.5	17.5	0.51	0.99	0.23	0.23
MCHC[7], g/L	365.4	365.9	2.59	366.2	366.3	2.60	0.11	0.97	0.89
Fibrinogen , g/L	3.4	3.3	0.67	3.9	4.2	0.67	0.80	0.42	0.54

[1]Diets were a TMR only (control) or control TMR replaced by 26% alfalfa pellets or control TMR replaced by 12% wheat barley pellets (grain pellets).

[2] WBC= White blood cell, [3] PCV = Packed cell volume, [4] RBC = Red blood cell, [5] MCV = Mean corpuscular volume, [6] MCH = Mean corpuscular hematocrit, [7] MCHC = Mean corpuscular hematocrit

SARA challenge" did not affect calcium, copper, potassium, MCV, MCH, MCHC, fibrinogen but tended to decrease ($P<0.10$) urea (6.0 vs 5.6 mmol/L) and also tended to increase ($P<0.10$) WBC (9.7 vs 10.9 X 10^9/L). The "grain-pellet SARA challenge" also did not affect did not affect calcium, urea, copper, potassium, MCV, MCH, MCHC, WBC and fibrinogen levels.

3.5 Discussion

Several researchers have suggested rumen pH thresholds between 5.5 and 6.0 for the diagnosis of SARA (Cooper et al., 1999; Garett et al., 1999; Duffield et al., 2004). These thresholds vary depending on the technique of rumen fluid collection and also on the time of sampling (Duffield et al., 2004). Gozho et al. (2005), has shown that, based on free lipopolysaccharide levels in the rumen fluid and acute phase proteins in the blood, SARA can be successfully induced when a rumen pH depression between 5.2 and 5.6 is obtained for more than 180 min/d. Two forms of SARA challenge i.e. the "alfalfa-pellet SARA challenge" and the "grain-pellet SARA challenge" were studied in the current experiment. Replacing 26% of the control TMR with alfalfa pellets in the "alfalfa-pellet SARA challenge" resulted in an increase of average duration of rumen pH < 5.6 from 43.0 to 234.3 min/d. Replacing 12% of control TMR with grain pellets in the "grain-pellet SARA challenge" resulted in an increase of average duration of rumen pH < 5.6 from 72.1 to 349.8 min/d respectively. These results are in agreement with the duration of rumen pH < 5.6 of 506 min/d reported by Krause and Oetzel (2005), and 309 min/d reported by Gozho et al. (2007) respectively. As the duration of average pH < 5.6 with

both methods of SARA induction was more than 180 min/d in the current study, we conclude that SARA was successfully induced in this study.

Neither the "grain-pellet SARA challenge" nor the "alfalfa-pellet SARA challenge", affected DMI significantly. In the literature, there is no agreement regarding the effect of SARA on DMI with reports showing unchanged DMI (Gozho et al., 2007), increased DMI (Krause and Oetzel, 2005; Gozho et al., 2006) or decreased DMI (Kleen et al., 2003; Plaizier et al., 2008) during SARA. Variability in DMI response during SARA may be related to the level of insulin prior to feeding of high starch diets to the cows. If the level of insulin is low before SARA induction, then depression in DMI is not noticed due to lack of negative feedback on hepatic gluconeogenesis (Bradford and Allen, 2007). This results in increased use of propionate for glucose production and unchanged DMI (Allen et al., 2005). Other reasons for this variable effect of SARA on DMI include differences in the contents of fiber and ensiled forages, in particle size and in starch fermentability among the diets used to induce SARA (Allen, 2000), as well as differences in endotoxin tolerance among individual cows (Hurley, 1995).

Milk yield was reduced by 0.7 kg/d and 2.5 kg/d in case of alfalfa pellet and the "grain-pellet SARA challenge", respectively. These results are in agreement with other studies (Kleen et al., 2003; Stone, 2004) which show that milk yield declines in SARA, and provide additional confirmation that SARA was induced in present study. Milk fat depression has long been associated with SARA (Nocek, 1997; Kleen et al., 2003; Stone, 2004). However, similar to our study, Keunen et al. (2002), Cottee et al. (2004) and Gozho et al. (2007) did not observe changes in milk fat content due to grain induced

SARA. It has also been suggested that the inconsistent response in milk fat in experimentally-induced SARA may be related to the duration of the bout(s) of SARA with shorts period being likely to have no effect on milk fat content (Krause and Oetzel, 2005) and the milk fat content of cows prior to the induction of SARA (Gozho et al., 2006). The increase in milk protein due to the induction of SARA in our study might be explained by the increase in rumen digestible organic matter due to the inclusion wheat barley pellets and alfalfa pellets in the diet, as this increase improves microbial protein production in the rumen (Grieve et al., 1986; National Research Council, 2001).

In this study, total VFA did not increase in response to the induction of SARA by either method. Allen (1997) reported that the rumen pH is primarily dependent on two factors i.e. total VFA concentration and rumen buffering. Further, the amount of buffer required in the rumen is not a constant, but instead is related to the amount of acid being produced, along with its absorption and passage rates (Allen, 1997). Therefore, we believe that the depression in rumen pH in the current study occurred due to inadequate rumen buffering due to reduction in saliva production because of smaller dietary particle size in both the diets used to induce SARA rather than increased total VFA concentration.

In the "alfalfa-pellet SARA challenge", the cows had increased drinking rates, higher number of drinking bouts, spent more time drinking and consumed 10.6 L/d more water on an average during SARA week, as compared to control week, in order to counter rumen pH depression. The accumulation of liquid inside the rumen contributes to water deficiency in animals with SARA (Slyter, 1976; Furll, 1994) causing dehydration (Dunlop and Hammond, 1965). The increased water intake in the "alfalfa-pellet SARA challenge" could be an attempt by cows to counter the ongoing systemic dehydration and

increased haemoconcentration. Water intake did not change in the "grain-pellet SARA challenge". The unchanged water intake in the "grain-pellet SARA challenge" might be due to systemic acidosis and intoxication in the blood forcing the cow to resent drinking (Enemark et al., 2002). The evidence to this systemic acidosis is the increased pCO_2 and lactate concentration in the blood.

Blood pH remained unaffected by both forms of SARA (Table 3.8), indicating that rumen pH depression in our study does not affect blood pH. A blood pH of 7.35 or below signals the presence of systemic acidosis (Owens et al., 1998) as the blood pH values in our study were well above 7.35, systemic acidosis did not occur. Depression in blood pH can occur in SARA only if there is accumulation of lactate in the rumen which subsequently leads to increased plasma lactate and hence lowered blood pH i.e. below 7.35 (Burrin and Britton, 1986; Krehbiel et al., 1995; Owens et al., 1998; Brown et al., 2000). As lactate concentration in the rumen fluid was very low in this study changes in blood pH were not observed.

Fecal pH was also not affected by either the "alfalfa-pellet" or the "grain-pellet SARA challenge" (Table 3.8). Several studies have reported that fecal pH remains unaffected during SARA (Enemark et al., 2004) unless large amounts of starch by-passes the rumen and results in high hindgut fermentation (Eastridge, 2000).

The mean difference in rumen pH samples between ST and RC, ST and RCT samples were 0.4, 0.6 units respectively (Fig 3.1). The mean difference observed between ST and RCT samples is quite close to that observed by Enemark et al. (2004). The differences can be attributed to the fact that the degree of rumen fill and thickness of fibre

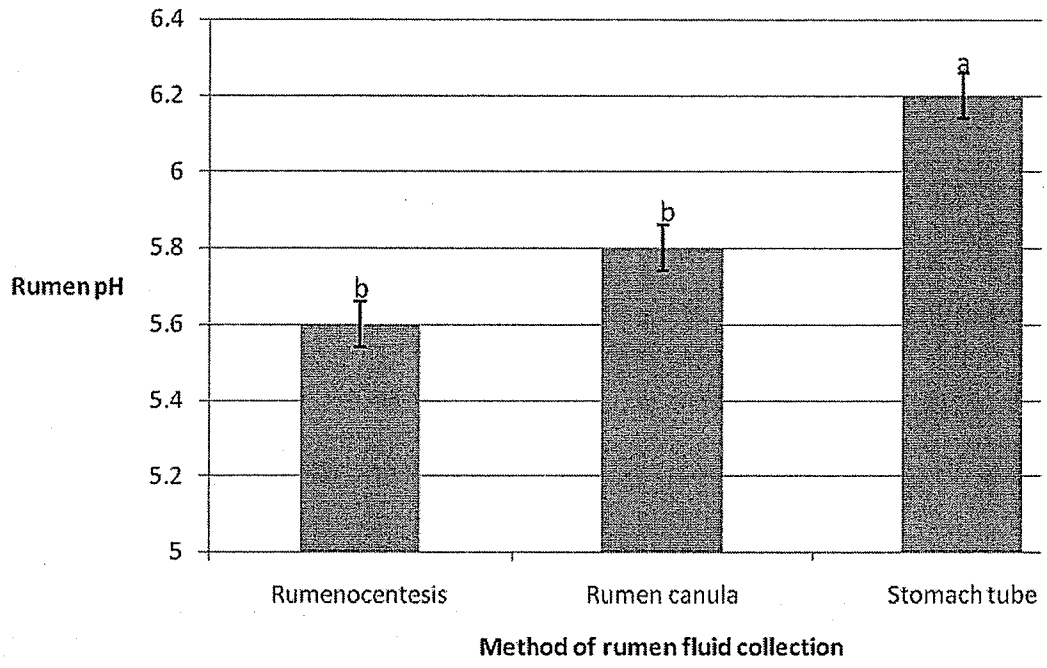


Fig 3.1:-Rumen pH (Mean ± SE) comparison by rumenocentesis, rumen canula and stomach tube.

(pH data of wk 5 presented. All the samples were collected 6 hrs after feeding)

a, b – LS means with different superscripts differ ($p<0.05$).

mat could influence the position of the stomach tube and also the samples collected via stomach tube are often contaminated with saliva (Bryant, 1968; Duffield, 2004; Enemark et al., 2004). Therefore, true rumen pH cannot be predicted based on the pH in rumen fluid samples obtained by stomach tube used in the present study due to considerable variation in the differences between RCT and ST sample values.

The urine pH and renal NABE did not change in the "grain-pellet SARA challenge" however, to our surprise the urine pH and renal NABE increased in the "alfalfa-pellet SARA challenge" despite low rumen pH. This increase in urine pH and renal NABE was unexpected as there was no effect of the "alfalfa-pellet SARA challenge" on the blood pH. We believe that the increase in urine pH and NABE in the "alfalfa-pellet SARA challenge" can be due to the ongoing systemic dehydration in the cows fed alfalfa pellet diet. The cows on alfalfa pellet diet were experiencing dehydration is supported by the increased blood PCV, WBC, Hb and RBC count. As mild diarrhea is also a consistent sign accompanying SARA (Kleen et al., 2003), water losses occurring during diarrhea also contributes towards the ongoing dehydration and its aggravation. It is possible that the cows on alfalfa pellet diet experienced urinary hypovolemia and an increased renal absorption of water in an attempt to conserve water leading to the passage of highly concentrated urine because of systemic dehydration. Such a reduction in urine volume would have increased urine pH as the urine pH of cows on control diet was already basic.

Subacute ruminal acidosis resulting diets with insufficient smaller particle size or diets rich in fermentable carbohydrates may cause reduction in the microbial protein

synthesis due to reduced growth of rumen microbes. In order to estimate the supply of microbial protein or the intestinal flow of microbial protein, daily excretion of purine derivatives (PD) in urine can be used (Lindberg et al., 1989; Chen et al., 1992; Balcells et al., 1993). Since, the rate of urinary excretion for creatinine has been reported as constant (Erb et al., 1977) for this reason creatinine has been used for the evaluation of urinary excretion rates of nitrogen (Albin and Clanton, 1966), steroids (Erb et al., 1970) and purine derivatives (Chen et al., 1995; Valadares et al., 1999). The difference of CP content between control TMR (19.5%) and both alfalfa-pellet TMR (18.1%) as well as grain-pellet TMR (18.7%) was quite small. We believe that the 'Spot test' technique used in the present study for estimation of microbial protein synthesis may not have sufficient sensitivity to detect the smaller difference in CP content of control and both SARA diets, and, also the little differences in microbial nitrogen supply to animals from these diets. Total urine collection is more suitable in cases where the difference in microbial N supply is small or the sensitivity of spot test technique can be improved if a less variable urine marker such as endogenous compound produced by the animal or an exogenous compound dosed to the animal is used in place of creatinine (Chen et al., 1995).

No significant effect of both forms of SARA was observed on clinical parameters like heart rate, respiration rate, rectal temperature and rumen motility. These observations lend credence to the fact that clinical signs associated with SARA are diffuse or often non-existent (Kleen et al., 2003; Underwood, 1992).

Subacute ruminal acidosis results in minor changes in systemic acid base status as there is intermittent depression of rumen pH during SARA and homeostasis of blood is

maintained (Horn et al., 1979; Goad et al., 1998; Brown et al., 2000). Acid-base imbalance along with amount of saliva secreted, rate of sodium, potassium secretion and/or sequestration in the abomasum, and the rate of renal excretion and absorption affect the serum electrolyte concentrations (Melvin, 1970; Blood and Radostitis, 1989). Hypernatremia (increased concentrations of sodium) were observed in blood under both forms of SARA indicating sodium retention in blood secondary to dehydration (Guard, 1993). Hypernatremia has been reported in ewes after they were subjected to a carbohydrate challenge (Irwin et al., 1979; Cao et al., 1987; Patra et al., 1995; Brown et al., 1999). Serum potassium levels have shown to decrease (Patra et al., 1995) or remain unchanged (Cao et al., 1987; Brown et al., 1999) following induction of acidosis. Our results generally agree with these previous observations. Blood sodium increased, whereas potassium levels seem to be unchanged in response to both forms of SARA. Plasma calcium has been reported to increase following induction of acidosis in ewes by glucose feeding (Irwin et al., 1979) and in cows when switched from high forage diet to high concentrate diet (Huntington and Britton, 1979). In contrast, plasma calcium has also been reported to decrease following induction of acidosis in ewes (Patra et al., 1995). In our study, calcium levels remained relatively unchanged. Increase of blood glucose in the "grain-pellet SARA challenge" could be attributed to lower acetate-to-propionate ratio in the "grain-pellet SARA challenge" and hence, more propionate availability and increased gluconeogenesis. In the "alfalfa-pellet SARA challenge" the increased blood glucose could be due to the high acetate ratios and insufficient availability of propionate for gluconeogenesis (Van Soest, 1994). In such cases where there is shortage of propionate increased blood glucose is from glucogenic amino acids (Van Soest, 1994).

Blood lactate concentration increased in the "grain-pellet SARA challenge", whereas blood lactate remained unchanged in the "alfalfa-pellet SARA challenge". In the "grain-pellet SARA challenge", rapid fermentation of large amounts of carbohydrates reduced the acetate-to-propionate ratio by increasing propionate. This might have been the result of changed microbial population in the rumen by increase of gram-positive bacteria and changing the fermentation to lactic acid fermentation, thus increasing blood lactate (Nikolov, 1996; Nikolov, 1998; Randhawa, 1987). In the case of "alfalfa-pellet SARA challenge", where SARA was induced by feeding smaller particle size forage and not by increasing readily fermentable carbohydrates in the diet, the reduction in acetate-to-propionate ratio was not noticed and hence increased blood lactate concentration was not present. This indicates slight metabolic acidosis in case of the "grain-pellet SARA challenge" and increased anaerobic generation of lactate (Howard and Smith, 1999) or reduced aerobic metabolism leading to exhaustion of compensatory metabolism (Burrin and Britton, 1986). Metabolic acidosis is a result of low rumen pH during episodes of SARA (Brown et al., 2000; Enemark, 2008). Increased blood lactate levels have also been observed in ewes by inducing acidosis after induction of acute acidosis (Cao et al., 1987; Patra et al., 1995; Brown et al., 1999). Blood urea concentration declined in the "alfalfa-pellet SARA challenge" while remaining unchanged in the "grain-pellet SARA challenge". The differences in blood urea concentration can be attributed to lower rumen degradability of the protein content of the alfalfa pellets and grain pellets, which led to decreased ammonia production in the rumen and increased urea production in the liver (Van Soest, 1994).

Accompanied by acidotic conditions in the rumen, the increased blood PCV, RBC, Hb, and increased water intake indicated severe haemoconcentration in case of the "alfalfa-pellet SARA challenge". Increases in these blood parameters may be attributed to state of dehydration due to passage of water from intravascular compartment as noticed in rumen acidosis (Parthasarthy and Philipson, 1952; Randhawa et al., 1989). Leucocytic counts also tended to be significantly more in "alfalfa-pellet SARA challenge" due to haemoconcentration. Increased PCV has also been observed in ewes by inducing acidosis following a sucrose challenge (Cao et al., 1987).

Multivariate discriminant analysis (Fig 3.2) of milk yield, milk protein, milk fat, urine pH, blood $C0_2$, sodium, glucose and PCV indicated that the most important indicator of the "grain-pellet SARA challenge" were blood glucose, blood CO_2, milk yield, milk protein and blood sodium while in the "alfalfa-pellet SARA challenge" it was urine pH , blood lactate and milk fat.

The results of the stepwise multiple linear regression (SMLR) indicated that out of eight independent variables included in the SMLR analysis (SAS, 2004) i.e. milk yield, milk protein, milk fat, urine pH, blood $C0_2$, sodium, glucose and PCV only three variables milk fat, milk protein and sodium appeared to be the best predictors of rumen pH (dependent variable). These three independent variables presented p-value lower than the default p-value of 0.15 in case of stepwise MLR. The R-square of the model was 0.48 with p-value of the model being <0.05.

According to the model rumen pH could be best predicted by the equation.

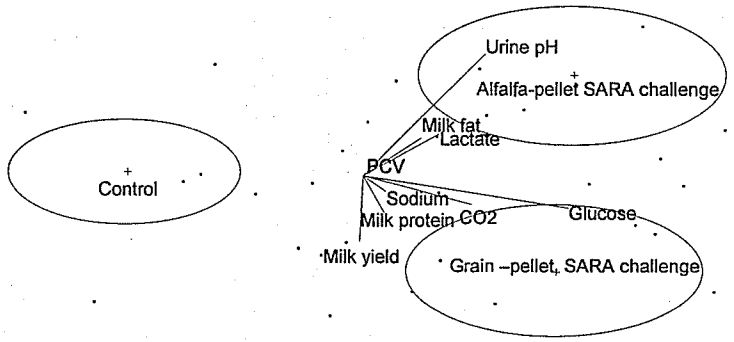


Fig 3.2:- Multivariate discriminant analysis for "grain-pellet SARA challenge" and "alfalfa-pellet SARA challenge".

Y (Rumen pH) = 13.63 - 0.03sodium - 0.27milk fat % – 0.40 milk protein %

Based on the results from SMLR it can be suggested that sodium, milk fat percentage and milk protein percentage could be potentially used as the indicators of SARA.

3.6 Conclusions

In this study, SARA was induced by feeding of alfalfa pellets and grain pellets, and, its effects were observed on selected biochemical markers in milk, urine, feces, blood that might be suitable for the diagnosis of this disease. SARA was induced by replacing 26% of the DM of control TMR with alfalfa pellets in the "alfalfa-pellet SARA challenge" and 12% of DM of control TMR with grain pellets in the "grain-pellet SARA challenge". The "alfalfa-pellet SARA challenge", increased the duration of rumen pH below 5.6 from 43.0 min/d to 234.3 min/d. The "grain-pellet SARA challenge" increased the duration of rumen pH below 5.6 from 72.1 min/d to 349.8 min/d. The "alfalfa-pellet SARA challenge" increased milk protein, total water intake per day, urine pH, net acid base excretion, blood sodium, blood PCV, RBC and hemoglobin while milk yield, milk fat, DMI, blood pH, fecal pH, microbial protein synthesis, blood lactate remained unaffected. The "grain-pellet SARA challenge" increased milk protein, blood sodium, blood lactate but decreased milk yield without affecting the DMI, blood pH, fecal pH, urine pH, NABE, microbial protein synthesis. Multivariate discriminant analysis indicated that the best predictor of "grain-pellet SARA challenge" was blood glucose, blood CO_2, milk yield, milk protein and blood sodium while in "alfalfa-pellet SARA

challenge" it was urine pH. The results of the stepwise MLR indicated that milk fat, milk protein and sodium were the best indicators of rumen pH. Dissimilarity in response of urine pH, NABE, water intake per day and certain blood parameters to SARA induction by both methods, further complicates the development of alternate markers for this disease. These dissimilar responses warrant further research in order to completely understand the effect of SARA induction on these parameters.

Short Communication: Effects of an In Vitro Model of Subacute Ruminal Acidosis on Rumen Free lipopolysaccharide and Fermentation Characteristics

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4.1 ABSTRACT

The effects of inducing SARA were investigated in a continuous fermenter (Rusitec), using a factorial design. Acidogenic conditions similar to SARA were obtained by simultaneous reduction in buffering capacity of the artificial saliva and decreasing the forage-to-concentrate (F:C) ratios of the diets used during the study. Both artificial saliva and the diets were supplied at three levels. The three levels in artificial saliva were strong (S1), medium (S2) and weak buffer (S3) based on the composition and buffering capacity of the artificial saliva. The dietary levels differed in F:C ratios with F:C ratio being 80:20, 50:50 and 20:80 for diet 1, diet 2 and diet 3 respectively. In general, the S3 artificial saliva reduced total VFA, in vitro dry matter digestibility of the diets with different F:C ratios, protozoa numbers but increased ammonia nitrogen. The diet with low F:C ratio (D3) decreased the pH of the fermenters but increased the total VFA, in vitro dry matter digestibility, ammonia nitrogen without affecting the protozoa. The lowest pH at 6 h after feeding and the highest dry matter digestibility was obtained at S3-D3 (weak buffer, 80% concentrate) treatment combinations. Average pH observed under this combination was 5.86. Induction of SARA did not affect the concentrations of free lipopolysaccharide and the number of coliform bacteria. However, SARA induction reduced the average daily pH (7.10 vs 6.85 and 5.86) and IVDMD of diets (54.54 vs 50.37 and 48.42 %) from S1 to S2 and S3. The detailed analysis of global rumen fluid microbiota in an in vitro model of SARA remains to be investigated.

Key words: Subacute ruminal acidosis, In vitro, Rusitec, Lipopolysaccharide

Subacute ruminal acidosis (**SARA**) is a metabolic disorder of ruminants that is associated with low rumen pH (Kleen et al., 2003). While there is still a debate on the threshold of rumen pH that is definitive of SARA (Plaizier et al., 2008), some believe that rumen pH has a low correlation with SARA symptoms, and that other indicators of rumen function must be included in definition of SARA (Enemark et al., 2004; Bramely et al., 2008). To refine this definition, Gozho et al. (2005) suggested that free rumen bacterial lipopolysaccharide (**LPS**) concentration should be considered. Recent studies with experimentally grain-induced SARA showed an increase in free rumen LPS concentration (Gozho et al., 2005; 2006; Emmanuel et al., 2008) which was accompanied with the translocation of LPS into peripheral blood (Khafipour et al., 2008a) and activation of inflammatory response (Gozho et al., 2007; Emannuel et al., 2008; Khafipour et al, 2008a). However, these studies did not indicate what concentrations of LPS are detrimental for SARA. On the other hand, as shedding of LPS increases due to both rapid growth and lysis of Gram-negative bacteria, it is not clear whether low rumen pH or high concentrate diets is the main trigger for the LPS release.

The use of an in vitro system allows studying the effects of pH on free LPS level under conditions where the type of diet, dilution rates and artificial saliva input are controlled. We hypothesized that reducing pH by changing artificial saliva composition and forage-to-concentrate (**F:C**) ratios of diets increases free rumen LPS. Hence, our objectives were to manipulate the rumen fluid pH in a Rusitec continuous fermenter to determine the effects of diet F:C ratio and saliva composition on the rumen pH and free LPS concentration. Moreover, the effects of diet F:C ratio and saliva composition on

rumen fermentation characteristics, protozoa numbers and coliform bacterial count were studied.

A 3 × 3 factorial experiment consisting of three artificial saliva (S1 = strong buffer, S2 = medium buffer, S3 = weak buffer) and three diets differing in F:C ratio (D1 = 20% concentrate, D2 = 50% concentrate , D3 = 80% concentrate) (Table 4.1, 4.2) was conducted using a rumen simulation technique (Czerkawski and Breckenridge, 1977) with a Rusitec continuous fermenter (Fig. 4.1) (Rusitec, Sanshin Industrial Co. Ltd., Japan). The fermenter was equipped with eight vessels, each made up of transparent acrylic resin and immersed in a water bath maintained at 39°C. Vessels had a working volume of approximately 800 mL with inner diameter (ID) of 75 mm, outer diameter (OD) of 85 mm, and 200 mm of height. Each vessel was sealed with a cap with a rubber packing and held in position by a screw flange. The cap had an inlet for artificial saliva infusion (0.85 mm of ID, 1.2 mm of OD), a sampling port closed with a rubber lid (6 mm ID and 10 mm OD), and an outlet for effluent (6 mm ID and 1.2 mm OD). Each vessel enclosed a feed container (73 mm OD and 90 mm of height) and the cap of each feed container was connected to the driving shaft. Each fermentation vessel was considered an experimental unit. Since the Rusitec has only eight vessels, it was not possible to test all nine treatments within one run. Thus, treatments were replicated in three independent 5-day incubation periods and randomly assigned to the vessels so that each treatment was tested at least in duplicate.

The vessels were supplied with dietary treatments, rumen fluid, rumen solid digesta, and artificial saliva. Diets were dried (60°C for 48 h) and grounded using a 1 mm mesh size screen and their nutrient composition were determined by wet chemistry as

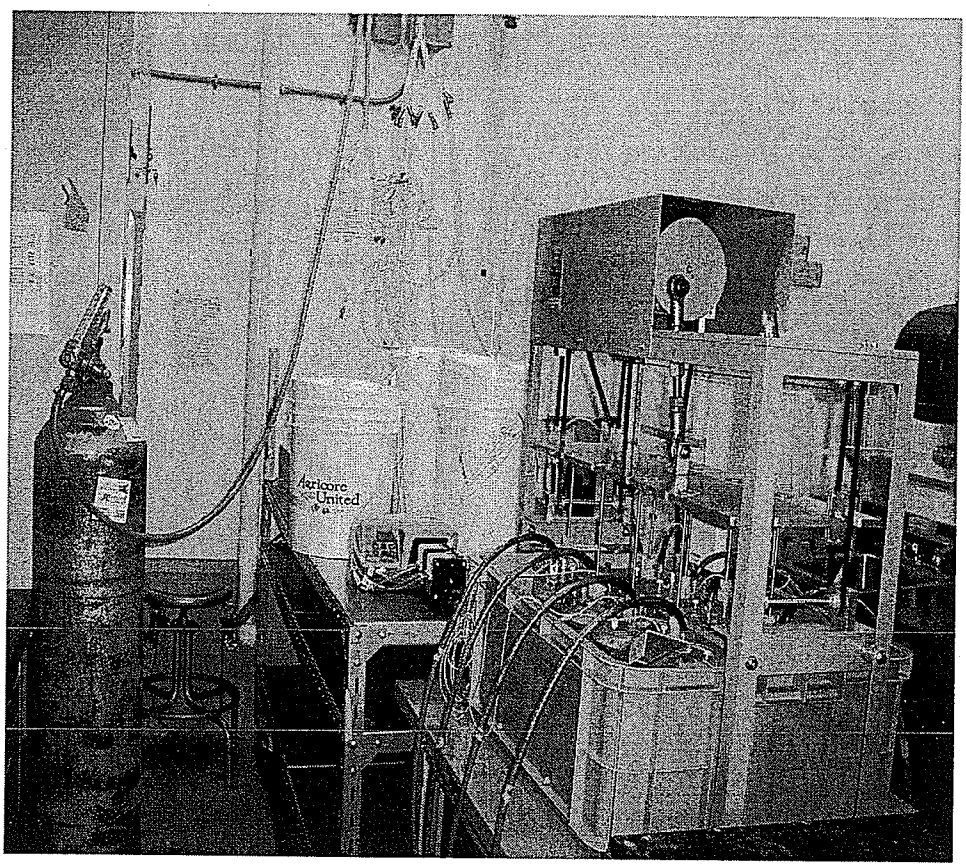


Fig 4.1: Rusitec continuous culture fermenter

described by Bhandari et al., (2007) (Table 4.2). Rumen fluid was obtained from two ruminally cannulated lactating Holstein cows that were fed a TMR ad libitum (Table 4.2). Cows were housed in individual tie-stalls in Glenlea Dairy Research station at the University of Manitoba (Winnipeg, MB, Canada) in accordance with the guidelines of Canadian Council on Animal Care (CCAC, 1993). Ruminal content (4.5 L of rumen liquor and 1 kg of solid digesta) was collected prior to morning feeding and strained through a four layered sterile cheese cloth into a bucket into which CO_2 gas had been flushed. Rumen fluid was kept bubbling with CO_2 until transferred to the Rusitec vessel. Then two nylon bags (10 × 20 cm, 100 μm pore size; Sanshin Industrial Co. Ltd.,Yokohamashi, Kanagewaken, Japan), one containing dietary sample (16 g DM) and the other containing the solid digesta (70 g wet weight) were placed into each of the feed container in the fermentation vessels. The vessels were then filled with strained rumen fluid and artificial saliva in equal proportion of 1:1 (Mc Dougall, 1948) to the overflow level and sealed.

The pH of the vessel was controlled by infusing artificial saliva (pH = 8.6) in which the principal buffering components (i.e. carbonate and phosphate) were decreased from the original McDougall's saliva (Mc Dougall, 1948). Artificial saliva was continuously bubbled with CO_2 throughout the incubation period to maintain anaerobic conditions in the Rusitec. A multi headed peristaltic pump (Model 323, Watson Marlow Ltd., Falmouth, Cornwall, England) was used for the infusion of artificial saliva via tubing (Cole – Parmer Instrument Co., Verner Hills, IL) into the vessels. The dilution rate was maintained at 3% / h. Effluent from each vessel was drained through a tube into a Duran media bottle having volume of 1 L.

Table 4.1 Chemical composition of different artificial saliva

Component (g/L)	Saliva*		
	S1	S2	S3
$NaHCO_3$	9.8	6.5	2.45
Na_2HPO_4	3.72	1.86	0.62
NaCl	0.47	0.47	0.47
KCl	0.57	0.57	0.57
$CaCl_2.2H_2O$	0.053	0.053	0.053
$MgCl_2.6H_2O$	0.128	0.128	0.128

*pH = 8.6 in S1, S2 and S3

Table 4.2 Ingredients, nutrients composition and F:C ratio of the TMR fed to the dairy cows that were used for rumen fluid collection and dietary treatments in Rusitec

Ingredients, % of DM	TMR	Dietary treatments		
		1	2	3
Alfalfa hay, %	-	40	25	10
Alfalfa silage, %	20	-	-	-
Corn silage, %	41	40	25	10
Faba bean silage, %	9.0	-	-	-
Energy supplement, %	24	6	13	20
Protein supplement, %	2.5	14	37	60
Soybean, %	3.5	-	-	-
F:C ratio		80:20	50:50	20:80
Nutrient composition, % of DM				
Dry matter, %	51	93.46	93.55	94.07
CP, %	18.9	14.75	17.51	20.73
Soluble protein, %	5.10	5.52	4.22	4.07
Insoluble protein, %		9.23	13.29	16.66
NDF, %	32.8	44.18	35.14	27.20
ADF, %		28.39	20.52	14.05
Non fibre carbohydrates, %	38	30.27	36.55	41.27
Total digestible nutrients, %	77	63.99	67.92	71.15
NE_L[1], Mcal/kg	1.68	1.44	1.54	1.62
Relative feed value		141	193	267
Calcium, %	1.05	0.94	0.98	1.12
Magnesium, %	0.34	0.31	0.33	0.34
Potassium, %	1.50	1.54	1.29	1.07
Phosphorus, %	0.62	0.34	0.53	0.68
Sodium, %	0.27	0.20	0.37	0.56

[1]NE_L = (Dry Matter TDN X 0.0245) - 0.12
where TDN = (Dry Matter DE / 4.4) X 100
DE = 3.44 - (0.022 X Dry Matter ADF)

Each 5-day incubation period consisted of two days of adaptation followed by three days of sampling. During each 24 h, the Rusitec was stopped for approximately 1.5 h and tubes for artificial saliva and effluent were disconnected, and, vessels were removed from the water bath. Vessels then were opened, and bubbled with CO_2 at the time of opening. Following the first 24 h, rumen solid digesta bags were replaced by new feed bags. These bags were placed underneath of the feed bags that were incubated for 24 h. Feed bags were replaced with new ones after 48 h incubation period, washed with 100 mL of respective artificial saliva and washings were poured into the fermentation vessel. The in vitro dry matter digestibility (IVDMD) of feed samples was determined after drying in the oven for at least 48 h at 60°C. In each run, rumen fluid was sampled at 6 h after daily morning feeding through the sampling port using a syringe and a long rumenocentesis needle, and pH was measured immediately (Accumet Basic 15 pH meter and Accumet gel filled polymer-body electrode; Fisher Scientific, Fairlawn, NJ). On day 3, 4, and 5 of each run rumen fluid was sampled for protozoal count (5 mL), ammonia nitrogen (5 mL), VFA (5 mL), and free LPS analysis (10 mL). On day 4, an additional sample was collected and kept on ice till used for total coliform count (10 mL).

To determine the protozoa numbers, 5 mL of rumen fluid sample was mixed with 5 mL of 10% formalin. A 2 ml aliquot of the well mixed formalinized sample was pipetted with a wide orifice pipette into a test tube containing 18 mL of glycerol-buffer-methyl green mixture (70: 30). The samples were then stored at room temperature (21°C) until protozoal counts were carried out by using Sedgewick Rafter Cell (PhycoTech Inc., St. Joseph, MI). Protozoa numbers were calculated at 10× magnification as described by Boyne et al. (1957), and Dehority, B.A. (1984). For enumeration of coliform bacteria, a

100 µL of rumen fluid samples were decimally diluted with 900 µL of 2% buffered peptone water (pH 7.2; Difco™, Beckton and Dickson, Sparks, MD) in a 2 mL sterile deep well plate and thoroughly mixed. Sterile pipette tips were used for each dilution. A total of 100 µL of 10^{-1} to 10^{-7} dilutions was inoculated onto Eosin Methylene Blue (EMB) media plates (Beckton and Dickson, Sparks, MD). Plates were inverted following drying and incubated at 37°C for 18 h. Colonies were manually counted and the countable dilution was determined as the dilution that gave 3 to 30 colonies per 100 µL of sample dispensed.

For free LPS analysis, samples from day 3, 4, and 5 of each run from each fermentation vessel were pooled and mixed thoroughly. Then a 10 mL of pooled sample was centrifuged at 12,000 × *g* for 30 min. The supernatant was passed through a disposable 0.22 µm sterile, pyrogen free filter (Millex, Millipore Corporation, Bedford, MA). The filtrate was heated at 100°C for 30 min and stored at -20°C until analyzed for free LPS. Concentration of free LPS was determined by a chromogenic *Limulus* amoebocyte lysate (LAL) end point assay (QCL-1000, Lonza group Ltd, Basel, Switzerland) as described by Gozho et al., (2005).

Frozen rumen fluid samples were thawed at room temperature and 0.5 mL of 25% meta-phosphoric acid solution was added to 2.5 mL of rumen fluid. The tubes were vortexed and placed in a −20°C freezer for 17 h. 0.2 mL of the 25% sodium hydroxide and 0.32 mL of 0.3 M oxalic acid were added to the thawed samples. Samples were centrifuged for 20 min at 3000 rpm. Approximately 2 mL of supernatant was decanted and placed into the autosampler device for analysis. Concentrations of VFA were determined by gas chromatography (Clarius 500, Perkin Elmer) using a 2.0-m packed

glass column (21179-U, Supelco, Oakville, Ontario, Canada). The injector and detector temperatures were set at 200°C, with initial and final column temperatures set at 175°C and 200°C respectively. The run time was 21.25 min followed by a 2-min thermal stabilization period. Ammonia nitrogen concentration was determined using a colorimetric technique as described by Bhandari et al. (2007).

Data were analyzed using PROC MIXED procedure of SAS (2004). The model included the fixed effects of diet, saliva, and diet × saliva and random effects of run, and day within run. Treatment means were compared by Tukey – Kramer multiple comparison test. Probability values were considered significant at $P \leq 0.05$. Trends were discussed at $P \leq 0.10$, if any.

The pH of rumen fluid was significantly affected by the composition of saliva and F:C ratio of the diet ($P < 0.01$; Table 4.3). The greatest decline in pH was observed during S3 (weak buffer) compared to S1 and S2 (5.86 vs. 6.85 and 7.10, respectively), and during D3 (80% concentrate) and D2 (50% concentrate) compared to D1 (6.50 and 6.60 vs. 6.69, respectively). There was a significant interaction ($P = 0.04$) between artificial saliva and dietary F:C ratio. The greatest rumen fluid acidity for S1, S2, and S3 was observed at the level of D3 (7.08 vs. 6.82 vs. 5.69, respectively; $P < 0.05$). In contrast, the greatest decline in pH for D1, D2, and D3 was obtained at the level of S3 (6.03 vs. 5.86 vs. 5.69, respectively; $P < 0.05$). The weak buffering capacity of saliva (S3) resulted in more rumen pH depression than high concentrate level of the diet (D3). The 5.69 to 6.03 range of pH obtained with S3 saliva were comparable to the rumen pH reported at 6 h after feeding during grain-induced SARA (Keunen et al., 2002; Krause and Oetzel, 2005). In these studies, the nadir pH was obtained at 10 h (Krause and

Oetzel., 2005) and 15 h after feed delivery (Keunen et al., 2002). Cows were fed twice daily in the study conducted by Keunen et al. (2002) which explains the observance of nadir pH at 15 h as compared to study Krause and Oetzel (2005) where the cows were fed once daily. Since the fluid samples were collected at 6 h after feeding in this study, the nadir pH might not have been obtained at the time of sampling and the rumen pH might have continued to drop after this point. This suggests that typical condition of SARA might have been induced at the S3 level of saliva in the fermentation vessels.

The VFA results are summarized in Table 4.3. Total VFA concentration declined from S1 to S2 to S3 (100.79 vs. 84.10 vs. 66.94 m*M*; $P < 0.01$), but increased from D1 to D3 (74.90 vs. 93.79 m*M*; $P=0.01$). Total VFA was lowest for S3 and highest for S1 where the lowest and highest pH was observed, respectively. Among VFA, acetate also decreased similarly from S1 to S2 to S3 (59.82 vs. 47.99 vs. 34.68 m*M*; $P < 0.01$) most likely due to reduced fibrolytic activity in pH below 6.0 (Russell and Dombrovski, 1980). In contrast, dietary F:C ratios did not affect acetate concentration as pH remained above 6 in all of three dietary treatments. Propionate, which is the most important sink of electron among VFA, was similar in S1 and S2 (22.8 and 19.83 m*M*, respectively) but declined in S3 (15.54; $P < 0.01$). Propionate concentration was significantly greater in D3 compared to D1 (22.45 vs. 16.55 m*M*; $P < 0.01$) due to increased dietary substrates. Valerate, which is the second important electron sink and has been suggested as SARA marker (Bramely et al., 2008), remained unaffected by change of artificial saliva, but increased from D1 to D3 (3.51 vs. 4.56 m*M*; $P = 0.05$). The acetate-to-propionate (**A:P**) ratio was significantly higher in S1 compared to S2 and S3 (2.63 vs. 2.45 and 2.47, respectively; $P < 0.01$), and in D1 compared to D2 and D3 (2.64 vs. 2.42 and 2.29, respectively; $P < 0.01$). In all

treatments, the A:P ratio remained above 2, while detrimental effects of SARA on DMI and milk fat are more associated with A:P ratio below 2 (Sauvant and Mertens, 1998).

Ammonia nitrogen level were similar among dietary treatments as well as in S1 and S2 (5.25 and 5.11 mg/dL, respectively), but tended to increase in S3 (6.78 mg/dL, $P = 0.09$). Although, the CP content of the diets increased from 13.79% in D1 to 16.38% in D2, and 19.50% in D3, the synchronization between energy and protein substrate avoided an increase in ammonia concentration. In contrast, as amylolytic bacteria are more proteolytic than fibrolytic bacteria and their resultant ammonia is the nitrogen source for the fibrolytic bacteria, the decline in fibrolytic activity under low pH condition could have resulted in accumulation of ammonia in the vessels (Wallace and Cotta, 1989).

The IVDMD decreased following reduction in the buffering capacity of saliva from S1 to S2 or S3 (54.54 vs. 50.37 and 48.42 %, respectively) due to pH decline. The decline in rumen pH < 6.2 results in reduced fibrolytic activity due to reduction in replication rate and in the ability of fibrolytic bacteria to attach to feed particles (Cheng et al., 1977; Russell and Dombrovski, 1980) leading to lowered digestibility of fiber and organic matter (Pitt et al., 1996). Increasing the concentrate ratio from D1 to D3 increased IVDMD (44.37 vs. 51.73 vs. 57.23 %, respectively) due to greater proportion of non forage based substrate.

Nakamura and Kurihara (1978) indicated that protozoa numbers is the most reliable indicator of rumen acidosis, as their numbers decline due to low rumen pH. In this experiment, protozoa numbers were similar in S1 and S2 (3,033 and 3,768 /mL, respectively) but decreased in S3 (2,495 /mL; $P < 0.01$), indicating that protozoa numbers were affected by the reduction in pH. Jorgensen et al. (1993) similarly reported that

protozoa populations may undergo partial defaunation at the pH levels between 6.2 and 5.3. Dietary treatments did not have a significant impact on protozoa numbers.

Although no significant difference was observed in coliform numbers and FREE LPS concentrations (Table 4.4), S3 had the lowest number of coliforms [4.65 $\log_{10}$ colony forming units (cfu)/mL] and the highest concentration of free LPS [23,514 endotoxin units (EU)/mL] among different artificial saliva and dietary treatments. The reported range of free rumen LPS during experimentally grain-induced animals was 4 to 6 fold greater than those observed in this study (Gozho et al., 2007; Emmanuel et al., 2008; Khafipour et al., 2008a). The total coliform numbers in the rumen fluid in this study were also one to two logs lower than those reported by Gozho et al. (2006) and Khafipour (2008b) during grain-induced SARA. However, as these authors did not observe a significant difference in coliform numbers between control and SARA-induced animals, it precludes the possibility that less free LPS concentration is due to lower number of coliform bacteria in this study. A detailed microbial analysis of rumen (Khafipour, 2008b) indicated that the proportion of phylum *Bacteroidetes*, which forms approximately 80% of rumen Gram-negative bacteria, declines during grain-induced SARA. Given that the size of the *Bacteroidetes* population is relatively of much larger magnitude than that of coliform bacteria, the authors concluded that the majority of free LPS in the rumen was likely from *Bacteroidetes*. Although we did not measure the proportion of *Bacteroidetes* in this study as it was not our aim to conduct a detailed microbial analysis, the low numbers of coliform bacteria and protozoa (one to two log) in this study compared to those of in vivo studies may suggest that the overall concentration of microbes in the rumen fluid in vitro was less than those of in vivo (Goad et al., 1998;

Nagaraja and Titgemeyer 2007). This difference might be due to the complexity of nutritional requirements of rumen microbes and interactions that exist among members of the community, which can not be easily simulated in vitro. Thus, the lower *Bacteroidetes* concentration might have resulted in less free LPS in the rumen fluid. Since the greater proportion of free LPS is released during logarithmic replication phase of Gram-negative bacteria than their lysis (Hurley, 1999; Anderson 2000), the lower number of Gram-negative bacteria can intensify the difference in the concentration of free LPS between in vitro and in vivo models of SARA. In conclusion, the aim of this study was to simulate the typical pH of SARA in vitro. The decline in pH at 6 h after feeding observed at S3 level (weak buffer) of artificial saliva was similar to those observed during experimentally grain-induced SARA. However, as pH was not measured continuously, it is difficult to conclude that SARA was successfully induced. In general, the S3 artificial saliva reduced total VFA, acetate, propionate, A:P ratio, dry matter digestibility of diets with different F:C ratios, and protozoa numbers, but increased ammonia nitrogen. The lowest pH at 6 h post feeding and the highest dry matter digestibility was obtained at S3-D3 (weak buffer, 80% concentrate) treatment combinations. Saliva compositions and the dietary treatments did not affect the free LPS concentration and total coliform counts, but these values were numerically lower in S3 than other treatments. The detailed analysis of global rumen fluid microbiota in an in vitro model of SARA remains to be investigated.

Table 4.3. Effects of artificial saliva composition and dietary treatments on rumen fermentation characteristics in Rusitec

Item	Treatments									
	Saliva			Diet				Effect, *P*-value		
	S1	S2	S3	D1	D2	D3	SEM	Saliva	Diet	Saliva×Diet
Average pH[1]	7.10[a]	6.85[b]	5.86[c]	6.69[*]	6.60[†]	6.53[†]	0.05	< 0.01	< 0.01	0.04
Total VFA, m*M*	100.79[a]	84.10[b]	66.94[c]	74.90[†]	83.11[*†]	93.79[*]	5.60	< 0.01	0.01	0.12
Acetate, m*M*	59.82[a]	47.99[b]	34.68[c]	43.68	46.40	52.38	4.72	< 0.01	0.07	0.07
Propionate, m*M*	22.80[a]	19.83[a]	15.54[b]	16.55[†]	19.17[*†]	22.45[*]	2.15	< 0.01	< 0.01	0.38
Butyrate, m*M*	12.58	11.24	12.09	10.23	12.46	13.22	1.16	0.49	0.06	0.47
Valerate, m*M*	4.43	4.28	3.55	3.51[†]	4.19[*†]	4.56[*]	1.16	0.10	0.05	0.94
Isobutyrate, m*M*	0.53[a]	0.42[b]	0.21[c]	0.41	0.42	0.44	0.03	< 0.01	0.80	0.10
Isovalerate, m*M*	0.65[a]	0.49[b]	0.37[b]	0.50	0.52	0.49	0.05	< 0.01	0.89	0.09
Acetate : Propionate	2.63[a]	2.45[b]	2.47[b]	2.64[*]	2.42[†]	2.29[†]	0.06	< 0.01	< 0.01	0.11
Ammonia nitrogen, mg/dL	5.25[b]	5.11[b]	6.78[a]	5.44	5.85	5.86	0.48	0.09	< 0.01	0.31
IVDMD[2], %	54.54[a]	50.37[b]	48.42[b]	44.37[‡]	51.73[†]	57.23[*]	1.71	< 0.01	< 0.01	0.37

[a, b, c] Means for saliva within a row with different superscripts differed ($P \leq 0.05$).

[*,†,‡] Means for diets within a row with different superscripts differed ($P \leq 0.05$).

[1] Average pH at 6 h after morning feeding.

[2] IVDMD = In vitro dry matter digestibility.

Table 4.4. Effects of artificial saliva composition and dietary treatments on free rumen LPS, and protozoal and coliform counts in Rusitec

Item	Treatments									
	Saliva			Diet				Effect, *P*-value		
	S1	S2	S3	D1	D2	D3	SEM	Saliva	Diet	Saliva×Diet
Protozoa, count/mL	3033[a,b]	3,768[a]	2,495[b]	2,706	3,549	2,398	543.40	< 0.01	0.34	0.29
Coliform, log_{10} CFU/mL[2]	4.91	5.07	4.65	5.03	4.79	4.81	0.28	0.69	0.41	0.26
LPS, EU/mL[1]	18,141	18,157	23,514	23,046	15,994	20,772	1,637	0.36	0.34	0.36

[a, b, c] Means for saliva within a row with different superscripts differed ($P \leq 0.05$).

[1]EU = Endotoxin unit

[2]CFU = Colony forming units

5.0 GENERAL DISCUSSION

This study comprised of two experiments. The primary aim of both experiments was to induce SARA, and study its effects on rumen, urine and fecal pH along with milk production, dry matter intake, net acid base secretion, drinking behavior, free LPS and certain blood parameters that could be potentially used for the diagnosis of this disease. The experiment 1 was an *in vivo* study and the second experiment was an *in vitro* study. The similarity between the two experiments was that in the first experiment, SARA was induced by reducing forage-to-concentrate (F:C) ratios of the diets and by reducing forage particle size of the diet resulting in insufficient rumen buffering, while in the second experiment, SARA was induced by combination of altering F:C ratios of diets and inadequate rumen buffering which was achieved by varying the artificial saliva composition.

In the first experiment, two different forms of SARA were studied, i.e. the "grain-pellet SARA challenge" and the "alfalfa-pellet SARA challenge". In the "grain-pellet SARA challenge", this disease was induced by replacing 12 % of control TMR with addition of wheat barley pellets to the diet. In the "alfalfa-pellet SARA challenge", this disease was induced by reducing the forage particle size of the diet by replacing long alfalfa hay with alfalfa pellets (26 %) in the diet without any resultant change in F:C ratio. In the first experiment, depression of rumen pH occurred as a result of inadequate rumen buffering, rather than increased VFA. The hypotheses behind the first experiment were that SARA decreases rumen, fecal, urine and blood pH, and thus it decreases milk production, dry matter intake, and affects blood parameters, drinking behavior and

microbial protein synthesis, and that these changes can be used for the diagnosis of SARA.

In the second experiment, SARA was induced in a "Rusitec" *in vitro* continuous culture system by varying composition of the artificial saliva to achieve inadequate rumen buffering and decreasing forage-to-concentrate ratios of the diets. In this experiment, both artificial saliva and the diets were supplied at three levels. The three levels in artificial saliva were strong (S1), medium (S2) and weak buffer (S3), based on the composition and buffering capacity of the artificial saliva. The concentration of the principal buffering components $NaHCO_3$ and Na_2HPO_4 decreased from S1 to S3. The dietary levels differed in F:C ratios with F:C ratio being 80:20, 50:50 and 20:80 for diet 1 (D1), diet 2 (D2) and diet 3 (D3) respectively. In the second experiment, inadequate rumen buffering and increased total VFA caused depression of pH. In the second experiment, it was hypothesized that reducing pH by changing artificial saliva composition and forage-to-concentrate (**F:C**) ratios of diets, increases free rumen LPS following lysis of gram-negative bacteria, reduces digestibility, and also affects the rumen fermentation.

In the first experiment, replacing 26% of the control TMR with alfalfa pellets in the "alfalfa-pellet SARA challenge" resulted in an increase of average duration of rumen pH < 5.6 from 43.0 to 234.3 min/d. Replacing 12% of control TMR with grain pellets in the "grain-pellet SARA challenge" resulted in an increase of the duration of rumen pH < 5.6 from 72.1 to 349.8 min/d respectively. As the duration of the pH < 5.6 with both methods of SARA induction was more than 180 min/d in the current study, we concluded

that SARA was successfully induced in this study. The average pH using the "S3" saliva in the second experiment was 5.86. These pH values are quite comparable to the pH of the rumen fluid 6 h after feeding reported by *in vivo* studies on SARA (Krause and Oetzel, 2005; Keunen et al., 2002). These authors have reported that the nadir pH is obtained at 10 h (Keunen et al., 2002) and 15 h (Krause and Oetzel, 2005) after feeding. In the second experiment, the samples were collected at 6 h after feeding, suggesting that nadir pH might not have been obtained at the time of sample collection and that the rumen pH could have continued to drop after the sample collection. This suggests that SARA was induced with the S3 saliva in the second experiment. The 5.69 to 6.03 range of pH obtained with S3 saliva was comparable to the rumen pH reported at 6 h after feeding during grain-induced SARA (Keunen et al., 2002; Krause and Oetzel, 2005).

In the first experiment, the DMI intake was not affected by both forms of SARA. The absence of an effect on DMI may be explained by the level of insulin prior to the feeding of high starch diets to the cows as it plays an important role in determining the DMI response of the cows to SARA induction. The DMI remains unchanged if the level of insulin is low before SARA induction, due to lack of negative feedback on hepatic gluconeogenesis (Bradford and Allen, 2007), thereby increasing use of propionate for glucose production and unchanged DMI (Allen et al., 2005). Another possible reason for this variable effect of SARA on DMI includes differences in the contents of fiber and ensiled forages (Allen, 2000) as high forage diets limit DMI by distension of reticulo-rumen and high concentrate diets limit DMI by metabolic feedback (Allen, 1997).

Differences in endotoxin tolerance among individual cows can also cause variability in the effect of SARA on DMI (Hurley, 1995).

In the first experiment, the total VFA concentration remained unchanged in both forms of SARA, suggesting that the depression in rumen pH in this experiment occurred due to inadequate rumen buffering and inadequate saliva production. However, in the second experiment, the maximum depression in rumen pH was observed with treatment combination of weak buffer (S3 saliva) and diet3 with F:C ratio of 20: 80, as this combination provided minimum rumen buffering and maximum amount of readily fermentable carbohydrates as compared to other treatment combinations. In the first experiment, the highest VFA concentration (93.79 m*M*) was recorded in the diet where the ratio of F:C was 20: 80. The acetate-to-propionate ratio in both the experiments was quite similar. The acetate-to-propionate ratio in the experiment 1 was lower for the "grain-pellet SARA challenge" (2.16) than "alfalfa-pellet SARA challenge" (2.54) whereas the acetate-to-propionate ratio in the second experiment was 2.47 for the S3 saliva and 2.29 for the dietary combination having F:C ratio of 20: 80. These ratios indicate the high concentrate diet that puts cows at risk of SARA (Van Soest, 1994). Also, in the first experiment the propionate concentration increased significantly in the "grain-pellet SARA challenge" (19.85 vs 26.61, m*M*), whereas propionate remained unchanged in the "alfalfa-pellet SARA challenge". In the second experiment, the propionate concentration (22.45 m*M*) was highest with diet containing F:C ratio of 20: 80. These results suggest similarity of responses in terms of VFA and acetate-to-

propionate ratio when diets containing excess of concentrates are fed for SARA induction.

In experiment 1, the "alfalfa-pellet SARA challenge" increased daily water intake of cows as compared to the "grain-pellet SARA challenge". We believe that the reason for this increased water intake was the accumulation of liquid inside the rumen that contributed to water deficiency and dehydration in animals with the "alfalfa-pellet SARA challenge" (Slyter, 1976; Furll, 1994). We assume that the cows attempted to counter systemic dehydration and increased haemoconcentration during episodes of reduced rumen pH by increasing daily water intake. Systemic dehydration was also reflected by the increased urine pH and renal NABE in the "alfalfa-pellet SARA challenge". In contrast to the "alfalfa-pellet SARA challenge", water intake did not change in the "grain-pellet SARA challenge" which we believe occurred due to systemic acidosis and intoxication in the blood forcing the cow to resent drinking (Enemark et al., 2002). Cows on the alfalfa pellet diet experienced urinary hypovolemia and an increased renal absorption of water in an attempt to conserve water leading to the passage of highly concentrated urine because of systemic dehydration. This reduction in urine volume would have increased urine pH as the urine pH of cows on control diet was already basic.

The total number of coliform bacteria in the rumen fluid in experiment 2 was one to two logs lower than those reported by Gozho et al. (2006) and Khafipour (2008b) during grain-induced SARA. These authors reported no interrelationship among free LPS and the total coliform numbers in the rumen fluid. Based on the results from these studies it was concluded that the low free LPS concentration (23,515 endotoxin units EU/mL) in

rumen fluid in the experiment 2 was not due to lower number of coliform bacteria. The proportion of phylum *Bacteroidetes*, which forms approximately 80% of rumen Gram-negative bacteria, declines during grain-induced SARA (Khafipour, 2008b). Hence, it would be logical to assume that the contribution of *Bacteroidetes* population towards free LPS pool in the rumen is of a much larger magnitude in comparison to that from coliform bacteria which form approximately 1% of the total rumen Gram-negative bacteria of phylum *Protobacteria*. In this study, it was concluded that the lower *Bacteroidetes* concentration might have resulted in less free LPS in the rumen fluid.

Subacute ruminal acidosis has a negative effect on the digestibility of fibre, rumen health and rumen microbial populations (Plaizier et al., 2001; Krajcarski-Hunt et al., 2002). Pitt et al. (1996) suggested that the fall in rumen pH below threshold levels of 6.2 results in lesser growth of fiber digesting microbes leading to lowered digestibility of fiber and organic matter. Fibrolytic bacteria have a reduced ability of attaching to feed particles at low pH (Cheng et al., 1977), and also their rate of growth is slower under low pH conditions (Russell and Dombrovski, 1980). At low pH, the increased cost of maintenance of bacteria due to the energy required to maintain the proton motive force across the membrane is one of the primary reasons for elimination of fibrolytic bacteria (Russell and Dombrovski, 1980). However, in the second experiment, reduction in the F:C ratio lead to increased digestibility due to the increase in concentrate component of the three diets as the concentrate-based diets are more digestible as compared to the forage-based diets. The number of protozoa decreased with SARA induction in the

second experiment. This agrees with previous reports that protozoal populations may undergo partial defaunation at pH levels between 6.2 and 5.3 (Jorgensen et al., 1993).

A mild acid base imbalance in blood has often been associated with SARA as there is only intermittent depression of rumen pH during SARA, and homeostasis of blood is maintained (Horn et al., 1979; Goad et al., 1998; Brown et al., 2000). Serum electrolyte concentrations depend on the degree of acid-base imbalance along with amount of saliva secreted, rate of sodium, potassium secretion and the rate of renal excretion and absorption (Melvin, 1970; Blood and Radostitis, 1989). The results of serum electrolytes concentration in the experiment 1 were on similar lines to those found in previous studies (Patra et al., 1995; Irwin et al. 1979; Cao et al., 1987; Brown et al., 1999). The blood sodium increased, whereas potassium and calcium concentration seemed to be unchanged in response to both forms of SARA. The increased blood glucose in the "grain-pellet SARA challenge" could be attributed to the higher propionate availability and increased gluconeogenesis, while in the "alfalfa-pellet SARA challenge" the increased blood glucose could be due to insufficient availability of propionate for gluconeogenesis but more availability of glucogenic amino acids (Van Soest, 1994). The increased blood lactate in the "grain-pellet SARA challenge" could be due to the increase of gram-positive bacteria and changing of the fermentation to lactic acid fermentation, thus increasing blood lactate (Nikolov, 1996; Randhawa, 1987; Nikolov, 1998). A similar rise of blood lactate was not observed in case of the "alfalfa-pellet SARA challenge", because in this case, SARA was induced by reducing forages particle size and not by increasing readily fermentable carbohydrates in the diet.

Blood glucose followed by blood CO_2, milk yield, milk protein and blood sodium in case of the "grain-pellet SARA challenge" and urine pH followed by blood lactate and milk fat presented the best indicators of the "alfalfa-pellet SARA challenge" in multivariate discriminant analysis amongst milk yield, milk protein, milk fat, urine pH, blood $C0_2$, sodium, glucose and PCV.

Milk fat%, milk protein% and sodium appeared to be the best indicators of rumen pH in SMLR analysis. These three independent variables presented p-value lower than the default p-value of 0.15 in case of stepwise MLR. The R-square of the model was 0.48. The overall p-value of the model was <0.05.

According to the model rumen pH could be best predicted by the equation.

Y (Rumen pH) = 13.63 - 0.03sodium - 0.27milk fat % – 0.40 milk protein %

Based on the results from SMLR it can be suggested that sodium, milk fat percentage and milk protein percentage could potentially be used as the indicators of SARA.

Also, a combination of measuring pH from the rumen fluid collected by rumenocentesis and the increased blood sodium, along with increased haemoconcentration (increased PCV, RBC, hemoglobin and leucocytes), altered drinking behavior, decreased milk production and milk fat yield can be used to assist the investigators in determining whether SARA is present in a dairy herd. The next step in the development of alternate markers for the diagnosis of this disease should be to evaluate the above mentioned parameters and determine their sensitivity and specificity under field conditions.

The increase of urine pH, NABE and water intake per day in the "alfalfa-pellet SARA challenge" and not in the grain-pellet induced warrants further research in order to completely understand the effect of SARA induction on these parameters. Future in vitro studies using Rusitec continuous culture system should focus on determining the effects of greater magnitude of decreased pH on free LPS, coliform numbers and microbial fermentation.

REFERENCES

Aceto, H., A. J. Simone, and J. D. Ferguson. 2000. Effect of rumenocentesis on health and production in dairy cows. J. Dairy Sci. 83 (1):40.

Albin, R. C., and D. C. Clanton. 1966. Factors contributing to the variation in urinary creatinine and creatinine-nitrogen ratios in beef cattle. J. Anim. Sci. 25:107–112.

Allen, M. S. 1997. Relationship between fermentation acid production in the rumen and the requirement for physically effective fiber. J. Dairy Sci. 80:1447–1462.

Allen, M.S. 2000. Effects of diet on short term regulation of feed intake by lactating dairy cattle. J. Dairy Sci. 83 (7):1598-1624.

Allen, M. S., B. J. Bradford, and K. J. Harvatine. 2005. The cow as a model to study food intake regulation. Annu. Rev. Nutr. 25:523-547.

Andersen, P. H. 2000. Bovine endotoxicosis: Aspects of relevance to ruminal acidosis. DFr. Vet. Sci. Thesis, The Royal Veterinary and Agricultural University, Copenhagen.

Aslan, V., S. M. Thamsborg, R. J. Jorgensen and A. Basse. 1995. Induced acute ruminal acidosis in goats treated with yeast (saccharomyces cerevisiae) and bicarbonate. Acta Vet. Scand. 36:65-77.

Balcells, J., M. Fondevila, J. A. Guada, C. Castrillo and J. C. E. Surra. 1993. Urinary excretions of purine derivatives and nitrogen in sheep given straw supplemented with different sources of carbohydrates. Anim. Prod. 57: 287-292.

Bauman, D. E., and J. M. Griinari. 2001. Regulation and nutritional manipulation of milk fat: low-fat milk syndrome. Livestock Prod. Sci. 70:15-29.

Beauchemin, K. A, W. Z. Yang, and L.M. Rode. 2001. Effects of barley grain processing on the site and extent of digestion of beef feedlot finishing diets. J. Anim. Sci. 79: 1925-1936.

Beauchemin, K. A., W. Z. Yang, and L. M. Rode. 2003. Effects of particle size of alfalfa based dairy cow diets on chewing activity, ruminal fermentation and milk production. J Dairy Sci. 86: 630- 643.

Bennink, M. R, T. R Tyler, G. M. Ward, and D. E. Johnson. 1978. Ionic milieu of bovine and ovine rumen as affected by diet. J. Dairy Sci. 61:315-323.

Bergen, W.G. 1972. Rumen osmolality as a factor in feed intake control of sheep. J. Anim. Sci. 34:1054-1060.

Bhandari, S. K., K. H. Ominski, K. M. Wittenberg and J. C. Plaizier. 2007. Effects of chop length of alfalfa and corn silage on milk production and rumen fermentation of dairy cows. J. Dairy Sci. 90:2355-2366.

Blood, D. C., and O. M. Radostitis. 1989. Acute carbohydrate engorgement of ruminants (rumen overload). Pages 246-253 In: Blood, D.C., Radostitis. O.M.(Eds), Veterinary Medicine, Bailliere Tindall.

Boyne, A. W., J. M. Eadie., and K. Raitt. 1957. The development and testing of a method of counting rumen ciliate protozoa. J. Gen. Microbiol 17:414-423.

Bradford, B. J. and M. S. Allen. 2007. Depression in feed intake by a highly fermentable diet is related to plasma insulin concentration and insulin response to glucose infusion. J. Dairy Sci. 90(8): 3838-3845.

Bramley, E., I. J. Lean, W. J. Fulkerson,M. A. Stevenson,A. R. Rabiee, and N. D. Costa. 2008. The definition of acidosis in dairy herds predominantly fed on pasture and concentrates. J. Dairy Sci. 91:308-321.

Britton R. A. and R. A. Stock. 1986. Acidosis, rate of starch digestion and intake. In: Symposium Proceedings: Feed Intake by Beef Cattle. Oklahoma State University. Pg. 125-137.

Brown, M. S., D. M. Hallford, M. L. Galyean, C. R. Krehbiel, and G. Duff. 1999. Effect of ruminal glucose infusion on dry matter intake, urinary nitrogen composition, and serum metabolite and hormone profiles in ewes. J. Anim. Sci. 77:3068–3076.

Brown, M. S., C. R. Krehbiel, M. L. Galyean, M. D. Remmenga, J. P. Peters, B. Hibbard, J. Robinson, and W. M. Moseley. 2000. Evaluation of models of acute and subacute acidosis on dry matter intake, ruminal fermentation, blood chemistry, and endocrine profiles of beef steers. J. Anim. Sci. 78:3155-3168.

Bryant, A. M. 1964. Variations in the pH and volatile fatty acid concentration within the bovine reticulo-rumen. N. Z. J. Agric. Res. 7:694–706.

Burrin, D. G. and R. A. Britton. 1986. Response to monensin in cattle during subacute acidosis. J. Anim. Sci. 63:888-893.

Calsamiglia, S., A. Ferret, and M. Devant. 2002. Effect of pH and pH fluctuations on microbial fermentation and nutrient flow from a dual-flow continuous culture system. J. Dairy Sci. 85:574-:579.

Canadian Council on Animal Care. 1993. Guide to the care and use of experimental animals. Volume 1. E. D. Olfert. B. M Cross and A. A. McWilliam, eds. CCAC, Ottawa, ON.

Cao, G. R., P. B. English, L. J. Filippich, and S. Inglis. 1987. Experimentally induced lactic acidosis in the goat. Aust. Vet. J. 64:367–370.

Cardozo, P. W., S. Calsamiglia, and A. Ferret. 2000. Effects of pH on microbial fermentation and nutrient flow in a dual flow continuous culture system. J. Dairy Sci. 83 (1): p. 265.

Carter, R. R. and W. L. Grovum. 1990. A review of the physiological significance of hypertonic body fluids on feed intake and ruminal function: salivation, motility and microbes. J. Anim. Sci. 68: 2811-2832.

Chen, X. B., Y. K. Chen, M. F. Franklin, E. R. Prskov and W. J. Shand. 1992. The effect of feed intake and body weight on purine xcretion and microbial protein supply in sheep. J. Anim Sci. 70:1534-1542.

Chen, X. B., A. T. Mejia, D. J. Kyle and E. R. Ørskov. 1995. Evaluation of the use of the purine derivative: creatinine ratio in spot urine and plasma samples as an index of microbial protein supply in ruminants: studies in sheep. J. Agric. Sci. 125:137–143.

Cooper, R., and T. Klopfenstein. 1996. Effect of Rumensin® and feed intake variation on ruminal pH. In: Scientific Update on Rumensin® / Tylan® / Micotil® for the Professional Feedlot Consultant. Elanco Animal Health, Indianapolis, IN. Pg. A-1 – A-14.

Cooper, R. J., T. J. Klopfenstein, R. A. Stock, C. T. Milton, D. W. Herold, and J. C. Parrott. 1999. Effects of imposed feed intake variation on acidosis and performance of finishing steers. J. Anim. Sci. 77:1093–1099.

Cottee, G., I. Kyriazakis, T. M. Widowski, M. I. Lindinger, J. P. Cant, T. F. Duffield, V. R. Osborne and B. W. McBride. 2004. The effects of subacute ruminal acidosis on sodium bicarbonate-supplemented water intake for lactating dairy cows. J. Dairy Sci. 87:2248-2253.

Crichlow, E. C. and R. K. Chaplin. 1985. Ruminal lactic acidosis: Relationship of forestomach motility to nondissociated volatile fatty acids levels. Am. J. Vet. Res. 46 (9): 1908-1911.

Cumby, J. L., J. C. Plaizier, I. Kyriazakis, and B.W. McBride. 2001. Effects of subacute ruminal acidosis on the preference of cows for pellets containing sodium bicarbonate. Can. J. Anim. Sci. 81:149-152.

Czerkawski, J. W., and Breckenridge. 1977. Experiments with the long-term rumen simulation technique (Rusitec); response to supplememtation of basal rations. Br. J. Nutr. 42:217-228.

Dado, R. G., and M. S. Allen. 1993. Continuous computer acquisition of feed and water intakes, chewing, reticular motility, and ruminal pH of cattle. J Dairy Sci. 76:1589-1600.

de Veth, M. J., and E. S. Kolver. 2001. Diurnal variation in pH reduces digestion and synthesis of microbial protein when pasture is fermented in continuous culture. J. Dairy. Sci. 84: 2066-2072.

Dehority, B. A. 1984. Evaluation of subsampling and fixation procedures used for counting rumen ciliate rotozoa. Appl. Env Micro 48(1):182-185.

Dirksen, G., 1965. Rumen acidosis in cattle. Vet. Med. Rev. (Bayer), 2: 98-125.

Dirksen, G. 1985. Tierarztliche Praxis 13:501. In: Braun, U., T. Rihs, and U. Schefer. 1992.

Dirksen, G. U., and M. C. Smith. 1987. Acquisition and analysis of bovine rumen fluid. Bovine Pract. 22:108–116.

Duffield, T., J. C. Plaizier, A. Fairfield, R. Bagg, G. Vessie, P. Dick, J. Wilson, J. Aramini and B. McBride. 2004. Comparison of techniques for measurement of rumen pH in lactating dairy cows. J. Dairy Sci. 87:59-66.

Dunlop, R. H., and P. B. Hammond. 1965. D-lactic acidosis of ruminants. Ann. NY Acad. Sci. 11:1109-1130.

Dunlop, R.H. 1972. Pathogenesis of ruminant lactic acidosis. Adv. Vet. Sci. Comp. Med., 16:259-302.

Eastridge, M. L. 2000. Guidelines for low forage diets. Pages 97-110 in Proceedings of the Tri-state Nutrition Conference, Fort Wayne, Indiana, US.

Eddy, R. G. 1992. Alimentary Conditions. In: Bovine Medicine - Diseases and Husbandry of cattle. A.H. Andrews, R. W. Blowey, H. Boyd & G. Eddy (ed.), Blackwell Scientific Publications, pp. 625-666.

Emmanuel, D. G. V., S. M. Dunn, and B. N. Ametaj. 2008. Feeding high proportions of barley grain stimulates an inflammatory response in dairy cows. J. Dairy Sci. 91:606-614.

Enemark, J. M. D., R. J. Jørgensen, and P. S. Enemark. 2002. Rumen acidosis with special emphasis on diagnostic aspects of subclinical rumen acidosis: a review. Veterinarija ir zootechnika. T. 20 (42). 2002

Enemark, J. M. D., R.J. Jorgensen, and N.B, Christensen. 2004. An evaluation of parameters for the detection of subclinical rumen acidosis in dairy herds. Vet. Res. Comm. 28:687-709.Enemark, J. M. 2008. The monitoring, prevention and treatment of sub-acute ruminal acidosis (SARA): A review. Vet. J. 176:32-43.

Enemark, J. M. 2008. The monitoring, prevention and treatment of subacute ruminal acidosis (SARA): A review. Vet. J. 176:32-43.

Ensminger, M. E. 1993. Dairy Cattle Science. / 3rd rev. ed. Interstate Publishers Inc. Danville, Illinois.

Erb, R. E., S. A. Tillson, G. D. Hodgen and E. D. Plotka. 1970. Urinary creatinine as an index compound for estimating rate of excretion of steroids in the domestic sow. J. Anim Sci. 30: 79–85.

Erb, R. E., A. H. Surve, R.D. Randel and H. A. Garverick. 1977. Urinary creatinine as an index of urinary excretion of estrogen in cows prepartum and postpartum. J. Dairy Sci. 60:1057–1063.

Erdman, R. A. 1988. Dietary Buffering Requirements of the Lactating Cow: A Review. J. Dairy Sci. 71:3246-3266.

Erfle, J. D., R. J. Boila, R. M. Teather, S. Mahadevan, and F.D. Sauer. 1982. Effect of pH on fermentation characteristics and protein degradation by rumen microorganisms in vitro. J. Dairy Sci. 65:1457-1464.

Fulton, W. R., T. J. Klopfenstein, and R. A. Britton. 1979a. Adaptation to high concentrate diets by beef cattle. I. Adaptation to corn and wheat diets. J. Anim. Sci. 49(3): 775.

Fulton, W. R, T. J. Klopfenstein and R.A Britton. 1979b. Adaptation to high concentrate diets by beef cattle. II. Effect of ruminal pH alteration on rumen fermentation and voluntary intake of wheat diets. J. Anim. Sci. 49:785-789.

Fürll, M. 1994. Diagnostik und Therapie chronischer Störungen des Säure-Basen-Haushaltes (SBH) bei Rindern. Der Prakt. Tierarzt, XXIV Coll. Vet. 75:49-54.

Garrett, E. F., K. V. Nordlund, W. J. Goodger, and G. R. Oetzel. 1997. A cross-sectional field study investigating the effect of periparturient dietary management on ruminal pH in early lactation dairy cows. J. Dairy Sci. 80(Suppl. 1):169.

Garrett, E. F., M. N. Pereira, K. V. Nordlund, L. E. Armentano, W. J. Goodger and G. R. Oetzel. 1999. Diagnostic methods for the detection of subacute ruminal acidosis in dairy cows. J. Dairy Sci. 82:1170-1178.

Geishauser, T. 1993. An instrument for collection and transfer of ruminal fluid and for administration of water soluble drugs in adult cattle. Bov. Pract. 27:38–42.

Ghorbani, G. R., D. P. Morgavi, K, A. Beauchemin, and J. A. Z. Leedle. 2002. Effects of bacterial direct-fed microbials on ruminal fermentation, blood variables, and the microbial populations of feedlot cattle. J. Anim Sci. 80:1977-1985.

Goad, D. W., C. L. Goad, and T. G. Nagaraja. 1998. Ruminal microbial and fermentative changes associated with experimentally induced subacute acidosis in steers. J. Anim. Sci. 76:234-241.

Gozho, G. N., J. C. Plaizier, D. O. Krause, A. D. Kennedy, and K. M. Wittenberg. 2005. Subacute ruminal acidosis induces ruminal lipopolysaccharide endotoxin release and triggers an inflammatory response. J. Dairy Sci. 88:1399-1403.

Gozho, G. N., D. O. Krause, and J. C. Plaizier. 2006. Rumen lipopolysaccharide and inflammation during grain adaptation and subacute ruminal acidosis in steers. J. Dairy Sci. 89:4404-4413.

Gozho, G. N., D. O. Krause, and J. C. Plaizier. 2007. Ruminal lipopolysaccharide concentration and inflammatory response during grain-induced subacute ruminai acidosis in dairy cows. J. Dairy Sci. 90:856-866.

Grant, R. J., V. F. Colenbrander, and D. R. Mertens. 1990. Milk Fat Depression in Dairy Cows: Role of Particle Size of Alfalfa Hay. J. Dairy Sci. 73: 1823-1833.

Grant, R. J. 1994. Influence of corn and sorghum starch on the in vitro kinetics of forage fiber digestion. J. Dairy Sci. 77:1563-1569.

Grieve, D. G., S. Korver, Y. S. Rijpkema, and G. Hof. 1986. Relationship between milk composition and some nutritional parameters in early lactation. Livestock Prod. Sci. 14(3):239-254.

Griinari, J. M., D. A. Dwyer, M. A. Mcguire, D. E. Bauman, D. L. Palmquist, and K.Y.V. Nurmela. 1998. Trans-Octadecenoic acids and milk fat depression in lactating dairy cows. J. Dairy Sci.81:1251-1261.

Griinari, J. M., and D. E. Bauman. 1999. Biosynthesis of conjugated lioleic acid and its incorporation into meat and milk in ruminants. Pages 180-200 in conjugated Linoleic acid: Biochemical and Nutritional, Chemical, cancer and Methodological aspects.

Grummer, R. R. 1991. Effect of feed on the composition of milk. J. Dairy Sci. 74, 3244-3257.

Guard, C. 1993. Disorders of the organ systems: diseases of the alimentary system. Pages 792-795. In: Smith, B.P. Tratado de medicina interna de grandes animais. São Paulo: Manole.

Hibbard, B., J. P. Peters, S. T. Chester, I. A. Robinson, S. Kotarski, W. I. Croom, and W. M. Hagler. 1995. The effect of slaframine on salivary output and subacute and acute acidosis in growing beef steers. J. Anim. Sci. 73:516-525.

Hoeben, D., C. Burvenich, E. Trevisi, G. Bertoni, J. Hamann, R. Buckmaier, and J. W. Blum. 2000. Role of endotoxins and TNFalpha in the pathogenesis of experimentally induced coliform mastitis in periparturient cows. J. Dairy Res. 67: 503–514.

Hollberg, W. 1984. Vergleichende Untersuchungen von mittels Schambye-Sørensen-Sonde oder durch Punktion des kaudoventralen Pansensacks gewonnenen Pansensaftproben. Dtsch. Tierärztl. Wochenschr. 91: 317-320.

Höltershinken, M., V. Kress, U. Rathjens, J. Rehage, and H. Scholz. 1997. Auswirkungen oral zu verabreichender Therapeutika auf Fermentationsvorgänge im Pansensaft ruminierender Rinder (in vitro). 7. Mitteilung: Wirkung von Trockenhefe bei chronischer Pansenacidose. Dtsch. Tierärztl. Wchschr. 104:317-320.

Hoover, W.H., and TK. Miller. 1992. Rumen digestive physiology and microbial ecology In Nutrition & Lactation in the Dairy Cow Bulletin 708T.

Horn, G. W., J. L. Gordon, E. C. Prigge, and F. N. Owens. 1979. Dietary buffers and ruminal and blood parameters of subclinical lactic acidosis in steers. J. Anim. Sci.48:683-691.

Howard, J. L., and R. A. Smith. 1999. Current veterinary therapy - food animal practice 4. Page 766 in 4th ed. W.B. Saunders, St. Louis.

Huber, T.L. 1976. Physiological effects of acidosis on feedlot cattle. J. Anim. Sci. 43:902-909.

Huntington, G., and R. Britton. 1978. Effect of dietary lactic acid content and energy level on rumen lactate metabolism in sheep. J. Anim. Sci. 47:241-246.

Huntington, G. B. and R. A. Britton. 1979. Effect of dietary lactic acid on rumen lactate metabolism and blood acid-base status of lambs switched from low to high concentrate diets. J. Anim. Sci. 49(6): 1569.

Hurley, J. C. 1995. Endotoxemia: Methods of detection and clinical correlates. Clin Microbiol Rev 8(2):268-292.

Hyldgaard-Jensen, J., and Simesen, M. G. 1966. Grutforgiftning hos kvaeg. Nordisk Veterinaemedicin. 18:73-94.

International Atomic Energy Agency. 1997. Estimation of rumen microbial protein production from purine derivatives in urine. Pages 22-24 in IAEA-TECDOC-945. IAEA, Vienna.

Ireland-Perry, R. L., and C. C. Stallings. 1993. Fecal consistency as related to dietary composition in lactating Holstein cows. J Dairy Sci. 76:1074-1082.

Irwin, L. N., G. E. Mitchell, R. E. Tucker, and G. T. Schelling. 1979. Histamine, tyramine, tryptamine and electrolytes during glucose induced lactic acidosis. J. Anim. Sci. 48:367–374.

Kelly, E.R., and J.D. Leaver. 1990. Lameness in dairy cattle and the type of concentrate given. Anim. Prod. 51:221-227.

Keunen, J. E., J. C. Plaizier, I. Kyriazakis, T. F. Duffield, T. M. Widowski, M. I. Lindinger and B. W. McBride. 2002. Effects of a Subacute Ruminal Acidosis Model on the Diet Selection of Dairy Cows, J. Dairy Sci. 85:3304-3313.

Khafipour, E., D. O. Krause, and J. C. Plaizier. 2008a. A Grain-based Subacute Ruminal Acidosis Challenge Causes Translocation of Lipopolysaccharide and Triggers Inflammation. J. Dairy Sci. Accepted Oct 8, 2008.

Khafipour, E. 2008b. Mechanistic relationships between immune and microbial responses of dairy cows subjected to subacute ruminal acidosis (PhD thesis). University of Manitoba.

Kleen J. L., G. A. Hooijer, J. Rehage, and J. P Noordhuizen. 2003. Subacute ruminal acidosis (SARA):a review. J. Vet. Med. A. 50:406-414.

Koenig, K. M., K. A. Beauchemin, and L. M. Rode. 2003. Effect of grain processing and silage on microbial protein synthesis and nutrient digestibility in beef cattle fed barley-based diets. J. Anim. Sci. 81:1057-1067.

Koers, W. C., R. A. Britton, T. J. Klopfenstein, and W. R. Woods. 1976. Ruminal histamine, lactate and animal performance. J. Anim. Sci. 43: 684-691.

Kovac, G., P. Mudron, and P. Bartko. 1986. Comparative studies on the pH of blood, rumen and abomasal fluid, feces and urine in cattle. Folia Veterinaria. 30: 21-35.

Krajcarski-Hunt, H., J. C. Plaizier, J. P. Walton, R. Spratt, and B. W. McBride. 2002. Short Communication: Effect of Subacute Ruminal Acidosis on In Situ Fiber Digestion in Lactating Dairy Cows. J. Dairy Sci. 85:570-573.

Krause, K. M. and G. R. Oetzel. 2005. Inducing subacute ruminal acidosis in lactating dairy cows. J. Dairy Sci. 88:3633-3639.

Krehbiel, C. R., R. A. Stock, D. H. Shain, C. J. Richards, G. A. Ham, R. A. McCoy, T. J. Klopfenstein, R. A. Britton, and R. P. Huffman. 1995. Effect of level and type of fat on subacute acidosis in cattle fed dry-rolled corn finishing diets. J. Anim. Sci. 73:2438–2446.

Kutas, F. 1965. Determination of net acid-base excretion in urine of cattle. Acta Vet. Acad. Sci. hung. 15: 147-153.

Lachmann, G. and Siebert, H. 1980. Die Bestimmung des Säure-Basen-Status in den Erythrocytten und im Lebergewebe beim Rind. Mh. Vet.-Med. 35:384-388.

Lane, G. T., C. H. Noller, V. F. Colenbrander, K. R. Cummings, and R. B. Harrington. 1968. Apparatus for obtaining ruminoreticular samples and the effect of sampling location on pH and volatile fatty acids. J. Dairy Sci. 51:114–116.

Leedle, J.; Coe, M. L. and Russel, A. F., 1995. Evaluation of health and ruminal variables during adaption to grain-based diets in beef cattle. Am. J. Vet. Res. **57**, 885-892.

Lindberg, J. E., H. Bristav, and A. R. Manyenga. 1989. Excretion of purines in the urine of sheep in relation to duodenal flow of microbial protein. Swedish Journal of Agricultural Research 19:45-52.

Loor, J. J., K. Ueda, A. Ferlay, Y. Chilliard, and M. Doreau. 2004. Biohydrogenation, duodenal flow, and intestinal digestibility of trans fatty acids and conjugated linoleic acids in response to dietary forage:concentrate ratio and linseed oil in dairy cows. J. Dairy Sci. 87:2472-2485.

Manson, F. J. and J. D. Leaver. 1988. The influence of concentrate amount on locomotion and clinical lameness in dairy cattle. Anim. Prod. 47:185-190.

Markusfeld, O. 1987. Aciduria in the Postparturient Dairy Cow. Br. Vet. J. 143:119-127.
Melvin, J. 1970. Duke's Physiology of Domestic Animals. 8th Ed. Cornell University.

Mertens, D. R. 1997. Creating a system for meeting the fiber requirements of dairy cows. J. Dairy Sci.80:1463-1481.

Morgante, M., C. Stelletta, P. Berzaghi, M. Gianesella and I. Andrighetto. 2007. Subacute rumen acidosis in lactating cows: An investigation in intensive Italian dairy herds. J. Anim. Physiol. Anim. Nutr. 91:226-234.

Mortensen, K. 1993. Laminitis in Cattle. Ph.d.-Thesis, Royal Veterinary and Agricultural University, Copenhagen. pp. 253.

Mosby. 1996. Pages 3-15 In Ruminant history, physical examination and records. Large animal internal medicine/ 2nd edition, St. Louis, Missouri, USA.

Nagaraja, T.G., E.E. Bartley, L.R., and H.D. Anthony. 1978. Relationship of rumen Gram negative bacteria and free endotoxin in the circulatory system of sheep and cattle after induced grain engorgement. Am. J. Vet. Res. 36:831-832.

Nagaraja, T. G., E. E. Bartley, L. R Fina, and H. D. Anthony. 1978a. Relationship of rumen gram-negative bacteria and free endotoxin to lactic acidosis in cattle. J. Anim. Sci. 47:1329-336.

Nagaraja, T. G. and E. C. Titgemeyer. 2007. Ruminal acidosis in beef cattle: The current microbiological and nutritional outlook. J. Dairy Sci. 90 (Suppl 1):E17-38.

Nakamura, F., and Kurihara, Y. 1978. Maintenance of a certainrumen protozoal population in a continuous in vitro fermentation system. Appl. Environ. Microbiol. 1978:500-506.

Nikolov, Y. 1996. Clinical experimental studies on acute rumen acidosis in buffaloes (*Bubalus bubalis* L.). II. Influence of acute rumen acidosis on biochemistry of rumen liquor. Vet. Sci. 4, 17-20.

Nikolov, Y. 1998. Clinical experimental studies on acute rumen acidosis in buffaloes (Bubalus bubalis L.). IV. Influence of acidosis on blood, rumen liquid and urine electrolytes. Vet. arhiv 68: 1-9.

Nocek, J. E. 1997. Bovine acidosis: Implications on laminitis. J. Dairy Sci. 80:1005-1028.

Nordlund, K. V., and E. F. Garrett. 1994. Rumeno centesis – a technique for collecting rumen fluid for the diagnosis of subacute rumen acidosis in dairy herds. The Bovine Prac. 28, 109–112.

Nordlund, K. V., E. F. Garrett, and G. R. Oetzel. 1995. Herdbased rumenocentesis: a clinical approach to the diagnosis of subacute rumen acidosis. Pages 48-56 in Compend. Contin. Educ. Pract. Vet. 17.

Nordlund, K. V., N. R Cook, and G. R Oetzel. 2004. Investigation strategies for Laminitis Problem Herds. J. Dairy Sci. 87:E27-E35.

Novozamsky, I., R. van Eck, J. C. van Schouwenburg, and I. Walinga. 1974. Total nitrogen determination in plant material by means of the indophenol-blue method. Neth. J. Agric. Sci. 22:3–5.

NRC. 2001. Nutrient requirement of dairy cattle. 7th rev.ed. National Research Council, Washington, DC. National Academy Press.

Oetzel, G. R., and K. V. Nordlund. 1998. Methods of evaluating ruminal pH in dairy cattle. Page 203 in Proc. 31st Annu. Conv.Am. Assoc. Bovine Pract., Rome, GA.

Oetzel, G. R., K. V. Nordlund, and E.F. Garett. 1999. Effect of ruminal pH and stage of lactation on ruminal lactate concentration in dairy cows. J Dairy Sci. 82(1):35.

Oetzel, G. R. 2000. Clinical aspects of ruminal acidosis in dairy cattle. Proc. Am. Assoc. Bov. Prac. 33:46-53.

Olfert, E. D., B. M. Cross, and A. A. McWilliam. 1993. Guide to the care and use of experimental animals. in CCAC. Vol. 1. Canadian council on animal care, Ottawa, ON, Canada.

Olson, J. D. 1991. Relationship of Nutrition to Abomasal Displacement and Parturient Paresis. Bovine Pract. 26:88-91.

Owens, F. N., and A L. Goetsch. 1988. Ruminal fermentation. In: The Ruminant Animal. Digestive Physiology and Nutrition. Edited by Church, D. C. p. 145-171.

Owens, F. N., D. S. Secrist, W. J. Hill, and D. R. Gill. 1998. Acidosis in cattle – a review. J. Anim. Sci. 76: 275–286.

Parthasarthy, D. and A.T. Philipson. 1952. The movement of potassium, sodium chloride and water across the rumen epithelium of sheep. J. Physiol. 121:452-469.

Patra, R. C., S. B. Lal, and D. Swarup. 1995. Biochemical profile of rumen liquor, blood and urine in experimental acidosis in sheep. Small Ruminant Res. 19:177-180.

Phy, T. S. and F. D. Provenza. 1998. Sheep fed grain prefer foods and solutions that attenuate acidosis. J. Anim. Sci. 76:954-960.

Pitt, R. E., J. S. Van Kessel, D. G. Fox, A. N. Pell, M. C. Barry, and P. J. Van Soest. 1996. Prediction of ruminal volatile fatty acids and pH within the net carbohydrate and protein system. J. Anim. Sci. 74:226-244.

Plaizier, J. C., J. E. Keunen, J. P. Walton, T. F. Duffield, and B. W. McBride. 2001. Effect of subacute ruminal acidosis on in situ digestion of mixed hay in lactating dairy cows. Can. J. Anim. Sci. 81:421-423.

Plaizier, J. C., A. M. Fairfield, P. A. Azevedo, A. Nikkhah, T. F. Duffield, G. H. Crow, R. Bagg, P. Dick and B. W. McBride. 2005. Effects of monensin and stage of lactation on variation of blood metabolites within twenty-four hours in dairy cows. J. Dairy Sci. 88:3595-3602.

Plaizier, J. C., D. O. Krause, G. N. Gozho, and B. W. McBride. 2008. Subacute ruminal acidosis in dairy cows: The physiological causes, incidence and consequences. Vet. J. 176 (1):21-31.

Randhawa, S. S. 1987. Evidence of toxic factors in the rumen liquor of healty and acidotic buffalo calves. Acta Vet. 56: 455-464.

Randhawa, S. S., P. P. Gupta, K. S. Roy, A. K. Ahuja and S. S. Rathore. 1989. Biochemical, pathological and histoenzymological studies on experimental acute lactic acidosis in buffalo calves (Bubalus bubalis). Buffalo J. 5(1):12-24.

Russell, J. B., and D. B. Dombrowski. 1980. Effect of pH on the efficiency of growth by pure cultures of rumen bacteria in continuuous culture. Appl. Environ. Microbiol. 39:604-610.

SAS. 2004. Sas / stat users guide, release 9.1.2. Vol. ed. No. SAS Inst., Inc., Cary, NC.

Sann, J., L. Creighto and A. Lehman. 2005. Page375-395 in JMP start statistics (3rd edition), Thomson learning Books/cole.

Sauvant, D., and D. R. Mertens. 1998. Dietary characteristics affecting ruminal acidosis. Pages 63–65 in Annual Research Summaries 1998. US Dairy Forage Research Center, Madison, WI.

Shaver, R. D., 1997. Nutritional Risk Factors in the Etiology of Left Displaced Abomasum in Dairy Cows: A Review. J. Dairy Sci. 80: 2449-2453.

Shi, Y., and P.J. Weimer. 1992. Response surface analysis of the effects of pH and dilution rate on Ruminococcus flavefaciens FD-1 in cellulose fed continuous cultures. Appl. Environ. Microbiol. 58:2583-2591.

Simesen, M. G. and Konggaard, S. P., 1970. Eksperimentelle undersøgelser vedrørende roeforgiftning hos kvæg. Nord. Vet. Med. 22:174-185.

Slyter, L. L. 1976. Influence of acidosis on rumen function. J. Anim. Sci. 43(4): 910-929.

Stock, R.A. and B.A. Britton. 1993. Acidosis in feedlot cattle. In: Scientific update on Rumensin/Tylan for the professional feedlot consultant. Elanco Animal Health, Indianapolis, IN. p. B1.

Stock, R. 2000. Acidosis in cattle: an overview. Proc. Am. Assoc. Bov. Prac. 33:30-37.

Stone, W.C. 1999. The effect of subclinical rumen acidosis on milk components. Proc. Cornell Nutr. Conf. 40-46.

Stone, W. 2004. Nutritional approaches to minimize subacute ruminal acidosis and laminitis in dairy cattle. J. Dairy Sci. 87:13-26.

Sutton, J. D., I. C. Hart, W. H. Broster, R. J. Elliott, and E. Schuller. 1986. Feeding frequency for lactating cows: effects on rumen fermentation and blood metabolites and hormones. Br. J. Nutr. 56:181-192.

Underwood, W. J. 1992. Rumen Lactic Acidosis. Part I. Epidemiology and Pathophysiology. Compendium on Continuing Education for the Practicing Veterinarian. 14:1127-1133.

Van Soest P.J. 1994. Nutritional Ecology of the Ruminant. 2nd ed. Cornell University Press. Ithaca, New York.

Verheiden, J. H. M., A. S. J. P. A.M. van Miert, A. J. H. Schotman, and C. T. M. van Duin. 1981. De pathogenese van coliforme mastitis. Tijdschrift v. Diergeneeskunde 106: 501–507.

Wallace, R. J., and M. A. Cotta. 1989. Metabolism of nitrogentaining compounds. Pages 217-250 in The Rumen Microbial Ecoosystem. P. N. Hobson, ed. Elsevier Applied Science, New York.

APPENDICES

I Analytic methods used to assess NABE in cattle urine

A. NABE assessment, using the reference method and an automated titrator. (Standard method)

1. 10 ml urine from a well mixed total sample is poured into a test tube. The sample is preferably taken from the same area in the total sample . The test tube is placed on a magnetic stirrer and a pH electrode is placed permanently in the sample (Titrino 702, *Metrohm,*)

2. The titrator is programmed to perform a 2 point titration starting with a titration to pH = 4.0 by adding 1 M HCl. As a result, all HCO3 - present will be converted to CO2 and water, and any phosphate sediments will be dissolved. Furthermore, all acids present will be left undissociated.

3. Gentle, short time (½ - 1 minute) boiling of sample using a hotplate, which will liberate and eliminate formed CO2. Subsequent cooling to room temperature using ice.

4. Addition of 10 ml 16-20% aqueous formalin solution, adjusted to pH between 6 to 8 using 0.1 M NaOH. Formalin will ensure that NH4 +-ions present, stay titratable by formation of Hexamethylenetetramin and simultaneously liberation of H+ (*Burton*, 1980). Added formalin does not affect pH of the analytic sample (*Jørgensen*, 1957).

5. Titration of urine sample with x ml of an 0.1 M NaOH solution until pH = 7.4. (done automatically by the titrator). This will expel any free or bound H+.

6. Hence, net acid base excretion in 10 ml urine can be calculated as follows:

number of ml x 1mmol/ml - number of ml 0.1 mol/ml = number of mol acid-or base in excess pr. 10 ml

urine. To convert this into NABE pr. litre of urine, the result above has to be multiplied by 100. Hence:

NABE (mol/l) = 100(y ml x 1 M HCl - y ml x 0.1 M NaOH)

Calculation is done automatically by the tirator.

7. Step 4 - 9 is repeated for each urine sample. Replicates are estimated for each sample.

B. On-farm method (NABE farm)

The analysis is performed corresponding to the reference method but without the help of the automated titrator, the pH metre is replaced by the colour indicator cresol purple and addition of hydrochloric acid and NaOH is done with an 1 ml disposable syringe. Due to practical reasons and in order to simplify titration towards pH = 7.4, 1 M NaOH is used instead of 0.1 M (reference method). The analytic sample is poured into 25 ml test tubes and pumice stones are added. Initially, 3 ml of 1 M hydrochloric acid is added to all samples as to standardize this analytic step. Subsequently, the sample is boiled for ½ - 1 minute using an Bunsen burner. After cooling, 10 ml of formalin solution and 6 drops of cresol purple are added. By adding 1 M NaOH, the urine sample is titrated back to the alkaline transition range of cresol purple. The number of ml 1 M NaOH added is recorded and NABE is calculated as follows:

NABE mol/l = 10(10(3 - ml 1 M NaOH))

Replicates are estimated for each sample.