EFFECTS OF SUBACUTE RUMINAL ACIDOSIS (SARA) ON RUMINAL LIPOPOLYSACCHARIDE RELEASE AND ON INFLAMMATION IN CATTLE

BY

GEORGE NHAMO GOZHO

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department of Animal Science
University of Manitoba
Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION

Effects of Subacute Ruminal Acidosis (Sara) on Ruminal Lipopolysaccharide Release and on Inflammation in Cattle

BY

George Nhamo Gozho

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

Doctor of Philosophy

To Dr Timothy Mutsvangwa

ABSTRACT

Subacute ruminal acidosis (SARA) is a common disease in dairy cows fed high concentrate diets. This disease can result in rumenitis, laminitis and sudden death syndrome, but the mechanisms responsible for this are not yet well understood. Roles of the concentration of free lipopolysaccharide (LPS), in the rumen and LPS translocation through the rumen epithelium, especially if the barrier function is compromised by SARA, and inflammation, have been suggested in these mechanisms. The primary objective of this study was to investigate the effects of grain induced SARA in cattle on free ruminal LPS concentration and on major acute phase proteins and other inflammation markers in cattle.

Subacute ruminal acidosis was induced using a nutritional model in which pellets made of equal amounts of wheat and barley were fed to cattle. Feeding protocols used included restricting other types of feeds during the time the pellets were fed, or mixing pellets to form part of the ration offered to cattle. The pellets were fed to steers adapted to an all forage diet (experiment 1), steers gradually adapted to 60% concentrate over 21 days (experiment 2) or to dairy cows after more than 17 wk of feeding a total mixed ration that contained 44% concentrate (experiment 3). Rumen pH was monitored continuously and summarized as average rumen pH, time with pH below 6.0 and 5.6 and area (time x pH) below pH 6 and area below pH 5.6.

Wheat-barley feeding decreased average rumen pH from 6.49 to 6.19 in experiment 1, from 6.72 to 6.14 in experiment 2, and from 6.24 to 6.01 in experiment 3. Wheat-barley feeding also increased the time with pH below 6.0 from 9 to 589 min/d in

experiment 1, from 22 to 600 min/d in experiment 2 and from 460 to 742 min/d in experiment 3. Time with pH below 5.6 increased from 5 to 187 min/d in experiment 1, from 0 to 219 min/d in experiment 2 and from 187 to 309 min/d in experiments 3 with wheat-barley feeding. Free ruminal LPS concentrations increased from 3714 to 12,589 EU/mL in experiment 1, from 6310 to 26,915 EU/mL in experiment 2, and from 22,908 to 128,825 EU/mL in experiment 3 when SARA was induced. Lipopolysaccharide was below detection limit of the assay in peripheral blood but serum amyloid A and haptoglobin concentrations increased from 33.6 to 170.7 μg/mL and 0.43 to 0.79 mg/mL when SARA was induced in experiment 1, and 38 to 163 μg/mL and 0.53 and 1.40 mg/mL when SARA was induced in experiment 2. In experiment 3 serum amyloid A concentrations increased from 286.8 to 498.9 μg/mL following induction of SARA, but haptoglobin and other markers of inflammation such as fibrinogen, serum copper and differential white blood cell counts were not affected.

These data demonstrate that inducing SARA in cattle resulting in 3 h or more with pH below 5.6 activates an inflammation response as the host animal's immune system responds to restore homeostatic balance. It is possible that such inflammation results from a combination of tissue injury in the gastrointestinal tract and an increased rate of free ruminal LPS translocation into the pre-hepatic blood circulation.

ACKNOWLEDGMENTS

I would like to thank my supervisors Drs J.C. Plaizier and D.O. Krause for their guidance, encouragement and patience with me during the course of my studies. I am forever indebt to Dr Plaizier for working tirelessly to facilitate my relocation to Canada, looking after me upon arrival and assisting me to get my family to join me in Canada. I also thank the members of my degree committee; Drs Wittenberg, Kennedy and Zhanel who offered their expertise and advice on different aspects of this study and helped to shape it.

I would also like to extend my warmest gratitude to the following: Robert Stuski, Dale Rosner and staff at Glenlea Research Station for looking after the animals used in the experiments, Terri Garner, Deanne Fulawka, Ricki Araneda, Prakash Sharma, Janice Haines,Karen Carette Akbar Nikkhah, Ehsan Khafipoor and Sanjiv Bandari for technical assistance; and Drs Gary Crow and Loreen Onischuk for help with statistical analysis.

I would like to acknowledge the financial support I received in the form of a Graduate student tuition waiver and the Cyril L. Anderson – Ridley Canada Limited Graduate Fellowship in Animal Nutrition. The support I got in the form of inspiration from my sons Munashe and Masimba, and encouragement from my extended family in Zimbabwe, my Kiwi parents Pamela and Len Blackwell, Brother Grant and Sisters Kerry and Sally made it possible for me to carry on when it was easy to find an excuse to give up. Finally I would like to thank the immense support I got from the most important person in my life, my wife Miriam who made it possible for me to see the light at the end of the tunnel. Thank you for being the anchor in my life and being there when I needed you the most.

FOREWARD

This thesis is written in manuscript style. The first manuscript has been published in 'Journal of Dairy Science'; the second manuscript has been submitted to 'Journal of Dairy Science' and the third manuscript has not yet been submitted to a journal

The authors of the manuscripts are:

I. G. N. Gozho, J. C. Plaizier, D. O. Krause, A. D. Kennedy and K. M. Wittenberg

II. G.N. Gozho, J.C. Plaizier and D.O. Krause

III. G.N. Gozho, J.C. Plaizier and D.O. Krause

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ac/Pr ratio = acetate to propionate ratio

APP = acute phase protein

APR = acute phase response

BW = body weight

CGH = chopped grass hay

CLA = conjugated linoleic acid

DIM = days in milk

DM = dry matter

DMI = dry matter intake

EU = endotoxin unit

F:C = forage to concentrate ratio

Hp = haptoglobin

IL-1 = interleukin-1

IL-6 = interleukin-6

LAL = limulus amoebocyte lysate

LPS = lipopolysaccharide

NDF = neutral detergent fiber

NSC = nonstructural carbohydrates

SAA = serum amyloid A

SARA = Subacute ruminal acidosis

SCC = somatic cell counts

SD = standard deviation

SE = standard error

TMR = total mixed ration

TNFα = tumor necrosis factor alpha

VFA = volatile fatty acid

WBP = wheat barley pellets

GENERAL INTRODUCTION

Subacute ruminal acidosis (SARA) is defined as a digestive disorder in cattle that is characterized by prolonged episodes of depressed rumen pH. Daily episodes of rumen pH between 5.2 and 5.6 have been used to define SARA (Cooper and Klopfenstein, 1996) although some disagreements exist in the literature on the precise pH range. It is generally agreed that within the pH range that characterizes SARA, lactic acid does not accumulate in the rumen and lactic acid concentrations are maintained below 10 m*M* (Burrin and Britton, 1986; Goad et al. 1998). Subacute ruminal acidosis has been shown to reduce fiber digestion in dairy cows (Plaizier et al., 2001; Krajcarski-Hunt et al., 2002), reduce milk yield (Krause and Oetzel, 2005), reduce milk fat percentage (Stone, 1999; Krause and Oetzel, 2005) result in reduced and erratic feed intake (Fulton et al 1979a, b; Cooper et al., 1999), and increased total amylolytic bacteria, anaerobic lactobacilli and lactic acid utilizing bacteria (Goad et al., 1998).

Subacute ruminal acidosis is estimated to cost the US dairy industry between US$500 million and US$1 billion dollars every year through reduced milk production and reduced longevity (Donovan, 1997). Economic losses due to SARA include decreased efficiency of milk production, reduced milk fat yield, increased veterinary costs, and high involuntary culling due to lameness-related problems (Nordlund et al., 1995; Nocek, 1997; Stone, 1999; Kleen et al., 2003).

The diagnosis of SARA is made on a herd basis and involves the analysis of feed intake, milk production records and rumen pH measurements of some animals (Nordlund et al., 1995; Enermark et al. 2002; Nordlund, 2002). Signs such as irregular feed intake, loss

of body condition, intermittent diarrhea, dehydration, abscesses, milk fat depression and laminitis are not specific to SARA, but form part of the diagnostic profile (Nordlund et al., 1995). Measuring rumen pH is currently the only tool used to confirm a diagnosis of SARA in a herd. Rumenocentesis and stomach tubing are the methods that can be employed in rumen fluid sampling for pH measurement in the field. However, problems such as abscesses and other infections around the puncture site, and a reduction in milk production are associated with rumenocentesis (Aceto et al., 2000). Although collecting rumen fluid samples by stomach tube is less invasive, the samples can be contaminated with saliva, and therefore may not be representative of rumen fluid (Nocek, 1997; Duffield et al., 2004). Therefore, only limited information on the prevalence of SARA is available. A survey of 15 dairy farms in Wisconsin reported the presence of SARA in 19% of early lactation cows and 26% of mid-lactation cows (Garrett et al., 1997). Another survey of 14 dairy farms in Wisconsin detected SARA in 20.1% of early and peak lactation cows (Oetzel et al., 1999). While there is no data on the prevalence of SARA in Western Canadian dairy herds, Plaizier et al. (2004) found that in at least 25% of the farms surveyed the particle size of feeds used in the total mixed rations (TMR) were finer than recommended (Heinrichs, 1996), which put these cows at risk of developing SARA.

Dairy cattle at high risk of developing SARA include transition dairy cows, cattle with high dry matter intakes, and cows that are subjected to a high degree of variability in ration composition and meal patterns (Stone, 2004). It has been shown that rumen pH depression occurs in these cows when volatile fatty acid (VFA) production exceeds absorption and outflow from the rumen and when there is insufficient rumen buffering (Stone, 2004). Feeding high concentrate rations increases the nonfiber carbohydrate content

of the diet and subsequently VFA concentration in the rumen from microbial fermentation (Allen, 1997). As well, insufficient fiber in these diets contributes to low rumen pH because of reduced chewing time during eating and rumination which decreases saliva production and subsequently rumen buffering (Shi and Weiner, 1992). High concentrate diets are used in dairy rations in order to meet the nutrient requirements for high yielding cows.

In Western Canada, dairy cow diets are commonly based on barley rather than corn. In comparison to corn, barley grain contains more neutral detergent fiber (NDF) than corn and barley starch is more rapidly fermented than cornstarch in the rumen which puts cows on barley-based diets at risk of SARA (McCarthy et al., 1989; Beauchemin and Rode, 1997). Barley based diets should contain at least 34% NDF to reduce the risk of SARA and milk fat depression (Beauchemin, 1991).

Feeding high concentrate diets is also associated with nutrition-related laminitis (Livesey and Fleming, 1987; Manson and Leaver, 1988; Kelly and Leaver, 1990). The mechanism through which these high concentrate diets cause laminitis is not well understood but research data has shown some association between laminitis and or laminitis and fiber and laminitis and the net energy contents of the diet (Donovan et al., 2004). Although the mechanisms that are involved in the etiology of laminitis are not clearly understood, it has been suggested that laminitis is initiated by the presence of vasoactive substances such as bacterial lipopolysaccharide (LPS) and histamine in the blood which cause damage to blood capillaries and result in sole hemorrhaging (Nocek, 1997). It has also been suggested that a group of enzymes called matrix metalloproteinase are important factors in the manifestations of laminitis (Johnson et al., 1998; Pollitt, 1994).

This link between nutrition and laminitis may start with changes in rumen microflora due to changes in rumen pH as a result of feeding high concentrate diets. The resultant low rumen pH increases the concentration of free ruminal LPS. Additionally, low pH can reduce the barrier function of the rumen wall by causing micro-lesions on the rumen epithelium (Kleen et al., 2003). Histamine produced in the rumen and from rumen mucosal tissue damage and free ruminal LPS that is translocated into blood circulation are vasoactive agents that can result in capillary damage and hemorrhaging in the digit which culminates in laminitis (Nocek, 1997). Free ruminal LPS can increase as a result of rapid growth, death and lyses of rumen gram-negative bacteria (Nagaraja et al., 1978a; Wells and Russell, 1996). To date, there have been no studies of the effects of SARA on free ruminal LPS concentration. Studies in which acute acidosis was induced by grain engorgement in cattle do not support the hypothesis that free ruminal LPS increases when rumen pH decreases (Andersen and Jarlov, 1990; Andersen et al., 1994a). These studies cannot be used to draw inferences on the effect of SARA on free ruminal LPS because acute acidosis results in rumen pH depression below 5.0 whilst SARA only results from rumen pH depressions in the range between 5.2 and 5.6. Rumen pH below 5.2 promotes the proliferation of *Streptococcus bovis* and *Lactobacillus* species as opposed to diverse rumen bacteria populations at higher pH (Schwartzkopf-Genswein et al., 2003).

It is hypothesized that, in the rumen pH range between 5.2 and 5.6, free ruminal LPS concentration increases from a combination of LPS shed from rapidly growing bacteria and through death and lyses of gram-negative bacteria. A low rumen pH also compromises the barrier function of the rumen wall and increases the rate of LPS translocation into blood circulation (Andersen, 2000). The presence of free ruminal LPS in blood circulation could

trigger the host animal's immune system to respond by stimulating macrophage to release pro-inflammatory cytokines, including interleukin-6 (IL-6), interleukin-1 (IL-1) and tumor necrosis factor (TNF)α (Klasing, 1988; Werling et al., 1996). These cytokines can initiate the acute phase response, which is indicated by dramatic increases in the hepatic synthetic activity of acute phase proteins (Colditz, 2002). It is further postulated that the free ruminal LPS and immune system responses are dependent on both the diet and the duration such a diet is fed before SARA is induced. This is because microbial adaptation is a gradual process that requires at least 3 wk for its completion (Mackie and Gilchrist, 1979). Therefore inducing SARA through grain engorgement in cattle after they have been on concentrate diets for less than or more than 3 weeks should produce different responses in terms of concentrations of free LPS in the rumen.

The general objective of the studies described herein were to investigate the effects of grain induced SARA on free ruminal LPS concentration in cattle after they had been on grain based diets for varying periods of time. Since SARA is also believed to compromise the barrier function of the rumen wall and to increase the translocation of LPS into blood circulation, the effects of grain induced SARA on acute phase protein concentration was also investigated.

LITERATURE REVIEW

1.0 Definition of Subacute Ruminal Acidosis

Subacute ruminal acidosis (SARA) is a digestive disorder that is characterized by prolonged daily episodes with rumen pH between 5.2 and 5.6 (Cooper and Klopfenstein, 1996). Apart from a low rumen pH SARA, shows no other discernible clinical signs. The rumen pH that defines SARA is also a matter of controversy because various threshold values have been used in the literature. For example, the upper pH threshold that defines SARA has been defined as 5.5 (Hibbard et al., 1995; Reinhardt et al., 1997), 5.8 (Beauchemin et al., 2001; Ghorbani et al., 2002; Koenig et al., 2003) and 6.0 (Bauer et al., 1995; Krehbiel et al., 1995a, b, c). Rumen pH depression during SARA is largely attributed to increases in VFA with lactic acid concentrations of less than 10 m*M* (Burrin and Britton, 1986; Goad et al., 1998). Ancillary clinical signs include erratic feed intake, high incidence of lameness, depressed milk fat percentage; high incidence of unexplained diarrhea or loose feces as well as unexplained body condition loss and abscesses in affected animals (Nordlund et al., 1995; Nocek, 1997; Enemark et al., 2002; Kleen et al., 2003). These signs are of limited diagnostic value because they are also associated with poor forage quality or poor bunk management (Nocek, 1997). Therefore SARA is often not thought of immediately as being the reason for poor productivity in affected herds.

Subacute ruminal acidosis is a major problem for beef cattle during adaptation to high grain finishing diets (Schwartzkopf-Genswein et al., 2003). In dairy cattle, SARA can be a consequence of maximizing energy intake, which requires provision of appropriate

levels of physical and chemical dietary components (Nocek, 1997). Groups of dairy cows at high risk of developing SARA include; i) transition dairy cows, ii) cows with high dry matter intakes, iii) cows that are subjected to high degree of variability in their ration and meal patterns, and iv) cows fed TMR formulated from feeds with inconsistent moisture content which produces differences in actual ration composition and the diet as represented by the ration formulation (Stone, 2004). A comparison of the economic impact of SARA in dairy and beef cattle has not been documented in the literature. However; it is likely to be greater in dairy than beef cattle because dairy cows are exposed to high concentrate diets for longer periods of time.

1.2 Signs Associated With SARA

1.2.1 Dry Matter Intake

Studies on SARA with beef cattle reported in the literature have used feeding protocols that simulate feedlot conditions where cattle consume large quantities of high concentrate diets (Krehbiel et al., 1995b, c; Hibbard et al., 1995; Reinhardt et al., 1997; Goad et al., 1998; Brown et al., 2000). When cattle were gradually adapted to diets that contained wheat and corn at 35, 55, 75 and 90% of dry matter, daily intake variation was more pronounced in both corn and wheat based diets at the 90% inclusion rate (Fulton et al., 1979a). In a subsequent study feeding the 90% coarsely rolled wheat diet to Hereford x Angus steers and maintaining rumen pH above 5.6 by intra-ruminal infusion of sodium hydroxide increased dry matter intake which illustrates that low rumen pH depresses voluntary feed intake in cattle (Fulton et al., 1979b).

Over time, dry matter intake can return to normal if SARA does not recur. Brown et al. (2000) showed that inducing SARA in Hereford x Angus crossbred steers and then giving them free access to feed in days following SARA induction resulted in a gradual increase in dry matter intake over a 3 d period to become similar to that of control animals. Depression in dry matter intake is believed to be caused by rumen hypomotility that is observed at low rumen pH (Huber, 1976; Kleen et al., 2003). Hypomotility is also believed to result from increased translocation of gram-negative bacteria lipopolysaccharide (LPS) from rumen bacteria as a result of low pH of rumen contents during SARA. The observed hypomotility during acute rumen acidosis and after infusion of LPS into the blood support this theory (Andersen, 2000). Bacterial LPS is the biologically active constituent of endotoxin from gram-negative organisms that play a critical role in initiating proinflammatory events that contribute to the pathogenesis of some diseases. Proinflammatory mediators such as the cytokines TNFα, IL-1 and IL-6 are also believed to suppress voluntary feed intake (Weingarten, 1996).

Rumen fluid osmolality increases in cattle after feed consumption. Inducing SARA increases rumen fluid osmolality beyond the 300 mOsm limit that is associated with normal rumen environment (Carter and Grovum, 1990). Osmolality of rumen fluid has been shown to increase from 300 to 700 mOsm / kg in cattle with acute acidosis (Andersen et al., 1994a). The increase in osmolality is due to dissolved minerals, and organic acid in the rumen. This results in an osmotic gradient that draws fluids into the rumen. Osmolality may also be registered by osmosensors in the reticulo-ruminal wall (Bergen, 1972). These sensors then send a negative feedback that is independent of rumen motility to the brain for the animal to stop eating (Carter and Grovum, 1990). Recent studies show that intraruminal

water infusions during water deprivation decreased rumen fluid osmolality and normalized feed intake suggesting that a combination of rumen fluid and plasma hypertonicity are major factors in feed intake depression (Burgos et al., 2000).

1.2.2 Laminitis

Laminitis is defined as the inflammation of the dermal layers inside the foot (pododermatitis aseptica diffusa) (Nocek, 1997). It is prevalent in dairy cows during early lactation and beef cattle on feedlot (Brent, 1976; Underwood, 1992). The true mechanistic causes of laminitis are poorly understood but nutritional factors are important to its etiology (Ruegg, 2000). Other factors such as season, housing type, and stall surface and stall type are also important in the etiology of laminitis (Wells et al., 1993; Cook, 2003) Diets with high proportions of digestible carbohydrates have been associated with the onset of nutrition mediated laminitis (Livesey and Fleming, 1987; Manson and Leaver, 1988; Kelly and Leaver, 1990; Greenough et al., 1990), particularly in early lactation (Prentice and Neal, 1972). One of the theories on laminitis that implicates nutrition postulates that rumen acidosis increases free ruminal LPS from gram-negative rumen bacteria. The low rumen pH leads to damage to the ruminal walls which subsequently leads to translocation of free ruminal LPS into blood circulation (Nocek, 1997). Ruminal LPS and other substances such as histamines act as vasoactive agents when they are released into blood circulation causing vascular changes within the dermal capillary beds of the corium (Nocek, 1997; Donovan et al., 2004). The vascular changes lead to blood seepage into the corium and subsequently to ischemia, inflammation, and necrosis of the corium-epidermal junction resulting in

laminitis (Nocek, 1997; Donovan et al., 2004). Repeated incidence of laminitis as a result of repeated bouts of SARA in the same animal can lead to lameness.

Surveys carried out over the last 30 years have demonstrated that lameness in dairy cows is a major cause of losses to the dairy industry. Data pertaining to the prevalence of SARA, lameness in general, and specific causes of lameness, along with losses caused by each of these disorders, are scant; therefore it is difficult to estimate the cost of these disorders (Stone, 2004). However, it is estimated that lameness is the third most important problem on many modern dairy farms after mastitis and reproductive failure, contributing to economic losses for farmers (Whitaker et al., 1983; Enting et al., 1997). Economic losses include cost of treatment, decreased milk production, decreased reproductive performance and increased culling due to feet and legs problems. Data on the incidence of laminitis that is included in surveys is often a result of diverse factors whose ultimate manifestation is lameness in cattle. Therefore care must be taken to try and separate the contribution of environmental from nutritional causes of lameness in order to avoid bias. However, the leading cause of lameness is laminitis (Frankena et al., 1992).

1.2.3 Milk Fat Depression

Milk fat depression or low milk fat syndrome is also associated with SARA. However, milk fat content is also influenced by stage of lactation, breed, the proportion of concentrate in the diet, the levels of unsaturated dietary fatty acids and processing of forages in the ration (Chouinard et al., 1999; Grant et al., 1990). The theory that low milk fat can result from SARA comes from the observed linear relationship between the ratio of acetate + butyrate to propionate in rumen fluid and milk fat content (Kaufman, 1976;

Sutton et al., 1986). This is because an increase in readily fermentable carbohydrates in the diet favors a shift in rumen fermentation toward propionate. This shift in ruminal VFA can affect milk fat synthesis through increasing supply of propionic acid at the expense of acetate in the rumen. Propionic acid is a precursor for glucose formation and therefore when its concentration increases more glucose is formed through gluconeogenesis. In lactating dairy cows glucose is an important precursor for lactose synthesis and hence an increase in glucose leads to higher milk yield without a concomitant increase in milk fat, which invariably depresses milk fat. Increased propionate production in the rumen can also lead to low intra-ruminal production of vitamin B_{12} which causes a reduction in the conversion of propionate to succinyl-coA. The resultant increase in the concentration of methyl-malonate in the blood inhibits fat synthesis in various tissues including the mammary gland (Van Soest, 1994).

Other evidence suggests that incomplete saturation of fatty acids in the rumen is the reason for milk fat depression. Infusion of 50, 100 and 150 g/d of conjugated linoleic acids (CLA) into the abomasum reduced milk fat content in Holstein dairy cows (Chouinard et al., 1999). Conjugated linoleic acids represent a mixture of positional and geometric isomers of octadecadienoic acid with conjugated double bonds, and represents incomplete biohydrogenation in dietary fatty acids. The CLA in ruminant milk and meat originates from CLA produced from incomplete biohydrogenation of dietary linoleic acid in the rumen, and endogenous synthesis of CLA from trans-11 octadecadienoic acid (Griinari and Bauman, 1999). Recent data show that biohydrogenation of polyunsaturated fatty acids in the rumen is reduced when high concentrate diets are fed (Loor et al., 2004). This partly explains the association between milk fat depression and high concentrate diets.

1.2.4 Diarrhea

Incidences of unexplained diarrhea are associated with SARA in a dairy herd (Nocek, 1997). It has been suggested that fecal consistency can be used as a diagnostic aide for SARA. For example, Ireland-Perry and Stalling (1993) found that cows consuming low fiber diets had feces that visually appeared to be of more liquid consistency but actually had greater DM content than those from cows on high fiber diets. Rumen pH was not recorded in this study and hence it is not possible to postulate whether this would be true under rumen conditions that define SARA. However, others (e.g. Nordlund et al., 2004) content that diarrhea is of limited usefulness in the diagnosis of SARA.

Qualitative evaluations of feces may yield important information. For example, feces from cows with SARA may appear brighter and yellowish (Kleen et al., 2003). Foamy feces and diarrhea suggest extensive hindgut fermentation which can be associated with SARA (Nordlund et al., 2004). Fermentation in the hindgut produces VFA and gases such as carbon dioxide. Whereas the VFA can be absorbed, microbial protein is excreted in feces and the gas produced appear as bubbles in feces giving feces the 'foamy' appearance. Hindgut fermentation also results in increased acidity of hindgut contents and feces and this leads to sloughing of epithelial cells in the large intestine (Hall, 2002). Protection from further damage is accorded the animal by mucous or fibrin that is secreted to protect the injured tissue (Argenzio et al., 1988). Mucin or fibrin casts found in the feces often have the tubular form of the gut which is evident that intestinal damage has occurred (Hall, 2002). Extensive hindgut fermentation may also contribute to diarrhea. This is because fecal consistency is determined by movement of water into the digestive tract when digesta become hypertonic to plasma as a result of SARA (Huber, 1976).

1.3 Patho-Physiology of SARA

Subacute ruminal acidosis is associated with inflammation of different tissue and organs (Kleen et al., 2003). Factors that initiate inflammation include physical damage to mucosal tissue and translocation of enteric bacteria such as *Fusobacterium necrophorum* into blood circulation. These pathogens ultimately end up in body organs such as heart, lungs and kidneys (Nordlund et al., 1995; Nocek, 1997). A combination of high concentration of free ruminal LPS and rumen mucosal tissue damage can increase free ruminal LPS translocation into blood circulation which results in inflammation (Nagaraja et al., 1978a, b; Andersen, 2000). Therefore changes in rumen fermentation that alters rumen bacterial populations and increases the acidity of rumen contents predisposes cattle to SARA-related diseases and conditions such as abscesses, laminitis and sudden death syndrome.

1.3.1 Rumen pH

Feed factors that predispose cattle to low rumen pH include amount and type of concentrate, grain processing, forage type and quality. High proportion of readily fermentable carbohydrates in the diet increases ruminal VFA production beyond the rumen's absorptive capacity leading to rumen pH depression (Stone, 2004) and inadequate buffering from saliva flow due to either insufficient forage particle size in the diet or low NDF content in the diet to promote chewing during eating and rumination (Beauchemin, 1991; Beauchemin and Rode, 1997; Mertens, 1997; Nocek, 1997). Additionally, cereal grains are digested more rapidly in the rumen than forages, which leads to more rapid VFA

production and diurnal variation in rumen pH in concentrate based diets compared to forage diets (Mertens, 1997).

Gradual introduction to high concentrate diets is necessary to allow rumen papillae to increase in size and density so that capacity of the rumen wall to absorb VFA increases sufficiently to prevent accumulation of VFA in the rumen (Stone, 2004). Gradual introduction of concentrate also allows both lactic acid-producing and lactic acid-utilizing bacteria increase. The increase in lactic-acid utilizing bacteria is important under these feeding conditions because lactic acid produced by lactic acid-producing bacteria can be converted to VFA which prevents its accumulation in the rumen. Gradually adapting dairy cows to high concentrate diets over 4 to 5 weeks increased the average surface area of rumen papillae from 10 mm^2 to 60 mm^2 and resulted in a fivefold increase in the absorption of acetic, propionic and butyric acid during the first hour of production (Dirksen et al., 1985). The increased absorptive capacity prevented accumulation of VFA and prevented rumen pH from falling below 5.6.

Studies by Mackie and Gilchrist (1979) showed that at least 21 days are required to adequately adapt sheep from high roughage to high concentrate diets in order to allow the slow growing lactic acid-utilizing bacteria to sufficiently increase in number to prevent accumulation of lactic acid produced by the more acid-tolerant lactic acid-producing bacteria. Allowing the population of lactic acid-utilizing bacteria to increase ensures that lactic acid concentration does not increase in the rumen fluid. Lactic acid is a more potent acid which can reduce the rumen pH more drastically compared to VFA because it has a lower dissociation constant; pKa = 3.8 compared to 4.8 for other ruminal acids (Owens et al., 1998). Taken together, data from the studies by Dirksen et al. (1985) and Mackie and

Gilchrist (1979) suggest that adaptation of ruminants to high concentrate diets must be taken in the context of allowing sufficient time for rumen papillary growth and lactic acid-utilizing bacteria to increase in number to prevent rumen pH depression by either an accumulation of VFA or lactic acid in the rumen.

The time taken to adapt cows to concentrate diets is important. For example, Nocek et al. (2002) showed that putting cows on 50, 60 or 70% grain diets and then switching them between these diets whilst ensuring that the change in each case did not result in more than 10% increase in grain, increased diurnal rumen pH variation and daily intake variation for at least one week after the change. In order to meet requirements for energy and protein for dairy cows in early lactation, the level of concentrate in the diet is increased and high quality forages are used in ration formulation. The dilemma is often whether increase nutrient density in the diet by increasing concentrate whilst reducing the forage content; or to ensure adequate forage levels in the diet and reduce the nutrient density in the ration. The latter would require the cows to mobilize their body reserves more rapidly in early lactation in order to meet nutrient requirements for milk production. Forage content is critical in maintaining rumen pH through saliva production. The ability of dietary forage to stimulate chewing is important in maintaining the flow of salivary buffers into the rumen, which are required to neutralize fermentation acids (Bailey, 1961; Mertens, 1997). Dietary fibre promotes chewing activity. The National Research Council (NRC, 2001) recommends a minimum of 25% DM as NDF and that 75% of it must come from forage sources, for diets containing corn grain. Diets that are low in fiber are often associated with ruminal acidosis, reduced rumination, saliva secretion and fiber digestion, low acetate to propionate ratio and milk fat depression (Mertens, 1997).

The physical form of the fibre is also important because the feed particles size in the diet affects saliva production during eating and rumination. Saliva supplies an estimated 70 to 90% of the fluid and buffering capacity entering the rumen (Bailey 1961). Soita et al., (2000), fed low and high concentrate diets with either short or long chop barley silage (either 4.68 mm or 18.75 mm, respectively) and found that forage particle size had no effect on dry matter intake but that eating and ruminating times per day were reduced by 30 min/d and 60 min/d, respectively for cows fed the short barley silage diets. There was no data in this experiment on the effects of the additional saliva buffer production with the long particle size on rumen pH. Theoretically, a physical form of the diet that promotes chewing and rumination will result in more saliva production per kg DM consumed and increase the ability of the diet to buffer rumen pH. However there appears to be a limit to this relationship. Dado and Allen (1994) showed that an increase in chewing time (min/day) in high producing cows was not proportional to the increase in DM intake, and total chewing and rumination times per kg of DM intake declined by 18% and 15%, respectively as milk production and DM intake increased by 30% and 24%, respectively. Total chewing and rumination times per kg of DM intake were negatively correlated with milk production. Thus, in high yielding dairy cows, it may be difficult to avoid conditions that predispose the cow to SARA.

Maekwa et al. (2002a) found that multiparous cows spent more time chewing and ruminating and had higher saliva production during resting than primiparous cows. However, the rate of saliva production during eating was not affected by parity. Total daily saliva production was similar between primiparous and multiparous cows. Diurnal rumen pH variations showed that multiparous cows were at greater risk of ruminal acidosis

because they ate more and probably had a higher ruminal total VFA concentration. Chewing activity is usually a good indication of rumen health because chewing stimulates saliva secretion.

In addition to the depressive effect on saliva production, processing feeds through grinding and pelleting are etiological factors in hyperkeratosis of rumen epithelium which compromises its ability to absorb VFA (Nocek and Kessler, 1980). Rumen papillae of 18 week old calves fed pelleted feed lacked uniformity in height and size, were broad and blunt while others were abnormally long, and there was evidence of erosion, crusting and of firmly embedded feed particles on the rumen mucosa in contrast to calves fed a chopped grass hay or corn silage-based diets. Resumption of conventional feeding in these calves (i.e., chopped hay or corn silage-based calf diet) resulted in papillary rejuvenation and development. Beharka et al. (1998) observed that when commercially rolled grains and ground alfalfa hay were fed to calves, shorter papillae with abnormal branching developed in the dorsal sac and dorsal blind sac of the rumen compared to when the commercially rolled grains were combined with chopped alfalfa hay. Grinding alfalfa hay also reduced the rumen pH below 5.5 from week 2 to 8 of age. This suggests that rumen buffering from saliva flow was lower in the calves on the ground alfalfa hay than in those fed chopped hay.

The type of feeds used in rations can affect the diet's inherent buffering capacity. This is because buffering capacity varies tremendously among feedstuffs. Generally forages and high protein feeds have more buffering capacity than grains, low protein feeds and grass forages (Jasaitis et al., 1987).

1.3.2 Volatile Fatty Acids

Volatile fatty acids produced when carbohydrates are fermented by rumen bacteria are passively absorbed through the rumen wall. The rate of absorption is dependent on volatile fatty acid chain length, pH, concentration and osmolarity (Hoover and Miller, 1992). Accumulation of total VFA in the rumen depresses pH. Data from a study by Goad et al., (1998) showed a negative correlation between rumen pH and ruminal VFA concentration when SARA was induced in hay and concentrate adapted steers. In the same study rumen VFA concentration peaked at 48 hours after SARA induction which also coincided with the time with the lowest pH in the rumen suggesting that total VFA played a major role in the reduction of rumen pH. When acute acidosis is induced lactic acid concentration increases and is the main determinant of rumen pH. This is because when rumen pH decreases below 5.0, fermentation by S*treptococcus bovis* is altered so that end products of fermentation change from a mixture of acetic acid, formic acid and ethanol to lactic acid only (Russell and Baldwin, 1979; Russell and Hino, 1985).

1.3.3 Rumen Fluid Osmolality

The osmotic pressure of rumen fluid has an important physiological significance for ruminal function and voluntary feed intake (Andersen, 1993). Water is drawn across membranes by osmosis into areas of high osmolality. The rate and extent of an increase in rumen fluid osmolality depends on factors such as diet, water intake microbial activity and time since the last meal (Carter and Grovum, 1990). Ruminal osmolality normally ranges between 260 and 360 mili-osmoles (mOsm) per kg (Hoover and Miller, 1992). When ruminal osmolality increases beyond this range, VFA absorption from the rumen decreases

(Oshio et al., 1984) because VFA absorption is through passive diffusion (Hoover and Miller, 1992). If osmolality of rumen contents becomes higher than that of body fluids and blood osmolality, there will be a net inflow of body fluids into the rumen. This rapid osmosis may lead to damage to rumen epithelium. Telle and Preston (1971) noticed massive hemorrhaging in many areas of the rumen mucosa of a ewe that died after intra-ruminal infusion with a racemic solution of D-L lactic acid probably due to corrosive action of low pH. Similar damage to rumen mucosa may occur in cases of SARA. Once damaged, these sites become focal points for enteric bacterial infection resulting in abscesses which may subsequently lead to invasion of body tissue and organs such as the liver, heart and lungs leading to formation of abscesses (Owen et al, 1998).

Rumen osmolality is strongly influenced by feed management prior to and at the time of measurement. Andersen (1993), found that rumen osmolality of grain engorged cows was influenced by pre-experimental diets. Osmolality increased from 290 mOsm/ kg to 320 mOsm/ kg, when cows were adapted from hay to high concentrate diets. Inducing acute rumen acidosis in these cows increased osmolality to 600 mOsm/ kg in concentrate adapted cows compared to 400 mOsm/ kg in hay adapted cows.

1.3.4 Rumen Micro-organisms

The end products of microbial fermentation in the rumen depend on the diet and the rumen microbial populations that are predominant. These end products of fermentation which are mainly VFA and sometimes lactic acid, also determine the rumen pH depending on their ruminal concentrations. This in turn determines the predominant types of rumen bacteria (i.e., either fibrolytic or lactolytic). An increase in readily fermentable

carbohydrates in the diet initially increases the growth of most rumen bacteria because substrate is available for the growth of these different groups of bacteria (Nocek, 1997). Grain processing makes starch granules, more available for microbial digestion. For example, fine grinding or heat and pressure treatments like steam flaking of cereal grains increases the rate of ruminal digestion of starch (Nocek, 1997; Owens et al., 1998) As fermentation proceeds and the concentration of products of fermentation such as acetate, propionate and butyrate increase, the rumen papillae absorptive capacity is reached and the accumulation of VFA in the rumen, decreases rumen pH. Within the pH range of 5.2 and 5.6, the fermentative capacity and growth rates of major lactic acid-producing bacteria such as *Streptococcus bovis* and lactic acid-utilizing bacteria such as *Megasphaera elsdenii* exist in equilibrium and therefore lactic acid produced is immediately utilized. Therefore decrease in rumen pH within this range is due to increases in total VFA rather lactic acid (Goad et al., 1998; Enemark, 2002).

In general, rumen acidity results in a reduction in cellulolytic bacteria and a shift in bacterial population so that gram-positive cocci and rods predominate even though gram-negative bacterial numbers also increase under these conditions (Nagaraja et al., 1978a; Goad et al., 1998). There is a lack of data on studies to identify the bacteria species that predominates within the rumen pH range that defines SARA. One possible reason is the complexity of studies involving rumen bacteria in terms of nutrient and environmental requirements such as pH and presence or absence of oxygen. This diversity in the requirements for different microbial groups does not allow enumeration of different species of bacteria on a single sample in the same medium. In one study, total coliform counts and D-lactate concentrations were determine in an *in vitro* experiment where inoculum was

obtained from hay- and concentrate-adapted steers to determine microbial changes that occur at different pH (Slyter and Rumsey, 1991). A microbial model that can be used to determine changes in rumen gram-negative bacteria populations that encompasses all enteric gram-negative bacteria is lacking. Table 1 is adapted from Russell and Rychlik (2001) and it contains the predominant rumen bacteria classified according to gram staining, substrate and end products of fermentation. Both gram-negative and gram-positive bacteria belong to the group of bacteria that uses both fibrous and starchy material as the main substrate. This may imply that in forage or concentrate based diets, gram positive and gram-negative bacteria exist in the rumen. However, it is not clear whether gram-negative or gram-positive species are predominant within a pH range between 5.2 and 5.6 in the rumen.

Some protozoa species such as entodiniomorphid are also important in maintaining rumen pH. This is because these protozoa engulf starch. Because starch is sequestered from bacteria, the fermentation of starch by lactic acid-producing bacteria is slowed down (Bonhomme, 1990). Other species of protozoa are susceptible to rumen acidity. For example, in a study by Goad et al. (1998) where SARA was induced in two groups of steers that had been adapted to either high grain or forage diet, the protozoa numbers decreased more in the forage adapted than a high grain diet adapted steers. The contribution from protozoa to the maintenance of rumen pH is small.

TABLE 1. Predominant ruminal bacteria classified according to gram staining, substrate and end products of fermentation.

Gram-negative	**Gram-positive**	**Ruminal niche**	**Fermentation products**
Fibrobacter succinogenes		cellulose	succinate, formate, acetate
	Ruminococcus flavefaciens	cellulose hemicellulose	succinate, formate, acetate, H_2
	Ruminococcus albus	cellulose hemicellulose	acetate, formate, ethanol, H_2
	Butyrivibrio fibrisolvens	starch, cellulose, hemicellulose, pectin, sugars	butyrate, formate, acetate, H_2
Prevotella ruminicola *Prevotella albensis* *Prevotella bryantii*		starch, pectin, xylans, sugars	succinate, acetate, formate, propionate
	Streptococcus bovis	starch, sugars	lactate, acetate, formate, ethanol
	Selenomonas ruminantium	starch, dextrins, sugars, lactate, succinate	lactate, acetate, propionate, butyrate, formate, H_2
	Eubacterium ruminantium	hemicellulose, dextrins, sugars	acetate, formate, butyrate, lactate
Megasphaera		lactate, sugars	propionate, acetate, butyrate, branched chain VFA, H_2
	Clostridium	amino acids	Branched chain VFA, acetate, butyrate
	Clostridium	amino acids	Branched chain VFA, acetate, butyrate
	Lactobacillus	starch, sugars	lactate, acetate, formate, ethanol
Anaerovibrio lipolytica		glycerol, sugars	acetate, succinate, propionate

TABLE 1. (continued) Predominant ruminal bacteria classified according to gram staining, substrate and end products of fermentation.

Gram-negative	**Gram-positive**	**Ruminal niche**	**Fermentation products**
Succinomonas amylolytica		starch	succinate, acetate, propionate
Ruminobacter amylophilus		starch	succinate, formate, acetate, ethanol
	Lachnospira multiparous[2]	pectin, sugars	lactate, acetate, formate, H_2
Succinivibrio dextrinosolvens		pectin, dextrin, sugars	succinate, acetate, formate, lactate
	Peptostreptococcus anaerobius	amino acids	branched chain VFA, acetate
	Metahonbrevibacter ruminantium	H_2, CO_2, formate	CH_4

Source: Adapted from Russell and Rychlik, 2001.

1.3.5 Systemic Manifestations Associated With SARA

Physiological changes that result from SARA are implicated in the etiology of some diseases particularly those associated with inflammation. Inflammation may be due to either tissue injury or invasion of the systemic circulation by rumen bacteria. Prolonged exposure of the ruminal epithelium to low rumen pH leads to hardening and enlargement of rumen papillae. This can be exacerbated by feeding pelleted feeds. When cows are repeatedly exposed to conditions that result in low rumen pH, papillae may adhere together to form bundles and subsequently contain excessive layers of keratinized epithelial cells, particles of food and bacteria (Nocek, 1997). This decreases the ability of the rumen wall to absorb VFA, and further exacerbates total VFA concentration in the rumen making affected animals more prone to SARA.

Progressive epithelial tissue damage and formation of lesions on the rumen wall lead to entrance of rumen micro-organisms most notably by *Fusobacterium nercrophorum* and sometimes by *Acanobacterium pyrogenes* into the blood circulation (Nocek, 1997; Nagaraja and Chengappa, 1998). These bacteria spread first to the liver where they form abscesses and, in some cases, they may spread to other tissue and organs (Nordlund et al., 1995). This often leads to generalized abscessation within the ruminant body (Kleen et al., 2003). The presence of micro-organisms within the body may also be manifested as subcutaneous abscesses that are not related to injections (Nordlund et al., 1995), or as incidences of hemoptysis and epistaxis especially when bacterial infection occur in the lungs (bacterial pneumonia) or in the caudal vena cava (Nordlund, et al., 1995). The term parakeratosis–rumenitis–liver abscesses complex has been proposed as being apt for the description of these patho-physiological manifestations that originate with exposure of

rumen mucosa to acidic rumen contents and then the invasion of body organs and tissue by pathogens from the rumen (Kleen et al., 2003).

1.4 Inflammation Response

One of the common physiological characteristic of the disease complexes described above is the involvement of the inflammatory response. This response is indicated by changes in concentrations of acute phase proteins synthesized in the liver. Immediately following infection, trauma or tissue injury, a complex series of reactions occur in order to prevent ongoing tissue damage, and to isolate and destroy infective organisms as well as to start the repair process to return the affected organism to normal functions (Kushner, 1982; Baumann and Gauldie, 1994). The cumulative homeostatic process is called inflammation and the cascades of reactions that are set off immediately in concert with the stimulus are known as the acute phase response (APR). These are a well orchestrated sequence of processes initiated at the site of infection or trauma and involved the release of soluble mediators such as cytokines, anaphylatoxins and glucocorticoids (Baumann and Gauldie, 1994; Kushner and Rzewnicki, 1994; Steel and Whitehead, 1994).

1.4.1 The Acute Phase Response

The initial cascade of events during the APR starts with the tissue macrophages and blood monocytes which are activated to produce a broad range of mediators (Baumann and Gauldie, 1994). Additionally, other events that complement the elevation of these mediators in the tissue and blood are also initiated. Notable among them are mast cell

degranulation and aggregation-induced platelet activation which results in the release of monocyte and macrophage chemo-tactic mediators such as transforming growth factor ß (Baumann and Gauldie, 1994 Kushner and Rzewnicki, 1994). Macrophages and monocytes are also activated by bacterial LPS or the byproducts of opsonins and thus contribute to the APR cascade (Petersen et al. 2004). The major mediators that are important in the APR are the cytokines IL-1, IL-6, and TNFα. These cytokines appear to be uniquely important for setting off the initial reactions in the cascade that characterizes APR (Baumann and Gauldie, 1994). In terms of the immune response, a number of responses are triggered by the initial wave of cytokines released at the reactive site. The cytokines amplify their own levels through the action of IL-1 and TNFα on stromal cells to cause a secondary wave of cytokines. This is achieved through the secretion of molecules that are highly chemo-tactic for neutrophils and mononuclear cells such as interleukin-8 and monocyte chemo-attractant protein, respectively (Baumann and Gauldie, 1994). In this way, leucocytes migrate into the injured tissue and synthesize mediators that are released during the secondary wave of cytokines (Lloyd and Oppenheim, 1992).

Another way in which the second wave of cytokines is produced involves endothelial cells of the blood vessels adjacent to damaged tissue communicating with the reactive tissue to facilitate the migration of leucocytes to the reactive tissue. Through the action of IL-1 and TNF, endothelial cells undergo major changes in gene regulation and surface expression of important adhesion and integrin molecules which interacts with neutrophils and other circulating leucocytes to slow their rate of flow and initiate trans-endothelial passage and subsequent migration to the reactive site (Rot, 1992; Baumann and Gauldie, 1994).

The second wave of cytokines is responsible for the systemic inflammatory response. The major manifestations of a systemic response are; elevation of the temperature set point in the hypothalamus with a resultant febrile response (Dinarello et al., 1991), and altered hepatic metabolism and subsequent changes in serum concentration of acute phase proteins (APP) (Kushner 1982; Petersen et al., 2004). The APP produced by hepatocytes are present in different concentrations. Acute phase proteins are generally classified by the magnitude of their response during an APR. The concentration of positive APP generally increases during an APR. These can further be classified into major, moderate or minor APP. The concentrations of negative APP generally decrease during an acute phase response. However, the term APP is usually used to refer to positive APP because these are the ones that are of interest as diagnostic aides in inflammatory diseases for livestock (Petersen et al., 2004; Hirvonen, 2000). Hirvonen (2000) categorized APP in cattle according to the magnitude of their response during an APR. Proteins that showed 10 – 100 fold increase were classified as major APP and this group includes haptoglobin (Hp) and serum amyloid A (SAA). Proteins that showed a 2-10 fold increase were classified as moderate APP and this group includes α_1-acid glycoprotein and α_1-proteinase inhibitor. Proteins showing a 1-5 fold increase during an APR were termed mild APP and this group includes fibrinogen, ceruloplasmin, α_2-macroglobulin, complement component 3 and bovine lipopolysaccharide binding protein.

1.4.2 Acute Phase Proteins in Cattle

The range of APP in human medicine (Kushner, 1982; Steel and Whitefield, 1994; Bauman and Gauldie, 1994; Kushner and Rzewnicki, 1994) and veterinary medicine (Petersen et al., 2004) has been reviewed in detail. In cattle, the major acute phase proteins are Hp and SAA and these have been studied the most in this species. Their role as diagnostic aides in various inflammatory diseases has been recognized and exploited particularly in studies on mastitis and bovine viral and bacterial respiratory diseases. The APP can potentially be useful in diagnosis of subclinical disease conditions where clinical signs cannot be used to detect diseases. The merits and demerits of the various APP will not be discussed here but discussion hereon will be focused on those proteins that may have a practical application in the present studies.

1.4.3 Serum Amyloid-A

Serum amyloid-A is the collective name given to a family of polymorphic proteins encoded by multiple genes in a number of mammalian species (Steel and Whitehead, 1994). Two members of this family have been identified in cattle (McDonald et al., 2001). Functionally, SAA is associated with lipoproteins in plasma and acts as a scavenger for cholesterol released at sites of inflammation (Meglia et al., 2005). Serum amyloid A may also increase the clearance of bacterial LPS because of its association with high density lipoprotein (Tobias et al., 1982).

Serum amyloid-A is released into the circulation in response to infection or injury. Within 24-36 hours after infection or injury, the blood concentrations of SAA can increase by as much as 10 times over basal concentrations (Heegaard et al., 2000). In cattle, SAA

concentrations increased as a result of various inflammation stimuli such as infection with a wide range of inflammatory diseases (Alsemgeest et al. 1994); in clinically and bacteriologically defined acute metritis (Hirvonen et al., 1999); experimental mastitis infections with either *Streptococcus uberis* (Pedersen et al., 2003) or intra-mammary infusion with *Escherichia coli* 0111:B4 LPS (Lehtolainen et al., 2004); experimental infections with bovine respiratory syncytial virus (Heegaard et al., 2000); in cattle housed in pens with slippery floors which induced stress (Alsemgeest et al. 1995); and as a result of physical injury (Connor et al., 1988).

Serum amyloid-A response to acute phase stimulants is faster than that observed for Hp. For example, Werling et al. (1996) reported an increase in SAA within 6 hours after an intravenous LPS infusion but found no increase in Hp within the same time frame. Horadagoda et al. (1994) also observed a more rapid SAA response compared to that of Hp in calves in experimental infections with *Pasteurella haemolytica.* The magnitude and the duration of response of SAA during an APR correlated positively with the severity of clinical signs in cattle undergoing experimental infection with bovine respiratory syncytial virus (Hirvonen et al., 1999; Heegaard et al., 2000). Lipopolysaccharide infusions at the rate of 10 ng and 100 ng / kg body weight in dairy cows resulted in a dose dependent increase in both SAA and Hp concentrations (Jacobsen et al., 2004). Serum amyloid-A can be more useful in distinguishing between acute and chronic inflammation than neutrophils and white blood cell counts (Horadagoda et al., 1999).

1.4.4 Haptoglobin

Bovine Hp consists of two subunits that are polymerized with albumin putting its total molecular weight in the range between 1 000 and 2 000 kDa (Morimatsu et al, 1991). The component subunits consist of one α-chain with a molecular weight of 16 – 23 kDa and a β-chain with a molecular weight of 35 – 40 kDa (Eckersall and Conner, 1990; Morimatsu et al, 1991). Large and heterogeneous molecular sizes with different degrees of polymerization have been reported (Morimatsu et al., 1991).

The primary function of Hp is to bind hemoglobin released from red blood cells during hemolysis and thus prevent the loss of iron by forming stable complexes with free hemoglobin in blood (Putnam, 1975). The hemoglobin is then transferred to liver cells for detoxification whereupon the iron is recycled in the body (Putnam, 1975). By restricting the availability of iron, Hp prevents the growth of some pathogenic bacteria that require iron for their metabolism (Eaton et al., 1982). Although Hp is a major APP in most species, serum concentrations can be influenced by factors other than the APR. Hp concentrations can increase under a variety of experimentally induced and naturally occurring disease conditions and trauma. Diseases such as coliform mastitis (Hirvonen et al., 1996; Ohtsuka et al., 2002) and trauma (Faulkner et al., 1992; Earley and Crowe, 2002; Ting et al., 2003) have been shown to increase Hp concentrations. Humblet et al. (2004) concluded that Hp and fibrinogen can be used as screening aides to identify calves suffering from bronchopneumonia when selecting animals for treatment. Haptoglobin is also increased in cattle suffering from pneumonic pasteurellosis (Dowling et al., 2002) or bovine respiratory syncytial viral disease (Heegaard et al., 2000). It has been suggested that Hp could also be used as a diagnostic aide for some reproductive problems in cattle after

parturition (Chan et al., 2004). Hirvonen et al. (1999) observed increased Hp concentrations in cows with severe cases of acute metritis. Non-infective metabolic conditions such as milk fever and ketosis do not increase plasma Hp concentration (Skinner et al., 1991).

The fact that Hp concentrations can be elevated within 72 h after infection or injury make it a potentially useful diagnostic tool for detecting conditions that result in an inflammation before the onset of clinical signs (Godson et al., 1996).

1.4.5 Fibrinogen

Fibrinogen is a mild APP whose concentration may increase 2-3 times during an acute phase response (Eckersall, 2000). Fibrinogen is an integral part of the coagulation pathway. The coagulation cascade comprises two independent pathways termed the intrinsic and extrinsic pathways which converge on the activation of factor x and subsequently becomes a single common pathway which leads to the formation of fibrin from fibrinogen (Nutescu et al., 2005). Fibrinogen concentrations increased after localized inflammation from tissue damage without infection when turpentine was injected subcutaneously in calves (Conner et al., 1988). Concentrations also increased as a result of trauma of surgery such as castration (Fisher et al., 1997; Earley and Crowe, 2002), or due to transportation (Arthington et al., 2003), and experimental infections with bovine herpes virus-1 (Arthington et al., 1996).

Fibrinogen concentration can also remain unchanged or decrease during acute inflammatory conditions in cattle. Fibrinogen concentrations may decrease because it is vulnerable to proteolytic enzymes other than thrombin and is constantly being broken down (Putnam, 1975). Fibrinogen may also decrease because of fibrinogen-fibrin conversion at

the inflamed area, and thus its consumption may transiently exceed production (Welles et al., 1993). Fibrinogen responses to acute phase stimulants may be exacerbated by copper deficiency in cattle (Arthington et al., 1996).

1.4.6 Hematological Analyses as a Diagnostic Tool For Inflammation

1.4.6.1 White Blood Cell Count: White blood cells (leukocytes) are made from stem cells in bone marrow. White blood cells (WBC) can be used in diagnosing infectious diseases and inflammatory conditions in most species. There are five types of leukocytes and these are the granulocytes which include neutrophils, eosinophils, and basinophils and two kinds of leucocytes that do not have granulocytes which include lymphocytes and monocytes (Swenson, 1984). They are all important components of the immune system. Neutrophils enter the tissue fluid by squeezing through capillary walls and phagocytize foreign substances. Lymphocytes play an important and integral part in the body's defense against infections. The two categories of lymphocytes are T-cells, which attack cells containing viruses and B-cells that produce antibodies (Tizard, 1982).

Total WBC are only slightly affected by inflammatory disease and are of limited usefulness in diagnosing inflammatory conditions. White blood cell counts do not increase very strongly with inflammatory diseases (Horadagoda et al., 1999). However, changes in the different types of leucocytes can be more informative in diagnosis than the total WBC. For example, cows respond to inflammation with an initial leukopenia which is an abnormal decrease in leukocyte, followed by a leukocytosis which involves the appearance of abnormal and immature cells in circulation. The leukopenia is a result of loss of

lymphocytes from circulation. This is because lymphocytes migrate to site of injury or infection during inflammation.

1.4.6.2 Platelet Count: Platelets are small, colorless, round or rod shaped bodies in the circulating blood of mammals. Platelets respond to damaged endothelium or almost any 'foreign surface' by adhering to the surface, altering their shape, releasing platelet constituents, and forming platelet aggregates (Gentry and Downie, 1984). Although their diagnostic value has not been evaluated, it has been suggested that changes in platelet counts may be a better diagnostic tool than WBC and that increased platelet count would indicate persistent bacterial infection (Hirvonen, 2000). Therefore tissue damage and pathogen invasion into tissue and organs can potentially elevate platelet counts in affected animals.

1.5 Incidence of SARA

The insidious nature of SARA has made documentation of its prevalence difficult. However, it is suspected to be widespread within the dairy industries of most major dairy farming nations where grains such as barley and corn are the major energy sources in the rations. The problem of diagnosing SARA is the absence of specific clinical signs. Additionally signs that are associated with SARA can also be attributed to poor management (Nocek, 1997). Only limited information on the prevalence of SARA is currently available. A survey on 15 dairy farms in Wisconsin showed the presence of SARA in 19% of early lactation cows and 26% of mid-lactation cows (Garrett et al., 1997).

lactation cows (Oetzel et al., 1999). The problems in diagnosing SARA, makes it difficult to assess its economic impact but it is believed to be significant. Data obtained from a case study on a 500 cow dairy herd in New York can be used to illustrate the economic importance of SARA. In this field study, SARA reduced milk yield, milk fat production and milk protein production by 2.7 kg/d, 0.3 percentage points and 0.12 percentage points, respectively (Stone, 1999). The percentage reduction on milk fat and milk protein may not seem great but applied to an entire lactation these reductions can amount to a financial loss of as much as $400 per cow /year . If costs such as culling due to SARA related problems and penalties incurred due to low milk fat are added, the estimated cost would be even higher than this estimate.

Currently, there is no data on the prevalence of SARA in western Canada. Since barley grain and barley silage are common ingredients in dairy diets in western Canada, and these feedstuffs are fermented more rapidly in the rumen than corn, barley-based diets pose a greater risk for SARA. Recent data also shows that some dairy herds in western Canada are likely to be at greater risk of SARA because of a potentially low buffering capacity of the some of the diets due to a shorter than recommended particle size in the diet. While it is recommended that at least 40% of feed particles of dietary ingredients in a TMR for dairy cows be longer than 8 mm (Heinrichs, 1996), a survey of Manitoba dairy farms revealed that TMR was finer than recommended on at least 25% of farms (Plaizier et al., 2004). Thus, cows on at least 25% of the farms surveyed are at risk for SARA.

1.6. Effects of SARA on Ruminal Lipopolysaccharide

1.6.1. Structure of LPS

Bacterial LPS is a component of the cell wall of gram negative bacteria. In general LPS is comprised of three main regions which are: the side chain, the core polysaccharide and the lipid A (Raetz and Whitfield, 2002). Different gram negative bacterial strains have different side chains. This enables LPS from different bacterial strains to be differentiated. The side chain is made of repeating units of oligosaccharides whose type, sequence and linkage determine the antigenic specificity of a particular LPS (Rietschel et al., 1996; Raetz and Whitfield, 2002). The side chain is known as 'the O-specific side chain' and is used for serological typing of gram-negative bacteria (Rietschel et al., 1996). The core polysaccharide is common to LPS from a one species of bacteria and it is structurally distinct. Two unusual sugars are usually present, heptose and 2-keto-3-deoxyoctonoic acid, in the core polysaccharide (Rietschel et al., 1996). The lipid A part of LPS is composed of a backbone of phosphorylated glucosamine-disaccharide with lipids attached to it. Lipid A is responsible for endotoxic activity of LPS (Galanos et al., 1984).

1.6.2. Ruminal Fluid Concentration of LPS

It has been suggested that LPS is released into the rumen from bacteria when they die and lyse or during rapid growth (Nagaraja et al., 1978a; Wells and Russell, 1996). It has also been suggested that as much as 60% of LPS in the rumen may be released during bacteria growth (Andersen, 2000). During rapid growth, autolytic enzymes are required for bacteria cells to expand and grow but excessive activity of these enzymes lead to bacteria

cell lysis. Studies with *Fibrobacter succinogenes* showed that autolysis was 10 times higher in rapidly growing cells compared to cells in the stationary phase (Wells and Russell, 1996). Efforts to relate free ruminal LPS concentration to the total number of gram negative bacteria in rumen fluid showed poor correlations (Nagaraja et al., 1978a). The number of gram negative bacteria relative to gram positive bacteria is larger in hay fed cattle than in grain fed cattle (Krogh, 1961). However, the total number of bacteria is greater in grain fed cattle and thus the total number of gram negative bacteria was found to be greater in grain fed cattle compared to hay fed cattle (Nagaraja et al., 1978a). Mullenax et al. (1966) were among the early scientists to investigate the presence of free LPS in the rumen. They extracted substances from ruminal fluid which, when injected in healthy sheep and cattle, exhibited symptoms similar to endotoxicosis. In addition to the induction of endotoxicosis they also found that symptoms were reduced if the animal had been previously exposed to LPS.

Nagaraja et al. (1978b) measured LPS in cell-free rumen fluid of two cows that were fed either all hay or all grain diets. Lipopolysaccharide concentration in rumen fluid was determined indirectly by determining the dose that would kill half the test population (LD_{50}), of mice that had been made tolerant to *E coli* endotoxin. Ruminal LPS was in the range of 24.57 - 30.71 μg/mL in the grain-fed cow and 6.47 - 11.62 μg/mL in the hay fed cow. Feeding grain has also been shown to increase both gram-negative and gram-positive bacteria in the rumen although the proportion of gram-positive bacteria is higher than when hay based diets are fed (Nagaraja et al., 1978a). As indicated above, when rumen fluid from a cow that had been adapted to hay was incubated *in vitro* with either alfalfa hay or ground extruded corn, the gram-positive bacteria increased from 10.7% and 19.1% to 28.9% and

45.6%, respectively and gram-negative bacteria decreased from 89.3% and 80.9% to 71.1% and 54.5% by 9 hours after the start of incubation (Nagaraja et al., 1978a). However, gram-negative bacteria numbers increased and were higher in rumen fluid incubated with corn than alfalfa hay. This led these authors to conclude that LPS resulted from a process in which rapidly growing gram-negative bacteria shed LPS into rumen fluid. Additionally, death and lyses in other gram-negative bacteria that cannot thrive due to low pH also contribute to free ruminal LPS.

Andersen et al. (1994a) observed that acute acidosis induced by grain engorgement caused an increase in free ruminal LPS only in grain adapted cows compared to those adapted to hay. These findings appear to be inconsistent with the well accepted premise that gradual introduction of concentrate prepares ruminants to deal with intakes of high concentrate diets. These researchers also used a modified *Limulus amoebocyte* lysate (LAL) technique that they described as a very sensitive and accurate technique for LPS analysis. Mochizuki et al. (1996) measured the ruminal concentration of LPS in dairy cows immediately after calving and found that free ruminal LPS increased slightly with declining pH but that the peak concentration varied with each animal. There was a tendency for higher yielding cows to have a higher ruminal LPS level. Grain adaptation appears to provide a greater potential for the release or production of free LPS in rumen fluid (Andersen et al., 1994a). Therefore, high yielding dairy cows on high concentrate diets in the transition period or during early lactation are more susceptible to ruminal acidosis and to high LPS concentration in the rumen.

1.6.3. Peripheral Blood Concentration of LPS

The role of LPS in the manifestation of clinical signs of ruminal acidosis is not clear. Studies to measure LPS in peripheral blood circulation in experiments in which acute acidosis was induced in cattle have yielded inconsistent results. Some studies reported the presence of LPS in peripheral circulation of cattle with experimental acute acidosis (Dougherty et al., 1975; Aiumlamai et al., 1992), while others (Andersen and Jarlov, 1990; Andersen et al., 1994b) were not able to detect any LPS. It is generally believed that LPS is not a contaminant of systemic blood in healthy animals and that its presence in peripheral blood indicates impaired liver function (Andersen, 1990).

Aiumlamai et al. (1992) used oats to induce acute acidosis in calves and then monitored plasma LPS. Four calves aged 5 months that had previously been adapted to a roughage diet were used. Liver function was tested prior to the experiment and was deemed normal in all animals. Blood samples were collected before acute acidosis was induced to determine baseline values of plasma LPS. Two calves had significantly elevated plasma LPS before grain engorgement. The LPS concentration increased in only one of the two calves that had shown high LPS concentration before ruminal acidosis. There was no change in LPS concentration of the other calves that showed high pre-engorgement LPS levels. The two calves that did not show LPS in plasma before acute acidosis was induced only showed a slight increase in LPS concentrations when acute acidosis was induced. There was no evidence of liver damage in all the animals. These researchers concluded that ruminal acidosis may be complicated by LPS from the gastrointestinal tract and that the response in LPS translocation varies among animals. Dougherty et al. (1975) also detected LPS in blood of three sheep and a steer after inducing acute ruminal acidosis. Their results

showed that LPS was present in peripheral blood before and after acute ruminal acidosis was induced.

The findings of these studies contradict those by Andersen and Jarlov (1990) who did not detect any LPS in plasma samples from non lactating Jersey cows that had been adapted to an all forage diet before acute rumen acidosis was induced. Instead they noticed a decrease in ruminal LPS concentrations 2-4 days after grain engorgement. Subsequently, Andersen et al. (1994b), using two fistulated jersey cows fitted with totally implanted indwelling catheters in the portal and hepatic veins and carotid artery, detected LPS in the portal and pre-hepatic circulation but not in the peripheral blood circulation. Hepatic and portal blood samples tested positive to LPS even before ruminal acidosis was induced. Starting 12 h after inducing acute acidosis, LPS was present in hepatic and portal blood samples. In one cow, only one blood sample collected 14 h after inducing acute ruminal acidosis was positive to LPS in arterial blood whilst in the other cow, arterial blood samples became positive 4 h after acute acidosis was induced and LPS could be detected for up to 12 h. Hepatic and portal blood LPS concentrations increased 2-4 times compared to concentrations in the same animals prior to inducing acute acidosis. The finding that hepatic and portal blood concentrations of LPS were unpredictable and intermittent in this experiment, may explain the inconsistencies reported in the literature on the effects of acute acidosis on LPS concentration in peripheral blood (Andersen et al., 1994b). In the same study, higher LPS concentration was detected in hepatic and portal blood but not in peripheral circulation in cows with acute ruminal acidosis. The presence of LPS in hepatic and portal circulation may imply that the rate of LPS translocation increases when acidosis is induced. However, LPS concentrations are often undetected in peripheral circulation due

to the ability of the liver to rapidly detoxified and remove LPS before it gets further into peripheral circulation (Andersen, 2000). Therefore, an absence of LPS from peripheral circulation must be interpreted cautiously as this does not necessarily prove that ruminal bacterial LPS was not translocated into blood circulation.

1.7. Summary

When cattle experience daily episodes of low rumen pH, there are immediate and long term effects on productivity and animal health. The immediate effects of SARA on cattle production include alteration in rumen microbial populations which lead to changes in rumen fermentation. Subacute ruminal acidosis impairs the activity of cellulolytic bacteria (Grant and Mertens, 1992), which results in decreased rate of fiber digestion in the rumen as demonstrated by *in vitro* (Grant and Mertens, 1992; Calsamiglia et al., 2002) and *in vivo* (Plaizier et al., 2001; Krajcarski-Hunt et al., 2002) studies. As a result, fermentation shifts towards production of more propionic acid and less acetic acid in the rumen. The altered rumen microbial populations also result in incomplete biohydrogenation of unsaturated dietary fats in the rumen which has been suggested to cause a decrease in milk fat content. Changes in the rate of rumen digestion also result in increased hindgut fermentation of dietary starch. This leads to decreased amino acid absorption through a concomitant decrease in rumen microbial protein which may subsequently lead to depressed milk protein content. Some studies have shown significant decreases in milk fat and protein contents when SARA was induced while others have not. This inconsistence may be due to the variation in the period for which experimental animals are subjected to

SARA in the different studies in the literature (Krause and Oetzel, 2005). The changes associated with altered rumen fermentation can be reversed if SARA is diagnosed and rectified.

Subacute ruminal acidosis in dairy cows is also associated with unexplained episodes of diarrhea that are often overlooked, subcutaneous abscesses, and sudden death syndrome (Nordlund et al., 1995). The initial effect of SARA is the parakeratosis of the rumen epithelium that leads to rumenitis and subsequent invasion of body tissue by pathogens from the rumen. These pathogens include *Fusobacterium necrophorum* and *Arcanobacterium pyrogenes* which have been isolated from SARA-related abscesses. Another long term effect of SARA is believed to be subclinical laminitis which subsequently leads to lameness. The high incidence of laminitis in cattle on high concentrate diets particularly during grain adaptation in beef cattle during introduction to finishing diets, has led to the presumption of a 'cause' and 'effect' relationship between concentrate based rations and laminitis. Central to this hypothesis is the role of vasoactive substances such as bacterial LPS and histamine. It has been suggested that rumen bacterial LPS can be translocated into blood circulation via damaged epithelial tissue when SARA is induced. Tissue damage, presence of pathogens and presence of LPS in the tissue and organs of animals are responsible for the perturbations that are associated with SARA.

Conditions that predispose cows to SARA include insufficient dietary physically effective fiber, or conversely too much nonstructural carbohydrates of the diet. Physically effective fiber is important because it stimulates saliva flow during chewing and rumination. Saliva is a major source of buffers that are needed to neutralize the low rumen pH that results from VFA production through microbial fermentation. There is a general

paucity of data in the literature on the incidence of SARA because of the absence of specific signs and simple diagnostic methods that can be used under field conditions. Presently, rumenocentesis and obtaining rumen fluid samples by stomach tubing for measuring rumen pH *in vitro* are the only methods that are available to diagnose SARA under field conditions.

It is generally believed that ruminal LPS concentration increases when SARA is induced because of rapid growth and death and lyses of some gram-negative bacteria. However, data from some experiments where acute acidosis was induced do not show results consistent with this theory. Rumen bacteria populations that are predominant under SARA and acute acidosis could be different. It is important to investigate the changes in ruminal LPS in order to better understand the factors that are involved in initiating disease conditions that are related to SARA such as laminitis. In this way, interventions can be designed that would minimize the negative effects of SARA, improve dairy production efficiency and improve the consumers' perception of the industry.

HYPOTHESES

The literature reviewed shows the central role of rumen bacteria in the development of SARA-related diseases. Based on this, the following hypotheses are made:

1. Inducing SARA can create conditions in the rumen that increases free ruminal LPS concentration. The changes in free ruminal LPS depend on the diet and the duration this diet is fed prior to induction of SARA.
2. Changes in free ruminal LPS and the damage to rumen epithelium as a result of low rumen pH activates a systemic inflammatory response that can be indicated by changes in concentrations of inflammation markers such as serum amyloid A, haptoglobin, fibrinogen, and white blood cells.
3. The inflammatory response that results from SARA will be different in cattle previously adapted to hay versus concentrate diet.

OBJECTIVES

In order to test the hypotheses, experiments were designed to investigate the following:

1) To determine changes in free ruminal LPS, serum Hp, and plasma SAA concentrations resulting from grain induced SARA in cattle.
2) To determine the effects of diets fed prior to SARA induction on free ruminal LPS concentration, serum Hp and plasma SAA in cattle.
3) To determine the ruminal LPS concentration and inflammatory response to grain induced SARA mid-lactation dairy cows.
4) To determine the level of concentrate feeding that elicits an acute phase response during gradual adaptation to concentrate diets in cattle.
5) To determine changes in total coliform counts in cattle during stepwise adaptation to a high concentrate diets and when SARA is induced in cattle adapted to concentrate diets.

MANUSCRIPT I

Subacute Ruminal Acidosis Induces Ruminal Lipopolysaccharide Endotoxin Release and Triggers an Inflammatory Response

G. N. Gozho, J. C. Plaizier, D. O. Krause, A. D. Kennedy and K. M. Wittenberg

Department of Animal Science, University of Manitoba, Winnipeg, Canada R3T 2N2

Corresponding author: J. C. Plaizier; e-mail: plaizier@ms.umanitoba.ca.

ABSTRACT

Subacute ruminal acidosis (SARA) was induced in 3 rumen fistulated Jersey steers by offering them different combinations of wheat-barley pellets and chopped grass hay. Steers were offered 4, 5, and 6 kg/d of pelleted concentrate and 6, 5, and 4 kg/d of chopped grass hay for diets 1, 2, and 3, respectively, during 5-d treatment periods and were fed chopped grass hay between treatment periods. Inducing SARA increased blood concentrations of haptoglobin and serum amyloid-A. Dry matter intake of concentrate and hay decreased from d 1 to 5 in each period. Subacute ruminal acidosis was induced in all steers during d 4 and 5 when concentrate was fed, with ruminal pH remaining below 5.6 for an average of 187 and 174 min/d on these days. Lipopolysaccharide concentration increased significantly during periods of grain feeding compared with times when only hay was fed. Inducing SARA by feeding wheat-barley pellets activated a systemic inflammatory response in the steers.

Key Words: steers • subacute ruminal acidosis • ruminal pH • acute phase response

Abbreviations: Hp = haptoglobin • **LAL** = Limulus amebocyte lysate • **SAA** = serum amyloid-A • **SARA** = subacute ruminal acidosis.

INTRODUCTION

Subacute ruminal acidosis (SARA) is a metabolic disorder characterized by episodes of low rumen pH between 5.2 and 5.6 (Cooper and Klopfenstein, 1996). Clinical signs of SARA are variable and, thus, it is often difficult to identify animals suffering from the disorder. Affected animals may become anorexic, develop intermittent diarrhea, become dehydrated, have unexplained abscesses, and develop laminitis (Nocek, 1997; Kleen et al., 2003). Some of these signs are not specific to SARA and this may lead to SARA being dismissed as other problems such as poor forage quality or poor bunk management (Nocek, 1997). Laminitis cannot be used to diagnose SARA because of the long time lapse between SARA and appearance of signs of laminitis.

Lipopolysaccharide from rumen gram-negative bacteria has been implicated in diseases that are related to feeding high concentrate diets such as sudden death syndrome, rumenitis, ruminal acidosis, and laminitis (Dougherty et al., 1975; Nagaraja et al., 1978a). There is a paucity of literature on changes in rumen fluid LPS concentration due to feeding high concentrate diets. Nagaraja et al. (1978a) demonstrated that feeding high concentrate diets increased ruminal LPS concentration compared to feeding only hay. However more a recent study in which acute acidosis was induced by grain engorgement did not find such a relationship (Andersen et al., 1994a). It has been suggested that the acidic rumen environment, changes in osmotic pressure, and ruminal LPS may render the rumen epithelium susceptible to injury (Brent, 1976; Enemark et al., 2002; Kleen et al., 2003). These changes may result in translocation of rumen LPS into the prehepatic bloodstream.

The presence of LPS in the bloodstream results in the production of multiple proinflammatory cytokines, reactive oxygen and nitrogen intermediates, and bioactive lipids, which affect the host's metabolic response to inflammation (Baumann and Gauldie, 1994). When released in large quantities, these mediators lead to an acute phase response (Kushner and Rzewnicki, 1994). Profiles of concentrations of some acute phase proteins are used as markers for inflammatory response (Baumann and Gauldie, 1994). Haptoglobin (Hp) and serum amyloid-A (SAA) are 2 such proteins that are used as inflammatory markers in cattle (Alsemgeest et al., 1994). Their concentrations are elevated because of tissue damage (Conner et al., 1988) or due to intradermal and intravascular LPS injections (Boosman et al., 1989), bacterial infections (Deignan et al., 2000), or viral infections (Heegaard et al., 2000).

Although acute phase proteins have been used as inflammatory markers in various situations where inflammation is the logical development, no studies have investigated inflammatory responses in cattle with SARA. We hypothesize that SARA leads to increased gram-negative bacterial lysis, which increases ruminal LPS concentration. Rumen wall damage associated with SARA further increases ruminal LPS translocation into the bloodstream, resulting in an inflammatory response. Therefore, the objective of this study was to determine changes in rumen fluid LPS, serum Hp, and plasma SAA concentrations in steers due to SARA induced by feeding wheat-barley pellets.

MATERIALS AND METHODS

Three ruminally fistulated adult Jersey steers were kept in metabolism crates at the Animal Science Research Unit at the University of Manitoba throughout the experiment, in accordance with the guidelines of the Canadian Council of Animal Care. The experimental design was a 3 x 3 Latin square with 21-d periods that were divided into a 5-d treatment period and 16-d rest period. During the treatment period, SARA was induced in the steers by offering diets 1, 2, and 3. Diets were combinations of wheat-barley pellets and chopped grass hay, respectively, in the following ratios (as-fed basis): diet 1 (4 kg: 6 kg); diet 2 (5 kg: 5 kg); and diet 3 (6 kg: 4 kg). The concentrate consisted of 50% wheat and 50% barley. Concentrate and hay were offered in separate meals. All animals were offered 1 kg of chopped grass hay at 0900 h followed by two-thirds of their allocation of concentrate at 1100 h. The remainder of the concentrate was offered at 1300 h. At 1700 h, concentrate not eaten was removed and the steers were offered chopped grass hay, to which they had access throughout the night. The steers were fed hay ad libitum during the 16-d rest period between treatments. The last 2 d of the rest period, when only hay was fed, were designated d –2 and d –1. Days when concentrate was fed were designated d 1 to 5. Dry matter (%), CP, and NDF (%DM) content were 84.8, 18.9, and 27.3% for chopped grass hay, and 93.0, 14.9, and 45.7% for wheat-barley pellets.

Rumen pH was measured continuously throughout the treatment period by placing one indwelling pH probe into the ventral sac of the rumen of each steer as described by Cumby et al. (2001). Measurements were taken every second and averaged over 60 s. Rumen fluid pH data were summarized as average pH, time below pH 6.0, and time below

pH 5.6, area (time x pH) below pH 6.0, and area (time x pH) below pH 5.6 for each 24-h period. Rumen fluid samples were collected into sterile plastic tubes from the ventral sac of the rumen at 0900 and 1400 h every day during the treatment period. Samples were mixed thoroughly before 25 mL was transferred into sterilized centrifuge tubes and centrifuged for 30 min at 10,000 x g. The supernatant was passed through a disposable 0.22-μm sterile, pyrogen-free filter (Millex, Millipore Corporation, Bedford, MA). Samples were further heated at 100°C for 30 min before being stored at –20°C for the determination of rumen LPS concentration using the Limulus amebocyte lysate (LAL) assay (Levin and Bang, 1964). The assay was performed using a 96-well microplate (BioWhittaker Inc., Walkersville, MD) with absorbance read at 405 nm using a microplate reader (BioRad model 3550, Hercules, CA). Samples were diluted 1000-fold using pyrogen-free water to determine the non-inhibitory dilution, with the final dilution being made of 50% diluted sample and 50% ß-glucan blocker (BioWhittaker kit number N190; component number: B50-700). ß-Glucan blocker blocks the reactivity of LAL to ß-1,3-glucans, conferring increased endotoxin specificity to the LAL test. Non-inhibitory dilution is achieved when 75 ± 25% of spike is recovered in positive control samples. An average recovery rate of 78.0 ± 8.6% (n = 8) was achieved in positive controls with a sample dilution of 1:60,000.

Two blood samples (7 mL) were collected by tail venipuncture from each steer on d –2, –1, 1, 2, 3, 4, and 5 into plain and sodium heparin coated plasma tubes at 0900 h. Serum and plasma were harvested by centrifuging samples at 3000 rpm for 30 min. Haptoglobin and SAA were determined in serum and plasma, respectively, using ELISA Tridelta Phase range kits (Tridelta Diagnostics Inc., Cedar Knolls, NJ; catalog numbers TP-801 and TP-802, respectively) (Makimura and Suzuki, 1982; McDonald et al., 1991). For

Hp, serum samples were diluted 1:5 with PBS and vortexed. Seventy-five microliters of diluted serum was added to duplicate wells of a 96-well microtiter plate. Stabilized hemoglobin diluted 1:1 with hemoglobin diluent, was added (100 μL) to the diluted sample. Chromogen and substrate, mixed in a ratio of 9:5 (140 μL), were added to the reaction mixture and incubated at room temperature (25°C) for 5 min. Absorbance was read immediately at 630 nm using a microplate spectrophotometer (Spectra Max 340 PC, Molecular Devices Corporation, Sunnyvale, CA). For SAA, plasma samples were diluted 1:500 in diluent buffer and vortexed. Fifty micro-liters were added to each well of a 96-well plate coated with 50 μL of biotinylated antiserum amyloid-A monoclonal antibody (diluted 1:100 in 1x diluent buffer). The microtiter plate was covered and incubated at 37°C for at least 1 h and then washed 4 times with diluted wash buffer to remove unbound material. Streptavidin-horseradish peroxidase conjugate was diluted 1:4000 in diluent buffer and added (100 μL) to each well. The plate was incubated at room temperature (25°C) in darkness for 30 min. The microtiter plate was washed (as described above) and tapped dry. Substrate (100 μL) was added to the plate, which was then incubated at room temperature (25°C) in darkness for an additional 30 min. Stop solution was added, and the plate was read in a microplate reader (BioRad model 3550) at 450 nm.

Statistical Analyses

Data were analyzed using mixed model analysis with the first-order autoregressive covariance structure in Proc Mixed in SAS (SAS Institute, 1996). The following model was used:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + D_k + (\alpha x \beta)_{ij} + T_l + (DxT)_{kl} + e_{ijkl}$$

where Yijkl = observations for dependent variables; μ = overall mean; αi = average effect of period i; ßj = average effect of the animal j; Dk = average effect of dietary treatment; (α x ß)ij = animal x period interaction, which was the main plot error; Tl = average effect of time in days for feed intake, or hours since the beginning of feeding concentrate for blood metabolites or endotoxin concentration; (D x T)kl = interaction between time and dietary treatment; and eijkl = subplot error. Lipopolysaccharide concentrations were log transformed because of nonhomogeneous residual error. Orthogonal contrasts were used to compare Hp, SAA, and log-transformed LPS concentrations during the period when only hay was fed with those when concentrate was fed to induce SARA. Mean differences for rumen pH data and DM intake were separated using Tukey's multiple comparison procedure.

RESULTS AND DISCUSSION

Average rumen fluid pH, time, and area with pH below 6.0 or 5.6 did not differ among the diets (Table 2). However, feeding concentrate decreased average daily pH from d 1 to a nadir on d 4. Both time below pH 6.0 and time below pH 5.6 increased from d 1 to a peak on d 4. Area below pH 6.0 increased from d 1 to a peak on d 4 but area below pH 5.6 showed only numerical increases (P = 0.096; Table 2). Because the steers spent 187 and 174 min/d with pH below 5.6 on d 4 and 5, respectively, we concluded that SARA was successfully induced on these days. The severity of SARA was less than that experienced by cows used in studies by Krajcarski-Hunt et al. (2002), who reported time and area below pH 5.6 of 594.4 ± 188.9 min/d and 228.0 ± 88.8 min x pH/d, respectively. The rumen fluid

pH that defines SARA is still a controversial issue, with various threshold values having been arbitrarily set to define SARA. For example, threshold values of 5.5 (Hibbard et al., 1995), 5.6 (Cooper and Klopfenstein, 1996), 5.8 (Beauchemin et al., 2001), and 6.0 (Kriehbiel et al., 1995a) have been used to define the upper bound of pH that defines SARA. The duration for which the pH must remain below this threshold has not been precisely defined with respect to the definition of SARA.

Offering different amounts of concentrate and hay was intended to induce SARA to different extents. Thus, diets were designed to differ significantly in their DM intake. Intake of both concentrate and hay was variable in all diets. However, offering 6 kg of concentrate resulted in more variable intake response among days compared with the other 2 diets. Dry matter intake of hay was more variable than that of concentrate and may be the reason for the diet x day interaction (Table 3). Animals went through cycles in which a high intake on one day was followed by low intake the following day. Averaged across diets, the decrease in DM intake was greatest between d 3 and 4 for concentrate and between d 2 and 3 for hay. The steers appeared to reduce hay intake in favor of concentrate on d 2 and 3. Variations in intake could explain the large variation in rumen pH among days that was also observed (Table 2).

Different levels of wheat-barley pellets in the diets did not affect LPS concentration. However, wheat-barley pellets increased (compared with hay-only days) LPS concentration from d 1 to a peak on d 4 ($P < 0.05$; Figure 1). The peak coincided with the day when the time with pH below 5.6 was greatest. Ruminal LPS increased from 3715 endotoxin units per mL for the 0900-h sample on d 1 (before concentrate was fed) to a peak of 12,589 endotoxin units/mL on d 4. This may have been due to an increase in free LPS under acidic

conditions. High concentrations of LPS with low rumen fluid pH may be due to increased lysis of dead bacterial cells or shedding of free LPS from rapidly growing gram-negative bacteria (Nagaraja et al., 1978a).

Haptoglobin concentration in blood serum was not affected by dietary treatments. However, inducing SARA increased concentrations of Hp from 0.43 ± 0.14 (when only hay was fed) to 0.79 ± 0.14 mg/mL on d 5 of the treatment period ($P < 0.05$; Figure 1). Inducing SARA resulted in higher Hp concentrations on d 3 and 5 ($P < 0.05$). Other researchers have reported that Hp concentrations are undetectable in healthy cattle, with concentrations only becoming detectable when there is an inflammatory response (Deignan et al., 2000). In the present study, low concentrations were detected during the 3 d prior to feeding wheat-barley pellets. The response in Hp concentration to SARA was low compared with concentrations seen in experimentally induced bacterial and virus infections, in which Hp concentrations increased 100-fold (Deignan et al., 2000; Heegaard et al., 2000).

Serum amyloid-A concentration was not significantly different among the different diets but concentrations in blood plasma increased from 33.6 ± 36.53 (when only hay was fed) to 170.7 ± 36.53 μg/mL on d 5 ($P < 0.001$), when concentrate was offered in addition to hay (Figure 1). Serum amyloid-A is the other major acute phase protein, and is a more sensitive marker of inflammatory challenge than is Hp (Horadagoda et al., 1999). The acute phase protein profiles obtained in the present study indicate that as time with pH below 5.6 increased, the intensity of the acute phase response increased (Figure 1). This could be due to formation of extensive microlesions on the ruminal epithelium leading to increased LPS translocation across the ruminal epithelium into the systemic circulation, which would

amplify the acute phase response associated with SARA. To our knowledge, this is the first study that has examined an acute phase response due to SARA.

In conclusion, offering steers wheat-barley pellets and chopped grass hay successfully induced SARA, particularly on d 4 and 5 of our study, although DM intake declined from d 1 to 5. Inducing SARA increased the concentration of LPS in rumen fluid, plasma SAA, and serum Hp concentrations. Although SAA concentrations increased within 24 h of feeding concentrate, Hp concentration did not change until the third day of feeding concentrate. Inducing SARA increased rumen LPS concentration and initiated an acute phase response. Causal factors for inflammatory response may be an increase in LPS and bacterial translocation into the pre-hepatic circulation. Further work needs to be done under controlled feed intake conditions to remove possible confounding of LPS concentration with variable feed intake.

TABLE 2. Rumen pH variables of steers offered different levels of wheat-barley pellets and chopped grass hay to induce subacute ruminal acidosis.

	Diet[1]				Day						Effect, *P* value		
	Diet 1	Diet 2	Diet 3	SEM	1	2	3	4	5	SEM	Diet	Day	Diet x Day
Average pH	6.39	6.29	6.26	0.05	6.49^{a}	6.20^{ab}	6.27^{a}	6.19^{b}	6.24^{ab}	0.10	0.19	0.006	0.057
Time <pH 5.6 (min/d)	42^{b}	117^{ab}	134^{a}	33.1	5^{b}	80^{ab}	78^{ab}	187^{a}	174^{a}	42.3	0.045	0.013	0.11
Time <pH 6.0, (min/d)	308	369	404	77.3	9^{b}	471^{a}	425^{a}	589^{a}	480^{a}	88.0	0.64	<0.001	0.14
Area <pH 5.6, (min x pH/d)	3	25	35	13.6	1	7	10	54	49	18.1	0.17	0.096	0.19
Area <pH 6.0, (min x pH/d)	71	124	125	28.9	2^{b}	104^{ab}	111^{ab}	189^{a}	170^{a}	37.5	0.20	0.005	0.13

[a,b]LSmeans for diets with different letter superscripts within a row were different ($P < 0.05$).

[c,d]LSmeans for days with different letter superscripts within a row were different ($P < 0.05$).

[1]Diet 1 = 4 kg of wheat-barley pellets and 6 kg of chopped grass hay offered daily; Diet 2 = 5 kg of wheat-barley pellets and 5 kg of chopped grass hay offered daily; Diet 3 = 6 kg of wheat-barley pellets and 4 kg of chopped grass hay offered daily.

TABLE 3. Feed intake of steers offered different levels of wheat-barley pellets and chopped grass hay to induce subacute ruminal acidosis.[1]

	Day						Effect, *P* value		
	1	2	3	4	5	SEM	Diet	Day	Diet x Day
Concentrate (kg of DM/d)									
Diet 1	3.72	2.68	3.14	2.62	2.84	0.75			
Diet 2	4.65	4.65	4.05	3.26	3.45	0.75	0.005	0.01	0.28
Diet 3	5.61^{a}	4.47^{a}	5.61^{a}	4.26^{a}	1.91^{b}	0.88			
Average across diets	4.66^{a}	3.93^{a}	4.27^{a}	3.38^{ab}	2.73^{ab}	0.56			
Chopped grass hay (kg of DM/d)									
Diet 1	5.06^{a}	4.69^{ab}	2.59^{b}	3.71^{ab}	3.50^{ab}	0.48			
Diet 2	4.24^{a}	4.40^{a}	4.08^{a}	2.78^{b}	2.08^{b}	0.48	0.085	<0.0001	0.036
Diet 3	3.72^{a}	4.25^{a}	3.35^{ab}	1.98^{ab}	0.83^{b}	0.59			
Average across	4.34^{a}	4.45^{a}	3.34^{b}	2.82^{bc}	2.14^{c}	0.28			

[a,b,c]Means with different superscripts within each row differed ($P < 0.05$).
[1]Diet 1 = 4 kg of wheat-barley pellets and 6 kg of chopped grass hay offered daily; Diet 2 = 5 kg of wheat-barley pellets and 5 kg of chopped grass hay offered daily; Diet 3 = 6 kg of wheat-barley pellets and 4 kg of chopped grass hay offered daily.

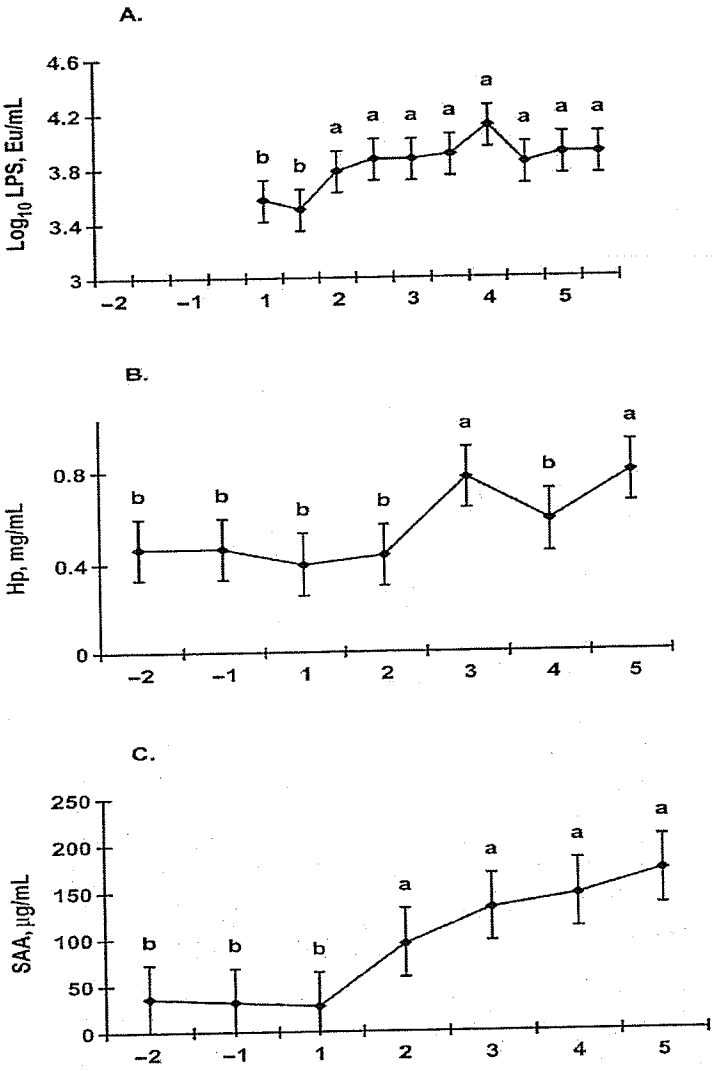

FIGURE 1. Concentrations of ruminal lipopolysaccharide (LPS; panel A), haptoglobin (Hp; panel B), and serum amyloid-A (SAA; panel C) in steers fed chopped grass hay only (d –2 and d –1) and during concentrate feeding (d 1 to 5). Orthogonal contrasts were used to determine which concentrations differed from baseline values. Lipopolysaccharide concentrations measured in rumen fluid samples collected before feeding concentrate on d 1 were taken as baseline. Bars indicate SE, and significance was declared at the 5% level of significance.

MANUSCRIPT II

Ruminal Lipopolysaccharide Concentration and Inflammatory Response During Gradual Stepwise Adaptation to Concentrate and Subacute Ruminal Acidosis Induction in Steers.

G.N. Gozho, J.C. Plaizier and D.O. Krause

Department of Animal Science, University of Manitoba, Winnipeg, Canada R3T 2N2

Corresponding author: J. C. Plaizier; e-mail: plaizier@ms.umanitoba.ca.

ABSTRACT

Three rumen fistulated Jersey steers were gradually adapted to a wheat-barley concentrate over a 4 wk period. Adaptation steps consisted of four 1 wk periods during which steers were fed diets with forage to concentrate (F:C) ratios of 100:0, 80:20, 60:40 and 40:60. The forage consisted of chopped grass hay and the concentrate consisted of ground and pelleted concentrate containing 50% wheat and 50% barley. Animals were fed the 100% forage diet *ad libitum* during week 1 and the average intakes were determined and used to predict intake for weeks 2 to 5. On two days that were set 3 d apart during week 5, SARA was induced in the steers by feeding them 1 kg of chopped grass hay at 0900 h followed by two meals of 3.2 kg each of wheat-barley concentrate at 1100 h and 1300 h and 1 kg of chopped grass hay at 1700 h. During stepwise adaptation, time with pH below 5.6 increased to an average of 121 min/d when the steers were on the 40:60 F:C ratio diet. Feeding the 20:80 F:C ratio diet induced SARA because the steers spent an average of 219 min/d with pH below 5.6. Ruminal lipopolysaccharide concentration increased from 6310 Eu/mL with the 100% forage diet to 18197 Eu/mL with the 40:60 F:C ratio diet. The ruminal LPS concentration increased to 26915 Eu/mL when SARA was induced. Total coliform counts increased during stepwise adaptation to a peak when 40% of the diet was wheat-barley concentrate. Subsequent additional concentrate in the diet resulted in a decrease in coliform numbers. Inducing SARA in concentrate adapted animals had no effect on coliform numbers. Serum haptoglobin increased from 0.53 mg/mL when steers were on the 100% forage diet to 1.90 mg/mL with the 40:60 F:C ratio diet and were not

further increased by inducing SARA. Serum amyloid-A was not significantly affected during concentrate adaptation but increased to 163 μg/mL when SARA was induced. Ruminal lipopolysaccharide concentrations were increased during stepwise adaptation and when SARA was induced in the steers.

(Key Words: steers, subacute ruminal acidosis, rumen pH, lipopolysaccharide, serum amyloid-A, haptoglobin, gram-negative bacteria)

Abbreviation key: Eu = endotoxin units, **F:C** = forage to concentrate ratio, **Hp** = haptoglobin, **LAL** = *Limulus* amebocyte lysate, **LPS** = lipopolysaccharide **SAA** = serum amyloid-A, **SARA** = subacute ruminal acidosis, **CGH** = chopped grass hay, **WBP** = wheat barley pellets.

INTRODUCTION

The following indicators of subacute ruminal acidosis (SARA) appear in the literature; daily episodes of low rumen pH between 5.2 and 5.6 for at least three hours (Cooper et al., 1999; Gozho et al., 2005a); erratic feed intake, diarrhea, abscesses, loss of body condition, and laminitis (Nordlund et al., 1995; Nocek, 1997). Apart from rumen pH, these signs are not specific for SARA and individually are of limited diagnostic value. However, if the clinical signs, production records, diet characteristics, and rumen pH, are considered together on a herd basis, SARA can be diagnosed (Nordlund et al., 2004).

One of the greatest impediments to the diagnosis of SARA is the measurement of rumen pH because of the difficulty in obtaining accurate data under field conditions. Therefore only limited information on its prevalence is available. A survey on 15 dairy farms in Wisconsin showed the presence of SARA in 19% of early lactation cows and 26% of mid-lactation cows (Garrett et al., 1997). Another survey on 14 dairy farms in Wisconsin detected SARA in 20.1% of early and peak lactation cows (Oetzel et al., 1999). Dairy cattle at high risk of developing SARA include transition dairy cows, cattle on high dry matter intakes, and cows that are subjected to a high degree of variability in ration composition and meal patterns (Stone, 2004). Suddenly switching cattle from high forage to high starch diets result in decreases in rumen pH that is characteristic of SARA because VFA accumulate in the rumen (Goad et al., 1998).

The increase in nonstructural carbohydrates in the diet of cattle during gradual grain adaptation results in microbiological changes in the rumen. Key among the changes is the increase in lactic acid utilizing bacteria like *Megashera elsdnenii* (Counotte et al., 1981).

These are relatively slow growing bacteria, found in low numbers in ruminants fed high forage diets, and only increase in numbers when lactic acid is a major end product of rumen fermentation. If the rate of concentrate inclusion in the diet results in lactic acid synthesis at a rate higher than the rate at which it can be utilized by adaptive increases in lactic acid utilizing bacteria, then lactic acid accumulates in the rumen and rumen pH is depressed more drastically than would be expected based on levels of volatile fatty acids (Owens et al., 1998). Fluctuation in the numbers of lactic acid producing and utilizing bacteria occur even in grain adapted cattle during the course of the day due to variation in nutrients and the range in substrate preferences of different microbial species (Dijkstra et al., 2002).

Inducing SARA by abruptly introducing concentrate in the diet of forage fed steers results in a marked increase in the concentration of ruminal lipopolysaccharide (LPS) (Gozho et al., 2005a). The increase in LPS in the rumen may be due to autolysis during rapid growth or death and lysis of gram-negative bacteria in the rumen (Nagaraja et al., 1978a; Well and Russell, 1996). Data on bacteriological changes (Mackie et al., 1978; Mackie and Gilchrist, 1979) and changes in ruminal LPS (Andersen et al., 1994a), have all been obtained in experiments in which acute ruminal acidosis was induced. Therefore inducing SARA may result in a response that differs from that observed with acute acidosis because the decrease in rumen pH is not as severe as that experienced with acute acidosis.

Feeding diets with graded levels of concentrate during adaptation to concentrate results in changes in numbers of both gram-negative and gram-positive bacteria. It is hypothesized that the ruminal LPS concentrations would be affected by the level of dietary concentrate inclusion and hence the inflammatory response is different during grain adaptation to concentrate and SARA induction in concentrate adapted cattle. Therefore the

objective of this study was to determine the effect of how gradual stepwise adaptation to concentrate and inducing SARA in animals adapted to a 60% concentrate diet affect ruminal LPS concentration and acute phase proteins. The level of concentrate feeding that elicits an acute phase response was also of interest. Rumen coliform bacteria were also monitored to determine trends that suggested changes in gram-negative bacteria.

MATERIALS AND METHODS

Animals and Diets

Three adult ruminally fistulated Jersey steers were adapted to a wheat-barley pellet (WBP) diet over a 4 wk period in a time series experimental design. The steers were maintained in metabolism crates in the Animal Science Research Unit building at the University of Manitoba, in accordance with the guidelines of the Canadian Council of Animal Care. The room that houses the crates is fitted with the Proportional Environment Control system (Model PEC, Phason, Winnipeg, MB, Canada). Ambient temperature in the room was set at 15°C for the duration of the experiment. The steers were kept indoors from Monday to Friday and were let out into a courtyard for 4 h to exercise on Saturdays and Sundays.

Steers were initially fed an all forage diet comprising chopped grass hay (CGH) *ad libitum* for 7 d. In subsequent weeks, the steers were adapted to concentrate diets. Each step was fed for a 7 d period and CGH was replaced with 0, 20, 40, and 60% (DM basis) of WBP during weeks 1 to 4, respectively. The amount of DM supplied to the steers was kept

constant at a level equal to the daily average DM intake achieved with the 100% forage diet in week 1. This was done to prevent erratic feed intake that can occur when high concentrate diets are introduced. During weeks 2 to 4, WBP were thoroughly mixed with CGH, and offered to the steers at 0900 h every day. On Monday and Friday of week 5, SARA was induced in the steers by feeding them 1 kg CGH at 0900 h followed by two meals of 3.2 kg each of WBP at 1100 h and 1300 h and 1 kg of CGH at 1700 h. During the other days of this week, steers were given the 40:60 F:C ratio diet. The chemical composition for WBP and CGH is given in Table 4.

Each 7 d period in the first 4 weeks during which a different concentrate diet was fed as well as the days that SARA was induced in week 5 were considered as separate treatments. Thus treatments were arranged in a time series design and diets with F:C ratios of 100:0, 80:20, 60:40 and 40:60 were designated treatments 1, 2, 3 and 4, respectively. The two days during which SARA was induced in week 5 was designated treatment 5 and the other days of this week during which the 40:60 F:C ratio diet was fed were assigned to treatment 4 for dry matter intake determination only. Weigh backs were determined and sampled for dry matter determination. Dry matter contents were 92.5% for chopped grass hay and 89.2% for wheat-barley pellets.

Rumen pH Measurement

Rumen pH was measured continuously for two 24 h periods during each week by inserting one indwelling pH probe into the rumen of each steer. For weeks 1 to 4, probes were inserted on Thursday at 0900 h and were taken out on Saturday at 0900 h. During week 5, probes were inserted on Monday at 0900 h and taken out on Tuesday 0900 h and

reinserted at 0900 h on Thursday and removed on Friday at 0900 h. The indwelling pH probes were placed into the ventral sac of the rumen of each steer and kept in position by means of a 0.5 kg weight. Rumen pH was measured every second and averaged over 60 s and the minutely averages were captured by a data logger and stored.

Rumen fluid pH data were summarized as average pH, time below pH 6.0 and time below pH 5.6, area (time x pH) below pH 6.0, and area (time x pH) below pH 5.6 for each 24 h period.

Rumen fluid samples were collected into sterile plastic tubes from the ventral sac of the rumen at 0900 h, 1200 h, 1500 h, 2100 h and 0300 h during weeks 1 to 4 starting on Thursday morning and ending on Saturday morning. In week 5 rumen fluid samples were collected for two 24 h periods so that sampling began on Monday morning and ended on Tuesday morning for the first 24 h period whilst the second 24 h sampling period started on Thursday morning and ended on Friday morning. The rumen fluid samples were processed for subsequent LPS determination as described previously (Gozho et al., 2005a). Another rumen fluid sample was collected from each animal into sterile plastic vial with airtight lids at 0900 h, 1200 h, 1500 h and 2100 h. This sample was used immediately to determine total coliform counts. A portion of this second sample was centrifuged at 1500 x g for 10 minutes and the supernatant was stored at -20°C for VFA analysis at a later stage.

TABLE 4. Chemical composition for wheat-barley pellets and chopped grass hay (DM basis).

Item	Chopped grass hay	WBP
DM, %	92.5	89.2
CP, % of DM	10.9	15.5
NDF, % of DM	64.0	17.6
ADF, % of DM	35.9	5.9
NSC, % of DM	15.2	62.2
Ca, % of DM	0.38	0.27
P, % of DM	0.27	0.35
K, % of DM	1.93	0.56
Mg, % of DM	0.20	0.16
Na, % of DM	0.01	0.03

Ruminal LPS and VFA Analyses

The *Limulus* amebocyte lysate (LAL) assay was used to determine LPS concentration (Levin and Bang, 1964). The assay was performed using a 96-well microplate kit (Cambrex Bio Science Walkersville Inc, MD) with absorbance read at 405 nm using a microplate reader (BioRad model 3550, Hercules, CA). Detailed procedures for sample preparation and method validation have been described previously (Gozho et al., 2005a).

Volatile fatty acids were determined in previously frozen rumen fluid samples. Samples were thawed at room temperature and 1 mL of a 25% meta-phosphoric acid solution was added to 5 mL of rumen fluid. The tubes containing the mixture were vortexed and placed in a -20°C freezer for 17 h after which samples were thawed and centrifuged for 10 min at 1500 x g. Approximately 2 mL of supernatant were decanted into a clean dry vial which was capped and placed into the auto-sampler device (model 8100; Varian, Walnut Creek, CA). Concentrations of VFA were determined by gas chromatography (model 3400 Star; Varian) using a 1.83-m glass column (model 2-1721; Supelco, Oakville, Ontario, Canada) (Erwin et al., 1961). The injector and detector temperatures were set at 170 and 195, with initial and final column temperatures set at 120°C and 165°C, respectively. The run time was 4 min followed by a 2 min thermal stabilization period.

Acute Phase Proteins

Blood samples were collected twice via extended use polyurethane catheters (Mila Cath®, Mila International, Florence, KY 41042 USA) fitted into the jugular vein of each steer. Samples were collected at 0900 h and 2100 h on the days that rumen pH was measured (Thursday and Friday for weeks 1 to 4 and Monday and Thursday for week 5). At each blood sampling, and at least once daily, the catheter sites were examined for inflammatory reactions, and catheters were flushed with approximately 15 mL of 0.9% sterile saline. After each sample collection, 5 mL of 0.9% sterile saline containing 50 IU of heparin (Sigma-Aldrich, St Louis, MO USA) was infused into the vein to prevent clot formation. Before blood collection, the heparinized saline solution was withdrawn along with approximately 10 – 15 mL of blood and discarded. Two blood samples (10 mL) were collected into serum and sodium heparin coated plasma tubes. Serum and plasma were harvested by centrifuging samples at 1500 x *g* for 30 minutes. Haptoglobin and serum amyloid-A were determined in serum and plasma, respectively, using ELISA Tridelta Phase™ range assay kits (Tridelta Diagnostics Inc, NJ, USA; catalogue numbers TP-801 and TP-802, respectively) as described by Gozho et al. (2005a).

Total Coliform Counts

Preparation of chromogenic medium (*Escherichia coli* / coliform medium catalogue number CM0956, Oxoid Inc. Nepean ON, Canada) was modified to include a step in which 20% of the distilled water was replaced with clarified rumen fluid. The clarification process for the rumen fluid included centrifuging the rumen fluid at 1500 x g for 10 minutes, followed by autoclaving the supernatant at 121°C for 15 minutes. The supernatant was

stored frozen at -20°C until required for preparing medium. The rumen fluid was collected from a fistulated cow fed on a diet comprising 100% CGH. The hay fed to the cow was from the same batch that was used in this experiment.

Buffered peptone water (pH 7.2) (Difco™) was prepared according to manufacturer's instructions. Carbon dioxide gas was bubbled through buffered peptone water and the chromogenic *E. coli* /coliform medium immediately after preparation and autoclaving in order to reduce the oxygen tension (Bryant, 1972). The coliform medium was used for culturing rumen coliform bacteria whilst buffered peptone water was used for the serial dilutions of rumen fluid samples prior to inoculation into Petri dishes. The chromogenic medium is normally used to differentiate between *E.coli* and other coliforms in cultures produced from food and environmental samples (Frampton et al., 1988).

Rumen fluid (100 µL) was inoculated into 900 µL of 2% buffered peptone water in a 2 ml sterile deep well plate and thoroughly mixed. Subsequently dilutions were made from this initial dilution whilst maintaining a dilution ratio of 1:10 until the appropriate dilution was reached. Sterile pipette tips were used for each dilution. Ten drops of 50 µL each were pipetted (Repeater™ 4780, Eppendorf) onto modified chromogenic *E coli* /coliform medium that had been prepared previous and allowed to set. After the drops on the agar dried, the plates were inverted and incubated at 37°C for 18 h. Colonies were manually counted and broadly classified according to color with purple being designated *E coli* and pink to brown colonies as other coliforms. The countable dilution was determined as the dilution that gave 3 to 30 colonies per drop of sample dispensed.

Statistical Analysis

Data were analyzed using SAS Mixed Procedure (SAS Institute Inc., 1996) as recommended by Wang and Goonewardene (2004) for the analysis of animal experiments with repeated measures. The effects of treatment, time (day for rumen pH and total VFA; or hour for SAA, Hp or LPS data), and their interactions were considered fixed. Animal and interactions of other factors with animal were considered random. Data for ruminal LPS and total coliform counts were log transformed because of non-homogenous residual error. The most appropriate covariance structure relative to the hourly or daily measurements that were tested included; simple, first order ante dependence, unstructured covariance, compound symmetry and first order auto regressive (Wang and Goonewardene 2004). Final mixed models were accepted only if the converge criteria was met, the estimated G matrix was positive definite and the degrees of freedom were the same as those obtained by running the same model using the general linear models procedure (SAS Institute Inc., 1996). The covariance structure that resulted in the lowest value for the fit statistic was chosen (Wang and Goonewardene 2004). Orthogonal contrasts were used to compare the 100:0 F:C diet with the 40:60 F:C ratio diet; and the 40:60 F:C with the 20:80 F:C ratio diet for all variables. However, where a diurnal variation of a variable was of interest and in the absence of a significant interaction, Tukey's multiple range test was used to separate means.

RESULTS

Dry Matter Intake

Average dry matter intakes were 5.9, 5.9, 5.9, 5.4 and 6.9 kg DM/d when steers were on the diets with F:C ratios of 100:0, 80:20, 60:40, 40:60 and 20:80, respectively (Table 5). Dry matter intake during week 5 was separated into the 5 days when the steers were fed the 40:60 F:C ratio diet and the two days when SARA was induced. Intake on the 40:60 F:C ratio diet in week 5 was added to that for week 4 in order to determine average daily intake when steers were on the same diet. The amount of feed allocated to each steer when SARA was induced in week 5 was higher than that for other treatments in the experiment. The intended and actual dietary F:C ratios were similar. Steers consumed all the feed offered during weeks 2 to 4. During week 5 intakes were slightly depressed following SARA induction and as much as 8.5 % of feed offered was recorded as orts. This is reflected as a lower intake for the 40:60 F:C ratio diet (Table 5).

Rumen pH, VFA and Ruminal LPS

Orthogonal contrasts revealed that the average rumen pH tended to be lower ($P = 0.06$) when steers were on the 40:60 F:C ratio diet compared to the 100% forage diet. Inducing SARA with the 20:80 F:C ratio diet decreased ($P = 0.04$) average rumen pH compared to the 40:60 F:C ratio diet (Table 6). Similarly, the time with pH below 6 tended to be higher ($P = 0.07$) with the 40:60 F:C ratio diet than the 100% forage diet but inducing SARA after concentrate adaptation did not increase ($P > 0.1$) time with pH below 5.6 compared to the 40:60 F:C ratio diet. The area under the pH x time curve for both the 5.6 and 6.0 pH

thresholds were not affected ($P > 0.1$) by stepwise adaptation to concentrate or inducing SARA. Compared to the 100% forage diet, the area under the pH x time curve tended to be higher ($P < 0.1$) when SARA was induced (Table 6). Total volatile fatty acids were measured to indicate changes in rumen pH and changes in fermentation patterns. The concentration of VFA was influenced by both treatment and time after feeding (Figure 2). VFA concentrations increased to peak around 1200 h during concentrate adaptation, but peaked around 1500 h when steers were fed to induce SARA during week 5.

Ruminal LPS concentration increased ($P < 0.0001$) when the 40:60 F:C ratio diet was fed compared to the 100% forage diet. Inducing SARA after concentrate adaptation increased ($P = 0.05$) LPS concentration compared to when the 40:60 F:C ratio diet was fed (Figure 3).There was also a ($P < 0.0001$) diurnal variation in LPS concentration and in the absence of a treatment x hour interaction; data were pooled across treatments to present diurnal variations. Ruminal LPS concentration increased from 4.039 Log_{10} EU/mL at 0900 h to a peak of 4.135 Log_{10} EU/mL at 1500 h (Figure 4).

TABLE 5. Dry matter intake (means ± standard deviation) of steers offered graded levels of wheat-barley concentrate and chopped grass hay.

Week[1]	1	2	3	4	5
Intended F:C ratio	100 : 0	80 : 20	60 : 40	40 : 60	20 : 80
	n = 21	n = 21	n = 21	n = 36	n = 6
Intake (As fed) (kg / d)					
Chopped grass hay	6.4±2.8	5.1±0.5	3.9 ± 0.3	2.5 ± 0.5	1.8 ± 0.4
Wheat-barley pellets	0	1.3 ± 0.1	2.6 ± 0.2	3.5 ± 1.0	6.0 ± 0.5
Intake (DM Basis) (kg / d)					
Chopped grass hay	5.9 ± 2.5	4.7 ± 0.4	3.6 ± 0.3	2.3 ± 0.4	1.6 ± 0.4
Wheat-barley pellets	0	1.2 ± 0.1	2.3 ± 0.3	3.1 ± 0.9	5.3 ± 0.4
Orts / d (% DM offered)	41	0	0	8.5	8.3
Total DM intake (kg / d)	5.9	5.9	5.9	5.4	6.9
Actual F: C Ratio	100 : 0	80 : 20	60 : 40	42 : 58	23 : 77

[1]Daily average feed intake for week 4 is the average week 4 intake and the 5 days in week 5 when steers were on the 40:60 F:C ratio and only week 5 intake is the average of two days during which SARA was induced.

TABLE 6. Rumen pH variables of steers offered graded levels of wheat-barley concentrate and chopped grass hay. [1]

Week	1	2	3	4	5		P value		
Intended F:C ratio	100:0	80:20	60:40	40:60	20:80	SEM	Trt	Day	Diet x Trt
Average pH	6.72^{a}	6.69^{ab}	6.64^{ab}	6.44^{b}	6.14^{c}	0.17	0.003	0.41	0.88
Time < pH 5.6, min / d	0^{b}	0^{b}	0^{b}	121^{ab}	219^{a}	61.6	0.069	0.71	0.99
Time < pH 6.0, min / d	22^{bc}	0^{c}	10^{bc}	344^{ab}	600^{a}	119.3	0.007	0.78	0.30
Area < pH 5.6, min x pH / d	0	0	0	17	38	15.2	0.34	0.23	0.68
Area < pH 6.0, min x pH / d	1	0	1	101	216	73.4	0.20	0.38	0.65

[a,b,c]Means with different superscripts within each row differed ($P \leq 0.05$).
[1]Orthogonal contrasts were used for mean separation for significant treatment effects.

Total Coliform Counts

The chromogenic medium used for culturing coliforms is based on the ability of *E. coli* specific glucuronidase to cleave a chromogenic substrate and produce purple colonies whilst a second chromogenic substrate is cleaved by galsctosidase, an enzyme produced by the majority of coliforms to produce pink colonies (Oxoid manual). Lipopolysaccharide from *E. coli* (0111:B4) is used as a reference standard endotoxin in determination of LPS with the *LAL* assay. In the absence of any other group of bacteria that could be used to relate changes in ruminal LPS concentration to gram-negative bacteria, coliforms numbers were monitored during the course of the experiment. Total coliform concentrations for samples collected at 0900 h from week 1 to 5 were used to determine the effects of stepwise concentrate adaptation on coliform numbers. Samples that were collected during the four time intervals during weeks 2 to 5 were used to determine diurnal variations in coliform counts. Using only the samples collected at 9:00, total coliform concentrations were 6.60, 6.71, 6.75, 6.48 and 6.62 $\log_{10}$ cfu/mL in rumen fluid samples from the 0, 20, 40, 60 and 80% diets, respectively (Figure 5). Coliform numbers varied with diet ($P = 0.05$) in that numbers for the 60% concentrate diet were less than the 40% and 80% concentrate diets.

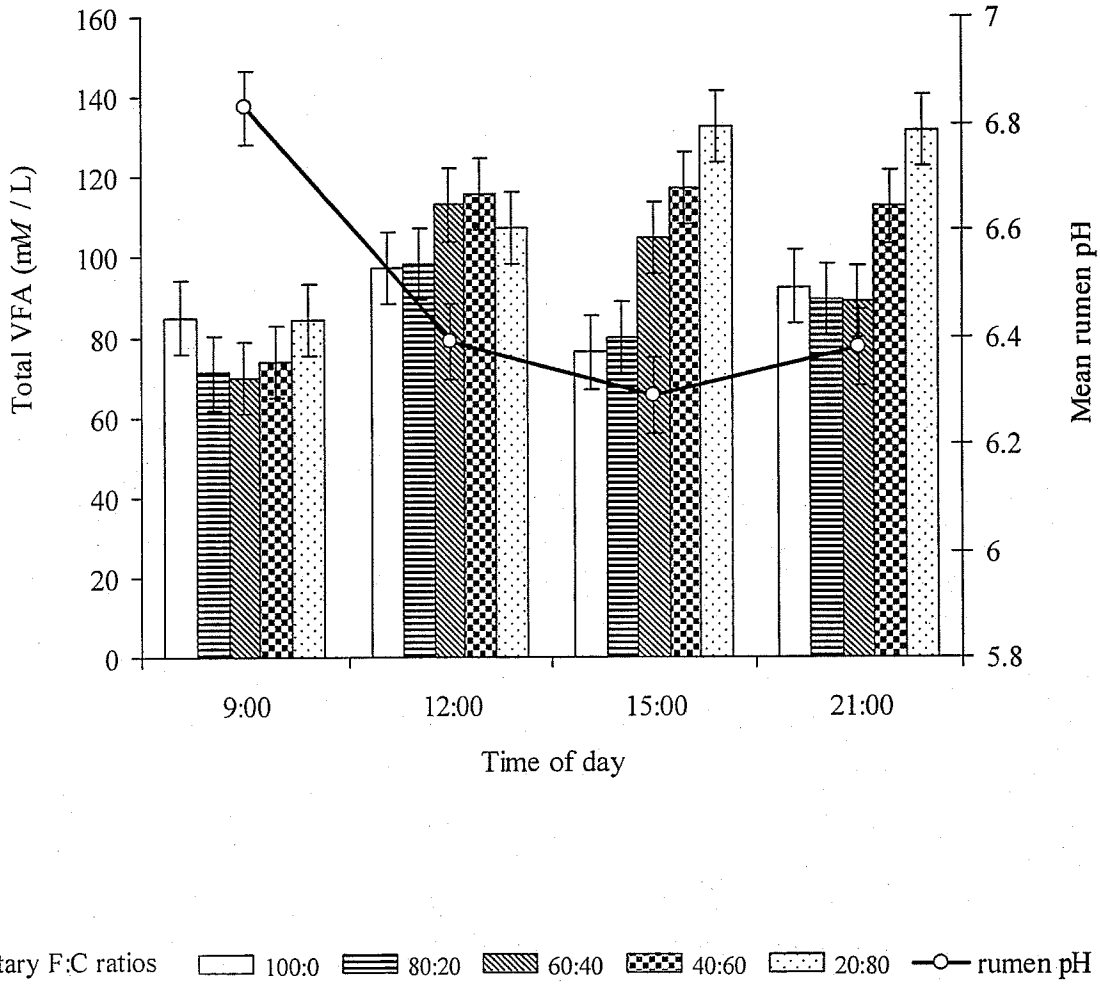

FIGURE 2. Changes in total VFA concentration (millimoles / mL) in rumen fluid of steers fed diets with different F:C ratios over time[1]. The average rumen pH (○) is also superimposed on the graph.

[1]Diet x time interaction was significant ($P<0.001$). Bars indicate SE

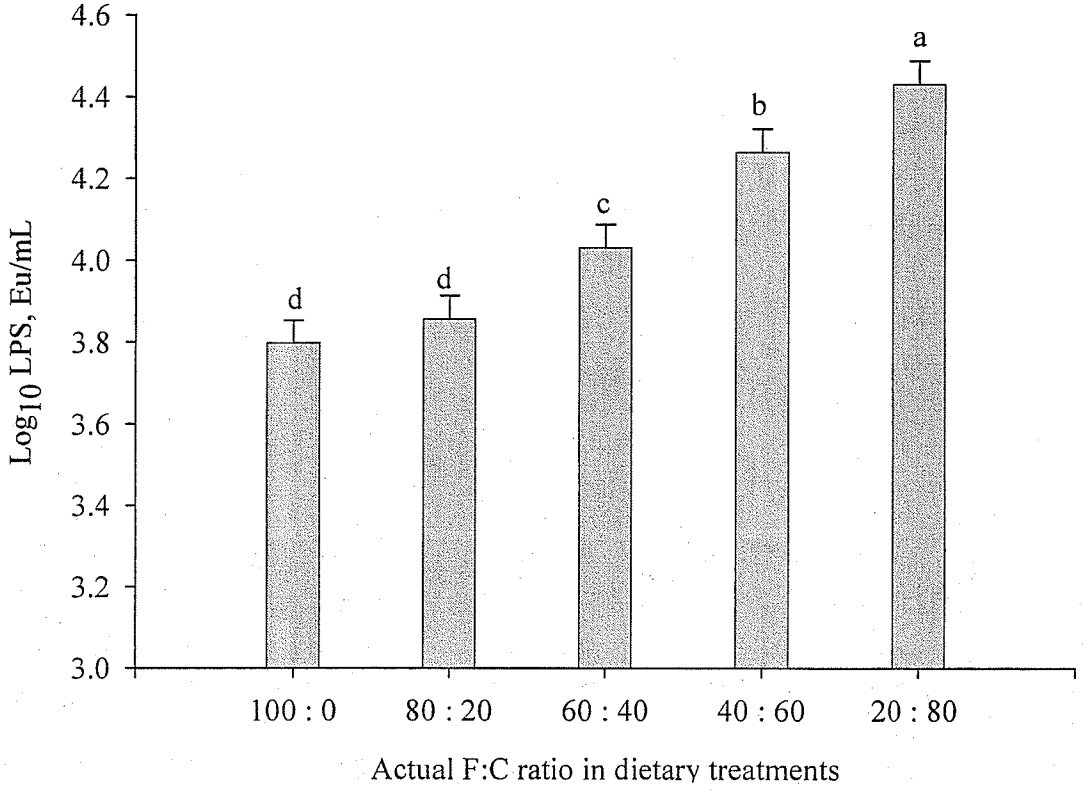

FIGURE 3. Concentration of ruminal LPS (log_{10} endotoxin units/ mL) in steers fed diets with different forage to concentrate ratios.
[a,b,c,d]Orthogonal contrasts were used to compare ruminal LPS in steers fed diets with different F:C ratios and statistical significance was declared at the 5% level of significance. Bars indicate SE.

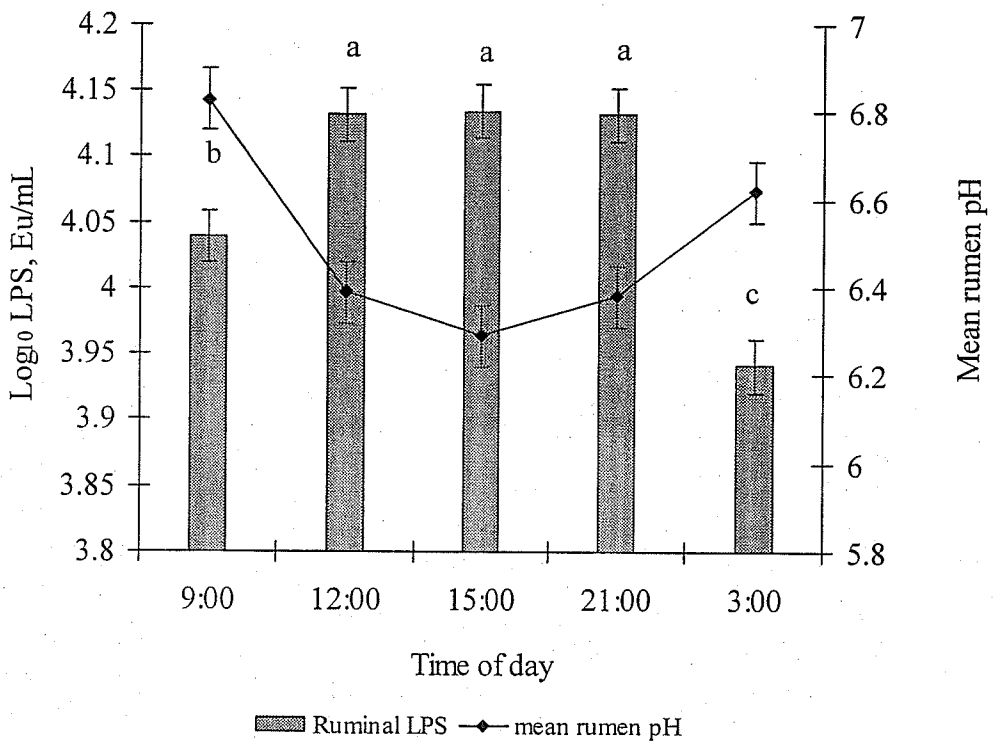

FIGURE 4. Diurnal changes in ruminal LPS concentrations (log_{10} endotoxin units/ mL) (bar graph) in steers fed diets with different forage to concentrate ratios. Mean rumen pH (line graph) over the same time is superimposed.
[a,b,c]Tukey's multiple comparison procedure was used to separate means and significance was declared at the 5% level of significance.
Bars indicate SE

Inducing SARA after adaptation to concentrate did not affect coliform numbers. There was no diurnal variation in coliform numbers in rumen fluid from the steers when the concentrate dietary inclusion rates were 20, 40, 60 or 80% of DM.

Acute phase proteins

Serum haptoglobin concentrations were higher ($P < 0.01$) for the 40:60 F:C ratio diet compared to the 100% forage diet. Inducing SARA after stepwise adaptation did not affect haptoglobin concentration. Haptoglobin concentrations were higher ($P = 0.0008$) for both the 40:60 and 20:80 F:C ratio diets compared to the 100% forage diet (Table 7). Haptoglobin concentrations increased from 0.53 mg/mL with the 100% forage diet to 1.90 and 1.40 mg / mL for steers consuming the 40:60 and 20:80 F:C ratio diets, respectively. Plasma concentrations of serum amyloid-A tended to be elevated ($P = 0.09$) in steers on the 40:60 F:C ratio diet compared to the 100% forage diet and were higher ($P < 0.0001$) in steers fed the 20:80 F:C ratio diet compared to the 40:60 F:C ratio diet (Table 7).

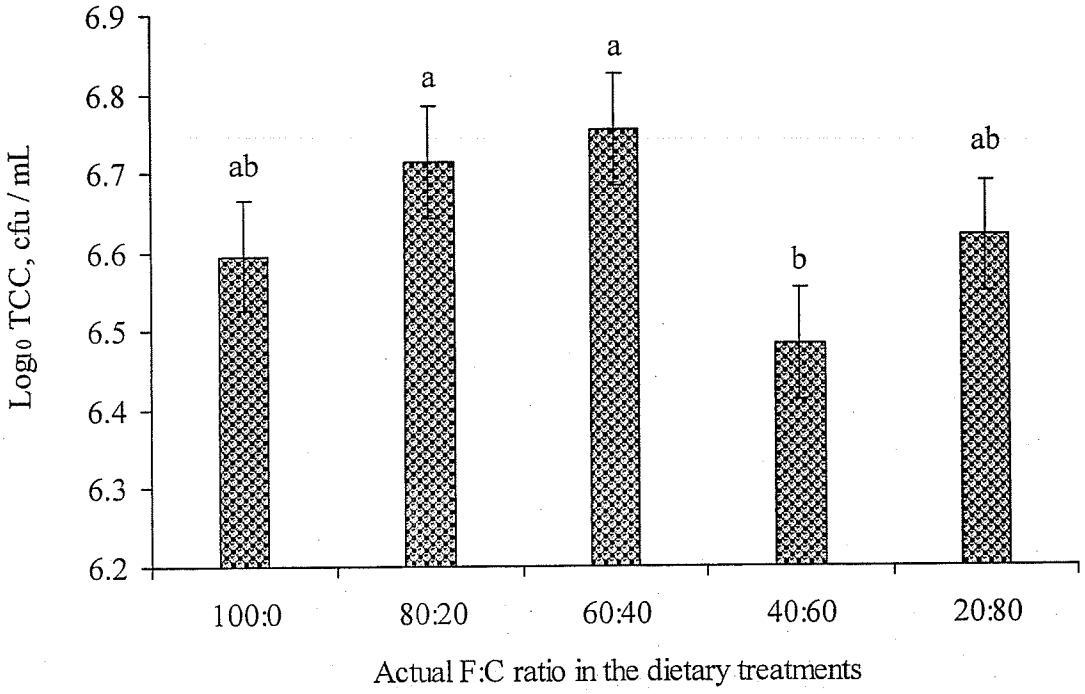

FIGURE 5. Total coliform counts (TCC) (log_{10} cfu / mL) in rumen fluid of steers fed diets with different forage to concentrate ratios.
[a,b,c,]Orthogonal contrasts were used to compare counts in steers fed diets with different F:C ratios and significance was declared at 5% level of significance.
Bars indicate SE

TABLE 7. Haptoglobin (Hp) and Serum Amyloid-A (SAA) concentrations in steers fed diets with different F:C ratios.[1]

Week	1	2	3	4	5		P value		
Intended F:C ratio	100:0	80:20	60:40	40:60	20:80	SEM	Trt	Time	Trt x Time
Hp *(mg/mL)*	0.53^{b}	0.54^{b}	0.56^{b}	1.90^{a}	1.40^{a}	0.23	0.0008	0.78	0.99
SAA *(μg/mL)*	38^{b}	49^{b}	55^{b}	71^{b}	163^{a}	13.83	<0.0001	0.23	0.44

[a,b]Means with different superscripts within each row differed ($P \leq 0.05$).
[1]Orthogonal contrasts were used for mean separation for significant treatment effects.

DISCUSSION

Dry matter supplied to the steers was kept constant to minimize variability in intake because erratic feeding patterns have been observed in studies when diets that induce SARA are fed *ad libitum* (Fulton et al., 1979). Only the possible maximum amount of feed that each animal could eat was predetermined in the present study. The average dry matter intake for each individual steer during week 1 was used to determine the feed supplied to each animal. It was anticipated that the 20:80 F:C ratio diet would result in erratic feed intake as was observed in a study with a similar treatment (Gozho et al., 2005a). In the present study, feed intake was slightly depressed during SARA induction and on subsequent days. Using a combination of feed restriction and complete withholding of feed, Brown et al. (2000) induced SARA in beef steers and were able to show that feed intake returned to normal by the third day after SARA was induced. In the present study, SARA was induced on Monday and Thursday of week 5 so that if animals became anorexic, due to SARA, there would be sufficient time for them to resume normal intake before the next bout of SARA induction.

Rumen pH progressively decreased as the proportion of concentrate increased (Table 6). This is because the amount of non-structural carbohydrates increased whilst the content of physically effective fibre in the diet decreased with each successive addition of concentrate. According to the definition of SARA of Cooper et al. (1999), it was only when the proportion of concentrate increased to 60 and 80% DM that the steers experienced SARA. However, according to recent studies by Gozho et al. (2005a), the time spent with rumen pH below 5.6 when SARA in induced determines the activation of an inflammatory

response as a result of SARA. Time with pH below 5.6 was higher (P = 0.02) when the F:C ratio in the diet decreased from 100:0 to 20:80. The 20:80 F:C ratio diet represents the nutritional model used to induce SARA. In the present study, SARA was only induced with the 20:80 F:C ratio diet because the steers spend an average of 219 min/d with pH below 5.6 when they were on this diet. In the rumen pH range that defines SARA, the decrease in pH is due to an increase total VFA concentration in the rumen (Krehbiel et al., 1995c; Goad et al., 1998; Stone, 2004). In the present study, total VFA increased with each successive increment in concentrate in the diet (Figure 2). The time taken for peak VFA concentration to be achieved differed among the diets leading to a significant diet x time interaction for VFA concentration.

The concentration of ruminal LPS increased significantly starting with the 60:40 F:C ratio diet and continued to increase with subsequent additions of concentrate to the diet (Figure 3). There is a paucity of data on the effects of SARA on ruminal LPS concentration and there also appears to be contradictions in the literature on the effect of acute acidosis on LPS concentration. For example, Andersen and Jarlov (1990) induced acute acidosis in two adult Jersey cows and measured both rumen pH and ruminal LPS concentrations. The LPS concentrations decreased with decreasing rumen pH. In another study, Andersen et al. (1994a) observed relative increases in LPS concentration after grain engorgement to induce acute acidosis only in cows that had previously been adapted to a concentrate diet. The reductions in rumen pH below 5.0 that occurred when acute acidosis was induced in the previous studies probably resulted in bacteriological alterations that differ from those experienced when SARA is induced. Additionally rumen pH was not measured and presented in a manner similar to that used in the current study, which makes it difficult to

speculate on possible reasons for the differences. More recently, Gozho et al. (2005a) showed that there was a 239% increase in ruminal LPS concentration when SARA was induced in steers without prior adaptation to concentrate diets. In the present study there was an increase of about 300% in ruminal LPS when the steers were on the 60% concentrate diet. When SARA was induced to concentrate adapted steers, there was only an increase of about 48% in ruminal LPS which was statistically significant. Osmolarity of rumen fluid is higher when acute acidosis is induced in grain adapted compared to forage adapted cattle (Andersen, 1993). It is possible that the changes in osmolarity may results in changes in the rumen gram-negative bacteria which lead to more LPS in grain adapted cattle.

The observations that the proportion of gram-negative bacteria in the rumen decreases when pH decreases although the numbers for both gram positive and gram-negative species increase has led some researchers to suggest that LPS in the rumen comes from either intact rapidly growing gram-negative bacteria, or they could also be from gram-negative bacteria death and lysis (Nagaraja et al., 1978a). It is also been suggested that as much as 60% of LPS in the rumen may be released during bacteria growth (Andersen, 2000). During rapid growth, autolytic enzymes are required for bacteria cells to expand and grow but excessive autolytic activity leads to bacteria cell lysis. Studies with *Fibrobacter succinogenes* showed that autolysis was 10 fold higher in rapidly growing cells compared to cells in the stationary phase (Wells and Russell, 1996). It is possible that rumen pH conditions for some part of the day resulted in rapid growth of some gram-negative bacteria and this contributed to increases LPS in the rumen during concentrate adaptation and SARA induction. The negative correlation between average rumen pH and LPS was

probably because changes in rumen pH affected the growth rate of some rumen bacteria so that these bacteria's contribution of LPS due to autolysis was low during periods with high rumen pH but was high when rumen pH was low (Figure 4).

Rumen fluid total coliform counts were unaffected by increasing grain during concentrate adaptation up to when the F:C ratio in the diet was 60:40 (Figure 4). Krause et al. (2003) also reported higher *E. coli* counts in the contents of rumen, ileum and upper colon of cattle after adapted to concentrate compared to those on an all forage diet. It is possible that with step-wise adaptation substrate supply and rumen environment continue to be favorable for coliforms to thrive although the change in numbers was not significantly altered. When concentrate increased beyond 40% of DM, the more acid prone coliforms were not able to proliferate. However, when SARA was induced in concentrate adapted steers, the coliform numbers were similar to those observed with the 100% forage diet probably because there was an increase in acid tolerant strains when concentrate was fed. Some strains of *E. coli* can develop tolerance if they are grown in the presence of un-dissociated VFA (Diez-Gonzalez and Russell, 1999).

Concentrations of acute phase proteins have been observed to increase when the time with pH below 5.6 increases to more than three hours per day (Gozho et al., 2005a). Increasing the proportion of WBP in the diet beyond 60% of DM activated an inflammatory response (Table 7). The high levels of concentrate depressed rumen pH and compromised animal health probably due to inflammation of the epithelial tissue of the digestive tract as well as increased LPS translocation into blood circulation due to a compromised digestive tract physical barrier. If LPS translocation is the main determinant of inflammation when SARA is induced, it is not clear how much LPS must be translocated

into bloodstream in order to increase the acute phase protein concentrations in peripheral circulation. Repeated episodes of SARA lead to rumen papillae inflammation as well as hyperkeratosis of the rumen wall which exacerbates the acidity in the rumen leading to further damage of the rumen epithelium, pathogen infiltration and abscessation of various organs in the body (Nocek, 1997; Kleen et al., 2003). This damage to rumen mucosal tissue may contribute to the observed inflammatory response in SARA -induced cattle.

Haptoglobin concentration increased (P = 0008) when the 40:60 and 20:80 F:C ratio diets were fed. Haptoglobin concentrations have been increased after experimentally induced inflammation (Conner et al., 1988), trauma (Earley and Crowe, 2002) and also after experimental infections with bovine respiratory syncytial virus (Heegaard et al., 2000) and natural diseases (Alsemgeest et al. 1994). Serum amyloid-A concentrations increased when steers were fed the 20:80 F:C ratio diet. The 20:80 F:C ratio diet was particularly successful in inducing SARA and SAA concentrations obtained with this treatment were comparable to those obtained during the latter stages when SARA was induced for 5-day periods at a time (Gozho et al., 2005a). In the present study, the SAA data suggest that relationships between the occurrences of SARA and acute phase proteins may be developed. However, this would only be possible in the absence of other acute phase stimulants such as mastitis, metritis in dairy cows, other tissue injury or viral or bacterial infections that induce an inflammatory response (Petersen et al., 2004). Acute phase proteins are released from hepatocytes upon stimulation by inflammation mediators which include interleukin-1, interleukin-6 and tumor necrosis factor (TNFα). These mediators are produced by tissue macrophages or blood monocytes at the site of injury or infection (Steel

and Whitehead, 1994). The quantification of acute phase proteins in this and a previous study indicate that SARA initiates an inflammatory response (Gozho et al., 2005a).

CONCLUSION

In this experiment, gradual stepwise adaptation to a wheat-barley concentrate resulted in a 300% increased in ruminal LPS concentration. Inducing SARA after adaptation resulted in a 48% increase in ruminal LPS. Coliform bacteria increased during stepwise adaptation up to when 40% of the diet was wheat barley concentrate. However, adding more concentrate resulted in a significant decrease in coliform numbers and subsequently inducing SARA in steers adapted to concentrate diets did not affect coliform numbers compared to the 100% forage diet. Inflammation occurred because the concentration of Hp and SAA which were used as inflammation markers increased when SARA was induced in the steers. These data show the importance of acute phase proteins in the early detection of SARA in the absence of overt signs. With wheat and barley as the main concentrate ingredients and alfalfa hay as the main forage, it appears that dietary F:C ratio greater than 40:60 result in a decrease in rumen pH that is sufficient to activate an inflammatory response. The data also suggests that the factors that determine the concentrations of ruminal LPS are still not clearly understood.

MANUSCRIPT III

Effects of Inducing Subacute Ruminal Acidosis on Ruminal Lipopolysaccharide and Inflammation in Holstein Cows.

G.N. Gozho, J.C. Plaizier and D.O. Krause

Department of Animal Science, University of Manitoba, Winnipeg, Canada R3T 2N2

Corresponding author: J. C. Plaizier; e-mail: plaizier@ms.umanitoba.ca.

ABSTRACT

The effects of grain induced subacute ruminal acidosis (SARA) in lactating dairy cows on free ruminal lipopolysaccharide and indicators of inflammation were determined. Four mid lactation dairy cows were divided into two groups with two cows each and used in a repeated switchover design. During each period SARA was induced in two animals for 5 subsequent days by replacing 25% of their total mixed ration (dry matter basis) with a concentrate made of 50% wheat and 50% barley. The other two cows acted as controls and were fed a TMR diet in which 44% of DM was concentrate. On average, inducing SARA increased the duration of rumen pH below 5.6 from 187 to 309 min/d and increases free ruminal lipopolysaccharide concentration from 22,908 EU/mL to 128,825 EU/mL. Serum lipopolysaccharide concentration in both control and SARA cows was less than the detection limit of <0.01 EU /mL for the assay. Induction of SARA elevated serum amyloid A concentrations from 286.8 to 498.8 μg/mL but did not affect haptoglobin, fibrinogen, ceruloplasmin or white blood cell profiles. These results suggest that inducing SARA in mid lactation dairy cows increases the lysis of gram-negative bacteria and activates an inflammatory response. However, this inflammatory response only increased serum amyloid A and did not affect other markers of inflammation.

Key Words: subacute ruminal acidosis, rumen pH, lipopolysaccharide, serum amyloid-A, haptoglobin, copper in serum, ceruloplasmin, fibrinogen,

Abbreviation key: **EU** = endotoxin units, **Hp** = haptoglobin, **LAL** = *Limulus* amebocyte lysate, **LPS** = lipopolysaccharide, **NSC** = nonstructural carbohydrates **SAA** = serum amyloid-A, **SARA** = subacute ruminal acidosis

INTRODUCTION

Subacute ruminal acidosis (SARA) is a common health problem in high yielding dairy cattle (Nordlund et al., 1995; Garrett et al., 1997), but data to quantify associated losses are scarce. A case-study of a 500 cow herd in New York State estimated losses due to SARA to be $1.12 per cow per day (Stone, 1999). Losses that are attributed to SARA include decreased milk yield, reduced milk fat, a general loss in efficiency of milk production and increased culling due to lameness (Nocek, 1997; Stone, 1999).

It has been suggested that translocation of free ruminal LPS into blood circulation is an important factor in diseases such as sudden death syndrome, rumenitis, ruminal acidosis, and laminitis that are related to feeding high concentrate diets (Dougherty et al., 1975; Nagaraja et al., 1978a, b). Free ruminal LPS increases as a result of rapid growth or death and lysis of rumen gram-negative bacteria (Nagaraja et al., 1978a; Andersen, 2000). Autolytic enzymes that facilitate growth can also result in bacterial lysis during the rapid bacteria growth phase (Wells and Russell, 1996). Feeding high concentrate diets may stimulate the growth of some gram-negative bacteria in the rumen and therefore increase free ruminal LPS. Abrupt induction of SARA by feeding excess grain pellets in steers on an all forage diet increased free ruminal LPS, SAA and Hp from 3,714 to 12,589 endotoxin units EU/mL, from 33.6 to 170.7 μg/mL and from 0.43 to 0.79 mg/mL, respectively (Gozho et al., 2005a). Gradual adaptations to a 60% concentrate diet over a 4 week period followed by inducing SARA increased free ruminal LPS, SAA and Hp from 6,542 to 32,275 EU/mL, from 36.5 to 131.3 μg/mL, and from 0.54 to 2.39 mg/mL, respectively (Gozho et al., 2005b). This shows that the composition of the diet prior to the induction of

SARA and the duration that this diets has been fed affect the increase in free ruminal LPS due to this induction. Lactating dairy cows receive high concentrate diets throughout most of their lactation As a result, the effects of grain induced SARA on ruminal free LPS and indicators of inflammation in mid-lactation dairy cows might differ from that in steers.

Serum amyloid A and Hp are the major inflammation markers in cattle whose concentrations have been shown to increase as a result of tissue injury (Conner et al., 1988), intravascular LPS injections (Jacobsen et al., 2004) or inflammatory diseases in cattle (Alsemgeest et al., 1994). Acute phase proteins such as fibrinogen and ceruloplasmin as well as white blood cells can also be used as markers of inflammation (Arthington et al., 1996; Horadagoda et al., 1999). Fibrinogen and ceruloplasmin show only minor increases in response to inflammation in cattle (Hirvonen, 2000). However, they can potentially be used to augment the diagnostic value of acute phase proteins to SARA under field conditions because blood samples for their analyses can be collected during routine visits by dairy practitioners.

The objectives of this study were to determine whether inducing SARA in dairy cows in mid-lactation changes free ruminal LPS, the concentration of LPS in peripheral blood, SAA, Hp, fibrinogen, serum copper concentrations and white blood cell profiles

MATERIALS AND METHODS

Animals and Diets

Four primiparous, ruminally fistulated Holstein dairy cows were housed in individual tie stalls in the Metabolism Unit of the Glenlea Dairy Research in accordance with the guidelines of the Canadian Council of Animal Care (1993). Cows averaged 538 ± 12.4 kg

of BW and 121± 8 (mean ± SD) DIM with an average milk yield of 30 ± 4.3 (mean ± SD) kg/d at the beginning of the experiment. The experiment was conducted as a switchover design with four periods of 1 wk. During each period, SARA was induced in two cows by replacing 25% of the TMR DMI measured in the previous period with an equal amount of DM from grain pellets (50% wheat:50% barley). The amount of pellets fed to each cow averaged 4.4 ± 0.4 (mean ± SD) kg/d and was offered in two meals with 67% of the daily allocation offered at 1100 h and the remaining 33% at 1400 h. The TMR was withheld between 1100 h and 1700 h. The other two cows received a TMR only (control). Treatments were switched in each subsequent week. Cows in both groups had *ad libitum* access to fresh water. The ingredients and chemical composition of the TMR and wheat barley concentrate are given in Table 8. Feed intakes were determined daily and representative feed and orts samples were taken three times per week. Samples were pooled by week and cow and DM content was determined by oven drying at 60°C for 48 h.

Ruminal Fluid pH Measurements and Analyses.

Rumen pH was measured continuously for 5 days in each period by inserting one in-dwelling pH probe into the rumen of each cow as described by Gozho et al. (2005a). Rumen pH was measured at 1-min intervals and stored for subsequent analysis. Rumen pH data were summarized by calculating average pH, time below pH 6, and time below pH 5.6 area (time x pH below pH 6 and area below pH 5.6) for each 24-h period. Time with rumen pH below 6.0 was monitored because microbial fiber digestion and nutrient digestibilities are reduced below this rumen pH (Calsamiglia et al., 2002).

TABLE 8. Ingredients and chemical composition of the total mixed diet and wheat-barley concentrate (DM basis).

Item	TMR[1]	WBP[2]
Ingredients, %		
Alfalfa silage	32.6	
Oat silage	22.4	
Energy supplement[3]	35.2	
Protein supplement[4]	8.8	
Ground wheat		50.0
Ground barley		50.0
Nutrient composition		
DM, %	63.4	89.2
CP, % of DM	18.5	15.5
NDF, % of DM	31.1	17.6
ADF, % of DM	23.3	5.9
NSC, % of DM	37.2	62.2
Ca, % of DM	1.25	0.27
P, % of DM	0.53	0.35
K, % of DM	1.72	0.56
Mg, % of DM	0.38	0.16
Na, % of DM	0.36	0.03

[1]total mixed ration
[2]wheat-barley pellets
[3]Energy supplement contained 0.13% vitamin ADE premix, 0.14% trace mineral premix, 2.6% soybean meal, 0.06% selenium, 39.1% wheat shorts, 5.0% distillers grain, 17.5% canola meal, 15.0% ground wheat, 1.7% dicalcium phosphate, 1.6% salt, 2.0% dynamate, 0.3% pellet binder, 1.0% cane molasses, 3.7% calcium carbonate, and 10.0% corn gluten meal.
[4]Protein pellets contained 42.0% dried distiller's grain, 7.0% fish meal, 22.8% canola meal, 20.0 soybean meal, 3.2% beet molasses, 0.3 % niacin (vitamin B_3), and 5.0% sodium bicarbonate.

Time with rumen pH below 5.6 was also used because this has been used as the upper pH bound for the onset of SARA (Cooper et al., 1999).

Rumen fluid samples were collected into sterile plastic tubes from the ventral sac of the rumen at 1500 h from Monday to Friday. Samples were also collected at 6-h intervals starting at 0900 h on Wednesday during each week. A portion (25 mL) of each sample of rumen fluid was processed for subsequent free ruminal LPS determination as described by Gozho et al. (2005a). The *Limulus* amebocyte lysate (LAL) assay was used to determine LPS concentration (Levin and Bang, 1964). The assay was performed using a 96-well microplate kit (Cambrex Bio Science Walkersville Inc, MD) with absorbance read at 405 nm on a microplate reader (BioRad model 3550, Hercules, CA). Detailed procedures for sample preparation and method validation have been described previously (Gozho et al., 2005a).

A second portion of rumen fluid (1.5 mL) was mixed with an equal volume of glycerin in plastic tubes and stored at -20°C until used for total coliform enumeration at a later stage. Total coliform counts were determined using modified chromogenic medium (Escherichia coli /coliform medium catalogue number CM0956, Oxoid Inc. Nepean ON, Canada) and buffered peptone water (pH 7.2) (Difco^{TM}) as a diluent for serial dilutions of rumen fluid samples before plating. The chromogenic medium was modified by replacing 20% of the distilled water with clarified rumen fluid. The clarification process for the rumen fluid included centrifuging the rumen fluid at 1500 x g for 10 minutes, followed by autoclaving the supernatant at 121°C for 15 minutes. Buffered peptone water (pH 7.2) (Difco^{TM}) was prepared according to manufacturer's instructions. Carbon dioxide gas was bubbled through buffered peptone water and the chromogenic *E. coli* /coliform medium

immediately after preparation and autoclaving in order to reduce the oxygen tension (Bryant, 1972).

The remaining portion of rumen fluid was centrifuged at 1500 x g for 10 minutes and the supernatant stored at -20°C for VFA analysis. A day before analysis rumen fluid samples were thawed at room temperature and 1 mL of a 25% meta-phosphoric acid solution was added to 5 mL of rumen fluid. The tubes containing the mixture were vortexed and placed in a -20°C freezer for 17 h after which samples were thawed and centrifuged for 10 min at 1500 x g. Approximately 2 mL of supernatant were decanted into a clean dry vial which was capped and placed into the auto-sampler device (model 8100; Varian, Walnut Creek, CA). Concentrations of VFA were determined by gas chromatography (model 3400 Star; Varian) using a 1.83-m glass column (model 2-1721; Supelco, Oakville, Ontario, Canada) (Erwin et al., 1961). The injector and detector temperatures were set at 170 and 195, with initial and final column temperatures set at 120°C and 165°C, respectively. The run time was 4 min followed by a 2 min thermal stabilization period.

Blood Sampling and Analyses

Blood was collected at 0900 h and 1500 h on Wednesday during each period into two10 mL plain, one 10 mL sodium heparin coated and one 5 mL K_3EDTA coated vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). Plasma fibrinogen was determined by refractometry (George, 2001). Copper in serum was analyzed by atomic absorption spectrophotometer (Varian AA240FS, Walnut Creek, CA). An automated hematology analyzer (Cell-Dyn 3500 system, Abbott Laboratories, Abbott Park, IL, USA)

was used for enumeration and delineation of white blood cells in whole blood. White blood cells were differentiated into neutrophils, eosinophils, lymphocytes, monocytes, basophils and bands where present. Platelet count was done using the manual method of Brecher and Cronkite (1950). These analyses were conducted at the Manitoba Veterinary Services laboratory using either serum or K_3EDTA-preserved whole blood.

The other sample in the serum tube was left to clot at room temperature, which was about 18°C, for 45 min and subsequently centrifuged at 1500 x g for 30 min. The sodium heparin-preserved blood sample was centrifuged immediately after collection at 1500 x g. The serum and plasma harvested was used in the determination of haptoglobin and serum amyloid-A, respectively, using ELISA Tridelta Phase™ range assay kits (Tridelta Diagnostics Inc, NJ, USA; catalogue numbers TP-801 and TP-802, respectively) as described by Gozho et al. (2005a).

Serum was also used to determine blood concentration of LPS using the *Limulus* amebocyte lysate (LAL) assay and the same method as in ruminal LPS analyses except that a metallo-modified polyanionic dispersants called Pyrosperse™ was added to the samples prior to analysis. Pyrosperse™ is recommended by the manufacturers (Cambrex Bio Science Walkersville Inc, MD) for use with serum to prevent endotoxin binding. Serum samples were diluted 1:5 with pyrogen-free water and the assay was also performed using a 96-well microplate kit with absorbance read at 405 nm on a microplate reader (BioRad model 3550, Hercules, CA).

Milk Yield and Composition

Cows were milked twice daily in their stalls, and milk production was determined using Tru Test regulation meters (Westfalia Surge, Mississauga, ON). Milk samples from 4 consecutive milkings 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 and milk protein content using the Milk-O-Scan 303AB (Foss Electric, Hillerød, Denmark) and for somatic cell counts using the Fossomatic 300 cell counter (Foss Electric, Hillerød, Denmark).

Statistical Analyses

Data were analyzed using SAS Mixed Procedures (SAS Institute Inc., 1996) as recommended by Wang and Goonewardene (2004) for the analysis of animal experiments with repeated measures. The effects of diet, time (which was either hour or day), and their interactions were considered fixed. Week effects were considered random. Compound symmetry was deemed the most appropriate covariance structure relative to the hourly or daily measurements that were tested. Tukey's multiple range test was used to separate means after a significant ($P \leq 0.05$) treatment or time effect.

RESULTS

Total DMI was lower in control than in SARA cows on first day of SARA induction but not on other days (Figure 6). However, control cows tended ($P = 0.14$) to consume more feed on day 5 of SARA induction and these changes led to a diet x day

interaction (P = 0.003). Dry matter intake averaged 18.0 and 18.3 kg/cow for control and SARA cows, respectively.

Somatic cell counts (SCC) were used to screen cows with subclinical mastitis, as this disease can also result in an inflammatory response. One cow had an abnormally high SCC for the first 3 weeks of the study which averaged 991,542 with a range of 16,000 to 4,087,000 cells /mL. Data on inflammation markers in blood samples from this cow were excluded from the analyses. The SCC for the other three cows averaged 98,125 cells /mL with a range of 21000 to 299,000 cells /mL. Milk yield and milk composition are given in Table 9. Milk yield, fat yield and protein yield did not differ between SARA and control cows. Milk fat content did not differ between SARA and control cows but was lower than the provincial herd average of 3.57% (WCDHIS, 2001) in both treatments.

Inducing SARA resulted in a decrease (P = 0.0008) in average daily rumen pH of 0.23 units in SARA cows compared to control cows (Table 10) and increased (P = 0.0008) the duration of time with pH below 6.0 from 459.5 min/d to 741.9 min/d and also increased (P < 0.0001) the duration of time with pH below 5.6 from 187.0 min/d to 309.4 min/d. The area (time x pH) below pH 6.0 increased (P < 0.0001) from 145.7 to 278.7 and the area (time x pH) below pH 5.6 increased (P = 0.001) from 28.4 to 67.0 when SARA was induced (Table 10). Total VFA concentrations in the rumen were not affected by treatment and were 137.2 and 129.7 m*M* for SARA and control cows, respectively. Ruminal propionic acid concentration increased (P < 0.0001) from 26.1 m*M* in control cows to 39.8 m*M* in SARA cows which also led to a lower (P < 0.0001) Ac/Pr ratio for SARA cows (Table 11). Average across periods and treatments, highest propionic and butyric acid concentrations were measured at 2100 h.

Averaged across sampling times, inducing SARA increased ($P < 0.0001$) free ruminal LPS 22,908 to 128,825 EU/mL (Table 12). There was a diurnal variation in free ruminal LPS and averaged across treatments, lowest concentrations were measured in samples collected at 1500 h. For the entire period of study free ruminal LPS concentrations were relatively higher during the second SARA challenge compared to the first (Figure 7). In order to determine the day to day variation, free ruminal LPS was measured in samples collected at 1500 h everyday. The difference in free ruminal LPS between SARA and control cows progressively increased up to the fourth day of treatment (Figure 8). Serum LPS concentration was below the detection limit of 0.1 EU /mL for the LAL assay.

Rectal temperature did not differ between treatments and were 38.4°C and 38.5 °C for control and SARA cows, respectively. Averaged across period and treatment, lowest ($P = 0.004$) rectal temperature within the 24 hour period were recorded at 1500 h. Haptoglobin, fibrinogen and platelet count did not differ between treatments and respective concentrations for Hp, Fibrinogen and platelet count were 0.24 mg/mL and 0.27 mg/mL, 4.5 mg/mL and 5.0 mg/mL and 476.0 x 10^9/L and 466.2 x 10^9/L, for control and SARA cows, respectively. The proportions of white blood cells did not differ between treatments and were within normal range for cows as defined by *The Merck Veterinary Manual.* Serum copper concentrations were not affected by treatments but inducing SARA tended ($P = 0.17$) to depress concentration by 0.06 mg/mL. This was probably due to differences in dietary copper levels in the two diets. Replacing TMR with WBP reduced dietary copper by 5%. Serum amyloid A increased ($P = 0.03$) from 286.8 μg/mL to 498.8 μg/mL when SARA was induced.

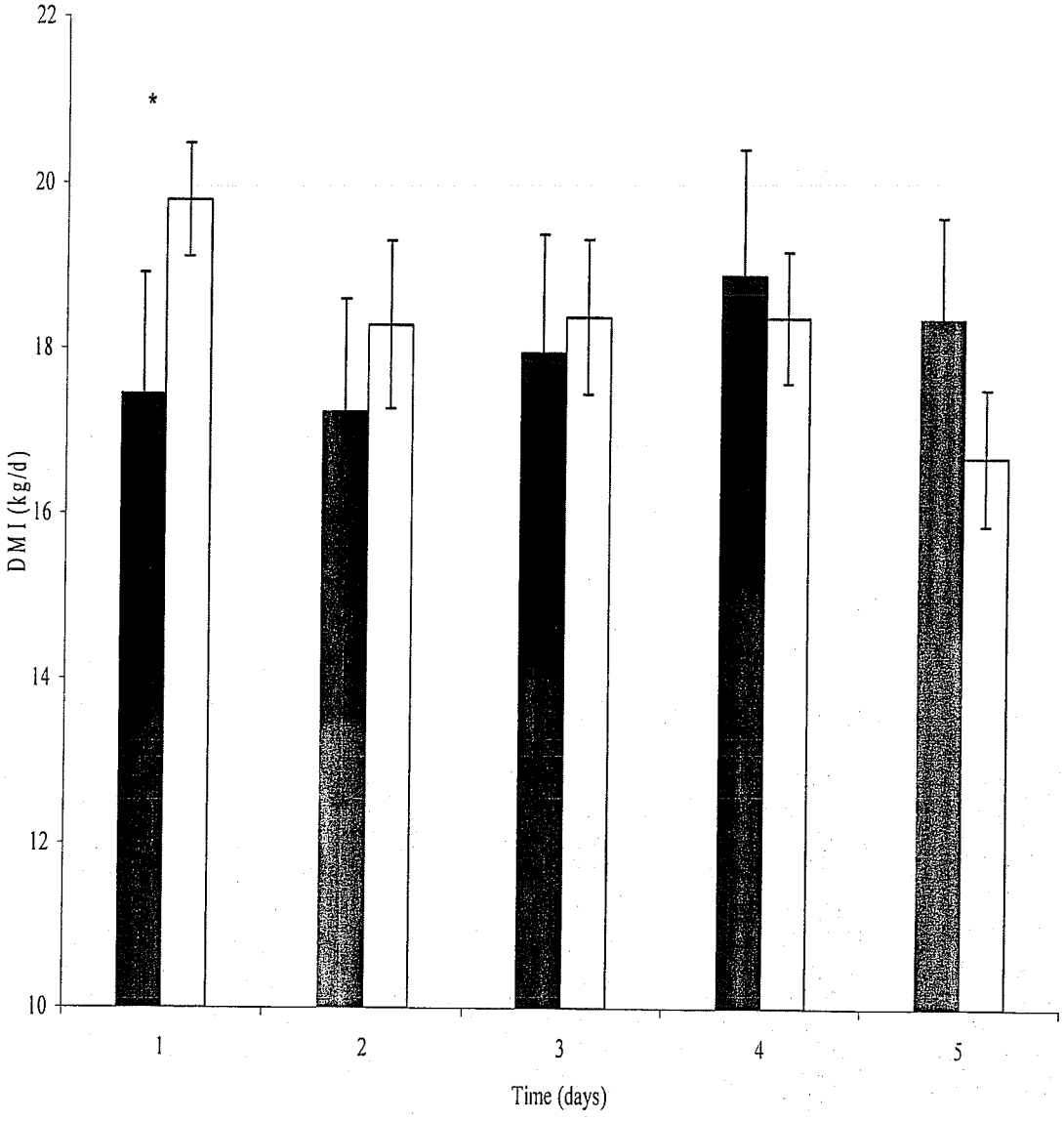

FIGURE 6. Total dry matter intake (kg / d) for dairy cows fed a TMR only (control) (■) or TMR and wheat-barley pellets to induce SARA (□) in four animals averaged across four periods. Each bar represents mean ± SEM total dry matter intake (n = 8). * = $P < 0.05$, Control vs. SARA;
Diet x day interaction was significant (P = 0.03)

TABLE 9. Dry matter intake, milk yield and composition of cows fed the control or SARA diet[1].

Item[2]	Diet		SEM	P value		
	Control	SARA		Diet	Day	Diet x Day
TMR dry matter intake (kg/d)	18.0[a]	14.0[b]	1.66	<0.0001	0.12	0.003
Total dry matter intake (kg/d)	18.0	18.3	1.62	0.28	0.13	0.003
Milk yield (kg/d)	27.5	28.6	1.83	0.27	0.81	0.69
Milk components						
Milk fat (%)	2.49	2.21	0.23	0.41	0.69	0.03
Fat yield (kg/d)	0.68	0.63	0.04	0.43	0.94	0.91
Milk protein (%)	3.60	3.39	0.14	0.27	0.69	0.02
Milk protein (kg/d)	1.01	0.98	0.08	0.77	0.86	0.76

[a,b]Means within the same row followed by different superscript letters differ significantly ($P \leq 0.05$).

[1]Diets were a TMR only (control) or TMR $\pm$ 25% wheat barley concentrate.

[2]Dry matter intakes were calculated for two animals on each treatment averaged across periods (n = 40) and for four animals averaged across periods and treatments for day effect (n = 16); milk yield and composition were from samples collected on two days during each week (n = 12) for treatment and for day effects (n = 4) and analyzed by analysis of variance.

Averaged across period and cows within treatment, coliform counts did not differ between treatments and were 6.77 $\log_{10}$ cfu/ mL and 6.72 $\log_{10}$ cfu/ mL for control and SARA cows, respectively. However, coliform numbers were lower ($P = 0.05$) in SARA cows than in control cows for samples collected at 2100 h (Figure 9).

TABLE 10. Rumen pH variables of cows fed the control or SARA diet[1]

Item[2]	Diet		SEM	P value		
	Control	SARA		Diet	Day	Day x Diet
Average pH	6.24[a]	6.01[b]	0.065	0.0008	0.75	0.69
Time < pH 5.6, min/d	187.0[b]	309.4[a]	32.1	<0.0001	0.59	0.99
Time < pH 6.0, min/d	459.5[b]	741.9[a]	61.5	0.0008	0.74	0.87
Area < pH 5.6, min x pH/d	28.4[b]	67.0[a]	9.7	0.001	0.12	0.95
Area < pH 6.0, min x pH/d	145.7[b]	278.7[a]	24.8	<0.0001	0.48	0.98

[a,b]Means within the same row followed by different superscript letters differ significantly ($P \leq 0.05$).

[1]Diets were a TMR only (control) or TMR ± 25% wheat barley concentrate.

[2]Time and area with pH below each rumen pH threshold was calculated for five days and in two animal on the same treatment averaged across periods (n = 40) and for four animals averaged across periods and treatments for day effect (n = 16) and analyzed by analysis of variance.

TABLE 11. Volatile fatty acid concentration (m*M*) of rumen fluid of dairy cows fed the control and SARA diets[1]

Variable[2]		Acetate	Propionate	Butyrate	Other[3]	Total	Ac/Pr ratio[4]
Time of day	0900 h	84.5	27.3^{b}	8.5^{c}	4.0^{a}	124.4	3.3^{a}
	1500 h	84.5	34.8^{a}	11.0^{ab}	3.5^{ab}	133.8	2.7^{b}
	2100 h	91.2	35.8^{a}	12.6^{a}	3.3^{b}	142.9	2.7^{b}
	0300 h	85.5	33.9^{a}	$9.8b^{c}$	3.5^{ab}	132.7	2.7^{b}
	SEM	6.76	3.53	1.12	0.36	10.8	0.18
Diet[5]	Control	88.9	26.1^{b}	10.9	4.0^{a}	129.7	3.5^{a}
	SARA	84.0	39.8^{a}	10.1	3.2^{b}	137.2	2.2^{b}
	SEM	6.22	3.30	1.11	0.31	9.84	0.20

[a,b,c]Means for variables in same part of a column followed by a different superscript letters differ significantly ($P \leq 0.05$).
[1]Diets were a TMR only (control) or TMR ± 25% wheat barley concentrate.
[2]Rumen fluid VFA concentration was averaged for two animal across periods (n = 16) for each sampling time and across sampling times and periods (n=32) for each treatment and analyzed by analysis of variance.
Other acids = isobutyric acid + isovaleric acid + valeric acid
[4]acetate: propionate ratio
[5]Dietary means were calculated across sampling times.

TABLE 12. Ruminal LPS, rectal temperature, serum amyloid A, haptoglobin and other blood chemistry variables in dairy cows fed the control or SARA diet.[1]

Item[2]	Diet		SEM	P value		
	Control	SARA		Diet	Hour	Diet x Hour
Ruminal LPS (Log_{10}Eu/mL)	4.36[b]	5.17[a]	0.116	<0.0001	0.20	0.99
Rectal temperature (°C)	38.4	38.5	0.19	0.14	0.005	0.20
Serum amyloid A (μg/mL)	286.8[b]	498.8[a]	85.6	0.03	0.61	0.68
Haptoglobin (mg/mL)	0.24	0.27	0.03	0.59	0.17	0.17
Fibrinogen (mg/mL)	4.5	5.0	0.29	0.24	0.99	0.40
Serum copper (μg/mL)	1.0	0.9	0.04	0.17	0.28	0.41
White blood cells (10^3 cells /mL)	5.7	5.2	0.24	0.20	0.12	0.09
Differential WBC count (% WBC)						
Neutrophils (%)	43.5	44.8	2.89	0.60	0.41	0.85
Eosinophils (%)	3.8	2.3	0.26	0.001	0.60	0.19
Lymphocytes (%)	46.2	45.5	2.61	0.57	0.65	0.91
Monocytes (%)	6.5	6.1	1.01	0.68	0.46	0.93
Basophils (%)	0.8	0.9	0.23	0.53	0.61	0.09
Bands[3] (%)	0.3	0.3	0.22	0.91	0.59	0.69
Thrombocytes	476.0	466.2	68.8	0.92	0.51	0.21

[a,b]Means within the same row followed by different superscript letters differ significantly ($P \leq 0.05$).

[1]Diets were a TMR only (control) or TMR ± 25% wheat barley concentrate.

[2]Ruminal LPS concentration and rectal temperature were calculated for the animals on each treatment averaged across periods and times of sampling (n = 32) and for time intervals (n = 16); blood chemistry variables (n = 16) for treatment effects and (n = 32) for time intervals and were analyzed by analysis of variance.

[3]Bands = immature white blood cells.

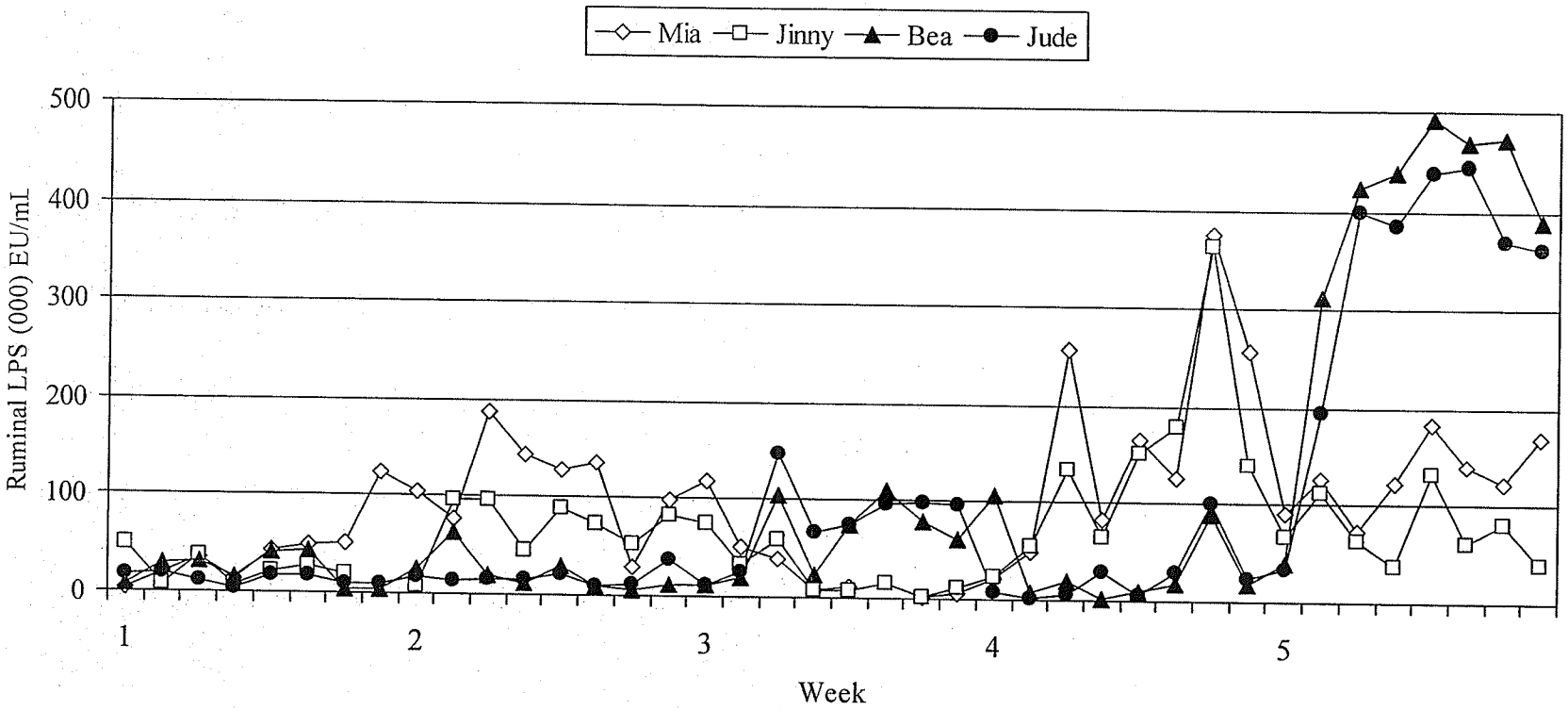

FIGURE 7. Ruminal LPS concentrations in cows during times when they were fed TMR only (control) or TMR and 4.4 ± 0.4 kg of wheat barley concentrate to induce SARA[1]

[1]Treatments were: Week 1 = all cows were fed TMR only, Week 2: Mia and Jinny = SARA and Bea + Jude = control, Week 3: Mia and Jinny = control and Bea + Jude = SARA, Week 4: Mia and Jinny = SARA and Bea + Jude = control, Week 5: Mia and Jinny = control and Bea + Jude = SARA.

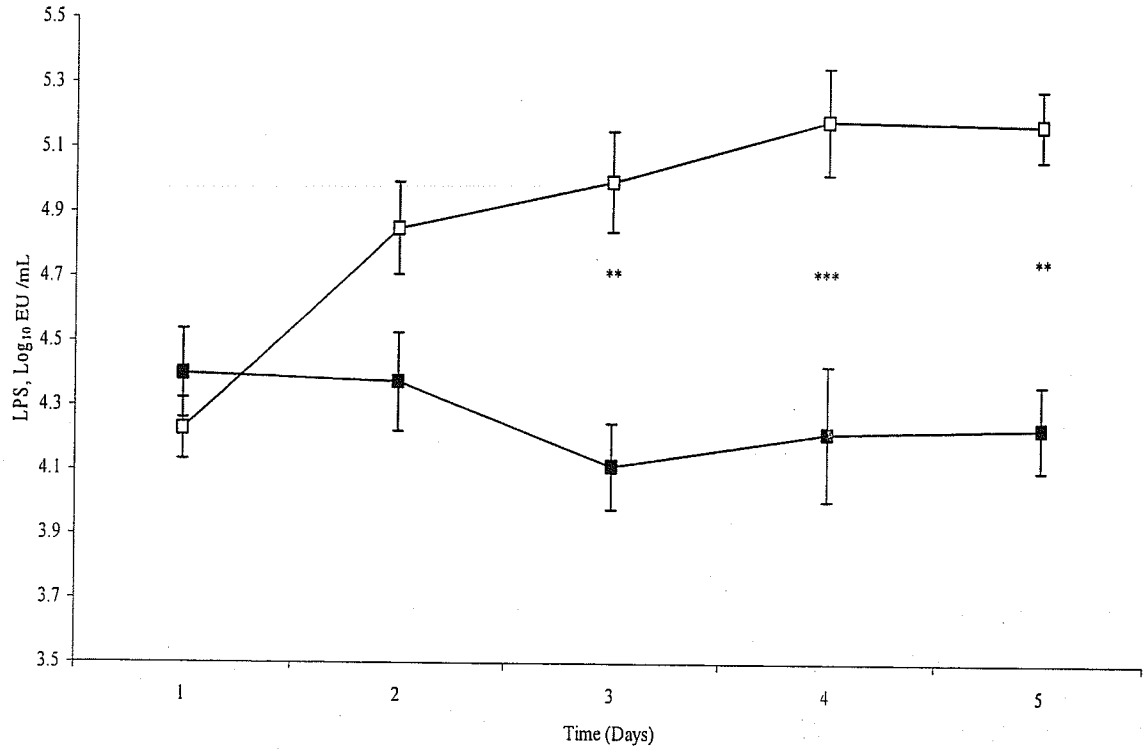

FIGURE 8. Ruminal LPS in dairy cows fed a TMR only (control) (●) or TMR and wheat-barley pellets to induce SARA (○) in two cows averaged across four periods. Each bar represents mean ± SEM LPS concentration in $\log_{10}$ Eu / mL (n = 8). ** = $P < 0.01$, Control vs. SARA; *** = $P < 0.001$, Control vs. SARA in samples collected at 1500 h every day.

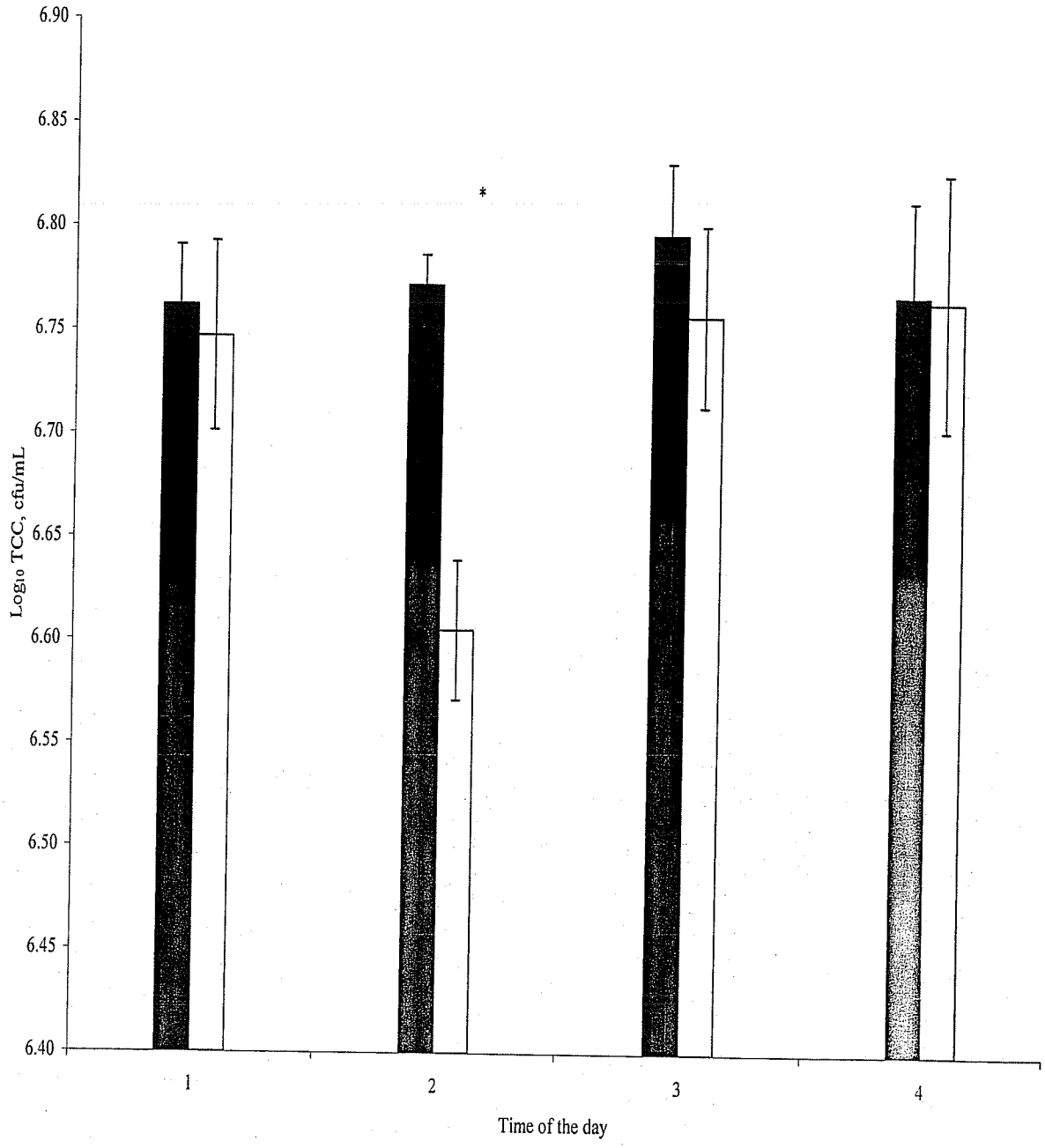

FIGURE 9. Total coliform counts (log_{10} cfu / mL) of cows fed a TMR only (control) (■) or TMR and wheat-barley pellets to induce SARA (□) enumerated in rumen fluid at 6 h intervals over 2 days and averaged across four periods. Each bar represents mean ± SEM Total coliform counts in log_{10} cfu /mL (n = 16). * = $P < 0.05$, Control vs. SARA.

DISCUSSION

Wheat-barley pellets were used in a model to induce SARA to simulate consumption of excess of nonstructural carbohydrates. In this study, inducing SARA by replacing 25% of the TMR with wheat barley pellets did not depress feed intake. Previous studies in steers have shown that daily episodes with rumen pH below 5.6 for at least 3 hours per day activate an inflammatory response (Gozho et al., 2005a). Cows in both treatments experienced SARA because they had more than 3 hours with pH below 5.6, but SARA increased this rumen pH depression. Beauchemin (1991) recommended that barley grain based diets must contain at least 34% DM of NDF to prevent SARA. Although the TMR was formulated to meet this recommendation, the analysis of the diets revealed NDF content was 31.1% DM. Hence, the low pH in the cows receiving the control diet might have been due to the low NDF content of the diet. Replacing TMR with pellets increased the concentrate content of the diet to 67% further reducing rumen buffering and probably reduced saliva flow (Maekwa et al., 2002b) leading to longer periods of time with pH below 5.6 in SARA cows. Total VFA concentrations in our experiment were similar to those observed with dairy steers with SARA (Gozho et al. 2005b).

Milk fat content depression is commonly associated with SARA (Nocek, 1997). Milk composition did not differ between SARA and controls in the current study. This might have been due to the low pH in the cows receiving the control diet. Reports in the literature indicate that milk fat depression does not always accompany SARA induction. For example, Cottee et al. (2004) and Keunen et al. (2002) did not observe that SARA results in milk fat depression but Krause et al. (2002) found a positive correlation between mean

rumen pH and milk fat content and a negative correlation between time with pH below 5.8 and milk fat content. 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). Reduction in rumen pH results in the production of trans-fatty acids, e.g. trans-10 C18:1 that limits milk fat synthesis and leads to milk fat depression (Griinari et al., 1998).

Free ruminal LPS was higher in the cows on a 54% forage diet than in steers on an all forage diet in previous studies (Gozho et al., 2005a, b). Inducing SARA in steers adapted to a hay diet or after gradual adaptation to a 60% concentrate diet increased ruminal LPS by 8874 and 8718 EU/mL, respectively (Gozho et al., 2005a, b). In the present study free ruminal LPS concentration was already higher than that of control animals in both these studies. Increases of over 400% in ruminal LPS when SARA was induced in the cows may also highlight the effects of basal diet, the duration the animals are on a diet before SARA is induced and level of dry matter intake on microbial populations in the rumen. Rapid growth of bacteria is associated with bacterial lysis due to excessive activity of autolytic enzymes during cell growth and division in the rapid growth phase (Wells and Russell, 1996). It has been suggested that as much as 60% of ruminal LPS is produced by rapidly growing gram-negative bacteria (Andersen, 2000). Introducing large proportions of nonstructural carbohydrates in the diet stimulates the growth of some ruminal bacteria. In the present study the basal diet already contained 37.2% of nonstructural carbohydrates which led to long periods when bacteria could grow rapidly and shed LPS into rumen fluid. Since the TMR and wheat barley pellets were offered at different times, this ensured that nonstructural carbohydrate availability for rumen

microbial growth was staggered and was available for longer times in SARA cows than controls. The total nonstructural carbohydrate content from TMR and pellets in the SARA diet was 43.5%. The higher ruminal LPS concentration observed during the second SARA challenge may be because the first week of SARA resulted in a rumen bacteria population shift towards those gram-negative bacterial species that grow rapidly at rumen pH lower than 5.6. Thus during the second SARA challenge there may have been larger numbers of these gram-negative bacteria present than at the beginning of the first SARA challenge. Within a 24-h period ruminal LPS were lowest when the lowest total coliform counts were recorded which may imply a slower growth rate in gram-negative bacteria during this time compared to all other sampling times.

SARA can result in damage to rumen epithelium which leads to pathogen infiltration and subsequently to abscessation of various organs in the body (Nordlund et al., 1995). The presence of pathogens and LPS in blood circulation sets off inflammatory responses (Andersen, 2000). In the present study, LPS was not detected in peripheral blood circulation. Dougherty et al. (1975) and Aiumlamai et al. (1992) reported the presence of LPS but Andersen and Jarlov (1990) and Andersen et al. (1994b) did not detect LPS in peripheral blood circulation when acute acidosis was induced. Free ruminal LPS that is translocated into hepatic portal circulation can be detoxified by the liver before it reaches the peripheral blood circulation (Andersen, 2000). However, the majority of cytokine receptors are found in the Kupffer cells in the liver (Bode and Heinrich, 2001) and therefore the first wave of proinflammatory cytokines may be initiated prior to detoxification.

Rectal temperature was recorded to determine the occurrence of fever. A febrile response does not appear to be a consistent clinical sign in ruminal acidosis related endotoxaemia (Andersen, 2000). Copper concentration was measured in serum because it is an integral component of ceruloplasmin and ceruloplasmin activity correlate closely with serum or plasma copper concentrations (Blakley and Hamilton, 1985). Therefore serum copper was used as an indirect measure of ceruloplasmin concentrations in control and SARA cows. Ceruloplasmin concentrations started to raise 3 days and peaked 6 days after turpentine injections to induce local inflammation in calves (Conner et al., 1988) which shows a delay in response after inflammation. The fibrinogen concentrations in our study are similar to concentrations obtained after experimentally induced infections with bovine herpes virus-1 (Arthington et al., 1996). This may suggest that both control and SARA cows responded to SARA induction with an inflammation response. Additionally blood samples were collected 48 h after the beginning of each period and this may have been too early for proteins such as ceruloplasmin and fibrinogen whose concentrations have been shown to peak 3 to 6 days after inducing inflammation (Conner et al., 1988).

In the present study, SAA in control cows was higher than that in steers with grain-induced SARA (Gozho et al., 2005a, b). This agrees with the rumen pH data that show that both control and SARA cows had a rumen pH below 5.6 for more that 3 hr/d. and the conclusion from Conner et al. (1988) and Alsemgeest et al. (1995) that healthy animals have very low levels of SAA. In previous studies Hp concentrations increased when SARA was induced in hay- and concentrate-adapted steers, respectively (Gozho et al., 2005a, b). Hp concentrations greater than 0.2 mg/mL suggest early or mild infection in cattle (Skinner et al., 1991). In the present study Hp concentrations were 0.24 and 2.70 mg/mL for control

and SARA cows and were, therefore, above the threshold suggested by Skinner et al. (1991). Hence, the data obtained in the present study suggests that inflammation occurred in both control and SARA cows. This agrees with rumen pH data that shows that SARA was induced in both the control and SARA cows. The disparity in the concentrations of Hp and SAA cows with SARA could be due to a difference in the cytokines involved in initiating the synthesis of these acute phase proteins (Jacobsen et al., 2004). SAA synthesis can be induced by either interleukin-6 or tumor necrosis factor-α but both these cytokines are required for haptoglobin to be synthesized (Alsemgeest et al., 1996). Therefore the combination of cytokines that is required for SAA synthesis may be different from the combination that activates haptoglobin synthesis (Jacobsen et al., 2004). The cows in the current experiment had received a high concentrate diet for at least 17 wk prior to the study. As a result, cows might have suffered from chronic SARA, whereas the steers in our earlier studies (Gozho et al., 2005 a, b) experienced acute SARA. The reasons the combinations of cytokines to activate Hp synthesis were not synthesized in dairy cows with chronic inflammation in this study are presently not known. SAA is also considered to be the more sensitive and to respond faster to inflammation stimuli because it is detected earlier in blood than haptoglobin (Horadagoda et al., 1994; Alsemgeest et al., 1994).

Further research is required to determine cytokine and acute phase protein responses as a result of acute or chronic SARA. This is because chronic inflammation most likely results from SARA that occurs in dairy cows compared to acute inflammation that results from experimentally induced SARA.

CONCLUSION

Grain induced SARA increased free ruminal LPS concentration in mid lactation in dairy cows, but did not result in detectable LPS in peripheral blood. Induction of SARA increased the duration with pH below 5.6 from 187.0 to 309.4 min/d. Milk yield and milk composition did not differ between control and SARA cows, but milk fat content was depressed in all cows. Inducing SARA increased SAA from 286.88 to 498.8 μg/mL. The concentrations of SAA are consistent with an inflammatory response in both control and SARA cows and also show that concentrations of SAA increase in proportion to the duration with pH below 5.6. Haptoglobin concentrations did not differ between treatments, but were higher than baseline values for all cows Serum copper concentration, fibrinogen and white blood cell profiles were also not affected by SARA. The absence of an effect of SARA on markers of inflammation, with the exception of SAA and Hp could be due to the chronic nature of SARA in lactating dairy cows.

GENERAL DISCUSSION

The primary aim of the study was to determine the ruminal LPS concentration and immune response when SARA was induced to cattle that had been on different feeding regimes that allowed varying degrees of adaptation to high grain diets. SARA was induced by feeding ground wheat and barley that was made into pellets and fed in combination with chopped grass hay in experiments 1 and 2 and or with a total mixed ration in experiment 3. The wheat barley pellets increased the dietary nonstructural carbohydrate, and chopping reduced feed particle size, therefore rumen pH depression resulted from increased ruminal VFA and low rumen buffering due to reduced saliva production from chewing during eating and rumination in experiments 1 and 2. Even though ruminal VFA concentrations were not measured in experiment 1, same combinations of wheat barley pellets and chopped grass hay were used in experiment 1 and 2. In experiment 3, a combination of high nonstructural carbohydrate and low dietary NDF depressed rumen pH because dietary NDF was lower than recommended for barley based diets (Beauchemin, 1991) (Manuscript 3). Wheat-barley pellets were fed separately in experiment 1 and 3 (Manuscript 1 and 3) or was mixed to form part of the diet in experiment 2 (Manuscript 2). Feeding the pellets alone increased the concentration of rumen readily fermentable carbohydrates in the diet which increased VFA production from rumen bacteria fermentation. This subsequently depressed rumen pH. Since the chopped grass hay or TMR was not fed at the same time with the pellets, rumen buffering from saliva flow was very low at the time of feeding wheat barley pellets. By using wheat-barley pellets to induce SARA in all experiments, the causes of rumen pH depression were similar across experiments. Steers were used in

experiment 1 and 2 because these studies involved feeding high forage diets which do not contain sufficient nutrients to meet the nutrient requirements for high yielding dairy cows.

In experiment 1, steers had been adapted to an all forage diet before SARA was induced abruptly (Manuscript 1) and therefore this represents a sudden introduction of grain in the diet without adaptation. In experiment 2, the steers were adapted to a 60% concentrate diet using three 20% incremental steps over a 3 wk period (Manuscript 2). However, prior to this study, the steers were also fed an all forage diet. This study represented a short adaptation period before SARA was induced. In experiment 3, mid-lactation dairy cows that had been on a 44% concentrate diet for 17 wk were induced to SARA. This experiment also represented situations in which cattle have been adapted to high grain diets and have been on these high diets for a long period of time before SARA is induced.

Abrupt induction of SARA in cattle on an all forage diet increased free ruminal LPS in the rumen from 3,714 to 12,589 EU/mL (Manuscript 1). Gradually increasing the concentrate inclusion in the diets of steers from 0% to 80% of dry matter increased free ruminal LPS from 6310 to 26,915 EU/mL (Manuscript 2). Inducing SARA feeding long periods of feeding a high grain diet increased free ruminal LPS from 22,908 to 128,825 EU/mL. A second induction of SARA after a 1 week period during which cows were fed the high grain diet increased free ruminal LPS up to 145,383 EU/mL. Despite of these large increases of free LPS in rumen fluid, no LPS was detected in the serum of peripheral blood (Manuscript 3).

These results highlight that gradual adaptation and durations of feeding high grain diets before SARA is induced has a significant effect on the free ruminal LPS response.

The model used to induce SARA in the studies described in this thesis could sustain the cattle in a subacute ruminal acidotic condition for five days. During the same period free ruminal LPS was maintained at levels that were higher than for control cows for as long as SARA was induced. This suggests that the larger percentage of free ruminal LPS under these conditions resulted from autolytic activity during the rapid growth phase of gram-negative bacteria. It has been suggested that as much as 60% of free ruminal LPS is shed as a result of rapid bacterial growth (Andersen, 2000). Rapidly growing gram-negative bacteria shed LPS into the rumen because the enzymes involved in the processes that facilitate cell expansion and division can also cause bacterial cell lysis (Wells and Russell, 1996). Studies with *Fibrobacter succinogenes* showed an association between rapid growth phase and high autolytic activity of these enzymes (Wells and Russell, 1996). Maintaining the rumen pH below 5.6 consistently increased free ruminal LPS which shows that some gram-negative bacteria species were able to proliferate within this pH range. In contrast, free ruminal LPS decreased when acute acidosis was induced in cattle by grain engorgement (Andersen and Jarlov, 1990) which illustrates the importance of rumen pH in maintaining the gram-negative bacteria populations. Therefore, a combination of a high growth rate and lysis of some of the bacterial cells ensures that species are maintained in the rumen to act as a continual source of LPS.

Low LPS is associated with high forage diets as shown by lower concentrations of free LPS in rumen fluid of cattle fed all forage diets in experiment 1 (Manuscript 1) and in experiment 2 (Manuscript 2). It has been argued that grain engorgement increases the proportion of gram-positive bacteria and decreases that of gram-negative bacteria (Nagaraja et al., 1978a). However, the important factors that may govern free ruminal LPS

may not be the size of the gram-negative population in relation to the rumen microbial pool, but their ability to grow rapidly and avoid washout from the rumen so that they continually contribute to free ruminal LPS. Observations that free ruminal LPS was higher in experiment 2 (Manuscript 2) and in experiment 3 (Manuscript 3) show the effect of grain on free ruminal LPS. In experiment 3, free ruminal LPS concentration during the second week of SARA induction were higher relative to the first SARA inductions which may imply that the first SARA induction acted as a primer in the cows.

Total coliform counts increased only during gradual concentrate adaptation Manuscript 2) but did not show trends that were similar to changes in free ruminal LPS when SARA was induced in experiment 2 and experiment 3. Coliform counts may therefore not be representative of changes in other gram-negative bacteria that are involved in the production of free ruminal LPS. Therefore until more information on the species that contribute the most free ruminal LPS are known, it is difficult to relate free ruminal LPS to any one group of gram-negative bacteria. In experiment 3, total coliform counts decreases coincided with the lowest free ruminal LPS and this may illustrate that at lower rumen pH, the production of free LPS decreases probably because the growth of gram-negative bacteria slows down. The low concentration of LPS was measured 5 h after the first meal of wheat barley pellets and therefore may have coincided with the rumen pH nadir (Duffield et al., 2004).

Serum amyloid A and Hp are major acute phase proteins that have been used as inflammation markers in cattle. Inducing SARA abruptly increased SAA and Hp in serum of peripheral blood from 33.6 to 170.7 μg/mL and from 0.43 to 0.79 mg/mL, respectively (Manuscript 1). Inducing SARA after gradual adaptation to high concentrate diets for 3 wk

increased SAA and Hp from 38 to 163 μg/mL and from 0.53 to 1.40 mg/mL, respectively (Manuscript 2). Inducing SARA after feeding a high grain diet for more than 17 wk increased SAA from 286.8 to 498.8 μg/mL but did not affect Hp concentration (Manuscript 3). The reasons for a lack of response in Hp when SARA was induced after a long period of feeding high concentrate in cattle are not clear. Acute inflammation resulted from abrupt induction of SARA in cattle that had been on an all forage diet and after a short adaptation period to grain. Chronic inflammation may have occurred in all cattle used in experiment 3. This is because the TMR contained insufficient NDF as revealed by chemical analyses of the diet after the experiment. This may have resulted from variations in chemical composition of the feeds used in formulating the TMR suggesting that all cattle used in the experiment may have experienced bouts of SARA even before the beginning of the SARA induction experiment. Additionally, daily episodes of rumen pH below 5.6 in control cows of more than 3 h also showed that both treatments were experiencing SARA in experiment 3. Chronic inflammation therefore resulted from SARA bouts in experiment 3 whilst acute inflammation occurred in experiments 1 and 2.

According to observations with bacterial, viral infections and LPS infusions into blood circulation, the acute phase response in the studies reported in this thesis were moderate. Inflammation in cattle with SARA can potentially arise from damage of the rumen wall due to low pH or a combination of this damage and free ruminal LPS translocation into blood circulation. The term translocation is used to describe the movement of free ruminal LPS from the gastrointestinal tract to other organs or the bloodstream as defined by Andersen (2000). The present studies were not designed to investigate how the inflammation is initiated when SARA is induced. However,

inflammation could occur from a combination of free ruminal LPS translocation into pre-hepatic circulation and tissue damage that varies from formation of micro-lesions to local spots superficial lesions to extensively damaged areas and micro abscesses on the rumen mucosa.

LPS was not detected in peripheral blood in experiment 3 but this does not rule out translocation of free ruminal LPS into pre-hepatic blood supply. This is because the LPS is detoxified in the liver hence peripheral blood may not show detectable concentrations (Andersen, 2000). The liver is recognized to have a huge capacity to detoxify LPS and peripheral detection of LPS occurs only when there is liver malfunction as can occur when there is lipidosis. The ability of the liver to recognized and detoxify LPS and the fact that the majority of cytokine receptors are found in the Kupffer cells in the liver (Bode and Heinrich, 2001) ensures elimination of LPS to be linked to activation of the synthesis cytokines that are the primary signals in an acute phase response. In all the experiments SAA concentrations consistently increased when SARA was induced. Hp responses were low particularly in experiment 3 where Hp concentrations were similar in control and SARA treatments. The control animals in experiment 3 spend as much as three hours with pH below 5.6 therefore SARA was induced in both treatments. Hp concentrations have been found to be very low or undetectable in healthy cattle in the absence of inflammation stimuli. Concentrations in controls in all studies in this thesis were greater than 0.2 mg/mL which is a threshold for mild inflammation (Skinner et al., 1991). There is no evidence that acute phase protein baseline concentrations are affected by gender and therefore the use of steers or cows in some experiments cannot explain the low Hp concentrations in both

treatments in experiment 3. The relative changes in the concentrations of the acute phase proteins are informative in determining the activation of an inflammation response.

In experiment 3, the lack of Hp response when SARA was induced compared to SAA may be related to differences in the cytokines involved in initiating the synthesis of these proteins. This is because SAA synthesis can be induced by either interleukin-6 or tumor necrosis factor-α but both these cytokines are required for haptoglobin to be synthesized (Alsemgeest et al., 1996). It may be that the combination of cytokines that is required for Hp was not achieved in the study with cows.

The rumen pH range that defines SARA has been set arbitrarily with pH upper bounds that range from 6.0 to 5.5 being used in the literature. Increasing the time with rumen pH below 5.6 from 0 to 187 min /d in experiment 1, from 121 to 219 min /d in experiment 2 and from 187 to 309 min /d in experiment 3, increased SAA by 400%, 329% and 74%, respectively. In all these experiments, when the duration with pH below 5.6 was 3 h or greater, an inflammatory response occurred. In experiment 3, even the control animals were experiencing SARA and SAA concentrations in these animals were comparable to the SARA treatment in experiments 1 and 2. Haptoglobin concentrations increased by 84, 164 and less than 10% in experiment 1, 2 and 3, respectively. Across experiments, there was no correlation between Hp and the duration with rumen pH below 5.6. Although Hp is recognized as a major acute phase protein, it has been shown that Hp serum concentrations do not always correlated well with future clinical cases of respiratory infections under feedlot conditions (Young et al., 1996). These studies also show that Hp is a poor predictor of inflammation caused by a subclinical stimulus like SARA.

Our research findings show free ruminal LPS increases when SARA is induced and gradually adapting cattle to high grain diets results in even higher concentrations when SARA is induced. Inducing SARA in cattle that are already adapted to high grain diets increased the free ruminal LPS concentrations by the greatest margin. This shows that ruminal gram-negative bacteria species that are responsible for shedding LPS in the rumen may actually thrive within the rumen pH that defines SARA. Our studies also show that the inflammation response from SARA depends on the duration with pH below 5.6. Daily episodes with pH below 5.6 of 3 h or more per day activate an inflammatory response. Serum amyloid A is sensitive and response faster to SARA-related inflammation than Hp and may offer a diagnostic method for detection of SARA in dairy herds. Coliform counts cannot be used to show trends in gram-negative bacteria changes in the rumen during SARA.

CONCLUSIONS

On the basis of the results obtained in this thesis it is concluded:

1. Inducing SARA increases free ruminal LPS concentrations in the rumen in cattle. The concentration of free ruminal LPS is dependent on diet and the duration these diet are fed prior to inducing SARA. Cattle on forage diets have lower free ruminal LPS compared to those on concentrate based diets. Feeding concentrate diets increases free ruminal LPS prior to inducing SARA but does not diminish the increase in LPS when SARA is induced.
2. An inflammatory response is activated when SARA is induced in cattle. However, haptoglobin may not be a very sensitive indicator of the inflammatory response that results from SARA. Serum amyloid-A showed consistent elevation of concentrations when cattle spent three or more hours with rumen pH below 5.6. The immune response as determined by SAA was similar in studies with both steers and cow in spite of differences in free ruminal LPS concentrations.
3. Concentrate inclusion rates higher than 60% may be detrimental to animal health because these high levels results in the activation of an acute phase response.
4. Gram-negative rumen bacteria other than coliforms are involved in the production of free ruminal LPS within the pH range between 5.2 and 5.6. Coliform numbers cannot be used as a representative group for rumen gram-negative bacteria that can show trends consistent with changes in ruminal LPS concentrations.

5. SARA must be defined in terms of the rumen pH and the duration spent with pH in below the threshold because daily episodes less that 3 h with pH below 5.6 in our studies did not constitute the occurrence of a subclinical disease condition in cattle.

These studies show that adapting the rumen environment to high grain diets provides greater potential for the production or release of free ruminal LPS. Subjecting cattle to a rumen pH below 5.6 for more than 3 hours through grain overload is accompanied by an inflammatory response. The origins of systemic inflammation are not presently known but possible sources include tissue damage on the rumen mucosa or other parts of the gastrointestinal tract, LPS translocation into portal circulation, or a combination of both these factors. Therefore there is a need for further research to determine the following:

a) To identify the inflammatory stimulus in the gastrointestinal tract that is responsible for the acute phase response. This can be done through experimentally inducing SARA repeatedly in one group of cows and feeding the second group to attain normal rumen pH before animals from both groups are slaughtered. Cows will be subjected to postmortem examination and histological examination of gut-associated lymphoid tissue from difference part of the gastrointestinal tract to qualitatively evaluate changes in immunological tissue.

b) To determine if there are differences in free ruminal LPS concentrations in cows subjected to SARA by grain engorgement or intra-ruminal infusion of VFA. Free ruminal LPS may be differ in these treatments because grain engorgement will promote rumen bacteria growth whilst VFA infusions will reduce rumen pH. Thus these two

models will help to determine if the inflammatory response is due mainly to tissue injury (VFA infusion) or tissue injury and higher rates of free ruminal LPS translocation into blood circulation.

c) Identify the rumen gram-negative bacteria species that could contribute to free ruminal LPS and develop methods for selectively culturing them.

d) To determine cytokines synthesized and present in blood in cattle with chronic and acute inflammation. This is important in determining why the acute phase response was different between the steers and the cows.

LITERATURE CITED

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

Aiumlamai, S., H. Kindahl, G. Fredriksson, L.E., Edqvist, L. Kulander, and O. Eriksson. 1992. The role of endotoxins in induced ruminal acidosis in calves. Acta Vet. Scand. 33:117-127.

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

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.

Alsemgeest, S. P. M., G. A. E. van't Klooster, A. S. J. P. A. M. van Miert, C. K. Hulskamp-Koch, and E. Gruys. 1996. Primary bovine hepatocytes in the study of cytokine induced acute-phase protein secretion in vitro. Vet. Immunol. Immunopathol. 53:179-184.

Alsemgeest, S.P., H.C. Kalsbeek, T. Wensing, J.P. Koeman, A.M. van Ederen, and E. Gruys. 1994. Concentrations of serum amyloid-A (SAA) and haptoglobin (HP) as parameters of inflammatory diseases in cattle. Vet. Q. 16:21-23.

Alsemgeest, S.P., I.E. lambooy, H.K. Wierenga, S.J. Dieleman, B. Meerkerk, A.M. van Ederen, and T.A. Niewold. 1995. Influence of physical stress on the plasma concentration of serum amyloid-A (SAA) and haptoglobin (Hp) in calves. Vet. Q. 17:9-12.

Andersen, P.H. 1990. Aspects of bovine endotoxaemia of possible relevance to lesions in the ruminant digit. Pages 59-73. In Proc. of the VIth Intl Symp Diseases of the Ruminant Digit. Liverpool, 1990.

Andersen, P.H. 1993. Is rumen osmolality of grain-engorged cows influenced by pre-experiment diet? Acta vet. Scand. Suppl. 89:147-149.

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

Andersen, P.H., and N. Jarlov. 1990. Investigation of the possible role of endotoxin, TXA2, PG12 and PGE2 in experimentally induced rumen acidosis in cattle. Acta Vet Scand. 31:27-38.

Andersen, P.H., B. Berelin and K. Christensen. 1994a. Effects of feeding regimen on concentration of free endotoxin in ruminal fluid of cattle. J. Anim. Sci. 72:487-491.

Andersen, P.H., M. Hesselholt and N. Jarlov. 1994b. Endotoxin and arachidonic acid metabolitesin portal, hepatic and arterial blood of cattle with acute ruminal acidosis. Acta Vet Scand. 35:223-234.

Argenzio, R.A., C.K. Henrikson, and J. A. Liacos. 1988. Restitution of barrier and transport function of porcine colon after acute mucosal injury. Am J Physiol Gastrointest Liver Physiol 255: G62-G71.

Arthington, J.D., L. R. Corah, and F. Blecha. 1996. The effect of molybdenum-induced copper deficiency on acute-phase protein concentrations, superoxide dismutase activity, leukocyte numbers, and lymphocyte proliferation in beef heifers inoculated with bovine herpesvirus-1. J. Anim. Sci. 74:211-217.

Arthington, J.D., S.D. Eicher, W.E. Kunkle, and F.G. Martin. 2003. Effect of transportation and commingling on the acute phase protein response, growth, and feed intake of newly weaned beef calves. J. Anim. Sci. 81:1120-1125.

Bailey, C.B. 1961. Saliva secretion and its relation to feeding in cattle: 3 The rate of secretion of mixed saliva in the cow during eating, with an estimate of the magnitude of the total daily secretion of mixed saliva. Brit. J. Nutr. 15:443-451.

Bauer, M. L., D. W. Herold, R. A. Britton, R. A. Stock, T. J. Klopfenstein, and D. A. Yates. 1995. Efficacy of laidlomycin propionate to reduce ruminal acidosis in cattle. J. Anim. Sci. 73:3445-3454.

Baumann, H., and J. Gauldie. 1994. The acute phase response. Immunol. Today 15:74-80.

Beauchemin, K. A. 1991. Effects of dietary neutral detergent fiber concentration and alfalfa hay quality on chewing, rumen function, and milk production of dairy cows. J. Dairy Sci. 74:3140-3151.

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.

Beauchemin, K.A., and L. M. Rode. 1997. Minimum versus optimum concentrations of fiber in dairy cow diets based on barley silage and concentrates of barley or corn. J. Dairy Sci. 80:1629-1639.

Beharka, A.A.,T.G. Nagaraja, J.L. Morrill, G.A. Kennedy and R.D. Klemm. 1998. Effects of form of the diet on anatomical, microbial, and fermentative development of the rumen of neonatal calves. J. dairy Sci. 81:1946-1955.

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

Blakley, B.R., and D.L. Hamilton. 1985. Ceruloplasmin as an indicator of copper status in cattle and sheep. Can. J. Comp. Med. 49:403-408.

Bode, J.G., and P.C. Heinrich. 2001. Interleukin-6 signaling during the acute-phase response of the liver. In The Liver, Biology and Pathobiology. 4th Edition. I.M. Arias, J.L. Boyer, F.V. Chisari, N. Fausto, D. Schachter, and D.A. Shafritz, eds. (Philadelphia, PA: Lippincott Williams and Wilkins), pp. 565-580.

Bonhomme, A. 1990. Remen cilates: their metabolism and relationships with bacteria and their hosts. Anim. Feed Sci. Technol. 30:203-266.

Boosman, R, T.A. Niewold, C.W.A.A. Mutsaers, and E. Gruys. 1989. Serum amyloid A concentrations in cows given endotoxin as an acute phase stimulant. Am. J. Vet. Res. 50:1690-1694.

Brecher, G., and E.P. Cronkite. 1950. Morphology and enumeration of human blood platelets. J Appl Physiol 3:365-377.

Brent, B.E. 1976. Relationship of acidosis to other feedlot ailments. J. Anim. Sci. 43:930-935.

Brown, M.S., C.R. Krehbiel, M.L. Galyean, M.D. Remmengas, 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, M.P. 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. Am. J. Clin. Nutr. 25:1324-1328.

Burgos, M.S. W. Langhans, and M. Senn. 2000. Role of rumen fluid hypertonicity in the dehydration-induced hypophagia of cows. Physiol. Behav. 71:423-430.

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.

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.

Chan, J.P., C.C. Chu, H.P. Fung, S.T. Chuang, Y.C. Lin, R.M. Chu, and S.L. Lee. 2004. Serum haptoglobin concentration in cattle. J Vet Med Sci. 66:43-56.

Chouinard, P.Y., L. Corneau, D.M. Barbano, L.E. Metzger, and D.E. Bauman. 1999. Conjugated linoleic acids alter milk fatty acid composition and inhibit milk fat secretion in dairy cows. J. Nutr. 129:1579-1584.

Colditz, I.G. 2002. Effects of the immune system on metabolism: implications for production and disease resistence in livestock. Livest. Prod. Sci. 75:257-268.

Colenbrander, V.F., C.H. Noller, and R.J. Grant. 1991. Effect of fiber content and particle size of alfalfa silage on performance and chewing behavior. J. Dairy Sci. 74:2681-2690.

Conner, J.G., P.D. eckersall, A. Wiseman, T.C. Aitchison & T.A. Douglas. 1988. Bovine acute phase response following turpentine injection. Res. Vet. Sci. 44:82-88.

Cook, N. B. 2003. Prevalence of lameness among dairy cattle in Wisconsin as a function of housing type and stall surface. JAVMA 223:1324-1328.

Cooper, R., and T. Klopfenstein. 1996. Effects of Rumensin and feed intake variation on ruminal pH. Update on Rumensin® / Tylan® / Micotyl® for the Professional Feedlot Consultant.

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.

Counotte, G. H. M., R. A. Prins, R. H. A. M. Janssen, and M. J. A. deBie. 1981. Role of Megasphaera elsdeniiMegasphaera elsdenii in the fermentation of dl-[2-13C]lactate in the rumen of dairy cattle. Appl. Environ. Microbiol. 42:649-655.

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.

Dado, R.G., and M.S. Allen. 1994. Variation in and relationships among feeding, chewing, and drinking variables for lactating dairy cows. J. Dairy Sci. 77:132-144.

Deignan, T., A. Alwan, J. Kelly, J. McNair, T. Warren, and C. O'Farrelly. 2000. Serum haptoglobin: an objective indicator of experimentally-induced *Salmonella* infection in calves. Res. Vet. Sci. 69:153-158.

Diez-Gonzalez, F., and J.B. Russell. 1999. Factors affecting the extreme acid resistance of Escherichia coli O157:H7. Food Microbiol. 16:367-374.

Dijkstra, J., J.A.N. Mills, and J. France. 2002. The role of dynamic modeling in understanding the microbial contribution to rumen function. Nutr. Res. Rev. 15:67-90.

Dinarello, C.A., J.G. Cannona, J. Mancillaa, I. Bishaib, J. Leesb and F. Coceanib. 1991. Interleukin-6 as an endogenous pyrogen: induction of prostaglandin E2 in brain but not in peripheral blood mononuclear cells. Brain Res. 562:199-206.

Dirksen, G.U., H.G. Liebich & E. Mayer. 1985. Adaptive changes of the ruminal mucosa and their functional and clinical significance. Bovine Practitioner 20:116-120.

Donovan, J. 1997. Subacute acidosis is costing us millions. Hoard's Dairyman: 660-666.

Donovan, G.A., C. A. Risco, G. M. DeChant Temple, T.Q. Tran, and H. H. van Horn. 2004. Influence of transition diets on occurrence of subclinical laminitis in holstein dairy cows. J. Dairy Sci. 87:73-84.

Dougherty, R. W., K. S. Coburn, H. M. Cook, and M. Allison. 1975. A preliminary study of the appearance of endotoxin in the circulatory system of sheep and cattle after induced grain-engorgement. Am. J. Vet. Res. 36:831-832.

Dowling, A.,J. C. Hodgson, A. Schock, W. Donachie, P. D. Eckersall, and I. J. Mckendrick. 2002. Experimental induction of pneumonic pasteurellosis in calves by intratracheal infection with Pasteurella multocida biotype A:3. Res.Vet. Sci. 73:37-44.

Duffield, T., J. C. Plaizier, 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.

Earley, B, and M. A. Crowe. 2002. Effects of ketoprofen alone or in combination with local anesthesia during the castration of bull calves on plasma cortisol, immunological, and inflammatory responses. J. Anim. Sci. 80:1044-1052.

Eaton, J. W., P. Brandt, and J.R. Mahoney. 1982. Haptoglobin: a natural bacteriostat. Science 215: 691-693.

Eckersall, P.D. 2000. Recent advances and future prospects for the use of acute phase proteins as markers of disease in animals Revue Méd. Vét. 151:577-584.

Eckersall, P.D., and J.G. Conner. 1990. Plasma haptoglobin in cattle (Bos taurus) exists as polymers in association with albumin. Comp Biochem Physiol B. 96:309-314.

Emery, R. S., J. S. Liesman, and T. H. Herdt. 1992. Metabolism of long chain fatty acids by ruminant liver. J. Nutr. 122:832-837.

Enemark, J.M.D., R.J. Jorgensen and P. Enemark. 2002. Rumen acidosis with special emphasis on diagnostic aspects of subclinical rumen acidosis: A review Vet Zootech 20:16-29.

Enting, H., D. Kooij, A.A. Dijkhuizen, R.B.M. Huirne, and E.N. Noordhuizen-Stassen. 1997. Economic losses due to clinical lameness in dairy cattle Livestock Prod. Sci. 49:259-267.

Erwin, E.S., G.J. Marco, and E.M. Emery. 1961. Volatile fatty acids analysis of blood and rumen fluid by gas chromatography. J. Dairy Sci. 44:1768-1776.

Faulkner, D.B., T. Eurell, W.J. Tranquilli, R.S. Ott, M.W. Ohl, G.F. Cmarik, and G. Zinn. 1992. Performance and health of weanling bulls after butorphanol and xylazine administration at castration. J Anim Sci. 70:2970-2974.

Fisher, A.D., M. A. Crowe, E. M. O'Nuallain, M. L. Monaghan, J. A. Larkin, P. O'Kiely, and W. J. Enrigh. 1997. Effects of cortisol on in vitro interferon-gamma production, acute-phase proteins, growth, and feed intake in a calf castration model. J. Anim.Sci. 75:1041-1047.

Frampton, E. W., L. Restaino, and N. Blaszko. 1988. Evaluation of the β-glucuronidase substrate 5-bromo-4-chloro-3-indolyl-b-D-glucuronide (XGLUC) in a 24-hour direct plating method for Escherichia coli. J. Food Prot.51:402-404.

Frankena K., K.A. Van Keulen, J.P. Noordhuizen, E.N. Noordhuizen-Stassen, J. Gundelach, D.J. de Jong, and I. Saedt. 1992. A cross-sectional study into prevalence and risk indicators of digital haemorrhage in female dairy calves. Prev. Vet Med 14:1-12.

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. 49:775-784.

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.

Galanos, C., V. Lehmann, O Luderitz, ET Rietschel, O Westphal, H Brade, L Brade, MA Freudenberg, T Hansen-Hagge, and T Luderitz. 1984. Endotoxic properties of chemically synthesized lipid A part structures. Comparison of synthetic lipid A precursor and synthetic analogues with biosynthetic lipid A precursor and free lipid A. Eur J Biochem 140:221-227.

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.

Gentry , P.A., and H.G. Downie. 1984. Bood coagulation. Pages 41-50. In Duke's Physiology of Domestic Animals. 10th ed. M.S. Swenson (ed). Cornell University Press.Ithaca, New York.

George, J.W. 2001. The usefulness and limitations of hand-held refractometers in veterinary medicine: An historical and technical review. Vet Clin Pathol. 30:201-210.

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

Godson, D.L. , M. Campos, S. K. Attah-Poku, ,M. J. Redmond , D.M. Cordeiro, M. S. Sethi, R. J. Harland, and L. A. Babiuk. 1996. Serum haptoglobin as an indicator of the acute phase response in bovine respiratory disease. Vet. Immunol. and Immunopathol. 51:277-292.

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

Gozho, G.N., J.C. Plaizier and D.O. Krause. 2005b. Effects of graded levels of wheat-barley concentrate on subacute ruminal acidosis (SARA), lipopolysaccharide endotoxin (LPS) and acute phase proteins in steers. J. Dairy Sci. 88 (Suppl. 1):434.

Grant, R.H., and D. R. Mertens. 1992. Influence of buffer ph and raw corn starch addition on in vitro fiber digestion kinetics. J. Dairy Sci. 75:2762-2768.

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.

Greenough. P.R., J.J. Vermunt, J.J. McKinnon, F.A. Fabby, P.A. Borg, and R.D.H. Cohen. 1990. Laminitis like changes in the claws of feedlot cattle. Canadian Vet J 31:202-208.

Griinari, J. M., and D. E. Bauman. 1999. Biosynthesis of conjugated linoleic 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.

M. P. Yurawecz, M. M. Mossoba, J. K. G. Kramer, G. Nelson and M. W. Pariza, ed. AOCS Press, Champaign, IL.

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

Hall. M.B. 2002. Rumen acidosis: carbohydrate feeding considerations. Pages 51-61 in Proc. 12th Int. Symp. Lameness in Ruminants, Orlando, FL.

Heegaard, P.M.H., D.L. Godson, M.J.M. Toussaint, K. Tjornehoj, L.E. Larsen, B. Viuff, and L. Ronsholt. 2000. The acute phase response of haptoglobin and serum amyloid A (SAA) in cattle undergoing experimental infection with bovine respiratory syncytial virus Vet Immunol Immunopathol. 77:151-159.

Heinrichs, J. 1996. Evaluating particle size of forages and TMRs using the Penn State Particle Size Separator. Pennsylvania State University, University Park. PA. pp 1-9.

Hibbard, B., J. P. Peters, S. T. Chester, J. A. Robinson, S. Kotarski, W. J. 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.

Hirvonen, J. 2000. Hirvonen's thesis on acute phase response in dairy cattle. University of Helsinki ISBN 951-45-9106-2.

Hirvonen, J., G. Huszenicza, M. Kulcsar & S. Pyoralla. 1999. Acute phase response in dairy cows with acute postpartum metritis. Theriogenology 51:1071-1083.

Hirvonen, J., S. Pyorala, and H. Jousimies-Somer H. 1996. Acute phase response in heifers with experimentally induced mastitis. J Dairy Res. 63:351-360.

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

Horadagoda, A., P.D. Eckersall, J.C. Hodgson, H.A. Gibbs, and G.M. Moon. 1994. Immediate responses in serum TNF alpha and acute phase protein concentrations to infection with Pasteurella haemolytica A1 in calves. Res Vet Sci. 57:129-132.

Horadagoda, N.U., K.M.G. Knox, H.A. Gibbs, S.W.J. Reid, A. Horadagoda, S.E.R. Edwards, and P.D. Eckersall. 1999. Acute phase proteins in cattle: discrimination between acute and chronic inflammation. Vet Rec. 144: 437-441.

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

Humblet, M.F., J. Coghe, P. Lekeux, and J.M. Godeau. 2004. Acute phase proteins assessment for an early selection of treatments in growing calves suffering from bronchopneumonia under field conditions Res. Vet. Sci. 77:41-47.

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.

Jacobsen, S., P. H. Andersen, T. Toelboell, and P. M. H. Heegaard. 2004. Dose dependency and individual variability of the lipopolysaccharide-induced bovine acute phase protein response. J. Dairy Sci. 87:3330-3339.

Jasaitis, D.K.,J.E. Wohlt, and J.L. Evans. 1987. Influence of feed ion content on buffering capacity of ruminant feedstuffs in vitro. J. Dairy Sci. 70:1391-1403.

Johnson, P.J., S.C. Tyagi, L.C. Katwa, V.K. Ganjam, L.A. Moore, J.M. Kreeger, and N.T. Messer. 1998. Activation of extracellular matrix metalloproteinases in equine laminitis. Vet. Rec. 142:392-396.

Kahn, C.M., and S. Line. 2003. Hematologic reference ranges. *In* The Merck Veterinary Manual. 8th Edition (online), Merck and Co. Inc., Whitehouse Station, NJ, USA

Kaufmann. 1976. Influence of the composition of the ration and feeding frequency on pH regulation in the rumen and on feed intake in ruminants. Livestock Prod. Sci. 3:103-114.

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, L. 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.

Klasing, K.C. 1988. Nutritional aspects of leukocytic cytokines. J. Nutr. 118:1436-1446.

Kleen, J.L., G.A., Hooijer, J. Rehage and J.P.T.M. 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.

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, D.O., W.J.M. Smith, L.L. Conlan, J.M. Gough, M.A. Williamson and C.S. McSweeney. 2003. Diet influences the ecology of lactic acid bacteria and Escherichia coli along the digestive tract of cattle: neural networks and 16S rDNA. Microbiol. 149:57-65.

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

Krause, K.M., D. K. Combs, and K. A. Beauchemin. 2002. Effects of forage particle size and grain fermentability in midlactation cows. II. Ruminal pH and chewing activity J. Dairy Sci. 85:1947-1957.

Kriehbiel, C. R., R. A. Britton, D. L. Harmon, T. J. Wester, and R. A. Stock. 1995a. The effects of ruminal acidosis on volatile fatty acid absorption and plasma activities of pancreatic enzymes in lambs. J. Anim. Sci. 73:3111-3121.

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. 1995b. Influence of grain type, tallow Level, and tallow feeding system on feedlot cattle performance. J. Anim. Sci. 73:2438-2446.

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. 1995c. Effect of level and type of fat on Subacute acidosis in cattle fed dry-rolled corn finishing diets. J. Anim. Sci. 73:2438-2446.

Krogh, N. 1961. Studies on alterations in the rumen fluid of sheep, especially concerning the microbial composition, when readily available carbohydrates are added to the food III Starch. Acta vet. Scand. 2:103-119.

Kushner, I and D.L. Rzewnicki. 1994. The acute phase response: General aspects. Bailliere's Clinical Rheumatol. 8:513-530.

Kushner, I. 1982. The phenomenon of the acute phase response. Annals of the New York Academy of sciences. 389:39-48.

Lehtolainen, T., S. Suominen, T. Kutila, and S. Pyöraöla. 2003 Effect of intramammary Escherichia coli endotoxin in early - vs. late-lactating dairy cows. J. Dairy Sci. 86:2327-2333.

Levin J., and F.B. Bang. 1964. A description of cellular coagulation in the *Limulus*. Bull. John Hopkins Hosp. 115:337-345.

Livesey, C.T., and F.L. Fleming. 1987. Nutritional influences on laminitis, sole ulcer and bruised sole in Friesian cows. Vet. Rec. 114:510-512.

Lloyd, A.R., and J. J. Oppenheim. 1992. Poly's lament: the neglected role of the polymorphonuclear neutrophil in the afferent limb of the immune response Immunol. Today. 13:169-172.

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.

Mackie, R.I. and F.M.C. Gilchrist. 1979. Changes in lactate-producing and lactate-utilizing bacteria in relation to pH in the rumen of sheep during stepwise adaptation to a high-concentrate diet. Appl. Environ. Microbiol. 38:422-430.

Mackie, R.I. F.M.C. Gilchrist, A.M. Roberts, P.E. Hannah, and H.M. Schwartz. 1978. Microbiological and chemical changes in the rumen during the stepwise adaptation of sheep to high concentrate diets. J. Agric. Sci. 90:241-254.

Maekwa, M., K.A. Beauchemin and D.A. Christensen. 2002a. Chewing activity, saliva production, and ruminal pH of primiparous and multiparous lactating dairy cows. J. dairy Sci. 85:1176-1182.

Maekwa, M. K.A. Beauchemin, and D.A. Christensen. 2002b. Effect of concentrate level and feeding manangement on chewing activities, saliva production, and ruminal pH of lactating dairy cows. J. Dairy Sci. 85:1165-175.

Makimura, S., and N. Suzuki, 1982. Quantitative determination of bovine serum haptoglobin and its elevation in some inflammatory diseases. J. Vet. Sci. 44:15-21.

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.

McCarthy, R. D., T. H. Klusmeyer, J. L. Vicini, J. H. Clark, and D. R. Nelson. 1989. Effects of source of protein and carbohydrate on ruminal fermentation and passage of nutrients to the small intestine of lactating cows. J. Dairy Sci. 72:2002–2016.

McDonald, T.L., A. Weber, and J.W. Smith. 1991. A monoclonal antibody sandwich immunoassay for serum Amyloid A protein. J. Immunol. Methods 144:149-155.

McDonald, T.L., M.A. Larson, D.R. Mack, A. Weber. 2001. Elevated extrahepatic expression and secretion of mammary-associated serum amyloid A 3 (M-SAA3) into colostrums.Vet Immunol. Immunopathol. 83:203-211.

Meglia, G.E., A. Johannisson, S. Agenas, K. Holtenius, and K. P. Waller. 2005 Effects of feeding intensity during the dry period on leukocyte and lymphocyte sub-populations, neutrophil function and health in periparturient dairy cows. Vet. J. 169:376-384.

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

Mochzuki, M.,N. Kamata, T. Itoh, G. Shimizu, Y. Yamada, T. Kadosawa, R. Nishimura, N. Sasaki and A. Takeuchi. 1996. Postparturient changes in endotoxin levels of ruminal fluid and serum in dairy cows J. Vet Med. Sci 58:577-580.

Morimatsu, M., B. Syuto, N. Shimada, T. Fujinaga, S. Yamamoto, M. Saito, and M. Naiki. 1991. Isolation and characterization of bovine haptoglobin from acute phase sera. J. Biol. Chem. 266:11833-11837.

Mullenax, C.H., R.F. Keeler, and M.J. Allison. 1966. Physiologic response of ruminants to toxic factors extracted from rumen bacteria and rumen fluid Am. J. Vet. Res. 27:857-868.

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 Chengappa, M.M. 1998. Liver abscesses in feedlot cattle: a review. J. Anim. Sci. 76:287-298.

Nagaraja, T.G., E.E. Bartley, L.R. Fina, H.D. Anthony, S.M. Dennis and R.M. Bechtle. 1978b. Quantitation of endotoxin in cell-free rumen fluid of cattle. J. Anim. Sci. 46:1759-1767.

Nocek, J. E., and E. M. Kessler. 1980. Growth and rumen characteristics of Holstein steers fed pelleted or conventional diets. J. Dairy Sci. 63:249-254.

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

Nocek, J.E., J.G. Allman, and W.P. Kautz. 2002. Evaluation of an indwelling ruminal probe methodology and effect of grain level on diurnal pH variation in dairy cattle J. Dairy Sci. 85: 422-428.

Nordlund, K. 2002. Herd-based diagnosis of subacute ruminal acidosis. Pages 70-74 in Proc. 12th Int. Symp. Lameness in Ruminants, Orlando, FL.

Nordlund, K.V., E.F. Garrett, & G.R. Oetzel. 1995. Herd-based rumenocentesis: A clinical approach to the diagnosis of Subacute rumen acidosis. Compend. Contin. Educat. Pract. Vet. Food Animal. 17:s48-s56.

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

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

Nutescu, E.A., N.L. Shapiro, A. Chevalier, and A. N. Amin. 2005. A pharmacologic overview of current and emerging anticoagulants. Cleveland Clinic J. Med. 72(Suppl 1):82-86.

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(Suppl 1):P35.

Oetzel, G.R. 2003. Introduction to ruminal acidosis in dairy cattle. Precovention seminar 7: Dairy herd problem investigation strategies. American association of bovine practitioners. 36th Annual conference, Sept 15-17 2003, Columbus OH.

Ohtsuka, H., K. Kudo, K. Mori, F. Nagai, A. Hatsugaya, M. Tajima, K. Tamura, F. Hoshi, M. Koiwa, and S. Kawamura. 2001. Acute phase response in naturally occurring coliform mastitis. J. Vet. Med. Sci. 63:675-678.

Oshio, S., and I. Tahata. 1984. Absorption of dissociated volatile fatty acids through the rumen wall of sheep. Can. J. Anim. Sci. 64(Suppl.):167.

Overton, T.R., and M. R. Waldron. 2004. Nutritional management of transition dairy cows: strategies to optimize metabolic health. J. Dairy Sci. 87:(E. Suppl.):E105-E119.

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.

Pedersen, L.H., B. Aalbaek, C.M. Rondveld, K.L. Ingvartsen, N.S. Sorensen, P.M.H. Heegaard, and H.E. Jensen. 2003. Early pathogenesis and inflammatory response in experimental bovine mastitis due to *Streptococcus uberis*. J. Comp. Path. 128:156-164.

Petersen, H.H., J.P. Nielsen, and P.M.H. Heegaard. 2004. Application of acute phase protein measurements in veterinary clinical chemistry. Vet Res. 35:163-187.

Plaizier, J.C., J. E., Keunen, J-P. Walton, T.F. Duffield and B.W. McBride. 2001. Short Communication: 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., T. Garner, T. Droppo, and T. Whiting. 2004. Nutritional practices on Manitoba dairy farms. Can. J. Anim. Sci. 84:501-509.

Pollitt, C.C. 1994. The basement membrane at the equine hoof dermal epidermal junction. Equine Vet. J. 28:38-46.

Prentice, D.E. & P.A. Neal. 1972. Some observations on the incidence of lameness in dairy cattle in west Cheshire. Vet. Rec. 91:1-7.

Putnam, F.W. 1975. The plasma proteins. Structure, function, and genetic control. 2nd ed. Vol. 2. Academic Press. New York. NY.

Reinhardt, C.D., R. T. Brandt, Jr, K. C. Behnke, A. S. Freeman, and T. P. Eck. 1997. Effect of steam-flaked sorghum grain density on performance, mill production rate, and subacute acidosis in feedlot steers J. Anim Sci. 75:2852-2857.

Rietschel, E.T., H. Brade, O. Holst, L.Brade, S. Muller-Loennies, U. Mamat,U. Zahringer, F. Beckmann, U. Seydel, K. Brandenburg, A.J. Ulmer, T. Mattern, H. Heine,J. Schletter, H. Loppnow, U. Schonbeck, H.D. Flad, S. Hauschildt, U.F. Schade, F. Di Padova, S. Kusumoto and R.R. Schumann. 1996. Bacterial endotoxin: Chemical constitution, biological recognition, host response, and immunological detoxification. In Pathology of Septic Shock. E.T. Rietschel & H. wagner, eds (Heidelberg: Springer) pp 40-81.

Rot, A. 1992. Endothelial cell binding of NAP-1/IL-8: role in neutrophil emigration. Immunol. Today 13:291-294.

Ruegg, P. 2000. Hooves: A laminitis history book. Proc. Ann. Conf. Am. Soc. Bovine Pract. 33:69-74.

Russell, J.B., and R.L. Baldwin. 1979. Comparison of maintenance energy expenditure and growth yields among several rumen bacteria growth yields among several rumen bacteria grown on continuous culture. Appl. Environ. Microbiol. 37:537-543.

Russell, J.B., and T. Hino. 1985. Regulation of lactate production in Steptococcus bovis: A spiraling effect that contributes to rumen acidosis. J. Dairy Sci. 68:1712-1721.

Russell J.B., and J.L. Rychlik. 2001. Factors that alter rumen microbial ecology. Science 292: 1119-1122.

SAS, 1996. SAS® System for Mixed Models 4th Edition. SAS® Inst., Inc., Cary NC.

Schwartzkopf-Genswein, K.S., K.A. Beauchemin, D.J. Gibb, D.H. Crews, Jr, D.D. Hickman, M. Streeter, and T.A. McAllister. 2003. Effect of bunk management on feeding behavior, ruminal acidosis and performance of feedlot cattle: A. review. J. Anim. Sci. 81(E. Suppl. 2):E149-E158

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 culture. Appl. Environ. Microbiol. 58:2583-2591.

Skinner, J.G., R.A.L. Brown, and L. Roberts. 1991 Bovine haptoglobin response in clinically defined field conditions. Vet. Rec. 128:147-149.

Slyter, L.L., and T.S. Rumsey. 1991. Effect of coliform bacteria, feed deprivation, and pH on ruminal D-lactic acid production by steer or continuous-culture microbial populations changed from forage to concentrates. J Anim Sci. 69:3055-3066.

Soita, H.W., D.A, Christensen, and J.J. McKinnon. 2000. Influence of particle size on the Effectiveness of fiber in barley silage J. Dairy Sci. 83:2295-2300.

Steel, D.M. & A.S. Whitehead. 1994. The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein. Immunol. Today 15:81-88.

Stone W.C. 1999. The effect of sub clinical rumen acidosis on milk components. Proc. Cornell Nutr. Conf. Feed Manuf pp 40-46.

Stone, W.C. 2004. Nutritional approaches to minimize subacute ruminal acidosis and laminitis in dairy cattle. J. Dairy Sci. 87: (E. Suppl.):E13-E26.

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.

Swenson, M.J. 1984. Physiological properties and cellular and chemical constituents of blood. Pages 15-40 In Duke's Physiology of Domestic Animals. 10th ed. M.S. Swenson (ed). Cornell University Press. Ithaca, New York.

Telle, P.P. and R.L. Preston. 1971. Ovine lactic acidosis: Intraruminal and systemic.J. Anim. Sci. 33:698-705.

Ting, S.T.L., B. Earley, and M. A. Crowe. 2003. Effect of repeated ketoprofen administration during surgical castration of bulls on cortisol, immunological function, feed intake, growth, and behavior. J. Anim. Sci. 81:1253-1264.

Tizard, I. R. 1982. An introduction to veterinary immunology 2nd ed. W.B Saunders Company. Toronto, Canada.

Tobias, P.S.,P. W. J. McAdam, and R.J. Ulevitch. 1982. Interactions of bacterial lipopolysaccharide with acute-phase rabbit serum and isolation of two forms of rabbit serum amyloid A. J. Immunol. 128:1420-1427.

Underwood, W.J. 1992. Rumen lactic acidosis. Part II. Clinical signs, diagnosis, treatment, and prevention. Compend. Contin. Educat. Pract. Vet. Food Animal. 14:1265-1270.

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

Wang, Z., and L.A. Goonewardene. 2004. The use of MIXED models in the analysis of animal experiments with repeated measures data. Can. J. Anim. Sci. 84:1-11.

WCDHIS. 2001. Western Canadian Dairy Herd Improvement Services: Herd Improvement Report. Edmonton AB. 2001.

Weingarten, H.P. 1996. Cytokines and food intake: The relevance of the immune system to the student of ingestive behavior. Neurosci. Biobehav. Rev. 20:163-170.

Welles, E.G., M.A. Williams, J.W. Tyler, and H.C. Lin. 1993. Hemostasis in cows with endotoxin-induced mastitis. Am J Vet Res. 54:1230-1234.

Wells, J.E., and J. Russell. 1996. The effect of growth and starvation on the lysis of the ruminal cellulolytic bacterium *Fibrobacter succinogenes*. Appl. Environ. Microbiol. 62:1342-1346.

Wells, S. J., A. M. Trent, W. E. Marsh, and R. A. Robinson. 1993. Prevalence and severity of lameness in lactating dairy cows in a sample of Minnesota and Wisconsin dairy herds. JAVMA 202:78–82.

Werling, D., F. Sutter, M. Anorld, G. Kun, P.C.J. Tooten, E. Gruys, M. Kreuzer, and W. Langhans. 1996. Characterisation of the acute phase response of heifers to a prolonged low dose infusion of lipopolysaccharide. Res. Vet. Sci. 61:252-257.

Whitaker, D.A., M.J. Kelly, and E.J. Smith. 1983. Incidence of lameness in dairy cows. Vet. Rec. 113:60-62.

Yang, W. Z., K.A. Beauchemin, and L.M. Rode. 2001. Effects of grain processing forage to concentrate ratio, and forage particle size on rumen pH and digestion by dairy cows. J. Dairy Sci. 84:2203-2216.

Young, C.R., T.E. Wittum, L.H. Stanker, L.J. Perino, D.D. Griffin, and E.T. Littledike. 1996. Serum haptoglobin concentrations in a population of feedlot cattle. Am. J. Vet. Res. 57:138-141.