Systemic immune responses to intestinal-derived lipopolysaccharide (LPS) during subacute ruminal acidosis (SARA) and their possible role in innate immunity

by

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ABSTRACT

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The effects of induced subacute ruminal acidosis (SARA) using grain pellet-based (GPI) and alfalfa pellet-based diet models on systemic immunological parameters were evaluated in nonlactating Holstein cows. The systemic immunological parameters analysed in this study included rectal temperature, blood cell leukogram, expression of lipopolysaccharide (LPS) recognition receptors on leukocytic cells, and plasma and serum proteins. Also, blood biochemistry was analysed. There were no significant differences in rectal temperature, blood cell leukogram, expression of LPS recognition receptors and fibrinogen or haptoglobin concentrations between control and SARA induction treatments. Concentrations of serum amyloid A and lipopolysaccharide-binding protein increased while total protein concentrations decreased in response to GPI SARA compared to control. Blood glucose and urea concentrations increased and decreased, respectively, with GPI SARA treatment. Grain pellet-induced SARA resulted in changes to serum proteins and acute phase proteins but did not affect other systemic immunological parameters suggesting a localized inflammatory response was initiated.

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ABBREVIATIONS

ANOVA	Analysis of variance
API	Alfalfa-pellet induced
APP	Acute phase protein
APR	Acute phase response
AST	Aspartate aminotransferase
BW	Body weight
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CK	Creatine kinase
Ct	Cycle threshold
DM	Dry matter
DNA	Deoxyribonucleic acid
EU	Endotoxin unit
GALT	Gut-associated lymphoid tissue
GGT	Gamma-glutamyl transferase
GIT	Gastrointestinal tract
GPI	Grain-pellet induced
Hp	Haptoglobin
IEC	Intestinal epithelial cell
LAL	*Limulus* amebocyte lysate
LBP	Lipopolysaccharide-binding protein

Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
mCD14	Membrane cluster of differentiation 14
MD2	Myeloid differentiation 2
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response gene 88
NCBI	National Center for Biotechnology Information
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
peNDF	Physically effective neutral detergent fibre
PMN	Polymorphonuclear
qRT PCR	Quantitative real time polymerase chain reaction
RBC	Red blood cell
SAA	Serum amyloid A
SARA	Subacute ruminal acidosis
sCD14	Soluble cluster of differentiation 14
TNF	Tumor necrosis factor
TOD	Time of day
TLR4	Toll-like receptor 4
tRNA	Total ribonucleic acid

VFA	Volatile fatty acid

1.0 GENERAL INTRODUCTION

The inclusion of grain into the diets of dairy cows has become a common practice in North America to facilitate energy demands associated with milk production. However, the high non-fibre carbohydrate content of grain increases the rate of microbial fermentation in the rumen thus increasing the production of volatile fatty acids (VFAs), reducing ruminal pH and changing the rumen microflora (Eusebio et al., 1959). Therefore, cattle switched from a forage-based diet or who are not well-adapted to a grain-based diet may experience metabolic problems such as subacute ruminal acidosis (SARA). Subacute ruminal acidosis can be defined as the reduction of ruminal pH below 5.6 for 3 hours daily (Gozho et al., 2005).

Several studies have observed considerable increases in the concentrations of free-endotoxin within the rumen of cattle after the induction of SARA (Gozho et al., 2007; Khafipour et al., 2009a; 2009b). These endotoxins, also known as lipopolysaccharides (LPS), are structural components of the gram-negative bacterium cell wall which are capable of triggering an immunological response within the host (Niemetz and Morrison, 1977). The lipid A region of the LPS structure is biologically active and toxic to mammalian cells (Niemetz and Morrison, 1977). Therefore, the presence of increased endotoxins within the gastrointestinal tract (GIT) may cause immunological stimulus to the animal. An increase in acute phase proteins (APP), including serum amyloid A (SAA), haptoglobin (Hp) and lipopolysaccharide-binding protein (LBP) concentrations, has been reported in cows induced with SARA, as compared to control cows fed a forage-based diet (Gozho et al., 2007; Khafipour et al., 2009a; 2009b).

However, the mechanisms involved with the SARA-associated acute phase response (APR) are not well understood. The presence of an APR could imply either local or systemic inflammation. Local inflammation may be triggered by the repeated exposure to low ruminal pH and VFA resulting in mucosal irritation and damage to the rumen epithelium (Kleen et al., 2003). Systemic inflammation may be caused by excessive presence of endotoxin in the blood. The increased concentrations of endotoxin in the GIT due to bacterial overgrowth, along with epithelial damage or immunosuppressive factors, such as T-cell depletion, lymphoma, leukemia and the production of corticosteroids, could facilitate the mobilization of beyond the GIT barrier (Berg, 1995, Khafipour et al., 2009a).

Several studies have infused LPS isolated from *Escherichia coli* into cattle, triggering physiological and immunological parameters such as increased body temperature and respiratory rate, increase in numbers of lymphocytes and decrease in neutrophil population (Werling et al., 1996; Waldron et al., 2003b, Jacobsen et al., 2007). However, rumen-isolated endotoxins do not exhibit the same degree of endotoxicity as *E. coli* LPS (Nagaraja et al., 1978a), therefore rumen-associated endotoxins may not result in the same biological response as that observed with *E. coli* LPS. Intestinal epithelial cells, as well as peripheral blood leukocytic cells, possess receptors including; cluster of differentiation (CD) 14, myeloid differentiation (MD) 2 and toll-like receptor (TLR) 4, which are imperative in the recognition of LPS (Sauter et al., 2007). Once recognized by leukocytes, an immune response is activated, resulting in the secretion of pro-inflammatory cytokines which regulate or stimulate other systemic parameters (Godson et al., 1995; da Silva Correia et al., 2001). Changes in physiological parameters,

including respiratory rate, heart rate and body temperature, and the production of APPs are down-stream inflammatory responses resulting from an endotoxin intravenous infusion (Werling et al., 1996; Jacobsen et al., 2005; Carroll et al., 2009).

The literature presented in this thesis outlines the systemic biological response within bovines to SARA induction, as well as the systemic response after exposure to *E. coli*-isolated LPS. The objective of the study was to determine the systemic immunological responses of nonlactating cows induced with SARA using two different models, grain pellet-induced (GPI) and alfalfa pellet-induced (API). The systemic response was determined by measuring rectal temperature, changes in peripheral leukocyte populations, APPs and changes in various other blood chemistry parameters. In addition, LPS-recognition receptors (CD14, TLR4 and MD2) located on peripheral blood cells were evaluated to determine the presence of endotoxins, potentially derived from the gut.

2.0 LITERATURE REVIEW

2.1 General introduction of subacute ruminal acidosis

Subacute ruminal acidosis has been characterized as a metabolic problem in ruminants associated with feeding diets rich in fermentable starches or deficient in physically effective neutral detergent fibre (peNDF) (Plaizier et al., 2008). These types of diets have become more prevalent with dairy cows in an attempt to meet the energy needs associated with milk production. Subacute ruminal acidosis is more common in cattle quickly transitioned from a forage-based diet to a concentrate-based diet. As clinical symptoms associated with SARA are difficult to characterize ruminal pH thresholds are used to diagnose SARA or when inducing SARA for experimental purposes. Ruminal pH thresholds ranging from 5.5 to 6.0, have been used to characterize SARA and vary with the method of rumen sample collection used (Plaizier et al., 2008). The fermentative process of non-structural carbohydrates prompts the growth of amylolytic and saccharolytic bacteria which are able to produce VFAs at a quicker rate than they are absorbed or buffered in the rumen, reducing the ruminal pH (Owens et al., 1998). The more extensive fermentative process of fibre stimulates rumination activity to prevent the accumulation of VFAs within the rumen. Along with the reduction in ruminal pH, a shift in the proportion of VFAs can also be observed. The concentration of propionate and acetate increase and decrease, respectively, while butyrate remains fairly constant (Erfle et al., 1982; Calsamiglia et al., 2002).

While the decrease in ruminal pH has been quite evident with SARA, much of the focus of SARA research has been on the increased concentrations of free-endotoxins (not attached to bacteria) within the rumen and increased APPs concentrations found in the blood (Gozho et al., 2007; Khafipour 2009a; 2009b). Alfalfa pellet-based diets and grain pellet-based models for SARA induction have shown increases in ruminal endotoxins, however the increase is much more intense with the latter model. The increased ruminal endotoxin concentrations have been associated with the shedding of LPS from rapid-growing gram-negative bacteria (Crutchley et al., 1967; Nagaraja et al., 1978b). The APP concentration increases in the blood with SARA is believed to be caused by the high concentrations of ruminal endotoxins due to their toxic properties. The implications of increased endotoxin concentrations in the rumen and the APR will be discussed in subsequent sections.

2.2 Rumen gram-negative microbes and associated endotoxins

Extensive studies have been conducted, beginning in the late 1950's; characterizing the microflora populations present in the rumen of cattle fed different diets. It is evident that gram-negative bacteria are ubiquitous in the rumen; however, Nagaraja et al. (1978b) along with others (Andersen et al., 1994; Emmanuel et al., 2008) have reported multiple-fold increases in concentrations of free-endotoxins in the rumen of cattle fed grain-rich diets. Increased ruminal endotoxin concentrations could have further ramifications on the health of the animals given that high endotoxin concentrations are not typically found in the rumen and the toxic properties of endotoxin.

Recently, research groups have been attempting to understand the significance of high ruminal endotoxin concentrations on the health of the animal. With the lack of analytical technology, it is difficult to characterize ruminal endotoxins to determine which bacteria species they originated from. As described in a review by Rietschel et al. (1994), endotoxins from different bacteria have varying degrees of toxicity believed to be associated with the three-dimensional conformation, phosphate composition and length of acyl chains of the lipid A region. The characterization of LPS structure and endotoxicity of various enteric gram-negative bacteria involved with human diseases has been completed in vitro (Erridge et al., 2002). Of the characterized enteric bacteria, LPS derived from *E. coli, Salmonella minnesota, Klebsiella pneumoniae* have been identified as the greatest endotoxic threat to hosts (Erridge et al., 2002). These bacteria can also be cultured from the GIT of ruminants however, the results must be confirmed in vivo as other confounding factors, such as bacterial and plasma proteins and LPS aggregates could affect the endotoxicity level of LPS within the host (Erridge et al., 2002).

Bacteroidetes are the most abundant gram-negative bacteria in the rumen and their associated-LPS have been found to express low endotoxic activity (Hungate, 1966; Hofstad, 1974; Nagaraja et al., 1978a). However, when cattle are switched from a forage-based diet to a grain-based diet, the shift in the ruminal microflora population may result in the increase of endotoxins. Endotoxins isolated from ruminal fluid from animals fed the grain-based diet tended to be more endotoxic than that of animals fed the hay-based diet when injected into rodents and chick embryos (Nagaraja et al., 1978a). Further, endotoxins isolated from ruminal fluid, from either animas fed hay-based or grain-based diets, expressed low endotoxic activity compared to *E. coli* LPS (Nagaraja et al., 1978a).

In a study characterizing the rumen microbiome composition of hay-based (control) and grain pellet-based (SARA) diet *Enterobacteriace*, the family of *E. coli*, did not represent a significant population, less than two-percent of all bacteria within the rumen of cows fed either diet (Khafipour et al., 2009c). Lipopolysaccharide isolated from *E. coli* has often been used for inoculation with in vitro and in vivo LPS-infusion studies. The use of *E. coli*-derived LPS results in the intense immunological responses in mammalians as mentioned before. Therefore, *E. coli*-derived LPS may not be the most useful endotoxin to understand the effects of increased ruminal endotoxin concentrations on local and systemic immunological responses in bovines. It is important to note that other endotoxic bacteria, besides *E. coli* may be present within the rumen. Further research is warranted to characterize the free-endotoxin in the rumen, to determine the contributing bacteria, and the toxicity level to subsequently understand its potential immunological implications on the host.

2.3 Ruminal and gastrointestinal epithelium barrier

The defence and protective mechanisms of the GIT are abundant and include mechanisms such as the presence of a protective mucous lining, highly-developed and specialized lymphoid tissue and secretion of antibodies (Pabst, 1987). The innate protective mechanisms of the gastrointestinal tract lining in ruminants are imperative as epithelial cells are continuously interacting with bacteria in the rumen and the hind-gut. Additionally, the four-layered epithelium of the rumen aids in the containment of microflora within the GIT (Steven and Marshall, 1970; Graham and Simmons, 2005).

The epithelia of the gut contain mucosa-associated lymphoid tissue (GALT). This GALT includes immune follicle-associated epithelium, such as the M cells of Peyer's patches and enterocytes which possess T and B lymphocytic cells, dendritic cells and macrophages (Pabst, 1987). The primary function of GALT is to maintain gut health by recognizing antigens and inducting immune response (Liebler-Tenorio and Pabst, 2006). The mucous lining the gut enterocytes provides protection against bacterial, toxin and viral attachment to the gut lining (Pabst, 1987). As well, immunoglobulin A (IgA) and IgG are secreted by B cells to enhance the protection barrier (Pabst, 1987).

Although the rumen epithelium consists of several stratified tissue layers, the effect of acidic conditions and changes in ruminal bacteria in response to changing diets on the integrity of these tissue layers and mucosal barrier is not well understood. Nocek and Kesler (1980) observed damaged, discoloured papillae and a thicker submucosal layer of steers after being fed a completely pelleted diet for 14 weeks. However, they did not observe significant rumen epithelium differences between steers fed pelleted and hay-concentrate diets after 18 weeks. These observations suggest that the adaptation of ruminal epithelium to diets which do not meet peNDF requirements for the rumen is possible, although it may take time. A case report by Steele et al. (2009) transitioning from a 100% forage diet to a 79% grain diet over 7 days showed damage to the ruminal epithelium and sloughing of the stratum corneum tissue layer. The Nocek and Kesler (1980) and Steele et al. (2009) studies found the submucosal tissue to be keratinized, an indication of parakeratosis. Parakeratosis has been characterized as the increased thickness and retention of nuclei within the stratum corneum (Wise et al., 1968). The keratinisation of ruminal epithelium could be a compensatory response to changing

ruminal conditions and act as a protective mechanism to undesirable ruminal conditions such as reduced pH levels and increased ruminal osmolality (Owens et al., 1998). However, under milder experimental conditions (i.e. pH 5.5 for short duration), it was found that induction of SARA did not affect the integrity of the ruminal epithelium in sheep but suggested that subsequent stress on the epithelium, such as acidic pH levels (i.e. rapid decrease to pH 5.2), could compromise epithelium integrity (Penner et al., 2010). In addition, an in vitro study using an Ussing chamber at a pH of 5.5, which is characteristic of SARA, did not have an effect on the permeability of isolated rumen tissue even in the presence of 500 μg/mL LPS (Emmanuel et al., 2007). The occurrence of severe ruminal conditions (i.e. acute ruminal acidosis) causes stress on ruminal epithelium compromising its integrity and permeability. Milder ruminal conditions (i.e. SARA), however, pose less of a threat as ruminal epithelium is able to maintain its function.

The presence of increased concentrations of ruminal endotoxins found in cows fed a grain-rich diet or induced with SARA, has led to the concern that compromised ruminal epithelium allows for the translocation of ruminal contents, such as bacteria and endotoxins, beyond the ruminal barrier. It has been proposed that endotoxin translocation of endotoxins at low levels is a natural process, present in healthy hosts (Berg, 1983). However, a healthy host exhibits hyporesponsiveness to natural inhabiting microflora, eliminating it before any response is triggered (Berg, 1983; 1995). Three mechanisms predisposing translocation of bacteria and/or bacterial-derived molecules from the GIT to the bloodstream or lymphatic system have been described and include bacterial overgrowth in the GIT, immunodeficiency of the host, and decreased epithelial and

mucosal integrity of GIT (Berg, 1995). Two routes of translocation of endotoxin across the intestinal barrier have been identified and include paracellular, translocation between intestinal epithelial cells, (Hietbrink et al., 2009) and transcellular, through the cell via receptor-mediated endocytosis (Neal et al., 2006). Tight junctions between cells tend to become more permeable to ruminal endotoxin contents during oxidative stress, metabolic stress and intestinal inflammation (Mani et al., 2012). In bovines, endotoxins have been detected in the plasma of cattle overfed grain (Dougherty et al., 1975) and cows induced with GPI SARA (Khafipour et al., 2009a). However, it is not well understood where or how endotoxins reached the circulation. Several factors have been proposed by Berg (1995) and Mani et al., (2012). The rapid change to a diet rich in non-structural carbohydrates that are highly fermentable by microbes leads to an increase in their rate of growth and production of VFAs. As a result of the diet change cows may be predisposed cows to metabolic and/or oxidative stresses especially when in negative energy balance post-parturition (Goff, 2006). As well, localized inflammation caused by the change in ruminal environmental conditions (decreased pH, increased endotoxins, and microbial changes) may occur (Kleen et al., 2003). It has also been suggested that translocation occurs in the hind gut which contains a monolayer epithelial barrier (Khafipour et al., 2009a). Contrary to the findings of Dougherty et al., (1975) and Khafipour et al., (2009a), other studies have not detected endotoxins beyond the GIT after inducing SARA or after feeding high-grain diets. Further, most studies feeding grain-rich diets and reporting increased ruminal endotoxin concentrations have not examined the presence of endotoxin in the peripheral blood.

2.4 Cellular recognition and interaction of LPS

The occurrence of endotoxin within the peripheral blood, or anywhere beyond the GIT, would be recognized by leukocytic cells. The most common pathway involved with recognition of LPS is the CD14-TLR4-MD2 pathway (da Silva Correia et al., 2001). As displayed in Fig. 2.1, the LPS molecule binds to LBP, due to the high affinity of LBP for LPS, which is then recognized by CD14 on the cell surface (Tobias et al., 1989; Wright, et al., 1990; Hailman et al., 1994). Cluster of differentiation 14, a glycosylphosphatidylinositol membrane-anchored protein, lacks a transmembrane domain (Haziot et al., 1988) therefore, TLR4 is required to transmit the signal across the cell membrane to intracellular responders (Hoshino et al., 1999). In addition, TLR4 requires physical association with MD2 to successfully stimulate intracellular pathways of the cell (Shimazu et al., 1999; Sauter et al., 2007). The two typical intracellular pathways include, myeloid differentiation primary response gene 88 (MyD88)-dependent and MyD88-independent which convey messages via intermediary signallers to the nucleus of the cell to synthesize pro-inflammatory cytokines (Kawai et al., 1999; Takeuchi et al., 2000; Kawai et al., 2001; Yamamoto et al., 2003). Pro-inflammatory cytokines relay information to different organs of the body to further the downstream inflammatory response.

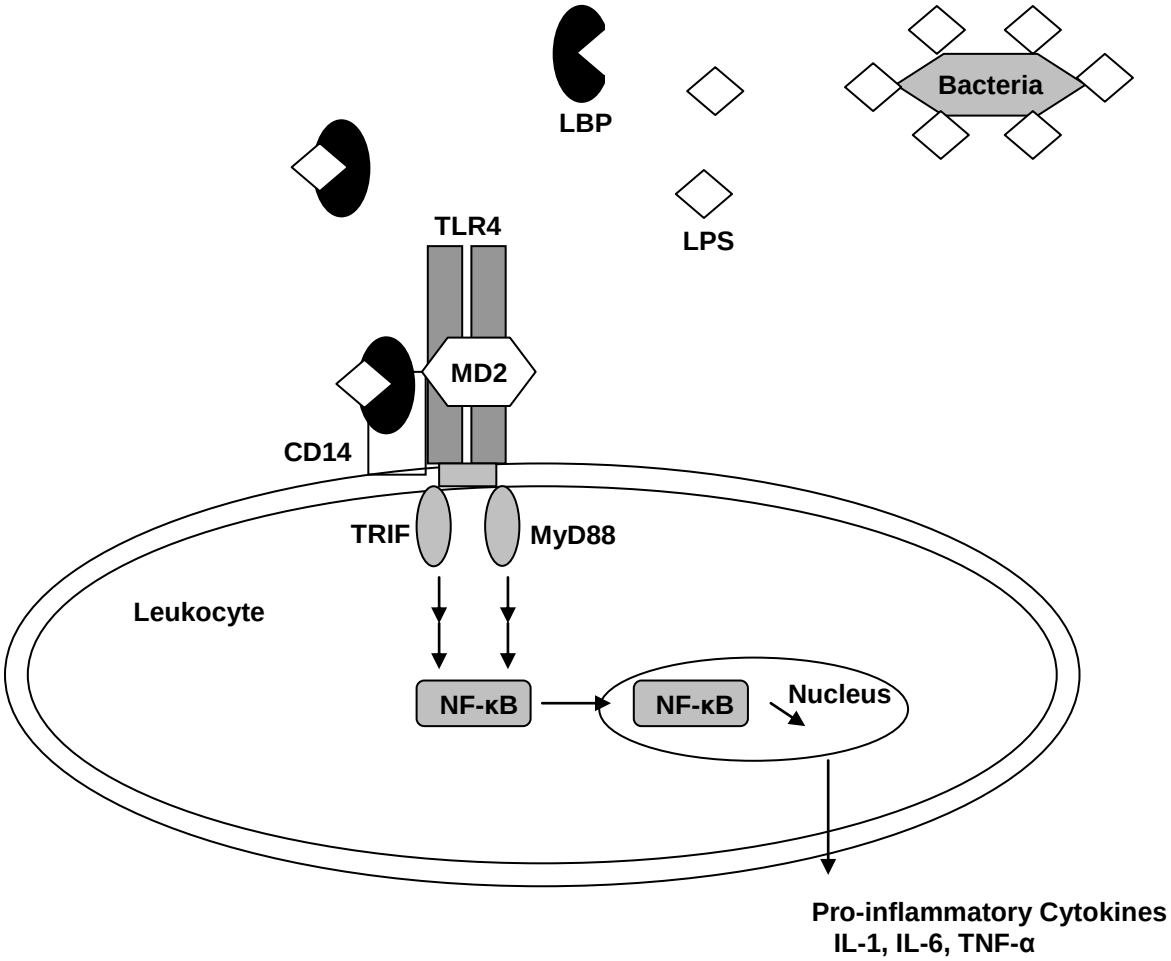

Figure 2.1 The activation mechanisms of the leukocytic pro-inflammatory response by gram-negative bacterium-associated lipopolysaccharide (LPS). The activation of toll-like receptor (TLR) 4 and myeloid differentiation (MD) 2 stimulates either the myeloid differentiation (MyD) 88-depedent or MyD88-independent TRIF signalling pathways which initiate the translation of nuclear factor (NF)-kappaβ and the secretion of pro-inflammatory cytokines, interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α. *Adapted from Akira and Takeda, 2004; Takeda and Akira, 2005.*

2.4.1. Lipopolysaccharide-binding protein in LPS recognition

Lipopolysaccharide-binding protein was first characterized as a LPS-specific protein. Lipopolysaccharide-binding protein contains a region which binds to lipid A, and gram-negative bacteria are the only organisms containing endotoxins with a lipid A region (Tobias et al., 1989; Schumann et al., 1990). Various *in vitro* studies in humans and bovines have shown the requirement of LBP for enhanced LPS recognition and cell activation (Heumann et al., 1992; Horadagoda et al., 1995; Muta and Takeshige, 2001). Plasma LBP concentrations increase rapidly after inflammatory challenge or bacterial exposure (Tobias et al., 1986; Schroedl et al., 2001). Due to the quick response, LBP is considered an acute phase protein which is regulated by pro-inflammatory cytokines (Schumann et al., 1996).

2.4.2 CD14 regulation

Cluster of differentiation 14 has been recognized as a co-receptor in the recognition of LBP-LPS complex. Cluster of differentiation 14 is expressed on the cell surface of monocytes, macrophages and to a lesser extent on polymorphonuclear (PMN) cells (Wright et al., 1990; Ibeagha-Awemu et al., 2008b). The down-regulation of membrane-bound CD14 (mCD14) has been reported in bovine and human leukocytes after several hours of incubation with LPS (Bažil and Strominger, 1991; Landmann et al., 1996; Sohn et al., 2007a). As well, the studies detected increased soluble CD14 (sCD14) concentrations when mCD14 expression was down-regulated (Bažil and Strominger, 1991; Landmann et al., 1996; Sohn et al., 2007a). Hiki et al., (1998) found similar results

in a human study when comparing healthy patients to septic patients. Peripheral blood mononuclear cells (PBMC) from healthy individuals displayed higher initial mCD14 expression than individuals with sepsis. After stimulation with endotoxin *ex vivo*; healthy patients had lower concentrations of sCD14 than septic individuals. The down-regulation of mCD14 expression, parallel with increased sCD14 concentrations, suggests that mCD14 may be shed from the cell surface. It has been hypothesized that sCD14 may act as an inhibitor, competing with mCD14 to bind to LBP-LPS complexes, thus preventing activation of leukocytes (Maliszewski and Wright, 1991). These same researchers have also proposed that sCD14 resulting from mCD14 shedding is unable to bind to LBP-LPS complexes and the down-regulation of mCD14 may be a mechanism of preventing hyperresponsiveness of cells (Maliszewski and Wright, 1991). Although the theories differ, both suggest that down-regulation of mCD14 and increased sCD14 concentrations are involved with the regulation of the immune response. The changes in CD14 expression suggest that co-receptors may have a significant role in immune response regulation upon stimulus of LPS.

2.4.3 TLR4 gene regulation

Toll-like receptor 4 plays a prominent role in signalling cell activation after LPS exposure, therefore, regulation of this receptor is important. Toll-like receptor 4 is considered to be one of the most stable TLRs when it comes to signalling which is likely attributed to the many factors that inhibit TLR4 regulation (Divanovic et al., 2007). The robustness of TLR4 is apparent from limited activity level of gene expression of

leukocytes after LPS stimulation. The messenger ribonucleic acid (mRNA) expression of TLR4 did not change in peripheral blood monocytes and monocyte-derived macrophages upon in vitro stimulation of *E. coli*-derived LPS at a concentration of 100-1000 ng/mL (Franchini et al., 2006; Guo et al., 2009). However in humans, *in vitro* LPS (10 ng/mL) stimulation of monocytes resulted in increased expression of TLR4 mRNA (Muzio et al., 2000). The regulation of the innate immune response is based upon the balance between stimulatory and inhibitory responses; therefore, the absence of change to TLR4 expression may be due to negative regulation or the inhibition of an inflammatory response. A review by Liew et al. (2005), described various negative regulation responses with regards to TLR4 which have been found in laboratory rodents and humans. These negative regulation mechanisms included: (1) soluble TLR4 which prevents cells from activation by endotoxins and intracellular molecules, (2) intracellular proteins which disrupt pathway activation, and (3) down-regulation of TLR expression. Also, Muzio et al., (2000) found that including an anti-inflammatory cytokine, interleukin (IL) -10, to LPS-stimulated cells down-regulated the expression of TLR4 in monocytes. Although it has not been proven, these regulating mechanisms could also be involved with the absence of TLR4 gene activity in bovine cells peripheral blood cells exposed to LPS.

An increase in TLR4 mRNA expression was reported in mammary-associated cells after in vitro stimulation of *E. coli* LPS (Ibeagha-Awemu et al., 2008a). Also, increased TLR4 gene expression was found in mammary tissue from dairy cows testing positive for gram-negative-associated mastitis (Goldammer et al., 2004). Intestinal epithelial cells (IEC), however, showed a different response to *E. coli* LPS than peripheral blood cells and mammary cells, as initial expression levels of TLR4 mRNA

were minimal and decreased after stimulation with LPS (Abreu et al., 2001; Böcker et al., 2003; Otte et al., 2004). It appears that the expression level of TLR4 may be profoundly dependent on the location of TLR4 and frequency of exposure to gram-negative bacteria or bacterial molecules. For example, IEC are frequently exposed to gram-negative bacteria, commensal to the gut, and therefore TLR4 expression levels remain low to prevent damage from constant exposure to inflammatory stimulators (Bäckhed and Hornef, 2003). Conversely, gram-negative bacteria (i.e. *E. coli*, *Klebsiella* spp., *Enterobacter* spp., and *Pseudomonas* spp.) are not ubiquitous to the mammary gland. As a result of the minimal exposure to gram-negative bacteria and the gland's many defence mechanisms, cells are less tolerant to bacteria and mastitis ensues rather quickly (Sordillo et al., 1997).

2.4.4 Cytokine regulatory response

In response to successful recognition of LPS by TLR4, leukocytic cells actively produce pro-inflammatory cytokines to further the immunological cascade. The in vitro stimulation of bovine peripheral blood and milk somatic cells with *E. coli*-isolated LPS has resulted in increased concentrations and gene expressions of IL-1β, IL-6 and tumor necrosis factor (TNF)-α (Prgomet et al., 2005; Sohn et al., 2007b). These results are consistent with the results reported after infusing LPS intravenously into cattle in which concentrations of IL-1β, IL-6 and TNF-α increased significantly in the blood (Werling et al., 1996; Steiger et al., 1999, Waldron et al., 2003b, Carroll et al., 2009).

Jacobsen et al., (2007) found that the highest percentage of cells expressing pro-inflammatory cytokines was 24-hours post-infusion. In addition the percentage of cells that expressed anti-inflammatory cytokines, IL-4 and IL-10 increased initially followed by a pro-inflammatory response as measured by an increase of TNF-α and IL-1β positive cells 24 hours post-infusion. The initial anti-inflammatory response followed by a pro-inflammatory response may be reflective of primary hyporesponsiveness to prevent an immune response from mounting (Jacobsen et al., 2007). The hyporesponsiveness, or tolerance, of pro-inflammatory cytokines after repeated exposure to LPS has been reported in humans (Granowitz et al., 1993; van der Poll et al., 1996). After a pro-inflammatory response has been triggered, it is the responsibility of anti-inflammatory cytokines, such as IL-4 and IL-10 produced by monocytes, to function as regulators and suppressors of pro-inflammatory cytokines (te Velde et al., 1990; de Waal Malefyt et al., 1991; Wang et al., 1995). Pro-inflammatory cytokines target hepatocytes in the liver to synthesize acute phase proteins.

2.5 Acute phase response

The APR is a non-specific response important for maintaining homeostasis and aiding in tissue repair in response to inflammation, infection and surgical trauma (Kushner, 1982). Cytokines produced by activated cells are the main molecules responsible for the initiating the production of APPs by the liver (Godson et al., 1995; Nakagawa-Tosa et al., 1995; Alsemgeest et al., 1996). The involvement and regulation of the inflammatory cytokines have been reviewed by Moshage (1997) and outlined in

Table 2.1. There are various APPs; SAA, Hp, LBP, fibrinogen, which perform different functions and therefore have different production patterns.

Several putative biological functions and mechanisms of action of the different APPs are known (reviewed by Petersen et al., 2004; Murata et al., 2004) and listed in Table 2.2. In general, the APPs have important functions in recruiting immune cells to the location of injury, suppressing bacterial growth and repairing damaged tissue.

Table 2.1. Putative biological functions of pro-inflammatory and anti-inflammatory cytokines.

Cytokine	Function	Reference
Pro-inflammatory (IL[1]-1β, IL-6, TNF-α[2])	Increase body temperature (fever)	Dinarello et al. (1986); Godson et al. (1995)
	Stimulate acute phase protein production (SAA[3], Hp[4])	Godson et al. (1995); Nakagawa-Tosa et al. (1995); Alsemgeest et al. (1996)
	Increase circulating leukocytes	Godson et al. (1995)
Anti-inflammatory (IL-4, IL-10)	Inhibit induction of acute phase proteins	Loyer et al. (1993)
	Inhibit synthesis of LPS[5]-induced pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) by monocytes	te Velde et al. (1990); de Waal Malefyt et al. (1991); Wang et al. (1995)

[1]IL = Interleukin; [2]TNF-α = Tumor necrosis factor alpha; [3]SAA = Serum amyloid A; [4]Hp = Haptoglobin; [5]LPS = Lipopolysaccharide

2.5.1 Serum amyloid A and haptoglobin

Acute phase protein concentrations have become a frequently used tool in many studies to monitor animal health and welfare. When management practices affect not only the performance of an animal but also the health of the animal, it becomes a welfare concern. However, it is difficult to use APPs to diagnose specific diseases or illnesses. Grain feeding (Emmanuel et al., 2008; Ametaj et al., 2009), SARA induction (Gozho et al., 2005; 2006; 2007; Khafipour et al., 2009a), mastitis (Nielsen et al., 2004), complex stress (Lomborg et al., 2008) and LPS infusion (Werling et al., 1996; Jacobsen et al., 2004; Carroll et al., 2009) have resulted in the increase of either, or both, SAA and Hp concentrations. However, APRs are not consistent across all concentrate feeding studies as Hp concentrations did not change in cattle fed grain over a longer period of time (González et al., 2008; Faleiro et al., 2010). The recognition and activation mechanisms of LPS derived from gram-negative bacteria have, for the most part, been described, but not all studies mentioned above have evidence to show that the observed APR was in response to a LPS stimulus. The confounding factors present from one study to another make it difficult to determine the exact causes and the mechanisms initiating the increases in APPs. Further research is warranted to understand the underlying mechanisms associated with the APR.

TABLE 2.2. Putative biological functions of acute phase proteins active in the acute phase response.

Acute Phase Protein	Function	Reference
Serum Amyloid A	Inhibit fever	Shainkin-Kestenbaum et al. (1991)
	Suppress immune responses (*in vitro*)	Benson and Aldo-Benson (1979); Aldo-Benson and Benson (1982)
	Attract monocytes, neutrophils and T lymphocytes to location of inflammation	Badolato et al. (1994); Xu et al. (1995)
Haptoglobin	Binding hemoglobin	Eaton et al. (1982)
	Inhibit bacterial growth	Eaton et al. (1982);
	Regulate immune effect	Murata and Miyamoto (1993)
Fibrinogen	Aid in tissue repair (fibrin formation)	Herrick et al. (1999)
	Intracellular signalling of phagocytes (via NF-κB[1])	Sitrin et al. (1998)
	Promote neutrophil activation and delay apoptosis	Rubel et al. (2001)
Lipopolysaccharide-binding protein (LBP)	Bind to lipopolysaccharide (LPS) with high affinity	Tobias et al. (1989)
	Bind to CD14[2] as LBP-LPS complex	Wright et al. (1990); Hailman et al. (1994)

[1] NF-κB = Nuclear factor – kappa beta; [2]CD14 = Cluster of differentiation 14

Serum amyloid A and Hp have been considered the most responsive and identifiable of the APPs produced by the liver in bovines (Murata et al. 2004). SAA and Hp have been acknowledged as proteins that can be used to highlight different stages of inflammation based upon the production patterns by the liver. In a study monitoring cows six months following parturition, it was suggested that SAA increases during the early stage of inflammation and Hp increases at later stages of inflammation, possibly due to its delayed synthesis rate (Humblet et al., 2006; Jahoor et al., 1996). Also, it has been proposed that acute and chronic inflammation can be differentiated based on increased SAA and Hp concentrations, respectively, in cattle (Alsemgeest et al., 1994). However, Horadagoda et al. (1999) demonstrated that all the cattle diagnosed with acute inflammations had SAA concentrations above reference ranges, and 68 percent of those cattle had increased Hp concentrations. The percentage of cattle with chronic inflammation that had increased concentrations of SAA and Hp, 54 and 24 percent, respectively; was lower than the acute inflammation percentages. However, more cattle had increased SAA concentrations than Hp concentrations in both inflammatory groups. According to Horadagoda et al., (1999), acute inflammation would be diagnosed when both SAA and Hp concentrations are increased, but chronic inflammation would be difficult to diagnose using SAA and Hp concentrations alone. Acute phase proteins do not indicate the specific cause of the inflammatory response, although it may be argued that increases in SAA and Hp do not necessarily indicate inflammation. Lomborg et al., (2008) reported increased APP, SAA and Hp, concentrations as a result of stress associated with 4 to 6 hours of transportation and change in housing facilities (ie, tie-stall,

slippery floors and social isolation). Similarly, two days of travel also resulted in increased Hp concentrations of six-month old calves (Murata and Miyamoto, 1993).

2.5.2 Lipopolysaccharide-binding protein

Lipopolysaccharide-binding protein acts as an APP, increasing rapidly after stimulation by an invader (Tobias et al., 1986; Schroedl et al., 2001). Several in vivo studies in bovines have reported increases in LBP in association with grain feeding (Emmanuel et al., 2008; Ametaj et al., 2009), SARA induction (Khafipour et al., 2009a), intratracheal inoculation of *Mannheimia haemolytica* Type A (Horadagoda et al., 1995; Schroedl et al., 2001), natural and induced clinical mastitis (Bannerman et al., 2003; Zeng et al., 2009). The studies in bovines involving induced mastitis and *M. haemolytica* Type A inoculation, gram-negative bacteria or LPS were putative causatives of increased LBP concentrations. But, in the case of the grain feeding and SARA induction studies it was not certain what caused the increase in LBP concentrations. The increase was assumed to be LPS-associated because concentration of endotoxin increased in the rumen and feces. However, in vitro studies using human cells have shown involvement of LBP in the recognition of gram-positive lipoteichoic acid (LTA) from *Bacillus subtilis*, *Streptococcus pneumoniae* and *Staphylococcus aureus* (Fan et al., 1999; Schröder et al., 2003). With the involvement of LBP for LPS recognition and potentially LTA recognition in bovines, further research is warranted to verify the specificity of LBP in cattle. If LBP were specific, it could be used as a LPS indicator and would be more convenient to measure than plasma LPS.

Although the liver is the primary site of acute phase protein synthesis, tissues elsewhere in the body have tested positive for the presence of LBP. A recent study by Rahman et al. (2010) characterizing the expression, both gene and protein, of LBP in tissues other than the liver in cattle found the presence of LBP throughout the GIT. Messenger RNA expression in the reticulum, rumen and omasum was higher relative to expression in the liver. The relative mRNA expression appeared to decrease the further along the GIT, away from the reticulum. Protein expression of LBP within the gut of the animals was positive but expression was higher in the thyroid gland and mammary gland epithelium. In addition, in vitro studies using gut-associated cell lines from humans and mice have reported the ability of the cells to synthesize and secrete LBP and SAA (Vreugdenhil et al., 1999; 2000). The results of the studies suggest tissues exposed to immune system antagonists, may have to ability to regulate responses locally without the involvement of the liver.

2.6 Physiological, haematological and biochemical changes associated with LPS infusion

In cattle, various studies have infused purified *E. coli* LPS intravenously and monitored the animal's response over the course of several days to understand the immunological response to the peripheral presence of LPS. Lipopolysaccharide was infused intravenously according to body weight (BW; 0.5 to 2.5 μg/kg BW), over a 100 minute period to mimic a realistic systemic infection (Werling et al., 1996; Steiger et al., 1999; Waldron et al., 2003b). However, it can be debated that the model used for infusion studies does not truly mimic systemic infections as exposure time to LPS may not be long

enough, purified LPS has more toxic properties and LPS concentrations used are too high.

Several physiological symptoms have been observed after peripheral LPS infusion. Rectal temperature and respiratory rate, potential physiological indicators of health, both increased shortly after infusion of LPS and then decreased back to initial levels five and three hours after challenge, respectively (Werling et al., 1996; Jacobsen et al., 2005; Carroll et al., 2009). Although blood cortisol levels increased in challenged animals the implication of this increase of cortisol is still unknown as increased cortisol concentrations may act as a stress indicator or may have an anti-inflammatory effect, for example, suppressing the endotoxin response (Andersen 2003; Fantuzzi and Ghezzi, 1993).

Serum amyloid A concentrations in the peripheral blood increased significantly in cattle infused with purified LPS, 0.5 to 2.5 μg / kg BW (Werling et al., 1996; Jacobsen et al., 2004; Carroll et al., 2009). A significant positive correlation between infused-LPS concentrations (10 to 1000 ng/kg BW) and SAA concentrations in the serum has been found in cattle (Jacobsen et al., 2004). Also, Jacobsen et al., (2004) reported significant increases in serum Hp concentrations in challenged cattle; however Werling et al., (1996) did not detect Hp changes. The differences in animals and experimental set-up could explain differences in Hp responses. Jacobsen et al., (2004) used non-pregnant, nonlactating cows infused with increasing LPS concentrations three weeks apart. Werling et al., (1996) used heifers and infused a higher concentration of LPS during a 100-minute time period. The response of APPs after LPS-infusion is important as, function to

eliminate LPS and control the immune response; to advance the body back to homeostasis.

Changes in blood leukocyte count, lymphocyte and neutrophil differentials, have been identified with the infusion of LPS both intravenously and intramammary (Jacobsen et al., 2007; Bannerman et al., 2003). Jacobsen et al. (2007) noted simultaneous changes, including the increase and decrease of lymphocytes and neutrophils, respectively, at 2-6 hours post-injection followed by the inverse response 24 hours post-injection. It was evident that systemic responsiveness was dependent upon the concentration of endotoxin infused (100 vs. 1000 ng/kg BW) and the time elapsed post-infusion. The change in the lymphocyte and neutrophil populations is attributed to the maturation and recruitment of lymphocytes from the lymphatic system into circulation and the attraction of neutrophils to the stimulation site (Deng et al., 2012). This type of response has also been identified with gland post-intramammary injection of 100 μg LPS (Bannerman et al., 2003).

The infusion of LPS into the cattle circulatory system affects blood biochemical and metabolites in addition to physiological and haematological effects. Blood glucose levels tend to increase initially after LPS-infusion and decrease thereafter (Werling et al., 1996; Steiger et al., 1999; Waggoner et al., 2009). These changes in glucose concentrations have been attributed to an increased demand for energy. Consequently, the utilization of glycogen and activation of gluconeogenesis results in increased glucose concentrations which are followed by a period where gluconeogenesis is unable to meet glucose demand (Werling et al., 1996; Waggoner et al., 2009). However, changes in blood glucose concentrations have not been observed in all studies (Waldron et al.,

2003b). Nonetheless, the studies agree that gluconeogenesis is an important process in cattle and potentially sensitive to endotoxins in the bloodstream.

In ruminants nitrogen is required for the growth of microbial protein which is digested and absorbed by the animal. Plasma urea concentrations are affected by the level of protein included in the diet (Preston et al., 1965). Waggoner et al., (2009) analyzed the effects of LPS-infusion on plasma urea concentrations in steers fed different protein levels. LPS treatment (1.5 μg LPS/kg BW) did not significantly affect plasma urea concentrations; however, with increased protein level in the diet, there was an increased urea concentration in the plasma. In addition to urea, several macrominerals have been analyzed in cattle after LPS-infusion. Plasma calcium and phosphorus concentrations decreased after infusing lactating cows with 0.5-1.5 μg LPS / kg BW (Waldron et al., 2003a). Even concentrations as low as 0.1 μg LPS / kg BW can cause decreased plasma calcium concentrations in cattle (Jacobsen et al., 2005). Similar types of responses have been reported in systemic disease and when feeding concentrate-rich diets to cattle (Wenz et al., 2001; Zebeli et al., 2010). The reason for the decrease in calcium and phosphorous concentrations is not well understood. However, increased pro-inflammatory cytokine concentrations have been associated with decreased macrominerals in the plasma of laboratory animals (Waldron et al., 2003a).

Infusions studies have revealed that the presence of LPS at concentrations from 10 ng / kg BW to 2.5 μg / kg BW in peripheral circulation initiates an immune response in cattle. As well, cattle possess the appropriate defence mechanisms to deal with foreign invaders, such as LPS. Throughout the studies, each animal was able to recover from the LPS experimental challenge, as mention previously, the host's response to endotoxin may

be more dependent upon the host's ability to deal with the endotoxin rather than the effect of the endotoxin itself (Hurley, 1995; Andersen, 2003; Jacobsen et al., 2004). Throughout the literature it was evident that cattle were able to deal with endotoxin, however, an immunocompromised animal may be slow or unable to mount the appropriate response. The detection of endotoxin within the peripheral blood of cattle is scarce (Dougherty et al., 1975; Khafipour et al., 2009) a further indication that bovines possess an effective immune system against endotoxins.

2.7 Summary

Many studies to date which have induced cattle with SARA using a grain pellet model or fed cattle a grain-rich diet have found significantly increased concentrations of endotoxins derived from gram-negative bacteria within the rumen as compared to cattle fed a forage-based diet (Gozho et al., 2007; Emmanuel et al., 2008; Khafipour et al., 2009a; Zebeli et al., 2010). In addition, these studies have reported increased concentrations of APPs within the peripheral blood of affected cattle. The explanation for the correlation between increased ruminal endotoxin and APP concentrations is not fully understood. Translocation of the endotoxins across the epithelial barrier has been proposed. Factors such as metabolic and oxidative stress, bacterial overgrowth and intestinal inflammation have been reported to affect the integrity of the epithelial barrier and predispose translocation (Berg 1995; Mani et al., 2012). However, cattle GIT possess protective and defence mechanisms to prevent the animal from bacterial and toxin related injury. As well, bovine leukocytic cells possess endotoxin-associated recognition

receptors on their cell surface to trigger defence and immune pathways to aid in endotoxin elimination.

Aside from grain- and SARA- associated studies, the immunological consequences of gram-negative endotoxins have been reported after infusing LPS, usually isolated from *E. coli*, intravenously into cattle. The systemic presence of LPS increases the concentrations of APPs (Werling et al., 1996; Jacobsen et al., 2007; Carroll et al., 2009), which has given reason to believe systemic presence of ruminal endotoxins causes APR in grain- and SARA- associated studies. However, other physiological changes have also been detected after intravenous infusion of LPS: increased body temperature, respiratory rate, cortisol concentrations and change in blood leukocyte counts (Werling et al., 1996; Steiger et al., 1999; Jacobsen et al., 2007, Carroll et al., 2009). As well, stimulation of peripheral blood leukocytes and IEC with LPS, in vitro, has resulted in changes to gene and protein expressions of LPS-associated recognition receptors: CD14 and TLR4 (Landmann et al., 1996; Abreu et al., 2001; Böcker et al., 2003; Sohn et al., 2007a).

There are noticeable physiological and cellular changes observed in LPS-infused cattle, therefore, if ruminal endotoxins triggered the APR, physiological and cellular changes should be observed within SARA-affected cows. It is important to understand the mechanisms involved with the increase in APP concentrations when cows are induced with SARA using the grain pellet model. By investigating changes to the recognition receptors and down-stream reactions, the mechanisms stimulating the APR can be determined.

2.8 HYPOTHESIS

An acute phase response has been identified with the onset or induction of SARA in cattle. It is believed to be initiated by the presence of intestinally-derived free-lipopolysaccharide in the blood. Therefore it is hypothesized that;

> The presence of putative intestinally-derived free-lipopolysaccharide in the peripheral blood induces immunological systemic changes to peripheral blood immunological parameters. These systemic immunological parameters include rectal temperature, blood cell leukogram, expression of lipopolysaccharide recognition receptors on leukocytic cells, and plasma and serum proteins.

2.9 OBJECTIVES

1. To evaluate the expression of cell surface markers associated with the recognition of lipopolysaccharide on peripheral blood leukocytes and the change in the leukocyte population.
2. To assess the systemic immunological parameters associated with free-lipopolysaccharide in the plasma of cows induced with subacute ruminal acidosis.

3.0 MATERIALS AND METHODS

3.1 Animals, experimental design and diets

Six nonlactating Holstein cows, fitted with a ruminal and caecal cannula, were used in a 3 X 3 Latin Square experimental design. Cow pairs and period made up the columns and rows, respectively, of the Latin square. Cows were paired randomly prior to the study and assigned to 1 of 3 treatments in 3 different time periods. Each experimental period consisted of 4 wks, 3 wks of adaptation and 1 wk of treatment. Data were collected during wk 3 and wk 4 of each experimental period. All cows were fed a total mixed ration control diet (Table 3.1) ad libitum during the adaptation period. During wk 4 of each experimental period, 1 group remained on the control diet while the other 2 groups received 1 of 2 SARA-induction diets, either the API or GPI (Table 3.1). Cows were transitioned from adaptation diet to SARA-induction diets over three days to prevent metabolic complications as a result of the change in diet (ie. rapid drop in ruminal pH). The cows were housed in individual tie-stalls in the 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). Cows were examined by veterinarians weekly to ensure good health. Rectal temperatures were recorded on d 3 and d 5 of wk 3 and wk 4. Feed intake was monitored via GrowSafe System (Airdrie, AB, Canada).

TABLE 3.1. Ingredient and nutrient composition of control diet, alfalfa pellet (API) and grain pellet (GPI) diets used for inducing subacute ruminal acidosis (SARA).

Item	**Control**	**API**	**GPI**
Ingredients, % of DM[1]			
Barley silage	40	33	28
Alfalfa hay	30	-	8
Supplement	30	30	30
Wheat-barley pellets[2]	-	-	34
Alfalfa pellets	-	37	-
Nutrient Composition			
DM, %	54.3	69.0	61.6
CP[3], % of DM	16.1	16.0	16.0
NDF[4], % of DM	35.6	34.5	22.9
ADF[5], % of DM	23.3	22.6	15.2
NFC[6], % of DM[3]	34.8	49.0	50.4
Starch, % of DM	14.2	15.9	33.7
Crude fat, % of DM	3.6	3.2	3.3
Ash, % of DM	9.9	7.3	7.4
Calcium, % of DM	0.85	0.13	0.64
Phosphorus, % of DM	0.35	0.35	0.41
Magnesium, % of DM	0.35	0.30	0.29
Potassium, % of DM	0.21	0.18	1.25
Sodium, % of DM	0.27	0.32	0.28

[1]DM = Dry matter; [2]50% wheat and 50% barley; [3]CP = Crude protein; [4] NDF = Neutral detergent fibre; [5]ADF = Acid detergent fibre [6]NFC = 100 – (NDF % + CP % + crude fat % + ash %)

TABLE 3.2 The nutrient composition (% of DM unless otherwise noted) of dietary ingredients used to formulate diets

Item	**Supplement**	**Barley silage**	**Alfalfa hay**	**Wheat-barley pellets**	**Alfalfa pellets**
DM[1], %	90.5	39.1	86.0	87.0	92.2
CP[2]	18.4	8.8	17.2	144.0	15.5
NDF[3]	14.7	53.3	59.4	16.2	57.9
ADF[4]	7.7	26.9	34.1	7.8	29.6
NFC[5]	56.3	26.7	13.3	61.9	17.9
Starch	35.6	19.5	0.9	48.7	1.5
Crude fat	3.9	2.9	2.3	3.2	2.1
Ash	6.7	8.3	7.8	4.3	6.6
Calcium	1.05	0.48	1.04	0.55	2.81
Phosphorus	0.67	0.29	0.08	0.47	0.14
Potassium	0.80	1.69	1.73	0.19	1.54
Magnesium	0.50	0.20	0.10	0.19	0.32
Sodium	0.53	0.17	0.05	0.35	0.14

[1]DM = Dry matter; [2]CP = Crude protein; [3]NDF = Neutral detergent fibre; [4]ADF = Acid detergent fibre [5]NFC = 100 – (NDF % + CP % + crude fat % + ash %)

3.2 Blood collection

Blood samples were collected from the coccygeal vein on d 3 and d 5 of wk 3 and wk 4 of each period. Blood was collected at 0900 h, before feed delivery, and at 1500 h, 6 hours post-feeding.

3.3 Haematological variable analyses

Blood was collected using one serum separator vacutainer (Corvac, Kendall, ON, Canada) and one K_2EDTA vacutainer (Becton Dickinson, Franklin Lakes, NJ, USA) and blood was analyzed by Manitoba Agricultural, Food and Rural Initiatives Vet Diagnostics Services for haematological parameters (automated haematology analyser, Cell-Dyn 3500 System, Abbott Laboratories, Abbott Park, IL, USA), fibrinogen concentrations (refractometry, Leica TS 400, Leica Microsystems Inc., ON, Canada) and blood chemistry (Vitros 250 Chemistry Analyzer, Ortho-Clinical Diagnostics, Markham, ON, Canada).

3.4 Acute phase protein and plasma lipopolysaccharide analyses

Blood collected in vacutainers containing sodium heparin were centrifuged at 3000 rpm for 15 min to isolate plasma. The extracted plasma was divided into 2 mL aliquots and stored at –20°C until used for plasma LPS, LBP and Hp analysis. Blood collected in serum vacutainers was allowed to clot at room temperature for 45 min before centrifugation at 3000 rpm for 15 min. Serum was separated and stored at –80°C until

used for SAA analysis. Serum amyloid A and Hp concentrations were measured at 1:500 and 1:5 dilutions, respectively, using commercial ELISA kits (TP-802 and TP-801, respectively, Tri-Delta Diagnostics Inc., Morris Plains, NJ, USA) as described by Gozho et al. (2005). Lipopolysaccharide-binding protein concentrations were analyzed using a commercial kit (HK503, HyCult Biotechnology, Uden, The Netherlands). Samples were diluted 1:1000 and analyzed in duplicate at 450 nm using a microplate spectrophotometer (Spectra Max 340 PC, Molecular Devices Corporation, Sunnyvale, CA). Lipopolysaccharide concentration in blood plasma was measured using chromogenic kinetic *Limulus* amebocyte lysate (LAL) assay (Kinetic-QCL, Lonza Group Ltd.) with a detection limit of 0.05 EU/mL. The analytical procedure was conducted as described by Khafipour et al. (2009a).

3.5 Cell preparation and flow cytometry analysis

Blood was collected into vacutainers containing sodium heparin anticoagulant (Becton Dickinson). Peripheral blood mononuclear cells and PMN cells were isolated via density gradient using Ficoll-Paque PLUS (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and red blood cell (RBC) lysis buffer as described by the University of Kansas Medical Center, Flow Cytometry Core Laboratory (Kansas City, KS, USA).

Blood was diluted with an equal volume of 1X phosphate buffered saline (PBS; Hyclone Laboratories, Thermo Scientific, Logan, UT), and then carefully layered on Ficoll-Paque and centrifuged at 2500 rpm for 25 min at 4°C. After centrifugation the

upper supernatant layer was discarded. The buffy coat containing PBMC was extracted and placed in a new microcentrifuge tube. Isolated PBMC were washed three times with 1X PBS then diluted in 1mL of 1X PBS for cell counting. The Ficoll-Paque layer was removed to access the granulocyte and erythrocyte layer. To every 1 mL of granulocyte and erythrocyte layer, 3.5 mL of RBC lysis buffer were added and incubated for 5 min at 37°C, centrifuged at 2000 rpm for 5 min and supernatant discarded. Isolated PMN cells were washed three times with 1X PBS then diluted in 1 mL of 1X PBS for cell counting.

The number of cells in the PBMC and PMN populations was determined using trypan blue staining (Gibco, Invitrogen Canada Inc., Burlington, ON, Canada). After counting, $1x10^6$ cells per 100 μL were placed into each well of a 96-well plate for antibody staining. Lipopolysaccharide-stimulated PBMC and PMN cells were included as a positive control. These cells were stimulated with 1000 EU/mL of purified *E. coli* O111:B4 LPS (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 1 hour at 37°C. The remaining cells were used for total ribonucleic acid (tRNA) extraction, as described later.

Anti-bovine monoclonal antibodies, against CD14 and CD11a (AbD Serotec, Raleigh, NC, USA) were used. Antibody CD11a was used to distinguish the desired cell populations (i.e. granulocytes, lymphocytes, monocytes and macrophages). Cells were labelled with monoclonal primary or isotype control antibodies at a 1:100 dilution. Labelled cells were incubated in the dark at 4°C for 30 minutes, and then centrifuged at 2000 rpm for 6 minutes. Cells were washed twice with 1X PBS to remove any unbound antibodies. Cells were fixated in one percent buffered-formalin, and left at 4°C overnight. For each sample 100,000 cells were run through a BD FACSCalibur flow cytometer

(Becton Dickinson) and analyzed for CD14 expression using Flowjo flow cytometry analysis software (v.7.5, Tree Star, Inc., Ashland, OR, USA) and figures were developed using Cyflogic software (v.1.2.1, CyFlo Ltd., Turku, Finland). Positive controls incubated with LPS were also observed for cell size and granularity using forward and side scatter channels, respectively.

3.6 RNA extraction and reverse transcription

Allocations of cells were left in 500 μL of RNAlater (Ambion, Applied Biosystems, Foster City, CA) at 4°C overnight. The cells were then centrifuged to remove the RNAlater and stored at –80°C until use. Total RNA was extracted the same week the samples were collected using TRI Reagent Solution following the manufacturer's protocol (Ambion, Applied Biosystems). The RNA pellets were resuspended in RNase/DNase free water (Invitrogen Canada Inc.) and stored at –80°C. Ten microliters of RNA was converted into complementary deoxyribonucleic acid (cDNA) using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems) as per the manufacturer's protocol. Complementary DNA was then stored at –80°C until quantitative real-time polymerase chain reaction (qRT PCR) analysis.

3.7 Relative quantification real-time polymerase chain reaction and analyses

The gene expression of TLR4 and MD2 were analyzed and β-actin was included as an endogenous control. The primers used for relative qRT PCR and for cloning

plasmid controls were designed with Primer Premier 5 Software (Premier Biosoft, Palo Alto, CA) and synthesized by University Core DNA Services (University of Calgary, Calgary, AB, Canada). The list of designed primers is located in Table 3.3. The National Center for Biotechnology Information (NCBI) GenBank was used to identify bovine mRNA sequences of β-actin, TLR4 and MD2 and NCBI BLASTN was used to verify the specificity of the primers.

Step One Real-Time PCR System (Applied Biosystems) and SYBR Green I dye chemistry were used for relative quantification of gene expression. SYBR green PCR Master Mix (12.5 μL; Applied Biosystems), 2 μL primers, 2 μL diluted cDNA template and 8.5 μL water were added into each well. All primers were standardized to find the optimal concentration to create a linear relationship between cycle threshold (Ct) value and the logarithm of cDNA amount (slope of -3.3 optimal). A concentration of 700 nM was used for the β-actin primer and 300 nM was used for TLR4 and MD2 primers. All samples were initially diluted to 1/25. If the cycle threshold (Ct) value was too low, samples were further diluted to get a Ct value between 15 and 30. A plasmid template of each gene was included as a control. Template-free wells were also included. All genes were run on the same plate in duplicate. The running settings were as follows; 95°C for 10 min, and 45 cycles of 95°C for 15 sec, 55°C for 30 sec and 60°C for 1 min. This was followed by a three step melt curve. Relative quantification of gene expression was calculated using an equation described by Pfaffl (2001).

3.8 Gene cloning and plasmid control preparation

Plasmids were prepared for each of the genes analyzed via qRT PCR and used for primer standardization, as well as a control in qRT PCR data analysis. Partial-length, indicated in Table 3.3, coding sequences of TLR4, MD2 and β-actin were amplified from PBMC cDNA using PCR Master Mix Kit (Promega Corporation, Fisher Scientific, Nepean, ON, Canada) and a 2720 Thermo Cycler (Applied Biosystems).

The PCR thermal profile for TLR4 and MD2 was initiated by a 1 min denaturation step at 94°C, followed by 35 cycles consisting of 1 min denaturation at 94°C, 45 sec annealing at 60°C and 45 sec elongation at 72°C. A final 10 min extension step was included at 72°C followed by a reduction in the temperature to 4°C. The thermal profile for β-actin started with a 1 min denaturation at 94°C, followed by 40 cycles consisting of 1 min denaturation at 94°C, 1 min annealing at 57°C and 1 min elongation at 72°C.

A final 15 min extension step was included at 72°C followed by a reduction in the temperature to 4°C. The PCR amplicons were then isolated on a 1.5% agarose gel, stained with ethidium bromide and purified using Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Fisher Scientific). The purified PCR product was ligated into a pGEM-T vector (Promega Corporation, Fisher Scientific) according to the manufacturer's protocol and transformed into *E. coli* competent cells (JM109; Promega Corporation, Fisher Scientific). Competent cells were added to ligated product, left on ice for 20 min, heat-shocked at 42°C for 45 sec and then put back on ice.

TABLE 3.3. Sequences of oligonucleotic primers used for plasmid inserts and relative quantification real-time polymerase chase reaction (qRT PCR) during the study.

Gene	Sense Primers (5' to 3')	Antisense Primers (5' to 3')	Size[1]	Accession	Purpose
TLR4[2]	CTGATCCCAGCCACGGCCAT	CCAAGTGCTCCAGGTTGGGCA	511	NM_174198	Plasmid insert
	CCATATCAACCAAGATGCTGGACC	CTGGATAGGGTTTCCCGTCAG	188	NM_174198	qRT-PCR
MD2[3]	GGGTCTGCAACTCCTCTGAC	CAGTGTTCCCCTCGATGGCT	360	DQ319076	Plasmid insert
	GCAACTCCTCTGACGCCAC	GAGTATTCCACGGCTTCCCT	110	DQ319076	qRT-PCR
β-actin	TCGACAACGGCTCCGGCATG	GCATTTGCGGTGGACGATGGAG	1094	NM_173979	Plasmid insert
	GTGCCCATCTATGAGGGGTACG	GTGGTGAAGCTGTAGCCACGCT	116	NM_173979	qRT-PCR

[1]Size indicates number of base pairs; [2]TLR4 = Toll-like receptor 4; [3]MD2 =Myeloid of differentiation 2

The transformed cells were plated on Luria-Bertani agar plates containing 50 µg/mL ampicillin, 100 m*M* isopropyl thiogalactoside and 20 mg/mL 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-gal) and left at 37°C overnight. Five putative colonies were then picked and transferred to liquid Terrific Broth media and incubated overnight at 37°C.

A basic alkaline minipreparation procedure was used to isolate plasmid DNA (Sambrook and Russell, 2001). Cells were centrifuged, supernatant discarded and pellets resuspended in 50 µL Tris-EDTA buffer containing 20 µg/mL of RNase A. The plasmid DNA concentration was determined at 260 nm (DU800 Spectrophotometer, Beckman Coulter Canada Inc., Mississauga, Ontario, Canada). Restriction enzyme digestion (BstZ1; Promega Corporation, Fisher Scientific) and PCR amplification with gene specific primers (Table 3.3) were used to verify gene insert in vector. Plasmids were stored at – 20°C.

3.9 Statistical analysis

Haematological parameters, flow cytometry and qRT PCR data were analyzed using a mixed model (Proc Mixed, SAS 9.1) as indicated below;

$$Y_{jklm} = \mu + c_j + p_k + t_l + e_{jkl} + d_m + e_{jklm}$$

Y_{jklm} = observation
μ = overall mean
c_j = mean effect of cow, j
p_k = mean effect of period, k
t_l = mean effect of treatment, l
e_{jkl} = whole plot error (defined as c*p*t interaction)
d_m = mean effect of day, m
e_{jklm} = residual error (defined as c*p*t*d interaction)

Metabolically associated blood chemistry parameters and APPs were analyzed using the following model;

$$Y_{jklmn} = \mu + c_j + p_k + t_l + e_{jkl} + d_m + e_{jklm} + s_n + s_n*t_l + e_{jklmn}$$

Y_{jklmn} = observation
μ = overall mean
c_j = mean effect of cow, j
p_k = mean effect of period, k
t_l = mean effect of treatment, l
e_{jkl} = whole plot error (defined as c*p*t interaction)
d_m = mean effect of day, m
e_{jklm} = subplot error (defined as c*p*t*d interaction)
s_n = mean effect of time of sampling, n
s_n*t_l = interaction of time of sampling, n, and treatment, l
e_{jklmn} = residual error defined as c*p*t*d*s

The mixed models included diet, period, time of day (TOD) and diet-TOD interaction as fixed variables. Animal and day were included as random variables. Covariance was also included in the model as a fixed variable for total protein, globulin and globulin to albumin ratio, gamma glutamyltransferase and APP analyses. Covariance was included if treatment means and slopes were similar and regression across treatments was greater than 0.75. Day and diet–day interactions were also included with qRT PCR analyses. Some parameters including white blood cell count, total neutrophils, lymphocytes, neutrophil to lymphocyte ratio, qRT PCR data, APPs were $\log_{10}$-transformed before analysis to meet analysis of variance (ANOVA) assumption for normal distribution of residual error. Tukey's multiple range test was used to identify differences between least square treatment means and Satterthwaite test was used to account for unequal variance between treatments. Effects were considered to be significant at $P < 0.05$. Data collected from one cow induced with alfalfa pellet SARA

during period one were omitted from blood cell differential and APP analyses due to clinical mastitis. For remaining parameters data was included as values did not have an effect on the significance of variables.

4.0 RESULTS

Subacute ruminal acidosis was induced using both the grain pellet and alfalfa pellet diet models based upon the criteria outlined by Gozho et al. (2005) with ruminal pH below 5.6 for greater than 3 hours per day. The induction of SARA resulted in increased duration of ruminal pH below 5.6 in API and GPI SARA, 225.2 and 298.7 min/d, respectively, as compared to control, 56.4 min/d. The concentrations of free-endotoxins were significantly increased in the rumen fluid in both SARA induction treatments in comparison to the control treatment, as described by Li et al. (2012). Endotoxins were not detected in the plasma when dry cows were either on the control treatment or on SARA induction treatments (Table 4.1).

4.1 LPS-associated recognition receptors

The expression of endotoxin-related recognition receptors did not change with the induction of SARA. The percentage of cells expressing CD14 on the cell surface did not differ between the SARA-induced treatments and control treatment (Fig. 4.1). The expression of CD14 was detected on the surface of PBMC but not on PMN cells. The expression of CD14 on LPS-stimulated PBMC, used as the positive control, was down-regulated as compared to control cells (Fig. 4.2). The down-regulation of CD14 was accompanied by a change in cell morphology based on the cell size (forward scatter) and granularity (side scatter). Both forward and side scatter of the CD14-positive population of cells expanded after LPS-stimulation indicating cell differentiation (Fig. 4.3).

TABLE 4.1. Immunologically-related concentrations and ratios of selected proteins from nonlactating dairy cows fed a control diet or induced with SARA[1] using the grain pellet- or alfalfa pellet- based diet models.

	Treatment				Effect, *P*-value		
Item	**Control**	**Grain pellet**	**Alfalfa pellet**	**SEM[2]**	**Diet**	**Period**	**TOD[3]**
Plasma LPS[4] (EU/mL)	ND[5]	ND	ND	---	---	---	---
LBP[6], mg/L[7]	8.36[b]	12.98[a]	9.34[ab]	1.13	0.003	0.03	0.31
Total Protein, g/L	81.77[b]	78.29[a]	80.83[ab]	0.77	0.02	0.39	---
Fibrinogen, g/L	6.67	6.08	6.92	0.44	0.17	0.04	---
Albumin, g/L	29.50	28.20	28.58	0.40	0.06	0.05	---
Globulin, g/L	52.27	50.61	52.32	0.64	0.09	0.43	---
Serum Amyloid A, mg/L[7]	38.39[b]	62.59[a]	35.64[b]	1.12	0.002	0.12	0.71
Haptoglobin, mg/L[7]	54.43	83.54	72.29	1.21	0.16	0.15	0.02

[a, b]Means within a row with different subscript letters differed ($P \leq 0.05$)

[1]SARA = Subacute ruminal acidosis; [2]SEM = Standard error of means; [3]TOD = Time of day, AM vs PM; [4]LPS = Lipopolysaccharide; [5]ND = Not detected; [6]LBP = Lipopolysaccharide-binding protein; [7]Statistical analyses were conducted on $\log_{10}$–transformed data

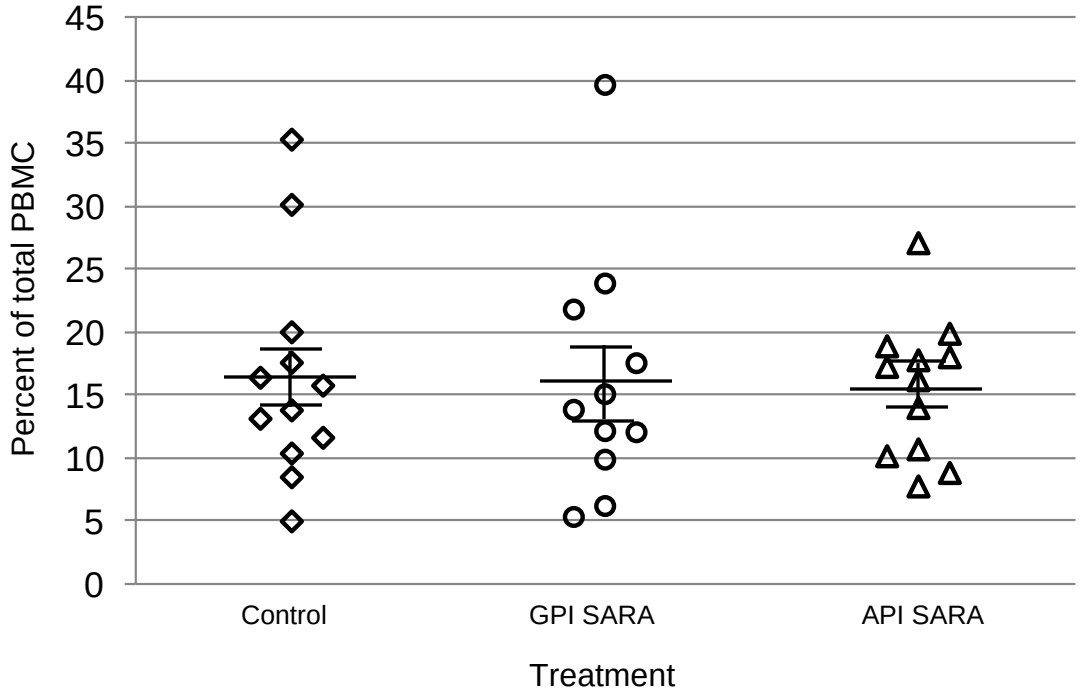

Figure 4.1 The percentage of peripheral blood mononuclear cells (PBMC) expressing CD14 on the cell surface after induction of SARA using grain pellet-induced (GPI) and alfalfa pellet-induced (API) models (mean and SEM are shown as horizontal lines). PBMC were isolated from blood collected six hours post-feeding. Statistical analysis completed using $\log_{10}$-transformed data. No significant differences were observed (P = 0.85).

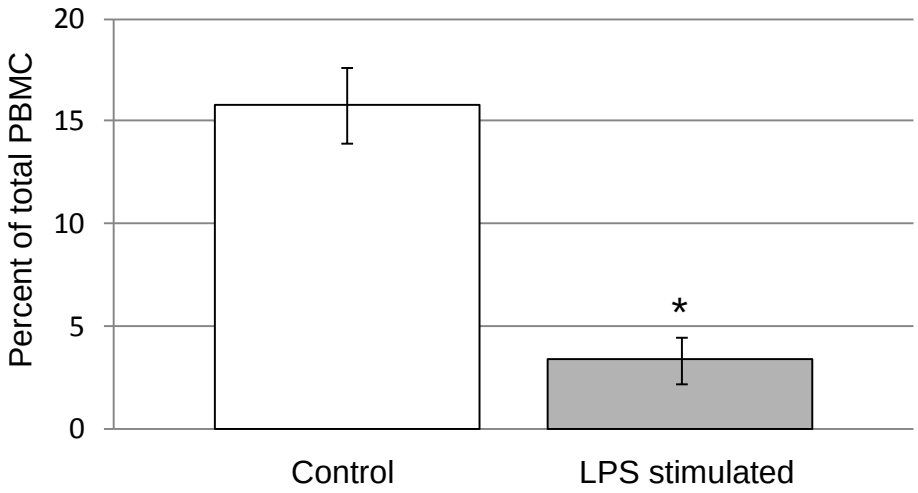

Figure 4.2 The percentage of peripheral blood mononuclear cells (PBMC) expressing CD14 on the cell surface after stimulation with 1000 EU/mL *Escherichia coli* O111:B4 LPS for 1-hr at 37ºC (n=10). *Treatment effect $P < 0.05$. Statistical analysis using paired Student's t-test.

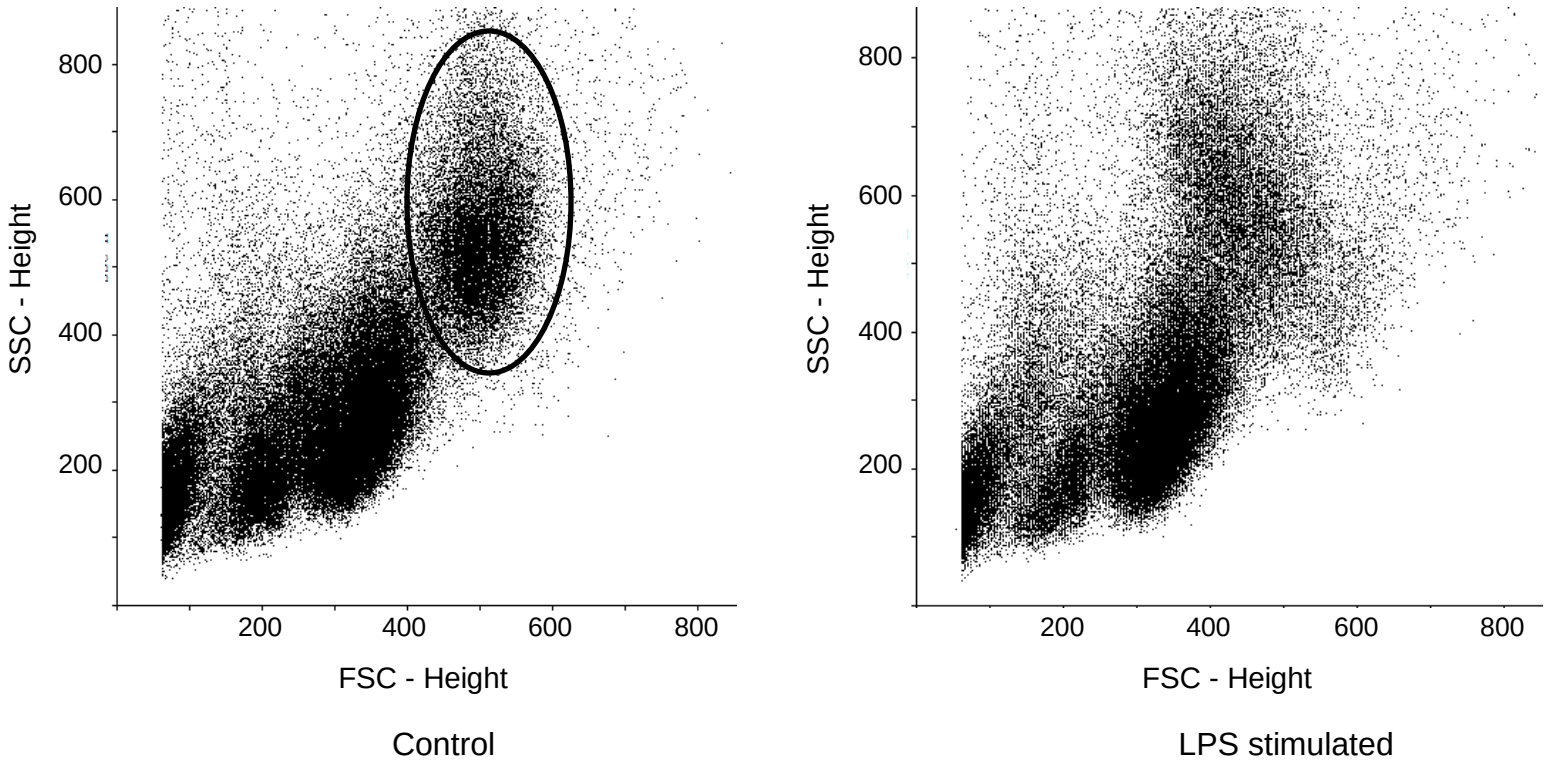

Figure 4.3 The morphological changes of CD14+ cells (circled) after stimulation with 1000 EU/mL *Escherichia coli* O111:B4 LPS for 1-hr at 37ºC. SSC: side scatter channel displays the granular content of a cell. FSC: forward scatter channel displays the size of a cell.

MD2 and TLR4 gene expression was detected in most PBMC and PMN samples. All samples expressed β-actin. Relative gene expression did not differ ($P > 0.05$) between each of the three treatments for either target gene (Fig. 4.4).

4.2 Plasma proteins and acute phase proteins

Changes in APPs were observed in cows induced with SARA using the grain pellet diet model but not the alfalfa pellet diet model (Table 4.1). Concentrations of serum LBP, SAA and Hp increased in the blood of cows induced with SARA using the grain pellet diet in comparison to control, but only LBP and SAA were statistically significant ($P < 0.01$). Serum amyloid A increased from 38.39 to 62.59 mg/L from control to GPI SARA, respectively. Plasma concentrations of LBP differed from control and GPI SARA, 8.36 verses 12.98 mg/L, respectively. Fibrinogen concentrations decreased in GPI SARA but were not significantly different from the control diet ($P > 0.05$). The concentrations of LBP, SAA, Hp and fibrinogen of cows induced with alfalfa pellet SARA were similar to the concentrations observed when fed control diet and were not statistically different ($P > 0.05$; Table 4.1).

Inducing cows with SARA using the grain pellet model resulted in a significant reduction in total protein concentrations in comparison to control (Table 4.1). Total protein concentrations decreased from 81.77 g/L in control to 78.29 g/L in GPI SARA treatment. In addition to decreased total protein concentrations, albumin and globulin concentrations tended ($P = 0.06$ and $P = 0.09$, respectively) to be reduced with GPI SARA.

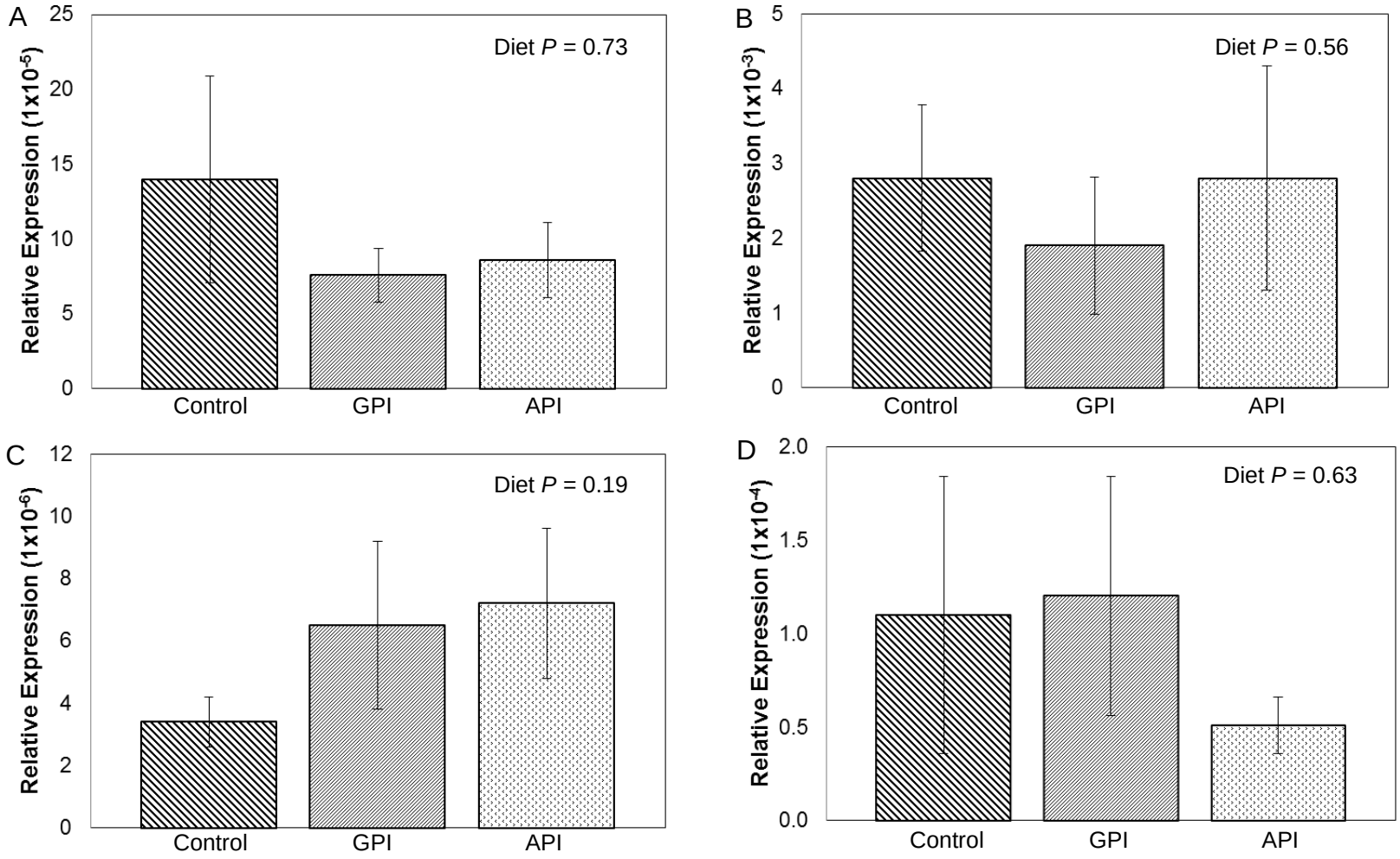

Figure 4.4 The relative expression of A) TLR4 in PMN cells B) MD2 in PMN cells C) TLR4 in PBMC D) MD2 in PBMC of nonlactating dairy cows after induction of SARA using grain pellet-induced (GPI) and alfalfa pellet-induced (API) models. Statistical analysis completed using $\log_{10}$-transformed data. Blood cells were isolated from blood collected 6-hr post-feeding.

However, significant differences were not found in cows induced with SARA using the alfalfa pellet model in comparison to control. Period had a significant effect on fibrinogen and albumin (Table 4.1). Albumin concentrations increased significantly from period 1 to period 3 ($P = 0.05$). Conversely, fibrinogen decreased significantly from period one to period three ($P < 0.05$). The total protein to fibrinogen ratios in both SARA induction treatments were reduced in comparison to the ratio of the control treatment, but were not significant (Table 4.2). The ratio of albumin to globulin, used to evaluate infection status, was similar between all three treatments (Table 4.2).

4.3 Rectal temperature, blood haematology and blood chemistry

Inflammatory and health indicators were examined to see if a systemic immune response could be identified. SARA induction did not have an effect on rectal temperatures which were similar across all three treatments (Table 4.2). White blood cell differentials of cows induced with SARA did not differ significantly from cows remaining on the control diet (Table 4.3). The ratio of neutrophil percentage to lymphocytes percentage of cows induced with the alfalfa pellet diet or the grain pellet diet were not found to be significantly different from the cows on the control diet (Table 4.2).

Blood chemistry parameters, metabolites, enzymes and plasma minerals, were identified in response to SARA induction (Table 4.4).

TABLE 4.2. Immunological parameters related to health status, systemic infection and inflammation of nonlactating dairy cows fed a control diet or induced with SARA[1] using the grain pellet- or alfalfa pellet- based diet models.

	Treatment				Effect, *P*-value	
Item	**Control**	**Grain pellet**	**Alfalfa pellet**	**SEM[2]**	**Diet**	**Period**
Temperature (°C)	38.40	38.37	38.42	0.07	0.90	0.01
TP[3] to Fibrinogen Ratio	12.82	12.65	11.59	0.68	0.23	0.08
Albumin to Globulin Ratio	0.57	0.56	0.55	0.007	0.39	0.09
Neutrophil to Lymphocyte Ratio[4]	0.77	0.77	0.76	1.16	1.00	0.45

[1]SARA = Subacute ruminal acidosis; [2]SEM = Standard error of means; [3]TP = Total protein; [4]Statistical analyses were conducted on $\log_{10}$–transformed data

TABLE 4.3. Blood cell differentials of nonlactating dairy cows fed a control diet or induced with SARA[1] using the grain pellet- or alfalfa pellet- based diet models.

	Treatment				Effect, *P*-value	
Item	**Control**	**Grain pellet**	**Alfalfa pellet**	**SEM[2]**	**Diet**	**Period**
White Blood Cells ($x10^9$/L)[4]	8.08	7.57	7.53	1.13	0.23	0.03
Total Neutrophils[3], %[4]	38.36	36.22	38.18	1.08	0.70	0.13
Lymphocytes, %[4]	49.82	49.27	49.79	1.08	0.98	0.74
Monocytes, %	5.83	5.92	4.88	0.83	0.61	0.64
Basophils, %	0.75	0.42	0.53	0.22	0.50	0.18
Eosinophils, %	3.08	4.97	3.69	0.85	0.28	0.22

[1]SARA = Subacute ruminal acidosis; [2]SEM = Standard error of means; [3]Total Neutrophils includes segmented and banded;
[4]Statistical analyses were conducted on $\log_{10}$–transformed data

TABLE 4.4. Blood chemistry of nonlactating dairy cows fed a control diet or induced with SARA[1] using the grain pellet- or alfalfa pellet- based diet models.

	Treatment				Effect, *P*-value			
Item	**Control**	**Grain pellet**	**Alfalfa pellet**	**SEM[2]**	**Diet**	**Period**	**TOD[3]**	**Diet*TOD**
Carbohydrates								
Glucose, mmol/L	3.96[b]	4.19[a]	3.81[b]	0.10	<0.01	<0.01	0.03	0.88
Renal Function								
Urea, mmol/L	5.51[b]	4.62[a]	5.13[b]	0.31	<0.01	0.17	0.09	<0.01
Creatinine, mmol/L	72.58	69.76	71.33	2.77	0.10	<0.01	<0.01	0.43
Liver Function								
CK[4], U/L	78.89	75.25	73.22	9.56	0.90	0.90	0.03	0.51
GGT[5], U/L	47.78	47.97	46.94	0.87	0.60	0.61	0.44	0.81
AST[6], U/L	85.17	95.56	75.96	10.26	0.13	0.76	0.05	0.57
Endocrine Function								
Calcium, mmol/L	2.35[b]	2.21[a]	2.32[b]	0.03	<0.01	0.81	0.71	0.81
Magnesium, mmol/L	0.86[b]	0.97[a]	0.82[b]	0.04	<0.01	0.79	<0.01	0.30
Phosphorus, mmol/L	2.25	2.21	2.14	0.10	0.76	0.15	<0.01	0.01
Electrolytes								
Sodium, mmol/L	138.17	137.61	137.37	0.45	0.44	0.05	<0.01	0.87
Potassium, mmol/L	4.53	4.45	4.43	0.11	0.37	0.29	<0.01	0.11
Chloride, mmol/L	100.75[b]	102.30[a]	101.65[ab]	0.61	0.04	0.10	<0.01	<0.01

[a, b]Means within a row with different subscript letters differed ($P \leq 0.05$)
[1]SARA = Subacute ruminal acidosis; [2]SEM = Standard error of means; [3]TOD = Time of day, AM vs PM; [4]CK = Creatine kinase; [5]GGT = Gamma-glutamyl transferase; [6]AST = Aspartate aminotransferase

Blood glucose concentrations were significantly higher ($P < 0.05$) in GPI SARA cows than cows induced using the alfalfa pellet diet and cows fed the control diet. Grain pellet-induced SARA significantly decreased ($P < 0.05$) blood urea concentrations in comparison to control diet, while API SARA did not have an effect on urea concentrations. Creatinine concentrations were reduced with SARA induction but were not statistically significant ($P > 0.05$). Glucose and creatinine concentrations decreased significantly from AM sampling to PM sampling. A diet and time of sampling interaction was found to be significant for urea ($P < 0.05$). Differences in diet and time of sampling interactions for urea were mainly in association with control and GPI SARA or API and GPI SARA. There were no significant differences in liver and muscle enzyme concentrations for aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) and creatine kinase (CK) upon SARA induction compared to control treatment. Time of day, AM verses PM, had a significant effect on CK and AST, increasing during the course of the day.

Plasma minerals calcium, magnesium, and chloride were significantly ($P < 0.05$) affected by GPI SARA, while phosphorus, sodium, and potassium, were not (Table 4.4). Calcium was reduced from 2.35 mmol/L in the control to 2.21 mmol/L in GPI SARA cows. Magnesium concentrations increased from 0.86 mmol/L in control to 0.97 mmol/L in GPI SARA. Plasma mineral concentrations in cows fed the alfalfa pellet diet remained similar to cows fed the control diet. Time of day, before feeding and six hours post-feeding, had a significant effect on all plasma minerals, except calcium, when all treatment samples were pooled together. Diet and time of sampling interaction was

significant for phosphorus and chloride ($P < 0.05$), however, the difference was only detected between API SARA AM and PM for phosphorus. For chloride, control AM concentrations were lower and therefore were significant when compared to all other diets and time of sampling values.

5.0 DISCUSSION

The induction of SARA as a result of feeding alfalfa pellet- and grain pellet-based diets was successful according to the definition used by Gozho et al. (2005). The daily reduction in ruminal pH is a prominent characteristic of SARA, remaining below pH 5.6 for more than 180 min/d. The significant increase in ruminal endotoxins in association with feeding high-grain diets (Nagaraja et al., 1978b; Andersen et al., 1994; Emmanuel et al., 2008) and induction of SARA (Gozho et al., 2007; Khafipour et al., 2009a; 2009b) have been clearly established. Similarly, in this study the induction of SARA using either the grain-based or alfalfa-based diet models found multiple-fold increases, 16-fold and 3 fold, respectively, in ruminal endotoxin concentrations (Li et al., 2012). The observed increase in ruminal endotoxins has been the focus of previous studies examining the APR as endotoxin, specifically the lipid A region, is capable of initiating an immune response (Niemetz and Morrison, 1977; Galanos et al., 1985). The increase of endotoxin concentrations, together with other factors such as reduced ruminal pH and increased ruminal osmolality may affect the GIT barrier integrity and could have further ramifications on the animal stimulating either a local inflammatory response within the gut or a systemic inflammatory response.

Although increased concentrations of endotoxins have been reported in various regions of the GIT, however endotoxins have rarely been detected beyond the GIT. The concentration of endotoxins in the plasma was analyzed in this study, but not detected in control or SARA-induced cows. Gozho et al., (2007) also did not detect endotoxins in the plasma; however, Khafipour et al. (2009a) reported the presence of endotoxins in the

plasma of GPI SARA cows. The presence of endotoxin in the peripheral plasma was assumed to be translocated from the GIT (Khafipour et al., 2009a). In other studies which have reported the detection of endotoxins in the plasma, the animals were under acute experimental conditions (Dougherty et al., 1975). Many studies feeding starch-rich diets which have reported increased concentrations of ruminal endotoxins have not analyzed plasma for the presence of endotoxins. Metabolic stress, oxidative stress, intestinal inflammation, and bacterial overgrowth have been reported to reduce the integrity of epithelial barrier, causing it to be susceptible to translocation of luminal contents (Berg 1995; Hietbrink et al., 2009; Mani et al., 2012). Ruminal changes (i.e. pH, microbe population, endotoxin concentration, and VFA composition) have consistently been reported after SARA induction and feeding of grain-rich diets, potentially predisposing the epithelial barrier to adverse changes.

Several studies have observed compromised rumen papillae and epithelia after a bout of ruminal acidosis or feeding a pelleted diet (Nocek and Kesler, 1980; Steele et al., 2009). The animals used in these studies were exposed to acute ruminal acidosis which is a more severe condition then SARA. In a study inducing sheep with a single dose of SARA, the rumen epithelial integrity was not affected; however, exposing rumen epithelia to a lower pH in vitro, following SARA, increased the permeability of ^{3}H-mannitol across the rumen epithelia (Penner et al., 2010). Mild and short exposure to reduced pH levels (i.e. pH 5.6 for less than 180 min) was not enough to cause damage to epithelial integrity; however, consecutive exposures to greater reduced pH levels (i.e. pH 5.2) could have a greater negative effect on epithelial integrity (Penner et al., 2010).

Therefore, the induction of SARA within this study may not have been severe enough to cause epithelial damage and the detection of endotoxins in the plasma.

Although endotoxins were not detected in the plasma, the concentration of LBP significantly increased in GPI SARA cows as compared to cows in the control treatment. Increased LBP concentrations have been reported in cattle fed high-grain diets and SARA-induced cows (Emmanuel et al., 2008; Ametaj et al., 2009; Khafipour et al., 2009a). An important biological role of LBP is to bind free-LPS and subsequently, be recognized by CD14 to initiate leukocyte activation via TLR4 and MD2 (Wright et al., 1990). In the case that plasma LPS was bound to LBP there might be evidence of activity of endotoxin-related receptors, such as CD14, TLR4 and MD2 on peripheral blood cells.

In PBMC positive controls, incubated with LPS (1000 EU/mL) isolated from *E. coli* O111:B4 for 1 hour at 37˚C, resulted in down-regulation in the percentage of cells expressing CD14. Despite the down-regulation of CD14 in positive control samples, the expression of CD14 on the cell surface of PBMC from GPI and API SARA treatments did not differ from control treatment. Similar results to the PBMC positive controls have been reported in bovine PMN cells (Sohn et al., 2007a) and in human monocytic cells several hours after stimulation with LPS (Bažil and Strominger, 1991; Landmann et al., 1996). These studies also detected an increase in the concentration of soluble CD14 in the supernatant, suggesting the down-regulation in membrane CD14 may be associated the shedding of CD14 from the outer membrane (Bažil and Strominger, 1991; Landmann et al., 1996; Sohn et al., 2007a). The increase in size and granularity of monocyte-derived macrophages in comparison to monocytes was observed by Guo et al., (2009) when monocytes were allowed to differentiate. Therefore, the down-regulation of mCD14 on

PBMC after incubation with LPS would signify the differentiation or maturation of monocytes into macrophages to strengthen immunological activity. The down-regulation of CD14 may be the result of shedding mCD14 from the outer membrane but is more likely to be the result of differentiation of monocytes to macrophages, based on the morphology observations. Similar expression levels of CD14 on PBMC of SARA-induced and control treatments denote that PBMC did not encounter endotoxins in the plasma after SARA induction.

Comparable with the results from CD14 analysis, SARA induction, compared to control, did not significantly affect the relative gene expression of TLR4 and MD2 in PBMC and PMN cells. These results are consist with in vitro studies which found that LPS, 100 - 1000 ng/mL, and other TLR agonists (i.e. LTA, peptidoglycan) did not affect the expression of TLR4 mRNA in bovine peripheral blood cells (Franchini et al., 2006; Guo et al., 2009). However, in human peripheral blood leukocytes, minute concentrations of LPS, 10 ng/mL, resulted in the up-regulation of TLR4 mRNA expression (Muzio et al., 2000). The authors of these studies suggested that bovine peripheral blood cells are more stable to TLR4 regulation than human cells and require a more rigorous stimulus or other factors, such as cytokines, to regulate the TLR4 gene (Abreu et al., 2002; Divanovic et al., 2007). Inhibitors of TLR4 signalling have also been recognized and it could be suggested that the inhibitors are more prominent in bovines than in humans (Liew et al., 2005).

The current study focused on the primary pathway for LPS recognition and cell activation. However, other proteins, such as albumin, high and low density lipoproteins and lactoferrin have shown to have LPS-binding ability and aid in the clearance of

endotoxins (Reviewed in Andra et al., 2009). A study by Cavaillon et al. (1990) showed that LPS bound to a low density lipoprotein mounted a weaker immune response, assessed by cytokine production, than free-LPS. This suggests that lipoproteins have the ability to reduce the endotoxic effect of LPS, preventing the binding of LPS to recognition receptors and ultimately inhibiting the stimulation of an inflammatory response. The presence of other LPS recognition mechanisms could help explain why changes were not found in association with the TLR4-MD2-CD14 pathway after the induction of SARA. Whatever the case may be, cytokines synthesized by activated leukocytes are responsible for initiating further immunological responses, such as the synthesis of APPs.

The increase of APPs have been reported upon the induction of grain pellet SARA and feeding concentrate-rich diets to dairy cattle (Gozho et al., 2007; Emmanuel et al., 2008; Khafipour et al., 2009a). Our results exhibited significant increases in SAA, but not Hp concentrations, upon GPI SARA as compared to control treatment. The possible cause of the APR under such conditions is not known, however, APPs possess biological functions involved with controlling the immunological response. The differences in biological roles could explain the increase of SAA but not Hp. Serum amyloid A functions by inhibiting fever stimulated by pro-inflammatory cytokines, enhancing migration of leukocytes to the site of inflammation and suppressing *in vitro* immune responses (Shainkin-Kestenbaum et al., 1991; Badolato et al., 1994; Benson and Aldo-Benson, 1979; Aldo-Benson and Benson, 1982), suggesting that a probable pro-inflammatory response may have occurred in cows on GPI SARA treatment. Also, the presence of SAA in the blood would serve to control increases in body temperature and

other immune response parameters in GPI SARA cows. Haptoglobin, which was present in all treatments, is involved with free-haemoglobin binding, inhibiting bacterial growth and regulating immunological effects such as suppressing lymphocyte differentiation in response to stimulus (Eaton et al., 1982; Murata and Miyamoto, 1993). Furthermore, SAA has been effective in diagnosing acute or early inflammation **(**Horadagoda et al., 1999; Humblet et al., 2006). According to the APR results of the current study, early stages of inflammation or an acute inflammatory response may have been present in cows induced with SARA using the grain pellet model. However, it is not possible to determine the location of the inflammation or to diagnose it specifically without further investigation.

Intestinal epithelial cells have the ability to synthesize and secrete pro-inflammatory cytokines and stimulate local production of APPs (Vreugdenhil et al., 1999; 2000; Rahman et al., 2010). Therefore, the increase in SAA and LBP detected in the plasma of GPI-SARA could be as a result of a localized inflammation within the GIT. Rahman et al., (2010) reported that the LBP gene was expressed in the epithelial lining throughout the GIT of bovines. Such a finding is imperative to understanding the protective capacity of the GIT; considering the presence of bacteria, particularly gram-negative within the rumen, as well as hind gut. Increased LBP concentrations have also been reported after recognition of LTA from the outer cell wall of various gram-positive bacteria (Fan et al., 1999; Schröder et al., 2003). The extensive presence of GALT and lymphoidic cells within the gut lining together with the IEC ability to synthesize APPs suggests that there is the capacity of tissues to regulate immunological responses without the involvement of the liver.

The concentrations of SAA, Hp and LBP reported in the current study were lower in the GPI SARA induced cows than in other studies. However, one study found the concentrations of plasma LPS increased from undetectable in control to 0.81 EU/mL 12 hours post-feeding in cows induced with the grain-pellet diet (Khafipour et al., 2009a). The presence of *E. coli* LPS in the blood of bovines initiates a strong APR, as proven in various LPS-infusion studies (Jacobsen et al., 2004; Carroll et al., 2009). A further distinction between the studies, the cows used for this study were not lactating whereas those used by Khafipour et al., (2009a; 2009b) and Gozho et al., (2007) were two to four months into lactation. Production demands create negative energy and protein balances, especially during early lactation, which results in metabolic problems and weakened immune responses which affect the well-being of the animal (Goff, 2006; Kehrli et al., 2008; Elsasser et al., 2000). A weaker APR would be expected in lactating cows compared to nonlactating cows. However, lactating cows may be more susceptible to the changes associated with SARA induction and therefore a greater APR would be observed.

Despite increases in APP concentrations, total protein concentrations significantly decreased in cows on GPI SARA treatment as compared to control treatment. The decrease was accompanied by reduced concentrations of albumin, globulin and fibrinogen. The period effects observed with albumin and fibrinogen concentrations were contrary to what would be expected with inflammatory conditions. According to period effects on albumin and fibrinogen concentrations, the cows became “healthier” throughout the duration of the trial. Three months prior to the experimental trial, cows had been dried up and fitted with rumen and caecum cannulas. From an immunological

perspective, considering the changes in albumin and fibrinogen levels, it may have been beneficial to wait several months before commencing with the experiment, although total protein and globulin concentrations did not change significantly over the course of the experimental trial. A reduction in total protein and albumin levels has also been observed in cattle with gastrointestinal parasites (Omer et al., 2003). Taking into consideration the effect of period on protein levels, it is anticipated that a chronic problem, such as recovery from cannulation, or parasitic conditions may have existed irrespective of SARA induction.

Elevated fibrinogen concentrations are often used to monitor inflammation in bovines. However, it is important to consider fibrinogen concentrations relative to total protein concentrations as total protein to fibrinogen ratios less than 10 have been used to indicate inflammation and over 15 indicate dehydration (Jones and Allison, 2007). Our results did not show any changes in total protein to fibrinogen ratio in SARA-induced cows compared to control cows. Albumin to globulin ratio can be a useful indicator of the presence of infection. Globulin concentrations increase, primarily due to immunoglobulin production by B lymphocytes, reducing the albumin to globulin ratio (Russell and Roussel, 2007). Although there tended to be changes in albumin concentrations in cows with GPI SARA, relative to globulin concentrations there were no differences across control and SARA-induced cows.

Rectal temperatures were monitored to identify systemic changes however differences were not detected in response to SARA-induction. Similarly, there was no effect of GPI SARA on rectal temperatures as reported by Gozho et al., (2007). The biological activity of SAA may have prevented changes to body temperature (Shainkin-

Kestenbaum et al., 1991). In LPS-infusion studies, concentrations ranging from 1.0-2.5 µg LPS/kg BW, increased temperatures were consistently detected a few hours post-infusion and showed mono- or bi-phasic peak pattern depending on concentration of LPS (Werling et al., 1996; Steiger et al., 1999; Jacobsen et al., 2005; Carroll et al., 2009). In the majority of LPS-infusion studies in cattle, LPS isolated from *E. coli* was used. *Escherichia coli* contains one of the most toxic structures of LPS (Erridge et al., 2002), therefore successfully triggering an inflammatory response. As well, ruminal endotoxins tend not to be as toxic as purified LPS; especially *E. coli*- isolated LPS (Nagaraja et al., 1978a). In the current study plasma LPS concentrations were not detectable and therefore were unable to stimulate systemic changes in body temperature.

There were no significant differences in the blood cell differentials of SARA-induced cows as compared to control cows. The study by Gozho and colleagues (2007) also did not find changes in blood cell differentials or total leukocyte count of GPI SARA cows. A change in blood leukocyte count, lymphocyte and neutrophil differentials have been identified with the infusion of LPS both intravenously and intramammary (Jacobsen et al., 2007; Bannerman et al., 2003). Typically neutropenia is evident during a severe inflammatory response as neutrophils are attracted to place of injury or stimulation (Jones and Allison, 2007). Period had a significant effect on total white blood cells, decreasing from period two to period three; however it is not certain what caused this decrease. Systemic changes were not observed in response to SARA with respect to blood cell populations.

Blood glucose concentrations were significantly higher in cows induced with SARA using the grain pellet model than in cows on control and API SARA treatments.

The increase of glucose has been reported previously in association with GPI SARA induction and with increasing levels of concentrates in the diet (Khafipour et al., 2009a; Ametaj et al., 2010; Zebeli et al., 2011). The induction of GPI SARA changes the VFA composition in the rumen; increasing propionate and decreasing acetate to propionate ratios (Goad et al., 1998; Gozho et al., 2007; Khafipour et al., 2009a). Conversely, in the current study blood urea concentrations were significantly decreased in GPI SARA treatment than control and API SARA treatments. Typically blood urea concentrations increase when diet protein levels increase (Russell and Rousell, 2007); however diet protein levels were similar across all the treatments in this study. Although diet protein levels were similar, the ingredients providing protein were different between treatments. The decreased urea concentrations in GPI SARA treatment may be due to the lower composition of NDF and ADF, as well as higher starch levels as compared to the other two diets (Table 3.2). Low levels of NDF and ADF would result in increased rate of fermentation by microbes; also an increased rate of passage through the rumen, therefore protein absorption would be reduced. Increased urea and creatinine concentrations in the blood are also indicators of kidney problems but further investigation would be needed to determine the cause (Russell and Roussel, 2007). Changes in glucose and urea concentrations observed with GPI SARA are associated with metabolic utilization of increased non-structural carbohydrates available in the diet.

SARA induction did not affect enzymes, CK, AST and GGT, associated with liver health and muscle metabolism. A study assessing the effects of acute coliform mastitis on systemic effects reported cows diagnosed with severe mastitis had significantly higher concentrations of AST and GGT in their blood then cows with mild

mastitis (Wenz et al., 2001). The cows diagnosed with severe mastitis most likely suffered from endotoxemia which has been associated with increased concentrations of liver-associated enzymes due to leaky hepatocytes (Russell and Roussel, 2007). In our results, changes were not identified in liver enzymes supporting the absence of a systemic response. However, the time of sampling affected CK and AST concentrations and increased concentrations post-feeding. The effect of time of sampling may be due to an increase in metabolic activity of the liver (i.e. gluconeogenesis, breakdown of ammonia to urea) several hours after eating, however this has not been reported before.

The concentrations of several macrominerals; calcium, magnesium and chloride, were significantly affected in cows induced with GPI SARA treatment. Calcium and magnesium decreased and increased, respectively and chloride concentrations of GPI cows increased comparatively to control cows. Recently, Zebeli et al. (2010) reported strong inverse correlations between ruminal endotoxin and plasma calcium concentrations as well as between SAA and plasma calcium. The correlation between SAA, ruminal endotoxins and serum calcium were not determined in this study, however, both SAA and ruminal endotoxin concentrations increased while calcium decreased in response to GPI SARA treatment as compared to control treatment. The decrease in calcium concentrations could be associated with the biological activity of calcium in SAA structure and stability (Wang and Colón, 2007). A study infusing *E. coli* LPS (0.5-1.5 µg LPS/kg BW) intravenously in bovines reported decreases in serum calcium and phosphorus concentrations, while magnesium concentrations remained unaffected (Waldron et al., 2003b). In another study which focused on the effects of natural occurring acute coliform mastitis on systemic macrominerals found that calcium and

magnesium concentrations decreased and increased, respectively in cows diagnosed with severe mastitis (Wenz et al., 2001). It is evident from the studies by Waldron et al., (2003b) and Wenz et al., (2001) that macrominerals may have prominent immunological roles and suggest that an immunological response may have been initiated during GPI SARA. The divalent characteristic of macrominerals, such as calcium, magnesium, zinc and copper, may be an important feature requiring their involvement in immunological responses (Rosen et al., 1958; Wang and Colón, 2007). Knowledge with regards to the biological roles of macrominerals is rather limited especially in association with SARA-induction and feeding grain-rich diets requires further investigation.

The macrominerals are essential for various biological functions in association with metabolism within the body; thus, the effect of time of sampling indicates metabolic changes in response to digestion, absorption and metabolism. Time of sampling had a significant effect on magnesium, phosphorus, sodium, potassium and concentrations. Although differences were detected in GPI SARA cows for some metabolic-associated parameters, it could be argued that those differences remained within the accepted range for cattle; therefore, the changes are not biologically relevant. However, it is important to consider the long term effect of such changes on the animal. The current study only looked at the short term effect of SARA but over a longer period of time imbalances could have adverse effects on health status.

6.0 CONCLUSION

In this study, the systemic immune responses of nonlactating cows induced with SARA using two different models, GPI and API was evaluated. A systemic immune response was defined as the changes in temperature, blood cell population and different plasma and serum proteins within experimental animals. The induction of SARA using grain pellet diet and alfalfa pellet diet models resulted in reduced ruminal pH below 5.6 for 298.7 and 225.2 minutes daily, respectively, and increased ruminal endotoxin concentrations (Li et al., 2012). However, changes in total protein and APPs, SAA and LBP, concentrations were only evident in cows induced with SARA using the grain pellet diet as compared to control diet. The changes in APPs suggest a localized inflammatory response may have been initiated. Grain pellet-induced SARA also affected metabolic-associated parameter concentrations; glucose, urea, calcium, magnesium and chloride. Cell-surface receptors responsible for the recognition of endotoxins on peripheral blood cells were not affected by SARA induction. Based on the definition of systemic immune response used for this study, there was not substantial evidence to conclude the GPI SARA triggered a systemic immune response, affecting the health status of the animals. However, it is important to stress that the dairy cows used in this study were nonlactating; therefore they did not have output energy demands and were not as affected by SARA induction as they may have been if they were lactating.

An important question which still needs to be addressed: do changes to APPs detected in the circulatory plasma indicate a systemic response on their own or are these changes indicative of a localized immune response? The presence of APP mRNA and

protein within the GIT tissue provides evidence of a local immune response rather than a systemic response produced by liver hepatocytes (Rahman et al., 2010). Since the increases in SAA and LBP were minor or "sub-clinical" relative to previous GPI SARA studies, a localized immune response may have been present.

There is a need for future research to focus on the endotoxic activity level of endotoxins isolated from the rumen under various diet challenges. Many studies propose that endotoxins, putatively translocated from the gut, are the cause of the APR observed with SARA induction and with the feeding starch-rich diets, however, endotoxins isolated from the rumen have not expressed strong endotoxic activity (Nagaraja et al., 1978a). Endotoxins isolated from the rumen should be infused in cattle rather than *E. coli*-isolated LPS to fully understand the effects of GIT-derived endotoxins on the bovine immune system. In addition, endotoxins isolated from the rumen can be used for in vitro stimulation of peripheral blood, liver and GIT cells to understand the pathway mechanisms and recognition receptors involved. There is also great potential to characterize the effects of SARA and feeding grain-rich diets on GIT mucosal and epithelial cells as biopsy techniques for obtaining tissue from the liver and the rumen have already been described (Kelly et al., 1993; Andersen et al., 2002; Vels et al., 2009; Steele et al., 2009).

Subacute ruminal acidosis has primarily been referred to as a metabolic problem which may have secondary immunological repercussions (Kleen et al., 2003). The extent of secondary effects due to SARA, or even feeding a grain-rich diet, depends upon the ability of an individual animal to cope with the pressures of feeding such diets (Jacobsen et al., 2004). From an applied standpoint, SARA should be observed from a system

biology perspective. There are many biological systems involved during SARA induction with respect to the metabolic, microbial and immunological systems. All of these biological systems interrelate within the bovine and cannot be accurately understood independently without knowledge of the other systems.

REFERENCES

Abreu, M. T., P. Vora, E. Faure, L. S. Thomas, E. T. Arnold, and M. Arditi. 2001. Decreased expression of toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. J. Immunol. 167: 1609-1616.

Abreu, M. T., E. T. Arnold, L. S. Thomas, R. Gonsky, Y. Zhou, B. Hu, and M. Arditi. 2002. TLR4 and MD-2 expression is regulated by immune-mediated signals in human intestinal epithelial cells. J. Biol. Chem. 277: 20431-20437.

Akira, S. and K. Takeda. 2004. Toll-like receptor signaling. Nat. Rev. Immunol. 4: 499-511.

Aldo-Benson, M., and M. D. Benson. 1982. SAA suppression of immune response in vitro: Evidence for an effect on T cell-macrophage interaction. J. Immunol. 128: 2390-2392.

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

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

Ametaj, B. N., K. M. Koenig, S. M. Dunn, W. Z. Yang, Q. Zebeli, and K. A. Beauchemin. 2009. Backgrounding and finishing diets are associated with inflammatory responses in feedlot steers. J. Anim. Sci. 87: 1314-1320.

Ametaj, B. N, Q. Zebeli, F. Saleem, N. Psychogios, M. J. Lewis, S. M. Dunn, J. Xia and D. S. Wishart. 2010. Metabolomics reveals unhealthy alterations in rumen metabolism with increased proportion of cereal grain in the diet of dairy cows. Metabolomics. 6: 583-594.

Andersen, J. B., D. G. Mashek, T. Larsen, M. O. Nielsen, and K. L. Ingvartsen. 2002. Effects of hyperinsulinaemia under euglycaemic condition on liver fat metabolism in dairy cows in early and mid-lactation. J. Vet. Med. A. 49: 65-71.

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

Andersen, P.H. 2003. Bovine endotoxicosis - some aspects of relevance to production diseases. A review. Acta Vet. Scand. 98(Suppl.): 141-155.

Andrä, J., T. Gutsmann, M. Müller, and A. B. Schromm. 2009. Interactions between lipid A and serum proteins. In: Lipid A in cancer therapy. Edited by Jeannin, J.-F. p. 39-51.

Bäckhead, F., and M. Hornef. 2003. Toll-like receptor 4-mediated signaling by epithelial surfaces: necessity or threat? Microbes Infect. 5: 951-959.

Badolato, R., J. M. Wang, W. J. Murphy, A. R. Lloyd, D. F. Michiel, L. L. Bausserman, D. J. Kelvin and J.J. Oppenheim. 1994. Serum amyloid A is a chemoattractant: Induction of migration, adhesion, and tissue infiltration of monocytes and polymorphonuclear leukocytes. J. Exp. Med. 180: 203-209.

Bannerman, D. D., M. J. Paape, W. R. Hare, and E. J. Sohn. 2003. Increased levels of LPS-binding protein in bovine blood and milk following bacterial lipopolysaccharide challenge. J. Dairy Sci. 86: 3128-3137.

Bažil, V., and J. L. Strominger. 1991. Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. J. Immunol. 147: 1567-1574.

Benson, M. D., and M. Aldo-Benson. 1979. Effect of purified protein SAA on immune response in vitro: Mechanisms of suppression. J. Immunol. 122: 2077-2082.

Berg, R. D. 1983. Bacterial translocation from the gastrointestinal tracts of mice receiving immunosuppressive chemotherapeutic agents. Curr. Microbiol. 8: 285-292.

Berg, R. D., 1995. Bacterial translocation from the gastrointestinal tract. Trends Microbiol. 3: 149-154.

Böcker, U., O. Yezerskyy, P. Feick, T. Manigold, A. Panja, U. Kalina, F. Herweck, S. Rossol, and M.V. Singer. 2003. Responsiveness of intestinal epithelial cell lines to lipopolysaccharide is correlated with toll-like receptor 4 but not toll-like receptor 2 or CD14 expression. Int. J. Colorectal Dis. 18: 25-32.

Carroll, J. A., R. R. Reuter, C. C. Chase, S. W. Coleman, D. G. Riley, D. E. Spiers, J. D. Arthington and M. L. Galyean. 2009. Profile of the bovine acute-phase response following an intravenous bolus-dose lipopolysaccharide challenge. Innate Immun. 15: 81-89.

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

Cavaillon, J. M., C. Fitting, N. Haeffner-Cavaillon, S. J. Kirsch, and H. S. Warren. 1990. Cytokine response by monocytes and macrophages to free and lipoprotein-bound lipopolysaccharide. Infect. Immun. 58: 2375-2382.

CCAC. 1993. Guide to the Care and Use of Experimental Animals. Vol. 1. 2nd ed. Canadian Council on Animal Care, Ottawa, ON, Canada.

Crutchley, M. J., D. G. Marsh, and J. Cameron. 1967. Free endotoxin. Nature. 214: 1052-1052.

da Silva Correia, J., K. Soldau, U. Christen, P. S. Tobias, and R. J. Ulevitch. 2001. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. J. Biol. Chem. 276: 21129-21135.

de Waal Malefyt, R., J. Abrams, B. Bennett, C. G. Figdor, and J. E. de Vries. 1991. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: An autoregulatory role of IL-10 produced by monocytes. J. Exp. Med. 174: 1209-1220.

Deng, Q., Harvie, E. A., and A. Huttenlocher. 2012. Distinct signaling mechanisms mediate neutrophil attraction to bacterial infection and tissue injury. Cell. Microbiol. 14: 517-528.

Dinarello, C. A., J. G. Cannon, S. M. Wolff, H. A. Bernheim, B. Beutler, A. Cerami, I. S. Figari, M. A. Palladino, J. V. O'Connor. 1986. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. J. Exp. Med. 163: 1433-1450.

Divanovic, S., A. Trompette, L. K. Petiniot, J. L. Allan, L. M. Flick, Y. Belkaid, R. Madan, J. J. Haky, and C. L. Karp. 2007. Regulation of TLR4 signaling and the host interface with pathogens and danger: the role of RP105. J. Leukocyte Biol. 82: 265-271.

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

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

Elsasser, T. H., K. C. Klasing, N. Filipov, and F. Thompson. 2000. The metabolic consequences of stress: targets for stress and priorities of nutrient use. In: The biology of animal stress: basic principles and implication for animal welfare. Edited by Moberg, G. P. and J. A. Mench. p. 77-110.

Emmanuel, D. G. V., K. L. Madsen, T. A. Churchill, S. M. Dunn, and B. N. Ametaj. 2007. Acidosis and lipopolysaccharide from *Escherichia coli* B:055 cause hyperpermeability of rumen and colon tissues. J. Dairy Sci. 90: 5552-5557.

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

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

Erridge, C., E. Bennett-Guerrero, and I. R. Poxton. 2002. Structure and function of lipopolysaccharides. Microb. Infect. 4: 837-851.

Eusebio, A. N., J. C. Shaw, E. C. Leffel, S. Lakshmanan, and R. N. Doetsch. 1959. Effect on rumen volatile fatty acids and rumen microbial dissimilation of glucose-C14 of corn meal when fed exclusively and in combination with hay or certain additives. J. Anim. Sci. 42: 692-697.

Faleiro, A. G., L. A. González, M. Blanch, S. Cavini, L. Castells, J. L. Ruíz de Torre, X. Manteca, S. Calsamiglia, and A. Ferret. 2010. Performance, ruminal changes, behaviour and welfare of growing heifers fed a concentrate diet with or without barley straw. Animal. 5: 294-303.

Fan, X., F. Stelter, R. Menzel, R. Jack, I. Spreitzer, T. Hartung and C. Schütt. 1999. Structures in Bacillus subtilis are recognized by CD14 in a lipopolysaccharide binding protein-dependent reaction. Infect. Immun. 67: 2964-2968.

Fantuzzi, G., and P. Ghezzi. 1993. Glucocorticoids as cytokine inhibitors: role in neuroendrocrine control and therapy of inflammatory diseases. Mediat. Inflamm. 2: 263-270.

Franchini, M., M. Schweizer, P. Mätzener, I. Magkouras, K. S. Sauter, J. Mirkovitch, E. Peterhans and T. W. Jungi. 2006. Evidence for dissociation of TLR mRNA expression and TLR agonist-mediated functions in bovine macrophages. Vet. Immunol. Immunopathol. 110: 37-49.

Galanos, C., O. Lüderitz, E. T. Rietschel, O. Westphal, H. Brade, L. Brade, M. Freudenburg, U. Schade, M. Imoto, H. Yoshimura, S. Kusumoto and T. Shiba. 1985. Synthetic and natural Escherichia coli free lipid A express identical endotoxic activities. Eur. J. Biochem. 148: 1-5.

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

Godson, D. L., M. E. Baca-Estrada, A. G. Van Kessel, H. P. A. Hughes, M. A. Morsy, J. Van Donkersgoed, R. J. Harland, D. E. Shuster, M. J. Daley, and L. A. Babiuk. 1995.

Regulation of bovine acute phase responses by recombinant interleukin-1β. Can. J. Vet. Res. 59: 249-255.

Goff, J. P. 2006. Major advances in our understanding of nutritional influences on bovine health. J. Dairy Sci. 89: 1292-1301.

Goldammer, T., H. Zerbe, A. Molenaar, H. J. Schuberth, R. M. Brunner, S. R. Kata and H. M. Seyfert. 2004. Mastitis increases mammary mRNA abundance of β-defensin 5, toll-like-receptor 2 (TLR2), and TLR4 but not TLR9 in cattle. Clin. Diagn. Lab. Immunol. 11: 174-185.

González, L. A., A. Ferret, X. Manteca, J. L. Ruíz-de-la-Torre, S. Calsamiglia, M. Devant, and A. Bach. 2008. Performance, behaviour, and welfare of Friesian heifers housed in pens with two, four, and eight individuals per concentrate feeding place. J. Anim. Sci. 86: 1446-1458.

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

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

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

Graham, C., and N. L. Simmons. 2005. Functional organization of the bovine rumen epithelium. Am. J. Physiol. Regul. Integr. Comp. Physiol. 288: R173-R181.

Granowitz, E. V., R. Porat, J. W. Mier, S. F. Orencole, G. Kaplanski, E. A. Lynch. K. Ye, E. Vannier, S. M. Wolff, and C. A. Dinarello. 1993. Intravenous endotoxin suppresses the cytokine response of peripheral blood mononuclear cells of healthy humans. J. Immunol. 151: 1637-1645.

Guo, Y., G. Zhao, S. Tanaka, and T. Yamaguchi. 2009. Differential responses between monocytes and monocyte-derived macrophages for lipopolysaccharide stimulation of calves. Cell. Mol. Immunol. 6: 223-229.

Hailman, E., H. S. Lichenstein, M. M. Wurfel, D. S. Miller, D. A. Johnson, M. Kelley, L. A. Busse, M. M. Zukowski, and S. D. Wright. 1994. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. J. Exp. Med. 179: 269-277.

Haziot, A., S. Chen, E. Ferrero, M. G. Low, R. Silber, and S. M. Goyert. 1988. The monocyte differentiation antigen, CD14, is anchored to the cell membrane by a phosphatidylinositol linkage. J. Immunol. 141: 547-552.

Herrick, S., O. Blanc-Brude, A. Gray, and G. Laurent. 1999. Fibrinogen. Int. J. Biochem. Cell Biol. 31: 741-746.

Heumann, D., P. Gallay, C. Barras, P. Zaech, R. J. Ulevitch, P. S. Tobias. M. P. Glauser, and J. D. Baumgartner. 1992. Control of lipopolysaccharide (LPS) binding and LPS-induced tumor necrosis factor secretion in human peripheral blood monocytes. J. Immunol. 148: 3505-3512.

Hietbrink, F., M. G. H. Besselink, W. Renooij, M. B. M. de Smet, A. Draisma, H. van der Hoeven, and P. Pickkers. 2009. Systemic inflammation increases intestinal permeability during experimental human endotoxemia. Shock 32: 374-378.

Hiki, N., D. Berger, C. Prigl, E. Boelke, H. Wiedeck, M. Seidelmann, L. Staib, M. Kaminishi, T. Oohara, and H. G. Beger. 1998. Endotoxin binding and elimination by monocytes: Secretion of soluble CD14 represents an inducible mechanism counteracting reduced expression of membrane CD14 in patients with sepsis and in a patient with paroxysmal nocturnal hemoglobinuria. Infect. Immun. 66: 1135-1141.

Hofstad, T. 1974. Endotoxins of anaerobic gram-negative microorganisms. In: Anaerobic bacteria: role in disease. Edited by Balows, A. p. 295-305.

Horadagoda, N. U., P. D. Eckersall, L. Andrew, P. Gallay, D. Heumann, and H. A. Gibbs. 1995. Characterisation of bovine lipopolysaccharide binding protein and the in vivo acute phase response to *Pasteurella haemolytica* type A. Vet. Immunol. Immunopathol. 49: 61-74.

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

Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: Evidence for TLR4 as the lps gene product. J. Immunol. 162: 3749-3752.

Humblet, M. F., H. Guyot, B. Boudry, F. Mbayahi, C. Hanzen, F. Rollin, and J. M. Godeau. 2006. Relationship between haptoglobin, serum amyloid A, and clinical status in a survey of dairy herds during a 6-month period. Vet. Clin. Pathol. 35: 188-193.

Hungate, R. E. 1966. The rumen and its microbes. Academic Press. New York, New York.

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

Ibeagha-Awemu, E. M., J. W. Lee, A. E. Ibeagha, D. D. Bannerman, M. J. Paape, and X. Zhao. 2008a. Bacterial lipopolysaccharide induces increased expression of toll-like receptor (TLR) 4 and downstream TLR signaling molecules in bovine mammary epithelial cells. Vet. Res. 39: 11-22.

Ibeagha-Awemu, E. M., J. W. Lee, A. E. Ibeagha, and X. Zhao. 2008b. Bovine CD14 gene characterization and relationship between polymorphisms and surface expression on monocytes and polymorphonuclear neutrophils. BMC Genet. 9: 50-60.

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

Jacobsen, S., T. Toelboell, and P. H. Andersen. 2005. Dose dependency and individual variability in selected clinical, haematological and blood biochemical responses after systemic lipopolysaccharide challenge in cattle. Vet. Res. 36: 167-178.

Jacobsen, S., P. H. Andersen, and B. Aasted. 2007. The cytokine response of circulating peripheral blood mononuclear cells is changed after intravenous injection of lipopolysaccharide in cattle. Vet. J. 174: 170-175.

Jahoor, F., B. Sivakumar, M. Del Rosario, and E. M. Frazer. 1996. Isolation of acute-phase proteins from plasma for determination of fractional synthesis rates by a stable isotope tracer technique. Anal. Biochem. 236: 95-100.

Jones, M. L., and R. W. Allison. 2007. Evaluation of the ruminant complete blood cell count. Vet. Clin. Food Anim. 23: 377-402.

Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity. 11: 115-122.

Kawai, T., O. Takeuchi, T. Fujita, J. Inoue, P. F. Mühlradt, S. Sato, K. Hoshino, and S. Akira. 2001. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. J. Immunol. 167: 5887-5894.

Kehrli, M. E. Jr., J. D. Neill, C. Burvenich, J. P. Goff, J. D. Lippolis, T. A. Reinhardt, and B. J. Nonnecke. 2008. Energy and protein effects on the immune system. In: Ruminant physiology: digestion, metabolism and impact of nutrition on gene expression, immunology and stress. Edited by Sejrsen, K., T. Hvelplund, and M. O. Nielsen. p. 455-471.

Kelly, J. M., B. W. McBride, and L. P. Milligan. 1993. In vitro ouabain-sensitive respiration and protein synthesis in ruminal epithelial papillae of Hereford steers fed either alfalfa or bromegrass hay once daily. J. Anim. Sci. 71: 2799-2808.

Khafipour, E., D. O. Krause, and J. C. Plaizier. 2009a. A grain-based subacute ruminal acidosis challenge causes translocation of lipopolysaccharide and triggers inflammation. J. Dairy Sci. 92: 1060-1070.

Khafipour, E., D. O. Krause, and J. C. Plaizier. 2009b. Alfalfa pellet-induced subacute ruminal acidosis in dairy cows increases bacterial endotoxin in the rumen without causing inflammation. J. Dairy Sci. 92: 1712-1724.

Khafipour, E., S. Li, J. C. Plaizier, and D. O. Krause. 2009c. Rumen microbiome composition determined using two nutritional models of subacute ruminal acidosis. Appl. Environ. Microb. 75: 7115-7124.

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

Kushner, I. 1982. The phenomenon of the acute phase response. Ann. N. Y. Acad. Sci. 389: 39-48.

Landmann, R., H. P. Knopf, S. Link, S. Sansano, R. Schumann, and W. Zimmerli. 1996. Human monocyte CD14 is upregulated by lipopolysaccharide. Infect. Immun. 64: 1762-1769.

Li, S., E. Khafipour, D. O. Krause, A. Kroeker, J. Rodriguez-Lecompte, G. N. Gozho, and J. C. Plaizier. 2012. Effects of subacute ruminal acidosis challenges on fermentation and endotoxins in the rumen and hindgut of dairy cows. J. Dairy Sci. 95: 294-303.

Liebler-Tenorio, E. M., and R. Pabst. 2006. MALT structure and function in farm animals. Vet. Res. 37: 257-280.

Liew, F. Y., D. Xu, E. K. Brint, and L. A. J. O'Neill. 2005. Negative regulation of toll-like receptor-mediated immune responses. Nat. Rev. Immunol. 5: 446-458.

Lomborg, S., L. Nielsen, P. Heegaard, and S. Jacobsen. 2008. Acute phase proteins in cattle after exposure to complex stress. Vet. Res. Commun. 32: 575-582.

Loyer, P., G. Ilyin, Z. A. Razzak, J. Banchereau, J.-F. Dezier, J.-P. Campion, C. Guguen-Guillouzo, and A. Guillouzo. 1993. Interleukin 4 inhibits the production of some acute-phase proteins by human hepatocytes in primary culture. FEBS Lett. 336: 215-220.

Maliszewski, C. R., and S. D. Wright. 1991. CD14 and immune response to lipopolysaccharide. Science. 252: 1321-1322.

Mani, V., T. E. Weber, L. H. Baumgard, and N. K. Gabler. 2012. Endotoxin, inflammation and intestinal function in livestock. J. Anim. Sci. (In press)

Moshage, H. 1997. Cytokines and the hepatic acute phase response. J. Pathol. 181: 257-266.

Murata, H., and T. Miyamoto. 1993. Bovine haptoglobin as a possible immunomodulator in the sera of transported calves. Br. Vet. J. 149: 277-283.

Murata, H., N. Shimada, and M. Yoshioka. 2004. Current research on acute phase proteins in veterinary diagnosis: An overview. Vet. J. 168: 28-40.

Muta, T., and K. Takeshige. 2001. Essential roles of CD14 and lipopolysaccharide-binding protein for activation of toll-like receptor (TLR)2 as well as TLR4 reconstitution of TLR2- and TLR4-activation by distinguishable ligands in LPS preparations. Eur. J. Biochem. 268: 4580-4589.

Muzio, M., D. Bosisio, N. Polentarutti, G. D'amico, A. Stoppacciaro, R. Mancinelli, C. van't Veer, G. Penton-Rol, L. P. Ruco, P. Allavena, and A. Mantovani. 2000. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J. Immunol. 164: 5998-6004.

Nagaraja, T. G., E. E. Bartley, L. R. Fina, H. D. Anthony, and R. M. Bechtle. 1978a. Evidence of endotoxins in the rumen bacteria of cattle fed hay or grain. J. Anim. Sci. 47: 226-234.

Nagaraja, T. G., E. E. Bartley, L. R. Fina, and H. D. Anthony. 1978b. Relationship of rumen gram-negative bacteria and free endotoxin to lactic acidosis in cattle. J. Anim. Sci. 47: 1329-1337.

Nakagawa-Tosa, N., M. Morimatsu, M. Kawasaki, H. Nakatsuji, B. Syuto, and M. Saito. 1995. Stimulation of haptoglobin synthesis by interleukin-6 and tumor necrosis factor, but not by interleukin-1, in bovine primary cultured hepatocytes. J. Vet. Med. Sci. 57: 219-223.

Neal, M. D., C. Leaphart, R. Levy, J. Prince, T. R. Billiar, S. Watkins, J. Li, S. Cetin, H. Ford, A. Schreiber, and D. J. Hackam. 2006. Enterocyte TLR4 mediates phagocytosis and translocation of bacteria across the intestinal barrier. J. Immunol. 176: 3070-3079.

Nielsen, B. H., S. Jacobsen, P. H. Andersen, T. A. Niewold, and P. M. H. Heegaard. 2004. Acute phase protein concentrations in serum and milk from healthy cows, cows with clinical mastitis and cows with extramammary inflammatory conditions. Vet. Rec. 154: 361-365.

Niemetz, J., and D. C. Morrison. 1977. Lipid A as the biologically active moiety in bacterial endotoxin (LPS)- initiated generation of procoagulant activity by peripheral blood leukocytes. Blood. 49: 947-956.

Nocek, J. E., and E. M. Kesler. 1980. Growth and rumen characteristics of holstein steers fed pelleted or conventional Diets1. J. Dairy Sci. 63: 249-254.

Omer, O. H., K. H. El-Malik, M. Magzoub, O. M. Mahmoud, E. M. Haroun, A. Hawas, and H. M. Omar. 2003. Biochemical profiles in Friesian cattle naturally infected with *Theileria annulata* in Saudi Arabia. Vet. Res. Commun. 27: 15-25.

Otte, J., E. Cario, and D. K. Podolsky. 2004. Mechanisms of cross hyporesponsiveness to toll-like receptor bacterial ligands in intestinal epithelial cells. Gastroenterology. 126: 1054-1070.

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

Pabst, R. 1987. The anatomical basis for the immune function of the gut. Anat. Embryol. 176: 135-144.

Penner, G. B., M. Oba, G. Gäbel, and J. R. Aschenbach. 2010. A single mild episode of subacute ruminal acidosis does not affect ruminal barrier function in the short term. J. Dairy Sci. 93: 4838-4845.

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

Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29: e45-50.

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

Preston, R. L., D. D. Schnakenberg, and W. H. Pfander. 1965. Protein utilization in ruminants. I. Blood urea nitrogen as affected by protein intake. J. Nutr. 86: 281-288.

Prgomet, C., H. Sarikaya, R. M. Bruckmaier, and M. W. Pfaffl. 2005. Short-term effects on pro-inflammatory cytokine, lactoferrin and CD14 mRNA expression levels in bovine immunoseparated milk and blood cells treated by LPS. J. Vet. Med. Ser. A. 52: 317-324.

Rahman, M. M., C. Lecchi, G. Avallone, P. Roccabianca, P. Sartorelli, and F. Ceciliani. 2010. Lipopolysaccharide-binding protein: Local expression in bovine extrahepatic tissues. Vet. Immunol. Immunopathol. 137: 28-35.

Rietschel, E. T., T. Kirikae, F. U. Schade, U. Mamat, G. Schmidt, H. Loppnow, A. J. Ulmer, U. Zähringer, U. Seydel, F. Di Padova, M. Schreier, and H. Brade. 1994. Bacterial endotoxin: Molecular relationships of structure to activity and function. FASEB J. 8: 217-225.

Rosen, F. S., R. C. Skarnes, M. Landy, and M. J. Shear. 1958. Inactivation of endotoxin by a humoral component. III. Role of divalent cation and a dialyzable component. J. Exp. Med. 108: 701-711.

Rubel, C., G. C. Fernández, G. Dran, M. B. Bompadre, M. A. Isturiz, and M. S. Palermo. 2001. Fibrinogen promotes neutrophil activation and delays apoptosis. J. Immunol. 166: 2002-2010.

Russell, K. E., and A. J. Roussel. 2007. Evaluation of the ruminant serum chemistry profile. Vet. Clin. Food Anim. 23: 403-426.

Sambrook, J., and D. W. Russell. 2001. Plasmids and their usefulness in molecular cloning. Pages 1.1-2.1 in Molecular Cloning: A Laboratory Manuel (3rd ed.), Cold Spring Harbor Laboratory Press, Cold Spring, New York, USA.

SAS Institute. 2004. SAS/STAT Users Guide. Release 9.1.2. SAS Inst. Inc., Cary, NC.

Sauter, K., M. Brcic, M. Franchini, and T. W. Jungi. 2007. Stable transduction of bovine TLR4 and bovine MD-2 into LPS-nonresponsive cells and soluble CD14 promote the ability to respond to LPS. Vet. Immunol. Immunopathol. 118: 92-104.

Schröder, N. W. J., S. Morath, C. Alexander, L. Hamann, T. Hartung, U. Zähringer, U. B. Göbel, J. R. Weber, and R. R. Schumann. 2003. Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. J. Biol. Chem. 278: 15587-15594.

Schroedl, W., B. Fuerll, P. Reinhold, M. Krueger, and C. Schuett. 2001. A novel acute phase marker in cattle: Lipopolysaccharide binding protein (LBP). J. Endotoxin Res. 7: 49-52.

Schumann, R. R., S. R. Leong, G. W. Flaggs, P. W. Gray, S. D. Wright, J. C. Mathison, P. S. Tobias, and R. J. Ulevitch. 1990. Structure and function of lipopolysaccharide binding protein. Science 249: 1429-1431.

Schumann, R. R., C. J. Kirschning, A. Unbehaun, H. Aberle, H.-P. Knopf, N. Lamping, R. J. Ulevitch, and F. Herrmann. 1996. The lipopolysaccharide-binding protein is a secretory class 1 acute-phase protein whose gene is transcriptionally activated by APRF/STAT-3 and other cytokine-inducible nuclear proteins. Mol. Cell. Biol. 16: 3490-3503

Shainkin-Kestenbaum, R., G. Berlyne, S. Zimlichman, H. R. Sorin, M. Nyska, and A. Danon. 1991. Acute phase protein, serum amyloid A, inhibits IL-1- and TNF-induced fever and hypothalamic PGE2 in mice. Scand. J. Immunol. 34: 179-183.

Shimazu, R., S. Akashi, H. Ogata, Y. Nagai, K. Fukudome, K. Miyake, and M. Kimoto. 1999. MD-2, a molecule that confers lipopolysaccharide responsiveness on toll-like receptor 4. J. Exp. Med. 189: 1777-1782.

Sitrin, R. G., P. M. Pan, S. Srikanth, and R. F. Todd. 1998. Fibrinogen activates NF-κB transcription factors in mononuclear phagocytes. J. Immunol. 161: 1462-1470.

Sohn, E. J., M. J. Paape, D. D. Bannerman, E. E. Connor, R. H. Fetterer, and R. R. Peters. 2007a. Shedding of sCD14 by bovine neutrophils following activation with bacterial lipopolysaccharide results in down-regulation of IL-8. Vet. Res. 38: 95-108.

Sohn, E. J., M. J. Paape, E. E. Connor, D. D. Bannerman, R. H. Fetterer, and R. R. Peters. 2007b. Bacterial lipopolysaccharide stimulates bovine neutrophil production of TNF-α, IL-1β, IL-12 and IFN-γ. Vet. Res. 38: 809-818.

Sordillo, L. M., K. Shafer-Weaver, and D. DeRosa. 1997. Immunobiology of the mammary gland. J. Dairy Sci. 80: 1851-1865.

Steele, M. A., O. AlZahal, S. E. Hook, J. Croom, and B. W. McBride. 2009. Ruminal acidosis and the rapid onset of ruminal parakeratosis in a mature dairy cow: A case report. Acta Vet. Scand. 51: 39-44.

Steiger, M., M. Senn, G. Altreuther, D. Werling, F. Sutter, M. Kreuzer, and W. Langhans. 1999. Effect of a prolonged low-dose lipopolysaccharide infusion on feed intake and metabolism in heifers. J. Anim. Sci. 77: 2523-2532.

Steven, D. H., and A. B. Marshall. 1970. Organization of rumen epithelium. In: Physiology of digestion and metabolism in the ruminant. Edited by Phillipson, A. T. p. 80-100.

Takeuchi, O., K. Takeda, K. Hoshino, O. Adachi, T. Ogawa, and S. Akira. 2000. Cellular responses to bacterial cell wall components are mediated through MyD88-dependent signaling cascades. Int. Immunol. 12: 113-117.

Takeda, K., and S. Akira. 2005. Toll-like receptors in innate immunity. Int. Immunol. 17: 1-14.

te Velde, A. A., R. J. Huijbens, K. Heije, J. E. de Vries, and C. G. Figdor. 1990. Interleukin-4 (IL-4) inhibits secretion of IL-1 beta, tumor necrosis factor alpha, and Il-6 by human monocytes. Blood 76: 1392-1397.

Tobias, P. S., K. Soldau, and R. J. Ulevitch. 1986. Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. J. Exp. Med. 164: 777-793.

Tobias, P. S., K. Soldau, and R. J. Ulevitch. 1989. Identification of a lipid A binding site in the acute phase reactant lipopolysaccharide binding protein. J. Biol. Chem. 264: 10867-10871.

van der Poll, T., S. M. Coyle, L. L. Moldawer, and S. F. Lowry. 1996. Changes in endotoxin-induced cytokine production by whole blood after in vivo exposure of normal humans to endotoxin. J. Infect. Dis. 174: 1356-1359.

Vels, L., C. M. Røntved, M. Bjerring, and K. L. Ingvartsen. 2009. Cytokine and acute phase protein gene expression in repeated liver biopsies of dairy cows with a lipopolysaccharide-induced mastitis. J. Dairy Sci. 92: 922-934.

Vreugdenhil, A. C. E., M. A. Dentener, A. M. P. Snoek, J. M. Greve, and W. A. Buurman. 1999. Lipopolysaccharide binding protein and serum amyloid A secretion by human intestinal epithelial cells during the acute phase response. J. Immunol. 163: 2792-2798.

Vreugdenhil, A. C. E., A. M. P. Snoek, J. W. M. Greve, and W. A. Buurman. 2000. Lipopolysaccharide-binding protein is vectorially secreted and transported by cultured intestinal epithelial cells and is present in the intestinal mucus of mice. J. Immunol. 165: 4561-4566.

Waggoner, J. W., C. A. Löest, J. L. Turner, C. P. Mathis, and D. M. Hallford. 2009. Effects of dietary protein and bacterial lipopolysaccharide infusion on nitrogren metabolism and hormonal responses of growing beef steers. J. Anim. Sci. 87: 3656-3668.

Waldron, M. R., B. J. Nonnecke, T. Nishida, R. L. Horst, and T. R. Overton. 2003a. Effect of lipopolysaccharide infusion on serum macromineral and vitamin D concentrations in dairy cows. J. Dairy Sci. 86: 3440-3446.

Waldron, M. R., T. Nishida, B. J. Nonnecke, and T. R. Overton. 2003b. Effect of lipopolysaccharide on indices of peripheral and hepatic metabolism in lactating cows. J. Dairy Sci. 86: 3447-3459.

Wang, L., and W. Colón. 2007. Effect of zinc, copper, and calcium on the structure and stability of serum amyloid A. Biochemistry 46: 5562-5569.

Wang, P., P. Wu, M. I. Siegel, R. W. Egan, and M. M. Billah. 1995. Interleukin (IL)-10 inhibits nuclear factor κβ (NFκβ) activation in human moncytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms. J. Biol. Chem. 270: 9558-9563.

Wenz, J. R., G. M. Barrington, F. B. Garry, R. P. Dinsmore, and R. J. Callan. 2001. Use of systemic disease signs to assess disease severity in dairy cows with acute coliform mastitis. J. Am. Vet. Med. A. 218: 567-572.

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

Wise, M. B., R. W. Harvey, B. R. Haskins, and E. R. Barrick. 1968. Finishing beef cattle on all-concentrate rations. J. Anim. Sci. 27: 1449-1461.

Wright, S. D., R. A. Ramos, P. S. Tobias, R. J. Ulevitch, and J. C. Mathison. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249: 1431-1433.

Xu, L., R. Badolato, W. J. Murphy, D. L. Longo, M. Anver, S. Hale, J. J. Oppenheim, and J. M. Wang. 1995. A novel biologic function of serum amyloid A. induction of T lymphocyte migration and adhesion. J. Immunol. 155: 1184-1190.

Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M Sugiyama, M. Okabe, K. Takeda, and, S. Akira. 2003. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science. 301: 640-643.

Zebeli, Q., S. M. Dunn, and B. N. Ametaj. 2010. Strong associations among endotoxin and acute phase proteins with plasma minerals in lactating cows fed graded amounts of concentrate. J. Anim. Sci. 88: 1545-1553.

Zebeli, Q., S. M. Dunn, and B. N. Ametaj. 2011. Perturbations of plasma metabolites correlated with the rise of rumen endotoxin in dairy cows fed diets rich in easily degradable carbohydrates. J. Dairy Sci. 94: 2374-2382.

Zeng, R., B. J. Bequette, B. T. Vinyard, and D. D. Bannerman. 2009. Determination of milk and blood concentrations of lipopolysaccharide-binding protein in cows with naturally acquired subclinical and clinical mastitis. J. Dairy Sci. 92: 980-989.

APPENDIX

I. The health history of the cows used for this study.

Cow	Age[1]	Lac[2]	Mastitis	Other Problems	Medications
Poppy	3y2m	1	no	Retained placenta, displaced abomasum	Cefa-Dri at dry-off
Mia	7y5m	1	no	-	-
		2	no	-	-
		3	no	Milk fever	-
		4	yes	Milk fever, foot abscess	Excenel, Cefa-Lak for 3 days for mastitis, Cefa-Dri at dry-off
Hope	4y2m	1	no	Foot problem - blocked	Cefa-Dri at dry-off
		2	no	-	Cefa- Dri at dry-off
Gilly	4y1m	1	no		Cefa-Dri at dry-off
		2	no	Lameness	After Calf bolus, Excenel 3 days for lameness, Cefa-Dri at dry-off
Pepsi	3y7m	1	no	-	Excenel, Cefa-Dri at dry-off, vaccination (DY, SGVIT)
		2	yes	-	Special Formula for 3 days for mastitis, Cefa-Dri at dry-off
Unity	3y1m	1	no	-	Excenel, Cefa-Dri at dry-off

[1] At the beginning of the experimental trial
[2] Lactation number