Mechanistic Relationships Between Immune and Rumen Microbial Responses of Dairy Cows Subjected to Subacute Ruminal Acidosis

by

Ehsan Khafipour

A Thesis submitted to the Faculty of Graduate Studies of

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in partial fulfilment of the requirements of the degree of

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ABSTRACT

Current definition of subacute ruminal acidosis (SARA) is based on low rumen pH typically generated on high starch or low coarse fiber diets. However, some researchers believe rumen pH has a low correlation with SARA symptoms, and that other indicators such as free rumen lipopolysaccharide (LPS) must be included in the definition of SARA. In this research, we developed two animal models of SARA, one based on wheat-barley pellets and the other based on alfalfa pellets, to determine if different nutritional models result in a similar increase in free LPS in the rumen fluid and inflammatory markers in peripheral blood, and if this inflammatory response is due to the LPS. Additionally, the rumen microbial community dynamics were compared in grain- versus alfalfa pellet-induced SARA.

Based on adopted threshold of SARA of at least 180 min/d of rumen pH < 5.6, SARA was successfully induced with both nutritional models. Rumen free LPS concentration increased during both grain- (28,184 vs. 107,152 EU/mL) and alfalfa pellet-induced SARA (38,019 vs. 165,959 EU/mL). In grain-induced SARA, this increase was accompanied by an increase in LPS and inflammatory indicators including serum amyloid-A, haptoglobin, and LPS-binding protein in the peripheral blood. However, LPS translocation or immune activation did not occur in alfalfa pellet-induced SARA. Rumen bacterial community analysis indicated that the proportion of phylum *Bacteroidetes*, which are likely the largest contributor to free rumen LPS pool, declined from 35% in alfalfa pellet- to 16% in grain-induced group. Moreover, the severity of SARA and inflammatory response were associated with an increase in *Escherichia coli* and not free LPS in the rumen. *E. coli* populations differed between the grain- and alfalfa pellet-

induced SARA. *E. coli* isolates from control and alfalfa pellet-induced SARA fell into one cluster, while isolates from grain-induced SARA grouped into a separate cluster. These results suggest that inflammatory responses observed with grain-induced SARA might be due to a sub-population of *E. coli* from grain based diet that could possess virulence features. Alternately, grain-induced SARA could have modified the hindgut microbiota by increasing the amount of starch in the intestine, and thus, LPS translocation might have occurred in the intestine rather than the rumen.

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FOREWORD

This dissertation is written in manuscript style and is composed of four manuscripts. The first and the second manuscripts have been accepted in the Journal of Dairy Science. The third and fourth manuscripts have not yet been submitted to a journal.

The authors of the manuscripts are:

Manuscript I:	E. Khafipoor, D. O. Krause, and J. C. Plaizier
Manuscript II:	E. Khafipoor, D. O. Krause, and J. C. Plaizier
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Manuscript IV:	E. Khafipoor, J. C. Plaizier, and D. O. Krause

* Authors contributed equally to the research.

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LIST OF ABBREVIATIONS

Ac:Pr = acetate to propionate ratio

ADF = acid detergent fiber

AFI = Acid fitness island

AFLP = amplified fragment length polymorphism

ARISA = automated ribosomal intergenic spacer analysis

BHBA = beta-hydroxy butyric acid

bp = basepair

BW = body weight

CP = crude protein

Cr = chromium

DGGE = denaturing gradient gel electrophoresis

DIM = days in milk

DM = dry matter

DMI = dry matter intake

ECOR = *Escherichia coli* collection of reference

EU = endotoxin unit

F:C = forage to concentrate ratio

GC = gas chromatography

GI = gastro-intestinal

Hp = haptoglobin

IL-1 = Interleukin -1

IL-6 = Interleukin -1

ITS = intergenic spacer region

LAL = limulus amebocyte lysate

LBP = lipopolysaccharide binding protein

LEE = locus of enterocyte effacement

LPS = lipopolysaccharide

MiCA = microbial community analysis

NDF = neutral detergent fiber

NEFA = non-esterified fatty acids

NFC = non-fiber carbohydrates

NF-κB = nuclear factor-kappa B

NO· = nitric oxide

NSC = non-structural carbohydrates

OD = optical density

OTU = operational taxonomic unit

PCR = polymerase chain reaction

qPCR = quantitative PCR

RAPD = random amplified polymorphic DNA

rDNA = ribosomal DNA

RDP = ribosomal database project

Rep-PCR = repetitive PCR

RFLP = restriction fragment length polymorphism

RISA = ribosomal intergenic spacer analysis

SAA = serum amyloid-A

SARA = subacute ruminal acidosis

SD = standard deviation

SED = standard error of difference between treatments

TGGE = thermal gradient gel electrophoresis

TLR-4 = toll like receptor 4

TMR = total mixed ration

TNF-α = tumor necrosis factor alpha

T-RF = terminal restriction fragment

T-RFLP = terminal restriction fragment length polymorphism

VFA = volatile fatty acid

WBP = wheat-barley pellets

WHC = water holding capacity

GENERAL INTRODUCTION

Subacute ruminal acidosis (SARA) is a metabolic disease of ruminants and is of particular concern in high producing dairy cattle in early and mid-lactation. Current definitions of SARA are based on rumen pH (Plaizier et al., 2008). However, there is no general agreement on the pH threshold that is definitive of SARA, as rumen pH of 5.5 (Garrett et al., 1999), between 5.2 and 5.6 (Cooper and Klopfenstein, 1996), 5.8 (Beauchemin et al., 2003) and 6 (Plaizier, 2004) have all been suggested based on criteria such as microbial activity in the rumen, health and function of the rumen epithelium, and the method of pH measurement. In addition, as rumen pH fluctuates within the day, the duration of period at which rumen pH is below a certain threshold affects the severity of SARA (Enemark et al., 2004). In our research group, we define SARA as daily episodes of rumen pH between 5.2 and 5.6 for at least 180 min/d that activates an inflammatory response (Gozho et al., 2005). Under these low rumen pH, animals experience erratic feed intake (Cooper et al., 1999, Brown et al., 2000), and decline in milk yield and milk fat (Stone, 1999, Enjalbert et al., 2008), along with the behavioral changes, such as anorexia, associated with the digestive tract disease (Kleen et al., 2003). In long term, SARA is linked with rumenitis, laminitis, and liver abscesses, which may appear several months after the initial insult (Enemark, 2008). As a result, SARA reduces the longevity of dairy cows in the herds (Nocek, 1997).

It is difficult to diagnose SARA in the field based on its clinical signs as they are either subtle, so they can be easily dismissed with other problems in the herd, or not present shortly after the pH insult (Kleen et al., 2003; Enemark, 2008). Therefore,

detection of SARA is currently based on the collection of rumen fluid and measurement of pH. Garret et al. (1999) suggested a screening method for the herd based diagnosis of SARA based on the sampling of 12 cows in early or mid-lactation groups. If three cows (> 33%) had a ruminal pH < 5.5, the herd was considered at risk of SARA. Based on this methodology, a survey in the United States (Garrett et al., 1997) and another survey in Germany and The Netherlands (Kleen, et al., 2004) estimated that the prevalence of SARA is 19% and 26%, and 11% and 18% in early and mid-lactation groups, respectively. Stone (1999) has estimated that only reduction in milk yield (2.7 kg/cow/day), milk fat (0.3% point) and milk protein (0.1% point) in SARA cows results in an economical loss of US$ 400 to 475 per cow per year.

The decline in rumen pH during SARA is due to the accumulation of volatile fatty acids (VFA) and reduction of rumen buffering capacity (Owens et al., 1998; Krause and Oetzel, 2005). Ingestion of large amount of low fiber high energy diets increases the growth rate of rumen bacteria, particularly amylolytic bacteria, resulting in greater rate of production of VFA in the rumen than its rate of absorption and passage (Nocek, 1997). In addition, intake of low fiber diet with small particle size reduces chewing activity, saliva production, and thus, rumen buffering (Beauchemin et al., 2008). Increased H^+ ions and decreased rumen buffering result in rumen pH depression, which is favorable for rapid proliferation of lactic acid producing bacteria like *Streptococcus bovis*. However, increase in lactic acid triggers the growth of lactate consuming bacteria like *Megasphaera elsdenii*. Under the rumen pH condition of SARA (5.2 to 5.6) the equilibrium between lactic acid producing and utilizing bacteria exists, preventing lactic acid accumulation, above 10 m*M* in the rumen (Goad et al., 1998).

The adverse effects of SARA are not solely due to the rumen acids. The dietary induced changes in gut microbiota and increase in its permeability is speculated as the potential cause of inflammation, laminitis, and liver abscesses seen in SARA cows (Nocek, 1997, Kleen et al., 2003, Plaizier et al., 2008). Disruption of gut barrier could allow the enteric bacteria, their cell components such as lipopolysaccharide (LPS), and their by-products like histamine to translocate into the lymph or blood circulation and trigger an inflammatory response. However, it is not clear how and under what circumstances the barrier impairment occurs. The barrier function of the rumen epithelium may be compromised by the parakeratosis of rumen epithelium, rumenitis and abscesses of the rumen wall that result from high rumen acidity (Kleen et al., 2003). Also, the high rumen osmolality that is seen during SARA can cause swelling and rupture of ruminal papillae, which will also reduce the barrier function of the rumen (Owens et al., 1998). Additionally, there is substantial evidence that SARA increases the content of free LPS in the rumen due to the increase in shedding of LPS during rapid growth and lysis of Gram-negative bacteria (Gozho et al., 2007; Nagaraja et al., 1978c). This increase in luminal LPS could increase gut permeability through production of nitric oxide ($NO^{\cdot}$) (Chin et al., 2006). Translocation of LPS or other immunogenic compounds into circulation then may trigger systemic inflammatory response (Horadagoda et al., 1999).

Previous research showed that grain-induced SARA increases the concentrations of the acute phase proteins serum amyloid A (SAA), and haptoglobin (Hp), which are markers of inflammation, in peripheral blood of cattle (Gozho et al., 2005; 2007). Despite this, no evidence of LPS in peripheral circulation of SARA cows has been found (Gozho et al., 2007). In addition, in these studies, no specific marker of LPS has been employed

to determine if the inflammatory reaction is due to LPS or other inflammatory stimuli. More importantly, the correlations between rumen pH, LPS and inflammation have been only studied when SARA was experimentally induced by increasing the content of starch in the diet. Therefore, it cannot be concluded that the clinical signs, particularly inflammation, that are associated with grain-induced SARA are due to low pH and high LPS in the rumen or due to the high starch content of the diet.

On the other hand, the microbiology of the rumen during SARA is not well studied (Goad et al., 1998, Nagaraja and Titgemeyer, 2007). The limited information that is available is provided by culture-based studies, which only allows for the isolation of a limited number of species of microbes, which might not necessarily be the only members of the rumen ecosystem (Krause et al., 2007). Moreover, in the complex ecosystem of the rumen, the interaction between microbial populations defines their overall function in the community; and thus, they may not necessarily perform the same activity in the pure cultures (Mackie et al., 2007). Hence, these aspects cannot be studied using culture-based methods.

In this research, we developed two animal models, one based on wheat-barley pellets and the other based on alfalfa pellets, to induce SARA in lactating dairy cows by increasing the dietary content of starch or reducing the particle size. Our objectives were to determine if different nutritional models of SARA result in similar increases in free LPS in the rumen fluid and in acute phase proteins in blood, and if this inflammatory response is due to translocation of LPS from the gut into peripheral circulation. Also, to understand the underlying cause of SARA, we used both culture-dependent and independent techniques to investigate the relationships between rumen pH conditions,

free rumen LPS, inflammation, and rumen microbial population dynamics in grain- versus alfalfa pellet-induced SARA.

LITERATURE REVIEW

1.0 Definition of Subacute Ruminal Acidosis

Subacute ruminal acidosis (SARA) is a metabolic disorder of ruminants that is associated with low rumen pH resulting from ingestion of diets that are high in energy and low in coarse forage particles (Krause and Oetzel, 2006). However, there is no general agreement on the pH threshold that defines SARA. Currently, rumen pH below 5.5 (Garrett et al., 1999), between 5.2 and 5.6 (Cooper and Klopfenstein, 1996), below 5.8 (Beauchemin et al., 2003) and below 6 (Plaizier, 2004) have been suggested as SARA. These thresholds have been considered based on criteria such as microbial activity in the rumen (Russell and Wilson, 1996), health and function of the rumen epithelium (Gabel et al., 2002), and the method of pH measurement (Garrett et al., 1997; Duffield et al., 2004). In our research group, we define SARA as daily episodes of low pH between 5.2 and 5.6 for at least 180 min/d (Gozho et al., 2005). The duration of time below pH 5.6 was particularly included in our definition as rumen pH fluctuates throughout the day (Enemark et al., 2004). We have observed that at least 180 min/d of pH < 5.6 is required to activate an inflammatory response during SARA (Gozho et al., 2006).

Depending on the magnitude and duration of rumen pH depression, SARA can reduce feed intake, milk yield, and milk fat (Plaizier et al., 2008), and can result in parakeratosis, rumenitis, and abscesses of the rumen wall, which impairs the barrier function of the epithelium (Nocek, 1997). As a result of barrier disruption, bacteria or immunogenic and biogenic compounds, such as lipopolysaccharide (LPS) and histamine,

can translocate into the circulation causing inflammation, laminitis, liver abscesses, and sudden death (Kleen et al., 2003)

Although the rumen pH has been used to define SARA, the aforementioned clinical signs cannot be explained by pH alone. To refine the definition of SARA based on rumen fluid analysis; Gozho et al. (2005) suggested that free rumen lipopolysaccharide (LPS) concentration needs to be considered. This view has been supported by the fact that grain-induced SARA increases the concentration of free rumen LPS (Gozho et al., 2005; 2007; Emmanuel et al., 2008). Since LPS is a known virulence factor in host-microbial interactions (Hurley, 1995; Sriskandan and Altmann, 2008), its translocation into circulation might be the cause of some of the signs of SARA, such as increase in inflammatory markers in blood and decrease in feed intake (Andersen, 2000). However, the possibility of translocation of free rumen LPS into blood and its role in inflammatory response has yet to be proven and quantified.

Given the rumen pH criteria of SARA, two distinct groups of cows are at risk of this disease: First, cows in early lactation that are not adapted enough to high energy diets and second, cows in mid-lactation that have high feed intake (Krause and Oetzel, 2006). In both groups, VFA production in the rumen can exceed its rate of absorption, neutralization, and passage, and that can result in VFA accumulation, and rumen microbial population shift causing different degree of SARA symptoms. These clinical signs, however, are variable among individuals, some are subtle and can be easily dismissed with other problems in the herd, and some are not present until several months after the initial insult. Therefore, rumen pH measurement is still recommended to diagnose SARA (Garret et al., 1999).

2.0 Prevalence and Cost of SARA

The current information that is available on the prevalence of SARA is based on the methodology developed by Garret et al. (1999). Authors suggested a screening method based on rumen fluid sampling of 12 cows in early or mid-lactation groups using rumenocentesis. If one-third or more of the cows had a ruminal pH < 5.5, then the herd was considered at risk of SARA, and if one-third or more of the cows had a ruminal pH < 5.8 the herd was considered marginally acidotic. Based on this methodology, authors conducted two surveys in 15 and 14 dairy farms in Wisconsin, and reported that in the first survey 19% of cows in early lactation and 26% of cows in mild-lactation, and in the second survey 20.1% of cows in early and peak-lactation groups were at risk of SARA (Garrett et al., 1997; Oetzel et al., 1999). Similarly, Stone (1999) conducted a case study on 500 dairy cows in New York State and reported that approximately 33% of cows in mid-lactation group (60-120 DIM) had a pH < 5.6 and more than 50% of cows had a pH < 5.8. Another survey in Germany and The Netherlands (Kleen et al., 2004) estimated that the prevalence of SARA is 11% and 18% in early and mid-lactation groups, respectively. Using similar methodology, Morgante et al. (2007) investigated the prevalence of SARA in early to peak-lactation cows (5-60 DIM) in 10 dairy farms of Italy and reported that 3 farms were at risk of SARA and 5 farms were at marginal level. In addition, O'Grady et al. (2008) assessed the prevalence of SARA in grazing Irish dairy cows in the mid-lactation (80-150 DIM) and found that from a total of 144 cows selected from 12 farms, 11% were at risk of SARA and 42% were marginally acidotic. Unfortunately there is no data on the prevalence of SARA in Canada. We speculate that SARA should be more prevalent, particularly in western Canada, as dairy diets are barley

based, which are more rapidly fermentable in the rumen than corn based diets (Beauchemin and Rode, 1997).

Stone (1999) has estimated that only reduction in milk yield (2.7 kg/cow/day), milk fat (0.3% point) and milk protein (0.1% point) in SARA cows results in an economical loss of US$ 400 to 475 per cow per year. However, there is no estimate of the financial loss due to health disorders, culling or veterinary costs that are associated with SARA.

3.0 Cause and Consequences of SARA

3.1 Rumen pH Depression

During SARA, excessive intake of rapidly fermentable carbohydrates results in rapid proliferation of rumen bacteria, and increases the production of VFA and lactic acid in the rumen. However, in the pH range of SARA the equilibrium between lactic acid producing bacteria and lactic acid consuming bacteria still exist, and therefore, lactic acid concentration remains below 10 m*M* in the rumen (Goad et al., 1998). Thus, the decline in rumen pH during SARA is mainly associated with accumulation of VFA and low rumen buffering capacity (Nocek, 1997; Enemark et al., 2002).

In early lactation cows, accumulation of VFA following grain engorgement is due to low absorptive capacity of rumen papilla (Van Soest, 1994), as size and density of rumen papilla can be reduced as much as 50% during dry period (Dirksen et al., 1985). In mid-lactation animals, due to high feed intake the rate of production of VFA exceeds the rate of absorption and passage, and therefore, VFA accumulate in the rumen (Nocek,

1997). In both scenarios, rumen pH declines if rumen buffers can not keep up with rumen acids. The main source of inorganic buffers in the rumen is saliva and its rate of production depends on chewing and rumination (Beauchemin et al. 2008). As a result, diets that contain coarse forage particles increase ruminating activity, saliva production, and therefore, rumen buffering (Beauchemin et al., 2008). More importantly, saliva increases fluid dilution rate, which increases passage rate of VFA from the rumen (Russell, 2002). Ingestion of diets with short particle size or sorting of animals against coarser particles in the diet could reduce rumen buffering, and therefore, rumen pH (Krause and Oetzel, 2006).

Rumen acidity can compromise the barrier function of the rumen epithelium and result in parakeratosis, rumenitis and abscesses of rumen wall (Gary, 2002). The epithelial damage can trigger a local inflammation, while translocation of bacteria or their immunogenic compounds to the circulation can result in systemic inflammation and liver abscesses (Nagaraja et al., 2007). In addition, fibrous tissue formation in the repaired area might reduce the capacity of the epithelium for VFA absorption in future (Krehbiel et., 1995).

High rumen acidity also affects the growth of rumen microorganisms (Russell and Diez-Gonzalez, 1998). The decline in rumen pH increases the degree of pH gradient (ΔpH) between rumen fluid and bacterial intercellular fluid, which is near neutral (Padan et al., 1981). This results in a flux of undissociated VFA, which are lipid-permeable, into the bacterial cells and release of H^+ ions inside the cells (Russell and Diez-Gonzalez, 1998). However, dissociated VFA are non-permeable to the cell membranes and accumulate inside the cells (Russell and Diez-Gonzalez, 1998). Every unit of ΔpH results

in approximately 10 fold accumulation of dissociated VFA inside the cells and that impairs the metabolism in the microorganisms that are dependent on proton motive force for ATP generation (Russell and Wilson, 1996). Gram-negative bacteria are more sensitive to acidic pH than Gram-positives (Russell and Diez-Gonzalez, 1998). This difference is due to the mechanism that bacteria employ to regulate their intracellular pH. Gram-negative bacteria maintain a constant intracellular pH and have a high ΔpH, therefore, are highly affected by anion accumulation. In contrast, Gram-positive bacteria generate a low ΔpH, as they are able to produce ATP and grow at lower intracellular pH, thus preventing anion accumulation (Russell and Diez-Gonzalez, 1998). However, the decline in intracellular pH might change their fermentative pathway and its end-products (Russell and Wilson, 1996). An example is *Streptococcus bovis,* which is able to reduce the intracellular pH to 5.4, but switches from acetate, formate, and ethanol production to lactate fermentation (Russell and Wilson, 1996).

As rumen pH fluctuates throughout the day, the duration of time and area below the SARA threshold determines the degree of adverse effects of pH on epithelial health and function, and microbial growth (Enemark et al., 2004). Gozho et al. (2005) indicated that at least 180 min/d of pH < 5.6 is required for the activation of inflammatory response seen during grain-induced SARA. Previous studies indicated that average durations of rumen pH < 5.6 was 506 (Krause and Oetzel, 2005), 412 (AlZahal et al., 2008) 328 (Bevans et al., 2005), and 309 min/d (Gozho et al., 2007), when SARA was induced by feeding high grain diets. In these studies SARA induction was achieved by replacing 20% (Krause and Oetzel, 2005), 28.4% (AlZahal et al., 2008), and 25% (Bevans et al., 2005;

Gozho et al., 2007) of the DM of the TMR with ground or pelleted wheat-barley or dry rolled barley.

3.2 Rumen Hyperosmolality

Rumen osmolality is a measure of concentration of substances such as minerals, VFA, lactate, and glucose in the rumen fluid, and affects the water transport into or out of the rumen and rumen bacterial cells (Owens et al., 1998). Osmolality of the rumen fluid is in the range of 240 to 265 mOsm/Kg in the forage based diets and 280 to 300 mOsm/kg in concentrate based diets (Owens et al., 1998). In contrast, blood osmolality is in the range of 285 to 310 mOsm/kg. During SARA, rumen osmolality may exceed blood osmolality. Brown et al. (2000) and Beavans et al. (2005) reported a rumen osmolality up to 330 and 357 mOsm/kg during grain-induced SARA. This increase in osmolality results in rapid flux of water from blood circulation into rumen epithelial cells and into rumen, and cause swelling and rupture of rumen papilla (Owens et al., 1998). Disruption of barrier function of the rumen epithelium following by a local inflammation is the initial consequences of increased rumen osmolality.

Increased rumen osmolality can also inhibit feed intake. However, the mechanism of action of osmoreceptors in the reticulo-rumen or their existence is under debate (Alen, 2000).

3.3 Feed Intake Fluctuation

Reduced or erratic feed intake is the most important sign of SARA in dairy cows (Kleen et al., 2003; Plaizier et al., 2008) and in beef cattle (Brown et al., 2000; Nagaraja and Lechtenberg, 2007). This reduction in DMI can be due to elevated levels of VFA in rumen, and glucose in blood (Oba and Allen, 2003), as well as the hyperosmolality of rumen, dehydration (Allen, 2000), and reduced rumen contraction (Kleen et al., 2003) that have been observed in SARA cows. However, there are several studies that reported no difference (Bevans et al., 2005; Gozho et al., 2007) or increased (Krause and Oetzel, 2005; Gozho et al., 2006) DMI during experimentally grain-induced SARA. Bradford and Allen (2007) and Allen (2000) discussed the multi factorial and complex regulation of feed intake suggesting that different DMI response to high starch diets is most importantly correlated to plasma insulin concentration of individual animals prior to treatment initiation and their insulin secretory ability in response to high starch diets. Animals with greater plasma insulin prior to high starch treatments or lower insulin secretion in response to glucose showed greater depression in feed intake (Bradford and Allen, 2007). Other reasons for the discrepancy in DMI response during SARA might include differences in the contents of fiber and ensiled forages, in particle size and in starch fermentability among the diets used to induce SARA (Allen, 2000), as well as differences in endotoxin tolerance among individual cows (Hurley, 1995).

Feed intake can be affected by VFA and Ac:Pr ratio in the rumen, as propionate but not acetate has hypophagic effects (Allen et al., 2005). However, when propionate is oxidized to acetyl CoA, DMI is reduced, but this does not occur if propionate is used for gluconeogenesis (Allen et al., 2005). Thus, factors that influence the pool of acetyl CoA,

demand for glucose, blood insulin and insulin sensitivity of the liver cells can alter the effect of propionate on feed intake (Allen et al., 2005; Bradford and Allen, 2007). Sauvant and Mertens (1998) have reported that an Ac:Pr ratio < 2 is associated with SARA.

Feed intake also can be reduced as a result of inflammation or endotoxemia in dairy cows (Waldron et al., 2003; Steiger et al., 1999). Gozho et al. (2005; 2006; 2007) and Emmanuel et al. (2008) reported that SARA is associated with an increase in acute phase proteins serum amyloid-A (SAA), and haptoglobin (Hp) in the peripheral blood. Release of these acute phase proteins is mediated by proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 (Sriskandan and Altmann, 2008). These mediators, when released, inhibit feed intake (Steiger et al., 1999). Alternatively, presence of free LPS in blood reduces the insulin sensitivity of the liver cells through the function of CD14, a cell membrane receptor of LPS, and increases blood glucose level (Cani et al., 2007). These changes can alter the direction of propionate metabolism in hepatocytes from gluconeogenesis to oxidation as acetyl CoA, which cause satiety and inhibits feed intake.

3.4 Altered Milk Composition

Induction of SARA is associated with decline in milk fat percentage and yield (Nocek, 1997; Kleen et al., 2003). The reduction in milk fat during SARA has been traditionally explained by the lower acetate + butyrate to propionate ratio in the rumen, as acetate and butyrate are precursors for *de novo* milk fat synthesis (Sutton et al., 1987). In cows in early lactation, increase in plasma insulin levels has been considered as the cause

of milk fat depression. This view is supported by the fact that insulin increases the utilization of acetate, β- hydroxy butyrate, and long chain fatty acids from dietary sources in adipose tissue and reduces lipolysis resulting in shortage of lipogenic precursors for milk fat synthesis (Bauman and Griinari, 2001). However, Bauman and Griinari (2003) examined a series of theories that might be the cause of diet-induced low milk fat and concluded that the above mentioned mechanisms are associated with low milk fat, but are not the cause of milk fat depression. The authors explained that the reduction in milk fat synthesis during ingestion of high-energy low-fiber diets is due to incomplete biohydrogenation of polyunsaturated fatty acids in the rumen under low pH conditions. These unique fatty acids are able to inhibit the synthesis of milk fat in the mammary glands. An example of these groups is *trans*-10, *cis*-12 conjugated linoleic acid that is associated with reduction in milk fat. Current research suggests that profile of milk fatty acids, particularly ratio of *trans*-10 C18:1 to *trans*-11 C18:1, might be considered as an additional indicator of SARA (Enjalbert et al., 2008)

In case of milk protein, experimentally grain-induced SARA is associated with increased protein percentage, which might be related to the increase in bacterial growth and microbial protein synthesis in the rumen in response to high energy diets (Plaizier, 2008). However, cows that develop SARA in the field are usually associated with a reduction in milk protein percentage (Stone, 1999), which might be due to the differences in the cause of pH depression.

3.5 Inflammation

Inflammation has recently been documented as a characteristic feature of grain-induced SARA in dairy and beef cattle (Kleen et al., 2003; Gozho et al., 2007; Emmanuel et al., 2008). This finding is supported by the fact that plasma levels of acute phase proteins SAA, and Hp increase shortly after induction of SARA (Gozho et al., 2005; 2006; Emmanuel et al., 2008). Acute phase proteins are serum proteins that are mainly synthesized by hepatocytes at steady state concentrations (Kushner and Rzewnicki, 1994). However, following an inflammatory stimulus, such as tissue injury or infection, circulating levels of acute phase proteins change substantially to contain the tissue damage and enhance the process of repair and resolution. Hence, the plasma concentrations of the acute phase proteins can be monitored as biomarkers of inflammation (Horadagoda et al., 1999). It is well established that initiation and magnitude of acute phase response is coordinated through production of pro- and anti-inflammatory cytokines such as tumor necrosis factor (TNF) -α, interleukin (IL) -1, IL-6, IL-12, and IL-8 (Sriskandan and Altmann, 2008). However, the molecular triggers for the excessive production of these mediators, which as a result triggers an acute phase response during grain-induced SARA, are not clear.

The dietary-induced changes in gut microbiota and increase in its permeability are speculated as the potential cause of inflammation seen in SARA-induced cows (Kleen et al., 2003, Emmanuel et al., 2007, Plaizier et al., 2008). Disruption of gut barrier function allows the enteric bacteria or immunogenic compounds, such as LPS and bioamines, to translocate into the lymph or blood circulation and trigger an inflammatory response (Plaizier et al., 2008). Gut barrier impairment might occur as a result of high rumen acidity and osmolality

as well as increased luminal LPS level during grain-induced SARA (Nocek, 1997; Chin et al., 2006).

3.6 Cytotoxicity of LPS

The LPS is a virulence factor in host-microbial interactions that is found in the outer membrane of Gram-negative bacteria and released either from intact cells during the logarithmic phase of growth or from the lysed cells (Hurley, 1995). Chin et al. (2006) in an in vitro study using *E. coli* LPS (026:B6) indicated that an increase in luminal LPS induces cell apoptosis, disrupts tight junction protein zonula occludens-1, and increases epithelial permeability in a dose and time dependent manner. In vitro experiments with *E. coli* LPS (0111:B4) also showed that luminal LPS alters the expression and/or localization of tight junctional structure and function through inducible nitric oxide ($NO^{\cdot}$) synthase (iNOS) and increases production of $NO^{\cdot}$, which somehow reduces Na^{+} transport activity and results in swelling and dysregulation of tight junctional protein expression, and therefore, results in barrier failure (Han et al., 2004). These findings are supported by other studies, which showed that *E. coli* LPS predisposes a variety of cell types, including epithelial cells in the lung and kidney as well as endothelial and colorectal cell lines, to apoptosis (Ortiz-Arduan et al., 1996; Schildberg et al., 2005; Yu et al., 2005; Neff et al., 2006). Moreover, excessive amount of $NO^{\cdot}$ can combine to superoxide anions (O_2-) to form toxic peroxynitrite ($ONOO^{-}$), which irreversibly blocks many components of the mitochondrial respiratory chain and trigger epithelial cell death by apoptosis (Bossy-Wetzel and Lipton, 2003). In addition, Cetin et al. (2004) demonstrated that *E. coli* LPS impairs the pH regulatory system of enterocytes by inhibition of sodium-proton pumps,

under extracellular acidosis condition, resulting in cytoplasmic acidification and cellular dysfunction.

Due to distinct differences in the composition of rumen epithelium with other epithelial structures it is not clear if that LPS can impair its barrier function. Epithelium in the intestine, lung or kidney has a simple monolayer structure with tight junctions at the apical pole of the cells, whereas rumen epithelium has a multilayer structure with tight junctions located in the middle layers, stratum granulosum and spinosum (Graham and Simmons, 2005). The external layer of the rumen epithelia has no tight junctions, but it may consist of up to 15 cell layers, which may limit the permeability of large molecules such as LPS (Baldwin, 1998).

The view that rumen epithelium might have a low permeability to LPS is supported by studies by Lassman (1980) and Anderson (1984), who reported that infusion of Cr-labeled *E. coli* LPS (0128-B12) into the rumen of steers under high forage or high concentrate diets or ruminal acidosis condition, did not result in LPS translocation into the thoracic duct (lymph) or the portal vein. In contrast, Emmanuel et al. (2007) used Ussing chambers to evaluate the translocation of *E. coli* LPS (B:055) across the rumen and colon tissue under low pH conditions. These authors indicated that LPS translocates across the rumen wall at a greater rate than the colon wall and this translocation was pH independent. However, the rate of translocation was numerically higher at pH 5.5 in the rumen and at pH 6.5 in the colon than other pH levels.

The concentration of *E. coli* LPS that was applied to the rumen or colon epithelium in the study of Emmanuel et al. (2008) was 500 μg/mL. This concentration is approximately 50 times greater than free rumen LPS concentration during SARA (Gozho

et al., 2007) or grain engorgement (Emmanuel et al., 2008). Chin et al. (2006) reported that the cytotoxic effect of *E. coli* LPS on enterocyte apoptosis occurred at a luminal concentration of 40 μg/mL after half an hour of administration of LPS and that these effects increased over time. Vreugdenhil et al. (1999) found that luminal *E. coli* LPS (055:B5) concentration up to 10 μg/mL did not increase the release of acute phase proteins by colonic adenocarcinoma Caco-2 cell lines that express structural and functional properties of the small intestine enterocytes. Therefore, the cytotoxic effect of LPS on epithelial permeability might be a function of its concentration in the lumen.

3.6.1 Ruminal and Blood Concentration of LPS

It has been shown that free rumen LPS concentration increases during grain-induced SARA. Gozho et al. (2007) reported a LPS range of 2,454 (control) to 12,882 (grain-induced SARA) ng/mL in Holstein dairy cows (538 kg, 121 DIM). Emmanuel et al. (2008) reported a LPS range from 790 ng/mL in control to 5,021 and 8,870 ng/mL when 30% and 45% barley was supplemented in the diet of Holstein dairy cows (650 kg, 60 to 140 DIM), respectively. Gozho et al. (2005; 2006) reported a lower range of LPS in Jersey steers (678 kg). In these studies, abrupt induction of SARA or gradual adaptation to 61% wheat-barley pellet in the diet, increased the rumen LPS from 375 to 887 ng/mL and from 631 to 871 ng/mL, respectively. Although rumen LPS concentration increased following SARA induction in these studies, it cannot be concluded that elevated concentrations of LPS in the rumen was due to high dietary content of starch or due to low rumen pH.

Among the aforementioned studies, only Gozho et al. (2007) measured LPS in the peripheral circulation. However despite a significant increase in free rumen LPS, no

evidence of LPS in the peripheral circulation was found. There is inconsistency in detection of LPS in peripheral blood during experimentally induced acute ruminal acidosis, which is more severe disorder than SARA. While some studies have detected LPS in peripheral blood (Andersen et al., 1994b, Dougherty et al., 1975), others were unsuccessful (Andersen and Jarlov, 1990). Andersen and Jarlov (1990) stated that endotoxemia is not necessarily related to presence of endotoxin in peripheral blood. Evidence in support of their view comes from studies that investigated the clearance rate of the LPS from the portal circulation by the liver (Andersen and Jarlov, 1990, Andersen, 1994); thereby, they attributed the clinical signs of endotoxemia to the release and presence of inflammatory mediators in circulation.

It must be stated that concentration of LPS in peripheral blood might be at the limit of sensitivity of the assay used for detection of LPS, which make the conclusion of previous studies regarding the absence of LPS in peripheral circulation questionable. This hypothesis is strengthened by the observations of the LPS concentration in peripheral blood during experimentally induced or naturally occurring *Escherichia coli* mastitis, which was in the range of 0.5 to 1.5 endotoxin unit (EU)/ml (Dosogne et al., 2002). Since SARA causes a low-grade inflammation compared to mastitis, a very low concentration of LPS in blood is expected.

3.6.2 LPS Structure and Biological Activity

Most of our information about LPS structure and biological activity was obtained from LPS of various *Enterobacteriaceae* (Brigham, 1994). Therefore, *Enterobacteriaceae* LPS is considered as a "gold standard" and LPS from other bacteria were compared to them.

The LPS molecule consists of a lipid and a polysaccharide portion. The lipid fraction of LPS is called lipid A and is responsible for all the biological and endotoxic activity of LPS. In contrast, polysaccharide portion consists of an O-specific and a core oligosaccharide. The O-specific chain consists of up to 50 repeating units of oligosaccharide and is highly specific for each bacterial strain. Core oligosaccharide consists of an inner core and outer core. While the inner core has a highly conserved structure; the outer core has an intermediate diversity. The inner core consists of at least one and at most two to three molecules of KDO (2-keto-3-deoxyoctonic acid) and two heptose residues (Brigham, 1994).

Based on the structure of O-specific chain, LPS can be divided into two groups: smooth (S)-LPS and rough (R)-LPS. Generally S-LPS have a colonial morphology, while R-LPS have defects in their O-specific chain or core biosynthesis. Therefore, R-LPS may not contain O-specific chain or parts of the core (Brigham, 1994).

The lipid A structure has least structural variability in the LPS molecule. Lipid A consists of 1) a e disaccharide backbone, which is highly conserved, and is mono or di-phosporilated, and 2) up to seven fatty acids of various lengths (C10, C12, C14, C15, C16, C17) that are attached to the D-glucosamine molecule. The difference in the biological endotoxicity of LPS is due to the nature, number, chain length, and location of fatty acids groups as well as the phosphorylation pattern (Brigham, 1994; Schletter et al., 1995). The lipid A of *E. coli* consist of β-(1,6)- linked D-*gluco*-hexos-amine disaccharide carrying two phosphoryl groups in position 1' and 4' with six acyl groups consist of 4 mol of (R)-3-hydroxy fatty acids (C10 to C16) and two secondary fatty acids as (R)-3-acyloxyacyl groups. A slight modification of lipid A composition or three dimensional

structure results in a significant reduction in endotoxic activity of *E. coli* LPS. The lipid A structure is different in *Bacteroides fragilis* from *E. coli* by the absence of one phosphoryl group in the position 4' and presence of only five acyl groups in the lipid A structure (Brigham, 1994). These differences might be the reason for lower endotoxic activity of *B. fragillis* LPS compared to *E. coli* LPS (Brigham, 1994; Weintraub et al., 1989; Kasper et al., 1976).

3.6.3 LPS and Acute Phase Response

Following barrier failure of the gut, LPS could translocate into blood circulation and interact with a specific acute phase protein called LPS binding protein (LBP). The LBP facilitates the transfer of LPS to lipoproteins, which results in neutralization of LPS (Wright et al., 1990). The LBP also facilitate the transfer of LPS to membrane-associate or soluble CD14 (Wright et al., 1990). The LPS-LBP-CD14 complex then can be recognized by toll like receptor (TLR)-4, which is involved in innate immunity (Guha and Mackman, 2001). Detection of LPS by TLR-4 initiates a cascade of events resulting in activation of nuclear factor kappa B (NF-κB), which results in production of pro-inflammatory cytokines and chemokine and finally acute phase response (Sriskandan and Altmann, 2008). Based on the severity of the gut barrier failure and the magnitude of LPS translocation, the immune response may remain local or expand to the systemic level.

3.7 Rumen Microbial Population Shifts

The classical view of acute ruminal acidosis in the rumen is that as grain increases so does the prevalence of starch fermenting rumen bacteria like *Streptococcus bovis*. As a

result VFA and lactate accumulate in the rumen and rumen pH declines to below 5. This pH condition further triggers the proliferation of other lactate producing bacteria such as *Lactobacillus* spp. (Russell and Hino, 1985; Owens et al., 1998) and result in more rumen pH depression. In case of SARA, rumen pH is in the range of 5.2 to 5.6, which might be generated due to excessive intake of rapidly fermentable carbohydrates or low coarse forage particles (Plaizier et al., 2008). If SARA is due to the greater availability of rapidly fermentable substrates, it may result in rapid proliferation of amylolytic and soluble sugar-fermenting bacteria like *Prevotella* spp., *Succinimonas amylolytica, Succinivibrio dextrinosolvens*, and *Ruminobacter amylophilus*, which are Gram-negative, as well as, *Streptococcus bovis, Selenomonas ruminantium*, *Butyrivibrio fibrosolvens*, and *Lactobacillus* spp., which are genetically Gram-positives (Nagaraja and Titgemeyer, 2007). In this scenario, the rapid proliferation of amylolytic bacteria results in greater production of VFA in the rumen. In contrast, *S. bovis, S. ruminantium,* and *Lactobacillus* spp. populations may result in greater production of lactate (Owens et al., 1998). However, in the pH range of SARA, the slower growing lactic acid consuming bacteria like *Megasphaera elsdenii* increase in number and convert lactic acid to VFA (Russell et al., 1981; Owens et al., 1998). In addition to *M. elsdenii*, *S. ruminantium* also can utilize lactic acids, and therefore, this bacterium contributes to both production and consumption of lactate (Nagaraja and Titgemeyer, 2007). Other lactic acid consuming species include *Propionibacterium acnes*, *Veillonella parvula,* and *Anaerovibrio lipolytica,* which are low in number compared to *M. elsdenii* and *S. ruminantium* (Nagaraja and Titgemeyer, 2007). Overall, lactic acid does not accumulate in the rumen during SARA and its concentration remains below 10 m*M* (Goad et al., 1998).

The low rumen pH condition is also associated with a decline in cellulolytic bacteria. Tajima et al. (2001) reported that a shift from forage-based diet to a grain-based diet reduced *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* populations by 10 and 20 fold. Similarly, ciliated protozoa are sensitive to low pH and their population declines in the pH range of SARA (Goad et al., 1998). As protozoa engulf starch, they have an important role in maintaining the rumen pH, and therefore, a reduction in their population may intensify the low pH conditions (Goad et al., 1998).

4.0 Methods for Studying Rumen Microorganisms

Microorganisms present in the rumen interact with each other, with their animal host, and with feed, resulting in a dynamic composition of microbiota (Yu and Forster, 2005). It is estimated that about 500 species of bacteria inhabit the gastrointestinal tract. Prior to advances in molecular biology, these microorganisms were studied using culture-dependent techniques. However, the fact that more than 70% of gut microorganisms are either uncultivable or unidentified makes culture-dependent methods limited (Dorigo et al., 2005; Yu and Forster, 2005).

Today, molecular approaches are widely used to assess the dynamics of the microbial composition of different ecosystems. These culture-independent techniques are more sensitive and more accurate tools than culture dependent methods for studying the composition and structure of the gut microbiota (Yu and Forster, 2005). These molecular tools often allow the monitoring of the dynamics of the microbial composition in a defined environment, as well as the impact of the specific factor on microbial composition (Dorigo et al., 2005). Among the numerous tools available, we will discuss

those that rely on the amplification of the target sequence of DNA or RNA extracted from gut samples. The most commonly used primers for the detection and quantification of rumen bacteria have been based on 16S rRNA gene sequences. This is mainly the result of the availability of a large number of 16S rRNA sequences in the public databases such as Ribosomal Database Project (RDP; Cole et al., 2005; Yu and Forster, 2005).

4.1 Real-time PCR

Real-time PCR or quantitative PCR (qPCR) is a technique based on the polymerase chain reaction, which is used to amplify and simultaneously quantify a targeted DNA molecule. Conventional PCR assays only detect the final product of amplification at the end of the PCR reaction, where exponential amplification is no longer achieved (Denman and McSweeney, 2005). In contrast, using real-time PCR or qPCR, quantification of the product is done at the end of each cycle during the exponential phase, where theoretically each cycle will result in a doubling of the amplicons. This allows viewing the entire reaction, monitor the product being generated, and quantify the amplicons more accurately (Denman and McSweeney, 2005). Real-time PCR can be used to assess the changes in the whole rumen microbiota (using universal primers) or in a specific species or genus of rumen microorganisms (using specific primers) (Yu and Forster, 2005). Real-time PCR assay is becoming the gold standard method of evaluating the changes in the abundance of DNA and RNA (Yu and Forster, 2005).

4.2 Clone Libraries and Sequencing

In this approach, 16S ribosomal genes of the total rumen bacteria are amplified (using universally conserved primers), cloned and randomly sequenced (Dorigo et al., 2005). Sequencing of 16S rRNA genes has become the standard procedure in the identification of bacterial isolates (Mackie et al., 2007). Analysis of the resulting sequences leads to the identification of the dominant rumen microorganisms. Comparing these sequences with those available in databases, reveals the information about the identity or relatedness of new sequences to known species (Dorigo et al., 2005). The sequence information can also be used to compare community composition and species richness, which is simply number of species present, and diversity, which is combination of richness and abundance of each species, in different samples (Dorigo et al., 2005). Despites the biases introduced by PCR and cloning procedure, sequencing of 16S rRNA gene clone libraries remain an accurate and reliable method for studying the bacterial composition of rumen (Mackie et al., 2007). However, in a high diversified ecosystem, this approach could be very laborious, time-consuming and expensive, and therefore, not suited for monitoring the dynamics of the microbial communities (Dorigo et al., 2005; Mackie et al., 2007).

4.3 Community Fingerprinting Techniques

Fingerprinting of the 16S rRNA genes is a suitable approach for studying microbial diversity, for monitoring the microbial shifts in a defined community, and for comparing different communities (Mackie et al., 2007; Kocherginskaya et al., 2005). Denaturing gradient gel electrophoresis (DGGE) and thermal gradient gel electrophoresis (TGGE) are two commonly used fingerprinting approaches based on the same principals. These routinely used methods involve amplification of 16S rRNA genes followed by the

separation of these fragments in a polyacrylamide gel. The DGGE and TGGE were developed based on the fact that the amplicons of the same size that differ by at least one nucleotide can be separated by electrophoresis through a gradient of increasing chemical denaturants of urea and formamide (DGGE), or through a temperature gradient (TGGE) (Dorigo et al., 2005). The resulted DGGE/TGGE pattern comprises a profile of multiple bands representing the sequence diversity within the bacterial community (Mackie et al., 2007). Most DGGE/TGGE studies focus on the number of the different bands to get an estimate of the community richness, but there have been few studies that also take into account the intensity of each band as an indicator for the relative abundance of that bands population (Dorigo et al., 2005). Likewise, the similarity indices, which are indicator of similarity of species between communities, can be calculated and the clustering analysis of the profiles can be performed (Mackie et al., 2005). The main limitations of these techniques are the optimization of the gel running conditions and the difficulty of comparing patterns across the gels (Dorigo et al., 2005). Also, DGGE and TGGE are not sensitive enough to represent the bacteria that make up less than 1% of the total bacterial community, which means that only most dominant bacteria will be detected by this approach (Mackie et al., 2007). The DGGE and TGGE are less labor intensive and biased than 16S rRNA clone libraries and allow rapid estimation of microbial diversity (Kocherginskaya et al., 2005).

Random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) are two molecular techniques not relying on the conserved 16S ribosomal gene for phylogenetic studies, but rather focusing on amplification of random genomic sequences (Denman et al., 2005). RAPD analysis is different from ribosomal-

based approaches in that no prior genetic information of the subject is required. A small random nucleotide primer of about 10 bp is employed under low stringency conditions for PCR amplification. Usually, several primers will be tested and the one which produces the highest level of community discrimination will be chosen (Denman et al., 2005). The AFLP is a DNA fingerprinting technique, which detects multiple DNA restriction fragments by the mean of PCR amplification. In this method, the community DNA will be digested, ligated with the adaptors, and selectively amplified using two primers complementary to the adapter and restriction site sequences. Although, only a subset of fragments is amplified using this technique, a typical AFLP profile contains 50-100 different bands. As was the case with RAPD, the application of the AFLP requires no prior sequence information. The AFLP profiles are analyzed for pattern similarities (Denman et al., 2005). Depending on the degree of polymorphism between samples, individual samples can be fingerprinted using a wide range of restriction enzymes in combination with different primers.

Ribosomal intergenic spacer analysis (RISA) involves the PCR amplification of the intergenic spacer region (ITS) located between the small (16S) and large (23S) subunits of the ribosomal gene. This region is highly variable in both size and sequence (Dorigo et al., 2005; Denman et al., 2005). Primers are designed to target the conserved regions of 16S and 23S genes. The amplification products are separated on the gel based on their size heterogeneity. The RISA is a particularly powerful tool to discriminate between closely related strains (Denman et al., 2005). The use of fluorescently labeled primers allows the analysis to be carried out on an automated capillary electrophoresis. This automated ribosomal intergenic spacer analysis (ARISA) is a more rapid, accurate and

efficient version of the RISA (Dorigo et al., 2005). The number of fluorescent peaks detected by capillary electrophoresis is an estimate of species richness within a given sample (Dorigo et al., 2005). Additionally, the sizes of the detected fragments can be compared to the ones available in databases, leading to potential identification of the amplicons population (Dorigo et al., 2005).

Restriction fragment length polymorphism (RFLP) and terminal restriction fragment length polymorphism (T-RFLP) are two rapid screening methods that present the ribosomal diversity patterns of complex communities, in a relatively easy and reproducible manner (Denman et al., 2005). In RFLP, a portion of the 16S rRNA gene is amplified from total bacterial DNA and then subjected to restriction digestion. Resulting products are separated on the gel and the pattern of the bands is a representative of the phylogenetic diversity within microbial populations (Denman et al., 2005). T-RFLP is an advancement of the RFLP technology, which implies the fluorescent labeling of one or both of the primers followed by the detection of terminal restriction fragment (T-RF) by a capillary electrophoresis. T-RFLP is a high-throughput and reproducible method of community structure analyses, both qualitatively and quantitatively (Dorigo et al., 2005). The number of the peaks represents the community richness and the area under each peak indicates the relative abundance of that fragment (Dorigo et al., 2005). One advantage of T-RFLP is its ability to detect the rarer members of the microbial community. Moreover, the comparison of T-RF sizes of the subjects to databases that predict T-RF sizes of known bacteria can lead to the phylogenetic assignment of these fragments (Dorigo et al., 2005). It has been shown that the choice of primers and restriction enzymes are important for obtaining an accurate evaluation of the microbial diversity. Also, there are concerns

about the formation of pseudo T-RFs, which can result in the overestimation of the microbial richness (Dorigo et al., 2005). Overall, T-RFLP seems to be a useful tool for assessing richness and diversity in microbial communities that are not extremely complex (Dorigo et al., 2005).

5.0 Summary

Grain-induced SARA is associated with an increase in free LPS in the rumen fluid. Grain-induced SARA is also associated with an increase in acute phase proteins SAA and Hp in the peripheral blood. In order to have a systemic effect, LPS must cross the gut wall into blood circulation. However, to date, no evidence of LPS in the peripheral circulation of SARA-induced cows has been found. The specific role of LPS in the inflammatory responses seen during SARA is also not clear, as SAA and Hp are general acute phase proteins and their concentration might also increase due to inflammatory stimuli other than LPS. On the other hand, the correlation between decreased rumen pH, increased free rumen LPS, and increased acute phase proteins as inflammatory markers in the peripheral blood have been only studied when SARA was experimentally induced by increasing the dietary content of starch. Therefore, it cannot be concluded that the clinical signs, especially inflammation, that are associated with grain-induced SARA are due to low pH in the rumen or due to high starch content of the diet. Moreover, microbiology of the rumen during SARA is not well studied and the limited information that is available is provided through culture-based studies, which only allows for the isolation of a limited number of species of microorganisms.

Application of culture-independent techniques brings new insights into understanding the underlying cause of SARA.

HYPOTHESES

1. Provision of substrate in the form of wheat-barley pellets or alfalfa-pellets can reduce rumen pH in magnitude and duration that is typical of SARA (< 5.6 for > 180 min/d)
2. Free rumen LPS concentration will be similar in grain-induced and alfalfa pellet-induced SARA if release of LPS is due to same pH depression.
3. Translocation of LPS from the gut into the peripheral blood is an outcome of SARA challenge.
4. Increase in the systemic LPS binding protein indicates that LPS translocated from the gut into the circulation.
5. Rumen microbial profile in the rumen in grain-induced SARA is different from alfalfa pellet-induced SARA.

OBJECTIVES

Two nutritional models of SARA, one based on wheat-barley pellets and the other based on alfalfa pellets were developed to test the following:

1. To determine if grain-induced SARA causes translocation of LPS from the gut into peripheral circulation.
2. To determine if inflammatory response seen during grain-induced SARA is associated with LPS.
3. To determine if SARA induced by feeding wheat-barley pellets or alfalfa pellets results in similar increases in free LPS in rumen fluid.
4. To determine if SARA induced by feeding alfalfa pellets results in translocation of LPS into peripheral blood, and if this method of SARA induction stimulates a systemic inflammatory response.
5. To compare the global microbial changes in the rumen between grain-induced and alfalfa pellet-induced SARA.
6. To quantify key microbial species of the rumen during grain-induced and alfalfa pellet-induced SARA.

MANUSCRIPT I

A Grain-based Subacute Ruminal Acidosis Challenge Causes Translocation of Lipopolysaccharide and Triggers Inflammation in Dairy Cows

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ABSTRACT

The effects of grain-induced subacute ruminal acidosis (SARA) on translocation of lipopolysaccharide (LPS) into the peripheral circulation, acute phase proteins in blood and milk, feed intake, milk production, and blood metabolites were determined in eight lactating Holstein cows. Between wk 1 and 5 of two successive 6 wk periods, cows received total mixed ration (TMR) ad libitum with a forage to concentrate (F:C) ratio of 50:50. In wk 6 of both periods, SARA was induced by replacing 21% of the DM of the TMR with pellets containing 50% wheat and 50% barley. Rumen pH was monitored continuously using indwelling pH probes in four rumen cannulated cows. Rumen fluid samples were collected 15 min before feeding and at 2, 4, 6, 12, 14, 16, 18, and 24 h after feeding for two days during wk 5 (control) and wk 6 (SARA). Peripheral blood samples were collected using jugular catheters 15 min before feeding and at 6 and 12 h after feed delivery on the same days of rumen fluid collections. Induction of SARA significantly reduced average daily pH from 6.17 to 5.97 and increased the duration of rumen pH below pH 5.6 from 118 to 279 min/d. Induction of SARA reduced dry matter intake (16.5 vs. 19 kg/d), milk yield (28.3 vs. 31.6 kg/d), and milk fat (2.93 vs. 3.30%, 0.85 vs. 0.97 kg/d), and tended to increase milk protein percentage (3.42 vs. 3.29%), without affecting milk protein yield (1.00 vs. 0.98 kg/d). Induction of SARA also increased the concentration of free LPS in rumen fluid from 28,184 to 107,152 endotoxin units (EU)/mL. This was accompanied by an increase in LPS in peripheral plasma (0.52 vs. < 0.05 EU/mL) with a peak at 12 h after feeding (0.81 EU/mL). Concentrations of the acute phase proteins serum amyloid A, haptoglobin, and LPS binding protein (LBP) in peripheral blood as well as LBP concentration in milk increased (438.5 vs. 167.4 μg/mL,

475.6 vs. 0 μg/mL, 53.1 vs. 18.2 μg/mL, 6.94 vs. 3.02 μg/mL, respectively) during SARA. Increased LBP provides additional evidence of translocation of LPS. Results suggest that grain-induced SARA resulted in translocation of LPS into the peripheral circulation, which triggered a systemic inflammatory response.

Key words: subacute ruminal acidosis, LPS translocation, acute phase response

INTRODUCTION

Grain-induced subacute ruminal acidosis (SARA) increases the concentrations of the acute phase proteins serum amyloid A (SAA), and haptoglobin (Hp) in peripheral blood of cattle (Gozho et al., 2007; Plaizier et al., 2008; Emmanuel et al., 2008). These increases in acute phase proteins, which are part of acute phase response, indicate that SARA causes inflammation (Horadagoda et al., 1999). This inflammation could be initiated by dietary induced damage to the gut mucosa or by translocation of immunogenic compounds into circulation, such as free lipopolysaccharide (LPS) (Horadagoda et al., 1999).

There is substantial evidence that SARA increases the content of free LPS in the rumen due to the increase in lysis of Gram-negative bacteria (Gozho et al., 2007; Nagaraja and Lechtenberg, 2007; Plaizier et al., 2008). This increase in luminal LPS could increase permeability of the gut for LPS (Chin et al., 2006). Also, the barrier function of the rumen epithelium may be compromised by the parakeratosis of rumen epithelium, rumenitis and abscesses of the rumen wall that result from high rumen acidity (Kleen et al., 2003). Additionally, the high rumen osmolality that is seen during SARA can cause swelling and rupture of ruminal papillae, which will also reduce the barrier function of the rumen. Despite this, no evidence of LPS in the peripheral circulation during SARA has been found (Gozho et al., 2007). There is also inconsistency in detection of LPS in peripheral blood during experimentally induced acute ruminal acidosis (Dougherty et al., 1975; Andersen and Jarlov, 1990; Andersen et al., 1994b). During recent years the sensitivity and accuracy of bioassays used to detect LPS in low concentrations has been substantially improved, which makes the conclusion of previous

studies regarding the absence of LPS in peripheral circulation questionable. The increases in the concentrations of the acute phase proteins SAA and Hp in peripheral blood do not prove translocation of LPS, as these concentrations can increase due to other inflammatory stimuli. However, as LPS interacts with a specific acute phase protein, LPS binding protein (LBP), an increase in LBP in peripheral circulation will provide evidence of the translocation of LPS (Sriskandan and Altmann, 2008).

The main objective of this study was to determine if grain-induced SARA causes translocation of LPS from the gut into peripheral circulation. This was achieved by measuring plasma LPS using a high sensitivity assay and by monitoring LBP levels in peripheral plasma and milk. The effects of grain-induced SARA on feed intake, milk production and composition, blood metabolites, and SAA and Hp in peripheral blood plasma and serum, respectively, were also determined.

MATERIAL AND METHODS

Animals, Diets, and Experimental Procedures

Eight lactating Holstein cows, four of which were ruminally cannulated, were used during two subsequent 6 wk periods. Cows were on average 84 ± 29 DIM (mean ± SD) with an average milk yield of 35 ± 3.9 kg/d and had an average BW of 615 ± 68 kg at the beginning and 634 ± 50 kg at the end of experiment. Cows were housed in individual tie-stalls in Glenlea Dairy Research Unit at the University of Manitoba (Winnipeg, MB, Canada) in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 1993). Animals were randomly allocated in two groups consisting of two

cannulated and two non-cannulated cows. During wk 1 to 5 of each 6 wk period, cows received a TMR ad libitum with a forage to concentrate (F:C) ratio of 50:50 (Table 1, 2, 3), with the intention of allowing for 5 to 10% orts. During wk 6 of both periods, SARA was induced by replacing 21% of the DM of the TMR with pellets containing 50% ground wheat and 50% ground barley, resulting in a F:C of 40:60 (Table 1). Cows had unlimited access to fresh water throughout the experiment. Data obtained from wk 5 of each period was considered as control and compared to wk 6 (SARA). Samples of diets, dietary ingredients, and orts were collected and analyzed by wet chemistry as described by Bhandari et al. (2007). The physical characteristics of wheat-barley pellets including dimension, bulk density and water holding capacity (WHC) were determined as described by Giger-Reverdin (2000).

Rumen pH Measurement

Rumen pH was monitored continuously for four consecutive days during wk 5 and wk 6 of both experimental periods in four rumen cannulated cows using indwelling pH probes as described by Gozho et al. (2006). The pH data were summarized as average pH, time spent below pH 6.0, time spent below pH 5.6, area (time × pH) spent below pH 6.0, and area spent below pH 5.6 for each 24 h period.

Table 1. Ingredients, nutrient composition, and forage to concentrate ratio (F:C) of the total mix ration (TMR) and wheat-barley pellets (WBP)

Item	Control diet	SARA Diet	WBP
	Ingredients, % of DM		
Alfalfa silage	25.0	20.0	-
Barley silage	25.0	20.0	-
Energy supplement	40.0	31.6	-
Protein supplement	10.0	7.8	-
Ground wheat	-	10.3	50.0
Ground barley	-	10.3	50.0
F:C	50:50	40:60	0:100
	Nutrient composition		
DM, %	52.4	60.1	89.2
CP, % of DM	16.9	16.5	14.8
NDF, % of DM	35.7	30.4	11.4
ADF, % of DM	24.3	22.7	5.7
NFC[1], % of DM	32.7	40.4	68.6
Starch, % of DM	26.1	33.4	60.7
Crude fat, % of DM	5.3	4.5	2.2
Ash, % of DM	9.4	8.2	3.0
Ca, % of DM	1.11	0.92	0.22
P, % of DM	0.53	0.52	0.54
K, % of DM	1.91	1.60	0.56
Mg, % of DM	0.32	0.32	0.16
Na, % of DM	0.32	0.26	0.03
Predicted NE_L[2], Mcal/kg DM	1.57	1.66	-

[1]Non fiber carbohydrates = 100 – (NDF% + CP% + Crude fat% + Ash%)

[2]NE_L values are predicted using CNCPS (Cornell Net Carbohydrate and Protein System) software version 5.0.4, Cornell University, Ithaca, NY.

Table 2. Nutrient composition of the forages and supplements

Item	Energy supplement	Protein supplement	Barley silage	Alfalfa silage
DM, %	90.4	91.7	26.5	57.5
CP, % of DM	18.4	31.1	11.1	19.6
NDF, % of DM	16.2	42.4	55.5	48.3
ADF, % of DM	9.2	15.5	40.9	33.7
NFC[1], % of DM	53.6	2.9	18.8	19.5
Starch, % of DM	35.9	2.5	11.3	12.2
Crude fat, % of DM	5.0	6.7	3.0	1.9
Ash, % of DM	6.8	16.9	11.6	10.7
Ca, % of DM	0.82	3.11	0.39	1.04
P, % of DM	0.68	1.07	0.39	0.41
K, % of DM	0.97	1.00	2.29	2.99
Mg, % of DM	0.30	0.29	0.31	0.37
Na, % of DM	0.30	1.70	0.06	0.12

[1]Non fiber carbohydrates = 100 – (NDF% + CP% + Crude fat% + Ash%)

Table 3. Ingredient composition of the energy and protein supplements (% of DM)

Ingredient	Energy supplement	Protein supplement
Rolled barley	54.0	-
Luprosil salt (calcium propionate)	0.2	-
Protein pellet[1]	1.8	-
Dairy supplement[2]	40.0	-
Vegetable oil	4.0	-
Corn dried distillers with solubles	-	42.0
Fish meal	-	7.0
Canola meal	-	22.7
Soybean meal	-	20.0
Beet molasses	-	3.0
Niacin	-	0.3
Sodium bicarbonate	-	5.0

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

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

Rumen Sampling and Analysis

Rumen fluid samples were collected from the ventral sac of the rumen of rumen cannulated cows 15 min before feeding and at 2, 4, 6, 12, 14, 16, 18, and 24 h after feeding on two consecutive days of each sampling week. Ruminal contents were strained through four layers of sterile cheesecloth and divided into two portions. The first portion of samples were transferred into 50 mL sterile tubes and kept in ice until transported to the laboratory for the initial processing prior to LPS determination as described by Gozho et al. (2005). In brief, rumen fluid samples were centrifuged at 10,000 × *g* for 45 min. The supernatant was aspirated gently to prevent its mixing with the pellet and passed through a disposable 0.22 μm LPS-free filter (Millex, Millipore Corporation, Bedfored, MA). The filtrate was collected in a sterile, depyrogenated glass tube (previously heated at 180°C for 4 h) and heated at 100°C for 30 min. Samples were cooled at room temperature (19°C) for 10 min and stored at - 20°C for subsequent LPS measurement. The second portion of the rumen fluid samples was immediately centrifuged at 1900 × *g* for 15 min and the supernatant was stored at -20°C until analyzed for osmolality, VFA, lactate, and ammonia.

Free Rumen LPS. Free rumen LPS content was determined by a chromogenic *Limulus* amebocyte lysate (LAL) end-point assay (QCL-1000, Lonza group Ltd, Basel, Switzerland). Pre-treated rumen samples were diluted until their LPS concentrations were in the range of 0.1–1 endotoxin units (EU)/mL relative to the reference endotoxin (*E. coli* O111:B4), and assayed as described by Gozho et al. (2005).

Rumen VFA, Lactate, Ammonia and Osmolality. Concentrations of VFAs (acetate, propionate, butyrate, valerate, isobutyrate, and isovalerate) and lactate were determined by gas chromatography (GC; Model 3400 Star, Varian, Walnut Creek, CA) as described by Bhandari et al. (2007) with some modifications. In brief, rumen fluid samples were thawed at room temperature and 1 mL of 25% meta-phosphoric acid solution was added to 5 mL of rumen fluid. The tubes were vortexed and stored at -20°C overnight. Samples were thawed again, and 0.4 mL of 25% sodium hydroxide and 0.64 mL of 0.3 *M* oxalic acid were added to the tubes and vortexed each time. The tubes were centrifuged at 3000 × *g* for 20 min and 2 mL of supernatants were transferred into GC vials for analysis. Ammonia nitrogen concentration of rumen fluid samples was determined using a colorimetric technique as described by Bhandari et al. (2007). Osmolality of rumen fluid samples was determined by freezing point depression using an automatic osmometer (Advanced single sample micro-osmometer Model 3300, Advanced Instruments Inc., Norwood, MA).

Blood Sampling and Analysis

Blood samples were collected using jugular catheters 15 min before feeding and at 6 and 12 h after feed delivery on two subsequent days in each sampling week. Catheters and their extenders were filled with sterile heparinized 0.9% saline (5000 IU/ 100 mL) to prevent clotting after each sampling. Samples were collected in a blank 10 mL vacutainer (Fisher Scientific, Fairlawn, NJ) for serum harvesting and in a 10 mL heparinized vacutainer (Fisher Scientific, Fairlawn, NJ) for plasma collection. The serum vacutainers were allowed to clot at room temperature (19°C) for 45 min, and then centrifuged at 1900 × *g* for 15 min. Subsequently, serum was aspirated and stored in -20°C until used for Hp

analysis. Plasma was harvested by centrifuging of heparinized vacutainers at 1900 × *g* for 15 min. A portion of plasma was transferred to pyrogen-free glass tubes and stored at –20°C for LPS analysis. The rest of plasma was divided into 2 mL aliquots and stored in -20°C until analyzed for SAA, LBP, glucose, and lactate. Plasma glucose and lactate were analyzed using a blood gas and electrolyte analyzer (Nova Stat Profile M, Nova Biomedical Corporation, Waltham, MA) as described by Bhandari et al. (2007).

LPS Analysis in Blood Plasma. The concentration of LPS in plasma was determined by a chromogenic kinetic LAL assay (Kinetic-QCL, Lonza group Ltd, Basel, Switzerland) with a minimum detection limit of 0.005 EU/mL. Samples were initially treated as recommended by Dosogne et al. (2002) and Hourly (1995) with some modifications to inactivate inhibitory factors in plasma. In brief, frozen plasma samples were thawed at 37°C, vortexed, and 100 µL of each sample were diluted at least 10 fold with LAL water. Diluted samples were incubated at 37°C for 30 min, then heated at 75°C for 15 min and cooled to room temperature (19°C) for 45 min. Then, a metallo-modified polyanionic dispersant called Pyrosperse (Lonza group Ltd, Basel, Switzerland) was added to the test samples at a ratio of 1/200 (vol/vol) prior to LAL testing. The kinetic LAL assay was performed in a 96-well microplate. The appearance of a yellow color was monitored over time using an incubating microplate spectrophotometer (Spectra Max 340 PC, Molecular Devices Corporation, Sunnyvale, CA) to determine the reaction time, which is defined as the time required for absorbance to increase 0.2 optical density (OD). A log/log linear correlation of the mean reaction time of each standard with its corresponding endotoxin concentration was performed to create a standard curve and predict endotoxin concentration of test samples. To verify the lack of product inhibition,

samples were spiked with 10 μL of 2 EU/mL standard. A recovery range between 50 to 150% of the spiked concentration was considered as non-inhibitory dilution. All samples were tested in duplicate and results were accepted when intra-assay CV was less than 10%. With regard to initial 10 fold dilution of samples, the minimum detection level of LPS in plasma was 0.05 EU/mL with this method.

Blood Acute Phase Proteins. Serum concentrations of Hp and plasma concentrations of SAA were determined using ELISA kits (TP-801 and TP-802, respectively, Tri-Delta Diagnostics Inc., Morris Plains, NJ) as described by Gozho et al. (2005). Plasma concentrations of LBP were measured using a commercially available kit (HK503, HyCult Biotechnology, Uden, The Netherlands). Samples were initially diluted 1:5 for Hp, 1:500 for SAA, and 1:1000 for LBP. Samples were analyzed in duplicate and absorbance values were read at 630 nm for Hp, and 450 nm for SAA and LBP using a microplate spectrophotometer (Spectra Max 340 PC, Molecular Devices Corporation, Sunnyvale, CA, and BioRad model 3550, Hercules, CA, respectively). The minimum detection limits of these assays were 50 μg/mL, 0.3 μg/mL, and 1.6 ng/mL for Hp, SAA, and LBP, respectively.

Milk Sampling and Analysis

Cows were milked twice daily in their stall at approximately 0400 h and 1600 h. Milk yield was recorded and milk samples from four consecutive milkings during each sampling week were collected and analyzed as described by Bhandari et al. (2007). The exception to this was that at each milking a second milk sample (10 mL) was taken without preservative and stored at -20°C for LBP analysis.

Milk LBP. Frozen milk samples were thawed at room temperature (19°C) and a 2 mL portion was centrifuged at 20,000 × g at 4°C for 1 h. After removing the fat layer, the samples were centrifuged two more times as indicated above, and the supernatant was collected and stored in -80°C until analyzed for LBP as described for plasma LBP.

Statistical Analysis

Data collected from wk 5 (control) and wk 6 (SARA) of each period were analyzed with repeated measures using PROC MIXED of SAS (2004). For intake, milk production and rumen pH parameters, the effect of diet (control or SARA) and period were considered fixed. As the effects of period were not significant, it was excluded from the statistical model. The effects of cow, period, period × cow, diet × period × cow, and day within diet and period were considered random. Day within diet and period was considered a repeated measure. For rumen and blood metabolites, additional effect of post-feeding hours and its interaction with diet were considered fixed, and hour × day interaction within diet and period were considered random. To obtain a normal distribution and homogeneous residual error, ruminal LPS data were log transformed. Tukey's multiple range test was used to compare the differences among treatment means. Effects were considered significant at a $P < 0.05$. Trends were discussed at $P < 0.10$.

RESULTS

The health of animals was monitored daily by checking feed consumption and rumen pH. Induction of SARA during the first day of period one resulted in an excessive reduction of DMI (> 50%) and rumen pH (< pH 5) in two of the cows. The data from d 1

and the next two consecutive days of those cows in period one were excluded from the statistical analysis.

Diets, Feed Intake, Milk Yield and Milk Composition

Replacing 21% of the DM of the TMR with wheat-barley pellets resulted in an average daily consumption of 3.5 kg (DM basis) of wheat-barley pellets during SARA weeks. This replacement changed the chemical composition of the diet toward higher DM (60.1 vs. 52.4%), NFC (40.4 vs. 32.7%) and starch (33.4 vs. 26.1%), and less CP (16.5 vs. 16.9%), NDF (30.4 vs. 35.7%) and ADF (22.7 vs. 24.3%) (Table 1). The mean and SD of wheat-barley pellets' length, diameter, bulk density, and WHC were 9.51 ± 2.83 mm, 4.15 ± 0.11 mm, 1.29 g/mL, and 2.21 g/g, respectively.

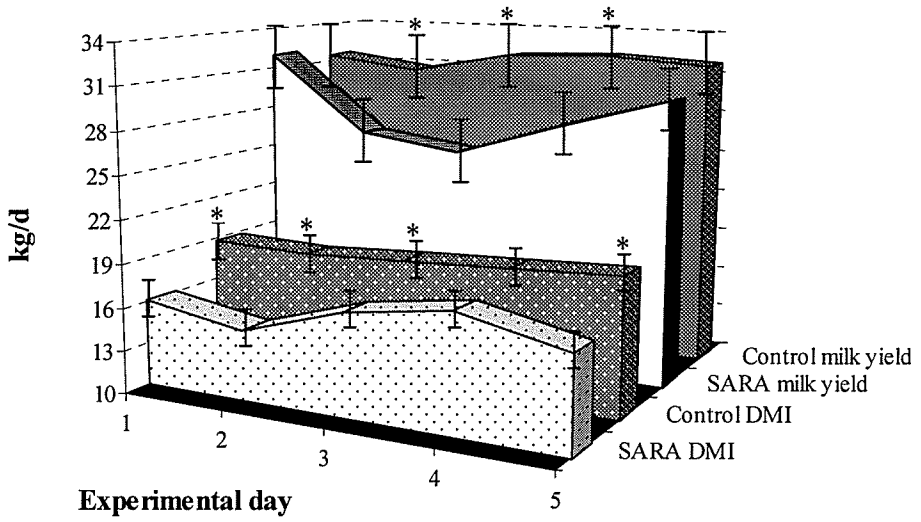
Dry matter intake was decreased by 15% following the inclusion of wheat-barley pellets in the diet ($P = 0.007$) (Table 4). The average DMI was 19.0 and 16.5 kg/d for the control and SARA cows, respectively. No significant effect of day or its interaction with diet with respect to DMI were detected ($P = 0.16$ and 0.49, respectively). Across cows, day-to-day variation in DMI was limited during the control weeks ranging from 18.6 to 19.6 kg/d (Figure 1). However, DMI fluctuation among days numerically increased and had a sinusoidal pattern during SARA. On average, the DMI declined during the first two days of SARA from 18.7 kg/d to 15.9 and 14.8 kg/d, respectively, but then increased to 17.1 and 18.2 kg/d during the next two consecutive days, and decreased again to 16.5 kg/d on d 5 of SARA (Figure 1).

Table 4. Feed intake, milk yield, and milk composition in dairy cows fed a basal TMR during control or TMR with wheat-barley pellets during subacute ruminal acidosis (SARA) treatment

Item	Diet		SED[1]	Effect, *P*-value		
	Control	SARA		Diet	Day	Diet × Day
Intake						
DM, kg/d	19.0	16.5	0.6	0.007	0.4	0.8
Kg of TMR DM/d	19.0	13.1	0.5	< 0.0001	0.33	0.8
Kg of WBP DM/d	0.00	3.45	0.16	< 0.0001	0.003	0.003
Orts, % of feed provided	12.6	28.3	2.1	0.0003	0.14	0.3
Milk						
Milk yield, kg/d	31.6	28.3	1.7	0.07	0.001	0.002
Fat, %	3.30	2.93	0.09	0.003	0.28	< 0.01
Fat yield, kg/d	0.97	0.85	0.05	0.03	0.05	0.47
Protein, %	3.29	3.42	0.07	0.08	0.09	0.08
Protein yield, kg/d	0.98	1.00	0.03	0.56	0.19	0.42

[1]SED = Standard error of difference between treatments.

Figure 1. Daily averages of DMI and milk yield in dairy cows fed a basal TMR during control or TMR with wheat-barley pellets during SARA treatment. Error bars indicate standard error of difference between treatments (SED). Within each day, * = $P < 0.05$



Milk yield tended to decline from 31.6 to 28.3 kg/d in response to SARA ($P = 0.07$) (Table 4). The effects of day ($P = 0.001$) and its interaction with diet on milk yield were significant ($P = 0.002$). Milk yield on the first day of SARA induction was similar to the control ($P = 0.82$) and averaged 31.8 kg/d across treatments, but declined until day 3 to 25.4 kg/d, and then increased during the last two days of SARA and reached 29.9 kg/d (Figure 1). Induction of SARA also significantly reduced milk fat (2.93 vs. 3.3%, $P = 0.003$) and fat yield (0.85 vs. 0.97 kg/d, $P = 0.03$), but tended to increase milk protein percentage (3.42 vs. 3.29%, $P = 0.08$) without affecting milk protein yield (1.00 vs. 0.98 kg/d, $P = 0.56$) (Table 4).

Ruminal Parameters

Continuous rumen pH monitoring during wks 5 and 6 indicated that SARA reduced average daily pH from 6.17 to 5.97 ($P = 0.03$) and increased the duration of rumen pH below 5.6 from 118 to 279 min/d ($P = 0.01$) (Table 5). This duration was above 180 min/d, which was taken as the threshold for SARA. Induction of SARA also increased the duration of rumen pH below 6 from 490 to 678 min/d ($P = 0.03$) and areas below pH 6 and 5.6 from 127 to 288 min×pH/d, and from 15 to 102 min×pH/d, respectively ($P = 0.02$ and 0.05, respectively). No significant effects of day or its interaction with diet on rumen pH parameters were observed (Table 5).

Feeding wheat-barley pellets increased the daily averages of rumen propionate (34.7 vs. 22.4 m*M*, $P = 0.0005$), butyrate (14.6 vs. 11.2 m*M*, $P = 0.04$) and valerate (2.6 vs. 1.4 m*M*, $P = 0.02$), but reduced rumen acetate (54 vs. 61.9 m*M*, $P = 0.02$) and the acetate to propionate ratio (1.61 vs. 2.86, $P = 0.0003$) (Table 6). The daily averages of total VFA and osmolality in the rumen tended to be higher (107.3 vs. 98.5 m*M* and 294.6

vs. 279.4 mOsm/kg, respectively) in SARA cows (P = 0.07) (Table 6, 7). However, addition of wheat-barley pellets to the diet did not affect the daily averages of total branch chain VFA (isobutyrate + isovalerate), lactate, and ammonia in the rumen, which averaged 1.58 m*M*, 1.97 m*M*, and 9.3 mg/dL across treatments, respectively. Concentrations of VFA, but not that of lactate, were significantly affected by hours post-feeding (Table 6) ($P < 0.0001$). The effect of day within week on rumen metabolites was not significant.

Across hours post-feeding, induction of SARA increased ruminal LPS concentration from 28,184 to 107,152 EU/mL (P = 0.004) (Table 7). The LPS concentration was also affected by hours post-feeding (P = 0.0005) and its interaction with diet (P = 0.025) (Table 7). During the control treatment, LPS concentration did not vary significantly throughout the 24 h period. However, during SARA, it increased from 73,283 before feeding to 109,320 EU/mL at 6 h post-feeding (P = 0.005), and reached a peak of 151,985 EU/mL at 12 h post-feeding (P = 0.02) (Table 7).

Table 5. Rumen pH variables in dairy cows fed a basal TMR during control or TMR with wheat-barley pellets during subacute ruminal acidosis (SARA) treatments

Item	Diet			Effect, *P*-value		
	Control	SARA	SED[1]	Diet	Day	Diet × Day
Average pH	6.17	5.97	0.07	0.03	0.83	0.82
Time < pH 5.6, min/d	118	279	47	0.01	0.53	0.28
Time < pH 6.0, min/d	490	678	69	0.03	0.77	0.63
Area < pH 5.6, min × pH/d	15	102	38	0.05	0.30	0.37
Area < pH 6.0, min × pH/d	127	288	52	0.02	0.42	0.36

[1]SED = Standard error of difference between treatments.

Table 6. Rumen fluid composition and blood metabolites of dairy cows fed a basal TMR during control or TMR with wheat-barley pellets during subacute ruminal acidosis (SARA) treatment

Item[1]	Diet		SED[2]	Effect, *P*-value		
	Control	SARA		Diet	Hour[3]	Diet × Hour
Ruminal parameters						
VFA, m*M*						
Total	98.5	107.3	3.7	0.07	< 0.0001	0.75
Acetate	61.9	54.0	2.1	0.02	< 0.0001	0.72
Propionate	22.4	34.7	2.3	0.0005	< 0.0001	0.26
Butyrate	11.2	14.6	1.1	0.04	< 0.0001	0.71
Valerate	1.4	2.6	0.4	0.02	< 0.0001	0.92
Total branch chain VFA[4]	1.69	1.47	0.15	0.23	< 0.0001	0.33
Acetate: Propionate	2.86	1.61	0.16	0.0003	< 0.0001	0.04
Lactate, m*M*	1.65	2.29	0.47	0.25	0.33	0.96
Ammonia, mg/dL	8.8	9.8	1.8	0.62	< 0.001	0.46
Blood parameters						
Glucose, mg/dL	66.7	73.0	1.0	0.0008	<0.001	0.1
Lactate, m*M*	1.4	0.8	0.5	0.27	0.07	0.68

[1]Daily concentration mean across sampling times.

[2]SED = Standard error of difference between treatments.

[3]Hour = Time of rumen fluid sampling before and at 2, 4, 6, 12, 14, 16, 18, and 24 h after feeding in two consecutive days during each sampling wk. The first day of sampling is three days after induction of SARA during SARA treatment.

[4]Total branch chain = isobutyrate + isovalerate

Blood and Milk Parameters

Plasma LPS concentration in control cows was below the minimum detection level of 0.05 EU/mL for the method (Table 7). Following SARA induction the average LPS in plasma increased to 0.52 EU/mL. Hours post-feeding tended to influence plasma LPS (P = 0.1) in SARA cows, as LPS declined to below pre-feeding levels (0.31 vs. 0.43 EU/mL) by 6 h post-feeding and then increased to 0.81 EU/mL at 12 h post-feeding (Table 7).

In response to SARA induction, blood concentrations of the acute phase proteins Hp, SAA, and LBP were increased from 0 to 475.6 μg/mL (P = 0.001), and from 167.4 to 438.5 μg/mL (P = 0.01), and from 18.2 to 53.1 μg/mL (P = 0.018), respectively (Table 8). In addition to blood acute phase proteins elevation, induction of SARA increased milk LBP concentration from 3.02 to 6.94 μg/mL (P = 0.02). The effects of hours post-feeding or its interaction with diet on Hp and SAA levels were not significant (Table 8).

Across hours post-feeding, SARA increased blood glucose concentration (73 vs. 66.7 mg/dL, P = 0.0008), but did not affect blood lactate (0.8 vs. 1.4 mM, P = 0.27) (Table 6). Averaged across treatments, glucose concentration increased from 66.5 mg/dL before feeding to 72.6 mg/dL at 12 h post-feeding (P = 0.0001).

Table 7. Plasma LPS, and rumen fluid LPS and osmolality in dairy cows fed a basal TMR during control or TMR with wheat-barley pellets during subacute ruminal acidosis (SARA) treatment

Item	Diet		SED[1]	Effect, *P*-value		
	Control	SARA		Diet	Hour[2]	Diet × Hour
Plasma LPS, EU/mL[3]						
0 h	< 0.05[4]	0.43				
6 h	< 0.05	0.31	0.18	0.001	0.16	0.1
12 h	< 0.05	0.81				
Rumen LPS, EU/mL[5]						
0 h	25,704	73,283				
6 h	29,383	109,320	34,590	0.004	0.0005	0.025
12 h	29,492	151,985				
Rumen osmolality, mOsm/kg						
0 h	263	257	7.1	0.07	0.0002	0.06
6 h	296	332				

[1]SED = Standard error of difference between treatments.

[2]Hour = Time of sampling before and at 6 and 12 h after feeding in two consecutive days of each period. The first day of sampling is three days after induction of SARA during SARA wks.

[3]EU= Endotoxin unit.

[4]The minimum detection level of plasma LPS with this method was 0.05 EU/mL.

[5] Statistical analyses were conducted on $\log_{10}$-transformed data.

Table 8. Serum amyloid A (SAA), haptoglobin (Hp), and LPS binding protein (LBP) concentrations in blood or milk of dairy cows fed a basal TMR during control or TMR with wheat-barley pellets during subacute ruminal acidosis (SARA) treatment

Item	Diet		SED[1]	Effect, *P*-value		
	Control	SARA		Diet	Hour[2]	Diet × Hour
Plasma SAA, μg/mL						
0 h	170.4	430.4	51.0	0.01	0.69	0.41
6 h	164.4	446.7				
Serum Hp, μg/mL						
0 h	ND[3]	468	48.0	0.001	0.33	0.33
6 h	ND	484				
Plasma LBP, μg/mL						
6 h	18.2	53.1	10.8	0.018	-	-
Milk LBP, μg/mL						
6 h	3.02	6.94	1.24	0.02	-	-

[1]SED = Standard error of difference between treatments.

[2]Hour = Time of sampling before and at 6 h after feeding. Samples are collected four days after induction of SARA during SARA wks.

[3]ND = Non-detectable with the minimum detection limit of 50 μg/mL

DISCUSSION

Earlier research has shown that successful induction of SARA, which is characterized by feed intake depression and inflammation, only occurs when a rumen pH depression between 5.2 and 5.6 was obtained for more than 180 min/d (Gozho et al., 2005). Replacing 21% of the DM of the TMR with wheat-barley pellets in this study resulted in average duration of rumen pH < 5.6 for 279 min/d, which was in agreement with 506 and 309 min/d reported by Krause and Oetzel (2005), and Gozho et al. (2007), respectively, for SARA induced cows. The area under pH 5.6 in the current study was 102 min × pH/d, which was close to 190 and 67 min × pH/d reported by Krause and Oetzel (2005), and Gozho et al. (2007), respectively. We, therefore, conclude that SARA was successfully induced in the present study.

Feeding wheat-barley pellets depressed DMI by 15% compared to the control group. This reduction in DMI can be due to hypophagic effects of elevated levels of propionate in the rumen and glucose in blood (Oba and Allen, 2003), as well as the hyperosmolality of rumen, dehydration (Allen, 2000), and endotoxemia (Waldron et al., 2003) that were observed during the current study. Our results are in agreement with those who reported that reduced feed intake is a consistent sign of SARA in dairy cows (Owens, et al., 1998; Kleen et al., 2003; Plaizier et al., 2008) and beef cattle (Nagaraja and Lechtenberg, 2007). However, several others did not observe that grain-induced SARA reduced DMI (Krause and Oetzel, 2005; Gozho et al., 2006; 2007). This discrepancy might be related to insulin, as cows with higher plasma insulin prior to high starch treatments or lower insulin secretion in response to glucose showed greater depression in feed intake (Bradford and Allen, 2007). Other reasons for this discrepancy

might include differences in the contents of fiber and ensiled forages, in particle size and in starch fermentability among the diets used to induce SARA (Allen, 2000), as well as differences in endotoxin tolerance among individual cows (Hurley, 1995).

The hypothesis that free rumen LPS concentration increases following grain engorgement (Andersen et al., 1994a; Emmanuel et al., 2008), especially during experimentally induced acute acidosis (Andersen and Jarlov, 1990; Andersen et al., 1994b) or SARA (Gozho et al., 2005; 2006; 2007), has been clearly proven. However, the reported range of free LPS in rumen fluid varied substantially in these earlier studies. For the purpose of comparison among studies, LPS concentrations were converted and expressed in ng/mL. Since 1 EU corresponds to 100 pg of LPS, the absolute concentration of LPS in our experiment varied from 2,818 ng/mL in control group to 10,715 ng/mL in SARA cows. Similar to our results, Gozho et al. (2007) reported a LPS range of 2,454 to 12,882 ng/mL in Holstein dairy cows (538 kg, 121 DIM) during periods of control and grain-induced SARA, respectively. A recent study by Emmanuel et al. (2008) reported a LPS range from 790 ng/mL in control to 5,021 and 8,870 ng/mL when 30% and 45% barley was supplemented in the diet of Holstein dairy cows (650 kg, 60 to 140 DIM), respectively. Gozho et al. (2005, 2006) reported a lower range of LPS in Jersey steers (678 kg). In these studies, abrupt induction of SARA or gradual adaptation to 61% wheat-barley pellet in the diet, increased the rumen LPS from 375 to 887 ng/mL and from 631 to 871 ng/mL, respectively. In contrast, Andersen et al. (1994b) reported a LPS range from 11.8 to 14.8 ng/mL with a hay-fed diet, which increased to 150 ng/mL during grain adaptation, and Andersen and Jarlov (1990) reported 25% decline in LPS concentration from the initial range of 150-160 ng/mL during control, after induction of

acute acidosis in non-lactating Jersey cows (320 kg). Earlier studies (Nagaraja et al., 1978a; 1978b) on rumen LPS in dairy cows were semi-quantitative and were based on extraction/purification of LPS from rumen fluid followed by biological assays, such as pyrogenicity in rabbit, or lethality in mice or chicken embryos. The reactivity of LPS from environmental or anaerobic bacterial sources with these biological assays is 1,000 times less than with LAL assay (Hurley, 1995). Therefore, a quantitative comparison cannot be made between the earlier studies with ones that are more recent. The discrepancy between our study and those of Andersen (1994b) and Andersen and Jarlov (1990) is probably due to differences in the method of LPS determination. The LAL assay was employed in all of these studies; however, over the past two decades, the accuracy of the assay has been improved substantially (Hurley, 1995). For example, the original gel clot assay of LAL provided a semi-quantitative result, while chromogenic endpoint LAL offers quantitative results with a sensitivity of 10 pg/mL (0.1 EU/mL) and exhibits less product interference than LAL utilizing the clotting protein. A more recent method is the kinetic chromogenic LAL, which provides a sensitivity as low as 0.5 pg/mL (0.005 EU/mL), and a wider range of detection up to 5 ng/mL (50 EU/mL) (Hurley, 1995). Factors such as abrupt, gradual or repeated induction of SARA, feed intake and rumen absorptive surface/capacity of individual animals, inclusion rate of NFC in the diet, starch fermentability, and feed particle size can affect the rumen pH, as well as the growth/lysis rate of Gram-negative bacteria, and, therefore, free LPS level in the rumen. Hence, apart from method of LPS measurement, several differences between animals and diets may also influence the range of free LPS in the rumen.

Despite general agreement that grain-induced SARA increases free rumen LPS, no evidence of LPS in peripheral circulation has been found (Gozho et al., 2007). There is also inconsistency in detection of LPS in peripheral circulation during experimentally induced acute ruminal acidosis. While some studies have occasionally detected LPS in peripheral circulation (Dougherty et al., 1975; Aiumlamai et al., 1992; Andersen et al., 1994b), others were unsuccessful (Andersen and Jarlov, 1990; Andersen et al., 1994a). The fact that LPS is present in low concentrations in peripheral plasma and at least a 10x dilution is required for the LAL assay to control the inhibitors and interfering factors in blood, creates challenges to the measurement of LPS when the sensitivity of the assay is low. Using a kinetic LAL assay, we reported here for the first time that SARA induction increased the peripheral plasma LPS from < 0.05 to 0.52 EU/mL (52 pg/mL) across the sampling hours. A continuous presence of a low amount of LPS in peripheral plasma of SARA induced cows could result in a metabolic endotoxemia that triggers a low-grade inflammation compared to acute disorders such as septicemia. In addition, it has been documented that metabolic endotoxemia can result in insulin resistance in the liver, hyperinsulinemia, higher blood glucose level, modification of the energy metabolism, and anorexia (Cani et al., 2007). Moreover, Cani et al. (2007) demonstrated that chronic endotoxemia changes the cells' insulin sensitivity through the function of CD14, a membrane receptor of LPS, and results in body weight gain without excessive energy intake in chronic LPS infused mice. This finding might similarly explain the gain in body condition in mid to late-lactation dairy cows experiencing SARA (Kleen et al., 2003).

In this study and consistent with the previous reports (Gozho et al., 2007; Emmanuel et al., 2008), the peripheral blood concentrations of SAA and Hp increased

during SARA and exceeded their physiological ranges for healthy animals. The basal ranges are < 50 μg/mL for Hp and < 25 μg/mL for SAA in cattle (Tri-Delta Diagnostics Inc., Morris Plains, NJ, Humblet et al., 2006). In response to blood LPS, the concentration of LPS specific acute phase protein LBP was also significantly increased in peripheral plasma and milk during SARA. The LBP concentration in SARA induced cows in our study (53.1 μg/mL) was higher than that reported by Emmanuel et al. (2008) for dairy cows supplemented with 45% barley (< 16 μg/mL). In control cows, blood SAA concentration was above the physiological level (167 vs. 25 μg/mL), while Hp was not detectable. SAA^{+}/Hp^{-} status is due to higher sensitivity of SAA to inflammatory stimuli than Hp and might be an indicator of an early stage of inflammation (Humblet et al., 2006). Plasma LBP levels of control cows were within the basal range of 10.8 to 37.8 μg/mL previously reported by Suojala et al. (2008) and Bannerman et al. (2003), respectively. These data support our hypothesis that grain-induced SARA increases translocation of LPS into the peripheral circulation and elevates peripheral plasma LBP that facilitate binding of LPS to cell membrane receptors, which then triggers a cascade of events toward a local or systemic inflammatory response and release of other acute phase proteins into the blood.

The present study indicated that LPS translocation occurs in grain-induced SARA cows. However, the mechanism and the site of translocation remain unknown. Chin et al. (2006) have conducted experiments using intestinal epithelial cell lines and concluded that an abnormal increase in luminal LPS induces cell apoptosis, disrupts tight junction protein zonula occludens-1, and increases epithelial permeability in a dose and time dependent manner by increasing the production of nitric oxide ($NO^{\cdot}$). In addition, Cetin

et al. (2004) demonstrated that LPS impairs the pH regulatory system of enterocytes by inhibition of sodium-proton pumps under extracellular acidosis conditions, resulting in cytoplasmic acidification and cellular dysfunction.

However, differences between the composition of epithelium of the rumen, and of the small and large intestine could result in differences in the effect of LPS on the barrier function of the epithelium among these parts of the gastro-intestinal tract. Epithelium in the intestine, lung and kidney has a simple monolayer structure with tight junctions at the apical pole of the cells, whereas rumen epithelium has a multilayer structure with tight junctions located in the middle layers, stratum granulosum and spinosum (Graham and Simmons, 2005). Although, the external layer of rumen epithelia has no tight junctions, it may consist of up to 15 cell layers, which may limit the permeability of large molecules such as LPS (Baldwin, 1998). A recent in vitro study by Emmanuel et al. (2007) indicated that LPS translocates across the rumen wall at a greater rate than across the colon wall, and this translocation is pH independent. However, the rate of translocation was numerically higher at pH 5.5 in the rumen and at pH 6.5 in the colon than at other pH levels. The concentration of LPS that was added to the mucosal side of the rumen or colon in this in vitro experiment was 500 μg/mL, which is 50 times more than ruminal free LPS concentration during grain-induced SARA. Hence, that may have disrupted the barrier function of the gut to a greater extent than what would have occurred physiologically.

Consistent with a previous report (Gozho et al., 2006), our data indicated that free rumen LPS concentration increased sharply by 6 h after feeding (10,965 ng/mL) and reached to its peak at 12 h post-feeding (15,135 ng/mL). In contrast, peripheral LPS

concentration in SARA-induced cows did not follow the same pattern and tended to decline to below pre-feeding levels until 6 hours post-feeding (31 vs. 43 pg/mL) and then started to increase until 12 h after feeding (81 pg/mL). The decline of plasma LPS to below pre-feeding levels until 6 h post-feeding and the delay in its increase until 12 h after feeding may suggest that the LPS translocation occurs from the lower gut rather than from the rumen. If LPS does not translocate into the blood circulation from the rumen, then peripheral LPS during grain-induced SARA must be translocated through the epithelium of the small or large intestine. However, before arriving to these sites, free rumen LPS will be detoxified in the duodenum, primarily by bile acids (Bertok, 1998). Therefore, the main source of free LPS in the lower gut must be originated from the bacterial community of the terminal ileum and the large intestine. If this hypothesis is correct, then the rate of LPS translocation and its peripheral concentration is independent of rumen pH and free rumen LPS concentration, but depends on factors such as bypassed starch that stimulate LPS release in the terminal ileum and large intestine. In this study, the diet used for the SARA challenge had higher content of starch than the control diet. The added starch in the SARA diet was in the form of wheat-barley pellets with physical characteristics, such as specific gravity >1.2 and <1.5 that categorize them in the range of particles with the highest rate of passage (Kaske et al., 1992). Therefore, a considerable portion of starch may have bypassed rumen fermentation. These conditions may intensify the release of free LPS in the lower gut and may trigger a cascade of events towards barrier failure of monolayer epithelial structure of the intestine and the colon as discussed before. Further research is necessary to understand if the barrier failure, LPS

translocation, and inflammatory responses that are seen after a grain-based SARA challenge occur ruminally or post-ruminally.

CONCLUSION

Induction of SARA by replacing 21% of the DM of TMR with wheat-barley pellet increased the duration of rumen pH below 5.6 to above 180 min/d, which was taken as the threshold for SARA. This induction reduced the DMI (15%), milk yield (3.3 kg/d), and milk fat (0.12% point), but increased the concentration of free LPS in rumen fluid from 2,818 to 10,715 ng/mL. Induction of SARA increased plasma LPS concentration from < 0.05 to 0.52 EU/mL. The peripheral blood concentrations of acute phase proteins SAA, and Hp, as inflammatory markers, increased during SARA and exceeded their physiological ranges for healthy animals. In response to blood LPS, concentration of LPS specific acute phase protein, LBP, was also increased in peripheral blood and milk during SARA. Our results suggest that grain-induced SARA increases the lysis of rumen Gram-negative bacteria, release of free LPS in the rumen and translocation of LPS into the peripheral circulation, and that triggers a systemic inflammatory response.

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MANUSCRIPT II

Alfalfa Pellet-Induced Subacute Ruminal Acidosis in Dairy Cows Increases Bacterial Endotoxin in the Rumen Without Causing Inflammation

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ABSTRACT

A study was conducted to determine if SARA induced by feeding alfalfa pellets results in increases in free bacterial lipopolysaccharide (LPS) in rumen fluid and peripheral blood, and acute phase proteins in plasma, and to determine the effect of alfalfa pellet-induced SARA on feed intake, milk production and composition, and blood metabolites. Eight lactating Holstein cows, four of which were ruminally cannulated, were used in a 6 wk experiment and were fed once daily at 900 h. During wk 1, cows received a diet containing 50% of DM as concentrate and 50% of DM chopped alfalfa hay. Between wk 2 and wk 6, alfalfa hay was gradually replaced with alfalfa pellets at the rate of 8% per week in order to reduce rumen pH. Rumen pH was monitored continuously in the rumen-cannulated cows using indwelling pH probes. Rumen fluid and peripheral blood were sampled 15 min before feed delivery and at 6 h after feed delivery. Based on adopted threshold of SARA of at least 180 min/d below pH 5.6, SARA was induced from wk 3 onwards. The most severe rumen pH depression was obtained in wk 5 (510 min/d and 110 min $\times$ pH/d $<$ pH 5.6). Replacing 40% of alfalfa hay with alfalfa pellets quadratically increased the DMI from 18.1 kg/d in wk 1 to 23.4 kg/d in wk 6. This replacement linearly reduced milk yield (32.7 vs. 35.9 kg/d) and milk fat (2.32 vs. 3.22 %, and 0.77 vs. 1.14 kg/d), and increased milk protein percentage and yield (3.80 vs. 3.04 %, and 1.23 vs. 1.07 kg/d, respectively). This gradual replacement also linearly increased the daily averages of total rumen VFA (90 to 121.9 m*M*), acetate (53.9 to 66.8 m*M*), propionate (21.5 to 39.6 m*M*) and osmolality (277.7 to 300.9 mOsm/kg), and decreased the acetate to propionate ratio in the rumen from 2.62 to 1.73. Replacing alfalfa hay with alfalfa pellets linearly increased blood lactate from 1.00 m*M* in wk 1 to a peak

of 3.46 m*M* in wk 5. Similar to grain-induced SARA, induction of SARA in this study increased free rumen LPS concentration from 38,019 endotoxin unit (EU)/mL in wk 1 to 165,959 EU/mL in wk 6. However, this increase was not accompanied by an increase in LPS (< 0.05 EU/mL) and in acute phase proteins serum amyloid-A (SAA), haptoglobin (Hp), and LPS-binding protein (LBP) in peripheral circulation. Results suggest that factors other than low pH and free LPS in the rumen are responsible for the inflammatory response seen during grain-induced SARA.

Key words: subacute ruminal acidosis, LPS, acute phase response

INTRODUCTION

Current definition of subacute ruminal acidosis (SARA) and recommendations for its diagnosis in dairy herds are mainly based on rumen pH that is derived from the spot sampling of rumen fluid or with continuous rumen pH measurement (Garrett et al., 1999; Plaizier et al., 2008). In addition to rumen pH, monitoring free lipopolysaccharide (endotoxin, LPS) concentration of rumen refines the diagnosis of SARA based on rumen fluid analysis. Rumen pH depression during SARA increases free LPS content of rumen (Gozho et al., 2007; Emmanuel et al., 2008) and this increase accompanies by translocation of LPS from the gut into blood circulation, and activation of an inflammatory response (Khafipour et al., 2008a). However, the correlation between decreased rumen pH, increased LPS in the rumen and increased acute phase proteins as inflammatory markers in blood have been only studied when SARA was experimentally induced by increasing the content of starch in the diet (Gozho et al., 2007; Khafipour et al., 2008a; Emmanuel et al., 2008). Therefore, it cannot be concluded that the clinical signs, especially inflammation, that are associated with grain-induced SARA are due to low pH in rumen or due to high starch content of the diet.

Evidence in support of this view comes from studies looking at the site of LPS translocation into the blood. While a recent in vitro study indicated that LPS translocates across the rumen and colon wall (Emmanuel et al., 2007), others believe that rumen epithelium has a low permeability to LPS as infusion of Cr-labeled LPS into the rumen of steers did not follow by LPS translocation into thoracic duct (lymph) or portal vein (Lassman, 1980; Anderson, 1984). If LPS does not translocate into the blood circulation from the rumen, then peripheral LPS during grain-induced SARA must be translocated

through the epithelium of the small or large intestine. However, before arriving to these sites, free rumen LPS is detoxified in the duodenum, primarily by bile acids (Bertok, 1998). Therefore, free LPS in the lower gut must be originated from the bacterial community of the terminal ileum and large intestine. Grain-induced SARA may trigger the release of LPS from Gram-negative bacteria in these compartments of gastro-intestinal (GI) tract by increasing the amount of starch in the intestine. This may increase the luminal LPS concentration to the levels that impairs the barrier function of monolayer epithelium of the small and large intestine, and that may initiate the inflammatory reaction seen during grain-induced SARA.

Diets that are low in coarse fiber can reduce the chewing activity, saliva production and rumen buffering, and result in similar rumen pH depression as grain-induced SARA (Beauchemin et al., 2003). In this study, we replaced chopped alfalfa hay with alfalfa pellets to induce low pH in the rumen without any changes in the starch content or in forage-to-concentrate (F:C) ratio of the diets. The objectives of this study were to determine if SARA induced by feeding alfalfa pellets results in similar increases in free LPS in rumen fluid and peripheral blood than grain-induced SARA, and if this method of SARA induction stimulates a systemic inflammatory response. The effects of alfalfa pellet-induced SARA on feed intake, milk production and composition, and blood metabolites were also determined.

MATERIAL AND METHODS

Animals, Diets, and Experimental procedures

Four rumen cannulated and four non-rumen cannulated dairy cows (175 ± 75 DIM) were used in a 6 wk study. Cows had an average BW of 648 ± 64 kg, and an average milk yield of 35.9 ± 5.4 kg at the beginning of the experiment. Cows were housed in individual tie-stalls in Glenlea Dairy Research Station at the University of Manitoba (Winnipeg, MB, Canada) in accordance with the guidelines of Canadian Council on Animal Care (CCAC, 1993). During wk 1, cows received a total mixed ration (TMR) that contained 50% of DM as concentrate and 50% of DM as chopped alfalfa hay (Table 9, 10, 11). From wk 2 to wk 6, alfalfa hay was gradually replaced with alfalfa pellets at the rate of 8% per week (DM basis) to induce SARA (Table 9). Cows were fed once daily ad libitum with the intention of allowing for 5 to 10% orts and had free access to fresh water for the duration of the experiment. The health of animals was monitored daily by checking the feed consumption and rumen pH. In addition, body temperature and milk somatic cell count were monitored weekly for the clinical signs of inflammation and mastitis as these could affect LPS and acute phase proteins concentrations in blood. Weekly samples of diets, dietary ingredients, and orts were collected and analyzed by wet chemistry as described by Bhandari et al. (2007). The physical characteristics of alfalfa pellets including dimension, bulk density and water holding capacity (WHC) were determined as described by Giger-Reverdin (2000).

Rumen pH Measurement

Rumen pH was monitored continuously for five consecutive days during each week of experiment in four rumen cannulated cows using indwelling pH probes as described by Gozho et al. (2006). The pH probes were placed in the ventral sac of the rumen, and their positions were checked twice a day at 0830 h and 1630 h. The pH data were summarized as average pH, time spent below pH 6.0, time spent below pH 5.6, area (time × pH) spent below pH 6.0, and area spent below pH 5.6 for each 24 h periods.

Rumen Fluid Sampling and Analysis

Rumen fluid samples were collected from ventral sac of the rumen, 15 min before feeding and at 6 h after feeding on Tuesdays, and Thursdays of each experimental week. Ruminal contents were strained through four layers of sterile cheesecloth and divided into two portions. The first portion of samples were transferred into 50 mL sterile tubes and kept in ice until transported to the laboratory for the initial processing prior to LPS determination as described by Gozho et al. (2005). In brief, rumen fluid samples were centrifuged at 10,000 × *g* for 45 min. The supernatant was aspirated gently to prevent its mixing with the pellet and passed through a disposable 0.22 μm LPS-free filter (Millex, Millipore Corporation, Bedfored, MA). The filtrate was collected in a sterile, depyrogenated glass tube (previously heated at 180°C for 4 h) and heated at 100°C for 30 min. Samples were cooled at room temperature (19°C) for 10 min and stored at -20°C for subsequent LPS measurement. The second portion of the rumen fluid samples was immediately centrifuged at 1900 × *g* for 15 min and supernatant stored at -20°C until analyzed for osmolality, VFA, and ammonia. Concentrations of VFAs (acetate,

Table 9. Ingredients, chemical composition and forage to concentrate ratio of TMR in a 6 wk period during which dietary alfalfa hay was gradually replaced with alfalfa pellets to induce low pH

Item	Week					
	Wk1	Wk2	Wk3	Wk4	Wk5	Wk6
Ingredients, % DM						
Energy Supplement	39	39	39	39	39	39
Protein Supplement	4.5	4.5	4.5	4.5	4.5	4.5
Roasted soybeans	6.5	6.5	6.5	6.5	6.5	6.5
Alfalfa hay	50	42	34.0	26	18	10
Alfalfa pellets	0.0	8	16	24	32	40
F:C	50:50	50:50	50:50	50:50	50:50	50:50
Nutrient Composition						
DM, %	74.5	74.4	74.3	74.3	77.4	73.3
CP , % of DM	17.1	18.0	18.7	18.3	18.7	17.3
ADF, % of DM	20.6	22.8	23.6	25.1	20.6	23.0
NDF , % of DM	36.3	35.2	34.0	35.2	36.9	35.4
NFC[1], % of DM	33.5	34.4	33.8	33.4	33.5	34.4
Starch, % of DM	21.8	22.4	22.0	21.7	21.8	22.4
Crude fat, % of DM	5.66	5.78	6.11	5.68	6.41	5.62
Ash, % of DM	7.4	6.6	7.4	7.4	7.0	7.1
Ca, % of DM	0.76	0.87	0.98	1.03	1.05	1.07
P, % of DM	0.51	0.52	0.54	0.51	0.49	0.46
K, % of DM	1.37	1.43	1.50	1.42	1.43	1.44
Mg, % of DM	0.26	0.27	0.29	0.30	0.30	0.29
Na, % of DM	0.22	0.23	0.23	0.26	0.25	0.23
Predicted NE_L[2], Mcal/kg DM	1.63	1.63	1.64	1.64	1.65	1.65

[1]Non fiber carbohydrates = 100 – (NDF% + CP% + Crude fat% + Ash%)

[2]NE_L values are predicted using CNCPS (Cornell Net Carbohydrate and Protein System) software version 5.0.4, Cornell University, Ithaca, NY.

Table 10. Chemical composition of TMR ingredients

Item	TMR Ingredients				
	Energy supplement	Protein supplement	Soybeans	Alfalfa hay	Alfalfa pellet
DM, %	90.4	91.7	95.7	89.5	94.1
CP, % of DM	17.2	28.9	26.7	11.2	15.3
ADF, % of DM	8.6	12.7	17.2	37.8	36.2
NDF , % of DM	19.4	28.9	20.5	54.7	50.0
NFC[1], % of DM	47.5	18.6	17.1	23.0	24.9
Starch, % of DM	34.2	3.9	4.7	1.2	1.3
Crude fat, % of DM	7.3	7.01	18.5	1.4	1.4
Ash, % of DM	2.0	14.5	4.8	7.5	7.9
Ca, % of DM	0.94	2.95	0.17	0.58	1.14
P, % of DM	0.71	1.07	0.65	0.23	0.20
K, % of DM	1.00	1.18	1.61	1.78	1.70
Mg, % of DM	0.34	0.33	0.25	0.18	0.18
Na, % of DM	0.33	1.57	0.01	0.03	0.03

[1]Non fiber carbohydrates = 100 – (NDF% + CP% + Crude fat% + Ash%)

Table 11. Ingredient composition of the energy and the protein supplement (% of DM)

Ingredient	Energy supplement	Protein supplement
Rolled barley	54.0	-
Luprosil salt (calcium propionate)	0.2	-
Protein pellet[1]	1.8	-
Dairy supplement[2]	40.0	-
Vegetable oil	4.0	-
Corn dried distillers with solubles	-	42.0
Fish meal	-	7.0
Canola meal	-	22.7
Soybean meal	-	20.0
Beet molasses	-	3.0
Niacin	-	0.3
Sodium bicarbonate	-	5.0

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

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

propionate, butyrate, valerate, isobutyrate, and isovalerate) were determined by gas chromatography as described by Bhandari et al. (2007). Ammonia nitrogen concentration of rumen fluid samples was determined using a colorimetric technique as described by Bhandari et al. (2007). Osmolality of rumen fluid samples was determined by freezing point depression using an automatic osmometer (Advanced single sample micro-osmometer Model 3300, Advanced Instruments Inc., Norwood, MA).

Free rumen LPS content was determined by a chromogenic *Limulus* amebocyte lysate (LAL) end-point assay (QCL-1000, Lonza group Ltd, Basel, Switzerland). Pre-treated rumen samples were diluted until their LPS concentrations were in the range of 0.1–1 EU/mL relative to the reference endotoxin (*E. coli* O111:B4), and assayed as described by Gozho et al. (2005).

Blood Sampling and Analysis

Blood samples were collected by tail venipuncture from each cow 15 min before feeding and at 6 h after feeding on Tuesdays, and Thursdays of each experimental week. Samples were collected in a blank 10 mL vacutainer (Fisher Scientific, Fairlawn, NJ) for serum harvesting and in a 10 mL heparinized vacutainer (Fisher Scientific, Fairlawn, NJ) for plasma collection. The serum vacutainers were allowed to clot at room temperature (19°C) for 45 min, and then centrifuged at 1900 × *g* for 15 min. Subsequently, serum was aspirated and stored in -20°C until used for haptoglobin (Hp) analysis. Plasma was harvested by centrifuging of heparinized vacutainers at 1900 × *g* for 15 min. A portion of plasma was transferred to pyrogen-free glass tubes and stored at -20°C for LPS analysis. The rest of plasma was divided into 2 mL aliquots and stored in -20°C until analyzed for

serum amyloid-A (SAA), LPS binding protein (LBP), glucose, and lactate. Plasma glucose and lactate were analyzed using a blood gas and electrolyte analyzer (Nova Stat Profile M, Nova Biomedical Corporation, Waltham, MA) as described by Bhandari et al. (2007).

LPS Analysis in Blood Plasma. The concentration of LPS in plasma was determined by a chromogenic kinetic LAL assay (Kinetic-QCL, Lonza group Ltd, Basel, Switzerland) with a minimum detection limit of 0.005 EU/mL. Samples were initially treated as recommended by Dosogne et al. (2002), Hourly (1995) and suppliers recommendations to inactive inhibitory factors in plasma. In brief, frozen plasma samples were thawed at 37°C, vortexed, and 100 µL of sample were diluted at least 10 fold with LAL water. Diluted samples were incubated at 37°C for 30 min, then heated at 75°C for 15 min and cooled down to room temperature (19°C) for 45 min. Then, a metallo-modified polyanionic dispersant called Pyrosperse (Lonza group Ltd, Basel, Switzerland) was added to the test samples at a ratio of 1/200 (vol/vol) prior to LAL testing. The kinetic LAL assay was performed in a 96-well microplate according to manufacturer's instructions. The appearance of a yellow color was monitored over time using an incubating microplate spectrophotometer (Spectra Max 340 PC, Molecular Devices Corporation, Sunnyvale, CA) to determine the reaction time, which is defined as the time required for absorbance to increase 0.2 optical density (OD). A log/log linear correlation of the mean reaction time of each standard with its corresponding endotoxin concentration was performed to create a standard curve and predict endotoxin concentration of test samples. To verify the lack of product inhibition, samples were spiked with 10 µL of 2 EU/mL standard. A recovery range between 50 to 150% of the

spiked concentration was considered as non-inhibitory dilution. All samples were tested in duplicate and results were accepted when intra-assay CV was less than 10%. With regard to initial 10 folds dilution of samples, the minimum detection level of LPS in plasma was 0.05 EU/mL with this method.

Blood Acute Phase Proteins. Serum concentrations of Hp and plasma concentrations of SAA were determined using ELISA kits (TP-801, and TP-802, respectively, Tri-Delta Diagnostics Inc., Morris Plains, NJ) as described by Gozho et al. (2005). Plasma concentrations of LBP were measured using a commercially available kit (HK503, HyCult Biotechnology, Uden, The Netherlands). Samples were initially diluted 1:5 for Hp, 1:500 for SAA, and 1:1000 for LBP and assayed according to the manufacturer's instructions. Samples were analyzed in duplicate and absorbance values were read at 630 nm for Hp and 450 nm for SAA and LBP using a microplate spectrophotometer (Spectra Max 340 PC, Molecular Devices Corporation, Sunnyvale, CA, and BioRad model 3550, Hercules, CA, respectively). The minimum detection limit of these assays was 50 μg/mL, 0.3 μg/mL, and 1.6 ng/mL for Hp, SAA and LBP, respectively.

Milk Sampling and Analysis

Cows were milked twice daily in their stall at approximately 0400 h and 1600 h. Milk yield was recorded using Tru Test regulation meters (Westfalia Surge, Mississauga, Ontario, Canada) with the accuracy of 5% or 0.3 L. Milk samples (50 mL) from four consecutive milkings during each sampling week were collected and preserved with 2-bromo-2-nitropropane-1,3 diol and stored at 4°C until analyzed for fat and protein at the laboratory of the Dairy Farmers of Manitoba (Winnipeg, Manitoba, Canada) by near-

infrared analysis using the Milk-O-Scan 303AB (Foss Electric, Hillerød, Denmark). The infrared analysis was calibrated by the Babcock method for milk fat analysis (AOAC 989.04, 1990) and the Kjeldahl method for nitrogen/protein nitrogen analysis in milk (AOAC 991.22, 1990).

Statistical Analysis

Six weeks data were analyzed as repeated measures using PROC MIXED of SAS (2004). For intake, milk production and rumen pH parameters, the effect of week, which corresponded with the inclusion rate of alfalfa pellets, was considered fixed. The effects of cow, and interaction between cow and week were considered random. The effect of day within week was considered random and analyzed as repeated measure. For rumen and blood metabolites, additional effect of post-feeding hours and its interactions with week were considered fixed. Interactions of hour and cow and hour × day within week were considered random. To obtain a normal distribution and homogeneous residual error ruminal and plasma LPS data were log transformed. Tukey's multiple range test was used to compare the differences among treatment means. Effects were considered significant at $P < 0.05$. Trends were discussed at $P < 0.10$.

RESULTS

Diets, Feed Intake, Milk Yield and Milk Composition

Replacing alfalfa hay with alfalfa pellets resulted in average daily consumption of 1.7, 3.5, 5.3, 7.5, and 9.5 kg (DM basis) of alfalfa pellets during wk 2 to wk 6. Although alfalfa pellets contained more DM (94.1 vs. 89.5%), CP (15.3 vs. 11.2% of DM) and Ca (1.14 vs. 0.58% of DM), and less NDF (50 vs. 54.7% of DM) than alfalfa hay, the

replacement of alfalfa hay with alfalfa pellets did not change the chemical composition of TMR among 6 wk of the experiment (Table 9). The mean and the standard deviation of physical parameters of alfalfa pellets including length, diameter, bulk density and WHC were 8.63 ± 2.91 mm, 6.56 ± 0.13 mm, 1.14 g/mL, and 6.55 g/g, respectively.

Replacing 40 % of alfalfa hay with alfalfa pellets during the 6 wk period quadratically increased the DMI from 18.1 kg/d in wk 1 to 23.4 kg/d in wk 6 ($P = 0.025$) (Table 12). This replacement linearly reduced milk yield, milk fat and fat yield from 35.9 to 32.7 kg/d ($P < 0.001$), from 3.22 to 2.32% ($P = 0.002$), and from 1.14 to 0.77 kg/d ($P = 0.0002$), respectively, but increased milk protein percentage and protein yield from 3.04 to 3.80% ($P = 0.0002$), and 1.07 to 1.23 kg/d ($P = 0.013$), respectively (Table 12).

Ruminal Parameters

Gradual replacement of alfalfa hay with alfalfa pellet linearly reduced the average daily pH from 6.35 in wk 1 to 5.78 in wk 6 ($P < 0.001$) (Table 13). From wk 3 onwards, rumen pH was lower than 5.6 for more than 180 min/d indicating that SARA was successfully induced during these weeks (Figure 2). This duration increased up to the maximum of 558 min/d in wk 4 and then declined ($P = 0.04$) to 510 and 447 min/d during wk 5 and wk 6, respectively (Figure 2). Based on the duration and area under pH 5.6, the most severe rumen pH depression was obtained in wk 4 and wk 5 (Table 13). During the 6 wk of the experiment, duration and area under pH 6 also linearly increased from 312 to 975 min/d ($P < 0.001$), and from 102 to 340 min × pH/d ($P = 0.001$), respectively.

Table 12. Intake, milk yield and milk components production of dairy cows in a 6 wk period during which dietary alfalfa hay was gradually replaced with alfalfa pellets to induce low pH

Item	Week						SED	Effect, *P value*		
	Wk1	Wk2	Wk3	Wk4	Wk5	Wk6		Week	Linear week	Quad. week
DM intake, kg/d	18.1	21.6	21.7	22.0	23.4	23.4	0.8	< 0.001	< 0.001	0.025
Orts, % of feed provided	12.7	9.6	13.6	12.4	10.4	8.5	2.0	< 0.06	< 0.16	0.21
Forage: Concentrate ratio (F:C)	50:50	50:50	50:50	50:50	50:50	50:50	50:50	-	-	-
Milk										
Yield, kg/d	35.9	35.7	33.9	33.8	32.2	32.7	0.9	0.001	< 0.001	0.50
Fat, %	3.22	3.19	3.10	2.89	2.53	2.32	0.25	0.029	0.002	0.22
Fat yield, kg/d	1.14	1.12	1.01	0.99	0.84	0.77	0.06	0.003	0.0002	0.32
Protein, %	3.04	3.14	3.24	3.40	3.60	3.80	0.14	< 0.01	0.0002	0.26
Protein yield, kg/d	1.07	1.10	1.09	1.15	1.17	1.23	0.05	0.13	0.013	0.44

[1]SED = Standard error of difference between treatments.

Replacing alfalfa hay with alfalfa pellets linearly increased the daily averages of total VFA (121.9 vs. 90.0 m*M*, $P < 0.001$), acetate (66.8 vs. 53.9 m*M*, $P = 0.003$), propionate (39.6 vs. 21.5 m*M*, $P < 0.001$), valerate (2.0 vs. 1.1 m*M*, $P < 0.001$), and osmolality (300.9 vs. 277.7 mOsm/kg, $P = 0.002$) in the rumen (Table 14, 15). This replacement quadratically increased butyrate concentration from 10.8 m*M* in wk 1 to 12.3 m*M* in wk 6 ($P = 0.009$), but did not affect total branch chain VFA (isobutyrate + isovalerate), which averaged 2.2 m*M* across all weeks. Feeding alfalfa pellets decreased the acetate to propionate ratio (Ac:Pr) in the rumen from 2.62 to 1.73, and tended to reduce the rumen ammonia from 7.0 to 5.5 mg/dL ($P = 0.06$) (Table 14). Concentrations of VFA were significantly increased by hours post-feeding (Table 14, Figure 3). The interaction of week, which corresponded with the inclusion rate of alfalfa pellets, with hours post-feeding on total VFA in rumen, is shown in Figure 3.

Across sampling times, gradual replacement of alfalfa hay with alfalfa pellets linearly increased free ruminal LPS concentration from 38,019 EU/mL in wk 1 to 165,960 EU/mL in wk 6 ($P < 0.001$) (Table 15). Rumen LPS concentration did not vary following feeding during wk 1 (control), but declined by 6 h post-feeding from wk 3 onwards ($P = 0.039$) when SARA was induced (Table 15). Averaged across wk 3 to wk 6, free rumen LPS concentration decreased from 145,725 EU/mL before feeding to 94,685 EU/mL at 6 h after feeding.

Blood Parameters

Regardless of the week of experiment and sampling time, peripheral LPS concentration remained below the minimum detection level of 0.05 EU/mL for the

Figure 2. Weekly averages of rumen pH and time spent below pH 5.6 of dairy cows in a 6 wk period during which dietary alfalfa hay was gradually replaced with alfalfa pellets to induce low pH. Error bars indicate standard error of difference between treatments (SED).

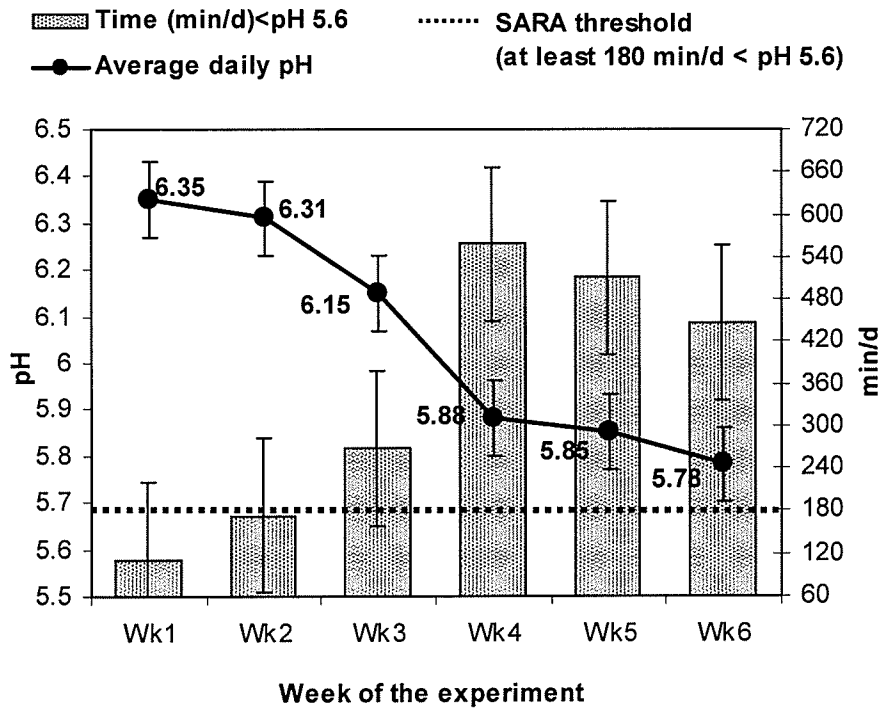


Table 13. Rumen pH variables based on continuous rumen pH monitoring using indwelling probes of dairy cows in a 6 wk period during which dietary alfalfa hay was gradually replaced with alfalfa pellets to induce low pH

Item	Week						SED	Effect, *P value*		
	Wk1	Wk2	Wk3	Wk4	Wk5	Wk6		Week	Linear week	Quad. week
Average daily pH	6.35	6.31	6.15	5.85	5.85	5.78	0.08	< 0.001	< 0.001	0.52
Time< pH 5.6, min/d	112	174	268	558	510	447	109.5	0.04	0.004	0.24
Time< pH 6, min/d	312	437	517	1002	940	975	108	< 0.001	< 0.001	0.28
Area<pH 5.6, min×pH/d	24	41	93	107	110	69	41	0.25	0.09	0.09
Area<pH 6, min×pH/d	102	163	223	424	408	340	69.4	0.01	0.001	0.18

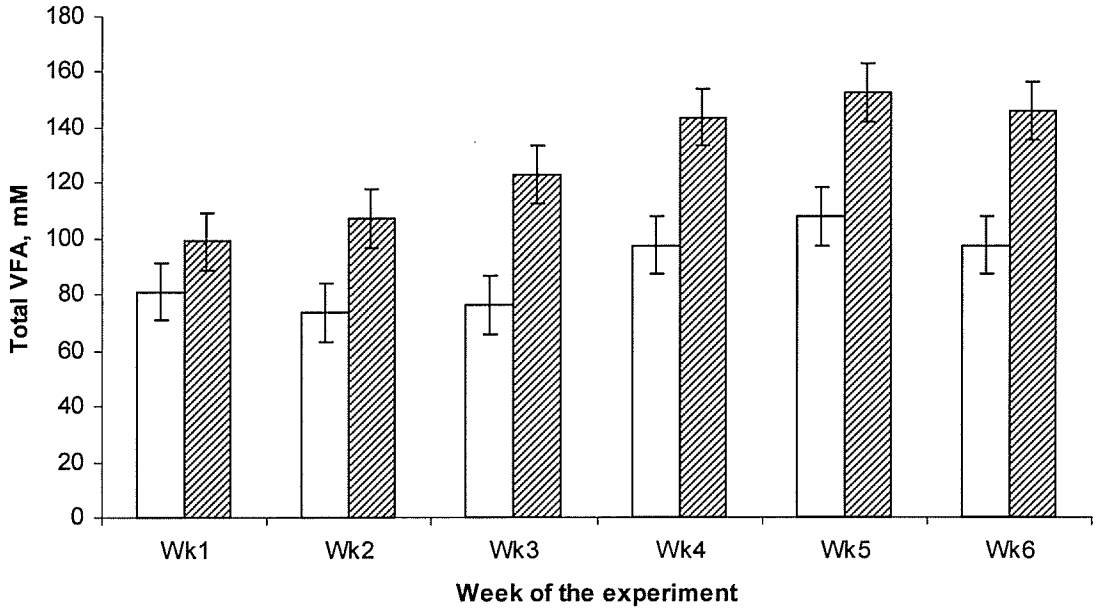
[1]SED = Standard error of difference between treatments.

Table 14. Rumen fluid composition and blood metabolites of dairy cows in a 6 wk period during which dietary alfalfa hay was gradually replaced with alfalfa pellets to induce low pH

Item[1]	Week						SED[2]	Effects, *P* value				
	Wk1	Wk2	Wk3	Wk4	Wk5	Wk6		Week	Linear week	Quad. week	Hour[3]	Hour × Week
Ruminal parameters												
VFA, m*M*												
Total	90.0	90.5	99.2	120.4	130.5	121.9	6.3	0.008	< 0.001	0.47	< 0.001	0.001
Acetate	53.9	52.7	55.0	67.4	74.5	66.8	3.7	0.02	0.003	0.69	< 0.001	< 0.001
Propionate	21.5	21.8	26.4	32.8	38.3	39.6	2.7	0.004	< 0.001	0.71	< 0.001	0.003
Butyrate	10.8	12.5	13.5	16.8	14.7	12.3	1.3	0.04	0.076	0.009	< 0.001	< 0.001
Valerate	1.1	1.3	1.6	1.9	2.1	2.0	0.2	0.009	< 0.001	0.18	< 0.001	0.42
Total branch chain[4]	2.8	2.4	3.6	1.9	1.2	1.2	0.6	0.17	0.51	0.30	0.02	0.23
Acetate: Propionate	2.62	2.55	2.22	2.21	2.00	1.73	0.18	0.02	< 0.001	0.68	< 0.001	< 0.001
Ammonia, mg/dL	7.0	8.1	7.4	6.4	5.7	5.5	0.9	0.16	0.06	0.43	< 0.04	< 0.01
Blood parameters												
Glucose, mg/dL	67.4	64.7	66.5	68.3	70.4	67.9	1.95	0.12	0.078	0.85	0.01	0.83
Lactate, m*M*	1.00	0.98	1.14	2.25	3.46	1.86	0.64	0.004	0.002	0.37	0.21	0.63

[1]Average concentration per day across sampling times.
[2]SED = Standard error of difference between treatments.
[3]Time of sampling 15 min before and at 6 h after feeding in two non-consecutive days during each wk. The first day of sampling was two days after diet change.
[4]Isobutyrate + isovalerate.

Figure 3. Rumen total VFA (m*M* /d) before (□) and at 6 h (⧄) after feeding in dairy cows in a 6 wk period during which dietary alfalfa hay was gradually replaced with alfalfa pellets to induce low pH. Error bars indicate standard error of difference between treatments (SED)



method (Table 15). Blood concentrations of the acute phase proteins Hp, SAA, and LBP linearly decreased during the 6 wk of the experiment from 56 to 12 μg/mL ($P < 0.0001$), from 23.1 to 6.9 μg/mL ($P = 0.003$), and from 7.2 to 2.6 μg/mL ($P = 0.038$), respectively (Table 16).

Across sampling times, replacement of alfalfa hay with alfalfa pellets did not affect blood glucose concentration, but linearly increased (P = 0.002) blood lactate (Table 14). The peak of lactate (3.46 mM) in blood was observed during wk 5.

Table 15. Rumen and plasma LPS, and rumen osmolality of dairy cows in a 6 wk period during which dietary alfalfa hay was gradually replaced with alfalfa pellets to induce low pH

Item	Week						SED[1]	Effects, *P* value				
	Wk1	Wk2	Wk3	Wk4	Wk5	Wk6		Week	Linear week	Quad. week	Hour[2]	Hour × Week
Plasma LPS, EU/mL[3]												
0 h	< 0.05[4]	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	-	-	-	-	-	-
6 h	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05						
Free rumen LPS, EU/mL[5]												
0 h	43,028	41,715	103,167	127,903	207,032	174,729	15,870	< 0.001	< 0.001	0.55	0.039	0.23
6 h	41,215	47,840	60,969	78,918	131,500	116,456						
Rumen osmolality, mOsm/kg												
0 h	267.8	269.4	264.9	271.4	276.1	264.6	7.55	0.01	0.002	0.23	< 0.01	0.05
6 h	287.5	309.1	307.8	316.8	337.1	323						

[1]SED = Standard error of difference between treatments.

[2]Time of rumen fluid sampling 15 min before and at 6 h after feeding in two non-consecutive days during each wk. The first day of sampling was two days after diet change.

[3]EU= Endotoxin unit.

[4]The minimum detection level of plasma LPS with this method was 0.05 EU/mL.

[5] Statistical analyses were conducted on $\log_{10}$-transformed data.

Table 16. Blood[1] concentrations of serum amyloid-A (SAA), haptoglobin (Hp), and LPS binding protein (LBP) of dairy cows in a 6 wk period during which dietary alfalfa hay was gradually replaced with alfalfa pellets to induce low pH

Item	Week						SED[1]	Effect, *P* value		
	Wk1	Wk2	Wk3	Wk4	Wk5	Wk6		Week	Linear week	Quad. week
Plasma SAA, μg/mL	23.1	12.3	7.8	12.3	9.6	6.9	3.9	0.013	0.003	0.11
Serum Hp, μg/mL	56	34	27	28	21	12	6.0	< 0.0001	< 0.0001	0.15
Plasma LBP, μg/mL	7.2	4.2	3.4	3.9	5.1	2.6	2.4	0.09	0.038	0.29

[1]Blood samples were collected at 6 h post feeding four days following diet change in each wk.
[2]SED = Standard error of difference between treatments.

DISCUSSION

The current definitions of subacute ruminal acidosis are based on rumen pH (Plaizer et al., 2008). There is no general agreement on the pH threshold that is definitive of SARA, as rumen pH of 5.5 (Garrett et al., 1999), between 5.2 and 5.6 (Cooper and Klopfenstein, 1996), 5.8 (Beauchemin et al., 2003) and 6 (Plaizier, 2004) have all been suggested as thresholds for SARA. These thresholds have been suggested based on criteria such as microbial activity in the rumen, health and function of rumen epithelium, and the method of pH measurement. We defined SARA as daily episodes of low rumen pH between 5.2 and 5.6 for at least 180 min/d (Gozho et al., 2005). Based on this definition, the replacement of 16% of the DM of alfalfa hay with alfalfa pellets induced SARA in this study (268 min/d of pH < 5.6). The replacement of 24% and 32% of the DM of alfalfa hay with alfalfa pellets further reduced rumen pH and resulted in average duration of rumen pH below 5.6 for 558 and 510 min/d, respectively. These durations were greater than 506, 412, 328, 309 and 279 min/d of rumen pH below 5.6 reported by Krause and Oetzel (2005), AlZahal et al. (2008), Bevans et al. (2005), Gozho et al. (2007), and Khafipour et al. (2008a), respectively, who induced SARA by feeding high grain diets.

The greater of rumen pH depression during alfalfa pellet-induced SARA compared to the grain-induced SARA in earlier studies and the differences among the rumen pH depression among these earlier studies were due to the dietary differences between the models of SARA induction. Krause and Oetzel (2005), Gozho et al. (2007), and Khafipour et al. (2008a) induced SARA by replacing 20%, 25% and 21% of the DM of the TMR with wheat-barley pellets, respectively. AlZahal et al. (2008) induced SARA by

replacing 18.9% and 9.5% of the DM of the TMR with ground wheat and ground barley, and Bevans et al. (2005) induced SARA by replacing 25% of the DM of the TMR with dry-rolled barley. Induction of SARA by increasing the content of starch and reducing the F:C ratio of the diet results in accumulation of organic acids in the rumen, reduction of chewing and rumination activity, leading to reduced rumen buffering, and increased rumen acidity (Plaizier et al., 2008). In contrast, in the current study SARA was induced by replacing alfalfa hay with alfalfa pellets without changing the F:C ratio and the dietary content of starch. As a result, the diets fed in wk 4 and wk 5 of our study, which induced the lowest rumen pH, contained 74% and 82% of concentrate and pelleted ground forages, and 26% and 18% of alfalfa hay, respectively. The size of alfalfa pellets were above the recommendations for particle length that could stimulate chewing and rumination (8.63 vs. 1.18 mm, respectively) (Poppi et al., 1980). However, pellets consisted of ground forages, which would have rapidly disintegrated in the rumen. Although we did not measure the fermentability of alfalfa pellets, the increase in rumen VFA to the levels similar to grain-induced SARA (Bevans et al., 2005; Gozho et al., 2007) suggests that alfalfa pellets were highly fermentable. Uden (1999) showed that cows that were consuming only ground pelleted hay did not ruminate. We assume that alfalfa pellets might not stimulate chewing, saliva production, and rumen buffering. In addition, the forage fraction of the diet in this study was consisted only of alfalfa hay and pellets, while the TMR fed during grain-induced SARA in the aforementioned earlier studies consisted between 30 and 40% of silages. The high DM content of hay and forage pellets compared to silages would have increased sorting against coarse particles

(Leonardi et al., 2005), and resulted in less rumen buffering and more pH depression in alfalfa pellet-induced SARA compared to grain-induced SARA.

Reduced or cyclic feed intake has been suggested as an indicator of SARA (Owens et al., 1998). These signs have been observed when SARA was experimentally induced by feeding a high grain diet (Bevans et al., 2005; Gozho et al., 2005; Khafipour et al., 2008a). Reasons for this reduction can include reduced pH and contractions in the rumen, and increased rumen osmolality and VFA, especially propionate (Owens et al., 1998; Kleen et al., 2003). However, in our study, a similar decline in rumen pH and similar increases in total VFA and osmolality compared to grain-induced SARA (Goad et al., 1998; Bevans et al., 2005; Khafipour et al., 2008a) did not reduce feed intake. Moreover, induction of SARA by replacing alfalfa hay with alfalfa pellets progressively increased the DMI during the 6 wk period. This increase could have been due to the change in the physical composition of the diet, which could affect the physical regulation of feed intake. Increasing the dietary content of forage pellets might have reduced the filling effect of the diet in the reticulo-rumen, and increased the DMI, as the density of ingested particles, and time for eating and ruminating was increased (Allen, 2000).

Feed intake also could have been affected by VFA composition and Ac:Pr ratio in the rumen, as propionate but not acetate has hypophagic effects (Allen et al., 2005). However, when propionate is oxidized to acetyl CoA, DMI is reduced, but this does not occur if propionate is used for gluconeogenesis (Allen et al., 2005). Thus, factors that influence the pool of acetyl CoA, demand of glucose, blood insulin and insulin sensitivity of the liver cells can alter the effect of propionate on feed intake (Allen et al., 2005; Bradford and Allen, 2007). In this study, replacing 32% of alfalfa hay with alfalfa pellets

from wk 1 to wk 5 progressively reduced the Ac:Pr ratio from 2.62 to 2.00, but increased feed intake from 18.1 to 23.4 kg of DM/d. Additional replacement of alfalfa hay with alfalfa pellets during wk 6 further reduced the Ac:Pr ratio to 1.76, which may inhibited the further increase in the DMI (23.4 kg of DM/d in wk 6). Bevans et al. (2005) reported a reduction in Ac:Pr ratio to 1.98 during grain-induced SARA, which was accompanied by a 13% decline in the DMI. Similarly, grain-induced SARA in the study of Khafipour et al. (2008a) reduced Ac:Pr ratio from 2.86 to 1.61 and resulted in a 15% decline in the DMI. In contrast, despite a decline in Ac:Pr ratio from 3.5 to 2.2, Gozho et al. (2007) did not observe a significant change in the DMI during grain-induced SARA. Although it appears that Ac:Pr ratio below 2 have more detrimental effect on feed intake, hypophagic effects of propionate must be considered along with other factors that influencing its fate in the liver (Bradford and Allen, 2007).

Alfalfa pellet-induced SARA increased the concentration of free rumen LPS from 38,019 to 165,960 EU/mL. This increase was greater than the reported range for grain-induced SARA, which were from 24,547 to 128,820 EU/mL, from 28,184 to 107,150 EU/mL, and from 7,900 to 88,700 EU/mL reported by Gozho et al. (2007), Khafipour et al. (2008a), and Emmanuel et al. (2008), respectively. The diurnal variation in rumen LPS appears to have a different rhythm during the alfalfa pellet-induced SARA in our study compared to grain-induced SARA in earlier studies. In the alfalfa pellet-induced SARA, the free rumen LPS content declined by 6 h post-feeding, while it sharply increased during grain-induced SARA (Gozho et al., 2006; Khafipour et al., 2008a). The concentration of free LPS increases in the rumen during the early hours post-feeding primarily due to the increase in shedding of LPS from rapidly growing Gram-negative

bacteria (Hurley, 1995; Wells and Russell, 1996; Andersen, 2000). The concentration of free LPS continues to rise later because of the increase in bacterial lysis due to the lower pH in the rumen (Nagaraja et al., 1978c). Provision of additional starch during grain-induced SARA could have triggered the rapid growth of starch/sugar fermenting Gram-negative bacteria, such as *Prevotella* spp., *Ruminobacter amylophilus, Succinimonas amylolytica, Succinivibrio dextrinosolvens, and Escherichia coli,* (Russell and Rychlik, 2001; Nagaraja and Titgemeyer, 2007) and therefore, increased the shedding of LPS during the early hours post-feeding. In contrast, in our study induction of SARA was achieved without adding starch, and, therefore, without providing additional starch to increase the growth of amylolytic Gram-negative bacteria. However, the ground pelleted forage could have triggered the growth of cellulolytic Gram-negatives such as *Fibrobacter succinogenes* as the surface area for the bacterial attachment was increased (McAllister et al., 1994). However, *F. succinogenes* population is less than five percent of the rumen bacteria (Tajima et al., 1999; Tajima et al., 2001). Hence, its contribution to the free rumen LPS pool is small compared to that of other Gram-negative bacteria, as a result, free rumen LPS declined during the early hours post-feeding.

Both alfalfa-pellet induced SARA in our present study and grain-induced SARA in earlier studies increased free rumen LPS (Gozho et al., 2007; Khafipour et al., 2008a; Emmanuel et al., 2008). However, when plasma LPS was monitored with a similar sensitive technique, only grain-induced SARA increased LPS in the peripheral plasma (Khafipour et al., 2008a). Despite greater free rumen LPS in alfalfa pellet-induced than grain-induced SARA, absence of LPS in the peripheral circulation suggests that free LPS in the rumen is not the cause of LPS translocation to the blood. This view is supported by

the findings of Lassman (1980), who reported that infusion of Cr-labeled LPS into the rumen of steers was not followed by LPS translocation into the lymph or portal circulation. Altogether, these results may suggest that rumen epithelium has a low permeability to LPS and the rate of LPS translocation through the rumen wall must be below its clearance rate by the liver (Andersen and Jarlov, 1990), so that translocation of small amounts of LPS prevents its accumulation in the peripheral blood. However, this hypothesis is challenged by recent observations showing that administration of 500 μg/mL of LPS to the epithelium of rumen resulted in translocation of LPS across the rumen wall and this translocation was pH independent (Emmanuel et al., 2007). However, the concentration of LPS that was applied to the mucosal side of rumen tissue in the study of Emmanuel et al. (2007) was 50 times more than free rumen LPS concentration during SARA (Gozho et al., 2007; Emmanuel et al., 2008), and that may have disrupted the barrier function of the rumen to a greater extent than what would have occurred physiologically.

Previous studies showed that abnormal increase in luminal LPS disrupts the barrier function of the epithelium and increases gut permeability through production of nitric oxide ($NO^{\cdot}$) (Chin et al., 2006). However, differences between the composition of the epithelium of the rumen, and of the small and large intestine could result in differences in the effect of LPS on the barrier impairment, and therefore, on the rate of LPS translocation from these parts of the gastro-intestinal tract (Graham and Simmons, 2005). If we assume that the rate of LPS translocation through the multilayer epithelium of the rumen is below its clearance rate by the liver, then peripheral LPS during grain-induced SARA must be translocated through the simple epithelium of the small or large intestine

(Chin et al., 2006). However, before arriving to these sites, free rumen LPS is detoxified in the duodenum, primarily by bile acids (Bertok, 1998). Hence, accumulation of LPS in the terminal ileum and in the large intestine must be due to shedding of LPS from Gram-negative bacterial communities in these components of the intestine. Allen (2000) indicated that up to 44% of starch intake can be digested postruminally. As a result, higher content of dietary starch during grain-induced SARA could have triggered the shedding of LPS from Gram-negatives in the terminal ileum and large intestine. Accumulation of free LPS in this part of the intestine probably resulted in barrier failure, which allowed LPS to translocate into the circulatory system and triggered an inflammatory response. In contrast, in alfalfa pellet-induced SARA, there was no additional starch added to the diet to modify the profile of bacterial communities of the intestine. This might explain why we could not find any evidence of LPS in the peripheral circulation. Lending support to this hypothesis, a recent study in our group (Li et al., 2008) found that LPS concentration in the feces was ~2.5 times more in grain-induced SARA than alfalfa pellet-induced SARA (82,842 vs. 34,179 EU/mL, respectively).

Additional support for our hypothesis is provided by the absence of increases in the acute phase proteins SAA, Hp and LBP in blood, which are inflammatory markers (Horadagoda et al., 1999). In particular, the lack of an LBP response supports this hypothesis, as it is a specific marker of LPS that binds to LPS to facilitate its transfer to membrane-associated receptors and enhance the immune response. Also, LBP mediates LPS transfer to lipoproteins resulting in neutralization of LPS (Sriskandan and Altmann, 2008). Previous studies showed that grain-induced SARA was accompanied by LPS

translocation into the bloodstream (Khafipour et al., 2008a) and increased SAA, Hp and LBP concentrations in the peripheral blood (Gozho et al., 2006; Khafipour et al., 2008a; Emmanuel et al., 2008). However, induction of SARA by replacing alfalfa hay with alfalfa pellets did not increase the concentration of LPS and acute phase proteins in peripheral blood. Despite the similarity in rumen pH and free rumen LPS between grain- and alfalfa pellet-induced SARA, these observations suggest that low pH and free LPS in the rumen are not the sole cause of the inflammatory response seen during grain-induced SARA.

We believe that the level of free LPS in the gut lumen that is required to impair the epithelial barrier function determines the site and rate of LPS translocation. Since rumen epithelium has multilayer structure and is more complex than monolayer epithelium of the small and large intestine (Graham and Simmons, 2005) a greater level of LPS would be required to disrupt the barrier function and result in LPS translocation. What minimum level of LPS is required to impair the barrier function of rumen or intestinal epithelium remains unanswered. Chin et al. (2006) reported that the cytotoxic effect of LPS on enterocytes apoptosis occurred at a luminal concentration of 40 μg/mL (400,000 EU/mL) after half an hour of administration of LPS and that these effects increased over time. However, these authors did not monitor the effect of lower concentrations of LPS during a longer time span. Vreugdenhil et al. (1999) found that luminal LPS concentration up to 10 μg/mL (100,000 EU/mL) did not increase the release of LBP and SAA by colonic adenocarcinoma Caco-2 cell lines that express structural and functional properties of small intestine enterocytes. This characteristic protects a host from a continuous inflammatory response to the high levels of LPS that is present in the lumen. The

reported concentration of free rumen LPS is in the range of 88,000 to 170,000 during SARA in dairy cows (Gozho et al., 2007; Khafipour et al., 2008a; Emmanuel et al., 2008). However, we have observed ruminal concentration of 250,000 to 350,000 EU/mL in some cows during SARA and we speculate that free rumen LPS concentration must be higher during acute rumen acidosis than SARA. Whether ruminal concentration of LPS was not high enough to disrupt the rumen barrier function during SARA and the accumulation of free LPS in the hindgut reached a level that impairs the intestinal barrier function during grain-induced SARA remains to be answered.

The initiation and magnitude of the acute phase response is coordinated by proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 (Sriskandan and Altmann, 2008). Release of these mediators in response to plasma LPS reduces feed intake (Steiger et al., 1999). Plasma LPS also reduces the insulin sensitivity of the liver cells through the function of CD14, a cell membrane receptor of LPS, and increases blood glucose level (Cani et al., 2007). These changes can alter the direction of propionate metabolism in hepatocytes from gluconeogenesis to oxidation as acetyl CoA, which cause satiety. While confirming earlier results these observations suggest that the differences in the DMI response between two models of SARA induction can be also in part due to the increased plasma LPS and proinflammatory mediators of acute phase response during grain-induced SARA (Khafipour et al., 2008a).

CONCLUSION

A rumen pH depression typical of SARA (at least 180 min/d of pH < 5.6) was created by increasing the dietary content of pelleted forage without changing the F:C

ratio and dietary content of starch. This induction was accompanied by increases in rumen VFA, osmolality, and LPS that were similar to those observed during grain-induced SARA. Also, the induction of SARA in our study reduced milk yield, and milk fat percentage and yield, similar to what has been observed during experimentally grain-induced SARA. However, in our study, the induction of low pH and high free LPS in the rumen was not accompanied by an inflammatory response, whereas grain-induced SARA commonly initiates such a response. Moreover, the induction of low pH or high LPS in the rumen in this study did not reduce DMI, which has been reported as one of the key symptoms of SARA. We speculate that high luminal concentrations of free LPS can disrupt the barrier function of the gut and cause translocation of free LPS into the blood circulation. However, free rumen LPS concentrations that are typical of SARA may not be high enough for this impairment of the barrier function. Inclusion of additional starch during grain-induced SARA may trigger the release of LPS from Gram-negative bacteria in the lower gut and increase the free luminal LPS to the levels that impairs the barrier function of monolayer epithelium of the large intestine, cause translocation of LPS in this part of the intestine, and initiate the inflammatory reaction that is seen during grain-induced SARA. The differences in the effects of the rumen pH depressions during alfalfa-pellet induced SARA and grain-pellet induced SARA challenge the current definition of SARA, which is currently only based on the rumen pH depression.

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MANUSCRIPT III

Rumen microbial community analysis using T-RFLP and real-time PCR in dairy cattle experimentally induced with subacute ruminal acidosis (SARA)

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ABSTRACT

Rumen microbial community composition in lactating dairy cows subjected to subacute ruminal acidosis (SARA) was analyzed using terminal restriction fragment length polymorphism (T-RFLP) and real-time PCR. The objective was to examine the relationships between microbial population shifts and free lipopolysaccharide (LPS) in the rumen, and the systemic inflammatory response. SARA was induced with two different nutritional models, one with grain and the other with alfalfa pellets. Based on the severity of SARA (rumen pH < 5.6, free rumen LPS, and serum haptoglobin) cows were divided into mild and severe groups only with grain-induced SARA. Indicators of SARA could not separate animals in alfalfa-pellet model. T-RFLP analysis demonstrated that at the phylum level SARA was associated with an increase in *Firmicutes* and a decline in *Bacteroidetes* ($P < 0.05$). The proportion of *Bacteroidetes* was greater in alfalfa pellet-induced SARA compared to mild or severe grain-induced SARA (35.4% vs. 26.0% and 16.6% respectively). Mild, control, and alfalfa pellet-induced SARA had the most similar microbial composition. Real-time PCR indicated that severe grain-induced SARA was dominated by *Streptococcus bovis* and *Escherichia coli*, mild grain-induced SARA was dominated by *Megasphaera elsdenii*, and alfalfa pellet-induced SARA was dominated by *Prevotella albensis* and *Ruminococcus albus*. Based on multivariate discriminative analysis, the severity of SARA and inflammation was highly correlated with the abundance of *E. coli* and not free LPS in the rumen. We thus suspect that a subset of *E. coli* might be the putative pathogenic trigger for SARA.

Key words: subacute ruminal acidosis, T-RFLP, real-time PCR

INTRODUCTION

Subacute ruminal acidosis (SARA) is less well recognized than acute lactic acid acidosis. Lactic acid acidosis occurs when ruminant animals are abruptly transitioned from a high forage diet to a high grain diet consisting predominantly of starch (Owens et al., 1998). This transition results in rapid proliferation of opportunistic bacteria like *Streptococcus bovis* and accumulation of lactic acid in the rumen (Russell and Hino, 1985). The consequences are acute ruminal and systemic acidosis due to pH decline in the rumen to below 5.0 and increased absorption of lactic acid into the blood (Nocek, 1997). If however, the adaptation to high grain diets happens gradually, the slower growing lactic acid consuming bacteria like *Megasphaera elsdenii* increase in number and convert the lactic acid to VFA (Nocek, 1997). Further accumulation of VFA reduces rumen pH to below 5.6, which is the threshold for SARA (Cooper and Klopfenstein, 1996). As rumen pH nears 5, the growth of lactic acid consuming bacteria is inhibited and the chance of lactic acid acidosis could increase. Thus, the borderline between acute lactic acidosis and SARA is narrow and could be depend on the equilibrium between lactic acid producing and utilizing bacteria.

The adverse effects of acute ruminal acidosis and SARA are not all due to the rumen acid production. Substantial evidence exists indicating that acute ruminal acidosis and SARA increase the concentration of free lipopolysaccharide (LPS) in the rumen (Andersen and Jarlov, 1990; Gozho et al., 2005; Emmanuel et al., 2008). This increase in luminal LPS can increase the permeability of the gut to LPS, which triggers systemic inflammatory response (Chin et al., 2006). We developed two animal models of SARA, one based on grain and the other based on alfalfa pellets. However, despite similarity in the rumen VFA and LPS levels only grain-induced SARA activated an inflammatory

reaction (Khafipour et al., 2008a; 2008b). These differences might to be associated with specific microbial changes that differ among diets.

In contrast to lactic acid acidosis the rumen microbiology of SARA is not well studied (Goad et al., 1998; Nagaraja and Titgemeyer, 2007). Moreover, the limited information that is available is provided through culture-based studies, which only allow for the isolation of a limited number of species of microorganisms, which does not reflect global rumen microbial changes. In this study, we examined the global microbial changes that occur with grain- versus alfalfa pellet-induced SARA using terminal restriction fragment length polymorphism (T-RFLP) of 16S rDNA genes, which is a valuable tool for comparing the composition of microbial communities within and between ecosystems (Liu et al., 1997). We also quantified key microbial species with real-time PCR.

MATERIAL AND METHODS

Animal experiments were conducted in Glenlea Dairy Research Unit at the University of Manitoba (Winnipeg, MB, Canada) in accordance to the guideline of the Canadian Council on Animal Care (CCAC, 1993). Data for rumen pH, feed intake, milk yield and composition, and rumen and blood metabolites for these experiments were reported previously (Khafipour et al., 2008a; 2008b). In this manuscript we recalculate the previous data in the light of the microbial information generated in this work.

Animal Models and Sampling

SARA was induced in four rumen cannulated lactating dairy cows using two different nutritional models, one with grain and the other with alfalfa pellets. In both

experiments, animals were fed once daily ad libitum at 09:00 h. In the first experiment (grain-induced SARA), cows received a basal diet with a forage-to-concentrate (F:C) ratio of 50:50 consisting of 25% alfalfa silage, 25% barley silage, 40% energy supplement and 10% protein supplements as dry matter (DM) basis. SARA was induced by abruptly by replacing 21% of the DM of the diet with pellets containing 50% wheat and 50% barley resulting in a F:C ratio of 40:60. In the second experiment (alfalfa pellet-induced SARA), cows received a basal diet that contained 50% of the DM as concentrate and 50% of the DM as chopped alfalfa hay. The concentrate fraction was consisting of 39% energy supplement, 4.5% protein supplement, and 6.5% roasted soybeans as DM basis. SARA was induced by gradually replacing 42% of the DM of alfalfa hay with alfalfa pellets during a six weeks period and without changing the F:C ratio or the dietary content of starch.

In both experiments, the severity of SARA in individual animals was determined based on three objective criteria including the duration of rumen pH below 5.6, free rumen LPS, and serum haptoglobin as an inflammatory marker (Gozho et al., 2005). Rumen fluid samples were collected from ventral sac of the rumen 15 min before feeding and at 6 h after feeding during the control period and four days after induction of SARA during the SARA periods. Ruminal contents were strained through four layers of sterile cheesecloth, transferred into 50 mL sterile tubes, and immediately frozen in liquid nitrogen. Samples were stored in -20°C until further analysis.

DNA Extraction

Rumen fluid samples were thawed at 32°C for 15 min and immediately centrifuged at 10,000 × *g* for 20 min. Supernatants were discarded and pellets were resuspended in

PBS in new sterile tubes. Then, approximately 150 mg of wet mass was washed in 1 mL of PBS and centrifuged at 10,000 × *g* for 2 min. The washing step was repeated twice. DNA was extracted from the pellets by using ZR Fecal DNA Kit (D6010, Zymo Research Corp., Orange, CA), which included a bead beating step for the mechanical lysis of the microbial cells. We followed the manufactuers instruction except that we increased the bead-beating step to 3 min. DNA concentration and purity were determined spectrophotometrically by measuring the optical density (OD) and $A_{260/280}$ (Beckman DU/800, Beckman Coulter Inc., Fullerton, CA).

PCR Amplification and T-RFLP

Microbial composition in the rumen fluid was assessed using T-RFLP as described by Sepehri et al. (2007) with some modifications. In brief, the V1 and V2 regions of the 16S rDNA genes were PCR amplified using universal bacterial primers 27f (5'-GAAGAGTTTGATCATGGCTCAG-3') and 342r (5'-CTGCTGCCTCCCGTAG-3') (Lane, 1991). Forward primers were fluorescently labeled (WellRED D4dye, Sigma-Proligo, St. Louis, MO) to allow detection of the fragments by capillary electrophoresis. The PCR reactions were as follows: 1 cycle of 94°C for 5 min; then 36 cycles of 94°C for 1 min; 56°C for 1 min; 72°C for 2 min; and a final extension at 72°C for 5 min. To produce terminal restriction fragments (T-RF), the PCR products were digested with *Hha*I (15 μL of PCR product, 10 units of *Hha*I, 1X *Hha*I buffer, and 20 μg of bovine serum, New England Biolabs, Ipswich, MA) at 37°C for 3 hours. The precise length of T-RF amplicons were determined on a CEQ 8800 Genetic Analysis System (Beckman Coulter Inc., Fullerton, CA). Then, 2 μL of fluorescently labeled fragments, 29.5 μL of sample loading solution, and 1 μL of 600 basepair (bp) DNA size standard (Beckman

Coulter Inc., Fullerton, CA) were mixed and applied to the capillaries. An electropherogram with peaks of different sizes was obtained for each rumen fluid sample, and each peak of unique size represents an operational taxonomic unit (OTU).

Fragment Analysis

CEQ software (Version 9.0; Beckman Coulter Inc., Fullerton, CA) was used to analyze the fragment data. A binning parameter of 2 bp was used and only peaks with relative abundances higher than 1% of the total were included. The incidence (presence/absence) data derived from the OTU profiles were grouped based on sampling times (0 h and 6 h) and dietary treatments and used for numerical analysis.

Richness, Diversity and Similarity

Chao2 incidence-based index of richness and Shannon incidence-based diversity estimator were calculated using EstimateS (Version 7.5; Colwell, 2006) to determine the richness and diversity of microbial community in each time/treatment group. An upper abundance limit of 5 was used to determine rare or infrequent species. The order of the samples was randomized 500 times for each run to reduce the effect of sample order. Similarities among microbial communities were also determined using SPADE (Version 2.1; Caho and Shen, 2008) based on Jaccard multiple incidence-based index.

Bioinformatic Analysis of T-RFLP Data

Only using T-RF does not allow for unequivocal identification of OTU to the genus or species level and for high resolution analysis sequencing reactions need to be done. To circumvent this problem, we have previously described a bioinformatic approach in

which we mined the Ribosomal Database Project (RDP, Cole et al., 2005) for all sequences that were found in the digestive tract (Sepehri et al., 2007; Bhandari et al., 2008). This was done be using the Boolean search terms: gut AND digestive AND rumen AND ruminant AND cow AND sheep AND goat AND herbivore AND pig AND swine AND avian AND mouse AND rat AND human, and applying it to the RDP search engine. The resultant sequence database was called the H.Q. 16S Gut Organisms. This database was then added to the Microbial Community Analysis (MiCA, Version 3; Shyu, et al., 2007) virtual digest (ISPaR) analysis database. We frequently update this database and it currently consists of 52,390 curated near full length sequences from the RDP that only come from the mammalian and avian gut.

As described by Sepeheri et al., (2007) an *in silico* reference database based on 27f and 342r plus *Hha*I restriction digestion was created and submitted to the Phylogenetic Assignment Tool (PAT; Kent et al., 2003) and compared to the experimentally produced data from capillary electrophoresis. The resultant libraries were then entered into the hierarchical browser of the RDP and converted to GenBank format. T-RF with multiple accession numbers were assigned to taxonomic rank according to phylum, class, order, and family. Based on this analysis, reported values were expressed as a proportion of phylogenetic lineage for each library (Tables 20, 21). We previously demonstrated that this analysis was not able to unambiguously identify peaks to the genus or family levels, but over 95% of T-RFs were unambiguous at the order level (Sepehri et al., 2007). This method provides a robust, quick, cost effective method of doing global community analysis of a large number of samples.

Primers and Real-time PCR

The PCR primers used are listed in Table 17. Primers were assembled from the literature or newly designed and tested for specificity *in silico*. Those primers that did not meet our selection criteria for specificity and performance were redesigned from sequence alignments. The oligonucleotides were synthesized by University Core DNA Services (University of Calgary, Calgary, AB).

Real-time PCR was carried out using an AB 7300 system (Applied Biosystems, Foster City, CA) and sequence detection software (Version 1.3; Applied Biosystems, Foster City, CA). Each reaction was run in triplicate in a volume of 25 μL in optical reaction plates (Applied Biosystems, Foster City, CA) sealed with optical adhesive film (Applied Biosystems, Foster City, CA). Amplification reactions were carried out with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) mixed with the selected primer set (Table 17) at a concentration of 0.5 μ*M* for each primer, and 2 μL (~12 ng) of genomic DNA. Different concentrations of forward and reverse primers only applied for *Prevotella brevis* and *Ruminobacter amylophilus*, which were 0.5 and 0.9 μ*M*, and 0.3 μ*M* each, respectively. Amplification consisted of one cycle of 95°C (10 min), and 40 cycles of denaturation at 95°C (15 sec), and annealing at 60°C (1 min). The only exceptions were for methanogenic archaea and ciliate protozoa that an annealing/extension of 63°C (30 sec)/72°C (30 sec) and 54°C (30 sec)/72°C (1 min) were applied, respectively. Final melting analysis was obtained by slow heating from 65°C to 95 °C. To evaluate the efficiency (E) of the amplification of each primer set, DNA templates were pooled (50 ng/reaction) and serially diluted 8 fold. Amplification efficiency was calculated from the slope of the standard curve

Table 17. Primers used for real-time PCR quantification

Target Organism	Primer set	Primer sequences (5' → 3')	T_m (°C)	G+C%	Amplicon size (bp)	Source of primer
Eubacteria	341-357F	CCTACGGGAGGCAGCAG	55.2	70.6	189	Muyzer et al., 1993
	518-534R	ATTACCGCGGCTGCTGG	56.2	64.7		
Prevotella albensis	ProAlb4F	GCGCCACTGACGCTGAAG	58.3	66.7	110	This study
	ProAlb4R	CCCCAAATCCAAAAGGACTCAG	56.6	50.0		
Prevotella brevis	PreBre2F	GCGAACTGGTTTCCTTGAGTGTATT	58.8	50.0	153	This study
	PreBre2R	ACCTTCGAGCTTTAGCGTCAGTTAT	57.6	40.0		
Prevotella bryantii	ProBry4F	GAAGGCAGCTCGCTGTAGTGTT	60.6	54.5	145	This study
	ProBry4R	CTTAACGCTTTCGCTTAGCCACT	59.4	47.8		
Prevotella ruminicola	PreRum92862F	GCGAAAGTCGGATTAATGCTCTATG	58.5	44.0	78	This study
	PreRum92862R	CCCATCCTATAGCGGTAAACCTTTG	59.3	48.0		
Succinimonas amylolytica	SucAmy2F	CGTTGGGCGGTCATTTGAAAC	55.2	52.4	139	This study
	SucAmy2R	CCTGAGCGTCAGTTACTATCCAGA	56.2	50.0		
Succinivibrio dextrinosolvens	SucDex1F	TAGGAGCTTGTGCGATAGTATGG	57.4	47.8	174	This study
	SucDex1R	CTCACTATGTCAAGGTCAGGTAAGG	58.4	48.0		
Ruminobacter amylophilus	RumAmy2F	CTGGGGAGCTGCCTGAAT	55.3	61.1	100	Stevenson and Weimer, 2007
	RumAmy2R	CATCTGAATGCGACTGGTTG	54.2	50.0		
Escherichia coli	EcoliFimH2F	GCCGGTGGCGCTTTATTTG	57.3	57.9	114	This study
	EcoliFimH2R	TCATCGCTGTTATAGTTGTTGGTCT	58.4	40.0		
Fibrobacter succinogenes	FibSuc4F	GGAGCGTAGGCGGAGATTCA	58.7	60.0	97	This study
	FibSuc4R	GCCTGCCCCTGAACTATCCA	58.5	60.0		
Anaerovibrio lipolytica	AnaLip2F	TGGGTGTTAGAAATGGATTCTAGTG	56.6	40.0	109	This study
	AnaLip2R	GCACGTCATTCGGTATTAGCAT	56.7	45.5		
Megasphaera elsdenni	MegEls1F	GACCGAAACTGCGATGCTAGA	57.7	52.4	129	Ozutsumi et al.,

	MegEls1R	CGCCTCAGCGTCAGTTGTC	58.2	63.2		2006
Selenomonas ruminantium	SelRum1F	GGCGGGAAGGCAAGTCAGTC	60.4	65.0	83	This study
	SelRum1R	CCTCTCCTGCACTCAAGAAAGACAG	61.1	52.0		
Ruminococcus albus	RumAlb1F	CCCTAAAAGCAGTCTTAGTTCG	54.3	45.5	176	Wang et al., 1997
	RumAlb1R	CCTCCTTGCGGTTAGAACA	53.8	52.6		
Ruminococcus flavefaciens	RumFla1F	CGAACGGAGATAATTTGAGTTTACTTAGG	57.5	34.5	132	(Denman and McSweeney, 2006
	RumFla1R	CGGTCTCTGTATGTTATGAGGTATTACC	59.3	42.9		
Butyrivibrio fibrisolvens	ButFib2F	ACCGCATAAGCGCACGGA	58.8	61.1	65	Stevenson and Weimer, 2007
	ButFib2R	CGGGTCCATCTTGTACCGATAAAT				
Lactobacillus spp.	Ulac16S1F	AGCAGTAGGGAATCTTCCA	51.5	47.4	345	Walter et al., 2001, Lan et al., 2004
	Ulac16S1R	ATTCCACCGCTACACATG	51.1	50.0		
Streptococcus bovis	SBovis1F	TTCCTAGAGATAGGAAGTTTCTTCGG	57.9	42.3	127	Stevenson and Weimer, 2007
	SBovis1R	ATGATGGCAACTAACAATAGGGGT	57.9	41.7		
Treponema bryantii	TrpBry1F	GAGAAACGCTTTGTGGTGACTGT	59.5	47.8	122	This study
	TrpBry1R	CCTACATGCCCTTTACGCTCAAT	58.7	47.8		
Methanogenic archaea	MB1174f	GAGGAAGGAGTGGACGACGGTA	60.6	59.1	232	Ohene-Adjei et al., 2007
	Arch1406-1389r	ACGGGCGGTGTGTGCAAG	60.0	66.7		
Ciliate protozoa	UPorCil1F	GCTTTCGWTGGTAGTGTATT	50.2	20.0	234	Sylvester et al., 2004
	UPorCil1R	CTTGCCCTCYAATCGTWCT	50.4	47.4		

generated from the plotting of the threshold cycle (CT) versus logarithmic values of different DNA concentrations using the following equation (Denman and McSweeney, 2005):

$$E=10^{-1/slope}$$

Relative quantitation is accomplished using following mathematical model (Pfaffl, 2001):

$$R_i= [(E_{target})\Delta CT_{target} (Control_i - SARA_i)]/[(E_{ref})\Delta CT_{ref} (Control_i - SARA_i)]$$

Where *target* is the 16S or 18S rDNA gene of species of interest, *ref* is the 16S rDNA gene of *Eubacteria*, ΔCT is CT deviation of SARA vs. Control, *i* is sampling time (0 h or 6 h after feeding), and R_i is the relative expression ratio of a target gene compared to a reference gene at a specific time point.

Statistical Analysis

Discriminant multivariate analysis was conducted using JMP IN (Version 5.1; SAS Institute Inc., Cary, NC) to examine possible relationships among models of SARA induction and duration of rumen pH below 5.6, free rumen LPS, rumen microbial community dynamics and serum haptoglobin as inflammatory marker. Statistical significance ($P < 0.05$) was calculated using the LSD multiple comparison test to detect significant differences among times/treatments groups (SAS, 2004).

RESULTS

A significant variation in the animals' response to SARA induction was observed with the grain-induced model but not with the alfalfa model (Tables 18, 19). Multivariate discriminate analysis (Figure 4) of time below pH 5.6, serum haptoglobin, and the

concentration of free rumen LPS divided cows into three groups: severe grain-induced SARA; mild grain-induced SARA, and alfalfa pellet-induced SARA. The major difference between groups was in the indicators of inflammation.

Grain versus alfalfa-pellet feeding and severity of inflammation were used to group T-RFLP data (Tables 20, 21). We did T-RFLP analysis with primers 27f and 342r, as well as 27f and 1100r, but the larger amplicon appeared to be less efficiently generated because it always resulted in fewer T-RFs. Based on our analysis nine predominant phyla including *Bacteroidetes, Proteobacteria, Firmicutes, Spirochaetes, Actinobacteria, Fusobacteria, TM7, Tenericutes,* and *Deinococcus-Thermus* were present in the rumen (Tables 20, 21). However, more than 98% of rumen bacteria fit into three phyla of *Bacteroidetes, Proteobacteria, and Firmicutes.* The phylum *Fibrobacteres* was not detected with the universal primers used in this study. We checked the 27 and 342 primers against *F. succinogenes* sequences and all the sequences available start upstream of the 27 priming site.

Mild and severe grain-induced SARA and alfalfa pellet-induced SARA had different profiles at the phylum level (Tables 20, 21). The major differences between mild and severe grain-induced SARA was a significant increase in the phylum *Firmicutes* (79.7% vs. 69.6%), and a significant decrease in the phylum *Bacteroidetes* (16.6% vs. 26%) in the severe versus mild (Tables 20, 21). Within the *Firmicutes*, the family most affected by severe form was the *Lachnospiraceae* (61.4% vs. 44.7%), which is richly populated with rumen bacteria. Alfalfa pellet-induced SARA also exhibited significant increases in the phylum *Firmicutes*, and a decrease in the phylum *Bacteroidetes* (Tables

20, 21). However, in the alfalfa pellet-induced model *Bacteroidetes* were 35.4% and *Firmicutes* were 61.5%

Table 18. Time spent below pH 5.6, rumen LPS, plasma LPS, and serum haptoglobin of dairy cows during control and grain-induced subacute ruminal acidosis (SARA)

Item	Diet		SED[1]
	Control	Grain-induced SARA	
Time < pH 5.6, min/d			
Mild	75[b]	217[a]	72
Severe	161[b]	337[a]	
Rumen LPS, EU/mL			
Mild	32,413[Bb]	100,175[Ab]	30,072
Severe	29,933[b]	179,762[a]	
Serum Haptoglobin, μg/mL			
Mild	0.0[c]	343.3[b]	35.8
Severe	0.0[c]	608.0[a]	

[a,b,c]Means within the same row or column with different superscripts differ ($P < 0.05$).
[A, B]Means within the same row or column with different superscripts differ ($P < 0.1$).
[1]SED = Standard error of difference between treatments.

Table 19. Time spent below pH 5.6, rumen LPS, plasma LPS and serum haptoglobin of dairy cows during control and alfalfa pellet-induced subacute ruminal acidosis (SARA)

Item	Diet		SEM[1]
	Control	Alfalfa pellet-induced SARA	
Time < pH 5.6, min/d	112[b]	510[a]	109.5
Rumen LPS, EU/mL	42,122[b]	169,266[a]	16,780
Serum Haptoglobin, μg/mL	56[a]	21[b]	6.0

[a,b]Means within the same row with different superscripts differ ($P < 0.05$).

[1]SED = Standard error of difference between treatments.

Figure 4. Discriminant analysis of the duration of time below pH 5.6 in rumen, free rumen LPS, and serum haptoglobin in response to SARA induction model.

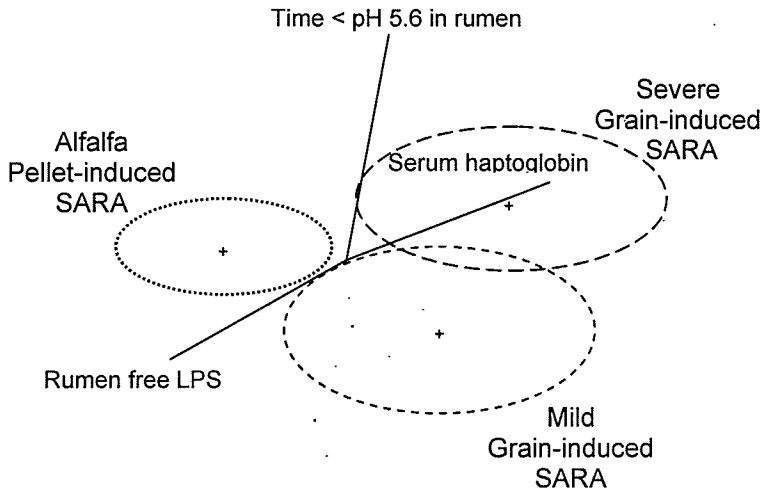


Table 20. Comparison of putative microbial distribution generated from terminal fragment (T-RF) libraries of rumen fluid samples 15 min before feeding during control, grain-induced, and alfalfa pellet-induced subacute ruminal acidosis (SARA)

Microbial level	Taxonomic rank (%)[1]					
	Control 0 h[2]			SARA 0 h		
	Grain-induced SARA		Alfalfa pellet-induced SARA	Grain-induced SARA		Alfalfa pellet-induced SARA
	Mild	Severe		Mild	Severe	
Phylum *Bacteroidetes*	28.18^{bc}	28.56^{bc}	85.50^{a}	25.78^{bc}	17.75^{c}	36.19^{b}
Class *Bacteroidetes*	27.81^{b}	28.25^{b}	85.28^{a}	25.53^{bc}	17.66^{c}	36.06^{b}
Order *Bacteroidales*	27.81^{b}	28.25^{b}	85.28^{a}	25.53^{bc}	17.66^{c}	36.06^{b}
Family *Prevotellaceae*	2.71^{c}	2.82^{c}	25.49^{a}	2.47^{c}	2.24^{c}	10.70^{b}
Phylum *Proteobacteria*	4.99	3.83	1.14	4.02	1.97	0.78
Class *Epsilonproteobacteria*	0.39	0.28	0.35	0.33	0.03	0.15
Order *Campylobacterales*	0.39	0.28	0.35	0.33	0.03	0.15
Class *Gammaproteobacteria*	2.21	2.10	0.51	1.93	1.37	0.23
Order *Aeromonadales*	0.02	0.02	0.03	0.02	0.00	0.01
Family *Succinivibrionaceae*	0.02	0.02	0.03	0.02	0.00	0.01
Order *Enterobacteriales*	1.47	1.53	0.03	1.35	1.21	0.01
Family *Enterobacteriaceae*	1.47	1.53	0.03	1.35	1.21	0.01
Phylum *Firmicutes*	64.74^{b}	66.03^{b}	12.44^{c}	68.53^{b}	79.74^{a}	62.34^{b}
Class *Clostridia*	59.53^{b}	60.91^{b}	11.02^{c}	64.08^{ab}	75.89^{a}	57.24^{b}
Order *Clostridiales*	57.49^{b}	58.84^{b}	10.11^{c}	63.08^{ab}	75.17^{a}	56.31^{b}
Family *Clostridiaceae*	0.69	0.72	0.00	0.64	1.11	0.01
Family *Eubacteriaceae*	0.03	0.03	0.00	0.03	0.03	0.03
Family *Lachnospiraceae*	35.86^{b}	36.23^{b}	1.60^{c}	43.60^{b}	60.14^{a}	39.12^{b}
Family *Peptostreptococcaceae*	0.03	0.02	0.05	0.02	0.00	0.02
Family *Ruminococcaceae*	15.55^{a}	16.29^{a}	3.70^{b}	14.15^{a}	10.83^{a}	11.72^{a}
Family *Veillonellaceae*	3.83	3.98	3.80	3.46	2.31	7.70
Class *Bacilli*	2.54	2.33	1.12	2.01	1.61	2.65
Order *Lactobacillales*	2.39	2.17	0.84	1.87	1.49	2.53
Family *Streptococcaceae*	1.93	2.00	0.56	1.77	1.42	1.81
Phylum *Spirochaetes*	0.01	0.01	0.00	0.01	0.00	0.00
Phylum *Actinobacteria*	1.43	1.29	0.53	1.12	0.64	0.29
Phylum *Fusobacteria*	0.02	0.02	0.00	0.02	0.00	0.00
Phylum *TM7*	0.01	0.01	0.00	0.01	0.01	0.00
Phylum *Tenericutes*	0.10	0.02	0.00	0.07	0.04	0.03

Phylum *Deinococcus-Thermus*	0.01	0.01	0.00	0.01	0.00	0.00
Unclassified Bacteria	0.50	0.21	0.38	0.42	0.03	0.29

[a,b,c]Means within a row with different superscripts differ ($P < 0.05$).

[1]Values are proportions of phylogenetic lineages for each library.

[2]Rumen fluid samples were collected 15 min before feeding four days after SARA induction during SARA periods.

Table 21. Comparison of putative microbial distribution generated from terminal fragment (T-RF) libraries of rumen fluid samples 6 h after feeding during control, grain-induced, and alfalfa pellet-induced subacute ruminal acidosis (SARA)

Microbial Level	Taxonomic rank (%)[1]					
	Control 6 h[2]			SARA 6 h		
	Grain-induced SARA		Alfalfa pellet-induced SARA	Grain-induced SARA		Alfalfa pellet-induced SARA
	Mild	Severe		Mild	Severe	
Phylum *Bacteroidetes*	24.58^{bc}	20.04^{bc}	47.41^{a}	26.27^{bc}	15.34^{c}	34.65^{ab}
Class *Bacteroidetes*	24.07^{bc}	19.81^{bc}	47.14^{a}	26.09^{bc}	15.29^{c}	34.46^{ab}
Order *Bacteroidales*	24.07^{bc}	19.81^{bc}	47.14^{a}	26.09^{bc}	15.29^{c}	34.46^{ab}
Family *Prevotellaceae*	2.41^{b}	2.03^{b}	13.53^{a}	2.60^{b}	7.65^{ab}	9.99^{a}
Phylum *Proteobacteria*	4.49	3.04	1.98	1.94	3.66	2.99
Class *Epsilonproteobacteria*	0.26	0.23	0.19	0.35	0.00	0.14
Order *Campylobacterales*	0.26	0.23	0.19	0.35	0.00	0.14
Class *Gammaproteobacteria*	2.03	1.45	0.62	0.40	2.96	1.92
Order *Aeromonadales*	0.02	0.02	0.03	0.00	0.00	0.02
Family *Succinivibrionaceae*	0.02	0.02	0.03	0.00	0.00	0.02
Order *Enterobacteriales*	1.31	1.12	0.03	0.37	1.22	1.28
Family *Enterobacteriaceae*	1.31	1.12	0.03	0.37	1.22	1.28
Phylum *Firmicutes*	69.33^{b}	75.65^{ab}	49.52^{c}	70.72^{ab}	79.58^{a}	60.68^{bc}
Class *Clostridia*	64.03^{bc}	71.83^{ab}	43.76^{cd}	66.11^{abc}	78.03^{a}	56.44^{c}
Order *Clostridiales*	62.23^{bc}	70.34^{ab}	42.78^{cd}	65.01^{abc}	78.19^{a}	55.46^{c}
Family *Clostridiaceae*	0.61	0.53	1.10	0.66	1.13	0.60
Family *Eubacteriaceae*	0.03	0.03	0.00	0.03	0.03	0.03
Family *Lachnospiraceae*	42.16^{bc}	54.85^{ab}	32.76^{c}	45.73^{bc}	62.60^{a}	37.00^{c}
Family *Peptostreptococcaceae*	0.08	0.00	0.03	0.02	0.00	0.07
Family *Ruminococcaceae*	13.90^{a}	11.69^{ab}	5.62^{b}	13.70^{a}	11.26^{ab}	13.30^{ab}
Family *Veillonellaceae*	4.09	2.14	2.66	3.69	2.34	3.37
Class *Bacilli*	2.90	1.67	2.46	2.11	0.41	1.93
Order *Lactobacillales*	2.77	1.56	2.29	1.97	0.29	1.80
Family *Streptococcaceae*	1.71	1.46	2.13	1.84	0.17	1.68
Phylum *Spirochaetes*	0.01	0.01	0.11	0.00	0.00	0.01
Phylum *Actinobacteria*	1.22	0.87	0.63	0.84	1.01	1.14
Phylum *Fusobacteria*	0.02	0.02	0.00	0.02	0.02	0.00
Phylum *TM7*	0.01	0.01	0.01	0.01	0.01	0.00
Phylum *Tenericutes*	0.10	0.02	0.12	0.00	0.08	0.11

Phylum *Deinococcus-Thermus*	0.01	0.01	0.01	0.01	0.00	0.01
Phylum Unclassified Bacteria	0.23	0.34	0.30	0.17	0.21	0.40

[a,b,c]Means within a row with different superscripts differ ($P < 0.05$).

[2]Values are proportions of phylogenetic lineages for each library.

[3]Rumen fluid samples were collected 6 hour after feeding four days after SARA induction during SARA periods.

of the population (Tables 20, 21). Microbial composition did not change over sampling hours with the exception of control period during alfalfa pellet-induced SARA that a significant shift from *Bacteroidetes* to *Firmicutes* was observed by 6h post-feeding (Table 21).

Species richness was not affected by SARA induction models but was numerically lower in SARA (both grain and alfalfa-pellet) than control (Table 22). Species diversity was significantly lower during mild grain-induced SARA and tended to be lower in severe grain-induced SARA, but was not different for alfalfa pellet-induced SARA (Table 22). The similarity between microbial communities was greatest for control, alfalfa, and mild grain-induced SARA than for severe grain-induced SARA (Table 23).

Relative quantification of major rumen bacteria, methanogenic archaea, and ciliate protozoa compared to controls are shown in Figure 5 and 6. At 0 hour, severe grain-induced SARA was dominated by *Escherichia coli*, *Streptococcus bovis, Megasphaera elsdenii*, and *Lactobacillus* spp.; mild grain-induced SARA was dominated by *M. elsdenii*, *Selenomonas ruminantium*, *Prevotella bryantii* and *Anaerovibrio lipolytica,* and alfalfa pellet-induced SARA was only dominated by *Prevotella albensis*. At 6 hour after feeding, the severe grain-induced SARA group was dominated *M. elsdenii* and *S. bovis,* while the mild group was dominated by *M. elsdenii, Succinivibrio dextrinosolvens, P. bryantii,* and *Ruminococcus flavefaciens.* In contrast the alfalfa-induced SARA group was dominated by *P. albensis, P. bryantii* and *Ruminococcus albus.* Multivariate discriminative analysis of real-time PCR data indicated that the best predictor of severe

grain-induced SARA was *E. coli*, of mild grain-induced SARA was *M. elsdenii*, and of alfalfa-induced SARA *P. albensis* (Figure 7).

Table 22. Richness and diversity indices calculated from T-RFLP incidence profiles of rumen fluid

Item	Control 0 h[1]	SARA 0 h	Control 6 h	SARA 6h	SEM[2]
Richness[3]					
Grain-induced SARA					
Mild	69.9	48.5	93.6	45.0	
Severe	46.8	29.6	48.1	49.2	35.3
Alfalfa pellet-induced SARA	41.3	31.7	25.2	42.5	
Diversity[4]					
Grain-induced SARA					
Mild	3.1^{a}	2.4^{b}	3.2^{a}	2.3^{b}	
Severe	2.8	2.0	2.8^{ab}	2.4	0.2
Alfalfa pellet-induced SARA	2.8	2.2	2.4^{b}	2.8	

[a,b]Means within the same row or column with different superscripts differ ($P < 0.05$).

[1]Rumen fluid samples were collected 15 min before feeding and at 6 hour after feeding four days after SARA induction during SARA period.

[1]SED = Standard error of difference between treatments.

[3]Based on Chao-2 richness estimator.

[4]Based on Shannon diversity estimator.

Table 23. Multiple incidence base similarity of rumen bacterial community between control, grain-induced and alfalfa pellet-induced SARA calculated from T-RFLP incidence profiles of rumen samples amplified using 27f and 342r primers

Similarity[1]			% of similarity ± SE											
			Grain-induced SARA								Alfalfa pellet-induced SARA			
			Control Mild		Control Severe		SARA Mild		SARA Severe		Control		SARA	
			0 h	6 h	0 h	6 h	0 h	6 h	0 h	6 h	0 h	6 h	0 h	6 h
Grain-induced SARA	Control Mild	0 h	100											
		6 h	77 ±8	100										
	Control Severe	0 h	72 ± 8	66 ± 9	100									
		6 h	73 ± 8	61 ± 9	74 ± 10	100								
	SARA Mild	0 h	69 ± 9	58 ± 9	77 ± 8	71 ± 10	100							
		6 h	59 ± 9	55 ± 8	73 ± 9	60 ± 9	86 ± 8	100						
	SARA Severe	0 h	31 ± 8	30 ± 8	38 ± 9	46 ± 10	42 ± 9	50 ± 10	100					
		6 h	29 ± 7	28 ± 7	29 ± 8	40 ± 10	41 ± 9	43 ± 10	67 ± 10	100				
Alfalfa pellet-induced SARA	Control	0 h	28 ± 8	28 ± 8	41 ± 9	31 ± 9	32 ± 8	39 ± 10	42 ± 11	42 ± 9	100			
		6 h	41 ± 9	44 ± 9	57 ± 10	46 ± 10	54 ± 10	57 ± 6	35 ± 10	36 ± 9	50 ± 11	100		
	SARA	0 h	40 ± 10	52 ± 9	54 ± 10	44 ± 9	46 ± 10	54 ± 10	33 ± 10	34 ± 9	48 ± 10	52 ± 10	100	
		6 h	54 ± 8	64 ± 8	44 ± 8	46 ± 9	47 ± 8	48 ± 9	38 ± 9	46 ± 8	30 ± 8	42 ± 9	45 ± 9	100

[1]Based on Jaccard index of similarity. Values are expressed as mean ± SD

Figure 5. Log-fold changes in predominant rumen microorganisms during mild (gray bars), and severe (black bars) grain-induced and alfalfa pellet- induced SARA (white bars) compared to control determined with real-time PCR at 15 min before feeding. The letters "a, b, c" indicate statistical differences within species, * $P < 0.05$; ** $P < 0.1$.

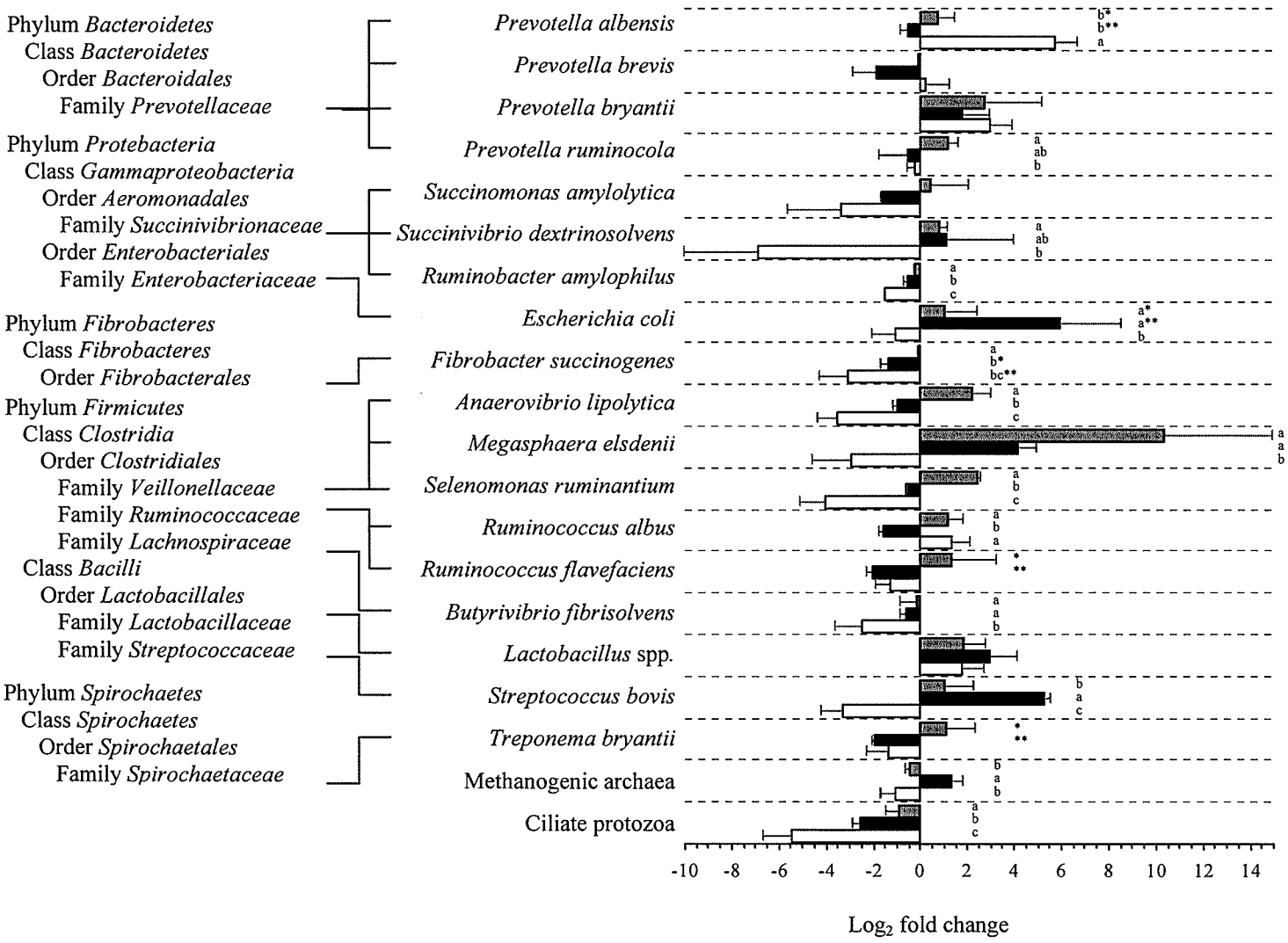

Phylum Bacteroidetes
Class Bacteroidetes
Order Bacteroidales
Family Prevotellaceae
Phylum Protebacteria
Class Gammaproteobacteria
Order Aeromonadales
Family Succinivibrionaceae
Order Enterobacteriales
Family Enterobacteriaceae
Phylum Fibrobacteres
Class Fibrobacteres
Order Fibrobacterales
Phylum Firmicutes
Class Clostridia
Order Clostridiales
Family Veillonellaceae
Family Ruminococcaceae
Family Lachnospiraceae
Class Bacilli
Order Lactobacillales
Family Lactobacillaceae
Family Streptococcaceae
Phylum Spirochaetes
Class Spirochaetes
Order Spirochaetales
Family Spirochaetaceae
Prevotella albensis
Prevotella brevis
Prevotella bryantii
Prevotella ruminocola
Succinomonas amylolytica
Succinivibrio dextrinosolvens
Ruminobacter amylophilus
Escherichia coli
Fibrobacter succinogenes
Anaerovibrio lipolytica
Megasphaera elsdenii
Selenomonas ruminantium
Ruminococcus albus
Ruminococcus flavefaciens
Butyrivibrio fibrisolvens
Lactobacillus spp.
Streptococcus bovis
Treponema bryantii
Methanogenic archaea
Ciliate protozoa
-10 -8 -6 -4 -2 0 2 4 6 8 10 12 14
Log2 fold change

Figure 6. Log-fold changes in predominant rumen microorganisms during mild (gray bars), and severe (black bars) grain-induced and alfalfa pellet- induced SARA (white bars) compared to control determined with real-time PCR at 6 h after feeding. The letters "a, b, c" indicate statistical differences within species $P < 0.05$; *, ** $P < 0.1$.

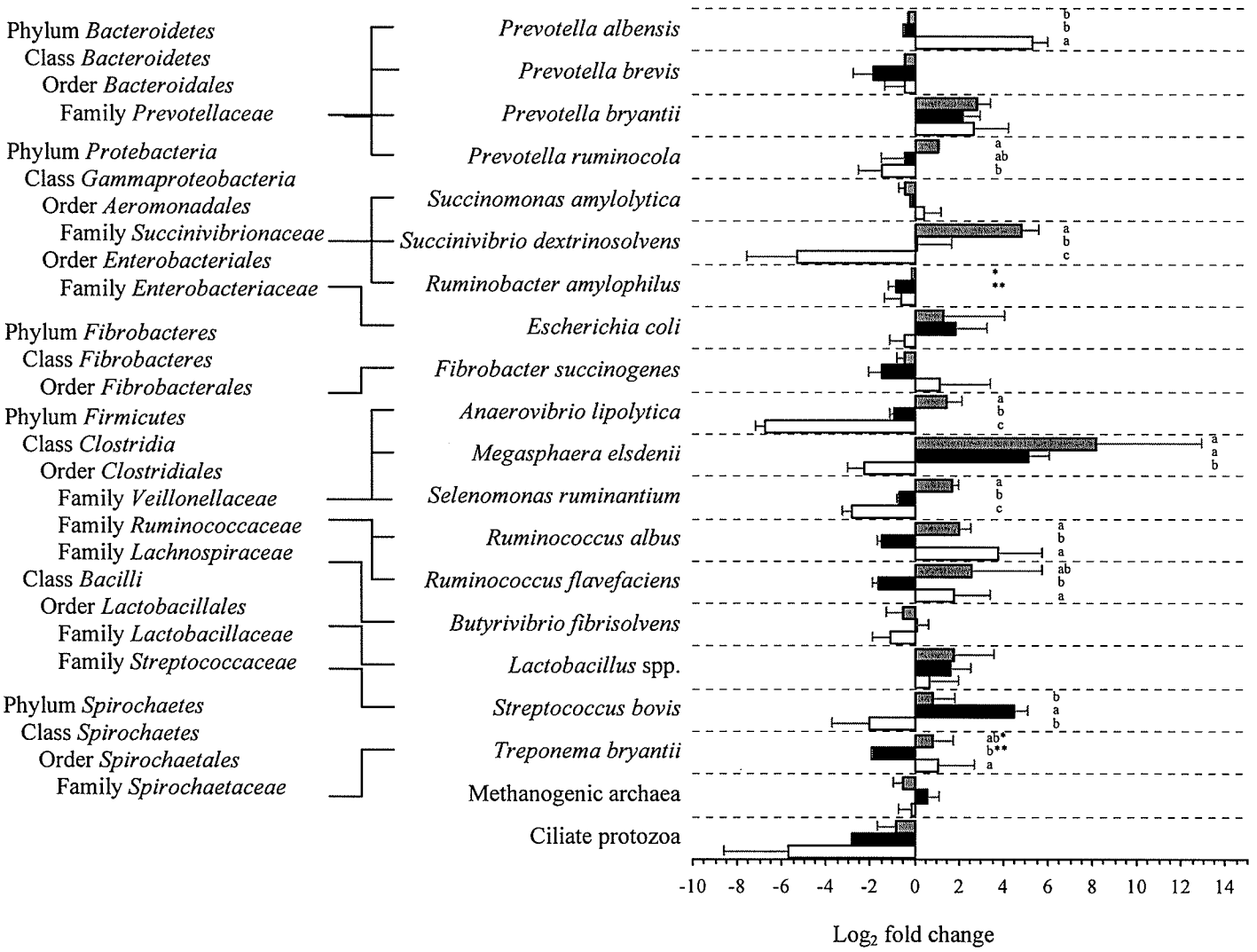
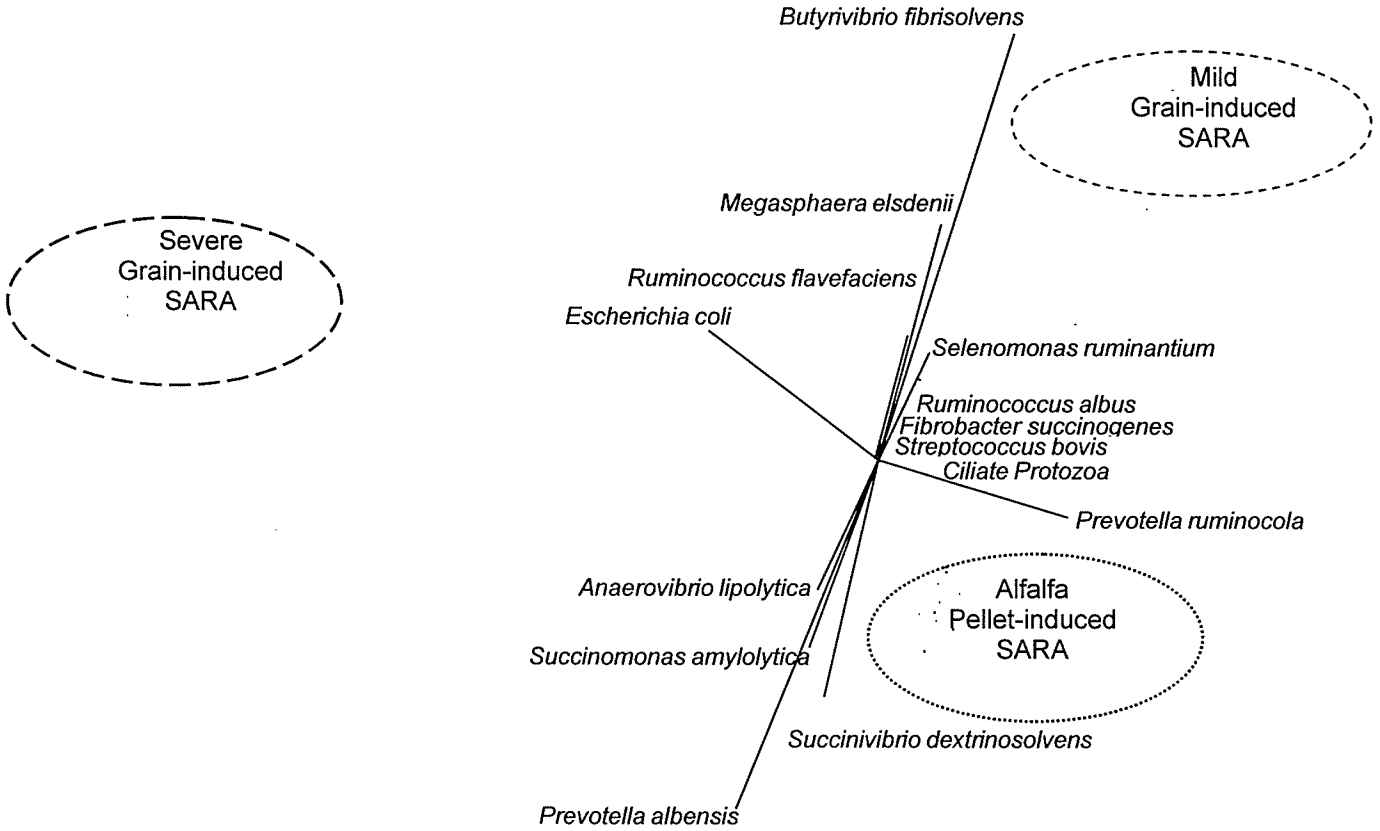

Phylum Bacteroidetes
Class Bacteroidetes
Order Bacteroidales
Family Prevotellaceae
Phylum Protebacteria
Class Gammaproteobacteria
Order Aeromonadales
Family Succinivibrionaceae
Order Enterobacteriales
Family Enterobacteriaceae
Phylum Fibrobacteres
Class Fibrobacteres
Order Fibrobacterales
Phylum Firmicutes
Class Clostridia
Order Clostridiales
Family Veillonellaceae
Family Ruminococcaceae
Family Lachnospiraceae
Class Bacilli
Order Lactobacillales
Family Lactobacillaceae
Family Streptococcaceae
Phylum Spirochaetes
Class Spirochaetes
Order Spirochaetales
Family Spirochaetaceae
Prevotella albensis
Prevotella brevis
Prevotella bryantii
Prevotella ruminocola
Succinomonas amylolytica
Succinivibrio dextrinosolvens
Ruminobacter amylophilus
Escherichia coli
Fibrobacter succinogenes
Anaerovibrio lipolytica
Megasphaera elsdenii
Selenomonas ruminantium
Ruminococcus albus
Ruminococcus flavefaciens
Butyrivibrio fibrisolvens
Lactobacillus spp.
Streptococcus bovis
Treponema bryantii
Methanogenic archaea
Ciliate protozoa
-10 -8 -6 -4 -2 0 2 4 6 8 10 12 14
Log2 fold change

Figure 7. Discriminant analysis of major rumen bacteria and ciliate protozoa in response to SARA induction model. The circles are independent variables and the distance between them reflects their dissimilarity. The straight lines are dependent variables and their length and angles between them are functions of the relative effects of independent variables.


Severe
Grain-induced
SARA
Butyrivibrio fibrisolvens
Mild
Grain-induced
SARA
Megasphaera elsdenii
Ruminococcus flavefaciens
Escherichia coli
Selenomonas ruminantium
Ruminococcus albus
Fibrobacter succinogenes
Streptococcus bovis
Ciliate Protozoa
Prevotella ruminocola
Anaerovibrio lipolytica
Alfalfa
Pellet-induced
SARA
Succinomonas amylolytica
Succinivibrio dextrinosolvens
Prevotella albensis

DISCUSSION

Current definition of SARA is based on low rumen pH typically generated on high starch diets (Plaizier et al., 2008). However, there is no general agreement on the pH threshold that defines SARA. Under low rumen pH conditions animals experience cyclic patterns of feed intake, a decline in milk yield, milk fat, and systemic inflammation (Plaizier et al., 2008). To refine the definition of SARA, Gozho et al. (2005) suggested that free rumen LPS concentration be considered. In our previous work (Khafipour et al., 2008a; 2008b) we demonstrated that feed ingredient type and physical form of the diet are also important. When we induced a low rumen pH with highly fermentable alfalfa-pellets inflammation was absent even though the free rumen LPS concentrations were high (Khafipour et al., 2008b). Thus, rumen fermentation conditions by themselves are not sufficient to explain SARA.

To understand the underlying causes of SARA, we examined the microbial changes that occur with grain- and alfalfa pellet-induced SARA from our previous experiments (Khafipour et al., 2008a; 2008b). A critical part of our analysis was to separate animals into mild and severe SARA groups based on objective criteria using multivariate statistical techniques (Table 18; Figure 4). Clustering of cows into mild and severe groups used animal as the independent variable and free rumen LPS concentration, time below pH 5.6, and serum haptoglobin as the dependent variables. The second phase of our analysis proceeded to associate microbial changes with these criteria of SARA severity.

We used T-RFLP to evaluate the structure, dynamics, and diversity of rumen microbial populations in mild and severe grain-induced SARA and alfalfa pellet-induced

SARA. The population shifts we noted in our analysis were similar to those obtained with the 16S clone library studies of Tajima et al. (1999) and Whitford et al. (1998). The most notable observations in our research were that both models of SARA induction resulted in a significant increase in the phylum *Firmicutes*, and a decrease in the phylum *Bacteroidetes*. However, the proportions of these populations were different among SARA models. In alfalfa pellet-induced SARA *Bacteroidetes* were 35.4% of the population, while in the grain-induced mild SARA group it was 26% but in the severe group only 16.6%.

Real-time PCR data (Figures 5 and 6) objectively evaluated with multivariate statistics (Figure 7) indicated that the major predictor of severe grain-induced SARA was *E. coli*. The T-RFLP prevalence analysis also indicated changes in the *Proteobacteria* (contains *E. coli*) although the differences between treatments was not significant. Given that rumen LPS concentration is high in both grain- and alfalfa pellet-induced SARA, and that only grain fed animals exhibited inflammation, this excludes the possibility that LPS alone is the cause of inflammation.

The most predominant shifts in microbial populations were the Gram-negative *Bacteroidetes*. This shift is evident from the T-RFLP prevalence data as well as real-time data for *P. albensis, P. brevis,* and *P. ruminicola,* which are members of the *Bacteroidetes*. We suspect that a majority of the LPS came from these bacteria. LPS from members of phylum *Bacteroidetes* (i.e. *B. fragilis*) is much less toxic than that from *E. coli* or *Salmonella* spp. (Kaspar, 1976; Stewart, 1977; Weintraub et al., 1989). A human study indicated that LPS from *Salmonella typhi*, *E. coli*, and *Pseudomonas aeruginosa* reached a toxicity threshold at ~0.1, 1.0 and 60 ng/kg of body weight, respectively

(Greisman and Hornick, 1969). In an earlier rumen study LPS was extracted from mixed rumen bacteria from either grain fed or hay-fed animals, and compared to *E. coli* LPS in a rodent endotoxin model. The most toxic LPS came from *E. coli*, followed by LPS from grain, and then hay fed animals (Nagaraja et al., 1979). *M. esldenii* is a Gram-negative bacterium and almost always in high abundance in grain fed animals. To test the possibility that the LPS from this bacterium was toxic its LPS was compared to that of *E. coli* in a rodent model and found to be much less potent (Nagaraja et al., 1979b).

The classical view of acidosis in the rumen is that as grain increases so does the prevalence of starch fermenting bacteria like *S. bovis* (Russell and Hino, 1985). Thus, there should be a high population of lactate consuming bacteria like *M. elsdenii* to prevent lactic acid acidosis (Russell et al., 1981). Our real-time PCR data indicated that the abundance of the amylolytic bacterium that most closely mirrored the severity of SARA was *S. bovis,* and other major amylolytic bacteria were less affected. *M. elsdenii* populations were in synchrony with the *S. bovis* numbers indicating that this bacterium was effectively eliminating rumen lactate. In contrast, even though the rumen pH conditions created by the alfalfa pellet diet were indicative of SARA, the levels of *S. bovis*, and consequently *M. elsdenii* were low.

Multivariate analysis (Figures 4, 7) indicated that there was a significant association between alfalfa fed animals, microbial populations, and the absence of inflammation. The microbial species most closely associated with this diet were *P. ruminicola, A. liplytica, S. amylolytica, P. albensis,* and *S. dextrinisolvens.* In particular *P. albensis* was in high abundance in this diet. Thus, *Prevotella* spp. may potentially be used as probiotics in SARA. Rodriguez (2003) selected a strain of *Prevotella* spp. that

grew rapidly on starch and produced succinate and propionate as major end-products. When inoculated into three lactic acid challenged goats they found that lactic acid levels declined. Chiquette et al. (2008) dosed *Prevotella* spp. into dairy cattle and observed small changes in lactic acid but the challenge they used was not sufficient to conclude that SARA had been induced.

Although we indicate that *Prevotalla* spp. are a probiotic target, our research provides evidence that *E. coli* might be another target for intervention. *E. coli* produce a range of anti-microbial peptides; the best studies of these are the colicins (Cascales et al., 2007). In human clinical medicine *E. coli* Nissel 1917 is often used as a probiotic to treat gastroenteritis (Schultz, 2008). Our laboratory has demonstrated that antimicrobial producing *E. coli* are highly effective at combating colibacillosis infections caused by *E. coli* K88 in weaned pigs (Krause et al., 2008; Setia et al., 2008)

In conclusion, we have demonstrated that grain- and alfalfa pellet-induced SARA result in different rumen microbial population structures even though rumen fermentation conditions are similar. We hypothesized that LPS concentrations in the rumen were not a predictor of SARA because high LPS levels could be created with an alfalfa pelleted diet but no inflammation occurred. Thus, the conclusion reached was that an alternate, more specific microbial etiology was implicated. The bacterium most closely associated with inflammation based on our population analysis was *E. coli*. We thus suspect that a subset of *E. coli* is putative pathogenic triggers for SARA. Future research should consider the population dynamics of *E. coli* in SARA and address potential virulence factors.

ACKNOWLEDGMENTS

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MANUSCRIPT IV

Molecular Population Analysis of *Escherichia coli* Associated with Subacute Ruminal Acidosis (SARA) in Dairy Cattle

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ABSTRACT

Grain-induced subacute ruminal acidosis (SARA) in dairy cattle is associated with inflammation; however, the cause of inflammation is not known. We hypothesized that diet induced changes to gut *Escherichia coli* populations may be partially responsible for this inflammatory response. To test this hypothesis, low rumen pH was induced by feeding a grain-pelleted diet or alfalfa-pelleted diet in two separate experiments. Rumen fluid samples were collected before and 6 h after feeding and cultured with or without acid shock on chromogenic medium for *E. coli* and total coliform enumeration. From each rumen sample, five *E. coli* strains were isolated, typed with rep-PCR DNA fingerprinting using BOX A1R primer, and genotyped using the ABD schema. DNA fingerprinting were assessed for presence or absence of 23 bands ranging from 420 to 3,300 bp and were subjected to discriminant function analysis and cluster analysis. In general, the numbers of *E. coli* in alfalfa induced SARA were significantly lower than in grain-induced SARA. Also, the fluctuation in the acid resistant populations was less during grain-induced SARA and a higher degree of variation occurred in the control animals. Cluster analysis indicated that isolates from control groups and alfalfa pellet-induced SARA tended to cluster together. In contrast, grain-induced SARA *E. coli* were members of distinctly separate *E. coli* populations. There was also a significant shift from an A type *E. coli* population to a B1 type population during grain-induced SARA. However, no significant differences occurred among *E. coli* using ABD typing when alfalfa-pellet induced SARA was compared to controls. These results suggest that only grain-induced SARA alters *E. coli* population in the rumen and suggests that *E. coli* from

grain based diets may possess virulence factors that could be involved in the inflammatory responses observed with SARA.

Key words: subacute ruminal acidosis, *Escherichia coli,* rep-PCR DNA fingerprinting, ABD genotyping

INTRODUCTION

Subacute ruminal acidosis is a metabolic disease of ruminants that results in a reduction in feed intake and milk production, and has traditionally being defined as a disease associated with low rumen pH (Plaizier et al., 2008). In recent years and additional hypothesis has emerged for SARA. This hypothesis suggests that during low rumen pH conditions Gram-negative bacteria release LPS and cause epithelial barrier function defects, which subsequently results in inflammation (Andersen et al., 1990; Gozho et al., 2007; Emmanuel et al., 2008). Thus, according to this hypothesis LPS is implicated in SARA and not only low pH of the rumen. Interestingly, the role of LPS in acute lactic acid acidosis was suggested more than 30 years ago (Dougherty, 1976; Huber, 1976).

To test this idea we created low rumen pH conditions with two different diets (Khafipour et al., 2008a; 2008b), one based on grain, and the other based on highly fermentable alfalfa pellets. Both diets resulted in pH conditions typical of SARA, and both diets resulted in increased free rumen LPS. However, only the grain fed animals had an inflammatory response, thus, precluding the possibility that free rumen LPS is necessarily the cause of inflammation.

More detailed molecular microbial community analysis demonstrated that the Gram-negative phylum *Bacteroidetes*, declined significantly during the grain fed inflammatory phase (Khafipour et al., 2008c). A major microbial population that was positively associated with inflammation was *E. coli*, which was in high abundance in the grain-induced SARA animals but was almost entirely absent in alfalfa pellet-induced SARA. Given that the size of the *Bacteroidetes* population was orders of magnitude

larger than that of *E. coli*, we concluded that the majority of LPS in the rumen was most likely from *Bacteroidetes*.

Members of the phylum *Bacteroidetes* currently consist of approximately 355 official species, the vast majority of which are non-pathogenic (RDP), while LPS from *E. coli* is clearly toxic (Fujimoto Y, 2005). Thus, we suspect that LPS in the rumen observed under low pH condition is not exclusively the cause of inflammation. We only observed translocation of LPS into the peripheral blood in the grain fed animals and not the alfalfa fed animals (Khafipour et al., 2008a; 2008b). From this data, we concluded that plasma LPS is indicative of a barrier function defect, and that the key to understanding SARA is to identify the microbial factors that result in increased epithelial permeability. If this is true then the genetic composition of the *E. coli* from grain-induced SARA animals should be different from the other groups in which inflammation is absent.

MATERIAL AND METHODS

Animal experiments were conducted in Glenlea Dairy Research Unit at the University of Manitoba (Winnipeg, MB, Canada) in accordance to the guideline of the Canadian Council on Animal Care (CCAC, 1993). The models for SARA, diets, and sampling procedures have been fully described in previous manuscripts (Khafipour et al., 2008a; 2008b). In brief rumen samples were taken from the ventral sac of the rumen, strained through four layers of sterile cheesecloth and mixed with an equal volume of 50% sterile glycerol into 15 mL sterile tubes and stored at -80°C for further analysis.

Enumeration and Isolation of E. coli

E. coli/Coliform counts were conducted on a chromogenic medium (CM0956, Oxide Inc., Nepean, ON, Canada). Buffered peptone water (pH 7.2, Difco Laboratories, Detroit, MI) was used to make decimal serial dilutions in 2 mL deep-well plates. These plates were covered with aluminum foil and sterilized at 121°C and 15 psi for 15 min. A 100 µL of rumen fluid from thawed glycerol stock was inoculated into 900 µL of a 2% buffered peptone water and serially diluted to 10^{-7}. Dilutions from 10^{-1} to 10^{-7} were plated by dispensing of 10 droplets (10 µL each) onto solid chromogenic medium. Following absorption of the droplets into the medium, plates were inverted and incubated at 39°C for 18 h. *E. coli* (purple colonies) and non-*E. coli* coliforms (blue, pink and white colonies) were counted in the dilutions that gave 3 to 30 colonies and an appropriate dilution factor was applied to determine the number of *E. coli* and total coliforms (cfu) per mL of undiluted rumen fluid.

From plates with at least 10 colonies five putative *E. coli* (purple colonies) were selected randomly and purified by repeated streaking on *E. coli*/Coliform chromogenic medium. Each single colony then was inoculated into 15 mL of Luria-Bertani broth (LB) and grown for 48 h at 37°C. One mL of fresh culture was added to one mL of 50% glycerol and stored in -80°C. The remainder was used for the DNA extraction. This procedure resulted in the recovery of 129 confirmed *E. coli* isolates that were used for further genetic analysis.

Acid Shock

Rumen fluid samples were diluted 10 fold into buffered peptone water that had been adjusted to pH 2.0 with HCl (6 N) or pH 5.2 with acetate (6 N). HCl and acetate-

shocked cultures were incubated at 37°C for 1 or 2 h, respectively. Acid resistant *E. coli* and coliforms count were determined by serial dilution and plating of acid-shocked samples as described before.

Genetic Analysis of E. coli

DNA Extraction. Cells were harvested by centrifugation at 3,000 × *g* for 15 min. Harvested cells were resuspended in 570 µL of 0.5X Tris-EDTA buffer (pH 8.0). Then, 30 µL of 10% SDS and 10 µL of proteinase-K (20 mg/mL) were added to each tube and incubated for 1 h at 37°C. Subsequently, 100 µL of 5 *M* NaCl and 80 µL of cetyl trimethylammonium bromide (CTAB) was added to the tubes, mixed thoroughly and incubated for 10 min at 65°C. Then, 600 µL of phenol:chloroform:isoamylalcohol (25:24:1) were added to the tubes, mixed and centrifuged at 10,000 × *g* at 4°C for 5 min. The supernatants were carefully transferred to fresh tubes, mixed with 600 µL of chloroform:isoamylalcohol (24:1), and respined at 10,000 × *g* at 4°C for 5 min. Supernatants were transferred again to fresh microfuge tubes and mixed with equal volume of isopropanol to precipitate nucleic acid. The tubes were left on ice for 15 min and then centrifuged at 10,000 × *g* for 10 min. Then, pellets were washed with 500 µL of 70% ethanol for 5 min and subsequently centrifuged at 10,000 × *g* for 10 min. The resulting nucleic acid pellets were redissolved in 100 µL of 0.5X Tris-EDTA buffer (pH 8.0) and stored in -20°C until further analysis. DNA concentration and purity were determined spectrophotometrically by measuring the optical density (OD) and $A_{260/280}$ (Beckman Coulter, DU/800 UV/VIS, Beckman Coulter Inc., Fullerton, CA).

Rep-PCR DNA Fingerprinting. Rep-PCR DNA fingerprinting was used to differentiate *E. coli* isolates (Dombek et al., 2000, Mohapatra et al., 2007). Each 20.5 µL

PCR reaction included 2.0 µL of 10X EconoTaq buffer (100 m*M* Tris-HCl, 500 m*M* KCl, 15 m*M* $MgCl_2$, 1% Triton X-100) (Lucigen Corporattion, Middleton, WI), 1 µL of 25 m*M* $MgSO_4$ (Fisher Scientific, Fairlawn, NJ), 0.4 µL of 10 m*M* nucleotide mix (Fisher Scientific, Fairlawn, NJ), 0.2 µL primer (25 mM) (BOX A1R, 5'-CTACGGCAAGGCGACGCTGACG-3') (Dombek et al., 2000), 1 U EconoTaq DNA polymerase (Lucigen Corporattion, Middleton, WI), 16.2 µL H_2O, and 0.5 µL DNA (~50 ng). A control reaction mixture containing 0.5 µL of mili-Q water (Millipore water purification system, Millipore corporate, Billerica, MA) instead of template DNA was included in each set of PCR reactions. Amplification was performed in a Techne-Genius thermocycler with initial denaturation of 94°C for 2 min; then 30 cycles of 94°C for 3 sec, 92°C for 30 sec, 53°C for 1 min, and 65°C for 3 min; followed by a single step extension at 65°C for 8 min (Dombek et al., 2000). The PCR products was separated on 1.5% agarose (Promega, Madison, WI) gels and were stained in 0.5 µg/mL ethidium bromide solution (Fisher Scientific, Fairlawn, NJ) for 30 min. The gel images were taken in a FluorChem SP (Alpha Innotech Corp., San Leandro, CA) and presence or absence of 23 bands ranging from 420 to 3,300 bp were analyzed by FluorChem SP (Ver. 5.0) software. The position of bands were normalized with external (1-kb DNA ladder) and internal (3 or 4 common bands between most of *E. coli* isolates) standards (Dombek et al., 2000).

ECOR Groups Assignment. *E. coli* isolates were assigned to one of the four main groups (A, B1, B2, and D) of the standard *E. coli* collection (ECOR) using the methods of Clermont et al. (2000) and (Kotlowski et al., 2007). In brief, isolates belonging to A or B1 groups were negative for the *chuA* gene. These isolates were classified as A or B1 if

they were negative or positive for TSPE4.C2 fragment, respectively. In contrast, B2 and D isolates were positive for the *chuA* gene can be separated into B2 and D groups if they were positive or negative for the *yjaA* gene, respectively. The *chuA* and *yjaA* genes and TSPE4.C2 fragment were amplified using following primer pairs: ChuA.1 (5'-GACGAACCAACGGTCAGGAT-3') and ChuA.2 (5'-TGCCGCCAGTACCAAAGACA-3'); YjaA.1 (5'-TGAAGTGTCAGGAGACGCTG-3') and YjaA.2 (5'-ATGGAGAATGCGTTCCTCAAC-3'); and TSPE4C2.1 (5'-GAGTAATGTCGGGGCATTCA-3') and TSPE4C2.2 (5'-CGCGCCAACAAAGTATTACG-3'), which generated 279, 211, and 152 bp fragments, respectively. The PCR reactions included 1 cycle of 94°C for 5 min; then 30 cycles of 94°C for 1 min; 70°C for 1 min; 72°C for 2 min; and a final extension at 72°C for 5 min. PCR products were subjected to electrophoresis using 2% agarose.

Statistical Analysis

Rep-PCR DNA fingerprints were clustered using maximum composite likelihood and the UPGMA (Unweighted Pair-Groups Method Average) method (MEGA, Ver. 4.0; Tamura et al., 2007). The fingerprints were also subjected to discriminant functional analysis with the "leave-one-out" classification method (SPSS, Ver 16.0; SPSS Inc., Chicago, IL) to determine the percentage of isolates from a given treatment that were correctly classified to its treatment group. Statistical differences calculated using Fisher's Exact Test and LSD multiple comparison test and were considered significant at $P < 0.05$ (SAS, 2004).

RESULTS

During grain-induced SARA total rumen coliforms and *E. coli* numbers were not different between control and SARA periods (Table 24). In contrast, in alfalfa pellet-induced SARA total coliforms were greater ($P < 0.05$) than in controls but *E. coli* did not differ (Table 24). In both grain- and alfalfa pellet-induced SARA, there was an effect of time caused by a decline in coliforms and *E. coli* (Table 24) at 6 h after feeding when the rumen pH was the lowest. In general the numbers of *E. coli* in alfalfa-induced SARA were significantly lower than in grain-induced SARA (Table 24).

The pH of rumen fluid samples was reduced to 5.2 with acetate for 2 h, or 2 with HCl for 1 h to induce acid shock to determine whether rumen conditions during grain-induced SARA would select for a subpopulation of *E.* coli. Acid shock was not performed on alfalfa pellet-induced SARA samples because *E. coli* numbers were extremely low, and there was no inflammatory response during SARA (Khafipour et al., 2008b). The greatest change in acid shocked *E. coli* occurred at pH 2, and the acid resistant population in SARA was substantially higher ($P < 0.05$), and more stable (0 h vs. 6 h) than in control animals (Figure 8). In contrast, changes in the total coliform populations were much smaller than *E. coli* populations (Figure 9).

Five *E. coli* colonies per cow (4 cows) per sampling time (0 and 6 h) were randomly selected from the rumen fluid samples collected during control, grain-, and alfalfa pellet-induced SARA periods. However, the number of isolates that were obtained from alfalfa pellet-fed group was less than grain fed group (49 vs. 80) because of the low numbers of *E. coli* in these samples. These isolates were subjected to rep-PCR DNA

Table 24. Total coliform and *E. coli* counts in rumen fluid samples taken during control, grain- and alfalfa pellet-induced SARA

Item	Diet		SED[1]	Effect, *P*-value		
	Control	SARA		Diet	Hour	Diet × Hour
Total coliform (log_{10} cfu/ml)						
Grain model						
0 h	5.37[b]	6.5[a]	0.21	0.3	0.026	<0.01
6 h	5.96[ab]	5.62[b]				
Alfalfa-pellet model						
0 h	5.19[b]	5.85[a]	0.13	< 0.01	< 0.01	<0.01
6 h	5.07[b]	5.06[b]				
E.coli (log_{10} cfu/ml)						
Grain model						
0 h	3.76[b]	5.55[a]	0.29	0.69	0.02	0.01
6 h	4.39[ab]	4.74[a]				
Alfalfa-pellet model						
0 h	2.07	2.86	0.57	0.23	0.07	0.89
6 h	1.45	2.35				

[a,b]Means within a row or column with different superscripts differ ($P < 0.05$).

[1]Standard error of difference between treatments.

[2]Time of rumen fluid sampling before and at 6 h after feeding. Samples are collected four days after induction of SARA during SARA period.

Figure 8. Total, acetate-resistant and HCl-resistant *E. coli* counts (log_{10} cfu/ml) in rumen fluid samples taken during control and grain-induced SARA before (a) or at 6 h after feeding (b). Error bars indicate standard error of difference between treatments. [a,b,c]Means with different superscripts differ ($P < 0.05$).

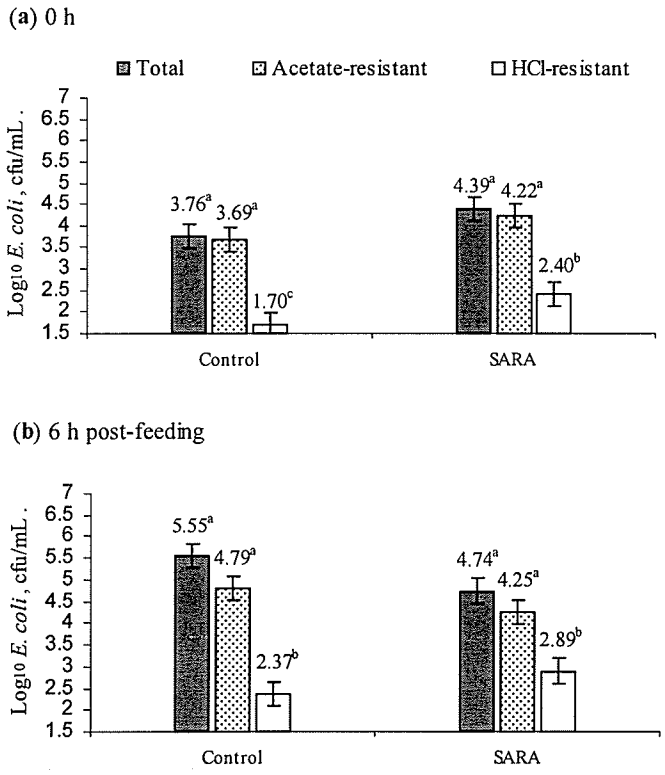
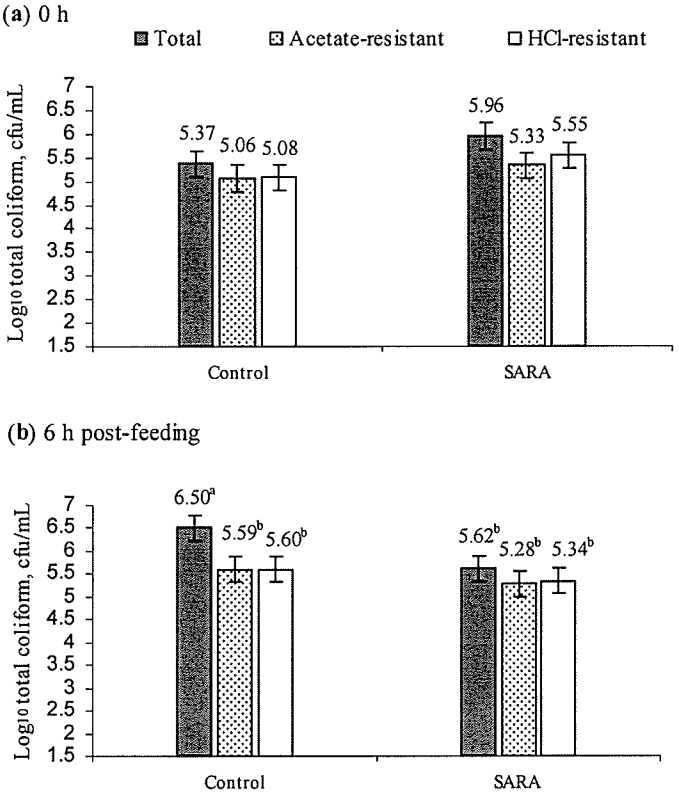


Figure 9. Total, acetate-resistant and HCl-resistant coliform counts (log_{10} cfu/ml) in rumen fluid samples taken during control and grain-induced SARA before (a) or at 6 h after feeding (b). Error bars indicate standard error of difference between treatments. [a,b,c]Means with different superscripts differ ($P < 0.05$).



fingerprinting using the BOX A1R primer. From a total of 129 isolates 6 isolates belonging to the grain fed group did not generate distinct banding patterns, and were eliminated. The DNA fingerprints of the remaining 123 isolates were analyzed for the presence or absence of 23 unique bands ranging from 420 to 3,300 bp, which resulted in a matrix of 0s and 1s. The matrix was subjected to cluster analysis. Using a clustering distance of 80% isolates were grouped into six clusters, while 95.1% of isolates fell into four clusters (clusters 1, 2, 3, and 4) or 73.9% of them fell into two major clusters (clusters 1 and 4) (Figure 10). Isolates from grain-induced SARA mostly (54%) grouped together in cluster 1. Similarly, isolates from control animals were more closely related, irrespective of the diet so that 46% and 70% of isolates from control grain and control alfalfa-pellet groups fell into cluster 4. Also, isolates from alfalfa pellet-induced SARA clustered mostly (53%) with the control isolates in cluster 4.

We also performed discriminant function analysis to evaluate how accurately rep-PCR fingerprints results were able to predict the source of *E. coli* (Table 25). Based on this analysis, the average rate of correct classification for all four treatment groups was 59.3%. The rate of correct classification of *E. coli* isolates from control grain model (59.5%), control alfalfa-pellet model (70%), SARA grain model (62.2%), and SARA alfalfa-pellet model (36.8%) was calculated. Using a discriminant two-function model, we plotted *E. coli* isolates with respect to their treatment groups (Figure 11). Function one exhibited a group difference of $\chi^2 = 211$ which was significant $P < 0.01$, and function two a group difference of $\chi^2 = 110$ which was significant at $P < 0.01$. The canonical correlation values for function one was 0.78 and for function two 0.71.

E. coli isolates were also assigned to one of the A, B1, B2, and D groups. The greatest shift was obtained in grain-induced SARA with a significant shift from A group ($P = 0.04$) to B1 group ($P = 0.07$)(Table 26).

Table 25. Percentage (number) of *E. coli* isolates assigned to the correct treatment group by using discriminant analysis (leave-one-out classification method) of rep-PCR DNA fingerprints

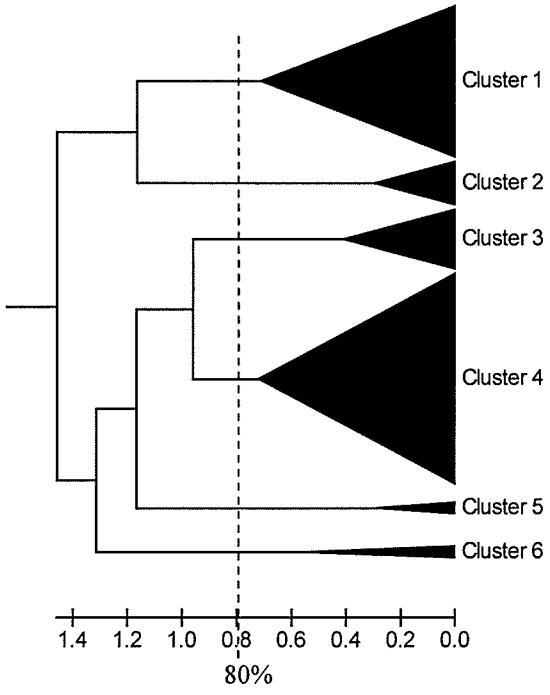
Treatment		% of E. coli isolates classified as[1]:				
		Control		SARA		Total
		Grain model	Alfalfa-pellet model	Grain model	Alfalfa-pellet model	
Control	Grain model	**59.5 (22)**	16.2 (6)	21.6 (8)	2.7 (1)	100 (37)
	Alfalfa-pellet model	6.7 (2)	**70.0 (21)**	6.7 (2)	16.7 (5)	100 (30)
SARA	Grain model	24.3 (9)	5.4 (2)	**62.2 (23)**	8.1 (3)	100 (37)
	Alfalfa-pellet model	5.3 (1)	31.6 (6)	26.3 (5)	**36.8 (7)**	100 (19)

[1]Values in boldface indicate the rate of correct classification. The average rate of correct classification was 59.3%.

Table 26. Percentage (number) of *E. coli* isolates assigned to A, B1, and B2+D ECOR groups in control, grain- and alfalfa pellet-induced SARA treatments

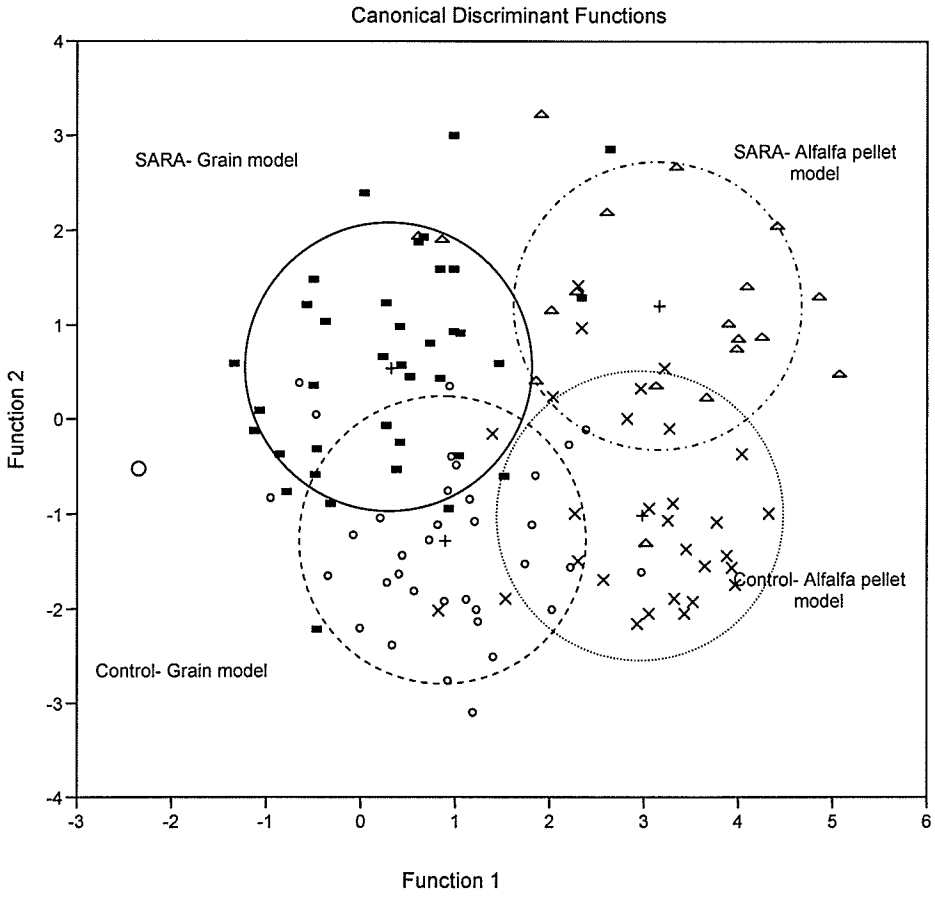
Item	Control	SARA	*P*-value
Grain model			
A	57.5 (23)	32.5 (13)	0.04
B1	37.5 (15)	57.5 (23)	0.07
B2+D	5.0 (2)	10 (4)	0.67
Total	100 (40)	100 (40)	
Alfalfa-pellet model			
A	50.0 (15)	47.4 (9)	1.00
B1	43.3 (13)	36.8 (7)	0.77
B2+D	6.7 (2)	15.8 (3)	0.36
Total	100 (30)	100 (19)	

Figure 10. The relatedness of *E. coli* strains isolated from rumen fluid of dairy cows in control, grain- and alfalfa pellet-induced SARA. Dendrograms were constructed from rep-PCR DNA fingerprints of *E. coli* isolates using maximum composite likelihood and UPGMA clustering methods. Values are representing the number of *E. coli* isolates in each cluster/treatment group.



	Control Grain model	Control Alfalfa-pellet model	SARA Grain model	SARA Alfalfa-pellet model
Cluster 1	8	5	20	5
Cluster 2	3	1	7	0
Cluster 3	8	1	5	1
Cluster 4	17	21	5	10
Cluster 5	1	1	0	1
Cluster 6	0	1	0	2
Total	37	30	37	19

Figure 11. Canonical discriminant function plot of rep-PCR DNA fingerprint of *E. coli* strains isolated from rumen fluid of dairy cows in control, grain- and alfalfa pellet-induced SARA.



DISCUSSION

Rumen *E. coli* numbers in grain-induced SARA cows were approximately two logs higher than in alfalfa pellet-fed animals (Table 24). In contrast, the total number of coliforms changed little among different dietary treatments (Table 24). Anderson (2000) suggested that as much as 60% of free rumen LPS is produced during the logarithmic phase of bacterial growth and the other 40% is from stationary growth. This might imply that a faster growing *E. coli* population is contributing more than other coliforms to the rumen LPS pool.

Even though the numbers of *E. coli* cultured from the rumen of cattle fed the alfalfa pellets were lower than with grain, the concentration of free LPS in the rumen was still higher (Khafipour et al., 2008a, 2008b). This indicates that a greater portion of free rumen LPS is produced by other Gram-negatives. However, it appears as if their LPS is less biologically active than *E. coli* LPS (Nagaraja et al., 1979a). We previously demonstrated that *Bacteroidetes* is probably contributing the majority of LPS to the rumen pool given the size of this population in contrast to the *Proteobacteria* (Khafipour et al., 2008c).

Cluster analysis grouped *E. coli* strains into six groups (Figure 10). There was a clear difference between *E. coli* strains depending on treatment. Cluster 1 (Figure 10) has 20 strains from the grain-induced SARA period; while controls (8 and 5 isolates) and alfalfa induced SARA had only 5 isolates. Thus inflammation can be correlated with the type of *E. coli.* We hypothesize that a subset of *E. coli* in cluster 1 has virulence properties important in SARA.

The shifts in *E. coli* populations observed with rep-PCR were further confirmed by ABD typing. We observed a shift from an A to B1 population during grain-induced SARA (Table 26). In comparison, there were no significant changes in the ABD typing groups in alfalfa pellet-induced SARA. The ABD analysis indicates that there is indeed a link worth perusing between the symptoms of SARA and the *E. coli* population. The original ABD typing system developed by Clermont et al. (2000) was designed as rapid typing method to separate virulent and non-virulent strains of *E. coli* isolated from humans involved in extra-intestinal infections. Based on this methodology, B2 and D genotypes are supposed to be the groups that represent virulent types, while most of commensal bacteria belong to A and B1. There is at present no conclusive evidence that *E. coli* pathogens of animal origin fall into the B2 and D groups. Having said this, it is still a robust typing method that indicates shifts in *E. coli* populations. Therefore, we could clearly demonstrate that the *E. coli* populations differed between the grain- and alfalfa pellet-induced SARA groups using rep-PCR and ABD typing.

The shifts in *E. coli* populations appeared to be associated with differences in the ability to resist low rumen pH conditions (Figure 8). Of interest was that the fluctuation in the acid resistant populations was less in the SARA induction period and higher with control animals. This would indicate that in the SARA period a larger acid tolerant population is being selected (Figure 8). Although acid shock with HCl is not physiological in the rumen, it is still a useful phenotype because it is indicative of clearly acid resistant strain. Using acetate shock, while more physiological, may detect marginally acid resistant strains.

We hypothesized that acid tolerance may be a significant factor driving putative virulence of *E. coli*. The ability to tolerate acidity was considered a major driver because SARA is traditionally defined as a disease associated with a low rumen pH (Plaizier et al., 2008). By extension, we are proposing that low pH conditions are selecting for a more virulent group of *E. coli*. A recent study indicated that twelve genes from the *E. coli* K-12 genome between positions 3652706 to 3665603 bp have been associated with acid tolerance and this genomic region is called the acid fitness island (AFI) (Bergholz et al., 2007). The AFI homologous region in the *E. coli* O157:H7 genome is between yhiF (ECs4378) and yhiD (ECs4388). The AFI region contains genes associated with transcriptional regulation and together with the other genes in this cluster are know as the locus of enterocyte effacement (LEE). The LEE is an extremely important cluster of genes usually associated with pathogenicity islands in *E. coli*. Thus, an environment that selects for an acid resistant phenotype would also select for a range of virulence genes that are part of the LEE.

A significant difference between the grain- and alfalfa pellet-induced SARA was that LPS translocated the gut wall in grain- but not in alfalfa pellet-induced SARA. Cetin et al. (Cetin et al., 2004) demonstrated that LPS impairs the pH regulatory system of enterocytes by inhibition of sodium-proton pumps under extracellular acidotic conditions. This results in barrier dysfunction and increased permeability of the mucosa. Chin et al. (2006) demonstrated that LPS impairs the barrier function of the gut through production of nitric oxide.

Emmanuel et al. (2007) used Ussing chambers to evaluate the translocation of LPS across the tissue under low pH conditions. They demonstrated that LPS translocated both

the rumen and colonic tissue. However, the LPS concentrations that were applied was ~50 times higher than that seen in the rumen during SARA (Gozho et al., 2007; Khafipour et al., 2008). In all these studies, the effect of LPS has been studied by using *E. coli* LPS as a gold standard. However, rumen LPS may originate from other Gram-negative phyla such as *Bacteroides* and *Fibrobacters*, which their LPS may be less biologically active than *E. coli* LPS (Kaspar, 1976; Nagaraja et al., 1979a; Nagaraja et al., 1979b; Weintraub et al., 1989). Therefore, current knowledge on the effect of LPS in barrier disruption of the gut has bias toward *E. coli* LPS.

In summary, we clearly demonstrated that grain-induced SARA had a unique population of *E. coli*. The acidic conditions of the rumen may select for a population of *E. coli* with enhanced virulence features. Future work should investigate potential virulence factor associated with these strains.

GENERAL DISCUSSION

SARA was induced in lactating dairy cows by feeding wheat-barley pellets or alfalfa pellets in two separate experiments to determine if increases in free LPS in rumen fluid and acute phase proteins in peripheral blood are due to low rumen pH or high dietary content of starch, and to assess if the inflammatory response is due to LPS. In addition, culture-dependent and independent techniques were employed to examine rumen microbial community dynamics in relation to rumen pH, free rumen LPS, and inflammation in grain- versus alfalfa pellet-induced SARA.

In both studies, animals were fed a control TMR consisting of 50% concentrate and 50% forage (DM basis). However, the forage fraction was a 50:50 mixture of alfalfa and barley silage in the first experiment (Manuscript 1) but only chopped alfalfa hay in the second experiment (Manuscript 2). In the first study, SARA was induced by increasing the dietary content of starch through replacement of 21% of the DM of the TMR with wheat-barley pellets resulting in a F:C ratio of 40:60. In the second study, SARA was induced by gradual replacement of 42 % of alfalfa hay with alfalfa pellets in a 6 wk period without changing the F:C ratio or the starch content. These replacements resulted in average duration of rumen pH below 5.6 for 279 min/d during grain-induced SARA (Manuscript 1) and 268, 558, 510, and 447 min/d from wk 3 to wk 6 of alfalfa pellet-induced SARA, respectively (Manuscript 2). Earlier research has shown that successful induction of SARA that is characterized by feed intake depression and inflammation only occurs when a rumen pH depression between 5.2 and 5.6 was obtained for more than 180 min/d (Gozho et al., 2005). Thus, based on this definition SARA was induced successfully in the first experiment and from wk 3 onwards in the second experiment.

The greater rumen pH depression in alfalfa pellet-induced SARA than in grain-induced SARA was likely due to the dietary differences. We assume that the small particle size forage of high quality in the form of pellets might not stimulate chewing, ruminating, saliva production, and thus, rumen buffering. This view has been supported by previous research (Uden, 1999) showing that cows that were consuming only ground pelleted hay did not ruminate. In addition, the TMR fed during grain-induced SARA contained silages, while the TMR in alfalfa pellet-induced model only contained dry chopped alfalfa hay and pellets, which could have increased sorting against coarse particles (Leonardi et al., 2005).

The decline in rumen pH was accompanied by accumulation of VFA but not lactate in the rumen. Grain- and alfalfa pellet-induced SARA increased rumen VFA concentration from 98.5 to 107.3 m*M*, and from 90.0 to a maximum of 130.5 m*M*, respectively (Manuscript 1 and 2). The rumen lactate concentration was only measured during grain-induced SARA and was negligible (2.29 m*M*). Rumen osmolality also increased from 279.4 to 294.6 mOsm/kg, and from 277.7 to 300.9 mOsm/kg during grain- and alfalfa pellet-induced SARA, respectively (Manuscript 1 and 2). The high rumen acidity that was observed with both methods of SARA induction could have compromised the barrier function of the rumen epithelium by the parakeratosis, rumenitis and abscesses of the rumen wall (Kleen et al., 2003). In addition, the high rumen osmolality could have caused swelling and rupture of ruminal papillae, which will also reduce the barrier function of the rumen. Since magnitude and duration of low pH-high osmolality condition dictates the level of damage (local spot or extensive area) to the

rumen epithelium (McManus et al., 1977), we speculate that the degree of epithelial damage must have been greater in the alfalfa than in the grain model of SARA.

In addition to rumen acids and osmolality, the gut barrier function can be compromised by the free LPS released from Gram-negative bacteria. Chin et al. (2006) in an in vitro study with intestinal epithelial cell line SCBN, which has a canine genotype, reported that increase in luminal LPS could induce cell apoptosis, disrupt tight junctions, and increase epithelial permeability by increasing the production of NO·. In our studies, SARA induction was accompanied by an increase in free rumen LPS from 28,184 to 107,152 EU/mL, and from 38,019 to a maximum of 165,960 EU/ml during grain- and alfalfa pellet-induced SARA, respectively (Manuscript 1 and 2). This similarly may suggest that the rumen epithelial damage must have been greater in the alfalfa than in the grain model of SARA. However, only grain-induced SARA resulted in translocation of LPS into the peripheral circulation (Manuscript 1 and 2). Using a high sensitivity assay (0.005 EU/mL), we showed that LPS concentration in peripheral plasma increased from < 0.05 EU/mL in control to 0.52 EU/mL in grain-induced animals, but was still < 0.05 in alfalfa pellet-induced animals.

We evaluated the inflammatory responses of the animals to grain- and alfalfa pellet-induced SARA by monitoring Hp, SAA, and LBP in the peripheral blood. In manuscript 1, we showed that grain-induced SARA increased serum concentration of Hp (0 to 475.6 μg/mL), and plasma levels of SAA (167.4 to 438.5 μg/mL), and LBP (18.2 to 53.1 μg/mL). In contrast, in alfalfa pellet-induced SARA (Manuscript 2), the peripheral concentrations of acute phase proteins was in the physiological levels ranging from 56 to 12 μg/mL for Hp, from 23.1 to 6.9 μg/mL for SAA, and from 7.2 to 2.6 μg/mL for LBP.

Taken together, these observations suggest that low pH, high osmolality, and high free LPS in the rumen are not the sole cause of inflammatory response seen only during grain-induced SARA, and the difference might be associated with unique microbial changes that differ among diets (Manuscript 3, and 4).

We examined the structure, dynamics, and diversity of rumen microbial populations in grain- and alfalfa pellet-induced SARA using T-RFLP of 16S rDNA genes (Manuscript 3). Based on this analysis, rumen bacteria were classified into 9 phyla including *Bacteroidetes, Proteobacteria, Firmicutes, Spirochaetes, Actinobacteria, Fusobacteria, TM7, Tenericutes,* and *Deinococcus-Thermus.* Approximately 98% of rumen bacteria fell into the three predominant phyla of *Bacteroidetes, Proteobacteria, and Firmicutes.* However, the phylum *Fibrobacteres*, which contains *F. succinogenes* that is an important rumen cellulolytic bacterium, was not detected with the universal primers used in this study as all the available sequences started upstream of our forward priming site.

Based on a multivariate discriminate analysis using animals as independent variables and duration of rumen pH below 5.6, serum haptoglobin, and the concentration of free rumen LPS as dependent variables, cows were divided into severe grain-induced SARA, mild grain-induced SARA, and alfalfa pellet-induced SARA (Manuscript 3). The major difference between groups was the extent of inflammation. We then evaluated the microbial shifts in the rumen in relation to these objectively derived groups of SARA.

The most notable observation in our T-RFLP data (Manuscript 3) was that both grain- and alfalfa pellet-induced SARA resulted in a significant increase in the phylum *Firmicutes*, which are Gram-positive, and a decrease in the phylum *Bacteroidetes*, which

are Gram-negative. However, the proportions of these populations were different among SARA models. In alfalfa pellet-induced SARA *Bacteroidetes* were 35.4% of the population, while in the grain-induced mild SARA group it was 26% but in severe group only 16.6%. Since, *Bacteroidetes* are the major Gram-negative phyla in the rumen (Hungate, 1966); we speculate that a greater proportion of them in the alfalfa-pellet fed group could contribute to a greater portion of free rumen LPS pool. However, LPS of *Bacteroides* spp. is much less toxic than LPS from *E. coli* or *Salmonella* (Kaspar, 1976; Stewart, 1977; Weintraub et al., 1989). Thus, it might not be the concentration of LPS *per se*, but the biological activity of those LPS that determines the degree of inflammatory response (Greisman and Hornick, 1969; Danner et al., 1990).

Our real-time PCR analysis of predominant rumen microorganisms (Manuscript 3) highlighted that severe grain-induced SARA was dominated by *E. coli*, *M. elsdenii*, and *S. bovis,* while mild grain-induced SARA was dominated by *M. elsdenii*, *S. ruminantium*, and *Ruminococcus flavefaciens.* In contrast the alfalfa-induced SARA group was dominated by *P. albensis,* and *Ruminococcus albus.* However, multivariate discriminative analysis of real-time PCR results indicated that the best predictor of inflammation was *E. coli,* which was highly abundant in severe grain-induced SARA. Thus, we suspect that a subpopulation of *E. coli* is putative pathogenic triggers of inflammation in grain-induced SARA.

In manuscript 4, we examined the genetic composition of a range of *E. coli* strains that were isolated from rumen fluid of dairy cows under control and both SARA conditions to evaluate the similarity of *E. coli* populations in grain- versus alfalfa pellet-induced SARA. Cluster analysis of *E. coli* isolates using rep-PCR DNA fingerprinting

revealed a significant difference in *E. coli* population according to diet. Based on this analysis, 73.9% of *E. coli* isolates grouped into two major clusters. Isolates from control animals from either the grain (46%) or alfalfa-pellet diets (70%), and isolates from alfalfa pellet-induced SARA (53%) tended to cluster together. In contrast, isolates from grain-induced SARA (54%) were grouped together in a separate cluster. Similarly, using a discriminant function analysis, we demonstrated a significant group difference among *E. coli* isolates with respect to their treatment groups. The shifts in *E. coli* populations observed with rep-PCR DNA fingerprinting were further enhanced by A, B1, B2, and D typing of all the isolates recovered from the rumen (manuscript 4). We observed a significant shift from A type to B1 type *E. coli* only in grain-induced SARA. Based on this classification, most of the commensal gut bacteria belong to A group and to lesser extent to B1, while B2 and D genotypes represent the virulent groups (Clermont et al., 2000). Acid shock analysis indicated that the acid resistant *E. coli* population in grain-induced SARA was significantly higher (2.65 vs 2.0 $\log_{10}$ cfu/mL) than in control animals. We speculate that the shift in *E. coli* population might be associated with the ability of this bacterium to resist the low pH conditions of the rumen (Manuscript 4). Taken together, our results may suggest that acid tolerance may be a significant factor driving putative virulence of *E. coli*, and thus, grain-induced SARA is selecting for a subpopulation of *E. coli*, which could potentially exhibit virulence factors that allow for an increased permeability of the gut wall allowing for the translocation of LPS into the circulation.

We do not exclude the possibility that LPS translocation might have occurred in the small or large intestine rather than in the rumen (Manuscript 2). This hypothesis is

supported by several observations: (a) the differences between the composition of epithelium of the rumen (multi layer), and of the small and large intestine (mono layer) could result in differences in the effect of LPS on the barrier impairment, and therefore, on the rate of LPS translocation from these parts of the gastro-intestinal tract (Graham and Simmons, 2005). (b) If rumen epithelium has a low permeability for LPS (Lassman, 1980; Anderson, 1984), then only small fraction of LPS may translocate from the rumen, whereas the remaining free rumen LPS will be detoxified in the duodenum by bile acids (Bertok, 1998). (C) The greater content of dietary starch in the grain model could have increased the amount of starch that bypasses rumen fermentation and reaches to the small and large intestine (Allen, 2000; Rowe, et al. 2004), which could have triggered the bacterial growth, and thus, release of LPS from Gram-negative bacterial community of the terminal ileum and large intestine. The present research provided support for this view but did not investigate the microbial dynamics of the intestine or translocation of LPS from these components of the gastro-intestinal tracts to the circulation, but this could be an interesting topic for future research.

CONCLUSIONS

Based on the results of present investigation it is concluded that:

1. A rumen pH depression typical of SARA (at least 180 min/d of pH < 5.6) can be created either by increasing the dietary content of starch and reducing the F:C ratio, or by increasing the dietary content of pelleted forage without changing the F:C ratio.

2. Inductions of SARA with both nutritional models were accompanied by similar increases in VFA, osmolality, and free LPS in the rumen fluid.

3. Only in grain-induced SARA, the decline in rumen pH and increase in free LPS was accompanied by translocation of LPS from the gut into the peripheral circulation and acute phase response. Thus, free LPS concentration in the rumen fluid is not a predictor of SARA.

4. The differences in the effects of the rumen pH depressions during alfalfa-pellet induced SARA and grain-pellet induced SARA challenge the current definition of SARA, which is only based on the rumen pH depression.

5. The phylum *Bacteroidetes*, which is the main Gram-negative phyla in the rumen, had a greater proportion in alfalfa pellet-induced than in grain-induced SARA. Hence, they may contribute to a greater portion of free rumen LPS pool.

6. A major microbial population that was positively associated with inflammation was *E. coli*, which was in high abundance in the grain-induced SARA but was almost entirely absent in alfalfa pellet-induced SARA. In addition, severe grain-induced SARA group had a unique population of *E. coli*, which may potentially exhibit virulence factors that allow for an increased permeability of the gut wall allowing for the translocation of LPS into the circulation.

7. Translocation of LPS from the gut may have occurred post-ruminally during grain-induced SARA. The greater content of dietary starch in the grain model could have increased the amount of starch that bypassed rumen fermentation and reached to the small and large intestine. This could have changed the lower gut microbiota and triggered LPS release, which could result in barrier failure of the monolayer epithelium of the intestine and translocation of LPS to peripheral blood.

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