# University of Alberta

**Novel Mucosal Vaccines to Improve Immune and Health Status of Periparturient Dairy Cows and Increase their Productive Potentials**

by

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**DEDICATION**

This thesis is dedicated to the one in whose hands my life is.

My humble dedication also goes to the greatest inspiration of my life whose love is over and above of my dear relations, and who advised strongly for acquisition of knowledge and to always strive to excel in virtue and truth.

My thesis is also dedicated to my parents Salma Parveen and Muhammad Iqbal for their endless love, support and prayers since the beginning of my life.

I also dedicate this thesis to my daughter Eshal Khurram Ahmed, and my husband, Khurram Ejaz Ahmed, for being there for me, supported me, and encouraged me throughout this degree.

I dedicate my dissertation work to my mentors-teachers for their guidance.

## ABSTRACT

The transition period is critical for the health and productivity of dairy cows. Feeding high-grain diets immediately after parturition is necessary to support high milk production. However, high-grain diets are associated with acidic rumen pH and alterations in the composition of rumen microbiota in favor of pathogenic Gram-negative and Gram-positive bacteria which releases considerable amounts of lipopolysaccharide (LPS) and lipoteichoic acid (LTA) in the rumen fluid. There is evidence for translocation of LPS into the host systemic circulation causing multiple metabolic and immune disturbances; however, no reports related to the fate of LTA in the rumen have been reported. Both LPS and LTA have been implicated in the etiopathology of multiple diseases in dairy cows and other animals. However, no strategies on how to prevent their deleterious effects have been explored so far in dairy cows. We hypothesized that mucosal (i.e., oral or nasal) vaccination against LPS and LTA before parturition, would stimulate mucosal immunity and confer protection to dairy cows. The overall aim was to enhance the immune competence and lower the occurrence of most frequent periparturient diseases after parturition. To reach this goal two studies were conducted. In study 1, 100 dairy cows were vaccinated several times oronasally either with saline (controls) or with increasing doses of LPS from *Escherichia coli* 0111:B4 before parturition. Thirty cows out of 100 were selected for intensive sampling. Results from this study indicated that oronasal treatment with LPS modulated plasma metabolic and immune profiles, increased total salivary and vaginal mucus immunoglobulin-A (IgA), lowered the incidence of selected

periparturient diseases, and improved overall productivity of dairy cows. In study 2, 30 transition dairy cows were treated several times orally before parturition either with saline (controls) or with increasing doses of LPS from *E. coli* 0111:B4 and a flat dose of LTA from *Bacillus subtilis*. Results from this trial demonstrated that the treatment increased salivary and vaginal mucus total IgA, modulated selected plasma metabolites, decreased acute phase proteins and pro-inflammatory cytokines as well as lowered the incidence of lameness, metritis, and retained placenta. Results of these studies are encouraging and warrant further research.

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**Summera Iqbal**

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| | |
|---|---|
| APCs | Antigen presenting cells |
| APP | Acute phase protein |
| APR | Acute phase response |
| ARA | Acute-ruminal acidosis |
| BALT | Bronchus-associated lymphoid tissue |
| BCR | B-cell receptor |
| BCS | Body condition score |
| BHBA | Ɓ-hydroxy butyric acid |
| BIC | Bayesian information criteria |
| BM | Bone marrow |
| CARD | Caspase recruitment domains |
| CGRP | Calcitonin-gene related peptide |
| CP | Crude protein |
| CRP | C-reactive protein |
| CTR | Cows administered with saline solution |
| DAP | Diaminopimelic acid |
| DC | Downer cow |
| DCAD | Dietary cationic-anionic ions |
| DCs | Dendritic cells |
| DCS | Downer cow syndrome |
| DRTC | Dairy research and technology centre |
| ECM | Energy-corrected milk |

| | |
|---|---|
| FA | Fatty acid |
| FAE | Follicle-associated epithelium |
| FCM | Fat-corrected milk |
| FO | Follicular |
| GALT | Gut-associated lymphoid tissues |
| G-CSF | Granulocyte-colony stimulating factor |
| GIT | Gastro-intestinal tract |
| GN | Gram negative |
| GNB | Gram negative bacteria |
| GP | Gram positive |
| GPB | Gram positive bacteria |
| GPI | Glycosylphosphatiddylinositol |
| HDL | High density lipoprotein |
| Hp | Haptoglobin |
| HRP | Horseradish peroxidise |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| iNOS | Inducible nitric oxide synthase |
| INT chloride | 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium |
| ISCOMS | Immunostimulating complex |
| Kdo | 2-keto-3-deoxyoctonic acid |

| | |
|---|---|
| LAL | *Limulus Amebocyte* lysate |
| LBP | Lipopolysaccharide binding protein |
| LDA | Left displaced abomasum |
| LPL | Lipoprotein lipase |
| LPS | Lipopolysaccharide |
| LRRs | Leucine rich repeats |
| LSM | Least-squares means |
| LTA | Lipoteichoic acid |
| LTB4 | leukotriene B4 |
| MALT | Mucosa-associated lymphoid tissue |
| MCP-1 | macrophage chemoattractant protein-1 |
| MDP | Muramyl dipeptide |
| MEC | Mammary epithelial cells |
| MEE | Milk energy efficiency |
| MEHA | 3-methyl-N-ethyl-N-β-hydroxy ethyl-O-aniline |
| MFE | Milk fat efficiency |
| MHC | Major histocompatibility complex |
| MLN | Mesenteric lymph nodes |
| MMP | Matrix-metalloproteinase |
| MPLA | Monophosphoryl lipid A |
| MUN | Milk urea nitrogen |
| MyD88 | Mmyeloid differentiation primary response gene 88 |
| MZ | marginal zone |

| | |
|---|---|
| NALT | Nasal-associated lymphoid tissue |
| NAs | Natural antibodies |
| NEB | Negative energy balance |
| NEFA | Non-esterified fatty acids |
| $NE_L$ | Net energy of lactation |
| NLRs | NOD like receptors |
| NO | Nitric oxide |
| NOD | Nucleotide oligomerisation domain |
| NRC | National research council |
| O-factors | O-antigenic epitopes |
| PC | Plasma cells |
| PDIFF | Probability difference |
| PEPCK | Phosphoenolpyruvate carboxykinase |
| PGE2 | Prostaglandin E2 |
| PRRs | pathogen recognition receptors |
| R-LPS | Rough lipopolysaccharide |
| ROS | Reactive oxygen species |
| RP | Retained placenta |
| SAA | Serum-amyloid A |
| SARA | Sub-acute ruminal acidosis |
| SCC | Somatic cell count |
| SCFA | Short chain fatty acids |
| SED | Sub-epithelium dome |

| | |
|---|---|
| SEM | Standard error of the mean |
| sIgA | Secretory IgA |
| S-LPS | Smooth lipopolysaccharide |
| TAG | Triacylglycerols |
| TD | T-cell dependent |
| TG | Triglyceride |
| TGF-Ɓ | Transforming growth factor |
| TI | T-cell-independent |
| TIRAP | TIR-containing adaptor protein, also known asMAL (MyD88-adaptor-like) |
| TLRs | Toll like receptos |
| TMR | Total mixed ration |
| TNF-α | tumour necrosis factor- α |
| TRAM | TRIF-related adaptor Molecule |
| TRIF | TIR-containing adaptor-inducing IFN (interferon)-$\beta$ |
| TRT | Cows administered saline with LPS or LTA |
| UE | Udder Edema |
| VLDL | Very low density lipoproteins |
| WBC | White blood cells |

# Chapter 1

# Introduction

In recent years, metabolic diseases in dairy cows have become a frequent problem affecting one in every two cows in a herd which results in enormous economic losses for dairy producers (Ingvartsen, 2006). Indeed, transition time frame of 3 weeks prepartum until 3 weeks postpartum often involves metabolic rearrangements which lead to multiple diseases postpartum. Periparturient abnormalities are commonly linked to some metabolic element (Wells et al., 1977); however, in recent years it is evident that feeding ruminants with a high-grain diet is associated with the release of large amounts of endotoxin in the rumen fluid (Emmanuel et al., 2008) and initiated major changes in the composition of rumen microbiota (Dunlop, 1972; Mackie et al., 1978). Earlier studies from our group and others indicated that feeding increasing proportions of cereal grains results in a 14- to 20-fold increase in the concentration of lipopolysaccharide [LPS; a cell wall component of Gram negative bacteria (GNB)] in rumen fluid (Andersen et al., 1994; Emmanuel et al., 2008). Also, a recent study from us revealed that higher grains based diet not only enhances release of rumen endotoxin but also initiated release of other plasma inflammatory markers (Zebeli et al., 2012a).

Recent research clearly demonstrated that endotoxin translocates through the rumen and colon tissues (Emmanuel et al., 2007) into the systemic circulation, and causing a variety of metabolic as well as immunologic alterations (Zebeli et al., 2011a; Khafipour et al., 2009a), and modulation of mineral metabolism

(Zebeli et al., 2010). Furthermore, LPS has been suggested to be involved in the etiology of multiple periparturient diseases of dairy cows such as lameness, fatty liver, retained placenta (RP), milk fever, and downer cow syndrome (DCS; Ametaj et al., 2010a). Most importantly, a new line of investigation by our team indicated a strong relation between rumen LPS and mediators of acute phase response (APR) with milk fat yield and efficiency in dairy cows fed high barley grain based diets (Zebeli and Ametaj, 2009). Furthermore, in a more recent study we showed that when cows were experimentally induced intermittent endotoxemia it modulated selected plasma metabolites, several acute phase proteins (APP), plasma minerals, and anti-LPS antibodies as well as it was associated with high incidence of left displaced abomasum (LDA) and RP (Zebeli et al., 2011b).

Analogous to LPS, lipoteichoic acid (LTA) is considered to be the counterpart of LPS and cell wall component of Gram positive bacteria (GPB). The concentration of LTA in the rumen might also increase during high-grain feeding, and has been suggested to induce inflammatory responses (Timmerman et al., 1995; Bone, 1994). A few recent studies indicated that intra-mammary infusions of LTA, from *Staphylococcus aureus*, induced clinical mastitis at the dose of 100 μg/quarter and a subclinical inflammatory response at 10 μg/quarter (Rainard et al., 2008). Another study reported that intra-mammary challenge with LPS from *E. coli* and LTA from *Staph. aureus*, induce different immune responses *in vivo* in mammary tissue (Wellnitz et al., 2011). In addition, a novel functional genomics approach was used to investigate the direct roles of *E. coli* derived LPS or *S.*

*aureus* derived LTA on mammary epithelial cells (MEC), and it was reported that both immunogens together elicit a complex and more robust immune response (Daly et al., 2009).

The major source of bacterial toxins in dairy cows is considered to be the mucosal surface of gastrointestinal tract, whereas other sources might be the uterus and mammary gland during infections related to GNB and GPB in transition dairy cows (Wenz et al., 2001a, b; Ametaj et al., 2010a). It is known that most conventional vaccines injected subcutaneously or intravenously are generally poor inducers of mucosal immune responses, thus there is need to target mucosal immunity which induces more efficient immune exclusion, mediated principally by immunoglobulin(Ig)-A antibodies (Levine, 2003; Brandtzaeg, 2003; Neutra and Kozlowski, 2006). In a pilot study we observed that cows administered orally with increasing doses of LPS stimulate humoral immune responses, and most importantly, they modulate concentrations of plasma anti-LPS antibodies in peri-parturient dairy cows (Ametaj et al., 2012b). Also, a companion study indicated that repeated oral challenge with increasing doses of LPS modulates profile of plasma metabolites and minerals postpartum in dairy cows (Zebeli et al., 2013). However, there has been no attempt to vaccinate cows with oral LTA and LPS in order to evaluate their protective effects against periparturient diseases in transition dairy cows. Furthermore, this was the first attempt to introduce nasal route of immunization along with oral challenge in dairy cows.

In these studies we treated dairy cows oronasally with LPS alone from *Escherichia coli*, and orally with LPS and LTA from *Bacillus subtilis*. These investigations were conducted into two phases. The first phase was conducted to evaluate whether repeated oronasal application of LPS before parturition would modulate plasma metabolites, increase humoral immune responses, enhance the overall health status and productivity and alleviate periparturient diseases related to endotoxin. In the second phase we tested the hypothesis that repeated oral treatment against both LPS and LTA would further lower the incidence of most prevalent periparturient diseases, which couldn't be addressed by the LPS alone, as well as further enhance humoral immunity, overall productivity and metabolic health of dairy cows.

# Chapter 2

# Literature review

## 2.1 Nutrition and composition of gastrointestinal microbiota

Bovine rumen constitutes a diverse and interdependent population of different kinds of microbiota, which are highly competitive. These microbes exhibit unique capabilities to digest cellulose-rich feedstuffs and to convert them into a wide range of compounds important for body maintenance and milk production. Moreover, keeping a stable and healthy rumen microbiota is critical for maintaining the health of the cows (Nocek, 1997; Emmanuel et al., 2008) and high milk quality (Jenkins and McGuire, 2006). Any disturbances in the balance of rumen ecosystem may lead to development of disease in the host.

Recent research indicates a strong association between feeding diets rich in readily available carbohydrates and major alterations in the composition of microbiota in the gastrointestinal tract (GIT; Tajima et al., 2001; Khafipour et al., 2009b). These types of diets also have been associated with greater incidence of periparturient diseases in cows or other mammals (Ametaj et al., 2005a; Amar et al., 2008; Cani and Delzenne, 2009a, b). Although, the exact mechanism(s) underlying the feeding of large amounts of cereal grains with the high incidence of periparturient diseases is not completely understood, there is increasing efforts to comprehend this relationship.

Recent data illustrated that dairy cows fed large amounts of dietary energy around parturition experience greater incidence of fatty liver and ketosis postpartum, which is associated with activation of acute phase response (APR;

Ametaj et al., 2005a; Loor et al., 2006). Earlier studies gathered considerable evidence that rumen of the cows fed high-grain diets becomes severely acidic as a consequence of a shift in bacterial population in favor of starch digesting amylolytic bacteria, predominantly lactate producing bacteria (i.e., *Streptococcus bovis* and *Lactobacillus* ssp.), which lower the ruminal pH to acidotic levels (i.e., below 5.5; Mackie et al., 1978; Russell and Rychilik, 2001). Furthermore, research shows that sub-acute ruminal acidosis (SARA) induced by grain based diets supports the growth of *Streptococcus bovis, Escherichia coli,* and *Megasphaera elsdenii* in the rumen fluid (Goad et al., 1998). In addition it was reported that high energy diets that favor an increase in the Gram-negative (GN) to Gram-positive (GP) ratio in the rumen initiate the release of considerable amounts of endotoxin and activation of an inflammatory response as reflected by activation of APR (Ametaj et al., 2005a; Loor et al., 2006; Emmanuel et al. 2008).

### 2.1.1 High energy diets and generation of harmful compounds in the rumen

Although there is a strong interrelationship between rumen metabolism and host health status, it is not yet clearly understood which of the compounds generated by the activity of rumen microbiota pose health risks to the cows under conditions of high grain feeding. Moreover, little is known with respect to microbial-mediated changes in the rumen metabolism during the feeding of high cereal grains in the diet and there is a need to explore the mechanisms involved. So far, most conventional studies addressing rumen metabolism, have focused on dietary effects on a single class of rumen compounds, such as short-chain fatty

acids (SCFA; Kristensen et al., 1998). On the other hand, published results related to rumen metabolome indicate that a whole variety of compounds including endotoxin, methylamine, ethanol, propionate, glucose, alanine, maltose, propionate, uracil, valerate, xanthine, lysine, leucine, phenylacetylglycine, nicotinate, glycerol, fumarate, butyrate, valine, phenylacetate N-nitrosodimethylamine, and dimethylamine are released in the rumen as a result of microbial shifts during high grain feeding, which might be involved in triggering an inflammatory state in the host (Ametaj et al., 2010b).

Out of these harmful compounds, our team has provided a direct evidence for involvement of endotoxin or its inflammatory mediators in multiple perturbations including metabolic responses as well as peripartum diseases of dairy cows (Ametaj et al., 2010a). In a series of recent experiments it was demonstrated that modulation of rumen metabolism, following high-grain engorgement, favors an increase in the GN to GP bacterial ratio in the rumen, which is associated with the release of a considerable amount of lipopolysaccharide (LPS) causing chronic metabolic endotoxemia (Ametaj et al., 2005a; Loor et al., 2006; Khafipour et al., 2009a). Recent studies conducted by our team and others demonstrated a 14- to 20-fold increase in the concentration of free endotoxin in the rumen fluid following high-grain feeding (Nagaraja et al., 1978; Andersen et al., 1994; Emmanuel et al., 2008; Ametaj et al., 2010a).

Besides the role of endotoxin in development of inflammatory responses, it is also evident that most Gram-positive bacteria (GPB) contain considerable amounts of LTA in their cell wall, which is a major immunostimulatory molecule

and attributed to a number of biological activities in the host as well, but its role in the pathogenesis of animal diseases remains unclear. It is thought to be a potential virulence factor and involved in the inflammatory processes; sharing many of its pathophysiological properties with LPS (Nau and Eiffert, 2002). Thus, it is speculated that these toxic compounds released by both Gram-negative bacteria (GNB) and GPB might be involved in the pathogenesis of many metabolic disease by initiating inflammatory processes in the body.

## 2.2 Structural features of bacterial endotoxins

Lipopolysaccharide and LTA are the main building blocks of the outer leaflets of bacterial cell wall membranes and essential for stability and growth of GNB and GPB, respectively. They also are considered to be both endotoxins.

### 2.2.1 Structural features of lipopoplysacharide

In the late 1800, Richard Pfeiffer was the first to describe endotoxin or LPS as a heat-stable, cell-associated material isolated from *Vibrio cholera*, which was able to induce toxic reactions in guinea-pigs (Pfeiffer, 1892). As a component of the outer cell membrane, LPS is essential for the survival of GNB, and protects the bacterium from host antibacterial compounds like bile acids and hydrophobic antibiotics. Lipopolysaccharide is commonly released during bacterial growth or lysis and plays a major role during severe GN bacterial infections in the infected host (Dal Nogare, 1991; Burvenich et al., 2007).

The cell wall of GNB is composed of three layers, i.e., the outer membrane having asymmetric bilayer structure containing porin proteins, receptor molecules, and LPS moieties. The outer membrane is connected to the

cytoplasmic membrane at adhesion sites and is tied to the peptidoglycan by lipoproteins. The area between the external surface of the cytoplasmic membrane and the internal surface of the outer membrane is referred to as the periplasmic space containing a variety of hydrolytic enzymes and lytic virulence factors. Whereas, the inner leaflet contain mostly phospholipids. The chemical components of the classic molecule of LPS are a hydrophobic domain, known also as lipid A, a core oligosaccharide, and an O-antigen (a distal polysaccharide; Raetz and Whitfield, 2002). Usually the O-specific chain is a heteropolysaccharide, and comprises repeated units of sugar monomers (between 2 and 8), which define the variety of O-antigenic epitopes (O-factors) and represent the binding sites of the highly specific endotoxin antibodies (Jann and Jann, 1984). One repeating unit might include several such O-factors or alternatively an O-factor might be determined by a single monosaccharide in the O-chain (Figure 2-1; Modified from Raetz et al., 1991; Rietschel, 1996).

The core polysaccharides consist mainly of *N*-acetylglucosamine, glucose, galactose, heptose, phosphate, and ethanolamine. The core polysaccharides are linked through their 2-keto-3-deoxyoctonic acid (Kdo) to the glucosamine of the lipid A moiety via a glycoside bond. The lipid A is a highly conserved structure of the LPS molecule and is attached to a backbone and contains a dimer of phosphorylated glucosamine bound to O- and N-linked saturated long-chain fatty acids or hydroxy fatty acids that account for about half the lipids in the outer bilayer (Rietschel et al., 1971; Lüderitz et al., 1973). It has been observed that small variations among different bacterial strains and natural and synthetic

variants of lipid A determine the endotoxic activity of endotoxin (Nau and Eiffert, 2002; Andersen, 2003).

### 2.2.2 Heterogeneity in the structure of the LPS and host response

Conservation within the LPS structure serves as the basis for the host identification and response by creating molecular signals. Recognition of the role of toll-like receptors (TLR) and co-associated molecules have revealed that even subtle differences in conserved LPS structures are recognized by the host cells and capable of blocking or down-regulating the response to other LPS forms. It is evident that naturally occurring LPS heterogeneities result from either altered bacterial growth or environmental conditions, leading to altered host immune responses (Dixon and Darveau, 2005). In the following sections we describe the main variations reported in the structure of the LPS molecule.

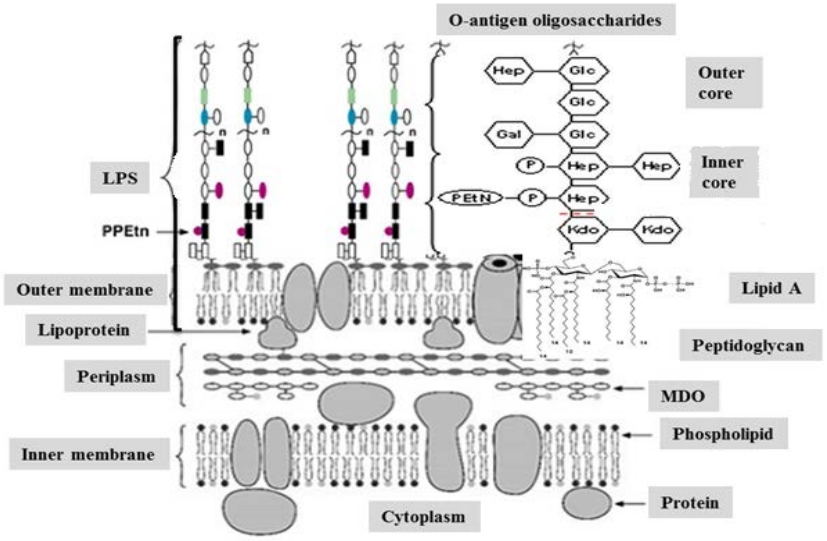


**Figure 2-1.** The composition of a Gram-negative bacterial membrane lipopolysaccharide having three distinct components; lipid A, oligosaccharide core, and O-antigen (Adapted from Raetz et al., 1991; Rietschel, 1996 with modifications); Kdo (2-keto-3-deoxyoctonic acid); MDO (membrane-derived oligosaccharides).

#### 2.2.2.1 Heterogeneity in O-antigen

Relative to the other structural components, the side chain (i.e., O-antigen) differs widely among GN bacterial strains and determines their antigenic specificity. The side chain is composed of repeating units of oligosaccharides; and their sequence, type, nature, ring form, substitution, and type of linkage vary among different bacterial strains (linear tri-saccharides or branched tetra- or penta-saccharides, may be more than 20) and all these differences contribute to the antigenic specificity of the LPS molecule. Therefore O-antigen is used for serological typing of GNB. Two distinctive phenotypes of LPS are recognized as smooth form of LPS (S-LPS) with fully expressed O-specific chain, and considered to be more virulent, whereas the rough form of LPS (R-LPS) has a substantially reduced or a missing outer core section and is known to be less virulent (Rietschel et al., 1982; Rietschel, 1996).

#### 2.2.2.2 Heterogeneity in oligosaccharide core

The last two sub-domains of the polysaccharide portion of the LPS are subdivided into the outer-core region that is proximal to the O-specific chain, containing common hexoses, and an inner-core region linked to the lipid A fraction, consistsing of one to three molecules of Kdo as well as two heptose residues, making this structure a more uniform one than the O-specific chain. However, it should be kept in mind that ethanolamine and phosphate residues might bring some variation among different strains of GNB (Sonesson et al., 1994).

#### 2.2.2.3 Heterogeneity in Lipid A structure

Lipid A is the endotoxic part of the LPS and it's considered to be the highly conserved structure of the molecule. However, recent studies with the LPS from a wide range of GNB strains revealed that the immunostimulatory capacity of the lipid A can be modulated by various compounds. For example, by substituting the phosphate groups with other chemical moieties (e.g., phosphoethanolamine), or modifies the disaccharide backbone as well as varying the acylation pattern. Furthermore, the length and number of acyl groups also contribute, given that these factors are associated with the charge and the three dimensional conformation of the lipid A. This indicates that all bacterial species carry unique LPS molecules with some of the variations residing in the lipid A moiety as well (Sonesson et al., 1994; Rietschel et al., 1996; van Amersfoort et al., 2003; Dixon and Darveau, 2005).

It is evident that the *E. coli* LPS molecule contains 6 fatty-acid residues (4 of these fatty acids carry a hydroxyl group on the third carbon, whereas the other 2 are not hydroxylated), which are linked to two phosphorylated glucosamine sugars and it is highly toxic (van Amersfoort et al., 2003; Dixon and Darveau, 2005). The conical-shaped structure of *E. coli* LPS binds strongly to lipopolysaccharide-binding protein (LBP) and stimulates TLR-4 for downstream signaling and cytokine production, whereas the strictly cylindrical-shaped LPS from *Rhodobacter sphaeroides* binds poorly to LBP and does not induce cytokine expression (Netea et al., 2002). Contrary to this, the LPS from *Porphyromonas gingivalis* has an intermediate conformation and causes differential stimulation of TLR-2, TLR-1/TLR-2 complexes, and the TLR-4 by *Salmonella* spp., resulting in

different gene transcription and cytokine induction (Figure 2-2; Modified from Netea et al., 2002).

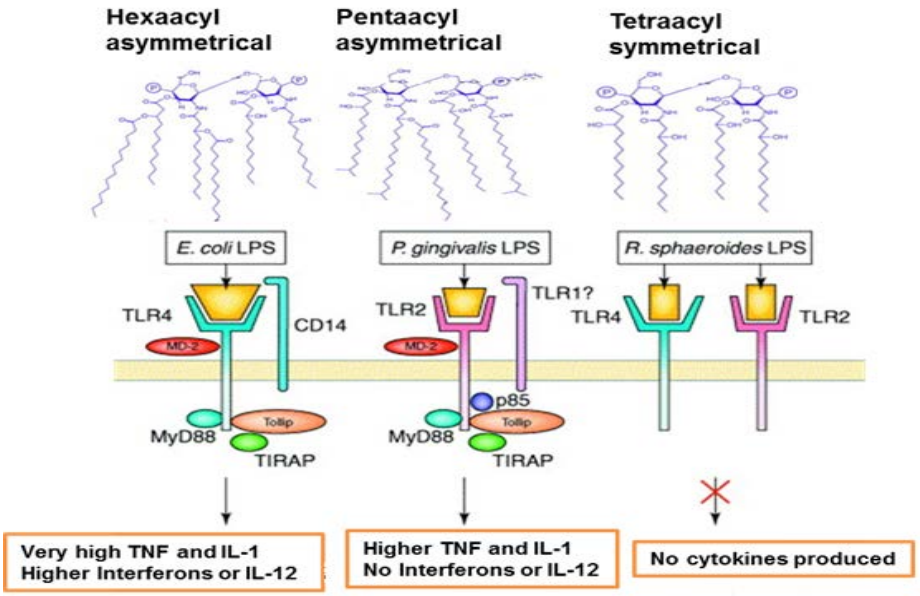


**Figure 2-2.** Three-dimensional conformation of different lipid A structures: (a) conical shape; (b) intermediary form; (c) cylindrical conformation bound differently to TLR's and consequently produce different immune response (Modified from Netea et al., 2002).

### 2.2.3 Structural features of lipoteichoic acid

Gram-positive bacteria possess a highly dynamic cell-wall structure, which is constantly remodeled and is critical for their survival. Most GPB contain LTA, which accounts for up to 50% of its dry weight, and is considered as the surface-reactive antigen, which mediates the attachment of certain bacteria to host tissues. Lipoteichoic acids are amphiphile molecules anchored in the cytoplasmic membrane by hydrophobic interactions. Although the LTAs are heterogeneous in their molecular composition their core structure is similar across different GPB species. Lipoteichoic acid resembles LPS in certain aspects and can therefore be considered the GP counterpart of the LPS. Comparison of the basic structure of

the LTAs from various species has revealed that it contains a single unbranched polyglycerophosphate chain phosphodiester-linked to the non-reducing hexapyranosyl residue of the diacylglycerol moiety. If we look into the details of this complex picture it contains glycerol residues joined through phosphodiester linkages, carrying one or more amino acid or sugar residues. The repeat units are joined by 1,3- or 1,2-linkages in the case of glycerol, and 1,5-linkages for ribitol. Out of all repeat units 10 or fewer are common. The glycerol or ribitol may also join to a sugar residue such as glucose, galactose, or *N*-acetylglucosamine. The GP bacterial cell wall is a porous structure (permeable to proteins as large as ~50 kDa) and is released into the environment during its growth, lysis, and presence of certain antibiotics (van Amersfoort et al., 2003).

### 2.2.4 Heterogeneity in the structure of LTA and host response

There is marked interspecies differences in the structure of LTA, particularly regarding the length of the acyl chains and carbohydrate composition of the glycerophosphate tail. It was observed that the number of the long tail repeating 1,3-linked glycerophosphate units, which is connected to the glucoside moiety, varies widely depending on the species, strain, and growth conditions. For example, it generally ranges between 4 and 30 glycerol-1-phosphate repeating units for the *S. aureus* and 22 for the *Bacillus subtilis*. Furthermore, *B. subtilis* strains are substituted to different extents at their repeating units with α-N-acetylglucosamine and D-alanine residues and are covalently linked to lipid anchors having a common structure, β-gentiobiosyldiacylglycerol. Moreover, the substitution of D-alanine at the 2 position of the glycerophosphate tail depends on

the species, strain, growth conditions and stage of growth (Figure 2-3; Modified from van Amersfoort et al., 2003).

## 2.3 Sources of bacterial endotoxins

### 2.3.1 Gastrointestinal tract

Bovine rumen is a complex anaerobic microbial ecosystem that is dependent on nutrient intake; and following high-grain feeding there are major shifts in the profile of rumen microbiota. It is well known that diets rich in starch favor the growth of several species of GPB and GNB like *Proteobacteria*, *Megasphaera elsdenii*, *Streptococcus bovis*, *Selenomonas ruminantium*, *E. coli*, and *Prevotella bryantii* (Nagaraja et al., 1978; Fernando et al., 2010; Ametaj et al., 2010a). Different sources of bacterial endotoxins are illustrated in Figure 2-4 (Suggested by Ametaj et al., 2010a).

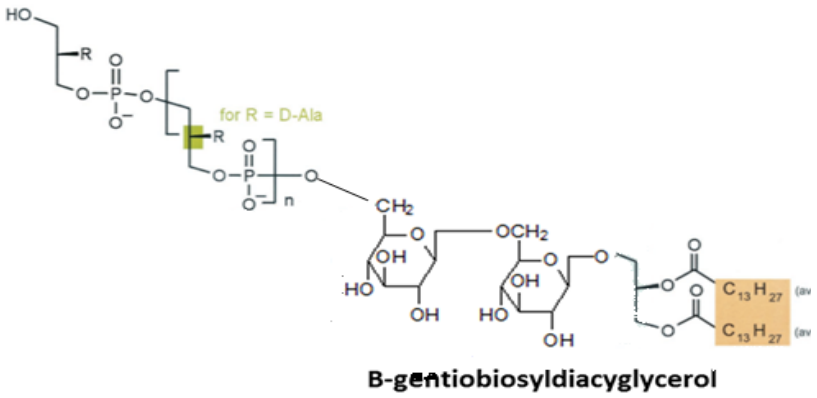


**Figure 2-3.** Structure of the LTA from *Bacillus subtilis*; Lipid anchor having a common structure β-gentiobiosyldiacylglycerol attach to fatty acid chains with an average composition of $C_{13}H_{27}$ and repeating unit of glycerol-1-phosphate, with the number of repeating units, "n", equal to about 22 (Modified from van Amersfoort et al., 2003).

These alterations in rumen microbiome due to abrupt dietary shifts towards high-grain diet generate the potential pool of free endotoxin in the rumen

fluid at the onset of lactation, and low ruminal pH is considered to be the major contributory factor. It is evident that more acidic pH in the rumen fluid (< 5.8) affects metabolic processes, change the cell membrane of various rumen bacteria and disturb bacterial ecological balances. This ultimately leads to the bacterial lysis and release of their immunogens in the rumen fluid as well as their subsequential entry into the body (Ametaj et al., 2010a; Dong et al., 2011). Our recent study revealed a strong negative relationship between preprandial rumen pH and endotoxin, which explains 64% of the variation in the concentration of endotoxin in the rumen fluid following high grain feeding (Ametaj et al., 2010a).

In a recent study we observed that during transition period the amount of endotoxin in the rumen fluid increased 8- to 14-fold during feeding of diets with 30 and 45% barley grain, respectively, compared to low-grain diets (0% and 15% barley grain on a dry matter basis; Emmanuel et al., 2008). In a similar study, cows were challenged by increasing the proportion of grain in the total mixed ration (TMR) from 50% to 60%, which resulted in a four-fold increase in the concentration of free endotoxin in the rumen fluid, from 28,184 to 107,152 endotoxin units (EU)/mL (Khafipour et al., 2009a).

### 2.3.2 Mammary gland

In an earlier study, Anderson (2003) stated that endotoxicosis in the systemic circulation could also derive from GNB infections during mastitis. Later, Rainard and Riollet (2005) observed that milk macrophages are stimulated by *E. coli* LPS. Furthermore, mammary epithelial cells (MEC) are in direct contact with invading bacteria and release chemotactic and inflammatory mediators following

bacterial encounter and recognition. Thus, not only local inflammatory signs are strongly related to presence of endotoxin, but also a systemic APR is associated with increased translocation of endotoxin from the mammary gland into the systemic circulation (Burvenich et al., 2007). Later, several LPS-mastitis models were able to show translocation of endotoxin from the mammary gland into the blood stream resulting in endotoxemia (Lehtolainen et al., 2003; Hiss et al., 2004).

It is well known that infection induced by *E. coli* is acute but normally cleared by immune system within a few days compared with that resulting from *Staphylococcus aureus*, which is less severe but chronic and persists for the life of the animal indicating the involvement of GPB in the severity of mammary gland disease (Strandberg et al., 2005). It has been shown that bovine MEC express mRNA for both TLR2 and TLR4, and their expression was increased in quarters suffering from subclinical, moderate or severe infections (Strandberg et al., 2005).

When LTA, purified from *Staphylococcus aureus*, was infused into the lumen of the mammary gland it was able to induce clinical mastitis at the dose of 100 μg/quarter, whereas a subclinical inflammatory response was observed at 10 μg/quarter (Rainard et al., 2008). Meanwhile, intramammary co-administration of both LPS from *E. coli* and LTA from *Staphylococcus aureus*, induce different immune responses *in vivo* in milk immune cells and mammary tissue (Wellnitz et al., 2011). A recent investigation indicated the direct roles of *E. coli*-derived LPS, or *S. aureus*-derived LTA on tammar MEC, stimulating a more complex and robust immune response and causing mastitis, suggesting that both these toxins are implicated in the etiology of the disease (Daly et al., 2009). Contrary to this,

previous *in vitro* study clearly demonstrated that stimulation of bovine primary MEC with LPS and LTA showed increased gene expression for LTA-stimulated cells. Furthermore, the unsustained cytokine response to LTA indicated its higher potential for chronic intra-mammary infection than GN infection. Thus, MEC have a strong but differential capacity to mount an innate immune response to different bacterial cell wall components (Boulanger et al., 2001).

### 2.3.3 Uterus

The healthy uterus is not a significant source of endotoxin in dairy cows; however, during uterine infections (i.e., metritis) and retained placenta (RP), the uterine lochia becomes a medium containing high concentrations of endotoxin. Dohmen et al. (2000) reported that cows with RP had 2.24 x $10^4$ endotoxin units (EU)/mL as compared to 0.10 x $10^4$ EU/mL in healthy cows (> 20-fold greater endotoxin concentration than healthy cows) immediately after calving. The same authors were not able to detect LPS in plasma. In line with these findings, Elmore et al. (1983) proved, in an experiment with ovulating ewes, that endotoxin did not translocate from its injection site intrauterine into the systemic circulation as indicated by the lack of alterations in the white blood cell counts (WBC), the *Limulus Amebocyte* lysate (LAL) assay, and rectal temperature at multiple sampling time-points after the injection. However, in a later study, Peter et al. (1990) found that endotoxin is readily absorbed from uteri of early postpartum cows (5 days postpartum), whereas at 20 d post-partum no endotoxin was found in the blood circulation. This leads to the conclusion that the uterus, for a short period of time post-partum, becomes vulnerable to endotoxin translocation.

## 2.4 Translocation of endotoxin into the systemic circulation

Previously, a number of studies showed no evidence of the ruminal absorption of endotoxin through lymph or blood following the ruminal infusion of $^{51}$Cr-labeled *E. coli* LPS, suggesting that the ruminal epithelium is impermeable to endotoxin (Nagaraja and Titgemeyer, 2007). However, other research showed that $Cr^{51}$ firmly attaches to the lipid A moiety of the endotoxin, rendering it much less diffusible by precipitating the LPS as an insoluble chromium complex (Sanford and Noyes, 1958; Lassman, 1980; Anderson, 1984). Thus, Ravin et al. (1960) when administered $^{125}$I-LPS, approximately 200 to 300 μg of the specific polysaccharide was recovered from the blood and liver following 8 to 10 hours after administration, showing that bioactive LPS is being absorbed from the gut and circulates through the body.

Translocation of LPS across the epithelium of GIT may occur across the epithelial cell membrane by an active endocytotic pathway (transcellular) or across the tight junction between cells through paracellular pathway (Wiest and Rath, 2003; Tomlinson and Blickslager, 2004). Chin et al. (2006) used intestinal epithelial cell lines in an *in vitro* study and concluded that an abnormal increase in luminal LPS from *E. coli* (O26:B6) enhanced nitric oxide (NO) production which converts into toxic peroxynitrite by combining with superoxide anions and inhibit the production of ATP; thus, leading to increased mucosal permeability. This also triggers epithelial cell death by apoptosis, which subsequently disrupts tight junction protein zonula occludens-1 and irreversibly block many components of the mitochondrial respiratory chain (Bossy-Wetzel and Lipton, 2003; Unno et al.,

1997). In addition, excessive NO levels lower $Na^+$ transport activity, which results in swelling and dysregulation of tight junctional protein expression and barrier failure (Han et al., 2004). Furthermore, under acidosis conditions, *E. coli* LPS impairs the pH regulatory system of enterocytes by impairing the activity of $Na^+$-proton pumps resulting in cytoplasmic acidification and cellular dysfunction (Cetin et al., 2004).

Deitch and Berg (1987) administered endotoxin intramuscularly or intraperitoneally and found its enhanced translocation from the gut to the mesenteric lymph node. They also observed a substantial difference in the effects of LPS on the barrier function of different parts of the GI tract due to different composition of epithelium.

Other less potential exposure routes for endotoxin are infected organs like udder and uterus. For example acute endometritis is known to be related to high LPS levels (Scholten et al., 1992; Dohmen et al., 1995), but the potential exposure takes place via translocation through a leaky GI barrier. Our *in vitro* study with an Ussing chamber system proved translocation of LPS through rumen walls at a higher rate than the colon tissue (Emmanuel et al., 2007).

### 2.4.1 Portal vein pathway

It is generally assumed that once endotoxin passes the mucosal barrier, it can freely enter into the systemic blood circulation. Endotoxin is then transported through the portal vein and into the liver where it is detoxified and removed from the blood circulation (Ametaj et al., 2005b).

#### 2.4.1.1 Activation of APR

Presence of endotoxin in the blood stream has been associated with a general, non-specific immune response known as APR (Werling et al., 1996). In the liver, macrophages known as Kupffer cells are the first to respond to inflammatory conditions through the release of a variety of cytokines such as tumour necrosis factor-alfa (TNF-α), interleukin(IL)-1, and IL-6, which contribute to the production of acute phase proteins (APP) by liver hepatocytes (Ametaj et al. 2005b). The two most important APP that directly participate in the detoxification and removal of endotoxin are serum amyloid A (SAA) and LBP (Gallay et al., 1994; Cabana et al., 1999). Another APP is haptoglobin (Hp), which scavenges hemoglobin to prevent iron utilization by bacteria; whereas C-reactive protein (CRP) opsonizes the harmful compounds to facilitate their clearance through complement pathway (Wassell, 2000). All these APP are increased during feeding of high-grain diets in dairy cows, indicating mounting of an endotoxin-related inflammatory response (Emmanuel et al. 2008).

#### 2.4.1.2 Pathways for endotoxin clearance from blood stream

Two pathways have been suggested to be involved in clearance of endotoxin from the bloodstream depending on its concentration in the blood (Kitchens and Munford, 1998; Ametaj et al., 2010). At low concentrations of endotoxin, the first pathway that is initiated involves activation of macrophages, whereas at higher concentrations a second pathway based on release of various APP and lipoproteins is initiated (Gallay et al., 1994). It is well known that endotoxin in the plasma is either in a monomeric or aggregate form. The aggregates of endotoxin are developed by binding to the high concentration of

calcium in the blood circulation (Munford et al., 1981; Rosen et al., 1958). These endotoxin aggregates, then, bind to macrophages through mCD14 and TLR4, in order to clear them from circulation. Macrophages are activated once endotoxin binds to its surface receptors and start releasing pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 to initiate the innate immune response and help in neutralization and removal of endotoxin from circulation. However, chronic cytokine production is associated with high temperature and general sickness (Elsasser et al., 2008). The lipoprotein pathway is activated when concentration of endotoxin in the plasma is large, which requires endotoxin to be in monomeric form (Gallay et al., 1994). Lipopolysaccharide binding protein and sCD14 play a role in monomerization of LPS molecules and their transport to lipoprotein particles (Levels et al., 2001). The clearance of endotoxin from circulation is achieved through endotoxin-SAA-lipoprotein complexes and removed by liver hepatocytes and into the bile (Ametaj et al., 2010a). Endotoxin clearance routes are explained in more detail in Figure 2-4 (Suggested by Ametaj et al., 2010a).

### 2.4.2 Lymphatic pathway

Another possible pathway that endotoxin may enter the body is by being incorporated into chylomicrons and as such is transported through the lymphatic system to the mesenteric lymph nodes (MLN). Chylomicrons themselves have a high affinity for LPS; and hence they not only transport dietary long-chain fatty acids, but likely also LPS, from the gastrointestinal tract (Harris et al., 1993; Goshal et al., 2009). Chylomicron formation in the Golgi complex of the epithelial cells with a pool of cell-associated endotoxin promotes the transport of

LPS through MLN and thereby might initiate a low grade inflammatory response in the MLN (Goshal et al., 2009). On the one hand, the binding of absorbed LPS with chylomicrons lowers LPS toxicity and enhances its hepatic clearance (Harris et al., 1993); however, LPS content might aggravate inflammatory properties of chylomicrons (Goshal et al., 2009). In the case of over-excessive fat feeding this pro-inflammatory effect on chylomicrons could result in extra-hepatic LPS exposure and perhaps in an increase in the risk of metabolic or inflammatory diseases (Goshal et al., 2009).

## 2.5 Downstream signaling of LPS and LTA

### 2.5.1 Signal transduction through LPS

It is established that LPS signals via LBP, CD14, and TLR4. Toll-like receptor-4 is a crucial receptor for recognition of LPS, and forms a tight complex with MD-2, which is a protein on the cell membrane and this complex is necessary for induction of pro-inflammatory cytokines (Miyake, 2007). Lipopolysaccharide binding protein is present as a soluble protein in the plasma and binds LPS; whereas CD14 is a glycosylphosphatidylinositol (GPI)-linked protein containing leucine rich repeats (LRRs), which binds LBP and delivers the LPS-LBP complex to the TLR4-MD-2 complex on macrophages (Miyake, 2007).

Cells lacking CD14 cannot recognize smooth LPS; however, they still respond to rough LPS or lipid A. Toll-like receptor-4 is known to activate two signaling pathways, the myeloid differentiation primary response gene 88 (MyD88) dependant and the TIR-containing adaptor-inducing IFN (interferon)-$\beta$

(TRIF)-dependant pathway (Figure 2-5; Modified from Mitchell et al., 2007; Kumar et al., 2009).

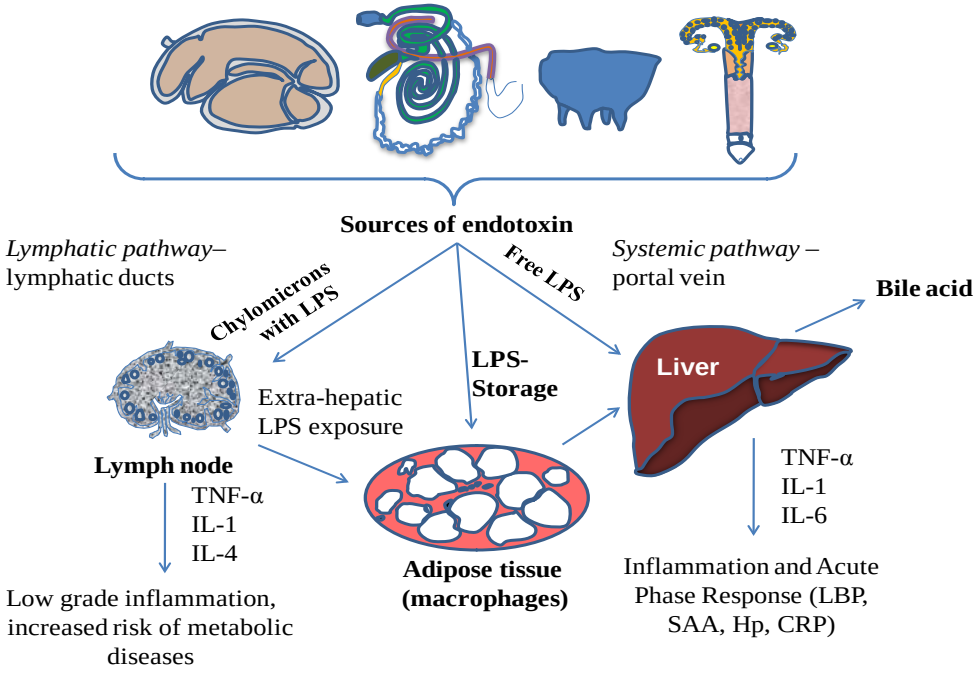


**Figure 2-4.** Sources of endotoxin translocation routes (Suggested by Ametaj et al., 2010a).

Lipid A as such can only signal through the MyD88-dependant pathway in the absence of CD14 (Jiang et al., 2005). Plaizier et al. (2012) suggested that the diversity of the structures of LPS among bacterial species might selectively influence activation of these pathways.

### 2.5.2 Signal transduction through LTA

Lipoteichoic acid induces signal transduction through the concerted action of CD36, CD14, LBP, and TLR2 (Hermann et al., 2002; Hoebe et al., 2005). There is diversity in the ligand recognition by TLR2 because it recognizes the ligands in association with structurally related TLR1 and TLR6. The downstream signaling of TLR2 is similar to that of TLR4 and they both use TIR-containing

adaptor protein (TIRAP) in addition to MyD88, which links TLR to MyD88. However, TLR4 uses TRIF and TRIF-related adaptor molecule (TRAM), whereas TRAM links TLR4 with TRIF, which is an additional feature for TLR4. The other additional component for the LTA signaling is class II scavenger receptor CD36, which is involved in phagocytosis and cytokine production in response to GPB and its cell wall components such as LTA suggesting that CD36 functions as a co-receptor of TLR2/6, as explained in Figure 2-5 (Modified from Mitchell et al., 2007; Kumar et al., 2009).

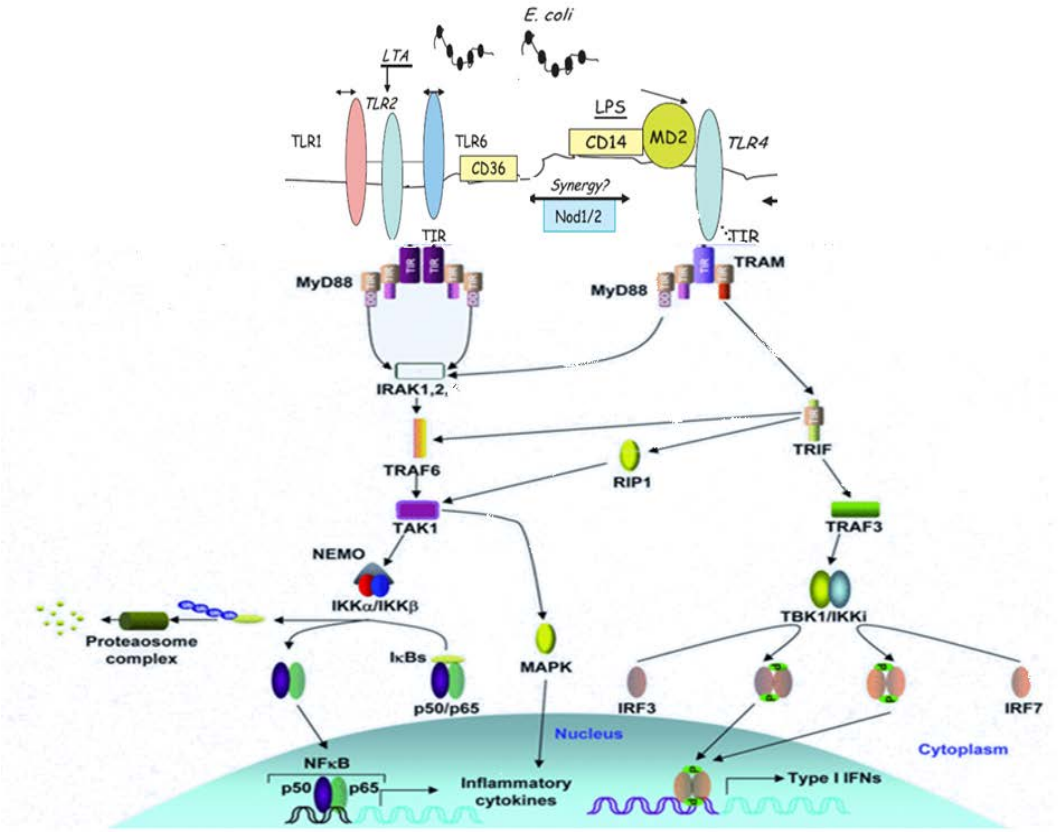


**Figure 2-5.** Signalling pathways employed by LPS from *Escherichia coli*, is manoeuvred to MD2 forming a complex that activates TLR4. LTA from *Staphylococcus aureus* is guided by CD36 to activate the TLR2/TLR6 complex. Synergy is often seen between TLR and NOD signalling pathways (Modified from Mitchell et al., 2007; Kumar et al., 2009).

## 2.6 Formation of inflammasome through LPS and LTA

Nucleotide oligomerization domain (NOD) proteins, containing leucine-rich repeats are called NOD like receptors (NLRs). NLRs are critically involved in sensing of pathogenic bacteria. They are proved to be pathogen recognition receptors (PRRs) for bacterial peptidioglycan and can recognize both GP and GN bacterial ligands. Two well-defined NLRs are NOD1 and NOD2. NOD1, also known as caspase recruitment domains (CARD4), senses diaminopimelic acid (DAP), which is mainly found in peptidioglycan molecules of GNB (Chamaillard et al., 2003). Whereas NOD2, also known as CARD15, mainly senses the muramyl dipeptide (MDP) present in both GP and GN peptdioglycan molecules (Inohara et al., 2003). Both NOD1 and NOD2 signal via adaptor protein RICK, which contains CARD like NOD1 and NOD2 that interact to form a signalling platform comprised of a number of proteins collectively known as the inflammasome. The downstream signalling cascade of inflammasome causes activation of caspase-1 and formation of pro-inflammatory cytokines such as IL-1β and IL-8 (Inohara et al., 2005; Mariathasan and Monack, 2007). The role of NLRs in pathogen sensing is suggested to be co-operation with TLRs (Takada and Uehara, 2006). NOD2 is important because of its main role in the maintenance of a healthy gut barrier, and it is evident that the lack of NOD2 compromises gut's barrier functions to bacterial invasion (Murillo et al., 2003, Abreu et al., 2005; Mitchell et al., 2007).

## 2.7 Innate and adaptive immune responses to LPS and LTA

After stimulation of the surface receptors upon invasion by GN and GP pathogenic bacteria, the complex downstream signaling cascade of the host response include production and release of proinflammatory and immunomodulating cytokines. Stimulation of surface receptors by LPS and LTA induce different patterns of cytokine release. It has been observed that LPS is a potent inducer of pro-inflammatory cytokines like IL-12, whereas LTA is a weaker inducer of pro-inflammatory cytokines and does not induce interferon (IFN)-γ; thus, does not mediate Th1-dependent responses (Hermann et al., 2002). The peak cytokine response in GPB infections occurs 50 to 75 h following the challenge, whereas it occurs 1 to 5 h after GNB infections, indicating that although LTA is similar with LPS in inducing inflammatory responses, its responses are delayed. Furthermore, LTA is a stronger inducer of chemoattractants such as IL-8, macrophage chemoattractant protein-1 (MCP-1), leukotriene B4 (LTB4), and the growth factor granulocyte-colony stimulating factor (G-CSF) than LPS (von Aulock et al., 2003). Moreover, LTA does not prime neutrophils for oxidative burst or degranulation (von Aulock et al., 2003); however, it induces pro-coagulant activity in monocytes (Mattsson et al., 2004) and activates the complement system via L-ficolin (Lynch et al., 2004).

**2.8 Alterations in rumen microbiota and metabolic diseases**

Epidemiological investigations have associated high concentrate feeding regimens to periparturient diseases and the main link between these two is the shift in rumen microbiome; however, information regarding the underlying mechanisms is scarce in ruminants. In this section, we will bring evidence

regarding the role of endotoxin in the etiopathology of periparturient diseases of dairy cows around parturition.

### 2.8.1 Acute- and sub-acute ruminal acidosis

Acute ruminal acidosis (ARA) occurs when ruminants consume excess amounts of grain or highly degradable starch, while the ruminal environment is not yet adapted to ferment and absorb the arising amounts of VFA adequately enough to keep the ruminal pH within physiological ranges, and may result in the death of the animal, severe illness, liver abscesses. Whereas, SARA is a well recognized digestive disorder that is characterized by extended periods of depressed ruminal pH below 5.5-5.6 (Kleen et al., 2003). It was established earlier that certain ranges of ruminal pH, after grain engorgement, are conducive to an increase in the free ruminal LPS concentration because of enhanced numbers of *E. coli* in the rumen (Diez-Gonzalez et al., 1998).

In recent experiments, two SARA models, one based on grain and the other on alfalfa pellets was developed, and it was noticed that only the grain model induced inflammatory responses although both models resulted in substantial accumulation of free LPS in the rumen, which excludes the possibility that all LPS are equally toxic. The authors reported that severe grain-induced SARA was dominated by *Streptococcus bovis* and *E. coli* in the rumen fluid (Khafipour et al., 2009 a, b). It is possible that the severity of SARA and the degree of inflammation might be influenced by the three-dimensional conformation of the LPS molecules from different GN bacterial species generated in the rumen. It is possible that the *E. coli* LPS, dominant in grain-based SARA

challenges, might be conical in shape and interacts differently with TLR and potentially intitiates stronger inflammatory responses (Khafipour et al., 2009b; Plaizier et al., 2012). On the other hand, *Bacteroidetes* phyla are the predominant microbiota in the rumen fluid, which release the majority of LPS potentially with low virulence, when animals are fed on high forage diet (Plaizier et al., 2012).

Earlier studies indicated that GPB might also be involved in the etiology of disease as indicated from *Streptococcus bovis* and other lactic acid-producing bacteria, which contribute to lowering of the rumen pH to acidic values by producing large quantities of lactic acid (Nocek, 1997; Owens et al., 1998). A recent study indicated that such disturbances in the rumen metabolism might lead to development of refractory states associated with immune suppression and increased susceptibility to various other diseases (Zebeli et al., 2012; Zebeli and Metzler-Zebeli, 2012). Thus, it is usually seen that SARA has been closely related with laminitis (Stone, 2004), displaced abomasum (Olson, 1991), and bloat (Cheng et al., 1998).

### 2.8.2 Fatty liver

Fatty liver is a metabolic disease of transition dairy cows, which is characterized by storage of fat (i.e., lipids) in the liver cells, which can occupy from 12 and up to 25% of the liver wet weight. Fatty liver is associated with inflammation, scarring, and hardening of the liver that frequently leads to the death of the cow (Jorritsma et al., 2000; Ametaj, 2005b). The conventional view considers fatty liver as excess triacylglycerols (TAG) arising from a negative energy balance (NEB) after parturition (Bauchart et al., 1998). This hypothesis

has been challenged recently, on numerous occasions, by our team. Most recently a review article published by Ametaj et al. (2010a) suggested that NEB develops in all cows around parturition, but only half of the cows are affected by fatty liver. In addition, fatty liver is observed in different periparturient diseases, which are not related to NEB (Ametaj, 2005a, b; Ametaj et al., 2010a). Furthermore, attempts to provide more energy to cows in NEB does not seem to prevent fatty liver but on the contrary increases the incidence rate of fatty liver (Ametaj 2005a, b; Grummer, 2008, Ametaj et al., 2010a).

Based on the latter observations, Ametaj et al. (2005a, b) hypothesized that endotoxin, might be involved in the etiology of fatty liver. Indeed cows suffering from fatty liver was reported to be in a state of inflammation as indicated by the presence of greater plasma concentrations of TNF-α and SAA (Ametaj et al., 2005a, b). It is known that once macrophages bind endotoxin, TNF-α and other pro-inflammatory cytokines are released (Gabay and Kushner, 1999; Ametaj et al., 2010a), which stimulate the release of APP like SAA, LBP, Hp, and CRP from hepatocytes. The most beneficial role of APP is to bind and neutralize endotoxin. Rapid removal of endotoxin from blood circulation through endotoxin-SAA-lipoprotein complexes and endocytosis of excessive lipoprotein particles by liver hepatocytes is the major cause behind the rapid accumulation of triglyceride (TG)-rich lipoproteins in the liver hepatocytes and possibly a major factor in the etiology of fatty liver (Ametaj et al., 2005a, b; Ametaj et al., 2010a). There are several recent observations supporting this newly established hypothesis. For example, Loor et al. (2005) detected increased expression of liver TNF-α in cows

suffering from fatty liver around parturition. Bradford et al. (2009) also recently demonstrated that consecutive injection of TNF-α for a week, even in cows in late lactation, induces TG accumulation in the liver and cause development of fatty liver.

### 2.8.3 Mastitis

Mastitis is inflammation of the mammary gland caused by multiple bacterial strains. The GP bacterium *Staphylococcus aureus* has a major influence on increasing somatic cell counts (SCC), which is indicative of the number of neutrophils in the milk and are commonly used as a determinant for the degree of inflammation in the mammary gland. However, a number of GNB, namely *Escherichia*, *Klebsiella*, and *Enterobacter* are etiological agents most often isolated from acute clinical cases of mastitis (Hogan and Smith, 2003).

Many studies used intra-mammary challenges with LPS to study the acute and clinical mastitis. During LPS-induced mastitis, elevated body temperature, increased heart and respiratory rates as well as increased milk SCC are commonly observed (Vels et al., 2009). Other responses to LPS include elevated plasma concentrations of Hp and immunoglobulin(Ig)-G, and decreased blood leukocyte counts and rumen motility (Perkins et al., 2002; Hiss et al., 2004).

In recent years it has been shown that when the lumen of the mammary gland is infused with purified LTA, from *Staphylococcus aureus*, symptoms of clinical mastitis at the dose of 100 μg/quarter and a subclinical inflammatory response at 10 μg/quarter develop (Rainard et al., 2008). However, when cows are infused intra-mammary with both LPS from *E. coli* and LTA from *S. aureus*, they

developed a more robust immune response against those bacterial antigens (Daly et al., 2009). There is a substantial difference in the gene expression profiles of bovine MEC to LPS and LTA, which suggests different activation pathways. This is evident from different cytokine responses measured in the milk from Holstein cows challenged with intra-mammary inoculations of *E. coli* or *S. aureus*. The lack of sustained proinflammatory cytokines responses following LTA challenge is consistent with the chronic nature of infections of the mammary tissue caused by GPB species (Strandberg et al., 2005).

### 2.8.4 Metritis, endometritis, and infertility

Metritis is the infection of the cavity, lining, and deeper layers of the uterus. Endometritis is a localized infection of the mucous membrane of the uterus, which during inflammation is seen covered with pus and excessive mucus as well as necrosis. Typically, 25-40% of the cows develop clinical metritis, in the first 2 weeks after calving, and the disease persists in up to 20% of animals in the form of clinical endometritis (Sheldon et al., 2008). The postpartum environment of the uterine lumen supports the growth of a variety of bacteria associated with the disease, but the most prevalent pathogens are *E. coli* (37%) and *A. pyogenes* (49%; Williams et al., 2005).

The delay in uterine involution and the following infertility due to endometritis has often been linked to endotoxin. It is evident that endotoxin in the uterine horns directly affects ovarian cell function. Williams et al. (2008a) measured *in vitro* that LPS and related cytokine TNF-α affects theca and granulusa cell and lowers androstenedione and estradiol production. In a follow

up *in vivo* experiment fewer animals ovulated following intrauterine infusion with LPS or TNF-α, which leads to the conclusion that LPS suppresses ovarian cell function (Williams et al., 2008b). Similarly, in the endometrium, *E. coli* LPS is responsible for prolonged luteal phase because of its association with prostaglandin E2 rather than prostaglandin F2α (Herath et al., 2009), thus lowering the probability of ovulation and conception. In a recent study LPS was used to treat mares suffering from persistent mating-induced endometritis by infusing 100 µg of LPS alone or together with two doses of oxytocin. The treatment increased the pregnancy rates of the mares compared to the untreated ones, where uterine inflammation persisted (Sharma and Dhaliwal, 2010). Different pathogens show varying effects on reproduction. For example, clinical mastitis caused by *Escherichia coli* and *Streptococcus* spp. is associated with a 50% lesser probability of conception (Wilson et al., 2008). An earlier study indicated that LTA also affects fertility in the same manner as LPS in mice; however, it required larger doses to show similar effects with those of LPS (Kajikawa et al., 1998).

### 2.8.5 Retained placenta

Retained placenta refers to the failure to expel the placenta within 24 hours postpartum (Kelton et al., 1998). The key event in the pathogenesis of RP is a failure of prompt breakdown of the cotyledon-caruncle attachment after delivery of the calf. Failure of placental detachment appears to be largely mediated by failure of the immune system to successfully degrade the placentomes at the end of the pregnancy (LeBlanc, 2008). Previous research work suggested the

involvement of endotoxin in the etiopathology of RP as evidenced from greater endotoxin levels in their uterine lochia compared to healthy cows postpartum (Dohmen et al., 2000).

Our research group reported an association of experimental intermittent endotoxemia with increased incidence of RP in postpartum dairy cows (Zebeli et al., 2011b). The causative role of LPS consists in the induction of reactive oxygen species (ROS), most importantly NO, particularly in bovine macrophages, which are directly related to the development of RP (Kankofer et al., 2005; Zelnickova et al., 2008). In an earlier study, Slama et al. (1994) investigated the effect of LPS on arachidonic acid metabolism in cows and confirmed that cows with RP, prostaglandin E2 (PGE2) became the most important metabolite secreted by allantochorion, especially in the presence of endotoxin.

It is also known that the infection of the uterus with *E. coli* appears to pave the way for subsequent infection with other bacteria or viruses as expression of caruncular endometrium of TLR2, TLR3, TLR4, TLR6, and TLR9 are increased 4-6 h postpartum (Sheldon et al., 2009). The latter findings indicate that these receptors might enhance the inflammatory process following binding to the bacterial ligands both from GPB and GNB species. The underlying mechanism might involve the reduced expression of cell adhesion molecule L-selectin following exposure to those bacterial ligands, which subsequently decreased the extravasations of neutrophils that play an important role in the expulsion of placenta (Ametaj et al., 2012a). The involvement of GNB, like *E. coli*, in cows

with RP has been established but further studies need to be conducted to address the role of LTA in the disease process.

### 2.8.6 Lameness

Lameness is the decrease in an animal's ability to bear weight on a limb. It also refers to the decrease in the normal mobility and function of a limb. Laminitis is one of the most common causes of lameness which is an inflammation of the dermal layers inside the foot. It occurs in acute, subclinical, and chronic forms. The subclinical form of the disease can be a long, slow, insidious process that is dependent upon persistency of low-grade insults (Nocek, 1997). The pathogenesis of lameness is believed to be associated with a disturbance in the microcirculation of blood in the corium, which leads to breakdown of the dermal-epidermal junction between the hoof and the third phalange (bone within the hoof; Pollit et al. 1998; Shearer and van Amstel, 2000).

Subacute ruminal acidosis and ARA are considered to be a major predisposing factor of lameness, and they presumably mediate its destructive effects through various vasoactive substances released in coincidence with the development of rumen acidosis (Shearer and van Amstel, 2000; Stone, 2004). Increases in the concentration of ruminal endotoxin were suggested, by some authors, in the etiology of lameness (Andersen, 2003; Ametaj et al., 2010a). In an earlier study, Boosman et al. (1991) investigated the effects of endotoxin administered systemically or locally in the hoof area and were able to develop laminitis in dairy cows. Later, it was shown that LPS infusion lowers digital perfusion. In this study, six horses were challenged with LPS and resulted in the

onset of digital hypoperfusion with increased levels of the platelet-derived mediators such as plasma serotonin and thromboxane B2 (Menzies-Gow et al., 2004). A recent report on acute laminitis, induced through feeding of oligofructose in an equine model, indicated the indirect involvement of endotoxin primarily in activating platelets and leukocytes, which in turn initiates the early inflammatory processes (Bailey et al., 2009).

It is becoming increasingly clear that LPS cannot fully trigger the aberrant signaling events leading to multi organ injury. In one study it was demonstrated that infusion of phenol-extracted LTA caused multiple organ failure in the anaesthetized rat, and this organ injury was associated with the induction of nitric oxide synthase (iNOS) and enhanced plasma concentration of cytokines like TNF-$\alpha$ and IFN-$\alpha$. It may be speculated that these bacterial components might damage the blood vessels through initiation of inflammatory processes in the hoof area. In addition, other factors such as methylamine and matrix-metalloproteinase-2 (MMP-2), and MMP-9 most likely contribute to the remodeling of the dermal layer of the lamina and blood vessel injury (Ametaj et al., 2010a). Therefore, a multicausal hypothesis is more likely to explain the etiology and etiopathogenesis of laminitis.

### 2.8.7 Displaced abomasum

Displaced abomasum is a repositioning of the abomasum from its normal position (Harris and Shearer, 2003). The risk factors in the etiology of displaced abomasum are: (1) a prepartum depression of feed intake followed by a slow increase postpartum causing lower ruminal fill; (2) lower forage to concentrate

ratio; and (3) increased incidence of other postpartum disorders such as rumen acidosis, fatty liver, RP, endometritis, and mastitis (LeBlanc et al., 2005). Wittek et al. (2004) hypothesized that metabolic endotoxemia is a major risk factor for left displaced abomasum (LDA) as this immunogen has a potential to decrease rumen motility (Shaver, 1997; Doll et al., 2009; Eades, 1997).

A number of studies indicated that the atony of abomasum during other metabolic diseases may be related to presence of endotoxin in the plasma of sick cows (Fürll and Krüger, 1999; Poike and Fürll, 2000). The motility of the abomasal smooth muscle might be inhibited directly or indirectly via induction of hypocalcemia during high loads of endotoxin. It is well known that high concentrations of calcium in the blood are involved in the development of endotoxin aggregates, which promote its clearance through macrophage pathway and are related to sickness and the inflammatory state in the body. Therefore, withdrawal of plasma calcium may help the process of monomerization in order to prevent the overactivation of macrophage pathway and promote endotoxin clearance through lipoprotein pathway, which is a protective mechanism in the body (Ametaj et al., 2010a). These findings are also supported by an experimental endotoxemia, which was found to be associated with hypocalcemia (Waldron et al., 2003). Also, Vlaminck et al. (1985) found a dose-dependent decline and inhibition of abomasal motility when *E. coli* LPS was infused intravenously or via a duodenal fistula. Furthermore, Kaze et al. (2004) reported decreased contractility in the muscle tissue derived from the abomasal antrum of cows treated with endotoxin. Recently, our research group indicated that the

intermittently induced endotoxemia was associated with increased incidence of displaced abomasum (Zebeli et al., 2011b).

Indeed, by no means, the lowered muscle contractility of the abomasum occurs by GNB infections only, suggesting that LPS might not be the only factor. It appears that GPB organisms might prove to be more virulent, as evidenced by the emergence of *S. aureus* cell wall LTA-induced activation of iNOS, located in the smooth muscle layer of human blood vessels, which may contribute to the massive generation of NO and depressed contractile function of arteries (Tsuneyoshi et al., 1996).

### 2.8.8 Milk fever

Milk fever originally was proposed to be an imbalance of calcium metabolism around parturition; however, later the balance of dietary cationic-anionic ions (DCAD) was thought to be the major factor in the development of this disease (Goff and Horst, 1997). Recently, our team observed that cows with milk fever had greater plasma SAA, suggesting presence of an inflammatory state in the effected cows (Ametaj et al., 2003). Additionally, there were lower concentrations of plasma calcitonin-gene related peptide (CGRP), an APP associated with decreased plasma calcium (Ametaj et al., 2003). These findings paved the way to a new interesting line of thought regarding the association of milk fever with endotoxin.

A decade back, Aiumlamai et al. (1992) reported for the first time about the potential role of endotoxin in the pathology of the milk fever; however, they did not explain the potential mechanism(s) of how endotoxin is involved in

development of hypocalcaemia and subsequently milk fever. Our research group proposed two possible mechanisms, which explained that hypocalcaemia during milk fever might be a combination of calcium-impaired mobilization or its withdrawal from plasma as a protective response of the host for safely removing endotoxin from blood circulation during conditions of endotoxemia (Ametaj et al., 2010a). These results were further confirmed by Waldron et al. (2003) who observed that the experimental endotoxemia is associated with hypocalcaemia. Furthermore, there is a need to take into consideration a multicausal hypothesis instead of one-causal agent that might be more likely to explain the etiopathogenesis of milk fever.

### 2.8.9 Downer cow syndrome

"Downer cow" (DC) is the description of a cow in sternal recumbency that suffers from hypocalcemia and at times complicates with other diseases such as milk fever, mastitis, metritis or calving paralysis, which makes the animal motionless to various degrees (Correa et al., 1993). Interestingly, our team recently had the opportunity to monitor prepartal changes (during days -14, -10, -7, and -4 before the expected day of parturition) in the plasma of a cow affected by downer cow syndrome (DCS) postpartum. Blood analysis revealed that the cow affected by the DCS on day -7 showed a 10-fold increase in the plasma LBP, which is closely related to presence of endotoxemia. Moreover, the sick cow had a lower concentration of anti-LPS IgG and greater levels of anti-LPS IgM. All latter variables are part of an innate immune response against endotoxin insult (Gallay

et al., 1994), and their prepartal alterations strongly suggest involvement of systemic inflammation triggered by endotoxin (Ametaj et al., 2010a).

The primary source of endotoxin, at this point prepartum, could be its translocation from the GI tract. This is supported by other research demonstrating that cows suffering from DC have a 3.3-fold greater prevalence of pathogenic *E. coli* strains in their colon than the healthy ones (Byrne et al., 2003). Our DCS-affected cow showed an altered metabolic profile starting at -14 days prior to parturition with greater concentrations of plasma non-esterified fatty acids (NEFA) and β-hydroxy butyric acid (BHBA) but lower cholesterol postpartum. Interestingly, our DCS-case study showed symptoms of subclinical ketosis starting at 7 days before calving (plasma BHBA > 1.2 mmol/L), and at the same time lower cholesterol throughout the testing period which is also indicative of an inflammatory condition in the cow (Ametaj et al., 2010a). Interestingly, the sick cow showed lower plasma Ca during all prepartum measurements (Ametaj et al., 2010a) and it is evident that LPS is associated with lower plasma Ca in dairy cows (Waldron et al., 2003). Ametaj et al. (2010a) postulated that endotoxemia might have played a role in the hypocalcemia of the sick cow in the case study. The GPB infections might involve similar hemodynamic dysfunctions, however, the pathogenic mechanisms involved need to be studied in more detail.

**2.9 Lipotechoic acid in infection and inflammation**

Since LTA shares many pathophysiological properties with LPS it might be expected to act in a similar way. There is some evidence that LTA induces pathological lesions in animals like LPS through generation of NO by activating

several NOSs, which might change the tone of the vascular bed, leading to hypotension and organ failure. At higher concentrations, LTA triggers generation of TNF-α, IFN-γ, and IL-1, -5, -6, and -8 but also the anti-inflammatory interleukins like IL-10 and -12, although much less IL-8 and TNF-α than LPS (Ginsburg., 2002). Thus, LPS is not the only exclusive pathgenic factor, and GPB induce tissue damage by elaborating many cytotoxic factors, of which LTA would be the most potent one. However, proof of a role of LTA as a pathogenic factor, at least in animal models, requires demonstration of its presence together with LPS and needs to be studied in more detail.

**2.10 Mucosal vaccination against bacterial infections**

It is believed that the major source of bacterial endotoxins in dairy cows is the GI tract and the mucosal surfaces of the uterus and mammary gland (Ametaj et al., 2010a). Thus, there is a need to develop an effective prophylactic intervention strategy for prevention of LPS and LTA translocation into the host's systemic circulation by immunizing the potential mucosal sites of the animal around the critical time of parturition. To date, only a few experiments have been conducted that make use of the mucosal route of vaccination (Karakus et al., 2007). These studies have offered insights into the following basic questions: advantages of mucosal over parental vaccination, the complex interconnectivity of inducer and effector sites of the mucosal surfaces in regards to antigens, and the necessary regimes for mucosal immunization (Neutra and Kozlowski, 2006). All the above questions will be discussed in more detail below.

## 2.11 Advantages of mucosal versus parental vaccination against endotoxin

The characteristics of the immune response induced through the mucosal route have several advantages over parental vaccination. Depending on the site of priming the immune response via the mucosal route can result in different outcomes. Inducing immune responses in mucosal tissues stimulates production of secretory IgA (sIgA) with little or no systemic responses or tolerance, depending on the activating signals provided with the antigen (Meeusen et al., 2004). The mechanism of priming and homing was proposed to explain mucosal immunization where immunization at a specific mucosal site can induce protection at several other distant sites because of the expression of mucosa-specific homing receptors for mucosally-primed lymphocytes. Furthermore, those mucosally primed lymphocytes induce systemic IgG responses that function as an additional defense against microorganisms or their products (DeMagistris, 2006).

In addition to sIgA and serum IgG antibodies, mucosal immunization can stimulate cell-mediated immune responses including helper $CD4^+$ T cells and $CD8^+$ cytotoxic T lymphocytes. The latter play an important role in intracellular pathogen defenses. Thus, mucosal vaccines bear the potential to activate all the different arms of the immune system. Moreover, sIgA might not only prevent toxic and infectious diseases but could also be important in eliminating the healthy carrier condition and transmission to non-protected animals (DeMagistris, 2006).

## 2.12 Secretory IgA against pathogenic bacteria

Secretory IgA is produced in a primitive T-cell-independent (TI) manner by plasma cells that are located throughout the lamina propria. Those antibodies are specific for the mucosal immune system, and are antagonistic to the growth of pathogenic bacteria (Macpherson et al., 2001). In the lamina propria of mucosal surfaces, sIgA is produced in the form of two or more (polymeric) IgA units linked together by an additional polypeptide, the J chain. This is different from the monomeric IgA produced in peripheral tissues. The J chain is used to attach the polymeric IgA to its receptor, expressed on the basolateral surface of epithelial cells, to transport it through the epithelial cells and secrete it at the luminal surface. After enzymatic cleaving of the receptor from the J chain a fraction remains bound to the IgA-J chain complex, the so-called secretory component. Secretory IgA secreted into the lumen therefore consists of a complex of dimeric or polymeric sIgA molecules, which are linked with two additional polypeptides: a chain and an epithelial-derived secretory component. This configuration adds properties to the sIgA-complex, including protease resistance (Crottet and Corthesy, 1998), antibody anchoring to mucus (Phalipon and Corthesy, 2003), and activation of effector cells such as eosinophils (Lamkhioued et al., 1995).

Local generation of sIgA constitutes the largest humoral immune system of the body and has multiple roles in mucosal defence. The important feature of this Ig is ‘immune exclusion’ by entrapment of antigens in order to prevent the direct contact of pathogens or toxins with the mucosal surface as well as sterically hinder the microbial surface molecules that mediate epithelial attachment. It might also intercept incoming pathogens within epithelial-cell vesicular compartments

while transporting across the cell. Interstitial fluids underlying the epithelial barrier of mucosal tissue also contains dimeric IgA produced by local IgA-secreting plasma cells, which entrapp pathogens that have breached the epithelial barrier and push them back into the lumen through pIgR-mediated transport or destroy them by mediating antibody-dependent cell-mediated cytotoxicity (Neutra and Kozlowski, 2006). Thus, sIgA persistently keep commensal bacteria outside the epithelial barrier targeting invasion of pathogens. An earlier study clearly indicated that resistance to LPS-producing bacteria such as *E. coli* appears to depend largely on sIgA (Rincheval-Arnold et al., 2002).

**2.13 Natural antibody response against bacterial toxins: T-cell independent response**

The naive B cell comprises four B cell subsets, i.e., follicular (FO), marginal zone (MZ), B-1a, and B-1b cells, which have different developmental program, phenotype, anatomical locations, and functions. LPS-stimulated animals produce B-1a cells, which reside in peritoneal cavity and spleen and differentiate rapidly into polyclonal antibody response based on ‘‘natural’’ serum IgM called natural antibodies (NAs) once infected with LPS (Yang et al., 2006). Furthermore, LPS-stimulated B1 cells are reactive in the absence of accessory signals and evoke a TI immune response within hours after infection. Therefore, neutralizing NAs titers are mounted early in contrast to T-cell dependent (TD) responses that usually require 6-8 days (Croft, 1994)).

This efficient and quick response of NAs efficiently controls the most infectious pathogens, which induce an immune response that is initially limited to

the mucosal surface itself and then to local lymph nodes. Furthermore, in some cases even these antibodies can cause direct neutralization of bacteria or viruses present in the circulation (Ochsenbein, 1999). Later on, the natural antibody-antigen complexes are very efficiently filtered in lymphoid organs, particularly spleen, in order to prevent further spread of the infection. Usually, NAs titers are low in serum (1:10-1:100), so large doses of infectious agents can overcome this first line of defence. Therefore, development of additional effector mechanisms including specific adaptive IgM and IgG antibody responses coevolve within a few days (Baumgarth et al., 2000; Ochsenbein and Zinkernagel, 2000).

## 2.14 Interconnectivity of the mucosal layers

### 2.14.1 Induction of immune response at inter-connected mucosal surfaces

The organized mucosa-associated lymphoid tissue (MALT) is an essential part of the mucosal immune system. Organized mucosal inductive sites are concentrated in areas which are potential sites for pathogen entry (for example, the palatine and lingual tonsils and adenoids in the oral and nasopharyx) and at sites of high microbial density (such as the lower intestinal tract). The presence of a mucosal lymphoid follicle causes differentiation of overlying specialized follicle-associated epithelium (FAE), containing M cells that form intraepithelial pockets into which lymphocytes migrate. These M cells sample antigen and delivere it directly into their pockets and to the underlying dendritic cells (DCs) by vesicular transport from the intestinal lumen (Neutra and Kozlowski, 2006).

Immature DCs in the sub-epithelium dome (SED) capture the antigen and then migrate to adjacent inter-follicular T-cell zones, where they upregulate the expression of maturation markers and major histocompatibility complex (MHC) molecules, although some DCs might also carry antigen to draining lymph nodes and interface with the systemic immune system. T cells activated in the organized mucosal lymphoid tissues upregulate the expression of different homing receptors and tissue-specific adhesion molecules that function to carry the lymphocytes back to the mucosa (Figure 2-6; Modified from Brandtzaeg and Pabst, 2004). This is achieved through recognition of counter-receptors in the mucosal vasculature. Some of those receptors and chemokines exhibit redundancy and are expressed at different mucosal sites, which explains the term common mucosal immune system. That is why mucosal immunization at one site can result in the secretion of specific IgA antibodies in other mucosal tissues.

### 2.14.2 Compartmentalization in the mucosal immune system

Contrary to common mucosal immune system, there is also a receptor-mediated recognition system based on the site where an antigen or pathogen is initially encountered. For example, plasma $IgA^+$ B cells primed in the intestinal inductive sites enter the bloodstream, but they express homing receptors that interact strongly with an ‘addressin’ expressed by venules in the small and large intestines, not in other mucosal tissues; so, preferentially migrate back into the intestinal mucosa. This remarkable regionalization showed by the mucosal immune system is explained by the specific adhesion molecules and chemokine receptors involved in local leukocyte and primed B cells extravasations (Neutra

and Kozlowski, 2006). It was observed that homing from gut-associated lymphoid tissues (GALT) takes place through a unique set of adhesion molecules and chemokine receptors, which differ from those directing B cells primed in bronchus-associated lymphoid tissue (BALT) and nasal-associated lymphoid tissue (NALT; including paired palatine tonsils and unpaired nasopharyngeal tonsil also called adenoids). The urogenital tract shows similar adhesion molecules and chemokine receptors as the upper respiratory and digestive tracts and therefore appears to receive primed immune cells from NALT, although to

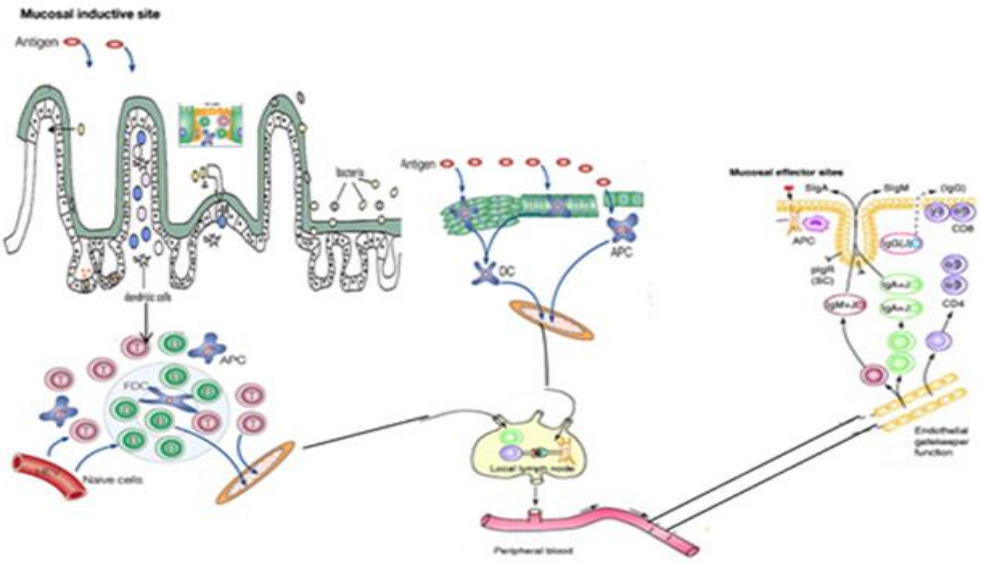


**Figure 2-6.** Depiction of mucosal immune response: Inductive sites for mucosal immunity are constituted by M-cell -containing follicle-associated epithelium through which exogenous antigens are transported actively to reach APCs. These APCs after capture antigens migrate via draining lymphatics to local/regional lymph nodes where they become active APCs, and stimulate T cells for productive or downregulatory immune responses. Naive B and T cells enter mucosal associated lymphoid tissues (MALT) and lymph nodes via HEVs. After being primed to become memory/effector B and T cells, they migrate from MALT and lymph nodes to peripheral blood for subsequent extravasation at mucosal effector. This process is directed by the local profile of vascular adhesion molecules and chemokines which are collectively called homing receptors (Modified from Brandtzaeg and Pabst, 2004).

some extent receives primed cells from GALT. In addition, lactating mammary glands receive plasma primed cells from both inductive tissues. It has been shown that immune cells from the gut may also show homing receptors for the inflamed synovial membrane (Figure 2-7; Neutra and Kozlowski, 2006; Brandtzaeg, 2007). Thus, immune cells preferentially home to effector sites corresponding to the inductive sites where they initially were stimulated by antigens, and MALT structures serve as routes for lymphocyte trafficking. Furthermore, the mucosal and systemic lymphoid cell systems are not completely segregated because several homing molecules are shared between the two systems. Such compartmentalization of mucosal immune system has to be taken into account in the development of local vaccines and in their application strategy (Neutra and Kozlowski, 2006).

**2.15 Interconnectivity of mucosal sites and vaccine efficacy**

This interconnectivity and compartmentalization of mucosal layers throughout the MALT provide an edge to induce effective immune responses at the desired effector sites, thus selection of the appropriate vaccination route plays an important role due to compartmentalization within the mucosal immune system. Holmgren and Czerkinsky (2005) conducted immunization studies in humans with cholera toxin B subunit through different mucosal routes and clearly indicated that immunization through oral route may induce substantial antibody responses in the small intestine (strong response in the proximal segment), colon, mammary, and salivary glands; however, it is relatively inefficient in inducing IgA antibody response in the distal part of the large intestines, tonsils or female

genital tract mucosa. Furthermore, nasal immunization in humans evokes antibody responses in the upper airway mucosa and regional secretions (i.e., saliva and nasal secretions); however, it is unable to generate immune responses in the gut. A notable exception with nasal mucosal immunization is the fact that it induces strong immune responses in the genital tract along with the respiratory tract. On the other hand, rectal immunization induces strong humoral IgA based immune responses in the rectum but the threshold of the response is very low in the small intestine and proximal colon. Finally, vaginal immunization gives rise to substantial IgA antibody responses in the cervico-vaginal mucosa.

**2.16 The preferred route of immumnization: oral and nasal routes**

Despite several advantages for the delivery of vaccines by mucosal routes, it is hard to administer a mucosal vaccine through the genital tract because of its unique immunological features, which alter dramatically in response to hormonal fluctuations during the menstrual cycle. In addition, the genial tract also lacks organized lymphoid follicles which are considered to be inductive mucosal sites. On the other hand, rectal immunizations have been shown to induce only modest and localized immune responses, which are not found to be very effective in larger animals and humans. Therefore, the routes of administration that appear to be more effective are those of oral and nasal administrations.

The epithelium of oral tract is unique and is multilayered squamous epithelial lining, whereas the respiratory tract varies from pseudoestratified to simple epithelium (Figure 2-7; Neutra and Kozlowski, 2006; Brandtzaeg, 2007). Some of the attractive features of the oral immunization include the ability to

induce both mucosal and systemic immune responses, safety, easiness in administration, and no strict need for sterile needles and syringes. Oral vaccines could be used easily in the developing countries, where access to trained medical professionals is frequently limited.

Nasally delivered vaccines also are easy to administer and show more promising results in inducing immune responses in both mucosal and systemic sites. Furthermore, nasal vaccines induce greater immune responses because of no enzymatic or acid secretions in the nasal mucosa. Moreover, both oral and nasal routes require a small quantity of vaccine for immunization compared to other routes where large surface areas might cause dilution problems, leading to the decrease in the immune response. Thus, the oral and nasal delivery of vaccines seems to be very convenient forms of immunization (Nedrud et al., 1997).

Hopkins et al. (1995) compared 4 different mucosal routes of vaccination using a mouse model for an attenuated *Salmonella typhimurium* vaccine: nasally, orally, rectally, and vaginally. They investigated specific anti-LPS IgA against *Salmonella typhimurium* in saliva, vaginal washes, and feces. Oral and nasal immunizations seemed to be the most effective in inducing immunity in saliva and the small and large intestines.

**2.17 Cell-mediated response of the mucosal immunization**

In addition to stimulating plasma IgG, IgM, and mucosal sIgA antibodies mucosal vaccination elicits cell-mediated immune responses involving $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T-lymphocytes (De Magistris, 2006). Dentritic cells cross-present antigens like endotoxin on MHC class I molecules to cytotoxic

CD8+ T cells (Burgdorf et al., 2008). The latter T-lymphocytes have the ability to fight intracellular pathogens. Again, this shows that mucosal immunization has the potential to activate all the different arms of the immune system.

**2.18 Mucosal adjuvants**

Adjuvant is an agent that may stimulate the immune system or is essential for enhancing and directing the adaptative immune response to the vaccine antigens. These formulations enhance the antigen uptake, processing and presentation by antigen presenting cells (APCs). Recent accumulating evidence indicates that new adjuvants based on the natural ligands or synthetic agonists for PRRs, either alone or with various formulations, enhance the production of proinflammatory cytokines/chemokines and type I IFNs that increase the host's ability to eliminate the pathogen (O'Hagan et al., 2001; Yuki and Kiyono, 2003).

Bacterial components are often used as potent cellular adjuvants (e.g. peptidoglycan, LTA, LPS, and other bacteria-derived substances) that mediate danger signals and can accelerate the induction of coadministered antigens through TLR2 and TLR4 receptors, thus evoking Th2 based immune responses (Yuki and Kiyono, 2003; Yoshino et al., 2000; Wang et al., 2003). Activated innate immunity, subsequently, leads to activation of effective adaptive immunity against infectious pathogens (Yuki and Kiyono, 2003).

Some well-known pathogen-derived immunostimulatory molecules used as strong oral mucosal adjuvants are bacterial toxins of *Vibrio cholera* and *E. coli* and their respective toxoids. Those adjuvants increase antigen presentation by B cells, B-cell differentiation to IgA secreting cells, interaction with T cells, and cyt-

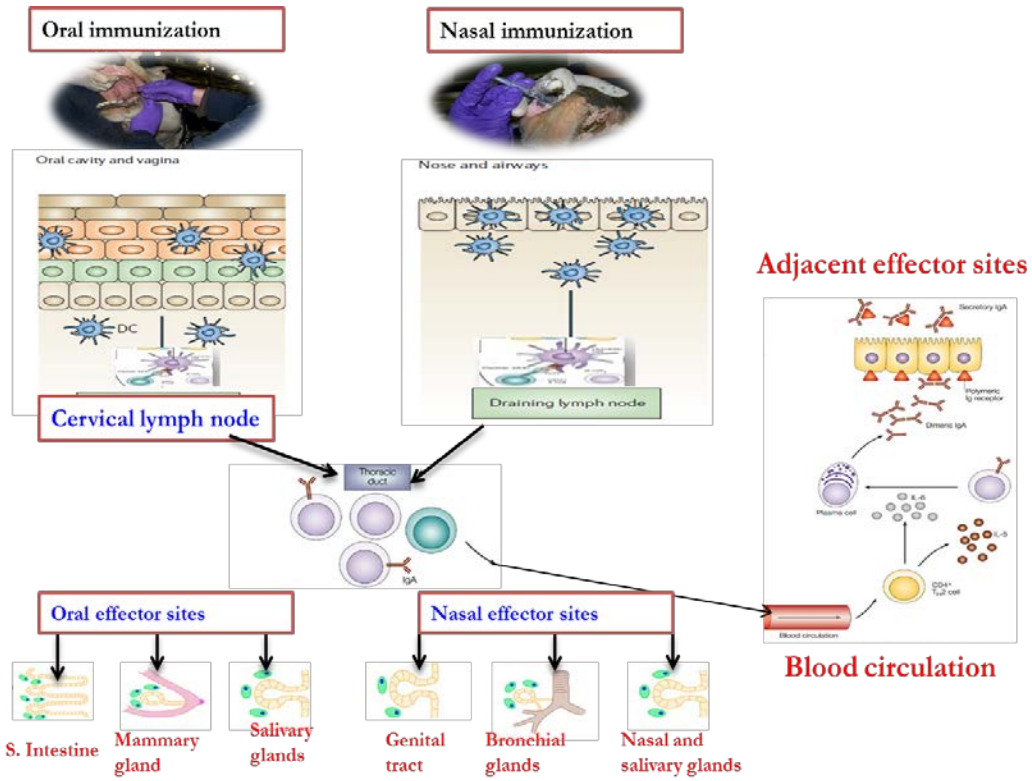


**Figure 2-7.** Model for regionalization and cellular communication between inductive and effector sites. Homing from oral mucosa is determined by a unique set of homing receptors which differ from the counterparts directing B cells primed in nasal-associated lymphoid tissue (NALT). NALT consists of palatine tonsils particularly the nasopharyngeal tonsil (adenoids). The urogenital tract receives primed immune cells from NALT. Lactating mammary glands receive primed cells from oral inductive tissue. Immune cells from the oral cavity may also home to small intestine (Modified from Brandtzaeg et al., 2007; Neutra and Kozlowski, 2006).

okine production. However, they suffer from high toxicity and induce strong immune responses against itself (Byun et al., 2001). Thus, the current challenge is to develop adjuvants, which are less toxic and can induce a strong Th1 bias immune response along with Th2, which is important for vaccines develop against certain viruses and infectious pathogens. This leads to the development of less toxic derivatives of monophosphoryl lipid A (MPLA), which are formulated along with alum and skew immune responses towards Th1 immune response. Further

research is warranted to explore other combinations of adjuvants with bacterial cell-wall components and other TLR-specific-bacterial associated ligands. Other natural compounds that facilitate the vaccine delivery are also classified under adjuvants like liposomes, virosomes, and immunostimulating complexes (ISCOMS; Yuki and Kiyono, 2003).

**2.19 Mucosal vaccines against bacterial toxins: T cell-independent B cell memory**

Vaccination with bacteria or their parts at the mucosal level has only recently been successful. Previously it was thought that obtaining memory to the B cell responses elicited by TI antigens such as bacterial polysaccharides was impossible and unable to produce amplified, accelerated, and affinity-matured antibody secondary immune responses. In a pioneering work, Hosokawa et al. (1984) indicated development of TI B-cell memory against haptenated polysaccharides and induction of an amplified secondary antibody response as a read-out for TI B cell memory. In most cases, the secondary antibody response to TI antigen could only be obtained when primed B cells are adoptively transferred to naive recipients before those cells are subjected to the secondary antigenic exposure (Hosokawa et al., 1984). However, the existence of TI memory B cells has been clearly confirmed by Obukhanych and Nussenzweig (2006) who reported that an amplified pool of quiescent antigenic-specific B cells persists 120 days after NP-Ficoll immunization. Recently, Yoshida et al. (2010) proposed that long-lived plasma cells (PC) can act as ‘memory PC’ because they contribute to the persistent modification of the system and enhanced protective state toward re-

infections. Manz et al. (1997), and Slifka et al. (1998) immunized mice with prototypic TD immunogens, and showed that plasma cells can survive in the bone marrow (BM) for a long period of time where they receive survival signals from stromal cytokines and chemokines. Interestingly, those cells seem to be maintained after immunization, even after depletion of memory B cells.

The issue of longevity of TI BM PC need to be addressed. This information was brought by an *in vivo* study conducted by Taillardet et al. (2009) who indicated that the pneumococcal capsular polysaccharide 3 (PS3) immunization generates a pool of BM PC that persists for at least 6 months. During adoptive transfer of these BM plasma cells from PS3-immunized mice to B cell-deficient recipients it induced PS3-specific IgM antibodies in the host that persisted in the serum for several months. In conclusion, the assumption that antigens that bypass T-cell help [strength of the B-cell receptor (BCR) signal and T-cell help are critical for generation of long-lived PC] are unable to generate memory PC proved to be wrong.

It is observed that TI antigens promote the extensive BCR cross-linking, which induces potent BCR; and that TLRs agonists or inflammatory cytokines substitute T-cell help for production of memory PC. It is evident that pure pneumococcal PS can generate four times amplified responses based on long-lived BM PC upon administration of a TLR-9 agonist indicating that BCR signals need to be complemented with TLR stimulation to generate TI memory (Taillardet et al., 2009). In the context of vaccine administration of pure bacterial PS, exogenous TLR agonists may be required to fully activate the TI arm of the

humoral immune response. Furthermore, it is suggested that TI memory PC may have an extended lifespan as compared to their TD counterparts. Defrance et al. (2011) concluded that the strength of the BCR-derived signals and the nature of the accessory stimuli received by B cells (T-cell help for TD Ag, danger signals for TI Ag) during their differentiation into effector cells dictates the lifespan of PC and subsequent memory for TI antigens.

**2.20 Tolerance to endotoxin**

Endotoxin tolerance is defined as prior exposure of innate immune cells to sublethal doses of LPS, which induces a transient hyporesponsiveness to subsequent endotoxin challenges. Indeed, animals that lack the ability to recognize endotoxin may die if they are infected by GNB, whereas animals reacting too vigorously might also die from overreaction to endotoxin. Thus, pathophysiological adaptations to regulate over-exuberant immune responses serve as an important protective mechanism against endotoxin shock (Heumann and Roger, 2002).

Many researchers have focused on the role of TLR4 in the tolerance phenomena (Medzhitov, 1997). Other endotoxin tolerance-related cell mechanisms are cell surface molecules, signaling proteins, proinflammatory and anti-inflammatory cytokines, and other mediator proteins (Ziegler-Heitbrock, 1995; West and Heagy, 2002). In fact, recent research identified the central role of anti-inflammatory mediators such as IL-10, transforming growth factor-β (TGF-β), and glucocorticoids in the temporarily refractoriness to secondary exposure to LPS (Fan and Cook, 2004). Tolerance to endotoxin is not a total shut-down of all

signaling pathways in the immune cells. LPS-tolerant animals and cells still show some responsiveness to repeated LPS exposures and have the ability to express specific genes and proteins (Learn et al., 2001).

A recent development has broadened this term to ‘microbial tolerance’, which indicates that endotoxin tolerance is also encountered to other TLR ligands such as those who give protection against GP bacterial sepsis. Such phenomenon is referred to as heterotolerance or crosstolerance. For example, prior exposure to TLR2 ligands such as to LTA renders macrophages not only tolerant to LTA but also to LPS, which is linked to defective TLR signalling involving downregulation of the activity of various signaling proteins and adaptor molecules (Sato et al., 2000; Dobrovolskaia et al., 2003; Buckley et al., 2006).

Endotoxin tolerance has two temporally distinct phases (i.e., early and late). Early tolerance is associated with non-antibody mechanisms and a transiently occurring refractory state; whereas late tolerance appears to be mediated by anti-endotoxin antibodies directed against both "O" and common core antigens (Biswa and Lopez-Collazo, 2009). Thus, the induction of LPS tolerance might be a useful tool in the prevention of endotoxin-related diseases or bacterial infections in dairy cows postpartum (Cavaillon, 1995; Salkowski et al., 1998; Gustafson et al., 1995).

**2.21 Summary**

The incidence rates of metabolic diseases in dairy cows are very high despite important advances during the last decades and the understanding that the potential causative role is bacterial endotoxin. Thus, there is a need to further

investigate the disease process and develop new immunization strategies targeting mucosal layers, which are the first port of entry for bacterial toxins. Mounting evidence suggests that oronasal mucosa is one of the most effective sites for triggering mucosal immune responses and generation of memory TI B-cell responses against bacterial polysaccharides. Based on these recent advances, we developed a novel hypothesis targeting oral and nasal inductive sites with LPS and LTA (from GN and GP bacteria, respectively) to induce immune responses and protect cows from postparturient diseases related to bacterial endotoxins. To evaluate this hypothesis, two experiments were designed. The main objectives were to produce not only appropriate antibody response at desired effector sites but also to increase the overall immune competence, health profile, and productivity of periparturient dairy cows.

**2.22 Research hypotheses**

There are two main hypotheses

1: Our first hypothesis assumes that repeated oral application of LPS from *E. coli* 0111:B4 in dairy cows starting at 4 wk before parturition would be able to prevent deleterious effects of LPS as reflected by improved immune responses, their overall health, metabolic status and productivity of periparturient dairy cows.

2: We hypothesized in the second study that repeated oral exposure of the prepartal dairy cows to increasing doses of LPS from *E. coli* 0111:B4 and a flat dose of LTA from *Bacillus subtilis* would be able to improve their immune responses, overall health and metabolic status, as well as their milk production and composition.

## 2.23 Overall objectives

To test our hypotheses two studies were designed with three objectives each as explaine below.

The first study aimed: 1) to ascertain whether repeated oronasal administration of LPS before parturition would modulate selected plasma metabolites related to carbohydrate and lipid metabolism and decrease the risk of periparturient diseases in transition dairy cows; 2) to investigate the innate and humoral immune responses in periparturient dairy cows against repeated oro-nasal LPS exposure before parturition; and 3) to establish the effect of repeated oronasal LPS administration on milk composition and overall productivity of dairy cows postpartum.

The objectives of the second study were: 1) to ascertain whether repeated oral administration of LPS and LTA would affects metabolic status and the incidence of periparturient diseases in periparturient dairy cows; 2) to evaluate whether repeated oral exposure of the periparturient dairy cows to LPS and LTA before parturition would modulate their innate and humoral immune responses of dairy cows; and 3) to determine whether repeated oral administration of LPS and LTA would modulate milk production and composition in dairy cows postpartum.

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# [1]Chapter 3

## Oronasal administration of lipopolysaccharide prepartum modulated serum metabolite patterns in periparturient dairy cows

### 3.1 Introduction

Initiation of lactation in dairy cows is associated with a major dietary shift from a high-forage low-grain diet before parturition to a diet consisting of highly degradable carbohydrates, to meet the energy demands for milk production. Frequently, these feeding practices are associated with development of subacute ruminal acidosis (SARA; Zebeli et al., 2008), leading to rumen dysbiosis (Khafipour et al., 2011) and the release of bacterial compounds with high immunogenic properties (Ametaj et al., 2010a; Saleem et al., 2012). One of these compounds is endotoxin or lipopolysaccharide (LPS), a component of the outer leaflet of the external membrane of all Gram-negative bacteria. Recently we demonstrated that feeding graded amounts of barley grain (i.e., 0, 15, 30, and 45% of the diet DM) resulted in a 6- to 14-fold increase in the concentration of endotoxin in the rumen fluid in cows fed 30 and 45% barley grain, respectively (Emmanuel et al., 2008).

It has been suggested that ruminal endotoxin that breaks through the mucosal barriers (Emmanuel et al., 2007), transfers into the portal vein or lymphatic system and is removed by macrophages located in the liver or other organs (Ametaj et al., 2010b). Activated macrophages initiate an overall immune response (Andersen, 2003; Ametaj et al., 2005, 2010b) that commonly is

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associated with perturbation of various plasma metabolites (Dougherty, 1976; Ametaj et al., 2005; Nagaraja and Lechtenberg, 2007). For example, our recent study demonstrated that oral administration of increasing doses of LPS, before and shortly after parturition, triggered changes in plasma metabolites with lowered concentrations of non-esterified fatty acids (NEFA), and β-hydroxy-butyrate (BHBA), but enhanced insulin and glucose in the plasma (Zebeli et al., 2013). However, we observed that when cows were challenged with intermittent endotoxemia by infusing intravenously increasing doses of LPS, it decreased concentration of cholesterol, whereas increased BHBA and cortisol, however no effect was observed on glucose, NEFA, lactate, and insulin (Zebeli et al., 2011b). Earlier studies regarding intravenous administration of endotoxin indicated modulation in the concentrations of plasma NEFA and BHBA of heifers (Werling et al., 1996; Steiger et al., 1999), and dairy cows (Waldron et al., 2003).

The interest in mucosal immune stimulation and most importantly in using the mucosally-induced tolerance as a form of immunomodulation to prevent against certain pathogens is increased recently. However, although our knowledge about a potential role of endotoxin in the etiopathogenesis of several periparturient diseases is increasing, there are only a limited number of investigations dealing with prevention of deleterious effects of translocated endotoxin on the host's health status. For example, pretreatment with LPS by the oral route was able to protect rats against sepsis through a regulation of anti-LPS antibody production (Márquez-Velasco et al., 2007). Moreover, intra-mammary mucosal pretreatment of cows with LPS was shown to protect against

experimental *Escherichia coli* mastitis (Petzl et al., 2011). In cows, oral administration of repeated doses of LPS, before and shortly after parturition, stimulated a humoral immune response against LPS (Ametaj et al., 2012). Because dairy cows are exposed to large amounts of cell-free LPS, in particular in gastrointestinal mucosal tissues, especially during the postpartum period, it would be of interest to evaluate whether exposure of cows to LPS long before this critical period might support metabolic status of the cows. Therefore, we hypothesized that repeated oronasal exposure of periparturient dairy cows to LPS from *E. coli* 0111:B4 2-4 wk before parturition will affect their energy and lipid metabolism as well as overall health status. Therefore the objectives of this study were to evaluate metabolic and some clinical responses of dairy cows to repeated oronasal administration of LPS during the 4 wk before and 4 wk after the expected day of calving.

## 3.2 Materials and Methods

### *3.2.1 Cows, Diets, and Experimental Design*

The trial consisted of two groups of 50 Holstein cows each allocated into a longitudinal study. Out of this pool (i.e., 100 cows) two intensive sampling sub-groups were established. Thirty Holstein dairy cows at 28 d before the expected day of parturition were randomly allocated to two treatment groups (n = 15) according to parity, milk production, disease susceptibility from previous year, and body condition scoring (BCS). Cows received an oral and a nasal treatment of 2 mL and 1 mL of sterile saline solution (control), respectively, or 2 mL oral and 1 mL nasal of sterile saline solution containing 3 increasing doses of LPS from *E.*

*coli* 0111:B4 as follows: 1) 0.01 µg/kg body weight (BW) on d -28, 2) 0.05 µg/kg BW on d -25, and -21, and 3) 0.1 µg/kg BW on d -18, and -14. The lowest dose of 0.01 µg/kg of BW was chosen because previous experiments have shown minimal changes in metabolism of dairy cows at this concentration, whereas dose 3 was selected due to a maximum host response at this dose (Werling et al., 1996; Waldron et al., 2003; Jacobsen et al., 2005). We recently established the effects of these doses on different immune parameters in dairy cows (Ametaj et al., 2012).

The crystalline *E. coli* LPS (Lipopolysaccharide-FITC from *E. coli* strain 0111:B4 supplied by Sigma-Aldrich Canada Ltd, Oakville, ON, Canada), containing 10 mg of purified LPS was dissolved in 10 mL of doubly distilled water, as suggested by the manufacturer, and stored at 4 °C. For administration to the animals the daily dose was dissolved in 3 mL of sterile saline and then introduced into the oral and nasal cavity of the cows using sterile 5 mL disposable syringes (Becton, Dickinson and Company, BD, Franklin Lakes, NJ). Similarly, the same amount of carrier (i.e., 3 mL of sterile saline supplied by Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) was sprayed oronasally to all cows in the CTR group.

Daily diet was offered as a total mixed ratio (TMR) and was formulated to meet or exceed the nutrient requirements of dry and early lactating cows as per NRC (2001) guidelines. The close-up diet fed to the dry cows was based on the 10% alfalfa hay, 63% barley silage and 27% diet supplement on the DM basis; whereas high ration fed to early lactating cows was based the on 10% alfalfa hay, 40.8% barley silage, and 49.2% dairy supplement. Ingredients and chemical

composition of the diets for the dry and early lactating cows are presented in Table 3-1 and 3-2, respectively. All experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Livestock and animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993). Veterinary supervision was provided to the animals throughout the experiment.

### *3.2.2 Sampling Procedure*

Blood samples were collected at 7:00 from the tail vein using serum vacutaniers (Becton, Dickinson and Company, BD, Franklin Lakes, NJ) on the following sampling days; -28, -25, -21, -14, and -7, before parturition and on d +7, +14, +21, and +28 after parturition. Blood samples were left to coagulate, and then centrifuged (Rotanta 460R, Hettrich Zentrifugen, Tuttlingen, Germany) at 3,000 × g and 4 °C for 20 min to collect serum, which was stored at -20 °C until analyses.

### *3.2.3 Clinical Monitoring of Animals*

Udder edema (UE) was estimated according to an evaluation scale of 1 - 3 with 1 = healthy, 2 = partial edema, and 3 = severe edema (Tucker et al., 1992). Evaluations were done on d -14, and -7, before parturition, then on the day of calving, as well as on d +7, and +14 after parturition.

Manure was evaluated and scored based on its consistency from scale 1-5 as follows; 1 = very liquid with consistency of pea soup, 2 = appeared runny, did not form a distinct pile; less than 2.5 cm in height and splattered when hitting the ground, 3 = optimal score, porridge-like appearance; stacked up at 4-5 cm, had

several concentric rings; plopping sound; sticked to shoe, 4 = thicker; sticked to shoe; stacked up more than 5 cm, 5 = firm fecal balls (Zaaijer et al., 2005). Evaluations were done on d -14, before parturition, and +7, +14, and +21 after parturition.

BCS was estimated by conformity of 3 trained individuals according to the evaluation system by Elanco Animal Health (a division of Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN). Estimations were undertaken on d -28, and -14, before parturition, then on the day of calving, as well as on d +14, and +28 after parturition.

Feed intake was recorded daily during the entire experimental period. All animals were at good health status when entering the experiment.

Disease and medication history was recorded for each cow throughout the entire experimental period. Data of disease incidences for different periparturient diseases including lameness, retained placenta, metritis, mastitis, milk fever, and left displaced abomasum (LDA) were collected for all dairy cows throughout the experimental period i.e., 28 days before and 28 days after parturition. Clinical monitoring for the disease incidence was conducted on each sampling day and every alternate day of the experimental period, and the person assessing clinical parameters was blinded (i.e., unaware) to the treatment groups.

### *3.2.4 Serum Metabolites*

Concentrations of glucose in the serum were quantified by an enzymatic method with a kit provided by Diagnostic Chemicals Ltd. (Charlottetown, PE). The procedure involves phosphorylation and oxidation of glucose in samples

resulting in the production of NADH, which produces a color proportional to the glucose concentration in the sample. All samples were tested in duplicate and serum glucose was then determined by reading on a microplate spectrophotometer (Spectramax 190, Molecular devices Corp., Sunnyvale, CA) at an optical density of 340 nm. According to the manufacturer's instructions the lower detection limit of the test was 0.06 mg/dL.

Serum NEFA was quantitatively determined by an enzymatic colorimetric method using kits provided by Wako Chemicals (Richmond, VA). The principle of the test involves acylation of coenzyme A by a fatty acid in the sample in presence of acyl-CoA synthase and production of hydrogen peroxide in presence of acyl-CoA oxidase. Hydrogen peroxide, in presence of peroxidase, permits the oxidative condensation of 3-methyl-N-ethyl-N-β-hydroxy ethyl-O-aniline with 4-aminoantipyrine to form a purple colored adduct, which is proportional to the amount of NEFA in the sample. The test was modified as described by Johnson and Peters (1993). Duplicates were used to read serum contents at an optical density of 550 nm, on a microplate spectrophotometer (Spectramax 190, Molecular devices Corp., Sunnyvale, CA). The measurable range of the kit was within 0.01-4.00 mEq/L NEFA.

Enzymatic measurement of BHBA by β-hydroxybutyrate dehydrogenase was used for measuring serum concentration of BHBA using a commercially available kit (Stanbio Laboratory, Boerne, TX). The principle of the test involves conversion of BHBA in the samples to acetoacetate and NADH at pH 8.5 by β-hydroxybutyrate dehydrogenase in the presence of NAD. The NADH produced

reacts with 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride (INT) in the presence of diaphorase to produce a color proportional to the concentration of BHBA in the sample. Controls and blanks were used in this procedure to eliminate the difference in the color intensity. Serum BHBA was measured in duplicates by reading on a microplate spectrophotometer (Spectramax 190, Molecular devices Corp., Sunnyvale, CA) at an optical density of 505 nm. The lower detection limit of the assay was 125 μmoL/L.

Concentrations of lactate in the serum were determined using commercially available lactate assay kits (Biomedical Research Service Center, Buffalo, NY). The principle of this test involves reduction of tetrazolium salt INT in a NADH-coupled enzymatic reaction of formazan, which exhibits a red color. The intensity of the red color is proportional to the concentration of lactate. The procedure was modified as described by Johnson and Peters (1993). The lactate standard provided in the kit was read at an optical density of 492 nm on a microplate spectrophotometer (Spectramax 190, Molecular devices Corp., Sunnyvale, CA).

Serum cholesterol was measured using kits provided by Diagnostic Chemicals Ltd. (Charlottetown, PE, Canada). The colorimetric method is based on the principle of hydrolyzing cholesterol esters to free cholesterol and oxidation of free cholesterol to cholest-4ene-3-one with simultaneous production of hydrogen peroxide. In the presence of peroxidase the hydrogen peroxide couples with 4-aminoantyine and p-hydroxybenzoate to yield a chromogen whose intensity is proportional to the concentration of cholesterol in the sample. Samples

were prepared in duplicates and the reading of serum cholesterol was conducted with an optical density of 505 nm on a microplate spectrophotometer (Spectramax 190, Molecular devices Corp., Sunnyvale, CA).

### *3.2.5 Statistical Analysis*

Data were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA Version 9.1.3) as describe by the following model:

$$Yijl = \mu + \gamma i + \alpha j + \beta l + (\alpha\beta)jl + \varepsilon ijkl$$

Where Yijkl is the observation for dependant variables, μ is the population mean, γi is the independent parameter for animal i, αj is a population parameter corresponding to treatment j, βl is the fixed effect of measurement time (relative to each LPS challenge) l, (αβ)jl is the effect of treatment by time interaction, and εijkl is the residual error. Degrees of freedom were approximated by the method of Kenward-Roger (1997). The measurements taken on the same cow but at different sampling hours and administration days were considered as double repeated measures in the model. The Kronecker product of a completely unrestricted variance-covariance matrix (for administration day) and a first-order autoregressive variance-covariance matrix (for time after LPS administration) was used to account for double repeated measures taken on individual cows across time (Zebeli et al., 2013). Multiple differences between treatments were tested by the option SLICE. The significance limit was declared at $P < 0.05$, while a tendency was considered at $P \leq 0.10$.

## 3.3 Results

### *3.3.1 Serum Metabolites*

The oronasal LPS group had greater concentrations of glucose in the serum ($P$ = 0.03; Figure 3-1). Furthermore, there was effect of measurement time ($P$ < 0.01). At d -14 before parturition concentrations of glucose started to decrease in both groups from 55 and 65 mg/dL to concentrations as low as 40 and 50 mg/dL for the control and the treatment groups, respectively. No interaction between the treatment and time was evidenced for both groups ($P$ = 0.33).

Although not quite reflected in the probability value, concentrations of NEFA in treated cows were numerically below the control group values ($P$ = 0.16; Figure 3-2). There was no effect in the time or treatment by time interactions regarding NEFA ($P$ = 0.33; $P$ = 0.89, respectively). However, Figure 2 clearly shows two distinct peaks in the curve of NEFA for the control group, which does not occur at the same extent in the LPS-treated group. The first increase in serum NEFA is between d -28 and -21 wk before calving and the second one at around d +14 to +21 postpartum.

Concentrations of BHBA in the serum did not differ among treatments ($P$ = 0.95; Figure 3-3). The effect of measurement time was evident ($P$ < 0.01); however no interaction between treatment and time ($P$ = 0.66) was observed. Nevertheless, in the first 7 d postpartum concentration of BHBA in the serum of the LPS group remained lower compared to the control group. At around +21 and +28 d postpartum concentrations of BHBA increased again in this group at an average concentration of 1,200 µmoL/L.

The overall concentration of lactate in the serum was numerically higher in the LPS group during the treatment period and the first wk postpartum; although it

did not reach the point of significance ($P$ = 0.11; Figure 3-4). Time had an effect on concentration of lactate, which was reflected in a decrease in the serum lactate in the control group 21 d before parturition, not observed in the LPS group. Along with parturition concentrations of lactate in the serum increased at a similar trend with the LPS group remaining higher within 3 d of calving ($P$ < 0.01). From a peak of 1,200 and 1,000 µmoL/L for LPS and control groups, respectively, at 1 - 3 days postpartum concentrations of lactate in the serum decreased to 900 µmol/L for both groups of cows. However, there was no interaction between the treatment and time in this study ($P$ = 0.86).

Concentrations of cholesterol in the serum were greater in the LPS group ($P$ < 0.01; Figure 3-5). Time also had an effect on both groups with concentrations of cholesterol declining by 25% from d -28 before calving to shortly after parturition and then recovering to original concentrations within a similar time span ($P$ < 0.01). Both the LPS and the control curves followed a similar dynamic and no treatment by time interaction was observed ($P$ = 0.79).

### *3.3.2 Clinical Parameters*

Body condition score was evaluated in 100 cows included in the trial. The overall effect of treatment was not significant ($P$ > 0.05; Figure 3-6) regarding BCS. The factor measurement time around parturition showed considerable effect on weight loss in both groups ($P$ < 0.01). Furthermore, there was no treatment by time interaction for this variable ($P$ > 0.05). During transitioning period, from d -14 before to d +14 after parturition, both groups of cows followed a similar BCS loss trends.

Udder edema was also evaluated in groups of 100 cows. Treatment as a single factor had no effect on UE ($P > 0.05$; Figure 3-7). However, time of measurement showed an influence as indicated from variation in the prevalence of UE during different time points around parturition ($P < 0.01$). Furthermore, interaction between treatment x time did not show an effect for this variable ($P > 0.05$).

Data showed no effect of treatment on the manure score ($P = 0.59$; Figure 3-8). Time around parturition affected the consistence of the manure, which was reflected in a lower average score after calving in treated cows ($P < 0.01$). Furthermore, there was no treatment by time interaction for manure score between both treatment groups ($P > 0.05$).

Data indicated that although oronasal administration of LPS did not show an influence on retained placenta and lameness ($P > 0.05$), it was numerically lowered in the LPS cows versus the CRT group for both diseases. We observed 6 cases in the treated cows versus 13 cases in control group for retained placenta; whereas for lameness it was 9 cases in the oronasally-administered LPS cows versus 15 in the control group. Treatment showed a tendency for cows with more than one disease to be lower compared to cows in the control group ($P = 0.06$). No effect on other clinical diseases was observed between the two treated groups (data not shown).

**3.4 Discussion**

In a recent study we showed that repeated oral administration of LPS, 2 wk before and 1 wk after parturition, modulated several serum metabolites and

improved the innate immune status of dairy cows postpartum (Ametaj et al., 2012; Zebeli et al., 2013). The present study aimed at evaluating whether repeated oronasal administration of LPS, for 3 consecutive wk, starting at 4 wk before parturition, has the same effects on metabolic and clinical responses in periparturient dairy cows in a larger cohort of experimental animals. In the present trial, we split the doses of LPS into oral and nasal applications versus oral only application in our previous trial, and included 100 Holstein dairy cows in order to evaluate the clinical variables. Another reason we decided to test this protocol is that dairy cows are exposed to large amounts of luminal cell-free LPS during the postpartum period. Therefore, we hypothesized that mucosal exposure of cows to LPS before this critical period (i.e., postpartum) might induce mucosal immunity against LPS and improve metabolic status of transition dairy cows. Several results of the energy metabolism obtained in the previous trial were confirmed in this experiment while others were not, and all these data are discussed below.

Results of the present study showed greater concentrations of serum glucose in the treatment group, which are in agreement with our previous research (Zebeli et al., 2013), and with other human and animal model studies (Bloesch et al., 1993; Michaeli et al., 2012). Research in cows has shown that the response of plasma glucose to single endotoxin challenge is biphasic and associated with marked increase in peripheral glucose uptake (Onozaki and Hashimoto, 1985; Elsasser et al., 2008). It is interesting that serum glucose did not follow a biphasic pattern in this study, but it showed a continued higher response without interruption in the oronasal LPS-treated cows, which also confirmed our earlier

findings where oral LPS administration around parturition induced sustained greater blood glucose (Zebeli et al., 2013). Although the exact mechanim(s) of how oronasal LPS causes greater blood glucose are not understood it is hypothesized that it might be either a stress response to repeated exposure to LPS or a better energy status of the cows as a result of increased immunity against LPS and prevention of its translocation into the host systemic circulation.

Another important finding of this study was greater serum cholesterol in cows administered oronasally with LPS. Recent research from our group showed that intermittent endotoxemia induced by infusing increasing doses of LPS via intravenous route is associated with decreased concentrations of cholesterol in the plasma of transition dairy cows (Zebeli et al., 2011b). This is in agreement with research in other species where LPS infusion subcutaneously for a period of 10 d showed a decrease in total plasma cholesterol and HDL cholesterol in cats (Michaeli et al., 2012), similar to humans experiencing endotoxemia (Fraunberger et al., 1999; Khovidhunkit et al., 2004). It is known that cholesterol is a precursor of bile acids, which are engaged in detoxifying endotoxin (Parlesak et al., 2007), by splitting the toxic endotoxin moiety into nontoxic fragments and may lead to its neutralization by gastrointestinal proteins as well as prevent its translocation into the host blood circulation (Bertok, 2004). So, greater serum cholesterol in the treated cows might indicate a better health status of those cows.

Data from this study showed that concentrations of lactate and NEFA in the serum were not different between the two treated groups. Although the LPS-treated cows had numerically greater lactate and lower serum NEFA, the

differences did not reach the point of significance. These data are not in accordance with our previous study where oral only administration of repeated and increasing doses of LPS in periparturient dairy cows increased lactate and lowered plasma NEFA (Zebeli et al., 2013). The discrepancy might be related to different routes of administration of LPS (oral vs oronasal) and splitting of the same dose into two different sites in the present study. It is a common finding in transition cows that low blood glucose is associated with greater serum NEFA (Ametaj et al., 2005; Duffield et al., 2009; Hammon et al., 2009). It is known that chronic infusion of endotoxin induces the release of various inflammatory cytokines like tumour necrosis factor (TNF)-α which enhances lipolysis in subcutaneous fat tissue and the release of NEFA in blood circulation (Gabay and Kushner, 1999; Kushibiki et al., 2003; Bradford et al., 2009). Waldron et al. (2003) reported a biphasic response for plasma NEFA following i.v. infusion of endotoxin in dairy cows, with an initial decrease and a subsequent increase.

Data also showed that concentrations of BHBA in the serum remained unchanged between the treated groups. In addition, our data are different with those reported by Steiger et al. (1999) and Waldron et al. (2003), who demonstrated enhanced plasma BHBA in transition cows, and then a decrease following a single i.v. infusion of LPS. The difference of the present results with the latter studies is related to differences in the route of administration, dose of LPS used, and the time-length of administration. Of note, concentrations of BHBA in the serum were within the normal ranges in both treatment groups.

Treatment did not affect manure score, UE, and BCS. Previously we reported that the same dose of LPS administered orally improved health status of the treated cows (Ametaj et al., 2012). The reason for the disagreement of results between the two studies might be related to the fact that the same dose of LPS was split between the nose and mouth in the present study. It is possible that splitting the dose of LPS might have lowered the required threshold dose to induce humoral immunity in experimental cows. Furthermore, oronasal application of LPS did not show beneficial effects on the incidence of clinical periparturient diseases of transition dairy cows. The reason for greater BCS in the treated cows, at some of the time points, is not clear at present and needs further investigation.

### 3.5 Conclusions

In conclusion, results of this study provided new evidence that oronasal administration of LPS was associated with enhanced concentrations of glucose and cholesterol. There was no effect of the treatment on serum NEFA, BHBA, and lactate; although NEFA was numerically lowered and lactate was numerically greater in the treated cows. No effect was observed on manure score and UE. Treatment had no effect on the incidence rates of retained placenta, lameness, metritis, mastitis, milk fever, and LDA. Cows in the treatment group showed more optimal BCS during the transition period. This warrants further research regarding the optimal dose of LPS and the best routes of administration.

### 3.6 Acknowledgement

We acknowledge the financial support of Alberta Milk (Edmonton, Alberta, Canada), Alberta Livestock Industry Development Fund (Edmonton, Alberta, Canada), and Natural Sciences and Engineering Research Council of Canada (Ottawa, Ontario, Canada). We are grateful to the technical staff at Dairy Research and Technology Center, University of Alberta for their help and care of the cows.

**Table 3-1.** Ingredients and chemical composition of the diet for dry cows

| Ingredients, % of DM | Prepartum diet |
|---|---|
| Alfalfa hay | 10.0 |
| Barley silage | 63.0 |
| Alfalfa silage | 00.0 |
| CUD[1] grain | 27.0 |
| Nutrient composition of cud grain (dietary supplement) % amount in 100 kg mix | |
| Barley grain, rolled | 55.0 |
| Canola meal | 7.5 |
| Dairy dry cow micro-premix | 6.2 |
| Limestone | 8.7 |
| Animate | 15.7 |
| Molasses | 0.9 |
| Canola oil | 4.1 |
| Yeast | 1.7 |

[1]CUD = giving more mineral especially Ca to avoid milk fever in early lactating cows

**Table 3-2.** Ingredients and chemical composition of the diet for early lactating cows

| Ingredients, % of DM | Early lactation diet |
|---|---|
| Alfalfa hay | 10.0 |
| Barley silage | 40.8 |
| Dairy supplement | 49.2 |
| Nutrient composition of dairy supplement, % amount in 100 kg mix | |
| ADE Vit Pak-30 Natural E | 0.056 |
| Ruminant TM Pak | 0.1025 |
| Selenium 1000 mg/kg (UNscr Fine Cr) | 0.065 |
| Custom TM Complex pmx | 0.060 |
| Di-calcium phosphate 21% | 1.25 |
| Co-op alantic Corn Dist | 10.0 |
| Corn ground | 25.0 |
| Corn rolled | 30.105 |
| Vit D- 10,000 KIU/kg | 0.015 |
| Diamond V XPC | 0.14 |
| Magalac/Enertia | 1.00 |
| Fermenten | 2.00 |
| Limestone | 1.50 |
| Mag Ox -56% | 0.37 |
| Canola meal | 15.5 |
| Hi bypass soy (Amino plus) | 2.75 |
| Soy bean meal-47.5% | 6.50 |
| Sodium bicarbonate | 1.00 |
| Salt | 0.113 |
| Pork-Tallow | 2.45 |
| Biotin 2%-Rovimix H-2 | 0.007 |
| ADM Vit E 405 Vegetable source | 0.015 |

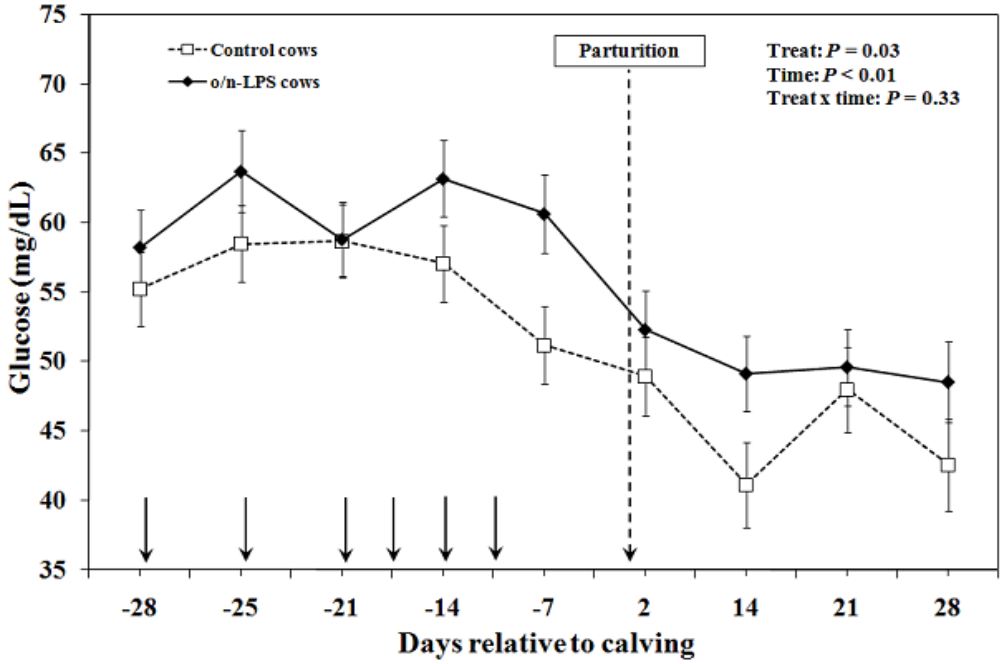


**Figure 3-1.** Concentration of glucose in the serum of periparturient Holstein dairy cows treated oronasally with increasing doses of lipopolysaccharide (o/n-LPS; ◆) or saline (Control □ ). Treat = Effect of treatment, Time = Effect of sampling day, Treat × time = Treatment by time interaction (LSM ± SEM; n = 15).

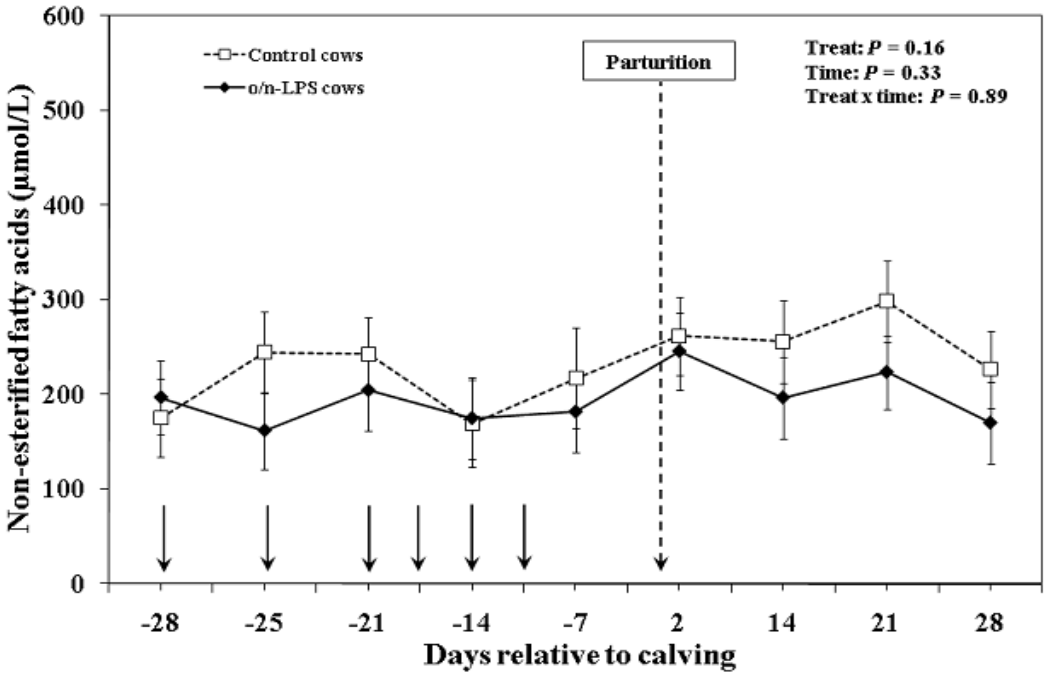


**Figure 3-2.** Concentration of non-esterified fatty acids in the serum of periparturient Holstein dairy cows treated oronasally with increasing doses of lipopolysaccharide (o/n-LPS; ◆) or saline (Control: □). Treat = Effect of treatment, Time = Effect of sampling day, Treat × time = Treatment by time interaction (LSM ± SEM; n = 15).

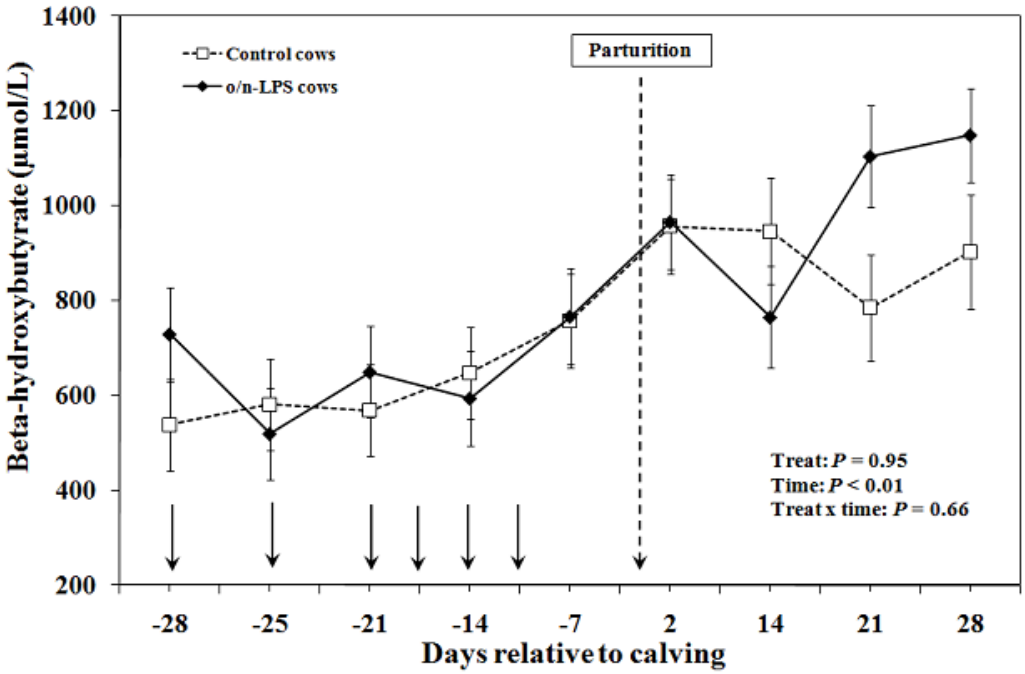


**Figure 3-3.** Concentration of beta-hydroxybutyrate in the serum of periparturient Holstein dairy cows treated oronasally with increasing doses of lipopolysaccharide (o/n-LPS; ◆) or saline (Control: □). Treat = Effect of treatment, Time = Effect of sampling day, Treat × time = Treatment by time interaction (LSM ± SEM; n = 15).

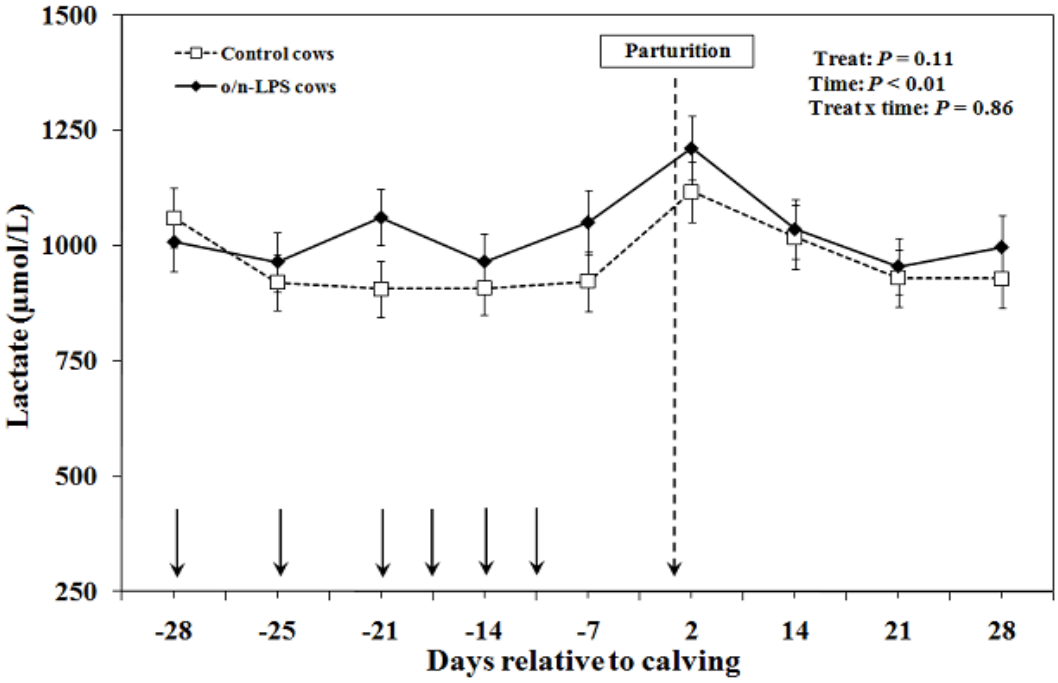


**Figure 3-4.** Concentration of lactate in the serum of periparturient Holstein dairy cows treated oronasal with increasing doses of lipopolysaccharide (o/n-LPS; ◆) or saline (Control: □ ). Treat = Effect of treatment, Time = Effect of sampling day, Treat × time = Treatment by time interaction (LSM ± SEM; n = 15).

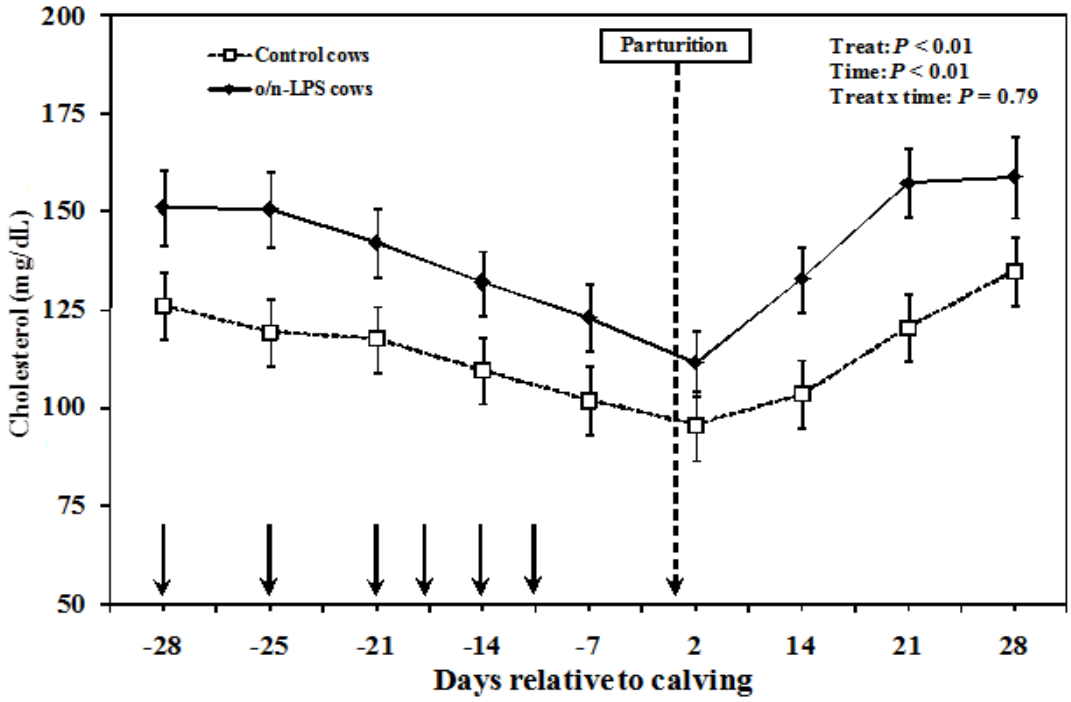


**Figure 3-5.** Concentration of cholesterol in the serum of periparturient Holstein dairy cows treated oronasal with increasing doses of lipopolysaccharide (o/n-LPS; ◆) or saline (Control: □). Treat = Effect of treatment, Time = Effect of sampling day, Treat × time = Treatment by time interaction (LSM ± SEM; n = 15).

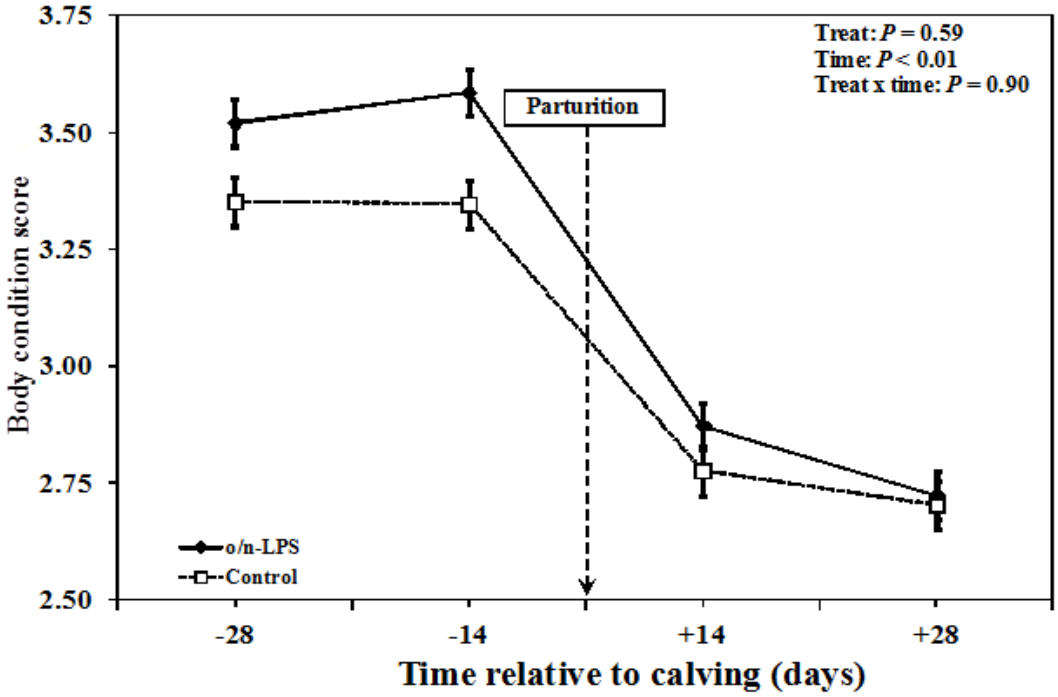


**Figure 3-6.** Body condition score (BCS) of periparturient Holstein dairy cows treated oronasal with increasing doses of LPS (o/n-LPS; ◆) or saline (Control: □). Treat = Effect of treatment, Time = Effect of measurement day, Treat × time = Treatment by time interaction (LSM ± SEM; n = 50).

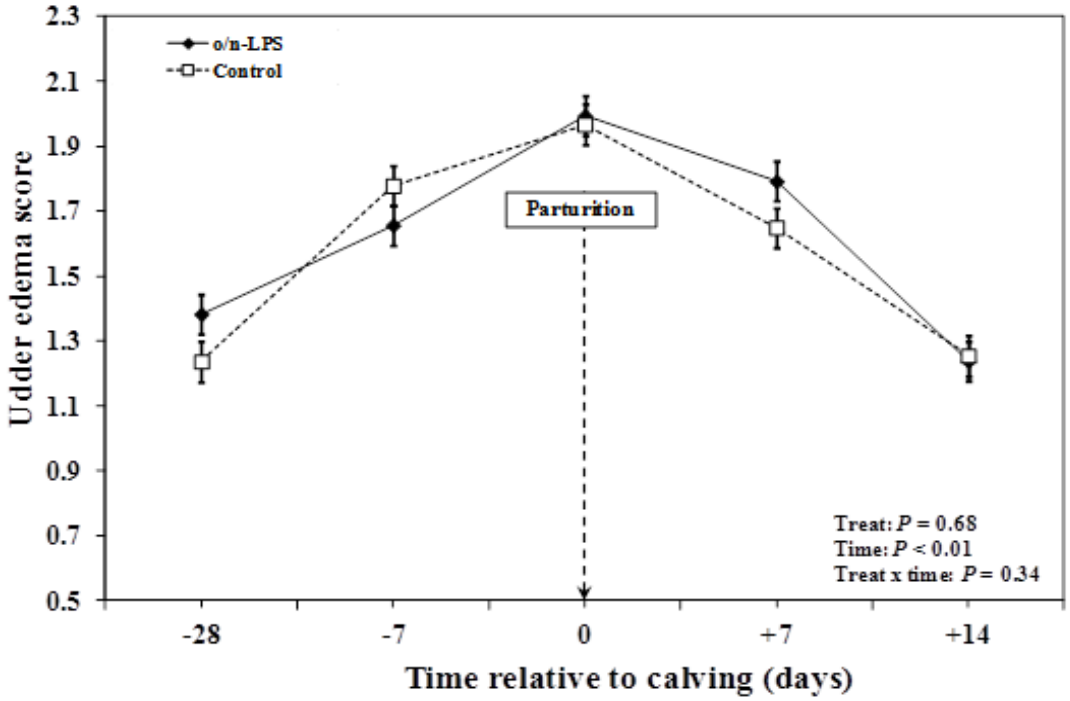


**Figure 3-7**. Udder edema (UE) scores of periparturient Holstein dairy cows treated oronasal with increasing doses of lipopolysaccharide (o/n-LPS; ◆) or saline (Control: □). Treat = Effect of treatment, Time = Effect of measurement day, Treat × time = Treatment by time interaction (LSM ± SEM; n = 50).

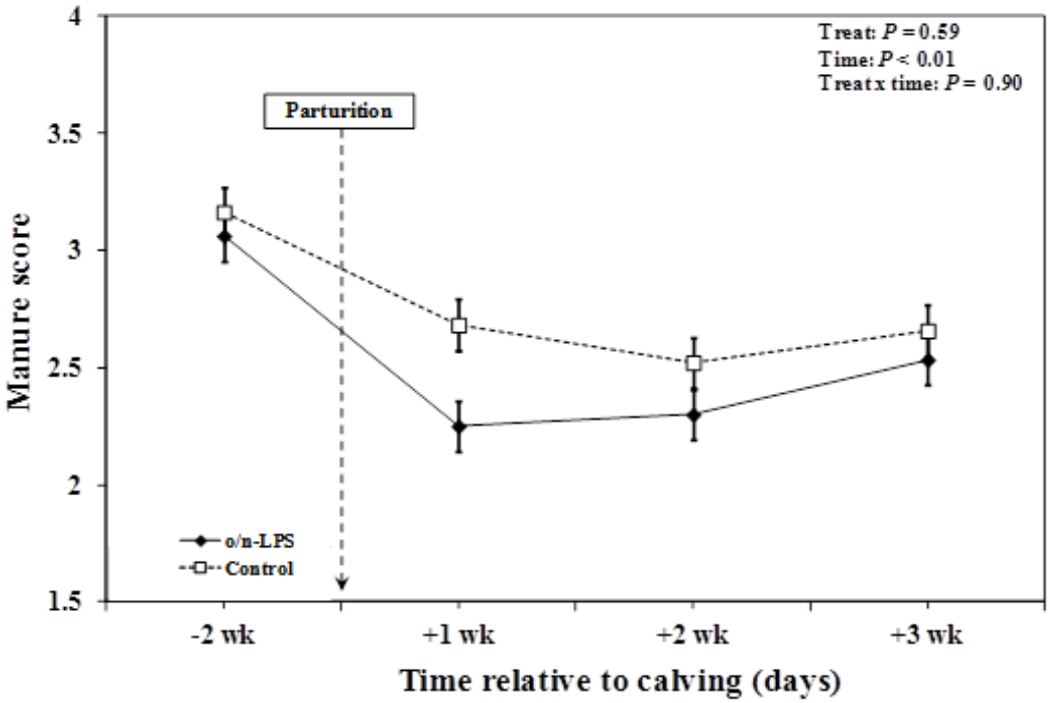


**Figure 3-8.** Manure score of periparturient Holstein dairy cows treated oronasally with increasing doses of lipopolysaccharide (o/n-LPS; ◆) or saline (Control: □ ). Treat = Effect of treatment, Time = Effect of measurement day, Treat × time = Treatment by time interaction (LSM ± SEM; n = 50).

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# Chapter 4

## Oronasal administration of lipopolysacharide prepartum induced mucosal IgA responses in periparturient dairy cows

### 4.1 Introduction

Transition period in dairy cows, including 3 wk before and 3 wk after parturition, is characterized by several immunological alterations that lead to a state of immunosuppression (Mallard et al., 1998). The decline in the immune status is a gradual process, which reaches its nadir immediately before calving (Ametaj et al., 2005; Lacetera et al., 2005). The exact mechanisms behind the lowered immune competence in periparturient dairy cows are not completely understood; however, the endocrinological changes and the increased metabolic stress around parturition are believed to play a role (Mallard et al., 1998; Rukkwamsuk et al., 1999). On the other hand, the presence of lipopolysaccharide (LPS), a cell-wall component of Gram-negative bacteria (GNB), has also been suggested as a factor playing a role in immunossupression of transition dairy cows (Bryn et al., 2008).

The LPS is continuously present in the mucosal sites of dairy cows; however, it is released in larger amounts in gastrointestinal tract when cows are switched from a high-forage to a high-grain diet immediately after parturition (Ametaj et al., 2005, 2010). Research also has demonstrated that the cell-free LPS in the rumen fluid translocates through rumen and colon tissues and it is found in the systemic circulation, triggering activation of an acute phase response (APR; Emmanuel et al., 2007, 2008; Khafipour et al., 2009; Ametaj et al., 2010). The

study conducted by Bryn et al. (2008) demonstrated that LPS induces monocytes to produce prostaglandin $E_2$ ($PGE_2$) that directly suppress T-cell functions and adaptive immune responses suggesting a role for LPS in the immunossuppression observed during transition period. Furthermore, free LPS in the uterine lumen during early postpartum also induces $PGE_2$ secretion by the uterine endometrium (Williams et al., 2005; Herath et al., 2006; Sheldon et al., 2008).

Mucosal surfaces comprise the first port of entry of bacterial LPS. Therefore, inducing humoral immunity against LPS in mucosal tissues before cows are exposed to high loads of LPS after parturition might prepare them immunologically to prevent harmful effects of LPS translocation. This type of immunomodulation potentially might increase production of secretory immunoglobulin-(Ig)A (sIgA), which is the dominant isotype synthesized by the mucosal immune system for neutralization of antigens at mucosal surfaces (Neutra and Kozlowski, 2006; Mantis et al., 2011; Ametaj et al., 2012a).

Recently, we primed periparturient dairy cows orally with increasing doses of LPS and observed an enhanced response of anti-LPS IgM antibodies in the plasma and improved overall immunity and metabolic health status (Ametaj et al., 2012b; Zebeli et al., 2013). In addition, a study in rats indicated that oral treatment with LPS provides protection against sepsis by increasing concentrations of anti-LPS IgM antibodies (Márquez-Velasco et al., 2007). Petzl et al. (2011) showed that intra-mammary infusion of LPS conferred protection against experimental *E. coli* mastitis in dairy cows. In another study it was shown that oral and nasal administration of monophosphoryl lipid A (MPL) induced

greater salivary and vaginal IgA responses to the group treated orally (Childers et al., 2000).

To our best knowledge the effects of repeated oronasal administration of LPS on innate and humoral immune responses and proinflammatory mediators in transition dairy cows are not known. Therefore, we hypothesized that repeated oronasal exposure of prepartum dairy cows to increasing doses of LPS might improve their innate and humoral immune responses against endotoxin in dairy cows. Thus, the objectives of this investigation were to evaluate the innate and adaptive immune responses of transition dairy cow to repeated oronasal exposure to LPS during the prepartum period.

## 4.2 Materials and Methods

### *4.2.1 Animals, Study Design, and Treatments*

One hundred pregnant Holstein dairy cows were randomly assigned to two treatment groups according to parity, body conditions score (BCS), milk production, and disease susceptibility from previous year. The average heifer and cow body weights (BW) were 600 ± 20 and 720 ± 30 kg, respectively. Out of 100 cows, 30 of them were randomly assigned to an intensive sampling (n = 15 per group) starting at -28 d before the expected day of parturition.

Cows (n = 50) assigned to the treatment group were orally and nasally administered 2 mL and 1 mL of sterile saline solution, respectively, containing 3 increasing doses of LPS from *Escherichia coli* 0111:B4 supplied by Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) as follows: 1) 0.01 µg/kg BW once per wk on d -28 before parturition, 2) 0.05 µg/kg BW twice per wk on d -25, and -

21 before parturition, and 3) 0.1 µg/kg BW twice per wk on d -18, and -14 before parturition. Cows (n = 50) allocated to the control group received an oral and a nasal treatment of 2 mL and 1 mL of sterile saline solution, respectively, on the same days as for the LPS treatments. Doses of LPS used were based on a previous research conducted by our team and the clinical responses to those doses (Ametaj et al., 2012b).

The initial crystalline *E. coli* LPS contained 10 mg of purified LPS, which was dissolved in 10 mL of doubly distilled water, as suggested by the manufacturer (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), and stored in the refrigerator at 4 °C until the time of administration. For oral and nasal administrations of LPS to the animals, the daily dose was dissolved in 2 mL and 1 mL of sterile saline solution, and then introduced into the oral and nasal cavity of the cows, respectively, using a disposable 5 mL plastic syringe (Becton, Dickinson and Company, BD, Franklin Lakes, NJ). Similarly, the same amount of carrier [i.e., 2 mL and 1 mL of sterile saline (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada)] was orally and nasally administered to all cows in the control group. The experiment lasted for 8 wks i.e., 28 d before the expected day of parturition and 28 d after calving. Cows were housed in tie stalls (48” x 79”), having free access to water throughout the experiment. Shortly before parturition cows were transferred to the maternity pens (22’ x 14 ½’) and returned to their stalls on the next day of parturition.

All animals were fed the same diet once daily at 0800 in their stalls, and daily ration was offered as total mixed ration (TMR) for ad-libitum intake to

allow approximately 5% feed refusals throughout the experiment. All animals were offered the close-up diet starting 3 wk prepartum (27% concentrate on DM basis), and they were gradually switched to the lactation diet (50% concentrate on DM basis) during the first 7 d after parturition. Diets were based on locally grown alfalfa hay and barley silage as forage sources, supplemented with rolled barley and corn grain as energy sources, and canola meal and soybean meal as protein sources, as well as mineral and vitamin supplements. These diets were formulated and offered to the cows to meet or exceed the energy and nutrient requirements of dry and lactating cows as per National Research Council (NRC) guidelines (2001). All experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Livestock and animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993).

### *4.2.2 Sample Collection*

Blood samples were collected from the coccygeal vein on d -28, -25, -21, -14, -7, +7, +14, +21, and +28 for plasma haptoglobin and on d -28, -7, +7, +28 for plasma serum amyloid A (SAA), lipopolysaccharide-binding protein (LBP), IgA, IgG, and IgM anti-LPS antibodies, tumour necrosis factor(TNF)-α, and interleukin-1 (IL-1) 15 min prior to administration of the treatment to the experimental cows. Blood samples of approximately 5-8 mL were collected in glass tubes with no additive for blood coagulation (BD Vacutainers 10 mL; Becton, Dickinson and Company, BD, Franklin Lakes, NJ). After collection, blood samples were put immediately on ice, and centrifuged within 20 min (Rotanta 460R, Hettich Zentrifugan, Tuttlingen, Germany) at 3,000 × *g* and 4 °C

for 20 min. The plasma was separated and stored at -20$^{o}$C until analyses. During blood withdrawal, no stress response was observed, as indicated from no agitation or overreaction from cows. Feed intake was recorded daily during the entire experimental period. All disease and medication history was recorded for each cow throughout the entire experimental period.

Saliva samples were collected from the oral cavity of the dairy cows on d -28, -7, +7, and +28 around parturition using sterile cotton gauze inserted between the cheek and the lower jaw, along the side of the mouth towards the back teeth until the gauze was soaked with saliva. The head movement of the animal was restrained for collection of saliva using conventional restraining technique (i.e., rope halter or held by a person). After collection, saliva was squeezed from the cotton gauze using 60 mL plastic syringe (Becton, Dickinson and Company, BD, Franklin Lakes, NJ), and then placed into a small sterile container, which was then sealed securely and stored at -86$^{o}$C until analyses for total IgA. No preservatives or additional material was added to the saliva samples.

Vaginal mucus samples were collected on d -28 and -7 before parturition and on d +7 and +28 after parturition using sterile insemination plastic pipettes (Becton, Dickinson and Company, BD, Franklin Lakes, NJ). Before sampling, the vulvar area was thoroughly cleaned with water and then disinfected with 30% (vol/vol) iodine solution (Iosan, WestAgro, Saint Laurent, Canada) prior to sampling. Approximately 1-2 mL of vaginal mucus were collected by inserting the insemination pipette into the vagina and close to the cervix by means of aspiration with a plastic syringe. Samples were placed into 1 mL of saline solution

in a plastic tube and transported to the laboratory in a refrigerated box. The tubes were sealed securely and stored at -20 $^{o}$C until analyses for total IgA. Before running assay, the samples were centrifuged (Rotanta 460R, Hettich Zentrifugan, Tuttlingen, Germany) at 1,000 × *g* and 4 °C for 20 min.

### *4.2.3 Sample Analyses*

Concentration of total saliva IgA was measured using a commercially available bovine ELISA kit (Uscn, Life Sciences Inc., USA). The basic principle involved the quantitative measure of IgA in bovine saliva through a sandwich enzyme immunoassay with a working time of 4 h. The microtiter plate pre-coated with antibodies was provided with the kit, and the standards and samples were added to the appropriate wells with a biotin-conjugated antibody preparation specific for IgA. Then avidin conjugated to the enzyme was added to the appropriate wells. The color developed by the substrate addition and was proportional to the concentration of IgA, biotin conjugated antibody, and enzyme conjugated avidin. The absorbance was measured at 450 nm, and the lower detection limit for this assay was 0.78 ng/mL. The inter- and intra-assay CV for all the samples tested for total salivary IgA was less than 10%.

Concentrations of vaginal mucus total IgA was measured using a commercially available bovine ELISA kit (Uscn, Life Sciences Inc., USA). The basic principle for *in vitro* quantitative measure of IgA in bovine biological fluids involved sandwich enzyme immunoassay with a working time of 4 h. The microtiter plate pre-coated with antibodies specific to the IgA was provided with the kit, and standards as well as samples were added to appropriate wells and

incubated. Then avidin conjugated to an enzyme was added to each microplate well and again incubated. The TMB substrate was added and the color developed was shown up only in those wells which contain IgA, biotin conjugated antibody, and enzyme conjugated avidin. The absorbance was measured at 450 nm with a spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA). The minimum detectable concentration of this assay was 0.78 ng/mL. The inter- and intra-assay CV for all the samples tested for total IgA in vaginal mucus was less than 10%.

Concentrations of anti-LPS core IgA, IgG, and IgM antibodies in the plasma were measured using commercially available ELISA kit EndoCab (HK504, Canton, MA, USA), as described recently by Ametaj et al.[16] In short, antibodies directed against the core structure of endotoxin (i.e., EndoCab) are cross-reactive against most types of LPS, and are measured using a solid phase ELISA based on the sandwich principle with a working time of 2.5 h. The anti-endotoxin core antibodies present in the sample determined the intensity of the colour developed, and the absorbance was measured at 450 nm with a microplate spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA). The minimum detection levels of IgA, IgG and IgM EndoCab antibodies were 0.156 AMU/ml, 0.0125 GMU/ml and 0.055 MMU/ml, respectively. The CV for the inter- and intra-assay analysis was less than 10% for all the samples tested.

Concentrations of plasma haptoglobin were measured with an ELISA kit provided by Tridelta Development Ltd. (Greystones C., Wicklow, Ireland). All samples were tested in duplicate, and the optical density was measured at 630 nm

on a microplate spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA). The minimum detection limit of the assay was 0.25 ng/ml. Inter- and intra-assay CV was less than 10% for this analysis.

Concentrations of LBP in the plasma were quantified with a commercially available ELISA kit, provided with antibody coated wells that cross-reacts with bovine LBP (Cell Sciences Inc., Norwood, MA; Emmanuel et al., 2007). Plasma samples were initially diluted 1:1,000; however the samples with lower optical density values than the range of the standard curve were reassayed according to the instructions of the manufacturer with a lower dilution of 1:500. All samples were tested in duplicate, and the optical densities were measured at 450 nm on a microplate spectrophotometer (Spectramax 190, Molecular Devices Corporation, Sunnyvale, CA, USA). The concentration of plasma LBP was calculated from a standard curve of the known LBP values in human plasma, and the minimum detection limit was 5 ng/ml. The inter- and intra-assay CV for the LBP analysis was less than 10%.

Concentrations of SAA in the plasma were determined using a commercially available ELISA kit (Tridelta Development Ltd., Greystones Co., Wicklow, Ireland) provided with the microtitre strips, coated with monoclonal antibodies specific for SAA as described previously by McDonald et al. (1991). In brief, initially samples were diluted 1:500; however if some of the samples had optical density values below the range of the standard curve, they were reanalyzed in lower dilutions. The inter- and intra-assay CV for the SAA analysis were less than 10%. All samples were tested in duplicate and the optical density values

were read on a microplate spectrophotometer (Spectramax 190, Molecular devices Corporation, CA, USA) at 450 nm. The minimum detection limit of the assay was 18.8 ng/mL.

A commercially-available ELISA kit was used to quantify concentrations of TNF-α in the plasma (Bethyl Laboratories, Inc. TX, USA). Initially, incubation of diluted samples and standards (100 μL) were done in the coated plate for 1 h, followed by washing. Then, the plate was incubated with 100 μL of detection antibody and horseradish peroxidase (HRP) substrate for 1 h and 30 min, respectively, and each of the incubations were followed by washings for 4 times. The detection antibody solution cross-reacts with the antibodies attached to the coated wells. The addition of 100 μL of TMB solution allow the enzymatic reaction, and the colour developed was proportional to the amount of anti-TNF-α antibodies present in the sample. The absorbances were measured at 450 nm with a spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA). The minimum detection limit of the assay was 0.078 ng/mL. The inter- and intra-assay CV for the analysis of TNF-α was less than 10%.

Concentrations of IL-1 in the plasma were determined by a commercially available bovine ELISA kit (Cusabio biotech Co., Ltd, Newark, USA), based on the competitive inhibition of an enzyme immunoassay technique. According to the manufacturer, antibodies specific to IL-1 were pre-coated onto microplate wells. Standards and samples were then incubated with biotin-conjugated IL-1, which leads to competitive inhibition reaction between IL-1 (standards or samples) and biotin-conjugated IL-1 with the pre-coated antibody specific for IL-

1. The avidin conjugated to HRP was added to each microplate well and incubated following the addition of substrate solution to the wells. The color developed was considered opposite to the amount of IL-1 in the sample. Further development of color was stopped by adding stop solution and the intensity of the color was measured at 450 nm with a spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA). The minimum detection level of bovine IL-1 was <125 pg/mL. The inter- and intra-assay CV for the IL-1 analysis was less than 10%.

### *4.2.4 Statistical Analysis*

Data were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA Version 9.1.3) as describe by the following model:

$$Yijl = \mu + \gamma i + \alpha j + \beta l + (\alpha\beta)jl + \varepsilon ijkl$$

Where Yijkl is the observation for dependant variables, μ is the population mean, γi is the independent parameter for animal i, αj is a population parameter corresponding to treatment j, βl is the fixed effect of measurement time (relative to each LPS challenge) l, (αβ)jl is the effect of treatment by time interaction, and εijkl is the residual error. Data are shown as least-squares means (LSM) and standard error of the mean (SEM). Multiple comparisons of LSM were conducted by probability difference (PDIFF) option of SAS. Measurements on the same animal were considered as repeated measures. The covariance structure of the repeated measurements for each variable was modeled separately according to the lowest values of fit statistics based on the Bayesian information criteria (BIC). The measurements taken on d -28 were considered as covariates in the analysis.

The significance limit was declared at $P < 0.05$, whereas a biologically relevant tendency was declared at $0.05 \leq P \leq 0.10$.

## 4.3 Results

### *4.3.1 Plasma, Saliva, and Vaginal Mucus Immunoglobulins*

Data showed differences with regards to saliva total IgA antibodies between the two treatment groups (Figure 4-1), with greater concentrations in cows treated oronasally with LPS than the control group ($P < 0.01$). There was also an effect of the sampling time on the concentrations of saliva total IgA ($P < 0.01$). However, no treatment by measurement time interaction was observed between the two groups, regarding saliva total IgA ($P = 0.22$; Figure 4-1).

The treated cows showed a tendency for greater total IgA antibodies in the vaginal mucus versus the control group ($P = 0.10$; Figure 4-2). The factor time of sampling had an effect on the vaginal mucus IgA ($P < 0.01$). No treatment by time interaction was evidenced regarding concentrations of total IgA in the vaginal mucus in the present study ($P = 0.15$; Figure 4-2).

Results indicated that oral administration of LPS had no effect on IgA anti-LPS antibodies in the plasma ($P = 0.74$; Figure 4-3a). The factor sampling time showed an influence on this variable ($P < 0.01$). No treatment by sampling time interaction was obtained regarding plasma IgA anti-LPS antibodies in this study ($P = 0.91$; Figure 4-3a).

Concentrations of IgG anti-LPS antibodies in the plasma were not different between the treated cows and those in the control group ($P = 0.40$; Figure 4-3b). Moreover, there was no effect of time of sampling around

parturition on this variable ($P$ = 0.19). No treatment by measurement time interaction with respect to this variable was obtained ($P$ = 0.30; Figure 4-3b).

No differences between the two treatment groups were found regarding plasma IgM anti-LPS antibodies ($P$ = 0.32; Figure 4-3c). It should be noted that the time of measurement showed an influence on this variable with values decreasing after parturition ($P$ = 0.01; Figure 4-3c). In addition, no treatment by sampling time interaction was observed regarding plasma IgM anti-LPS antibodies in this study ($P$ = 0.98; Figure 4-3c).

### *4.3.2 Plasma Acute Phase Proteins*

Concentrations of plasma haptoglobin were not different between the two treatment groups ($P$ = 0.54; Figure 4-4). Also, the factor of sampling time had no effect on plasma haptoglobin ($P$ = 0.96; Figure 4-4). An interaction was evident between the treatment and time of measurement for this variable ($P$ < 0.01).

Data indicated no overall effect of treatment on plasma SAA ($P$ = 0.36; Figure 4-5a). It should be noted that there was a sampling time effect with regards to plasma SAA ($P$ = 0.02). Also, data showed a treatment by time interaction for plasma SAA ($P$ < 0.01; Figure 4-5a), with greater concentrations in the treatment group at day -28 before parturition, which declined almost 2-fold the wk after parturition, and remained at this level until the end of the experiment (i.e., 254 μg/mL at -28 day versus 130 μg/mL at +28 day around parturition). Concentrations of plasma SAA in the control cows increased from 160 μg/mL to 210 μg/mL from -28 to +28 day around parturition, respectively (Figure 4-5a).

The treatment did not have an effect on plasma LBP in this study ($P$ = 0.75; Figure 4-5b). Furthermore, neither sampling time alone ($P$ = 0.11) nor treatment by time interaction showed an effect for this variable ($P$ = 0.32; Figure 4-5b).

### *4.3.3 Plasma Cytokines*

There were no differences in the plasma concentrations of TNF-α between the treated and the control cows ($P$ = 0.66; Figure 4-6). Also, no effect of time of sampling around parturition was obtained with regards to plasma TNF-α ($P$ = 0.54). No overall treatment by time interaction was obtained for plasma TNF-α ($P$ = 0.95; Figure 4-6).

There was no difference in the concentrations of plasma IL-1 between the two groups ($P$ = 0.19; Figure 4-7). There was a sampling time effect for plasma IL-1 and a treatment by time interaction for this variable ($P$ = 0.01), with almost 2-fold decrease from day -28 before parturition to day +28 after calving. On the other hand, the control cows showed enhanced plasma IL-1 from day -28 to -7 before calving, and then there was a slight decrease immediately after parturition at day +7 (Figure 4-7).

## 4.4 Discussion

The present study investigated whether repeated administration of increasing doses of LPS in the oral and nasal mucosa would affect cow's ability to mount an effective immune response against LPS during the periparturient period. The results of this study demonstrated that oronasal LPS modulated mucosal immunity, but had no effect on innate and humoral immune variables in the

plasma. To the best of our knowledge, this is the first study which associates the oronasal LPS challenge with mucosal humoral responses in dairy cows.

The most interesting finding of this study was that oronasal LPS increased concentrations of total salivary IgA antibodies in the treated cows around parturition. Secretory IgA constitutes the largest humoral immune response and serves as the first line of defence predominantly in the epithelial sites through a process known as the immune exclusion (Neutra and Kozlowski, 2006; Mantis et al., 2011). In the mucosal sites, the sIgA promotes the clearance of antigens by blocking their access to epithelial receptors, entrapping them in mucus, and facilitating their removal by peristaltic and mucociliary activities (Mantis et al., 2011). Lipopolysaccharide has been demonstrated to directly activate local B lymphocytes and induce sIgA secretion in a T-cell independent way (Wang, 2001; Bergqvist et al., 2010). Our data are in agreement with a previous study that showed that oral immunization with GNB *Brucella melitensis* enhanced salivary anti-LPS IgA responses (Izadjoo et al., 2004). The underlying mechanism might be related to priming of local plasma $IgA^+$ B cells. The primed lymphocytes enter into the bloodstream and preferentially home to various mucosal surfaces via the common mucosal immune system, and then differentiate into IgA-producing cells at adjacent mucosal layers (Thurnheer et al., 2003).

Results also showed a tendency for greater concentrations of total IgA antibodies in the vaginal mucus of cows treated oronasally with LPS. This indicates that oronasal LPS, at the dose used in this study, was able to induce a short-lived IgA response in the vagina of dairy cows. Induction of an immune

response in the genital tract of transition cows is of particular importance since uterine infections are very common and cause infertility and are the number one reason for culling of dairy cows. Previous studies demonstrated that intranasal immunization with cholera toxin B subunit alone or mixed with bacterial protein stimulated IgA antibody responses in the vaginal secretions of human volunteers and in rhesus monkeys (Russell et al., 1996; Bergquist et al., 1997). Besides nasal antigenic stimulation, results from studies in animal and human models have shown that oral immunization with GNB also induces a pronounced immune response in the genital tract and in the vaginal secretions (Cui et al., 1991; Kantele et al., 1998). Although oral and nasal mucosae are anatomically separate regions immunization at those sites induces specific sets of mucosal homing receptors during the interaction of T and B cells with antigen which create functional connectivity and can stimulate effector T and B cell responses at adjacent mucosal tissues such as the intestinal and urogenital tracts (Kiyono and Fukuyama, 2004; Kiyono et al., 2008).

Data showed that plasma IgA, IgG, and IgM anti-LPS antibodies were not affected by the oronasal administration of LPS. There was a sharp increase of plasma anti-LPS IgG concentration in the treated cows immediately after parturition. Qadri et al. (1999) observed that LPS from *Vibrio Cholera* enhanced IgG antibody responses of all four IgG subclasses in infected patients and that IgG antibodies showed important antibacterial activity and capacity to bind with complement. Interestingly, a new line of investigation indicated the TI memory B cells are established during administration of pure bacterial polysaccharide

(Gabay and Kushner, 1999). Our results suggest that oronasal treatment with LPS may have induced secondary humoral immunity in periparturient dairy cows.

Repeated oral challenge with LPS did not have an overall effect on concentrations of haptoglobin, SAA, and LBP in the plasma; however, cows in the treated group had lower plasma SAA after parturition. It is well established that plasma APP are non-specific immune responses and are produced when liver hepatocytes are stimulated by pro-inflammatory cytokines (Vels et al., 2009). A number of studies have indicated that SAA released into the systemic circulation binds, neutralizes, and removes LPS from systemic circulation through liver hepatocytes (Kushner, 1982; Opal et al., 1994, Cabana et al., 1999; Ametaj et al., 2010).

Results also indicated that oral challenge with repeated doses of LPS had no effect on plasma TNF-α. Furthermore, treatment did not show an overall effect on plasma IL-1, although it was lower in the treated cows around calving. The lower levels of IL-1 in the treated cows during 2 wk around calving might be related to prevention of LPS translocation into the systemic circulation of the treated cows during the transition period (Bone, 1991; Mackiewicz et al., 1991; Vels et al., 2009). Humblet et al. (2006) showed that calving is associated with enhanced concentration of proinflammatory cytokines in dairy cows. Interleukin-1 is produced by liver macrophages when they bind LPS (Gabay and Kushner, 1999). Furthermore, IL-1 cascade has an early and important role in neutrophilic recruitment in response to LPS challenge at the site of inflammation Basran et al.,

2013. Thus, lower plasma concentrations of IL-1 around calving suggest prevention of inflammatory responses in the treated cows.

### 4.5 Conclusions

In conclusion, data from this study showed that repeated oronasal challenge with LPS from *E. coli* 0111:B4 enhanced concentrations of total salivary IgA and had a tendency for greater total IgA in the vaginal mucus. There were no effects of the oronasal LPS on plasma IgA, IgM, and IgG anti-LPS antibodies in the treated cows. Results also demonstrated that APP including haptoglobin, SAA, LBP, and TNF-α were not affected by the treatment; however, IL-1 was lowered in the treated cows around calving compared to the control animals. Overall, data indicated that LPS affected mucosal IgA secretion but had little effect on blood variables related to innate and humoral immunity.

### 4.6 Conflict of Interest Statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

### 4.7 Acknowledgments

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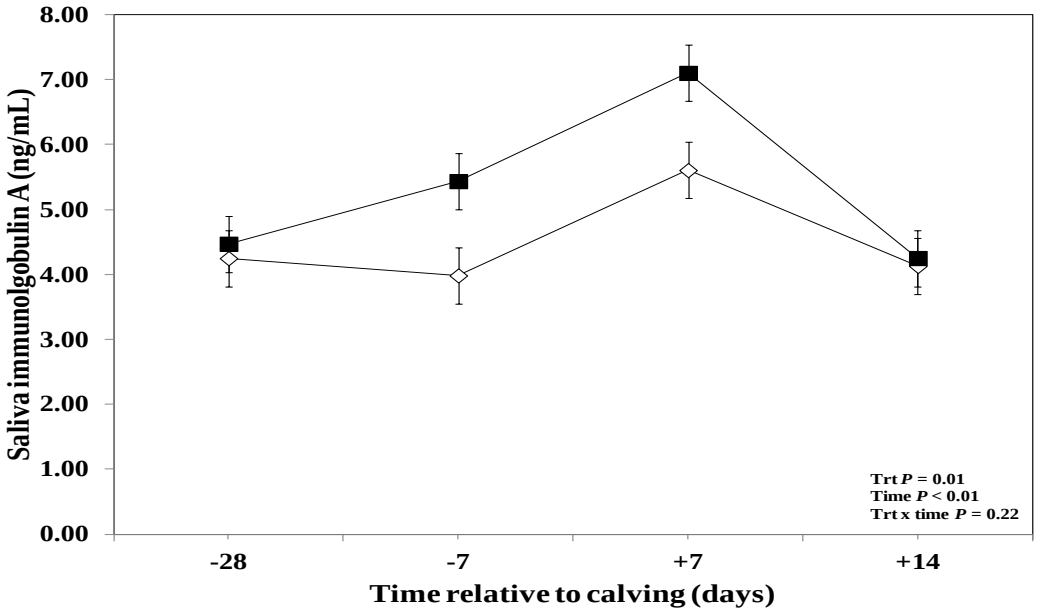


**Figure 4-1.** Concentrations of total salivary IgA in periparturient Holstein cows challenged oronasally with LPS (TRT; ■) or saline (Control; ◇) for 3 wk starting at -28 d before the expected day of parturition. Cows in the treatment group were administered orally and nasally 2 mL and 1 mL of sterile saline solution (0.15 M of NaCl), respectively, containing 3 increasing doses of LPS from *E. coli* 0111:B4 as follows: 1) 0.01 µg/kg body weight (BW) on d -28, 2) 0.05 µg/kg BW on d -25, and -21, and 3) 0.1 µg/kg BW on d -18, and -14, whereas control cows received 2 mL oral and 1 mL nasal sterile saline solution. (LSM ± SEM; n = 15; Trt = effect of treatment; Time = effect of sampling day, Trt x time = effect of treatment by sampling day).

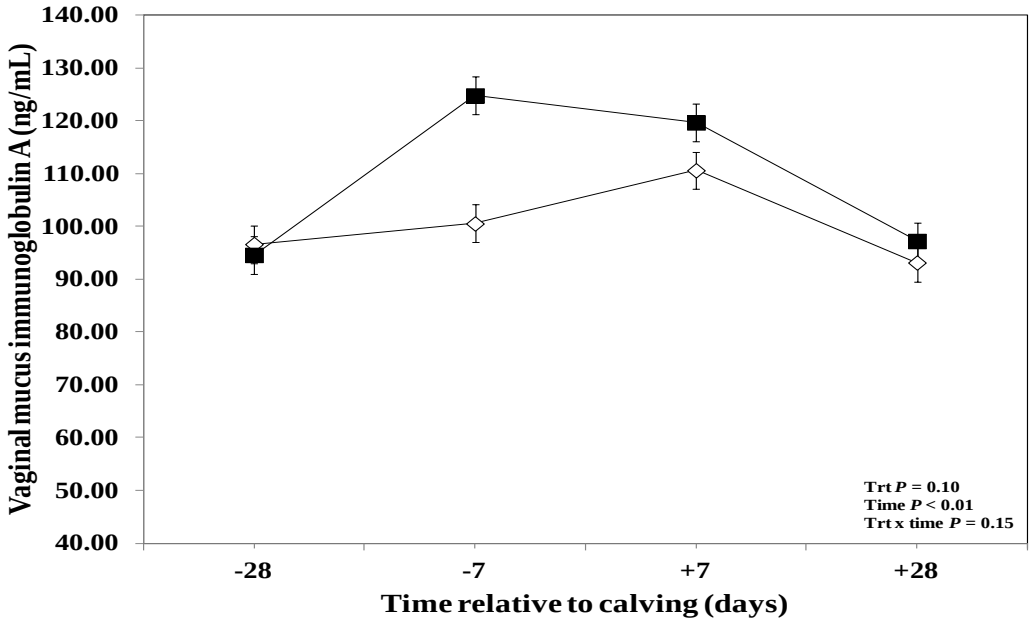


**Figure 4-2.** Concentrations of total vaginal mucus IgA in periparturient Holstein cows challenged oronasally with LPS (TRT; ■) or saline (Control; ◇) for 3 wk starting at -28 d before the expected day of parturition. Cows in the treatment group were administered orally and nasally 2 mL and 1 mL of sterile saline solution (0.15 M of NaCl), respectively, containing 3 increasing doses of LPS from *E. coli* 0111:B4 as follows: 1) 0.01 µg/kg body weight (BW) on d -28, 2) 0.05 µg/kg BW on d -25, and -21, and 3) 0.1 µg/kg BW on d -18, and -14, whereas control cows received 2 mL oral and 1 mL nasal sterile saline solution. (LSM ± SEM; n = 15; Trt = effect of treatment; Time = effect of sampling day, Trt x time = effect of treatment by sampling day).

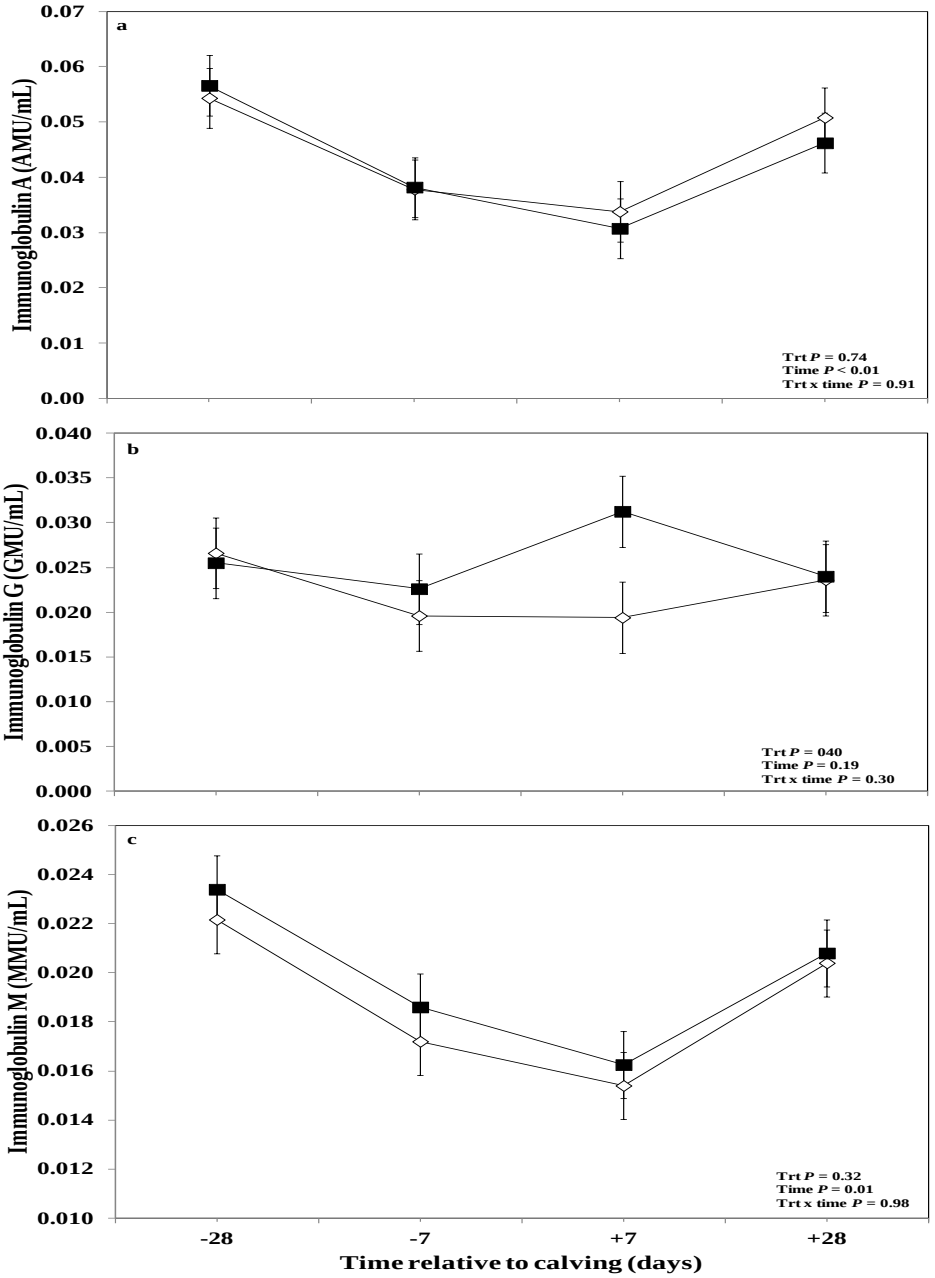


**Figure 4-3.** Concentrations of plasma immunoglobulin A (a), immunoglobulin G (b), and immunoglobulin M (c) in periparturient Holstein cows challenged oronasally with LPS (TRT; ■) or saline (Control; ◇) for 3 wk starting at -28 d before the expected day of parturition. Cows in the treatment group were administered orally and nasally 2 mL and 1 mL of sterile saline solution (0.15 M of NaCl), respectively, containing 3 increasing doses of LPS from *E. coli* 0111:B4 as follows: 1) 0.01 µg/kg body weight (BW) on d -28, 2) 0.05 µg/kg BW on d -25, and -21, and 3) 0.1 µg/kg BW on d -18, and -14, whereas control cows received 2 mL oral and 1 mL nasal sterile saline solution. (LSM ± SEM; n = 15; Trt = effect of treatment; Time = effect of sampling day, Trt x time = effect of treatment by sampling day).

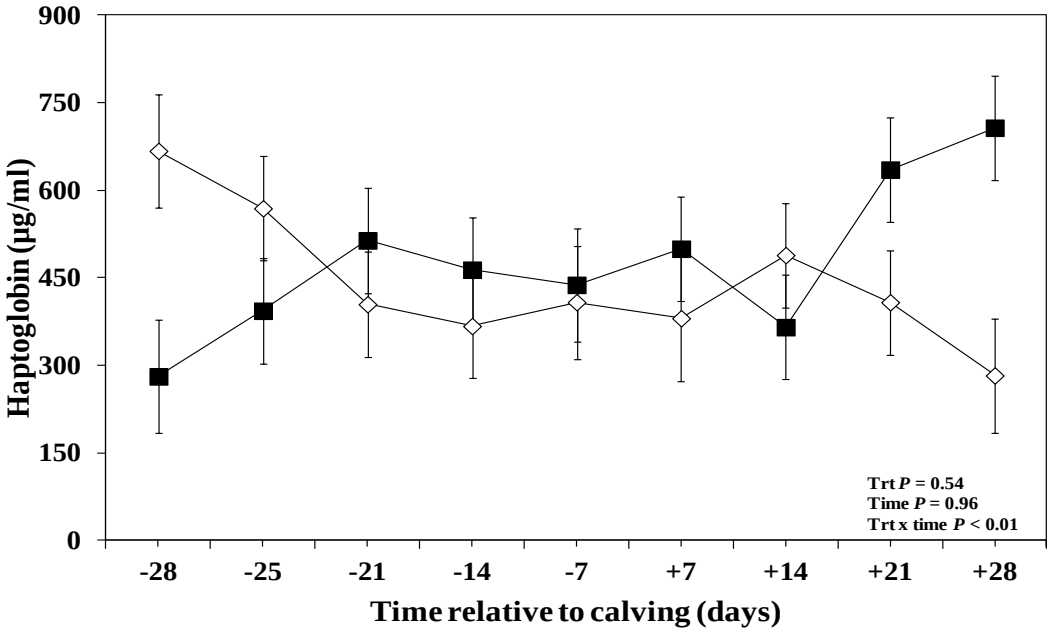


**Figure 4-4.** Concentrations of plasma haptoglobin in periparturient Holstein cows challenged oronasally with LPS (TRT; ■) or saline (Control; ◇) for 3 wk starting at -28 d before the expected day of parturition. Cows in the treatment group were administered orally and nasally 2 mL and 1 mL of sterile saline solution (0.15 M of NaCl), respectively, containing 3 increasing doses of LPS from *E. coli* 0111:B4 as follows: 1) 0.01 µg/kg body weight (BW) on d -28, 2) 0.05 µg/kg BW on d -25, and -21, and 3) 0.1 µg/kg BW on d -18, and -14, whereas control cows received 2 mL oral and 1 mL nasal sterile saline solution. (LSM ± SEM; n = 15; Trt = effect of treatment; Time = effect of sampling day, Trt x time = effect of treatment by sampling day).

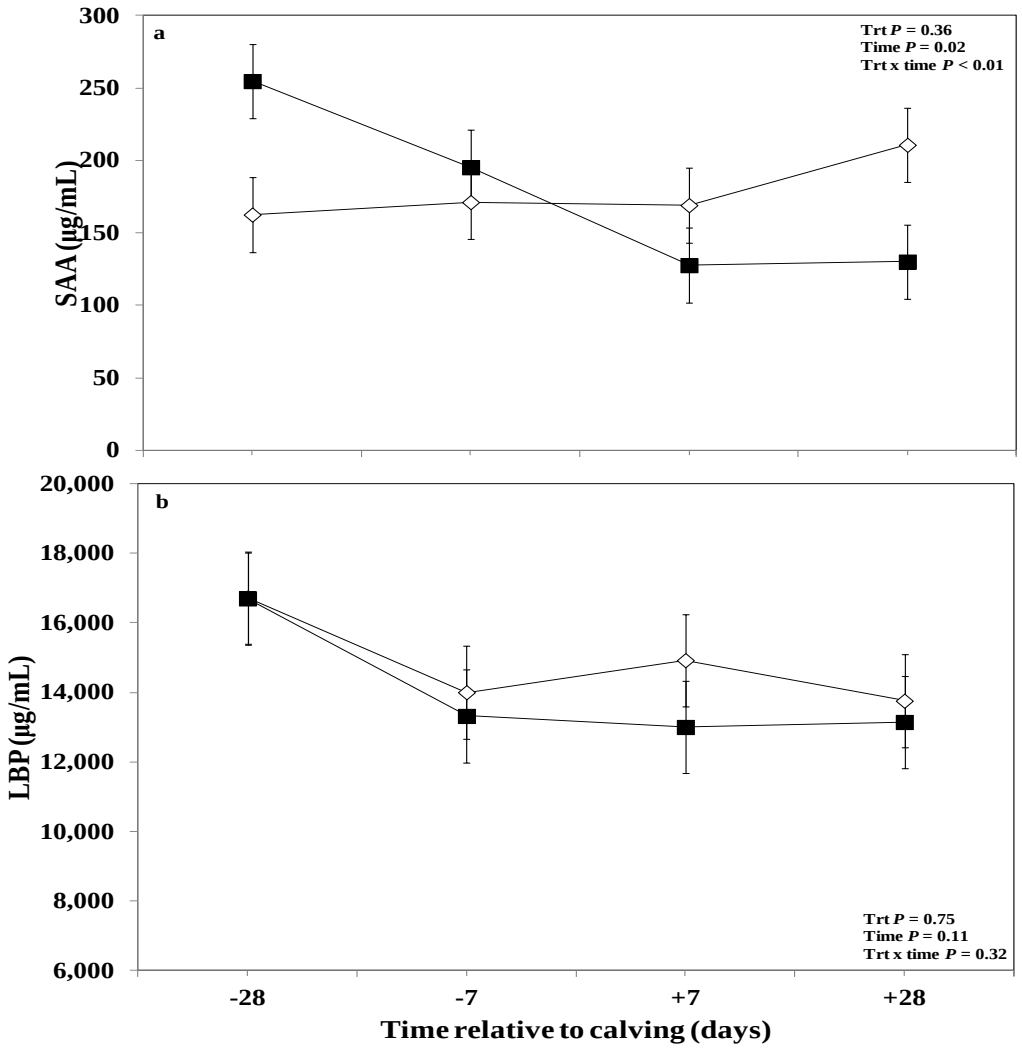


**Figure 4-5.** Concentrations of plasma serum amyloid A (a), and lipopolysaccharide binding protein (b), in periparturient Holstein cows challenged oronasally with LPS (TRT; ■) or saline (Control; ◇) for 3 wk starting at -28 d before the expected day of parturition. Cows in the treatment group were administered orally and nasally 2 mL and 1 mL of sterile saline solution (0.15 M of NaCl), respectively, containing 3 increasing doses of LPS from *E. coli* 0111:B4 as follows: 1) 0.01 µg/kg body weight (BW) on d -28, 2) 0.05 µg/kg BW on d -25, and -21, and 3) 0.1 µg/kg BW on d -18, and -14, whereas control cows received 2 mL oral and 1 mL nasal sterile saline solution. (LSM ± SEM; n = 15; Trt = effect of treatment; Time = effect of sampling day, Trt x time = effect of treatment by sampling day).

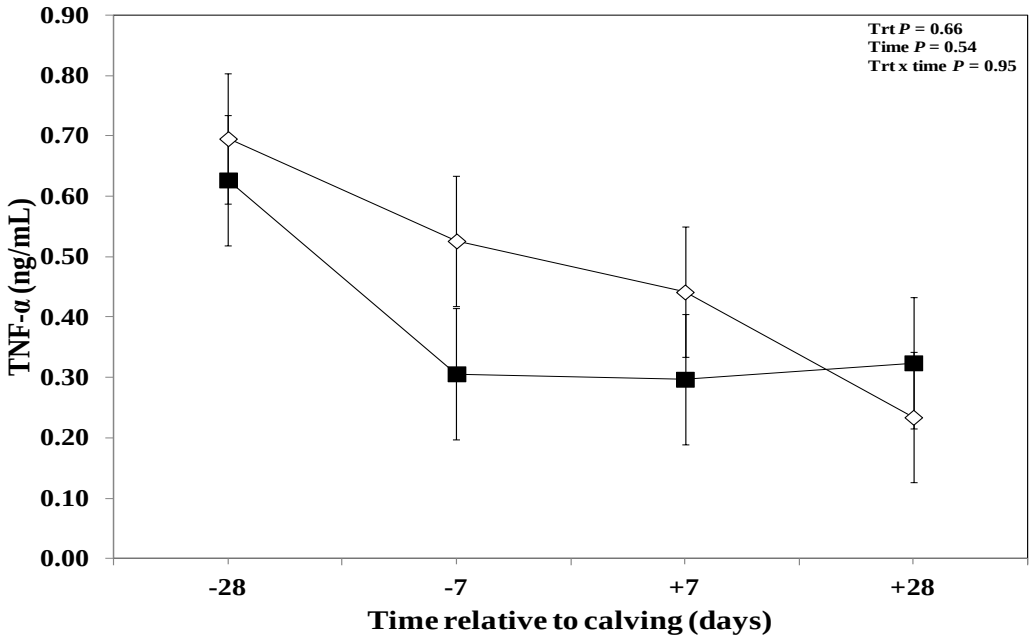


**Figure 4-6.** Concentrations of plasma tumour necrosis factor-α in periparturient Holstein cows challenged oronasally with LPS (TRT; ■) or saline (Control; ◇) for 3 wk starting at -28 d before the expected day of parturition. Cows in the treatment group were administered orally and nasally 2 mL and 1 mL of sterile saline solution (0.15 M of NaCl), respectively, containing 3 increasing doses of LPS from *E. coli* 0111:B4 as follows: 1) 0.01 µg/kg body weight (BW) on d -28, 2) 0.05 µg/kg BW on d -25, and -21, and 3) 0.1 µg/kg BW on d -18, and -14, whereas control cows received 2 mL oral and 1 mL nasal sterile saline solution. (LSM ± SEM; n = 15; Trt = effect of treatment; Time = effect of sampling day, Trt x time = effect of treatment by sampling day).

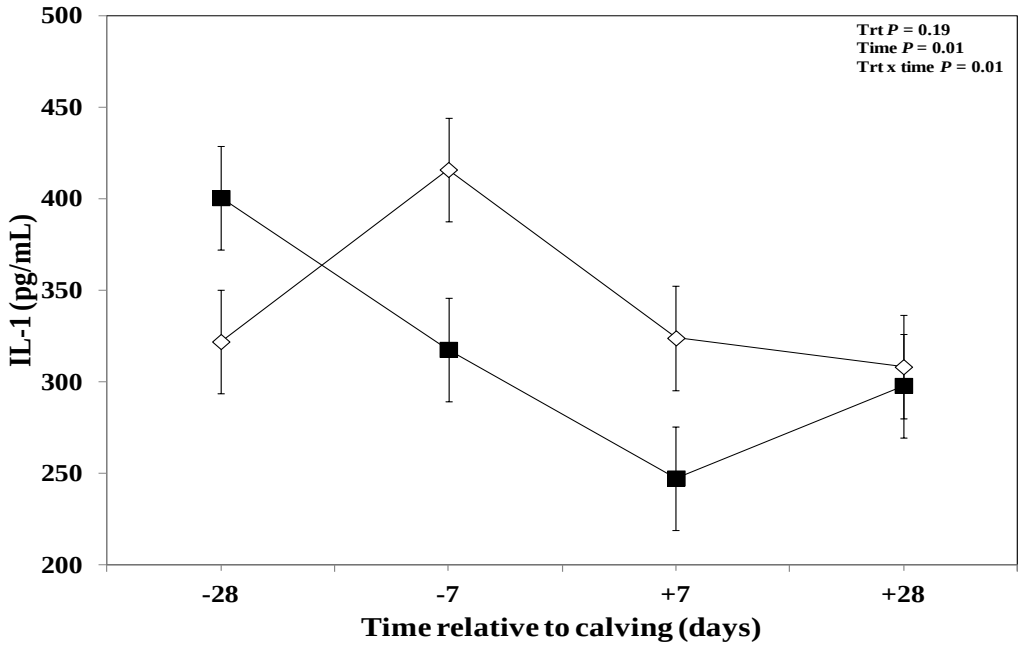


**Figure 4-7.** Concentrations of plasma interleukin-1 in periparturient Holstein cows challenged oronasally with LPS (TRT; ■) or saline (Control; ◇) for 3 wk starting at -28 d before the expected day of parturition. Cows in the treatment group were administered orally and nasally 2 mL and 1 mL of sterile saline solution (0.15 M of NaCl), respectively, containing 3 increasing doses of LPS from *E. coli* 0111:B4 as follows: 1) 0.01 µg/kg body weight (BW) on d -28, 2) 0.05 µg/kg BW on d -25, and -21, and 3) 0.1 µg/kg BW on d -18, and -14, whereas control cows received 2 mL oral and 1 mL nasal sterile saline solution. (LSM ± SEM; n = 15; Trt = effect of treatment; Time = effect of sampling day, Trt x time = effect of treatment by sampling day).

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# [1]Chapter 5

## Effects of prepartal oronasal administration of lipopolysaccharide on milk composition and productivity of transition Holstein dairy cows

### 5.1 Introduction

The normal function of the bovine mammary gland is disrupted during microbial infections and by the harmful toxins that they release early postpartum (Oliver and Calvinho, 1995). Among the various harmful bacterial toxins lipopolysaccharide (LPS), a cell wall component of all Gram-negative bacteria (GNB), commonly known as endotoxin, has received much attention (Oliver and Calvinho, 1995; Zebeli and Ametaj, 2009). The potential source for LPS could be the mammary gland itself following GN infection, which caused pathological changes in the mammary tissue, and developed local inflammatory conditions (Wenz et al., 2001; Ametaj et al., 2012a). Lipopolysaccharide also might originate from other sources such as gastrointestinal tract and uterus (Wenz et al., 2001). During transition period, rumen carries high loads of free LPS especially when cows are switched from the dry off period diets low in grain to high-grain diets early postpartum. The latter diets are known to cause sudden depression of ruminal pH with the onset of subacute ruminal acidosis and consequently disrupting the balance of multi-species microbiota with a major shift towards GNB (Ametaj et al., 2010; Dong et al., 2011). It is also evident that rumen LPS translocates into the bloodstream and further into the mammary gland where it

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suppresses the functions of the mammary epithelial cells (MEC; Dong et al., 2011; Emmanuel et al., 2007).

A recent study from our group evidenced a strong association between rumen free LPS with systemic inflammatory responses and milk-fat depression in lactating dairy cows (Zebeli and Ametaj, 2009). This indicates that the supply of nutrients for synthesis of milk components is hampered following endotoxin exposure. Several previous studies showed that endotoxin decreases de novo fat synthesis of the mammary gland by suppressing the activity of fatty acid (FA) synthetase and acetyl-CoA carboxylase (Pekala et al., 1983; Lopez-Soriano and Williamson, 1994) as well as down-regulating lipoprotein lipase (LPL), which lowers the uptake of FA for milk fat synthesis (Khovidhunkit et al., 2004). In addition, endotoxin causes various changes in other milk components and suppresses milk yield (Shuster et al., 1991). For example, intramammary injection of endotoxin induces a 100-fold increase in milk SCC and a 50% elevation in the concentration of protein content (McFadden et al., 1988).

Despite major progress in understanding the role of LPS in the etiopathology of periparturient diseases in transition dairy cows there is a lack of effective prophylactic strategies against its translocation into the host systemic circulation. Various parenteral vaccines have been designed to combat GN bacterial infections, especially those caused by *E. coli*, such as J5 core antigen against mastitis (Hogan et al., 1992). However, in the case of parenteral vaccines, immunoglobulin (Ig)G and IgM humoral responses are induced, which work by attacking intruding bacteria. Mucosal vaccines have received much attention

during recent years, and it is well known that by priming the immune response via one mucosal route generally stimulates production of secretory IgA (sIgA) at various adjacent mucosal layers (Neutra and Kozlowski, 2006). Several recent studies have shown that repeated intra-mammary infusion of sub-lethal doses of *E. coli* LPS mimics intra-mammary inflammation caused by GN infection (Lohuis et al., 1990; Schmitz et al., 2004; Beutler and Rietschel, 2003; Petzl et al., 2008), without having any deleterious effects on milk production in dairy cows (Shuster and Harmon,1991). Recent data from our group indicated increased anti-LPS IgM antibodies and better overall immune and metabolic health status in dairy cows treated orally with LPS (Ametaj et al., 2012b). To our best knowledge, the effects of oronasal administration of LPS have not been studied in transition dairy cows yet. We hypothesized that repeated oronasal administration of increasing doses of LPS in prepartum dairy cows might affect milk production and composition as well as the number of SCC in early lactating dairy cows. Therefore the objectives of this study were to treat cows oronasally with LPS for a period of 3 weeks starting at 4 weeks before parturition and monitor milk production, its composition, and the SCC in transition dairy cows.

## 5.2 Materials and Methods

### *5.2.1 Animals and Treatments*

One hundred pregnant Holstein dairy cows were randomly assigned to two treatment groups (n = 50 per group) according to parity, milk production, body condition score (BCS), and disease susceptibility from previous year. Thirty cows out of 100 (i.e., 10 primiparous with average weight of 600 kg and 20 multiparous

cows with average weight of 720 kg) were assigned to intensive sampling (n = 15 per group) starting at -28 d before the expected day of parturition.

Fifty cows in the treatment group were orally and nasally administered 2 and 1 mL of sterile saline solution, respectively, containing 3 increasing doses of LPS from *E. coli* 0111:B4 supplied by Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) as follows: 1) 0.01 µg/kg body weight (BW) once per week on d -28 before parturition, 2) 0.05 µg/kg BW twice per week on d -25 and -21 before parturition, and 3) 0.1 µg/kg BW twice per week on d -18 and -14 before the expected day of parturition. Doses of LPS used were determined from a previous investigation conducted by our research group and based on the clinical and pathological responses to those doses in order to prevent lethal endotoxemia and induction of a refractory state against endotoxin (Ametaj et al., 2012b). For preparation of the LPS doses, initial crystalline *E. coli* LPS containing 10 mg of purified LPS was dissolved in 10 mL of doubly distilled water (as suggested by Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), and stored in the refrigerator at 4 °C until the time of administration. For oral and nasal administrations of LPS, the daily dose was dissolved in 2 and 1 mL of sterile saline solution, and then introduced into the oral and nasal cavity of the cows, respectively, using a disposable 5 mL syringe (Becton, Dickinson and Company, BD, Franklin Lakes, NJ). Similarly, the same amount of carrier (i.e., 2 and 1 mL of sterile saline supplied by Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) was administered orally and nasally to all cows in the control group, on the same days as for the LPS treatments. All experimental procedures were approved by the University of

Alberta Animal Care and Use Committee for Livestock and animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993).

The experiment started 28 d before the expected day of parturition and lasted until 28 d after parturition. Cows were housed in tie stalls and transferred to the maternity pens shortly before parturition and returned to their stalls on the next day of calving. Cows were having free access to water throughout the experiment. The daily ration was offered as total mixed ration (TMR) to all animals in their stalls at 0800 for ad-libitum intake to allow approximately 10% feed refusals throughout the experiment. The composition of the TMR was based on the close up dry period diet, and started being fed to all cows at 21 d before the expected day of parturition. Then, all cows were switched to the lactation diet gradually during the first 7 d after parturition. All diets were formulated to meet or exceed the nutrient requirements of dry and early lactating cows as per the NRC (2001) guidelines. Ingredients and chemical composition of the diets are presented in Table 5-1 and 5-2.

### *5.2.2 Feed Intake and Milk Composition*

Feed intake and milk production were recorded daily during the 4 week before and 4 week after parturition. Feed intake was calculated for each individual cow by the difference between the total daily feed given with that of the feed refusals of the next morning. Milk samples were collected once per week at 0500 and 1500 and analyses were done for milk fat, crude protein (CP), milk urea nitrogen (MUN), somatic cell count (SCC), and lactose content by mid-infrared

spectroscopy (MilkoScan 605; A/S N Foss Electric, Hillerød, Denmark) at Central Milk Testing Laboratory in Edmonton, Alberta. Daily milk energy output was calculated from measured milk yield and concentrations of milk fat, CP, and lactose according to the NRC (2001) equation: Net energy of lactation ($NE_L$; Mcal/kg milk) = 0.0929 * fat % + 0.0547 * protein % + 0.0395 * lactose %. The $NE_L$ intake was estimated from measured DMI and $NE_L$ content of the diets, whereby the latter was determined as the sum of $NE_L$ content of individual feeds in a feeding level at 3 times maintenance (NRC, 2001). The ratio of $NE_L$ milk output:$NE_L$ intake was calculated to evaluate the effect of treatment on milk energy efficiency.

### *5.2.3 Statistical Analyses*

Data were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA Version 9.1.3) and considering the random effect of the study (St-Pierre, 2001), according to the following model:

$$Y_{ijkl} = \mu + \gamma_i + \alpha_j + \beta_k + \kappa_l + (\alpha\beta)_{jk} + (\alpha\kappa)_{jl} + (\beta\kappa)_{kl} + (\alpha\beta\kappa)_{jkl} + \varepsilon_{ijkl}$$

where $Y_{ijkl}$ is the observations for the dependent variables, $\mu$ represent the population mean, $\gamma_i$ is the independent parameter for animal i, $\alpha_j$ is a population parameter corresponding to treatment j, $\beta_k$ is the fixed effect of measurement week (relative to each LPS challenge) k, $\kappa_l$ is the fixed effect of parity l, $(\alpha\beta)_{jk}$ is the effect of treatment by measurement week interaction, $(\alpha\kappa)_{jl}$ is the effect of treatment by parity interaction, $(\beta\kappa)_{kl}$ is the effect of measurement week by parity interaction, $(\alpha\beta\kappa)_{jkl}$ is the effect of three way interaction of treatment by measurement week by parity, and $\varepsilon$ijkl is the residual error. Data are shown as

least-squares means (LSM) and standard error of the mean (SEM). Multiple comparisons of LSM were conducted by probability difference (PDIFF) option of SAS. Measurements on the same animal were considered as repeated measures. The covariance structure of the repeated measurements for each variable was modeled separately according to the lowest values of fit statistics based on the BIC (Bayesian information criteria). The significance limit was declared at $P < 0.05$, whereas a biologically relevant tendency was declared at $0.05 \leq P \leq 0.10$.

## 5.3 Results

### *5.3.1 Feed Intake and Milk yield*

Overall results regarding feed intake, milk yield, and milk efficiency are given in Figure 5-1. Data showed that treatment did not alter this variable between the treated and control cows ($P > 0.05$; Figure 5-1a). However, the factor measurement week showed an influence on feed intake ($P < 0.01$; Figure 5-1a) with numerical lower values in control cows after parturition. There was no treatment by week interaction regarding feed intake ($P > 0.05$; Figure 5-1a).

No differences between the control and treated cows were obtained for milk yield in the overall analysis of data ($P > 0.05$; Figure 5-1b), although an effect of week was evidenced by ANOVA ($P = 0.01$; Figure 5-1b). Milk yield declined numerically in the saline treated cows after parturition and returned to the level of the treated cows afterwards at week 3 and then slightly increased at week 4. In addition, there was no treatment by week interaction regarding milk yield in this study ($P > 0.05$; Figure 5-1b). Overall results indicated no differences regarding milk efficiency between the control and treated animals ($P > 0.05$;

Figure 5-1c). Also, there were no week effects or interaction between treatment and week in this study ($P > 0.05$; Figure 5-1c).

Data evidenced an effect of parity regarding milk efficiency. Treated primiparous cows showed a tendency for better milk efficiency than their respective control group animals, indicating that they produced more milk relative to their feed intake ($P < 0.10$; Table 5-3). Multiple comparisons of data indicated that this effect was more pronounced at week 1 following parturition ($P = 0.01$) with greater values in primiparous treated cows than the control ones. The factor week also had an effect for this variable ($P = 0.02$; Table 5-3); there was a tendency for treatment by week interaction for milk efficiency between the treated and control heifers ($P < 0.10$; Table 5-3). A treatment by week interaction for milk energy in primiparous cows was also obtained ($P = 0.03$; Table 5-3), and multiple comparison test indicated that this variable tended to be greater in oronasally LPS challenged heifers at week 3 than their respective control animals ($P < 0.10$). There was no week effect or treatment by week interaction regarding milk energy for this group of cows ($P > 0.05$; Table 5-3).

### *5.3.2 Milk Composition*

Overall results obtained with regards to milk fat variables are shown in Figure 5-2. Milk-fat content was not affected by treatment or treatment by week interaction between the treated and control cows ($P > 0.05$; Figure 5-2a). However, a week effect was evidenced for milk-fat content ($P = 0.01$; Figure 5-2a) with this variable showing numerically lower concentrations on week 1 after parturition in oronasal LPS treated cows, which then reached the level of control

cows on week 2, and staying higher for the remainder of the experiment in this group.

Interestingly, in this dataset, the effect of the parity on milk-fat content was found to be one of the most important factors, and milk-fat content tended to be higher in oronasally treated primiparous cows compared to saline treated heifers ($P = 0.09$; Table 5-3). This effect was more pronounced at week 3 post calving with higher values in the treated heifers ($P = 0.02$). Also measurement week showed an effect ($P = 0.02$; Table 5-3); however, no treatment by week interaction was obtained for this variable ($P > 0.05$; Table 5-3).

Overall ANOVA indicated no differences between control and treated cows for milk fat yield ($P > 0.05$; Figure 5-2b). Furthermore, factor of measurement week alone and treatment by week interaction was not evident regarding milk fat yield in the current study ($P > 0.05$; Figure 5-2b).

Overall mean results of the ANOVA for fat corrected milk (FCM) and energy corrected milk (ECM) revealed that the factor treatment did not show any effect ($P > 0.05$; Figure 5-3a). There was no effect of sampling week ($P > 0.05$; Figure 5-3a), and no treatment by week interaction for these variables ($P > 0.05$; Figure 5-3a). There was a tendency for a treatment by week interaction in the treated primiparous cows for FCM ($P < 0.10$; Table 5-3) and ECM ($P = 0.08$; Table 5-3). The multiple comparison analysis indicated a tendency for greater values for FCM ($P < 0.10$) and ECM ($P < 0.10$) in the treated heifers at week 3 following parturition. However, the factor measurement week or treatment alone

did not show any effect for these cows compared to their respective control group animals ($P > 0.05$; Table 5-3).

Milk-fat efficiency (MFE) was not different among the control and treated cows in the overall results ($P > 0.05$; Figure 5-3c). There was a tendency for a week effect ($P = 0.08$; Figure 5-3c), with this variable showing peak values 1 week after parturition in oronasally LPS administered group, slightly decreasing, and staying higher in those cows until week 3 postpartum, decreasing again to the level of control cows at week 4 postpartum. No treatment by week interaction was obtained for MFE in this dataset ($P > 0.05$; Figure 5-3c).

The overall analysis showed that protein content was not affected by oronasal LPS, or the interaction of treatment by week ($P > 0.05$; Figure 5-4a). Whereas, effect of factor week alone was obtained for this variable ($P < 0.01$; Figure 5-4a), with lower values in the treated cows at week 1 after parturition, which started to increase at the second week until week 3, and returned to the level of control cows at week 4.

Results obtained from the overall ANOVA, regarding milk protein yield, indicated no effect of factors oronasal LPS challenge, and sampling week relative to LPS administration or the interaction between these two for this variable ($P > 0.05$; Figure 5-4b). Although there seemed to be an effect of oronasal administration of LPS on MUN in the overall dataset, and it started to be lowered in the treated cows from week 2 postcalving till the end of the experiment; however, overall data showed that treatment did not alter this variable to the point of significance ($P > 0.05$; Figure 5-4c). Furthermore, the factor week of

measurement did not influence MUN ($P > 0.05$; Figure 5-4c), and also there was no effect for a treatment by week interaction regarding this variable in this study ($P > 0.05$; Figure 5-4c).

The overall profile of milk lactose content and yield was not affected by oronasal LPS challenge ($P > 0.05$; Figure 5-5a,b), or the interaction of treatment and measurement week ($P > 0.05$; Figure 5-5a,b). Also, analysis of the overall data did not reveal any effect of the factor measurement week alone for milk lactose content ($P > 0.05$; Figure 5-5b), although it reached the point of significance for milk lactose yield ($P < 0.01$; Figure 5-5a). Mean lactose content, by parity category is shown in Table 5-3, and it revealed a tendency for treatment by week interaction for heifers ($P < 0.10$), with a tendency for higher values in the treated primiparous cows at week 1 immediately after calving then their control group ($P = 0.08$).

No effect of treatment or interaction between treatment and week was evidenced for total solid contents of the milk as indicated from the overall means of ANOVA in the current study ($P > 0.05$; Figure 5-5c); although there was an effect of measurement week alone regarding this variable ($P = 0.05$; Figure 5-5c).

The total solid contents, categorized by parity are given in Table 5-3. Results showed a tendency for greater values in the treated primiparous cows than their control counterparts ($P = 0.06$). Furthermore, heifers showed only an influence of measurement week ($P = 0.02$; Table 5-3), with no interaction between treatment and week for this variable ($P > 0.05$; Table 5-3).

Interestingly, statistical processing of the overall data regarding milk SCC revealed that the factor oronasal LPS challenge tended to effect this variable and the values started to decrease in the treated cows from the week immediately after parturition till the end of the experiment ($P < 0.10$; Figure 5-6). No week effect or treatment by week interaction was evident for milk SCC ($P > 0.05$; Figure 5-6).

**5.4 Discussion**

This study was undertaken to evaluate whether repeated oronasal administration of LPS in prepartum dairy cows would be able to modulate the profile of milk components and milk SCC. Indeed, the most interesting finding of the present study was a tendency for lower milk SCC in the cows administered oronasally with LPS. It is evident that GNB invasion and the LPS shedding due to the breakdown of the infectious microbes, especially *Escherichia coil spp*, are often the cause of mammary gland inflammation (Hill et al., 1981; Guidry et al., 1983; Erskine et al., 1989). Several earlier studies indicated that the mammary gland of cows is highly susceptible to very low doses of LPS injection, resulting in marked influx of immune cells (especially neutrophils), reflected as high SCC in the milk (Carroll et al., 1964; Lengemann and Pitzrick, 1986; Mattila et al., 1989). In a more recent study, Lehtolainen et al. (2003) reconfirmed that intramammary challenge with LPS results in a marked increase in SCC in early lactation cows. More interestingly, cows inoculated with enterotoxigenic *E. coli* vaccine, followed by intramammary infusion with LPS 3 weeks later, had lower SCC due to suppression of innate immune factors in the milk and production of specific antibodies against the vaccine (Morimoto et al., 2012).

The lower SCC in the treated cows in the present study suggest that oronasal administration of LPS induced humoral immunity which lowered proinflammatory mediators and prevented subsequent translocation of large amounts of LPS. This hypothesis is supported by our finding that oral LPS administration enhanced the overall salivary IgA secretion (see previous chapter). Determination of IgG concentration in the milk, might be a valuable indicator whether LPS treatment improved humoral immune responses in the mammary gland.

Another important finding of this investigation was a tendency for greater milk-fat content and a treatment by week interaction for FCM with greater values in week 3 in the treated primiparous cows. Lipopolysaccharide has been shown to directly suppress key enzymes for the de-novo milk-fat synthesis like fatty acid synthetase and acetyl-CoA carboxylase (Pekala et al., 1983; López-Soriano and Williamson, 1994). In addition, LPS has been shown to inhibit the activity of LPL, the key enzyme for incorporation of fatty acids into the milk fat (Sweet and Hume, 1996). Moreover, Shuster et al. (1991) found a decline in milk-fat content following intra-mammary infusion with LPS. A recent investigation by Zebeli and Ametaj (2009) suggested that feeding dairy cows high-grain diets around parturition triggers an inflammatory response with enhanced C-reactive protein (CRP), which was negatively correlated with milk-fat content, milk-fat yield, and 3.5% FCM. The authors suggested that CRP inhibits interaction of LPL with apoC-II and as a result lowers the uptake of FA from the mammary gland.

A tendency for better milk efficiency and greater milk energy in primiparous cows was obtained in this study. Zebeli and Ametaj (2009) demonstrated a strong negative correlation between rumen free LPS with MEE in dairy cows. Better MEE also was reported in cows fed diets treated with lactic acid and heat that lowered the amount of LPS in the rumen fluid (Iqbal et al., 2012). Shuster et al. (1991) demonstrated reduced lactational performance of dairy cows administered intravenously with LPS.

The relationship between endotoxin and low milk synthetic capacity is explained by the fact that endotoxin in the mammary gland influences the metabolism of this tissue by eliciting local immune responses, and directing more nutrients to support immune reactions instead of being consumed as precursors for milk components. On the other hand, activated immune cells in mammary gland produce large quantities of antibacterial molecules like reactive oxygen species that have been associated with tissue damage. Furthermore, endotoxin exerts a direct harmful effect on MEC by down-regulating their functional and proliferation capacities (Dong et al., 2011). Indeed, better milk efficiency in the treated heifers in the present study indicates the importance of age in relation with infection and its resolution.

Repeated and increasing oronasal doses of LPS showed a tendency for increased ECM with a particular influence at week 3 postpartum in primiparous cows. In another study, ECM increased from 25.0 kg/d in control cows fed barley grain based diets to 27.4 kg/d in cows fed a similar diet processed with lactic acid and heat, which lowered rumen LPS content (Iqbal et al., 2012). In support of this

hypothesis are other studies that show that primiparous cows cope better with LPS and *E. coli* challenges as evidenced from more moderate inflammatory responses compared with multiparous cows (van Werven et al., 1997; Hoeben et al., 2000; Vangroenweghe et al., 2004). It is postulated that the LPS treated primiparous cows in our study were probably more efficient in clearance of bacteria and their toxins from mammary gland, which resulted in better milk production outcomes. However, more research is warranted to prove these assumptions.

Data revealed a tendency for milk lactose and total solid contents to be greater in the treated primiparous cows and this effect was more pronounced immediately after parturition. The precise mechanism by which this variable is higher in the treated primiparous cows is not clear at present. A number of studies have shown decreased milk lactose and casein during LPS-induced inflammation (Kitchen, 1981) by interfering with cellular secretion of specific milk components (Patton, 1978; Oliver and Smith, 1982; Sordillo et al., 1984). Our data are in line with those of Werner-Misof et al. (2007) who reported decreased milk lactose content when LPS was infused intramammary between 12.5 to 100 μg. It was demonstrated that LPS damaged tight junctions leading to increased permeability of the mammary gland epithelium and leakage of milk lactose in dairy cows. The release of proinflammatory cytokines, following endotoxin challenge, might be another potential reason for necrosis and severe vascular leakage as well as alterations in milk components in dairy cows (Oliver and Calvinho, 1995).

**5.5 Conclusions**

In conclusion, repeated oronasal administration of LPS from *E. coli* 0111:B4 modulated milk composition and SCC in periparturient dairy cows. Overall analysis demonstrated that cows challenged oronasally with LPS had a tendency for lower milk SCC. The parity class had an effect for better milk energy, milk efficiency, milk-fat content, FCM, ECM, lactose content, and total solid contents in the primiparous treated group. More research is warranted to study the mechanism(s) by which LPS affected SCC and milk composition and the reason(s) why primiparous cows responded better to this treatment compared to multiparous cows.

**5.6 Acknowledgments**

Appreciation is expressed to Alberta Milk (Edmonton, Alberta, Canada), Alberta Funding Consortium (Edmonton, Alberta, Canada), and Natural Sciences and Engineering Research Council of Canada (Ottawa, Ontario, Canada) for their financial support. Contributions of S. Pohl, M. Beal, and L. Sanderson in collection of samples from experimental animals is recognized. We also are thankful to the technical staff at the Dairy Research and Technology Center, University of Alberta, for their help and care to the cows.

**Table 5-1.** Ingredients and chemical composition of the diet for dry cows

| Ingredients, % of DM | Prepartum diet |
|---|---|
| Alfalfa hay | 10.0 |
| Barley silage | 63.0 |
| Alfalfa silage | 00.0 |
| CUD[1] grain | 27.0 |
| Nutrient composition of cud grain (dietary supplement) % amount in 100 kg mix | |
| Barley grain, rolled | 55.0 |
| Canola meal | 7.5 |
| Dairy dry cow micro-premix | 6.2 |
| Limestone | 8.7 |
| Animate | 15.7 |
| Molasses | 0.9 |
| Canola oil | 4.1 |
| Yeast | 1.7 |

[1]CUD = giving more mineral especially Ca to avoid milk fever in early lactating cows

**Table 5-2.** Ingredients and chemical composition of the diet for early lactating cows

| Ingredients, % of DM | Early lactation diet |
|---|---|
| Alfalfa hay | 10.0 |
| Barley silage | 40.8 |
| Dairy supplement | 49.2 |
| Nutrient composition of dairy supplement, % amount in 100 kg mix | |
| ADE Vit Pak-30 Natural E | 0.056 |
| Ruminant TM Pak | 0.1025 |
| Selenium 1000 mg/kg (UNscr Fine Cr) | 0.065 |
| Custom TM Complex pmx | 0.060 |
| Di-calcium phosphate 21% | 1.25 |
| Co-op alantic Corn Dist | 10.0 |
| Corn ground | 25.0 |
| Corn rolled | 30.105 |
| Vit D- 10,000 KIU/kg | 0.015 |
| Diamond V XPC | 0.14 |
| Magalac/Enertia | 1.00 |
| Fermenten | 2.00 |
| Limestone | 1.50 |
| Mag Ox -56% | 0.37 |
| Canola meal | 15.5 |
| Hi bypass soy (Amino plus) | 2.75 |
| Soy bean meal-47.5% | 6.50 |
| Sodium bicarbonate | 1.00 |
| Salt | 0.113 |
| Pork-Tallow | 2.45 |
| Biotin 2%-Rovimix H-2 | 0.007 |
| ADM Vit E 405 Vegetable source | 0.015 |

**Table 5-3.** Data of milk composition and production efficiency of lactating primiparous cows administered orally with saline (CTR) or saline containing increasing doses of LPS (TRT)

| Variable | Treatment groups[1] | | | | | |
|---|---|---|---|---|---|---|
| | Primiparous cows | | | Overall Effect,[2] *P*-value | | |
| | **TRT** | **CTR** | **SEM** | **Trt** | **wk** | **Trt x wk** |
| FCM[3] | 36.3 | 36.2 | 1.56 | 0.97 | 0.87 | <0.10 |
| ECM[4] | 35.2 | 35.0 | 1.39 | 0.92 | 0.84 | 0.08 |
| Fat content, % | 4.54 | 4.20 | 0.13 | 0.09 | 0.02 | 0.33 |
| Lactose content, % | 4.52 | 4.49 | 0.04 | 0.68 | 0.70 | <0.10 |
| Total solids, % | 13.1 | 12.7 | 0.16 | 0.06 | 0.02 | 0.50 |
| Milk efficiency | 1.45 | 1.13 | 0.14 | <0.10 | 0.02 | <0.10 |
| Milk energy,[5]Mcal/d | 23.1 | 22.9 | 1.02 | 0.87 | 0.72 | 0.03 |

[1]CTR = vows administered orally with saline solution; TRT = cows administered orally with increasing doses of lipopolysaccharide.
[2]Effect of treatment (Trt), measurement week (wk), and treatment by week interaction (Trt x wk).
[3]FCM = Milk amount (kg) * (0.4255 + 16.425 * % fat/100).
[4]ECM = Milk amount (kg) * (0.327 + 7.2 * % protein/100 + 12.96 * % fat/100).
[5]Milk energy (Mcal/kg milk) = 0.0929 * % fat + 0.0547 * % CP + 0.0359 * % lactose.

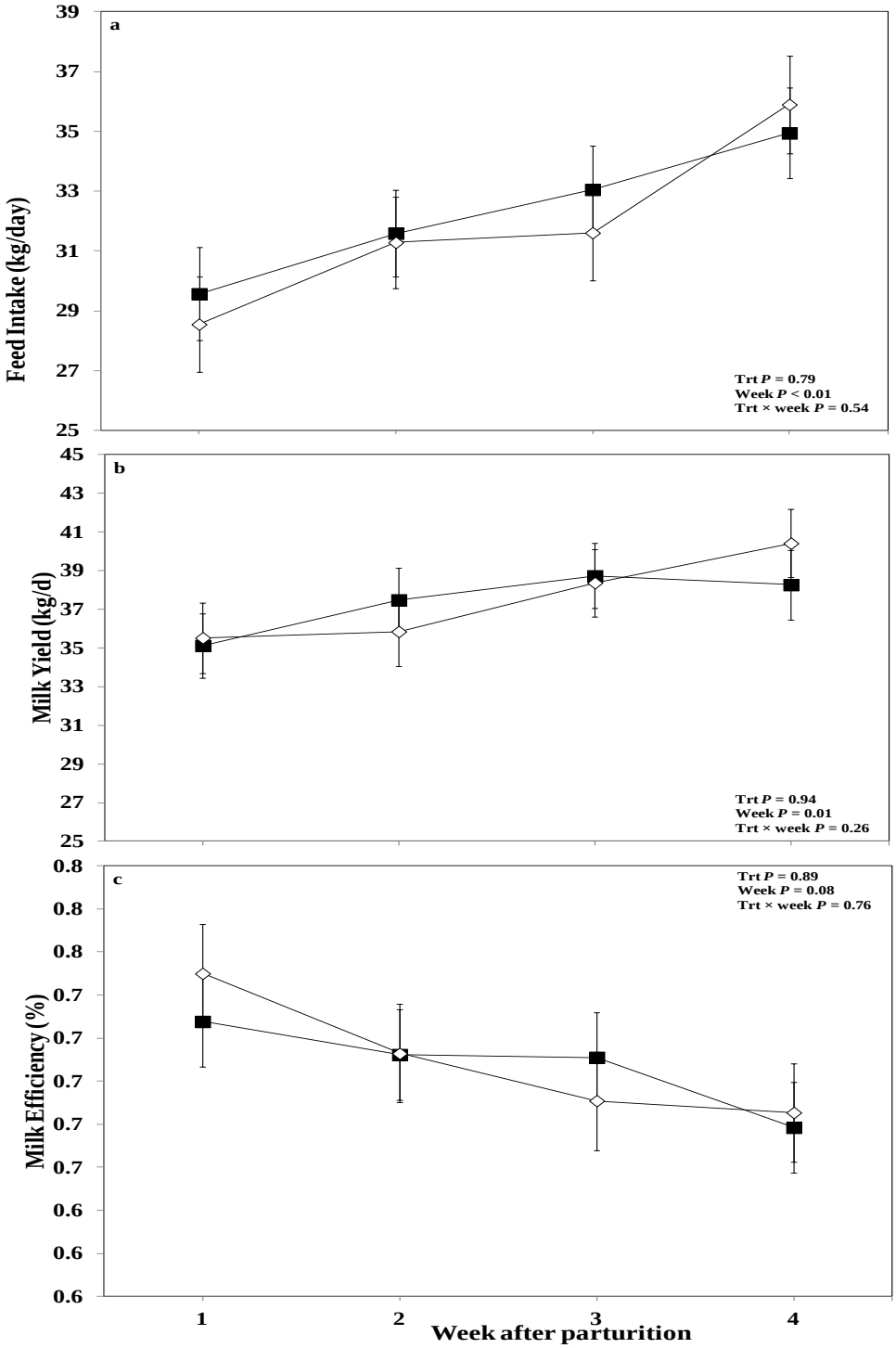


**Figure 5-1.** Weekly variations in milk (a) feed intake, (b) milk yield, and (c) milk efficiency of multiparous and primiparous Holstein cows challenged with oro-nasal treatment of LPS (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 16; Trt = effect of treatment; Week = effect of sampling week, Trt x week = effect of treatment by sampling week).

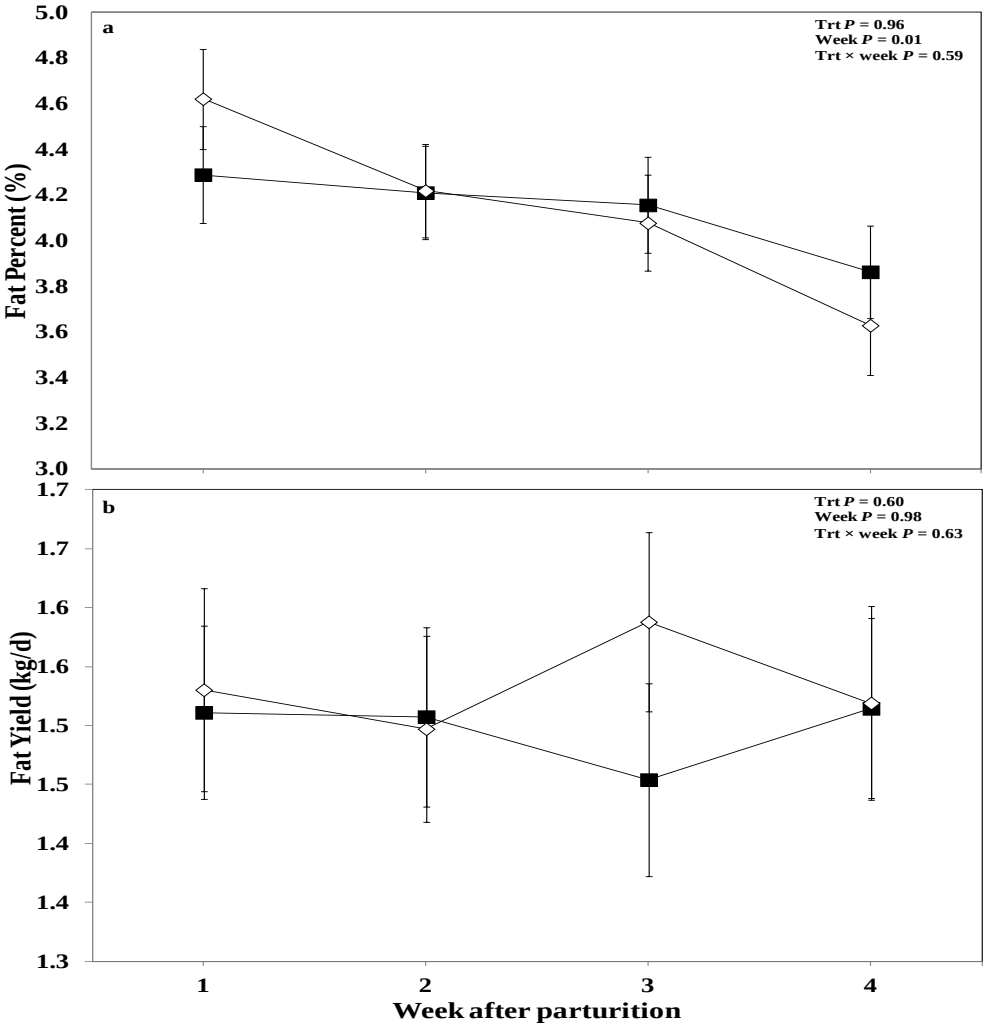


**Figure 5-2.** Weekly variations in milk (a) fat content, and (b) fat yield primiparous of multiparous and primiparous Holstein cows challenged with oro-nasal treatment of LPS (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 16; Trt = effect of treatment; Week = effect of sampling week, Trt x week = effect of treatment by sampling week).

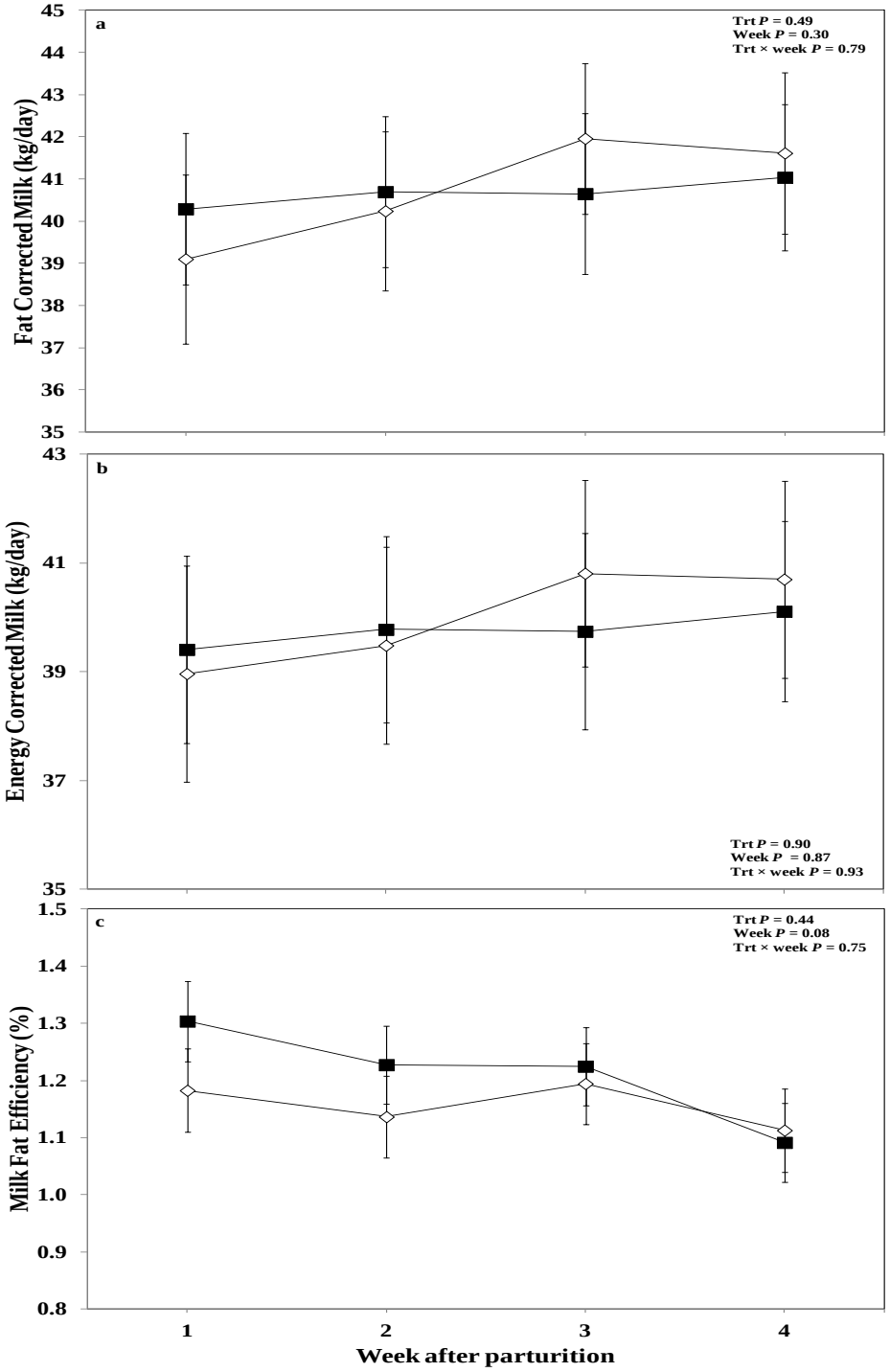


**Figure 5-3.** Weekly variations in milk (a) fat corrected milk, (b) energy corrected milk, and (c) milk fat efficiency of multiparous and primiparous Holstein cows challenged with oro-nasal treatment of LPS (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 16; Trt = effect of treatment; Week = effect of sampling week, Trt x week = effect of treatment by sampling week).

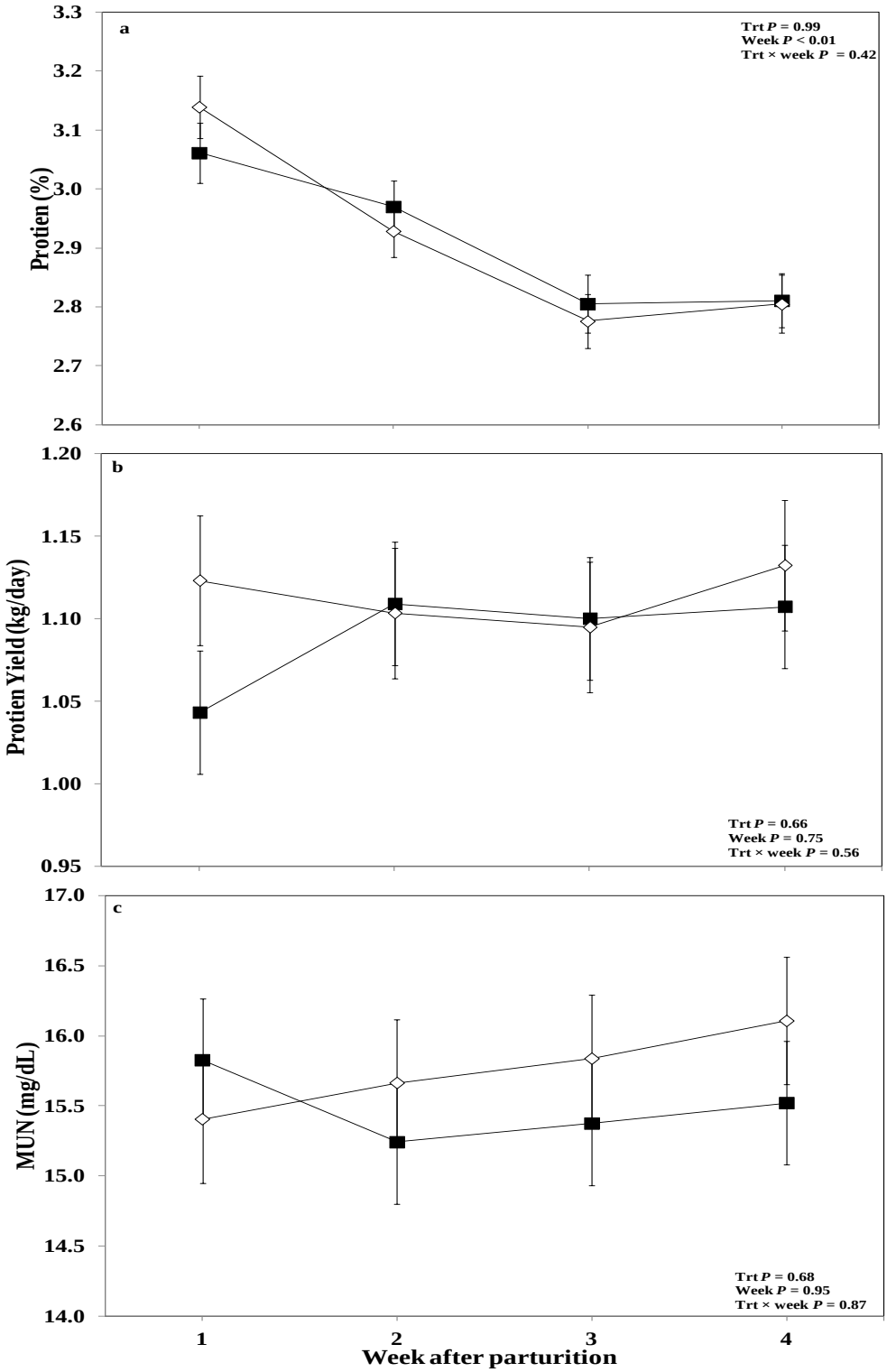


**Figure 5-4.** Weekly variations in milk (a) protein content, (b) protein yield, and (b) milk urea nitrogen of multiparous and primiparous Holstein cows challenged with oro-nasal treatment of LPS (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 16; Trt = effect of treatment; Week = effect of sampling week, Trt x week = effect of treatment by sampling week).

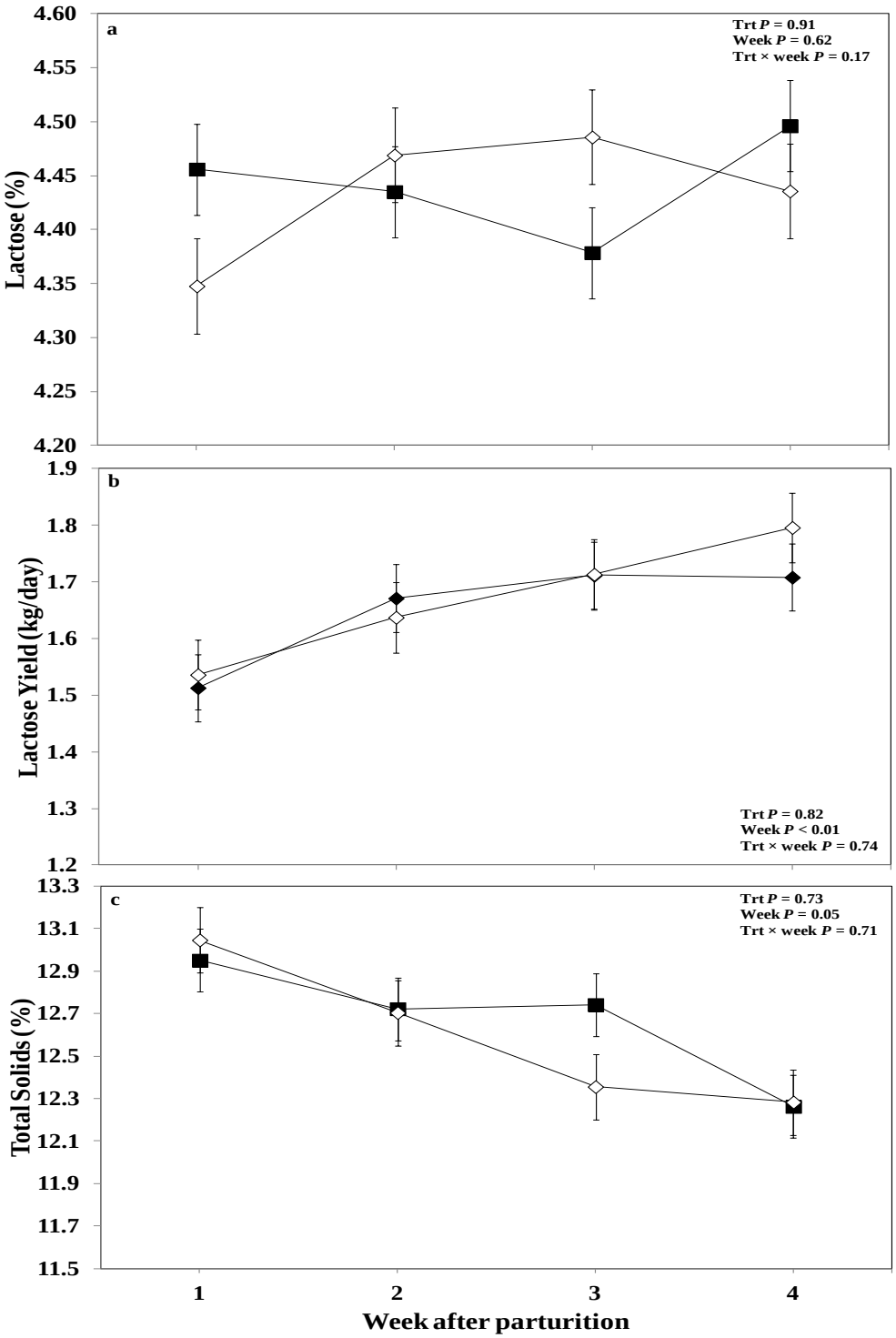


**Figure 5-5.** Weekly variations in milk (a) lactose content, (b) lactose yield, and (b) total solids content of multiparous and primiparous Holstein cows challenged with oro-nasal treatment of LPS (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 16; Trt = effect of treatment; Week = effect of sampling week, Trt x week = effect of treatment by sampling week).

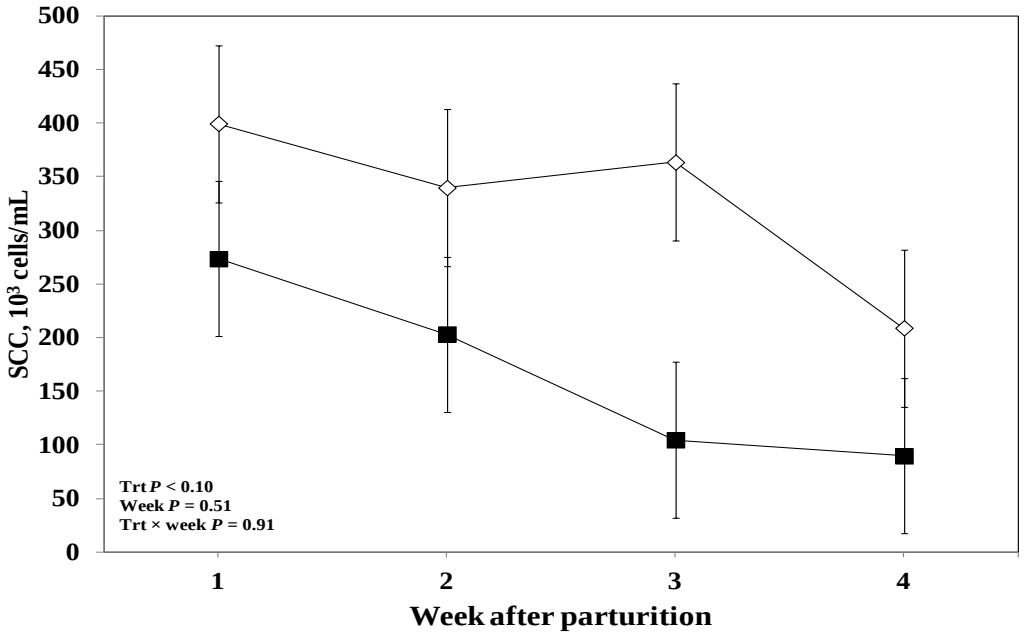


**Figure 5-6.** Weekly variations in milk somatic cell count of multiparous and primiparous Holstein cows challenged with oro-nasal treatment of LPS (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 16; Trt = effect of treatment; Week = effect of sampling week, Trt x week = effect of treatment by sampling week).

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# [1]Chapter 6

## Prepartal oral administration of lipopolysaccharide and lipoteichoic acid modulates uterine immunity and lowers the risk of periparturient diseases in dairy cows

### 6.1 Introduction

The incidence of periparturient diseases in dairy cows is high and has further increased during the last decade, affecting almost half of the animals in a herd with enormous economic losses to the dairy industry (Ingvartsen, 2006; Ametaj et al., 2012a). Increasing evidence indicates that feeding dairy cows diets high in easily-digestible carbohydrates is associated with high incidence of various periparturient diseases (Ametaj et al., 2005; Goff, 2006). Research conducted by us and others has demonstrated that the underlying cause might be alterations in the composition of rumen microbiota and accumulation of large amounts of bacterial harmful compounds like lipopolysaccharide (LPS) or endotoxin during feeding of high amounts of grain in the diet (Emmanuel et al., 2008; Khafipour et al., 2009; Ametaj et al., 2010). The high load of endotoxin in the rumen is derived from the outer cell wall of Gram-negative bacteria (GNB), and the major source of these bacterial toxins in dairy cows is believed to be the mucosal layers, particularly, the gastrointestinal tract and infected uterus and mammary gland (Linder et al., 1988; Ametaj et al., 2010).

Recently we showed that LPS translocates through the rumen and colon tissues of cattle and possibly enters into the host blood circulation, associated with

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perturbation of various metabolite networks and immune status (Emmanuel et al., 2007; Ametaj et al., 2010). Translocation of endotoxin into the systemic circulation causes various perturbations in the metabolism of the host. Increasing evidence demonstrates that both intravenous and intra-mammary infusion of endotoxin in cattle is associated with increased concentration of lactate and lowered circulating levels of beta-hydroxy butyric acid (BHBA), with a nadir between 3 and 4 h after the treatment. Furthermore, LPS modifies concentrations of plasma non-esterified fatty acid (NEFA) and results in a biphasic response of glucose and development of insulin resistance (Steiger et al., 1999; Werling et al., 1996; Waldron et al., 2006). In addition, endotoxin might also translocate through mucosal layers of mammary gland and uterus as indicated from systemic inflammation following endotoxin-related infections in these organs. For example, coliform mastitis results in permeation of LPS into the bloodstream and increases concentrations of various plasma inflammatory mediators (Hoeben et al., 2000). Likewise, metritis is associated with an elevated acute phase response particularly increased haptoglobin in periparturient cows (Huzzey et al., 2009).

An increasing body of evidence has established an association between endotoxin and a number of periparturient diseases of dairy cows; however, a recent investigation has shown that endotoxin might not be the only bacterial compound implicated in disease development during the periparturient period (Ametaj et al., 2012a). We hypothesized that lipoteichoic acid (LTA), a Gram-positive bacterial toxin, analogous to LPS, might also be involved in the etiopathology of periparturient diseases in dairy cows (Ametaj et al., 2012a).

Lipoteichoic acid is a cell-wall component of Gram-positive bacteria (GPB) and shares many pathophysiological effects with LPS (Ginsburg, 2002). In a recent study it was demonstrated that intra-mammary infusion of purified LTA in dairy cows induced clinical mastitis at the dose of 100 μg/quarter (Rainard et al., 2008). Moreover, cows challenged with both LPS from *E. coli* and LTA from *S. aureus* induced a greater inflammatory response suggesting that both of these toxins are implicated somehow in development of the disease (Daly et al., 2009). Despite progress made in our understanding regarding the contribution of bacterial toxins in the etiopathology of various periparturient diseases of dairy cows there are no available prophylactic strategies against those pathologies.

Since mucosal surfaces comprise the first port of entry for bacterial toxins, various investigators have studied the effects of mucosal exposure of various experimental animals to bacterial toxic compounds aiming to lower their translocation and their harmful effects on the host (Linder et al., 1988; Ametaj et al., 2010). An earlier study indicated that mucosal administration of antigens is important in inducing mucosal immunity particularly through oral route (Kofler et al. (1996). More specifically, coupling of mucosal inductive and effector sites indicates that priming of oral mucosa is effective in inducing immunoglobulin A (IgA) antibody responses in the gastrointestinal, respiratory, and urogenital tract (Neutra and Kozlowski, 2006; Cui et al., 1991).

Thus, based on the potential role of LPS and LTA in the etiopathology of multiple periparturient diseases in dairy cows we hypothesized that repeated oral administration of LPS and LTA might affect mucosal immunity and various

metabolic pathways and the incidence of periparturient diseases in transition dairy cows. Therefore, the main objectives of this study were to investigate the effects of increasing oral doses of LPS with a flat dose of LTA prior to parturition on vaginal mucus IgA, selected plasma metabolites involved in carbohydrate and lipid metabolism, and the incidence of periparturient diseases in transition dairy cows.

## 6.2 Materials and Methods

### *6.2.1 Cows, Diets, and Experimental Design*

Thirty pregnant multiparous and primiparous Holstein dairy cows with average body weight of 720 ± 30 and 600 ± 20 kg, respectively, were blocked by parity, and then randomly allocated to two treatment groups according to milk production, body condition score (BCS), disease susceptibility from previous year, and the anticipated day of calving. Fifteen cows [10 multiparous (average number of lactation 2.5) and 5 primiparous] were randomly allocated to each group, at -28 d before the expected day of parturition. Cows were orally administered 2 mL of sterile saline solution (controls), or 2 mL of sterile saline solution containing LPS from *E. coli* strain 0111:B4 at three increasing concentrations as follows: 1) 0.01 µg/kg body weight (BW) on d -28, 2) 0.05 µg/kg BW on d -25 and -21, and 3) 0.1 µg/kg BW on d -18 and -14, as well as a flat dose of LTA from *Bacillus subtilis* (i.e. 120 µg/animal) for 3 consecutive weeks on the same days as for LPS treatments.

The *E. coli* LPS (Lipopolysaccharide-FITC from *E. coli* strain 0111:B4) and LTA from *B. subtilis* were supplied by Sigma-Aldrich Canada Ltd. (Oakville,

Ontario, Canada) containing 10 mg of purified LPS (LPS solution is purified by gel filtration and contains endotoxin levels of not less than 500,000 EU) and 10 mg of LTA. The powder forms of LPS and LTA were then dissolved in 10 mL of double distilled water as suggested by the manufacturer and stored in the refrigerator at 4 °C. For administration to the animals the daily dose of LPS and LTA were dissolved in 2 mL of sterile saline and then introduced into the oral cavity of the cows using disposable sterile plastic syringes (Becton, Dickinson and Company, BD, Franklin Lakes, NJ). Similarly, the same amount of carrier (i.e., 2 mL sterile saline; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) was orally administered to all cows in the control group.

The three doses of LPS used were based on a previous research conducted by our team with dairy cows and on clinical and pathological responses to those doses (Ametaj et al., 2012b). The dose of LTA was based on another clinical study that determined the safe clinical dose of oral LTA to be used (Iqbal et al., 2011). The lowest dose of LPS i.e., 0.01 μg/kg BM was chosen because previous experiments have shown minimal changes in the metabolism of dairy cows at this concentration (Zebeli et al, 2013), whereas the highest dose was also selected owing to a maximum host response at this dose observed previously (Zebeli et al, 2013).

The experiment lasted for 8 wk (i.e., -28 d before and +28 d after parturition) and cows were housed in tie stalls with free access to water throughout the experiment. Shortly before parturition cows were transferred to the maternity pens and returned to their stalls on the next day of parturition. Daily

ration was offered as a total mixed ratio (TMR) for ad libitum intake to allow approximately 10% feed refusals throughout the experiment. Animals were fed once daily at 0800. Cows and heifers were fed a close-up (i.e., dry off) diet as part of a TMR starting at 3 wk before the expected day of parturition. After parturition, all cows were gradually switched during the first 7 d to a lactation diet (i.e., high ration). All diets were formulated to meet or exceed the nutrient requirements of dry and early lactating cows as per NRC (2001) guidelines. The close up diet fed to the dry cows was based on the 10% alfalfa hay, 63% barley silage and 27% diet supplement on the DM basis; whereas high ration fed to early lactating cows was based the on 10% alfalfa hay, 40.8% barley silage and 49.2% dairy supplement. The CP content of the diet for dry cows was 18.2% (DM basis), whereas the energy content (i.e., NEL) was 1.84 Mcal/kg DM. However, for the early lactation cows the CP content was 20.3% (DM basis), and the NEL was 1.91 Mcal/kg DM. Ingredients and chemical composition of the diets for the dry and early lactating cows are presented in Table 1 and 2, respectively. All experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Livestock, and animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993). Veterinary supervision was provided to the animals throughout the experiment, and the sick cows were treated according to the symptoms and severity of the disease by a veterinary specialist.

### *6.2.2 Sample Collection*

All blood samples were withdrawn before administration of treatments on d -28 , -25 , -21 , -14 , and -7 before the expected day of parturition and on d +7 , +14 , +21 , and +28 after parturition for plasma glucose, lactate, NEFA, cholesterol, and BHBA. For plasma insulin, blood samples were collected once per week on d -28 , -14 , and -7 , before parturition, as well as on d +7 , +14 , and +28 after parturition; whereas for plasma cortisol blood samples were collected on d -28 and -7 before parturition and on d +7 and +28 after parturition. In the control group, cows calved from -3 to +4 days from the expected day of parturition. Whereas, in the treatment group this average ranged between -5 to +4 days.

Blood samples were taken from the coccygeal vein using serum vacutaniers (Becton, Dickinson and Company, BD, Franklin Lakes, NJ) and were put immediately on ice, centrifuged (Rotanta 460R, Hettich Zentrifugan, Tuttlingen, Germany) within 20 min at 3,000 × *g* and 4 °C for 20 min to separate plasma, which was then stored at -20 $^{o}$C and analyzed for glucose, lactate, NEFA, cholesterol, BHBA, insulin, and cortisol.

Vaginal mucus samples were collected on d -28, -7, +7 , and +28 around parturition. The vulva of the cow was washed with disinfectant solution, rinsed with clean water, and dried. Vaginal mucus samples (~ 1-2 mL) were collected by aspiration with an insemination pipette and a 10-mL plastic syringe. These samples were placed into 1 mL of saline solution in a plastic tube and transported to the laboratory in a refrigerated box. Vaginal mucus sample tubes were sealed securely and stored at -20 $^{o}$C until analyses for total IgA. Before the assay,

samples were centrifuged (Rotanta 460R, Hettich Zentrifugan, Tuttlingen, Germany) at 1,000 × *g* and 4 °C for 20 min to remove any particulates.

### *6.2.3 Clinical Variables*

Disease incidence for different periparturient diseases including metritis, clinical or subclinical mastitis, lameness, retained placenta (RP), left displaced abomasum (LDA), clinical or subclinical ketosis, and milk fever were recorded for all dairy cows during - 28 d before and + 28 d after parturition.

The severity of metritis was scored according to Williams et al. (2005) based on the vaginal mucus character and odour as follows: type 1 - normal, healthy (mucus containing flecks of white or off-white pus), type 2 - slight metritis (< 50 mL exudate containing ≤ 50% white or off-white mucopurulent material), type 3 - severe or hard metritis (> 50 mL exudate containing purulent material, usually white or yellow, but occasionally reddish brown and in worst case fetid). In case of occurrence of the metritis, the cows were treated according to the severity of the disease by a veterinary specialist.

The clinical cases of mastitis were based on the visual abnormal milk secretion from one or more quarters with or without signs of the inflammation of the udder (Kelton et al., 1998). During clinical mastitis, the milk somatic cell count (SCC) reached the threshold of ≥ 1,000,000 cells/mL. Whereas, the threshold level for sub-clinical cases of mastitis was considered to be ranged between 250,000 to 300,000 cells/mL (on an average ≥ 280,000 cells/mL), however visual inspection of milk appeared normal (Dohoo and Meek, 1982;

Smith, 1996), however visual inspection of milk appeared normal. The sick cows with clinical and sub-clinical mastitis were treated accordingly.

The locomotion scoring system developed by Sprecher et al. (1997) was followed to determine the severity of the lameness. Cows were considered having lameness after monitoring them for an abnormal gait and presence of a swollen, inflamed, and painful coronary band. In addition, cows were observed for shifted weight when standing and curvature of the spine.

All cows were checked within 12 to 24 h after calving for RP. They were considered having RP if they failed to expel their fetal membranes within 24 h of parturition. Cows with retained placenta were treated by a veterinarian according to the severity of the case.

Similarly all cows were monitored for udder edema around parturition by pitting-test. Udder edema was scored by pressing the skin against two fingers to see if it makes a pit into the skin and how long the skin stays in that position. Based on the severity of udder edema the disease was classified as: 1 - slight edema (small part of the udder involved), 2 - medium edema (edema covering most of the udder), and 3 - brisket filled udder with liquid (adopted with modifications from Tucker et al., 1992). The treatment of udder edema cases was done according to the extent of fluid retained in the udder.

All animals entering the experiment had no visible signs of disease. Clinical monitoring for the incidence of periparturient diseases was conducted on each sampling day and every alternate day of the wk. The person assessing the clinical parameters was blinded (i.e., unaware) to the treatment groups.

### *6.2.4 Sample Analysis*

Concentrations of total IgA in the vaginal mucus were measured using a commercially available bovine ELISA kit (Uscn, Life Sciences Inc., USA). The procedure involves the basic principle of sandwich enzyme immunoassay for the *in vitro* quantitative measurement of IgA in bovine biological fluids with a working time of 4 h. The microtiter plate, provided with the kit, was precoated with antibodies specific to the IgA. The standards and samples were then added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific for IgA, and then avidin conjugated to an enzyme was added to each microplate well and incubated. The color developed by the substrate addition was shown up only in those wells which contained IgA, biotin conjugated antibody, and enzyme conjugated avidin. The absorbance was measured at 450 nm with a spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA). The detection range of IgA antibodies was 0.78-50 ng/mL.

Concentrations of glucose in the plasma were quantified by an enzymatic method using a commercially available kit (Diagnostic Chemicals Ltd., Charlottetown, Prince Edward Island, Canada). In short, the procedure involves phosphorylation and oxidization of glucose, which causes the production of NADH, and produces a color proportional to the glucose concentration in the sample. All samples were tested in duplicate for glucose concentration in plasma and values were determined by reading on a microplate spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA) at an optical density

of 340 nm. According to the manufacture's instructions, the lower limit of detection for plasma glucose was 0.06 mg/dL.

Plasma concentrations of lactate were determined using a commercially available assay kit (Biomedical Research Service Center, Buffalo, NY). Briefly, the protocol involves reduction of tetrazolium salt INT in a NADH coupled enzymatic reaction to formazan, which exhibits a red color, and the intensity of this colour indicates the concentration of lactate. The test was modified as described by Johnson and Peters (1993). The lactate standards provided with the kit were diluted from 125 to 1,000 μ*M* in order to set this detection range for measuring plasma samples for lactate. All samples were tested in duplicate and the lactate concentration was determined by reading the optical density values on a microplate spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA) at 492 nm.

An enzymatic colorimetric method was used for the quantitative determination of plasma NEFA by using a commercially available assay kit (Wako Chemicals, Richmond, VA). The procedure involves acylation of coenzyme A by fatty acids in the sample in presence of acyl-CoA synthetase and production of hydrogen peroxide in presence of acyl-CoA oxidase. Hydrogen peroxide, in presence of peroxidase, permits the oxidative condensation of 3-methyl-N-ethyl-N-β-hydroxy ethyl-O-aniline (MEHA) with 4-aminoantipyrine to form a purple adduct, and the color intensity is proportional to the concentration of NEFA in the sample. The test was modified as described by Johnson and Peters (1993). All samples were tested in duplicate and the optical density values were

measured at 550 nm on a microplate spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA). The lower limit of detection of the assay for plasma NEFA was 0.50 mEq/L.

Plasma cholesterol was also measured using a commercially available assay kit (Diagnostic Chemicals Ltd., Charlottetown, Prince Edward Island, Canada). The principle of colorimetric method involves hydrolyzing of cholesterol esters to free cholesterol and oxidation of free cholesterol to cholest-4ene-3-one with simultaneous production of hydrogen peroxide. The hydrogen peroxide couples with 4-aminoantyrine and p-hydroxybenzoate in the presence of peroxidase to yield a chromogen, the intensity of which is proportional to the concentration of cholesterol in the sample. All samples were tested in duplicate and plasma cholesterol was determined by reading the optical density values on a microplate spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA) at 505 nm. The lower limit of detection of the assay for plasma cholesterol was 1.2 mg/dL.

Enzymatic measurement of BHBA by β-hydroxybutyrate dehydrogenase was used to quantify plasma concentrations of BHBA using a commercially available kit (Stanbio Laboratory, Boerne, TX). The procedure involves the conversion of BHBA in the samples to acetoacetate and NADH at pH 8.5 by β-hydroxybutyrate dehydrogenase in the presence of NAD. The NADH produced reacts with INT in the presence of diaphorase to produce a color proportional to the concentration of BHBA in the sample. Controls and blanks were used to eliminate the difference in color intensity. Plasma BHBA was measured in

duplicates by reading on a microplate spectrophotometer (Spectramax 190, Molecular devices Corp., Sunnyvale, CA) at an optical density of 505 nm. The lower limit of detection of the assay was 0.125 μmol/mL.

Plasma insulin was measured by a commercially available bovine ELISA kit (Mercodia AB, Winston Salem, NC), which involves a solid phase 2-site enzyme immunoassay. This assay is based on the direct sandwich technique in which 2 monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. The procedure involves the reaction of insulin in the sample with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the microtitration wells during incubation. It follows the simple washing procedure which removes unbound enzyme-labeled antibodies, whereas the bound conjugate is detected by reaction with 3,3',5,5'-tetramethylbenzidine. The reaction was stopped by the addition of stop solution, and the colorimetric endpoint is read spectrophotometrically. All samples were tested in duplicate and plasma insulin was determined by reading the optical density values on a microplate spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA) at 450 nm. The detection limit of the assay was 0.025 μg/L.

Concentrations of cortisol in the plasma were measured using a commercially available bovine ELISA kit (Uscn, Life Sciences Inc. Wuhan, China). The kit was precoated with biotin-conjugated polyclonal antibody specific to cortisol. In brief, all samples and standards were added to the appropriate microtiter plate wells. Then, avidin conjugated to horseradish peroxidase was

added to each well and incubated. Finally, TMB substrate was added to each well and the colour developed was proportional to the cortisol, biotin-conjugated antibody and enzyme-conjugated avidin. The enzyme-substrate reaction was stopped by the addition of a sulphuric acid solution and the change in color was measured by spectrophotomer at a wavelength of 450 nm$\pm$2 nm. The detection range for this assay was 6.25-400 ng/mL.

### *6.2.5 Statistical Analyses*

Data of continuous variables were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA Version 9.1.3) as describe by the following model:

$$Y_{ijkl} = \mu + \gamma_i + \alpha_j + \beta_k + \kappa_l + (\alpha\beta)_{jk} + (\alpha\kappa)_{jl} + (\beta\kappa)_{kl} + (\alpha\beta\kappa)_{jkl} + \varepsilon_{ijkl}$$

where $Y_{ijkl}$ is the observations for the dependent variables, $\mu$ represent the population mean, $\gamma_i$ is the independent parameter for animal i, $\alpha_j$ is a population parameter corresponding to treatment j, $\beta_k$ is the fixed effect of measurement week (relative to each LPS challenge) k, $\kappa_l$ is the fixed effect of parity $(\alpha\beta)_{jk}$ is the effect of treatment by measurement week interaction, $(\alpha\kappa)_{jl}$ is the effect of treatment by parity interaction, $(\beta\kappa)_{kl}$ is the effect of measurement week by parity interaction, $(\alpha\beta\kappa)_{jkl}$ is the interaction of treatment by measurement week and parity, and $\varepsilon$ijkl is the residual error assumed to be normally distributed. Data are shown as least-squares means (LSM) and standard error of the mean (SEM). Multiple comparisons of LSM were conducted by probability difference (PDIFF) option of SAS. Measurements on the same animal at different times were considered as repeated measures. The covariance structure of the repeated

measurements for each variable was modeled separately according to the lowest values of fit statistics based on the Bayesian information criteria (BIC). The measurements taken on d -28 were considered as covariates in the analysis. The significance limit was declared at $P < 0.05$, whereas a biologically relevant tendency was declared at $0.05 < P \leq 0.10$.

Categorical data of the disease incidence of cows were analyzed using the categorical modeling procedure (PROC CATMOD) of SAS (SAS Inst. Inc). The model statement contained treatment as the main effect.

## 6.3 Results

### *6.3.1 Vaginal Mucus Immunoglobulin-A*

Oral administration of cows with LPS and LTA increased concentrations of total IgA in the vaginal mucus compared to the control counterparts ($P < 0.01$; Figure. 6-1). The factor sampling time around parturition also influenced this variable ($P = 0.02$). However, there was no treatment by time interaction for the concentrations of total IgA in the vaginal mucus in this study ($P > 0.05$).

### *6.3.2 Plasma Metabolites*

There were no differences in the concentration of glucose in the plasma between the treated and the control group of cows ($P > 0.05$; Figure 6-2). Concentrations of glucose in the plasma were lower following parturition, which was reflected by an effect of time on this variable ($P < 0.01$). No treatment by time interaction was obtained for plasma glucose ($P > 0.05$).

Results indicated differences for plasma lactate between the two treatment groups with lower concentrations in cows treated orally with LPS and LTA

compared to the control group (2.58 vs. 3.67 mmol/L; $P < 0.01$; Figure 6-3). However, no time effect or interaction between the treatment and time were evidenced for plasma lactate ($P > 0.05$).

The overall concentrations of NEFA in the plasma were not different between the cows treated orally with LPS and LTA or saline ($P > 0.05$; Figure 6-4). However, time had an effect on plasma NEFA ($P < 0.01$). Furthermore, no treatment by time interaction was observed for this variable ($P > 0.05$).

Results revealed that cows treated with LPS and LTA had a tendency for greater overall concentrations of plasma cholesterol compared to cows treated with saline ($P = 0.08$; Figure 6-5). There was an effect of time for this variable ($P < 0.01$). No treatment by time interaction was obtained for plasma cholesterol ($P > 0.05$).

Concentrations of BHBA in the plasma were not affected by oral LPS and LTA, and no interaction of treatment by time ($P > 0.05$; Figure 6-6) was obtained for this variable. However, the time of the measurement showed a tendency for plasma BHBA ($P < 0.10$).

Oral treatment with LPS and LTA showed greater concentrations of plasma insulin compared to the control group ($P < 0.01$; 0.67 vs. 0.28 μg/L; Figure 6-7). There was also an effect of both time ($P < 0.01$), and a treatment by time interaction ($P < 0.01$) for this variable. The analysis revealed that plasma insulin showed a peak at 2 wk before parturition in the treated cows.

Concentrations of cortisol in the plasma were not affected by the oral LPS and LTA administration, and no interaction of treatment by time was obtained for

this variable ($P > 0.05$; Figure 6-8). However, there was a time effect obtained for plasma cortisol ($P < 0.01$).

### *6.3.3 Metabolic Diseases*

Results of this study demonstrated that treatment with LPS and LTA lowered the risk of metritis in the treated cows ($P < 0.05$; Table 8-3). We observed that 4 out of 15 cows in the control group were affected by hard metritis. However, only 1 out of 15 cows from the LPS and LTA group were affected with hard metritis.

Data indicated that oral treatment with LPS and LTA lowered the risk of lameness ($P < 0.05$; Table 8-3). Two out of 15 cows were effected by lameness in the LPS and LTA treated group and 5 cows out of 15 in the control cows. Furthermore, when examined by the number of days which indicated the length of the time that cows were affected by the clinical signs of lameness, there was a tendency for the treatment group to have lower duration of the disease compared to the control animals ($P < 0.10$; Table 8-3).

Also, data showed differences regarding the risk of RP between the two treatment groups, which tended to be lower in cows treated orally with LPS and LTA ($P = 0.10$; Table 8-3). The overall analysis indicated that the incidence of RP was lowered to the extent that no clinical cases were observed in the treated group versus 2 cases in the control one.

The number of days that multiple periparturient diseases affected a single cow was lowered in the treatment group ($P < 0.01$; Table 8-3).

No differences in clinical or subclinical mastitis, LDA, clinical or subclinical ketosis, milk fever, and udder edema ($P > 0.05$) were obtained between the two groups in this study (Table 8-3).

**6.4 Discussion**

We hypothesized that oral treatment with LPS and LTA will influence vaginal humoral immunity as well as various plasma metabolites and the incidence rate of periparturient diseases in transition dairy cows. Results of this investigation indicated that repeated oral administration of LPS and LTA enhanced humoral immune responses in the vaginal mucus, modulated several plasma metabolites, and lowered the risk of various periparturient diseases in transition dairy cows.

Data showed that total IgA antibodies in the vaginal mucus were greater in cows treated with LPS and LTA, with concentrations of IgA remaining greater than those of the control animals during the whole post-treatment period. Although the precise mechanism of how oral treatment with LPS and LTA affected vaginal IgA secretion is not understood, it is known that lymphocyte precursors are induced in inductive mucosal lymphoid tissues following initial sensitization with an antigen, which subsequently enter into the circulation, and move to various mucosal effector sites through homing receptors (McDermott and Bienenstock, 1979; Mestecky, 1987). It has been established in various studies that mucosal vaccination with microbial antigens when administered orally leads to local production of IgA antibodies against various pathogens that colonize the surfaces of the gastrointestinal, respiratory, and urogenital tracts and has resulted

in the development of several oral human vaccines (McDermott and Bienenstock, 1979; Holmgren and Svennerholm, 1992; Holmgren et al., 1994). A few studies with animal models also suggest that oral vaccination is able to induce local immunity in the genital tract. For example, when mice were orally immunized against *Chlamydia trachomatis*, immunoglobulin A activity was detected in the genital tract (Cui et al., 1991).

Results also showed that oral treatment with both LPS and LTA lowered concentrations of plasma lactate. Although the exact mechanism of this effect is not understood well it is possible that enhanced mucosal immunity by repeated challenge with LPS and LTA has increased the host capacity to prevent endotoxin translocation into the host. In a recent study, we indicated that 93% of the variation of plasma lactate was explained by rumen endotoxin (Zebeli et al., 2011). Translocation of endotoxin into the systemic circulation stimulates the release of several proinflammatory cytokines that enhance glycogenolysis (Elsasser et al., 2008) and lowers the capacity of the extrahepatic tissues to utilize lactate (Steiger et al., 1999). In a companion paper we reported lower concentrations of cytokines like TNF-α, lipopolysaccharide-binding protein (LBP), and haptoglobin in LPS- and LTA-treated animals (Iqbal et al., 2013). It is speculated that translocation of endotoxin in the control cows might be associated with enhanced glycolysis and pyruvate production and convertion of pyruvate into lactate (Hughes, 2004). Caton et al. (2009) reported that endotoxin prevents utilization of lactate for glucose production through gluconeogenesis by downregulation of mitochondrial phosphoenolpyruvate carboxykinase.

Furthermore, there is increased expression of hypoxia-inducible transcription factor-1 (HIF-1α) from immune cells (Lu et al., 2008), which increase secretion of lactate in septicemic patients (Nguyen et al., 2004; Levy 2006).

Another finding of this study was a tendency for greater concentrations of plasma cholesterol in cows administered orally with LPS and LTA. The observed decrease in concentrations of plasma cholesterol in the control cows might be associated with the general inflammatory state triggered by translocation of endotoxin into the systemic circulation. Recently we observed a strong association between rumen endotoxin and decreasing concentrations of plasma cholesterol in response to feeding of large amounts of concentrate (Zebeli et al., 2011). One reason for lower plasma cholesterol in the control animals might be that cholesterol is the precursor metabolite for synthesis of bile acid (Parlesak et al., 2007). It is known that inflammatory conditions mediated by LPS are associated with alterations in bile acid synthesis by influencing production of cholesterol 7-alpha hydroxylase (Dikopoulos et al., 2003). Secreted bile acids cause fragmentation of endotoxin into non-toxic compounds and prevent its translocation into the host systemic circulation (Bertok, 2004). It is speculated that hypocholesterolemia in the control cows in the present study might be an attempt of the host to lower endotoxin translocation. Of note, cholesterol values in our experimental cows were a little greater than previously reported at the beginning of the experiment, but within the normal ranges immediately before and after parturition. For example, normal cholesterol concentrations for healthy periparturient dairy cows are reported to be between 65-114 mg/dL for dry cows

and 63-253 mg/dL for early lactation cows (Anderson and Rings, 2009). One explanation could be that the day-to-day levels of blood cholesterol related to fluctuations of the short-term feed intake patterns. The LPS and LTA treated cows had lower feed intake with greater milk and milk-fat efficiencies compared to the control group, which might contribute to different metabolism of blood cholesterol in those cows.

Concentrations of both NEFA and BHBA were not different between the two treated groups. Endotoxin challenge is commonly associated with enhanced production of proinflammatory cytokines like TNF-α, IL-1, and IL-6 from activated macrophages, which cause lysis of fat depots and the release of large amounts of free fatty acids (i.e., NEFA), which subsequently is converted into ketone bodies (i.e., BHBA) in liver hepatocytes (Drackley, 1999). However, high plasma BHBA in both groups was not related to the greater plasma NEFA in the present study. Concentrations of NEFA in the plasma were on the lower side but within normal ranges in both treatment groups. Normal ranges for dry cows are between 30-460 μEq/L; whereas for fresh cows those normal values range between 10-520 μEq/L (Anderson and Rings, 2009). There are conflicting reports regarding the effects of endotoxin on plasma NEFA. We observed an inverse relationship between the rise of rumen endotoxin and plasma NEFA (Zebeli et al., 2011). However, no effect of endotoxin infusion was observed in heifers (Steiger et al., 1999), and a biphasic response in dairy cows (Waldron et al., 2003). Recently, we reported that transition cows treated orally with LPS had lower plasma NEFA and BHBA early postpartum (Zebeli et al., 2013). The discrepancy

between the two studies might be related to the fact that in the present study cows were treated with two bacterial immunogens (i.e., LPS and LTA) instead of LPS only in our previous study. Regarding plasma BHBA, earlier studies indicated that intravenous administration of LPS in non-lactating heifers or mid-lactating cows decrease concentrations of BHBA in blood (Steiger et al., 1999; Waldron et al., 2003), which is in agreement with numerically lowered BHBA values in the treated cows in the present study. Greater plasma BHBA at the beginning or the end of the experiment in both treatment groups in this study might be due to some underlying diseases or inflammatory conditions, which were not clinically apparent. Furthermore, the feeding time should be taken into consideration when evaluating daily patterns of various plasma metabolites particularly NEFA and BHBA (Ametaj et al., 2009).

Data indicated a rise in plasma insulin, in cows orally challenged with LPS and LTA, before parturition. One possible explanation for the increase of insulin in the treated cows might be the gluconeogenic effects of administered endotoxin (Waldron et al., 2003). Interestingly, the increase in plasma insulin was not associated with increased plasma glucose, which suggests an increased consumption of glucose from the host. For example, Fukuzumi et al. (1996) showed that during endotoxemia the amount of glucose consumed by macrophages was increased together with enhanced expression of RNA for GLUT1. Insulin is known to induce expression of GLUT1 (Sargeant and Pâquet, 1993), a glucose transporter, in various tissues. In a previous investigation we reported that oral treatment of prepartum cows with similar doses of LPS was

associated with increases of both plasma glucose and insulin (Zebeli et al., 2013). Addition of LTA to the treatment might have changed the host metabolic responses. Those toxins might disturb the insulin and glucose dynamics transiently with no evident risk of insulin resistance for the cows.

Concentrations of cortisol in the plasma were not different between the two treatment groups, which are in line with another study indicating that repeated endotoxin challenge exert no effect on plasma cortisol (Borges et al., 2007). It is known that concentrations of cortisol in the plasma are affected by endotoxin challenge shortly after the treatment. An earlier study from our group showed that plasma cortisol is greater in cows administered orally with increasing doses of LPS only during the first exposure to LPS and up to 3 h post-treatment, whereas there were no differences between the treatment and the control animals in weekly doses that followed the first treatment (Zebeli et al., 2013). In this study, there was a greater concentration of plasma cortisol in both groups at the beginning of the experiment, which could be a direct effect of the handling and restraining stress occurring during the sampling of the cows in general. However, with repeated handling exposure, concentrations of cortisol declined and the treated cows had numerically lower values than the control ones until the end of the experiment. This might be an indication that those cows were not under stress for prolong periods of time and that the two bacterial immunogens did not exert additional stress in the treated animals.

An important finding of this investigation was greater IgA antibody responses in the vaginal mucus of the cows treated orally with LPS and LTA. The

postpartum uterine lumen usually harbours many pathogenic bacteria such as *Arcanobacterium pyogenes*, *Escherichia coli*, *Fusobacterium necrophorum*, and *Prevotella melaninogenicus* (Sheldon et al., 2004). In this context, oral immunization with GNB of both animal and human models induces immune responses in the vaginal secretions (Cui et al., 1991; Kantele et al., 1998). This effect is achieved through the induction of specific sets of mucosal homing receptors when antigen comes in contact with T and B cells at oral mucosa, and then the primed immune cells home to preferential effector sites such as the intestinal and urogenital tracts (Kiyono and Fukuyama, 2004; Kiyono et al., 2008).

The results of this study also showed that administration of LPS and LTA in the oral cavity lowered the risk of metritis. This is in agreement with the other finding of this study that the oral administration of LPS and LTA increased concentration of total IgA in the vaginal mucus of the treated cows. This is an interesting finding that needs to be confirmed in a larger cohort of animals. Similarly, the treated cows showed a lowered risk for lameness and RP, with a shorter time-length for which the cows remained sick with lameness. There are indications from research in horses that LPS lowers perfusion in the hoof area (Menzies-Gow et al., 2004) due to initiation of the inflammatory changes (Bailey et al., 2009). We also observed high concentrations of inflammatory markers (see next chapter) following the oral LPS and LTA administration (Iqbal et al., 2013). Lipopolysaccharide also has been suggested to be involved in the induction of reactive oxygen species, most importantly nitric oxide, and this oxidative stress is

directly related to pathogenesis of RP (Kankofer et al., 2005). Although there was a strong numerical difference in the incidence rates of RP between the two groups (treated cows were not affected versus 13% of cases in the control group), the lower number of animals made the data failing to reach the point of significance.

The number of days that each cow was affected by multiple diseases also was lowered in the treatment group. This suggest that bacterial immunogens used to treat the cows play a role in multiple diseases in dairy cows and that preparing cows immunologically against those two bacterial immunogens might have affected the time of recovery from postpartal diseases. This is an interesting topic to be followed in the future research to better understand the reason behind this finding. However, this finding needs to be validated further in a larger cohort of animals.

**6.5 Conclusions**

In conclusion, results of this study provided evidence that repeated oral administration of LPS from *E. coli* 0111:B4 and LTA from *B. subtilis* enhanced total IgA antibodies in the vaginal mucus. Treatment also modulated plasma concentrations of lactate, insulin, and cholesterol around parturition. Results also showed that treatment lowered the risk of metritis, lameness, and RP and had no effect on other periparturient diseases monitored during this study. Altogether, the data suggest that oral treatment with LPS and LTA prepartum might serve as an immune booster to lower the risk of some of the periparturient diseases in dairy cows. More research is warranted, with a larger cohort of animals, to confirm these results in the near future.

## 6.6 Acknowledgement

We acknowledge the financial support of Alberta Milk (Edmonton, Alberta, Canada), Alberta Livestock Industry Development Fund (Edmonton, Alberta, Canada), and Natural Sciences and Engineering Research Council of Canada (Ottawa, Ontario, Canada). We are grateful to the technical staff at Dairy Research and Technology Centre, University of Alberta for their help and care of the cows.

**Table 6-1.** Ingredients and chemical composition of the diet for dry cows

| Ingredients, % of DM | Prepartum diet |
|---|---|
| Alfalfa hay | 10.0 |
| Barley silage | 63.0 |
| Alfalfa silage | 00.0 |
| CUD[1] grain | 27.0 |
| Nutrient composition of cud grain (dietary supplement) % amount in 100 kg mix | |
| Barley grain, rolled | 55.0 |
| Canola meal | 7.5 |
| Dairy dry cow micro-premix | 6.2 |
| Limestone | 8.7 |
| Animate[2] | 15.7 |
| Molasses | 0.9 |
| Canola oil | 4.1 |
| Yeast | 1.7 |

[1]CUD = giving more mineral especially Ca to avoid milk fever in early lactating cows

[2]Animate = Primary source of anionic salts (chloride and sulfur), with supplemental protein, minerals, trace minerals and vitamins

**Table 6-2.** Ingredients and chemical composition of the diet for early lactating cows

| Ingredients, % of DM | Early lactation diet |
|---|---|
| Alfalfa hay | 10.0 |
| Barley silage | 40.8 |
| Dairy supplement | 49.2 |
| Nutrient composition of dairy supplement, % amount in 100 kg mix | |
| ADE Vit Pak-30 Natural E | 0.056 |
| Ruminant TM Pak | 0.1025 |
| Selenium 1000 mg/kg (UNscr Fine Cr) | 0.065 |
| Custom TM Complex pmx | 0.060 |
| Di-calcium phosphate 21% | 1.25 |
| Co-op alantic Corn Dist | 10.0 |
| Corn ground | 25.0 |
| Corn rolled | 30.105 |
| Vit D- 10,000 KIU/kg | 0.015 |
| Diamond V XPC | 0.14 |
| Magalac/Enertia | 1.00 |
| Fermenten | 2.00 |
| Limestone | 1.50 |
| Mag Ox -56% | 0.37 |
| Canola meal | 15.5 |
| Hi bypass soy (Amino plus) | 2.75 |
| Soy bean meal-47.5% | 6.50 |
| Sodium bicarbonate | 1.00 |
| Salt | 0.113 |
| Cattle-Tallow | 2.45 |
| Biotin 2%-Rovimix H-2 | 0.007 |
| ADM Vit E 405 Vegetable source | 0.015 |

**Table 6-3.** Data of incidence and length of disease in lactating multiparous and primiparous cows administered orally with saline (CTR) or saline containing increasing doses of LPS and a flat dose of LTA (TRT)

| [3]Diseases | Treatment groups[1] | | | | | |
|---|---|---|---|---|---|---|
| | Disease incidence, % (No) | | Length of disease (d) | | Overall Effect, *P*-value[2] | |
| | **TRT** | **CTR** | **TRT** | **CTR** | **Incidence** | **Length** |
| Milk fever | 0 (0) | 6.6 (1) | 0 | 1 | 1.00 | 1.00 |
| Retained placenta | 0 (0) | 13.3 (2)* | 0 | 4.5 | 0.10 | 0.16 |
| Metritis | 6.6 (1) | 26.6 (4)* | 9 | 13 | 0.05 | 0.32 |
| Lameness | 13.3 (2) | 33.3 (5)* | 5 | 11.8* | 0.05 | 0.10 |
| Clinical masitis | 6.6 (1) | 6.6 (1) | 5 | 6 | 1.00 | 1.00 |
| Sub-clinical mastitis | 6.6 (1) | 6.6 (1) | 14 | 14 | 1.00 | 1.00 |
| Clinical ketosis | 0 (0) | 0 (0) | 0 | 0 | 1.00 | 1.00 |
| Sub-clinical ketosis | 0 (0) | 0 (0) | 0 | 0 | 1.00 | 1.00 |
| [4]LDA | 0 (0) | 6.6 (1) | 0 | 2 | 1.00 | 1.00 |
| No. of days affected single cow with > 1 disease | | | 5.8 | 15.6* | | <0.01 |
| Udder edema | 1.45 | 1.49 | 7 | 9.5 | 0.82 | 0.22 |

[1]CTR = cows administered orally with saline solution; TRT = cows administered orally with increasing doses of lipopolysaccharide and flat dose of lipoteichoic acid.
[2]Effect of treatment (Trt) for disease incidence and length.
[3]Data across wk -4, to +4.
[4]LDA = Left displaced omasum.
*The significance limit was declared at $P < 0.05$, whereas a biologically relevant tendency was declared at $0.05 < P < 0.10$; however the inadequate number of cows per group suggested that the statistical differences are not significant between two treatment groups.

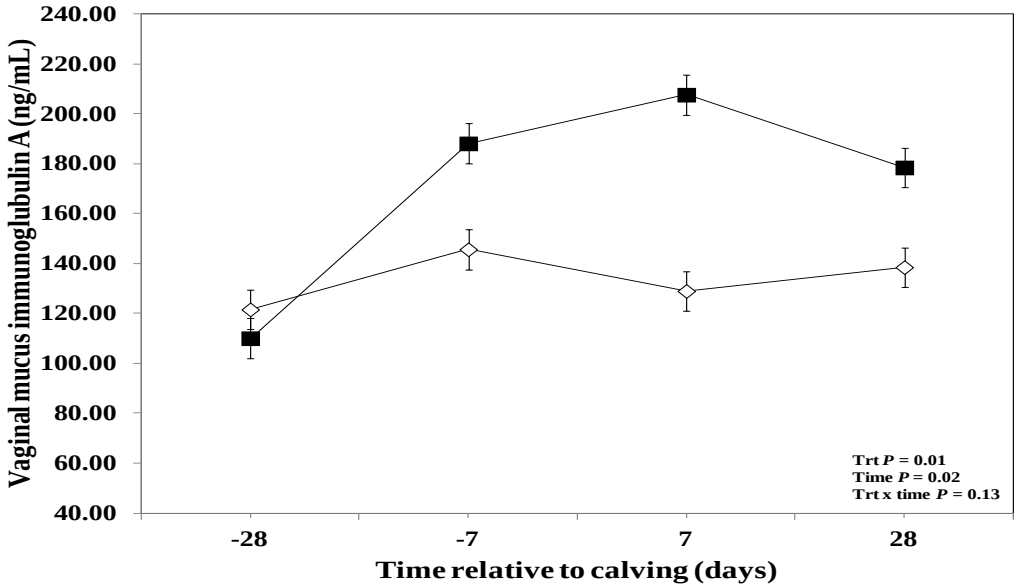


**Figure 6-1.** Concentration of immunoglobulin A in vaginal mucus of Holstein dairy cows challenged with oral treatment of LPS-LTA (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 15; Trt = effect of treatment; Time = Effect of sampling day, Trt x time = effect of treatment by sampling day).

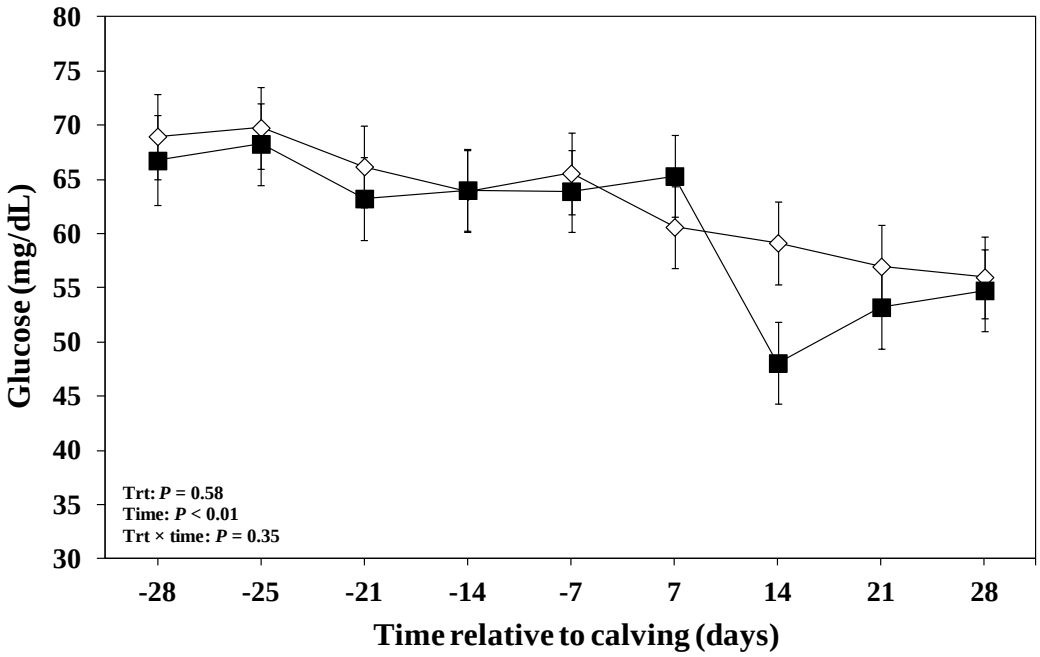


**Figure 6-2.** Concentration of glucose in plasma of Holstein dairy cows challenged with oral treatment of LPS-LTA (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 15; Trt = effect of treatment; Time = Effect of sampling day, Trt x time = effect of treatment by sampling day).

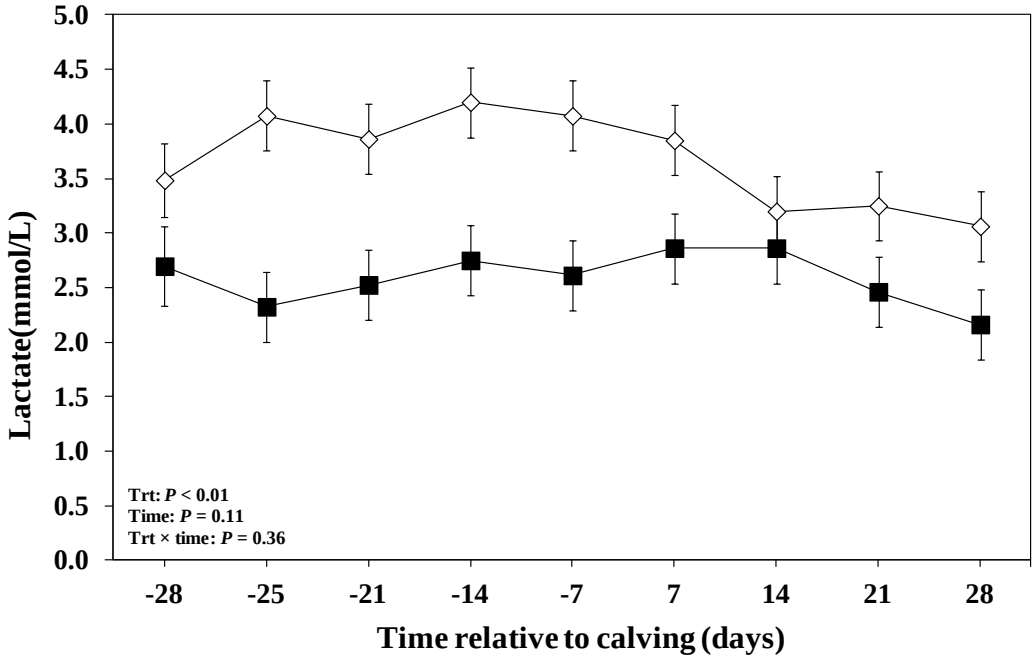


**Figure 6-3.** Concentration of lactate in plasma of Holstein dairy cows challenged with oral treatment of LPS-LTA (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 15; Trt = effect of treatment; Time = Effect of sampling day, Trt x time = effect of treatment by sampling day).

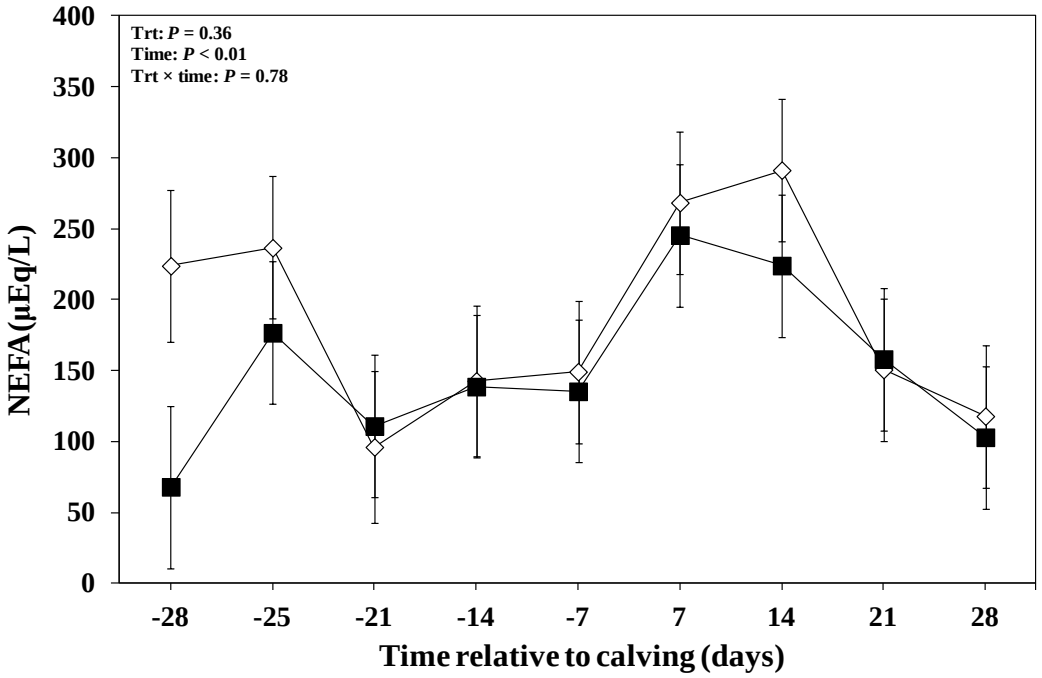


**Figure 6-4.** Concentration of non-esterified fatty acids in plasma of Holstein dairy cows challenged with oral treatment of LPS-LTA (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 15; Trt = effect of treatment; Time = Effect of sampling day, Trt x time = effect of treatment by sampling day).

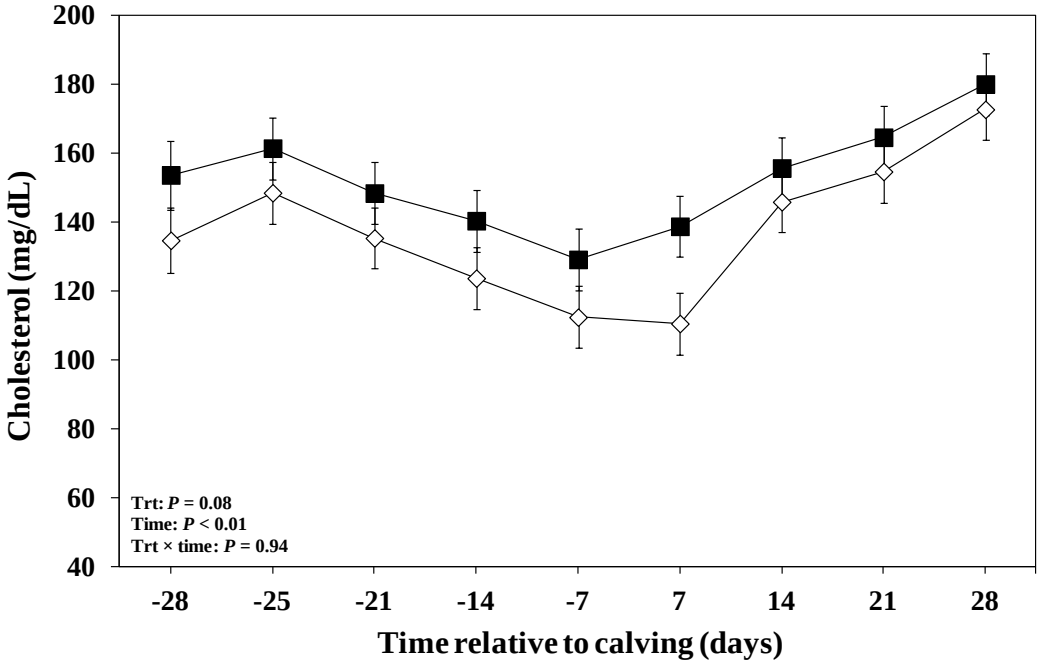


**Figure 6-5.** Concentration of cholesterol in plasma of Holstein dairy cows challenged with oral treatment of LPS-LTA (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 15; Trt = effect of treatment; Time = Effect of sampling day, Trt x time = effect of treatment by sampling day).

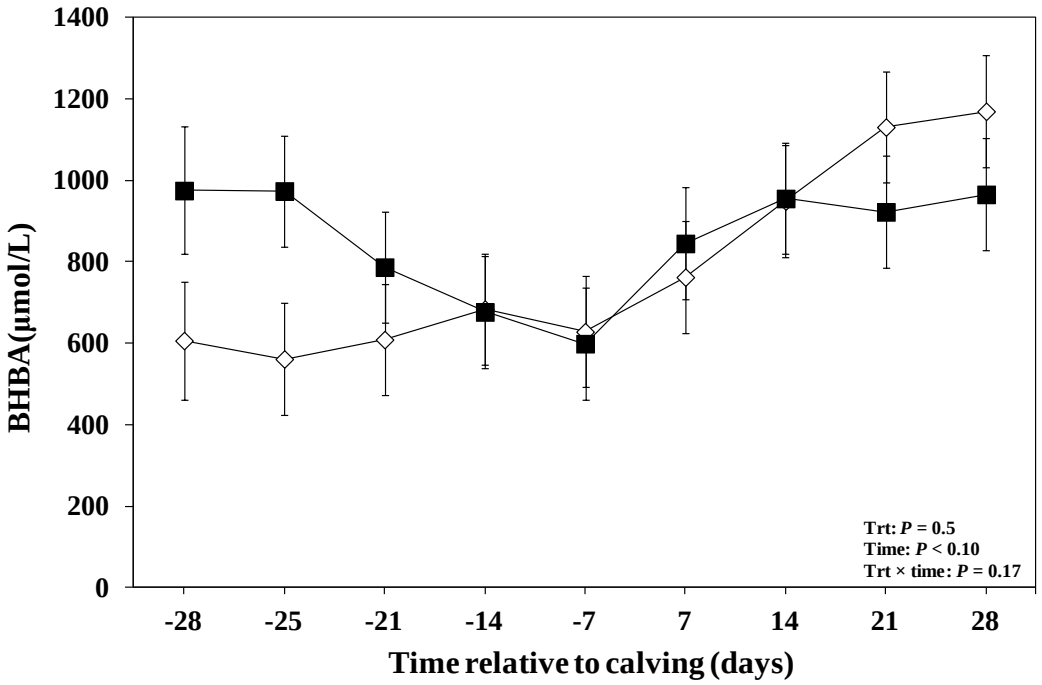


**Figure 6-6.** Concentration of β-hydroxy butyric acid in plasma of Holstein dairy cows challenged with oral treatment of LPS-LTA (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 15; Trt = effect of treatment; Time = Effect of sampling day, Trt x time = effect of treatment by sampling day).

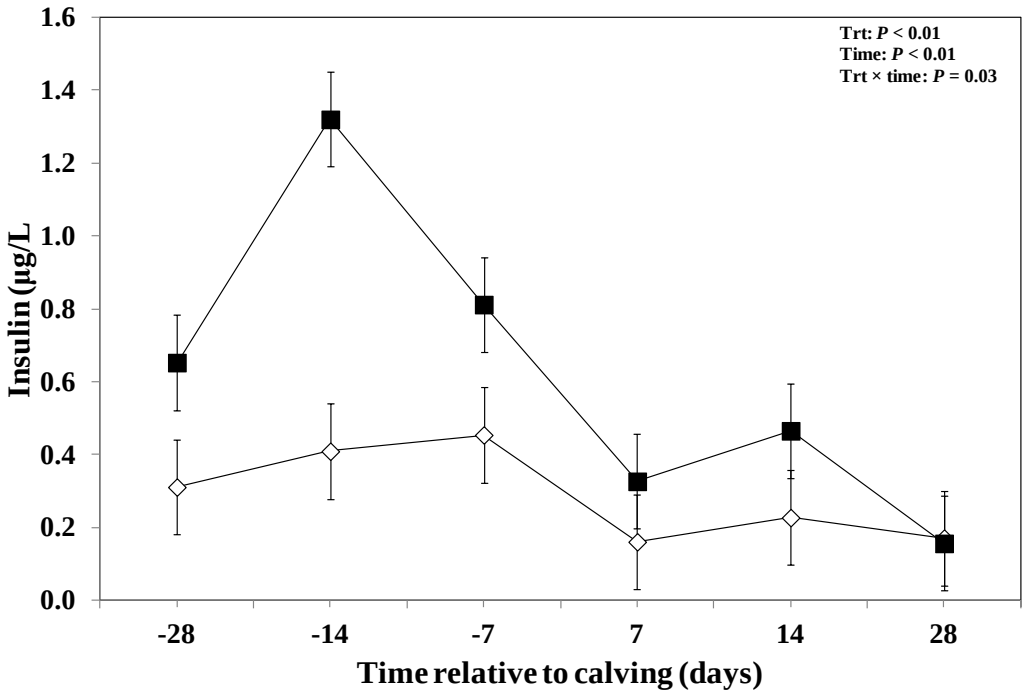


**Figure 6-7.** Concentration of insulin in plasma of Holstein dairy cows challenged with oral treatment of LPS-LTA (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 15; Trt = effect of treatment; Time = Effect of sampling day, Trt x time= effect of treatment by sampling day).

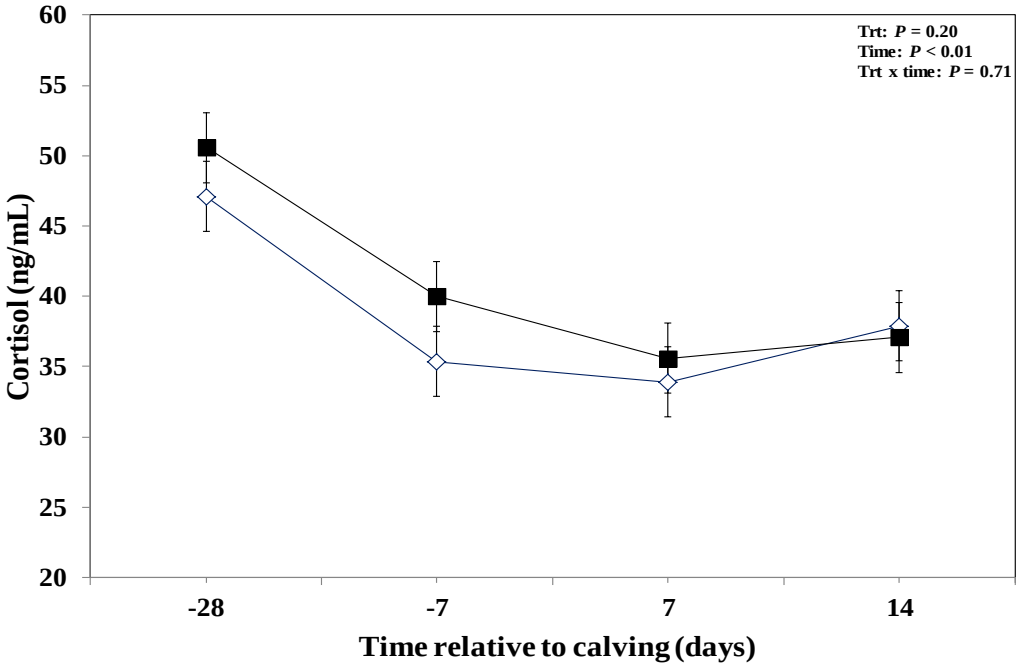


**Figure 6-8.** Concentration of cortisol in plasma of Holstein dairy cows challenged with oral treatment of LPS-LTA (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 15; Trt = effect of treatment; Time = Effect of sampling day, Trt x time = effect of treatment by sampling day).

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# [1]Chapter 7

## Oral administration of lipopolysacharide and lipoteichoic acid prepartum modulated reactants of innate and humoral immunity in periparturient dairy cows

### 7.1 Introduction

Dairy cows experience a state of immunosuppression during the periparturient period, which increases their susceptibility to various peripartal diseases. Furthermore, this attenuated immune function during the peripartum period increases the susceptibility to mastitis postpartum, which causes significant production loses to the dairy industry (Mallard et al., 1998). The reason(s) behind immunosupression in periparturient dairy cows is not well understood yet; however, several lines of evidence indicate that immune responsiveness decreases gradually in the prepartum period and reaches its lowest level immediately before parturition (Goff and Horst, 1997; Ametaj et al., 2005). There is a need to stimulate the immune competence of cows during the periparturient period, because health status during this period is critical for health and productivity of cows during the whole lactation (Ametaj et al., 2005; Lacetera et al., 2005).

Early postpartum, cows encounter various immunogenic substances such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA), which are important components of the Gram-negative and Gram-positive bacterial cell wall, respectively (Ametaj et al., 2010). Additional exposure of mucosal layers to LPS and LTA occurs due to accumulation of cell-free LPS or LTA in the rumen when

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cows are switched from a roughage-rich to a grain-rich diet (Emmanuel et al., 2008; Khafipour et al., 2009), at the onset of lactation. However, the virulence properties of those immunogenic compounds are highly influenced by the sudden switch in diet postpartum. For example, there is an abundance of *E. coli* with highly virulent LPS moiety during high-grain feeding immediately after parturition, and it strongly binds to LBP to induce a high inflammatory response (Netea et al., 2002). Furthermore, the *E. coli* infection paves the way for infection from other pathogens, and interacts with lactic acid resistant bacteria such as *B. subtilis*, which produce LTA as a major immunostimulatory component (Krause et al., 2003).

Mucosal surfaces comprise the first port of entry for bacterial endotoxins and LTA (Ametaj et al., 2012a). Thus, developing a prophylactic treatment targeting the mucosal immune responses by co-stimulating with LPS and LTA might be of great interest. Mucosal immunity is primarily mediated by antibodies of the immunoglobulin A (IgA) class, which is by far the most prominent isotype synthesized by the immune system (Lamm, 1997). The mucosal immune responses have been shown to strongly depend on the production of secretory IgA (sIgA) molecules (Lamm, 1997; Medaglini et al., 1998). In fact, the interest on inducing mucosal immunity and most importantly in administering immunoantigens on the mucosal layers has increased recently. Furthermore, there is strong evidence that the mucosal sites of immunogen challenge influence the location of the IgA response. A recent report demonstrated that oral immunization induces protective mucosal immune responses, but suppresses systemic

immunologic reactivity (Brandtzaegss, 2013). This kind of immunization stimulates secretory IgA responses at distant mucosal layers and develops sub-populations of regulatory T lymphocytes within the gut-associated lymphoid tissues, which inhibit the subsequent systemic responses to the same antigen (Keren et al., 1988). Recently we showed that oral treatment of cows with LPS was able to influence the proinflammatory responses and modulate production of anti-LPS IgM antibodies in the plasma as well as metabolic health status (Ametaj et al., 2012b). In addition, intra-mammary administration of LPS protected cows against experimental *Escherichia coli* mastitis (Petzl et al., 2011).

Despite tremendous progress in understanding the role of LPS on cow health only a few investigations have addressed the role of LTA on the etiopathogenesis of periparturient diseases of dairy cows. A recent study examined the effects of LTA from *Staphylococcus aureus* LTA, on initiation of clinical mastitis at the dose of 100 μg/quarter, and a subclinical inflammatory response at 10 μg/quarter (Rainard et al., 2008). Interestingly, another study showed that a challenge with *E. coli*-derived LPS and LTA from *S. aureus* induced a complex and robust immune response to pathogens (Daly et al., 2009). Data from this work also showed enhanced IgA responses in the vaginal mucus of cows when they were orally challenged with LPS and LTA.

To our best knowledge, there are no reports dealing with cow responses to oral administration of LPS and LTA, as a prophylactic strategy against deleterious effects of those bacterial endotoxins. Therefore, we hypothesized that repeated oral exposure of the periparturient dairy cows to increasing doses of LPS and a

flat dose of LTA before parturition might improve their innate and humoral immune responses against LPS and LTA and improve subsequent health status of dairy cows.

## 7.2 Materials and Methods

### *7.2.1 Cows and Experimental Design*

Thirty pregnant multiparous and primiparous Holstein dairy cows with average body weight of 720 ± 30 and 600 ± 20 kg, respectively were blocked by parity, milk production, body condition score (BCS), disease susceptibility from previous year, and the anticipated day of calving. Fifteen cows (10 multiparous and 5 primiparous) were randomly allocated to each group, at 28 d before the expected day of parturition. Cows were orally administered either 2 mL of sterile saline solution (CTR), or 2 mL of sterile saline solution containing LPS (TRT) from *E. coli* strain 0111:B4 at three increasing concentrations as follows: 1) 0.01 µg/kg body weight (BW) once on d -28, 2) 0.05 µg/kg BW twice on d -25, and -21, and 3) 0.1 µg/kg BW twice on d -18, and -14 along with a flat dose of LTA from *Bacillus subtilis* (i.e. 120 µg/animal) for 3 consecutive weeks on the same days with LPS treatments. The initial crystalline *E. coli* LPS (from *E. coli* strain 0111:B4) and *B. subtilis* LTA (both supplied by Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada), containing 10 mg of purified LPS and LTA, were then dissolved in 10 mL of doubly distilled water each, as suggested by the manufacturer, and stored in a refrigerator at 4°C. For administration to the animals, the daily dose was dissolved in 2 mL of saline and then introduced into the oral cavity of the cow using a disposable 5 mL syringe (Becton, Dickinson

and Company, BD, Franklin Lakes, NJ). Similarly, the same amount of carrier (i.e., 2 mL sterile saline; Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) was orally administered to all cows in the control group. Doses of LPS used were based on previous research conducted with dairy cows by our team and on clinical and pathological responses to those doses (Ametaj et al., 2012b), whereas a dose study was conducted to determine the safe clinical dose of oral LTA to be used (Iqbal et al., 2011). The lowest dose of 0.01 μg/kg BW was chosen because previous experiments have shown minimal changes in the metabolism of dairy cows at this concentration (Ametaj et al., 2012b), whereas the highest dose was also selected owing to a maximum host response at this dose observed previously (Ametaj et al., 2012b). Furthermore, the induction of endotoxin tolerance is dose dependent, and LPS is more effective in inducing endotoxin tolerance with increasing doses than LTA. The flat dose of LTA was selected based on a dose study conducted by us indicating no effects to cow's temperature, respiration rate, rumen contraction rate, and feed intake (Iqbal et al., 2011).

The experiment lasted for 8 weeks (i.e., 4 weeks before and 4 weeks after parturition) and cows were housed in tie stalls (48" x 79") with free access to water throughout the experiment. Shortly before parturition, cows were transferred to the maternity pens (22' x 14 ½') and returned to their stalls on the next day of parturition. Animals were fed once daily at 0800 and milked twice at 0500 and 1530 in their stalls. All cows were fed the same close up diet starting at 3 weeks before the expected day of parturition. The close up diet is usually offered to the dairy cows when they are close to parturition and contained

approximately 20% concentrate on dry matter basis. After parturition, cows were gradually switched during the first 7 days to a fresh-lactation diet with higher proportion of grain (up to 50% on dry matter basis) to meet the energy demands for high milk production. All diets were formulated to meet or exceed the nutrient requirements of dry and early lactating cows as per National Research Council (NRC) guidelines (2001). Daily ration was offered as total mixed ration (TMR) for ad libitum intake to allow approximately 10% feed refusals throughout the experiment. All experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Livestock, and animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993). Veterinary supervision was provided to the animals throughout the experiment.

### *7.2.2 Sample Collection*

Blood samples were collected from coccygeal vein on d -28, -25, -21, -14, -7, +7, +14, +21, and +28 around parturition for plasma haptoglobin, and once per week on d -28, -7, +7, and +28 around parturition for plasma serum amyloid A (SAA), lipopolysaccharide-binding protein (LBP), anti-LPS plasma immunoglobulin (Ig)A, IgG, IgM, tumour necrosis factor (TNF)-α and interleukin (IL )-1. Blood samples of approximately 5-8 mL were collected in 10 mL of glass tubes (BD Vacutainers) with no additive (Becton, Dickinson and Company, BD, Franklin Lakes, NJ). Blood samples were put immediately on ice, and centrifuged within 20 min (Rotanta 460R, Hettich Zentrifugan, Tuttlingen, Germany) at 3,000 × *g* and 4 °C for 20 min. The plasma was separated and stored at -20$^{o}$C until

analyses. No agitation or overreaction from cows was observed during the blood withdrawal. Feed intake was recorded daily during the entire experimental period. All disease and medication history was recorded for each cow throughout the entire experimental period.

Saliva samples were collected on d -28, -7, +7, and +28 around parturition using cotton swabs inserted between the cheek and the lower jaw, along the side of the mouth towards the back teeth until the swab was soaked. For collecting saliva, head movement of the animal was restrained using conventional restraining techniques (e.g., rope halter and held by a person). After collection, saliva samples were extracted from the cotton gauze using 60 mL plastic syringe (Becton, Dickinson and Company, BD, Franklin Lakes, NJ), and then placed in a small sterile container which was sealed securely and stored at -86$^{o}$C until analyses for total IgA. No preservatives or additional material was added to the saliva samples. Before assay, samples were centrifuged (Rotanta 460R, Hettich Zentrifugan, Tuttlingen, Germany) at 1,000 × *g* and 4 °C for 20 min to remove any particulates.

### *7.2.3 Sample Analyses*

Concentrations of anti-LPS core IgA, IgG, and IgM in the plasma were measured using a commercially available ELISA kit EndoCab (HK504, Canton, MA, USA), using the methods described previously by Zebeli et al (2011). In brief, antibodies directed against the core structure of endotoxin (EndoCab) are cross-reactive against most types of LPS, and are measured using commercial sandwich EndoCab ELISA kit which is a solid-phase enzyme-linked

immunosorbent assay with a working time of 2 and a half hours. The color developed was proportional to the amount of anti endotoxin core antibodies present in the sample. The absorbance was measured at 450 nm with a spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA). The minimum detection concentrations of IgG, IgM, and IgA EndoCab antibodies were 0.0125 GMU/mL, 0.055 MMU/mL, and 0.156 AMU/mL, respectively. The inter- and intra-assay CV for the IgA, IgG, and IgM anti-LPS antibodies analysis were less than 10%.

Concentrations of haptoglobin in the plasma were measured with an ELISA kit provided by Tridelta Development Ltd. (Greystones C., Wicklow, Ireland). According to the manufacturer, the minimum detection limit of the assay was 0.25 ng/mL as defined by the linear range of the standard curves. All samples were tested in duplicate, and the optical density was measured at 630 nm on a microplate spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA). The CV for the inter- and intra-assay analysis was less than 10% for all the samples tested.

Concentrations of LBP in the plasma were quantified with a commercially available ELISA kit (Cell Sciences Inc., Norwood, MA). The antibody coated in the walls cross-reacted with bovine LBP. Plasma samples were initially diluted 1:1,000 and samples with optical density values lower than the range of the standard curve were tested with a lower dilution (1:500). The minimum detection limit of the assay was 5 ng/mL as calculated from a standard curve of the known LBP values in human plasma. Samples were tested in duplicate, and the optical

density was measured at 450 nm on a microplate spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA). Inter- and intra-assay CV was less than 10% for this analysis.

Concentrations of SAA in plasma were determined by commercially available ELISA kits (Tridelta Development Ltd., Greystones Co., Wicklow, Ireland) with monoclonal antibodies specific for SAA coated on the walls of the microtitre strips originally described by McDonald et al (1991). Samples were initially diluted 1:500 and if some of the samples had optical density values below the range of the standard curve they were reanalyzed in lower dilutions. The inter- and intra-assay CV for the SAA analysis was less than 10%. All samples were tested in duplicate and the optical density values were read on microplate spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA) at 450 nm. The minimum detection limit of the assay was 18.8 ng/mL.

Concentrations of TNF-α in the plasma were measured using commercially available bovine ELISA kits (Bethyl Laboratories, Inc. TX, USA). Diluted samples and standards (100 μL) were incubated in the coated plate, followed by washing and incubation with 100 μL of detection antibody and horseradish peroxidise (HRP) substrate for 1 h and 30 min, respectively. The incubation with each of these reagents was followed by washings for four times. The detection antibody solution cross reacts with the antibodies attached to coated wells. The addition of 100 μL of TMB solution allow the enzymatic colour reaction, and the colour developed was proportional to the amount of anti TNF-α antibodies present in the sample. The absorbance was measured at 450 nm with a

spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA). The minimum detection limit of TNF-α was 0.078 ng/mL. The inter- and intra-assay CV for the analysis of TNF-α was less than 10%.

Plasma IL-1 was determined by commercially available bovine ELISA kits (Cusabio biotech Co., Ltd, Newark, USA). The assay is based on the competitive inhibition of an enzyme immunoassay technique. An antibody specific to IL-1 was pre-coated by manufacturer onto microplate wells and standards and samples were incubated with biotin-conjugated IL-1, which leads to competitive inhibition reaction between IL-1 (standards or samples) and biotin-conjugated IL-1 with the pre-coated antibody specific for IL-1. Then avidin conjugated to HRP was added to each microplate well and incubated after the substrate solution was added to the wells. The color developed was in opposite to the amount of IL-1 in the sample. Further development of color was stopped by adding stop solution and the intensity of the color was measured with a spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA) at 450 nm. The minimum detectable concentration of bovine IL-1 was at <125 pg/mL. The inter- and intra-assay CV for the IL-1 analysis was less than 10%.

Concentrations of total IgA in the saliva were measured using a commercially available bovine ELISA kit (Uscn, Life Sciences Inc., USA). The procedure involved the basic principle of sandwich enzyme immunoassay for the quantitative measure of IgA in bovine saliva with a working time of 4 h. The microtiter plate provided with the kit was pre-coated with antibodies specific to the IgA. The standards and samples were then added to appropriate microtiter

plate wells with a biotin-conjugated antibody preparation specific for IgA, and then avidin conjugated to HRP was added. The color developed by the substrate was shown only in those wells which contain IgA, biotin conjugated antibody, and enzyme conjugated avidin. The absorbance was measured at 450 nm with a spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA). The minimum detectable concentration of this assay was 0.78 ng/mL. The inter- and intra-assay CV for all the samples tested for total salivary IgA was less than 10%.

### *7.2.4 Statistical Analyses*

Data were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA Version 9.1.3) as describe by the following model:

$$Y_{ijkl} = \mu + t_i + w_j + tw_{ij} + \varepsilon_{ijkl}$$

where $Y_{ijkl}$ is the observations for the dependent variables, $\mu$ represent the population mean, $t_i$ is the fixed effect of treatment, $w_j$ is the fixed effect of week, $tw_{ij}$ is the interaction between treatment and week, and $\varepsilon_{ijkl}$ is the residual error assumed to be normally distributed. The probability difference (PDIFF) option of SAS was used to compare the least square means (LSM). Measurements on the same animal were considered as repeated measures. The covariance structure of the repeated measurements for each variable was modeled separately according to the lowest values of fit statistics based on the BIC (Bayesian information criteria). The significance limit was declared at $P < 0.05$, while a tendency was considered at $0.05 < P \leq 0.10$.

## 7.3 Results

### *7.3.1 Plasma and Saliva Anti-LPS Immunoglobulins*

Oral treatment of cows with LPS and LTA had an effect on the profile of total IgA in the saliva (Figure 7-1). Treated cows showed overall greater concentrations of salivary IgA than their control counterparts ($P < 0.01$). The factor time of sampling ($P = 0.04$), and treatment by week interaction ($P < 0.01$) also affected salivary concentrations of IgA in this study. The control cows showed an unchanged and flat response of IgA in the saliva, whereas this variable was greater in the TRT cows starting from week -1 onward (Figure 7-1).

Results of the effects of oral LPS and LTA administration on plasma concentrations of anti-LPS IgA antibodies are shown in Figure 7-2a. Results indicated differences between the groups with the control group having greater concentrations of plasma anti-LPS IgA antibodies ($P < 0.01$). However, the factor measurement week when blood samples were taken did not show an effect on plasma IgA ($P = 0.22$). Furthermore, no treatment by week interaction was observed between the two treated groups regarding plasma anti-LPS IgA antibodies ($P = 0.74$).

The group of cows treated orally with LPS and LTA had lower concentrations of plasma anti-LPS IgG antibodies ($P < 0.01$; Figure 7-2b). However, no effect of week ($P = 0.13$), or treatment by week interaction was obtained regarding plasma anti-LPS IgG antibodies ($P = 0.75$).

Concentrations of anti-LPS IgM antibodies in the plasma were different between the treatment groups ($P < 0.01$; Figure 7-2c). The group of cows treated orally with saline had greater concentrations of plasma anti-LPS IgM antibodies

compared to the treated group. There was no effect of sampling week ($P$ = 0.19), or treatment by week interaction with respect to plasma endotoxin IgM antibodies ($P$ = 0.94).

### *7.3.2 Plasma Acute Phase Proteins*

Treating dairy cows orally with LPS and LTA showed a tendency for lower concentrations of haptoglobin in the plasma ($P$ = 0.10; Figure 7-3). Furthermore, no interaction between treatment and week of sampling was evidenced for the concentrations of haptoglobin in the plasma ($P$ = 0.27). Additionally, measurement week did have an effect on plasma haptoglobin ($P$ < 0.01).

No differences between the two treatment groups were observed regarding concentration of SAA in the plasma ($P$ = 0.17; Figure 7-4a), although sampling week alone had an effect on plasma SAA ($P$ < 0.01). However, data indicated no treatment by measurement week interaction for plasma SAA ($P$ = 0.60).

Data also demonstrated a tendency for the concentrations of LBP in the plasma to be lower in the treatment group ($P$ = 0.06; Figure 7-4b). Moreover, results showed a tendency for the effect of week of sampling on the concentrations of LBP ($P$ = 0.10); although no treatment by week interaction was obtained for the plasma LBP ($P$ = 0.16).

### *7.3.3 Plasma Cytokines*

Results indicated differences for plasma TNF-$\alpha$ between the two treated groups with the treatment group having lower concentrations before and after calving ($P$ = 0.02; Figure 7-5). However, no effect of week of sampling was

observed for plasma TNF-α ($P = 0.87$). Also no treatment by measurement week interaction was observed between the two treated groups regarding this variable ($P = 0.89$).

Concentration of IL-1 in the plasma did not differ between groups ($P = 0.60$; Figure 7-6). However, results showed an effect of time of sampling on plasma IL-1 ($P < 0.01$). Also, analysis of data indicated no treatment by sampling time interaction for plasma IL-1 ($P = 0.93$).

**7.4 Discussion**

The present study investigated whether repeated oral administration of LPS and LTA would affect the innate and humoral immune responses of periparturient dairy cows. Both LPS and LTA have been implicated in multiple metabolic and immune perturbations as well as various metabolic and infectious diseases of dairy cows (Ametaj et al., 2013a). Lipopolysaccharide and LTA are cell-wall components of Gram-negative and Gram-positive bacteria, respectively, with different mechanisms of action on host immune cells. Lipopolysaccharide is known as a TLR-4 ligand and LTA as a TLR-2 ligand. Immune responses to those ligands are also different (Kumar et al., 2009). It is widely accepted that during the postpartum period dairy cows are suddenly exposed to increased concentrations of these immunogenic compounds particularly LPS, and the underlying cause is the high-grain engorgement, which alters rumen microbial ecology (Dong et al., 2011). For this reason administration of LPS and LTA was done in the oral mucosa of dairy cows before this critical period in order to enhance the immune responsiveness of the cows by inducing antibodies in the

adjacent mucosal layers. Furthermore, repeated oral challenge with bacterial immunogens at different time points aimed at developing the state of mucosal immune tolerance and to lower responsiveness of the immune system when these cows are exposed to high concentrations of bacterial toxins postpartum. Indeed data from this investigation supported our hypothesis and demonstrated modulation of several innate and humoral immune variables in dairy cows treated orally with both LPS and LTA. The mechanism(s) related to improved innate and humoral immune status of the treated cows deserves further investigation; however, the findings are discussed in more detail below.

The most important finding of this research was that total salivary IgA antibodies were greater in the treated cows during the whole post-treatment measurement period. It is well known that oral immunization usually stimulates production of secretory IgA in the mucosal membranes, which are capable of inhibiting attachment of bacterial antigens to the luminal surface of the mucosal epithelia (Lycke, 2012). It is well known that IgA is the principal mucosal antibody class, which is synthesized by local plasma cells coming from the Peyer's patches (Lamm, 1997). The results of the present study indicated development of a better oral immunity in the LPS- and LTA-treated cows. While a dichotomy exists between the systemic and mucosal immune responses (Vázquez-Padrón et al., 2000), and evidence is accumulating that mucosal immunity consists in stimulation of secretory IgA responses at different mucosal sites and inhibition of systemic responses including plasma IgA production (Mestecky et al., 2007). This might be the case in the present study since

circulatory IgA were lower in the treated cows. Thus, it might be speculated that following the oral administration of LPS and LTA, a protective immunity associated with the induction of mucosal antibodies was elicited. On the other hand, it is obvious that treatment suppressed circulatory IgA antibodies by effectively controlling the entrance of bacterial toxins at the mucosal sites without involvement of systemic primary humoral response. An earlier study demonstrated that repeated exposure to oral dosing with LPS dampens the Th1-type immune responses of the gut, and promotes oral tolerance in rats (Bellmann et al., 1997). In addition, it was also observed that secretory IgA antibody responses were associated with systemic suppression of the humoral and T cell responses to that specific antigen (Challacombe and Tomasi, 1980).

Another interesting finding of this study was that plasma anti-LPS IgG antibodies were lesser in the LPS- and LTA-treated cows. These data are in agreement with previous findings from our team indicating that transition dairy cows treated repeatedly with increasing oral doses of LPS have lower anti-LPS IgG antibodies (Ametaj et al., 2012b). It is speculated that the phenomena involved might be related to the prevention of translocation of those bacterial toxic compounds into the systemic circulation and the loss of systemic reactivity which might involve not only the humoral responses but also T cell-mediated reactions in the treated cows. It has been reported that regulatory T cells, which suppress systemic T-cell responses are involved in systemic tolerance and prevent the exhaustion of the immune system by the abundance of this antibody (Mestecky et al., 2007). Furthermore, it is well accepted that plasma IgG is not as

important in protection of the mucosal layers as is IgA, although some of its fractions may do so (Bouvet and Fischetti, 1999).

Data indicated lower concentrations of plasma anti-LPS IgM antibodies, indicating the potential of this treatment to modulate primary humoral immune responses. Although the exact mechanism of action is not clear at present, it is speculated that the oral vaccination might down-regulate the proinflammatory immune reactivities and primary humoral immune responses by repeated exposure to two different bacterial stimulants. Again the mechanisms behind might be related to prevention of translocation of bacterial toxins into the host's bloodstream. Interestingly, a recent study confirmed the existence of T-cell independent (TI) memory B cells in the context of vaccine administration of pure bacterial polysaccharides, which requires exogenous TLR. It was suggested that TI memory plasma cells may have an extended lifespan (Defrance et al., 2011). Results of the present study confirm the existence of memory B-cells after oral administration of bacterial polysaccharides. However, further research needs to be done to address the phenomena of the longevity of memory against these bacterial toxins.

This study also showed lowered plasma TNF-$\alpha$ in the LPS-LTA treated cows. Results also showed that plasma IL-1 was numerically lesser in the treated cows; however, the difference did not reach significance. These data are indicative of a lower systemic inflammatory response in the treated cows and are in agreement with results of the salivary IgA and plasma anti-LPS IgA, IgG, and IgM antibodies. It is known that translocation of the luminal bacterial toxic

compounds into the systemic circulation stimulates the release of proinflammatory cytokines such as TNF-α, IL-1, and IL-6 by liver macrophages (Gabay and Kushner, 1999), resulting in a state of inflammation in cattle (Emmanuel et al., 2008). Recent evidence suggests that mediators of inflammation are released from liver macrophages when activated by binding of endotoxin (Elsasser et al., 2008). This results in enhanced secretion of a number of acute phase proteins (APP) like LBP, SAA, and C-reactive protein whose functions are to neutralize the effects of circulating LPS (Emmanuel et al., 2008).

Our data support the hypothesis that repeated oral application of LPS and LTA played an important role in attenuation of the acute phase response (APR) as indicated from the lower concentrations of LBP and haptoglobin in the plasma of the treated cows (Ametaj et al., 2011). It is well established that plasma APP are part of a general non-specific immune response, and translocation of endotoxin into the systemic circulation stimulates the release of those proteins from liver hepatocytes under the influence of proinflammatory cytokines like IL-1, IL-6, and TNF-α ( Gabay and Kushner, 1999; Emmanuel et al., 2008).

The main role of LBP in the plasma is to bind circulating endotoxin and facilitate its clearance either by the macrophage or lipoprotein pathways (Ametaj et al., 2005). Interestingly, the lower concentrations of haptoglobin in the treated cows in the present study is indicative of reduced translocation of bacterial toxins into the bloodstream immediately before and after parturition showing the ability of this new approach to maintain the mucosal barrier functions against these bacterial toxins.

Haptoglobin has the ability to selectively antagonize LPS effects by suppressing monocyte production of TNF-α, IL-10, and IL-12 and increased concentrations during an APR may generate a feedback that attenuates the release of cytokines, protecting against endotoxin harmful effects (Arredouani et al., 2003).

Repeated oral treatment with LPS and LTA showed no effect on the overall concentration of SAA in the plasma, although it should be pointed out that there was lower concentrations of SAA during the weeks following parturition. The greater concentrations of SAA in the control group are indicative of presence of an inflammatory state in those cows potentially related to increased circulating endotoxin. The main function of SAA is to bind and neutralize LPS and transport it to the liver hepatocytes for excretion through bile (Levels et al., 2001; Ametaj et al., 2005).

**7.5 Conclusions**

In conclusion, repeated oral administration with LPS from *E. coli* 0111:B4 and LTA from *B. subtilis* modulated innate and humoral immune responses in transition dairy cows. Treatment enhanced the total concentration of salivary IgA antibodies and lessened the concentrations of several plasma markers of APR including LBP and haptoglobin in peripartarturient dairy cows. Moreover, cows treated orally with LPS and LTA had lesser concentrations of plasma anti-LPS IgA, IgG, and IgM antibodies and the pro-inflammatory cytokine TNF-α. Altogether, data suggested that oral application of LPS and LTA before

parturition might play a role as a booster of mucosal immunity against two main bacterial endotoxins in periparturient dairy cows.

**7.6 Acknowledgements**

We are grateful to the technical staff at Dairy Research and Technology Centre, University of Alberta for their help and care of the cows.

**7.7 Funding**

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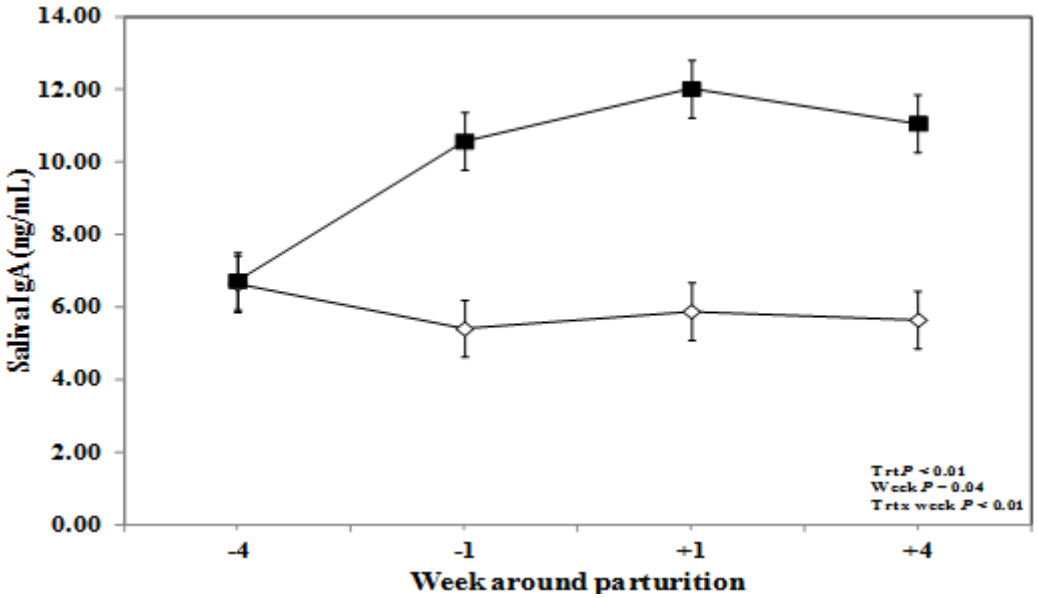


**Figure 7-1.** Weekly variations of total immunoglobulin A in saliva of multiparous and primiparous Holstein cows challenged with oral treatment of LPS-LTA (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 10; Trt = effect of treatment; Week = effect of sampling week, Trt x Week = effect of treatment by sampling week).

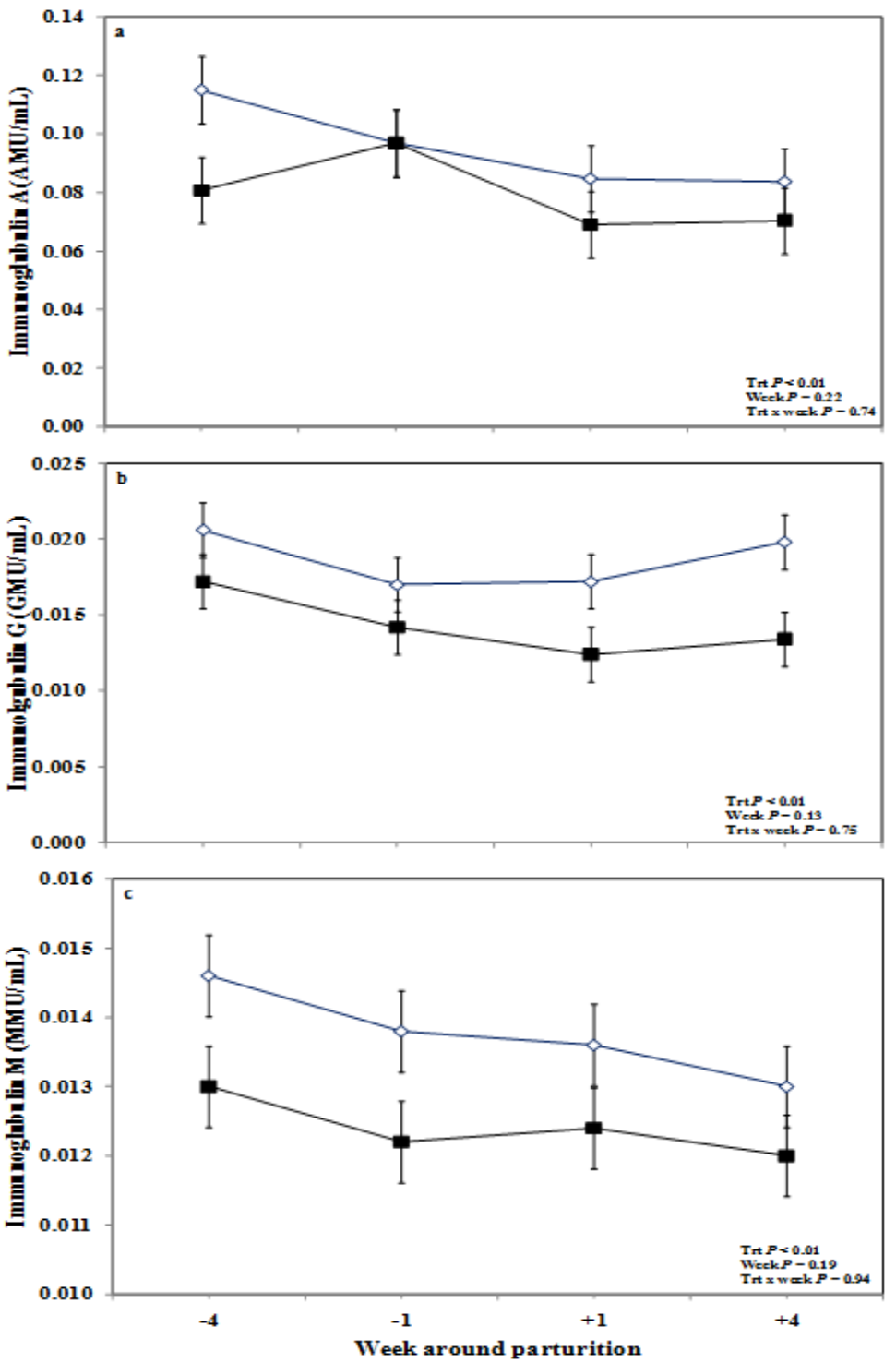


**Figure 7-2.** Weekly variations of anti-LPS immunoglobulin A (a), immunoglobulin G (b), and immunoglobulin M (c) in plasma of multiparous and primiparous Holstein cows challenged with oral treatment of LPS-LTA (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 10; Trt = effect of treatment; Week = effect of sampling week, Trt x Week = effect of treatment by sampling week).

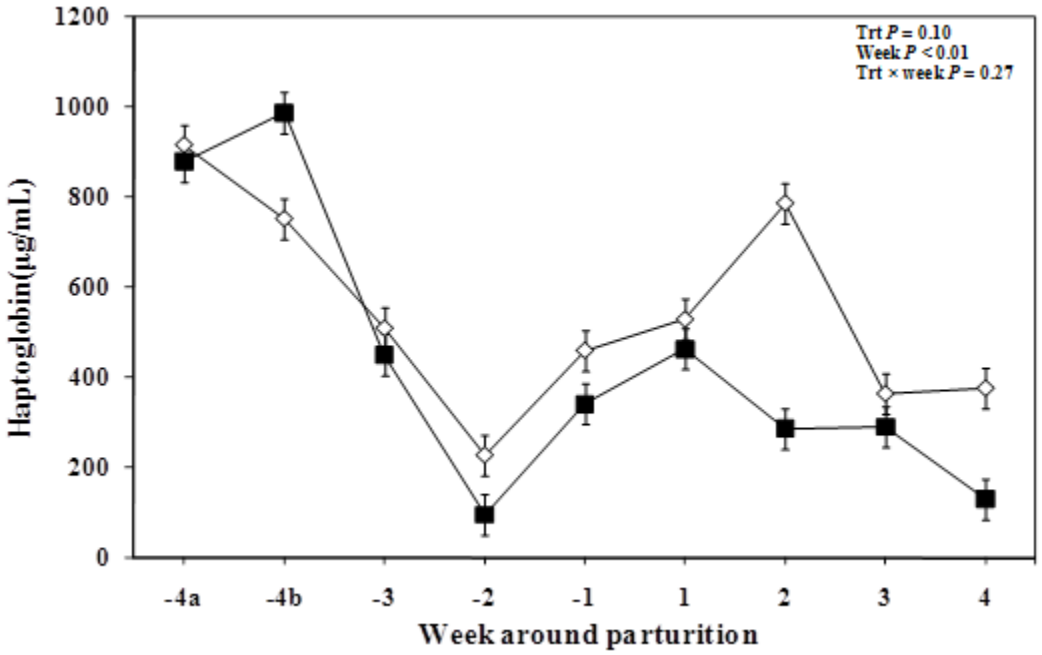


**Figure 7-3.** Weekly variations of haptoglobin in plasma of multiparous and primiparous Holstein cows challenged with oral treatment of LPS-LTA (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 10; Trt = effect of treatment; Week = effect of sampling week, Trt x Week = effect of treatment by sampling week).

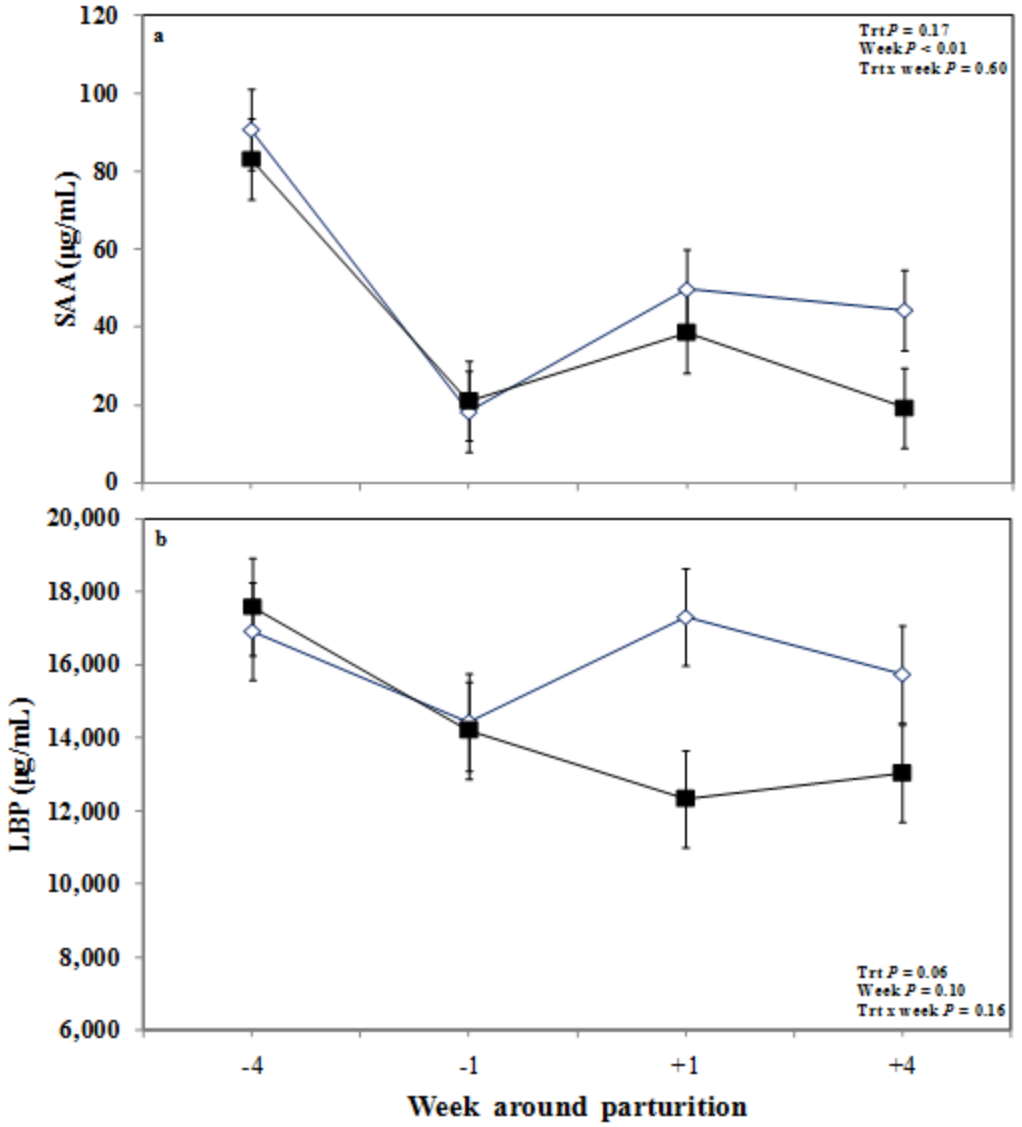


**Figure 7-4.** Weekly variations of serum amyloid A (a), and lipopolysaccharide binding protein (b), in plasma of multiparous and primiparous Holstein cows challenged with oral treatment of LPS-LTA (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 10; Trt = effect of treatment; Week = effect of sampling week, Trt x Week = effect of treatment by sampling week).

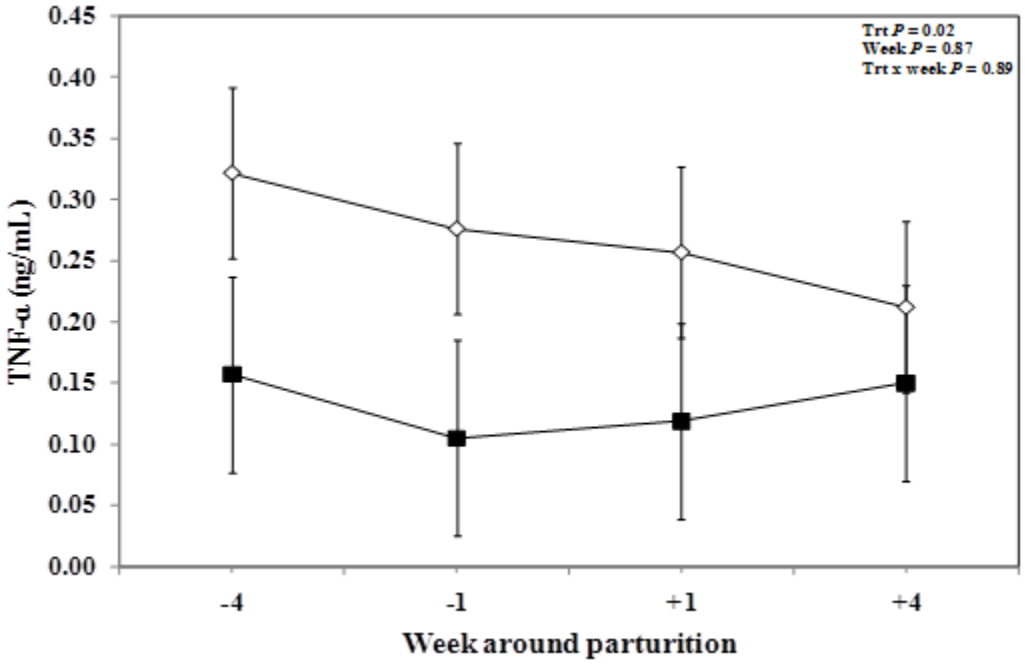


**Figure 7-5.** Weekly variations of tumour necrosis factor-α in plasma of multiparous and primiparous Holstein cows challenged with oral treatment of LPS-LTA (TRT; ■) or saline (CTR; ◇) (LSM ± SEM; n = 10; Trt = effect of treatment; Week = effect of sampling week, Trt x Week = effect of treatment by sampling week).

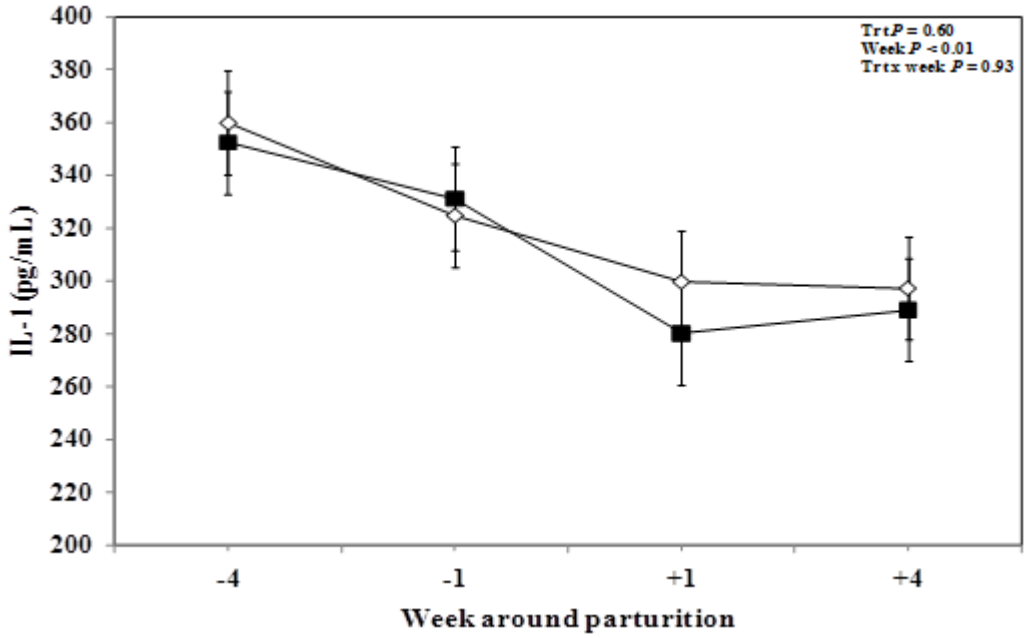


**Figure 7-6.** Weekly variations of interleukin-1 in plasma of multiparous and primiparous Holstein cows challenged with oral treatment of LPS-LTA (TRT; ■) or saline (CTR; ◇) (LSM ± SEM; n = 10; Trt = effect of treatment; Week = effect of sampling week, Trt x Week = effect of treatment by sampling week).

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# Chapter 8

## Oral administration of lipopolysaccharide and lipoteichoic acid prepartum increased milk-fat efficiency and the overall productive performance of dairy cows

### 8.1 Introduction

The inflammatory condition developed around parturition affects the overall health and productivity of dairy cows (Ametaj et al., 2012a). Recent reports support the interrelationship of the host inflammatory responses to the presence of increased amounts of lipopolysaccharide (LPS), a cell wall component of all Gram-negative bacteria (GNB), in the rumen fluid (Ametaj et al., 2010). Furthermore, recent investigations support previous assumptions of translocation of endotoxin from rumen into the systemic circulation and potentially to the mammary gland where it might influence repartition of nutrients as part of inflammatory processes and lower the availability of milk precursors (Emmanuel et al., 2007; Dong et al., 2011).

We reported negative correlations between the amount of rumen endotoxin and plasma C-reactive protein (CRP) with concentration of milk fat and milk-energy efficiency (MEE) in early lactating dairy cows. In fact, rumen LPS explains almost 69% of the decrease in milk-fat content and yield in dairy cows (Zebeli and Ametaj, 2009). It was suggested that the underlying mechanism might involve blockage of lipoprotein lipase (LPL) activity, a key enzyme in the uptake of fatty acids for milk-fat synthesis, by CRP (Khovidhunkit et al., 2004; Zebeli and Ametaj, 2009). Other investigations also support this line of thought

indicating that LPS influences de-novo fat synthesis by impeding the activity of fatty acid synthetase and acetyl-CoA carboxylase (Pekala et al., 1983; Lopez-Soriano and Williamson, 1994).

Another potent bacterial endotoxin, synergistic to LPS, is lipoteichoic acid (LTA), a cell wall component of Gram-positive bacteria (GPB). Infections of the mammary gland by GPB are associated with significant losses in milk production and synthesis of milk components (Schukken et al., 2011). Recently it was shown that mammary epithelilal cells (MEC) respond differently to *Staphilococcus aureus* LTA and *E. coli* LPS, which alter expression of unique chemokines and cytokines that may account for the respective acute and chronic nature of mastitis and the drop in milk production to different extent (Daly et al., 2009; Mount et al., 2009; Schukken et al., 2011). It's been reported that repeated exposure to LPS and LTA can lead to a subsequent refractory state (Schukken et al., 2011), and a decrease in the impact that those bacterial immunogens have on milk yield (Shuster et al., 1991a, b).

It is known that the major sources of bacterial toxins in dairy cows are mucosal layers including those of the gastrointestinal tract, uterus, and mammary gland (Linder et al., 1988; Emmanuel et al., 2008; Dohmen et al., 2000; Wenz et al., 2001). Despite progress in understanding the role of LPS and LTA in the etiopathology of multiple diseases in transition dairy cows, there is a lack of effective prophylactic strategies against translocation of those bacterial toxins into the host systemic circulation and their harmful effects. Thus, it would be of great interest to develop a mucosal immunization against those toxins and their side

effects. In an earlier study, Kofler et al. (1996) observed enhanced concentrations of immunoglobulin(Ig)-A and IgG antibodies in the lungs of mice following oral immunization with LTA from *Streptococcus pyogenes* and LPS from *Klebsilella pneumonia.*

Recently we treated dairy cows with repeated oral doses of LPS from *E. coli* 0111:B4 and were able to increase plasma anti-LPS IgM antibodies and lower anti-LPS IgG antibodies (Ametaj et al., 2012b). Those findings suggest that oral administration with bacterial toxins might be a preferred route of mucosal immunization. In the present study, we hypothesized that repeated oral administration with increasing doses of LPS from *E. coli* 0111:B4 and a flat dose of LTA from *B. subtilis* might modulate milk production and composition in Holstein dairy cows. Therefore, the objective of this study was to evaluate whether oral application with LPS and LTA before calving would affect milk production and its components in transition dairy cows.

## 8.2 Material and Methods

### *8.2.1 Cows, Diets, and Experimental Design*

Thirty clinically healthy pregnant Holstein dairy cows (n = 10 multiparous and n = 5 primiparous cows per group) were included in this study at the Dairy Research and Technology Centre dairy farm, University of Alberta. Cows were blocked by parity, body condition score (BCS), and the anticipated day of calving and were randomly allocated to 2 groups (n = 15) 28 d before the expected day of parturition. Cows in the control group (Control) were orally administered 2 mL of sterile saline solution (0.9% w/v NaCl; Sigma-Aldrich Canada Ltd., Oakville, ON,

Canada). Whereas, the treatment group were orally administered with the same amount of sterile saline with 3 increasing doses of LPS from *E. coli* strain 0111:B4 (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) as follows: 1) 0.01 µg/kg BW on d -28 and -24, 2) 0.05 µg/kg BW on d -21 and -18, and 3) 0.1 µg/kg BW on d -14 along with a flat dose of LTA from *B. subtilis* (i.e. 120 µg/animal; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) twice per week for 3 consecutive weeks starting from week 4 prepartum. Doses of LPS used were based on their clinical and pathological responses from previous trials conducted with dairy cows (Werling et al., 1996; Waldron et al., 2003; Jacobsen et al., 2005; Ametaj et al., 2012b); whereas the dose of LTA was determined based on a dose study conducted by us in order to determine the proper dose of LTA (Iqbal et al., 2011). The initial crystalline *E. coli* LPS and *B. subtilis* LTA containing 10 mg of purified LPS and LTA, respectively, were dissolved in 10 mL of doubly distilled water as suggested by the manufacturer and stored at 4 °C. The required dose of LPS or LTA was dissolved in 2 mL of sterile saline and then introduced into the oral cavity of the cow using disposable sterile plastic syringes (Becton, Dickinson and Company, BD, Franklin Lakes, NJ). Similarly, the same amount of carrier (i.e., 2 mL sterile saline) was orally administered to the cows of the control group.

The experiment lasted for 8 weeks (i.e., 4 weeks before and 4 weeks after parturition) and cows were housed in tie stalls with free access to water throughout the experiment. Cows were transferred to the maternity pens shortly before parturition and returned to their stalls on the next day of parturition. Animals were fed once daily at 0800 throughout the experiment, and ration was

offered as total mixed ration (TMR) for ad libitum intake to allow approximately 10% feed refusals. All cows were fed the same dry off diet starting at week 3 before the expected day of parturition, and then gradually switched during the first seven days after parturition into a lactation diet. All diets were formulated to meet or exceed the nutrient requirements of dry and early lactating cows as per NRC (2001) guidelines. Ingredients and chemical composition of the diets for close up and high rations are presented in Table 8-1 and 8-2. Cows were milked twice daily at 0500 and 1530 in their stalls. All experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Livestock and animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993). Veterinary supervision was provided to the animals throughout the experiment.

### *8.2.2 Feed Intake and Milk Composition*

Individual feed intake and milk production were recorded daily during the 4 week before and 4 week after parturition. Feed intake was calculated by the difference between the total daily feed given to each cow with that of the feed refusals of the next morning. Milk samples were collected once per week at 0500 and 1500 and analyzed for milk fat, crude protein (CP), milk urea nitrogen (MUN), somatic cell count (SCC), lactose content, and total solids by mid-infrared spectroscopy (MilkoScan 605; A/S N Foss Electric, Hillerød, Denmark) at Central Milk Testing Laboratory in Edmonton, Alberta. Daily milk energy output was estimated from measured milk yield and concentrations of milk fat, CP, and lactose according to the NRC (2001) equation: Net energy of lactation

($NE_L$; Mcal/kg milk) = 0.0929 * fat % + 0.0547 * protein % + 0.0395 * lactose %. The $NE_L$ intake was calculated from measured DMI and $NE_L$ content of the diets, whereby the latter was determined as the sum of $NE_L$ content of individual feeds in a feeding level at 3 times maintenance (NRC, 2001).

### *8.2.3 Statistical Analyses*

Data were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA Version 9.1.3) and considering the random effect of the study (St-Pierre, 2001), according to the following model:

$$Y_{ijkl} = \mu + \gamma_i + \alpha_j + \beta_k + \kappa_l + (\alpha\beta)_{jk} + (\alpha\kappa)_{jl} + (\beta\kappa)_{kl} + (\alpha\beta\kappa)_{jkl} + \varepsilon_{ijkl}$$

where $Y_{ijkl}$ is the observations for the dependent variables, $\mu$ represent the population mean, $\gamma_i$ is the independent parameter for animal i, $\alpha_j$ is a population parameter corresponding to treatment j, $\beta_k$ is the fixed effect of measurement week (relative to each LPS challenge) k, $\kappa_l$ is the fixed effect of parity l, $(\alpha\beta)_{jk}$ is the effect of treatment by measurement week interaction, $(\alpha\kappa)_{jl}$ is the effect of treatment by parity interaction, $(\beta\kappa)_{kl}$ is the effect of measurement week by parity interaction, $(\alpha\beta\kappa)_{jkl}$ is the effect of three way interaction of treatment by measurement week by parity, and $\varepsilon$ijkl is the residual error. Data are shown as least-squares means (LSM) and standard error of the mean (SEM). Multiple comparisons of LSM were conducted by probability difference (PDIFF) option of SAS. Measurements on the same animal were considered as repeated measures. The covariance structure of the repeated measurements for each variable was modeled separately according to the lowest values of fit statistics based on the

BIC (Bayesian information criteria). The significance limit was declared at $P < 0.05$, whereas a biologically relevant tendency was declared at $0.05 \leq P \leq 0.10$.

## 8.3 Results

### *8.3.1 Feed Intake and Milk yield*

Data of feed intake are shown in Figure 8-1a. Results obtained showed a tendency for the treated cows to have lower feed intake compared to the CTR animals ($P < 0.10$). There was an effect of the treatment week for feed intake, which was more pronounced at 1 and 3 week after calving ($P < 0.01$). No week by treatment interaction was obtained for this variable ($P > 0.05$). Parity affected feed intake with treated heifers taking more feed than their control counterparts ($P = 0.02$; Table 8-3).

Although milk yield was numerically lowered in the treated cows, overall no difference between the two treatment groups was observed during the 4 week after parturition ($P > 0.05$; Figure 8-1b). Nonetheless, measurement week had an effect on milk yield ($P < 0.01$); milk yield was slightly lowered in the CTR cows until 3 week after parturition and then it was numerically increased at 4 week compared to the treated cows. No overall treatment by week interaction was demonstrated for milk yield in this study ($P > 0.05$). Parity affected milk production with heifers treated orally with LPS and LTA producing more milk compared to their respective control groups ($P = 0.04$; Table 8-3).

Results indicated lower $NE_L$ intake in the treated multiparous cows compared to their respective control group ($P < 0.01$; Table 8-3). Also, the factor week showed an influence for this variable ($P < 0.01$); however, there was no

treatment by week interaction for this variable ($P > 0.05$). On the other hand, this variable showed greater values in the treated heifers ($P = 0.02$; Table 8-3), with a week influence as well ($P < 0.01$). No treatment by week interaction was observed regarding $NE_L$ intake in primiparous cows ($P > 0.05$).

The net energy output, also known as milk energy was higher in the treated primiparous cows ($P = 0.02$; Table 8-3), which indicated better conversion of the $NE_L$ intake into milk energy by treated cows compared to the control ones of this group. Furthermore, there was a treatment by week interaction for this variable ($P = 0.04$); although no influence of the factor of measurement week alone ($P > 0.05$).

Data showed a tendency for milk efficiency to be greater in cows treated orally with LPS and LTA vs cows treated orally with saline ($P < 0.10$; Figure 8-1c). There was also a week effect on milk efficiency, which stayed greater in the treated cows with a more pronounced effect on week 1, and this group continued having greater values particularly on week 3 after calving ($P < 0.05$). No treatment by week interaction was observed between the two treated groups for this variable ($P > 0.05$).

### *8.3.2 Milk Composition*

Results of milk fat content revealed no effect of treatment on this variable ($P > 0.05$; Figure 8-2a). However, data demonstrated an effect of week on milk fat ($P < 0.01$). In fact, the fat content of milk was greater in the group of cows administered orally with LPS and LTA throughout the 4 week after calving compared to the CTR cows. However, this effect was more pronounced in the

treated cows especially in the week immediately after parturition and on week 2. Furthermore, data indicated no treatment by week interaction for milk fat content ($P > 0.05$).

Results demonstrated that milk-fat yield was influenced by parity and it tended to be greater in the treated primiparous cows than in the CTR group ($P = 0.07$; Table 8-3). In addition, week and treatment by week interaction showed no effect regarding this variable for primiparous cows ($P > 0.05$).

Data obtained by ANOVA showed differences between fat to protein ratio between the two treatment groups, with the LPS/LTA group having greater values ($P < 0.05$; Figure 8-2b). There was an effect of measurement week on fat to protein ratio ($P < 0.05$) with values continuously greater following parturition, especially, during week 1, 2, and 4. No treatment by week interaction was observed between the two treated groups for this variable ($P > 0.05$).

Statistical processing of the data showed no difference in the amount of fat-corrected milk (FCM) between the cows administered orally with LPS and LTA and those of the CTR group ($P > 0.05$; Figure 8-3a). Also, no week effect, or treatment by week interaction was obtained regarding FCM ($P > 0.05$). Interestingly, treatment showed a tendency for the FCM in primiparous cows with higher values in the treatment group ($P = 0.07$; Table 8-3), whereas week alone and treatment by week interaction showed no influence for this variable ($P > 0.05$).

Although, the overall data showed no effect of treatment, measurement week, or treatment by week interaction on energy-corrected milk (ECM; $P > 0.05$;

Figure 8-3b); however, this variable was greater in the LPS/LTA treated primiparous cows ($P = 0.03$; Table 8-3). Moreover, there was a tendency for the treatment by week interaction regarding ECM in primiparous cows ($P = 0.09$), although no effect of week alone on ECM was obtained ($P > 0.05$).

Cows challenged orally with LPS/LTA showed greater milk-fat efficiency (MFE) than the CTR cows ($P < 0.05$; Figure 8-3c). In addition, there was an effect of measurement week for MFE ($P < 0.01$) and this variable was continuously greater in cows challenged orally with LPS and LTA through 1 to 4 week after parturition. However, no treatment by week interaction was acquired for this variable ($P > 0.05$).

No difference between the two treatment groups was obtained regarding milk protein content in the overall data ($P > 0.05$; Figure 8-4a). However, week had an effect for this variable ($P < 0.01$); particularly on week 2 after parturition. Analysis of data indicated no overall treatment by week interaction for milk protein content ($P > 0.05$). Interestingly, parity showed an effect on milk protein yield with lower values in the LPS/LTA treated multiparous cows, and a tendency to be greater in the primiparous treated animals ($P = 0.04$ and $P = 0.07$, respectively; Table 8-3). Furthermore, no week effect or treatment by week interaction regarding milk protein yield was obtained for primiparous or multiparous cows ($P > 0.05$).

The overall data for MUN showed no influence of treatment on this variable ($P > 0.05$; Figure 8-4b). Also, no week effect or treatment by week interaction was observed regarding MUN in the present study ($P > 0.05$). On the

other hand, multiparous treated cows had greater values for MUN ($P < 0.01$) than their respective CTR group animals. Week or treatment by week interaction did not influence this variable in multiparous cows ($P > 0.05$).

Treatment with LPS and LTA had no effect on milk lactose content ($P > 0.05$; Figure 8-5a). Week relative to parturition showed an influence on lactose content of the milk ($P < 0.01$), with greater lactose on week 3 in the treated cows. No treatment by week interaction was evidenced for milk lactose ($P > 0.05$). However, milk lactose yield was effected by parity with primiparous cows administered orally with LPS and LTA having greater yield of lactose ($P = 0.01$). Measurement week showed an effect ($P < 0.01$); whereas treatment by week interaction tended to influence this variable ($P = 0.09$).

Data showed no effect of treatment or treatment by week interaction on total milk solids ($P > 0.05$; Figure 8-5b); however, there was an influence of week on total milk solids, especially after parturition ($P < 0.01$). Results also demonstrated no effect of treatment on concentration of milk SCC ($P > 0.05$; Figure 8-6); although the week showed an influence on this variable with lower values ($P < 0.01$) in the treated cows, immediately after parturition. Moreover, no treatment by week interaction was observed for milk SCC ($P > 0.05$).

**8.4 Discussion**

Results of this study showed that repeated oral challenge with increasing doses of LPS and a flat dose of LTA, in dairy cows around parturition, modulated feed intake and milk composition in the treated cows. The overall analysis revealed that treated cows showed a tendency for lower feed intake. Exposure of

cows to repeated and increasing doses of LPS is associated with activation of immune cells and the release of proinflammatory cytokines. The latter have been shown to affect feed intake in animals. In support of this hypothesis are data published by Swiergiel and Dunn (1999), which indicated that tumor necrosis factor-alpha (TNF-α), interleukin (IL)-2, and IL-6 suppressed feed intake in experimental animals.

Interestingly, the lower feed intake was associated with better MEE in the treated cows, suggesting a better utilization of feed in those cows. These data are in agreement with those reported by Zebeli and Ametaj (2009) who observed a decrease in MEE in dairy cows following high-grain feeding and increased rumen LPS. Recent research explains the potential mechanism of how bacterial toxins translocate through the rumen mucosa into the blood circulation and further to the mammary gland decreasing nutrient flow and synthesis of milk components (Emmanuel et al., 2008; Dong et al., 2011). Bacterial-derived toxins directly depress functions and proliferation of the MEC (Daly et al., 2009), which subsequently lower the synthetic capacity of the mammary gland. Treated cows showed better immune status to these bacterial toxins as evidenced from enhanced humoral immune responses (see chapter 7) and potentially prevented their translocation into the systemic circulation. This might explain why those cows had better MEE.

Although the overall analysis of data showed no influence of the treatment on milk production of the cows, parity affected this variable with primiparous treated cows producing more milk than the control counterparts. There was no

difference in milk production between multiparous cows in this study. The reason for this discrepancy is not understood at present. However, the difference in the incidence rates of metritis between primiparous and multiparous cows might be one of the reasons. The infection of the uterus postpartum is known to lower milk yield (Rajala and Grohn, 1998), and multiparous cows have been reported to have greater incidence of the disease that lasts for a longer time compared with primiparous cows (Ostergaard and Grohn, 1999; Wittrock et al., 2011).

Another important finding of this research was greater fat to protein ratio and a better MFE in the LPS/LTA treated cows versus the controls. Parity also showed an effect on milk-fat yield, FCM, and ECM, being greater in the treated primiparous cows. These data are in agreement with recent research work conducted by our team, which pinpointed a strong negative correlation between free rumen LPS with milk-fat production and efficiency as well as FCM in the primiparous cows. The underlying mechanism, suggested by Zebeli and Ametaj (2009), is that LPS-induced plasma CRP might block the interaction of apoC-II, on very low density lipoproteins (VLDL), with LPL a key enzyme involved in the uptake of fatty acids from circulating triacylglycerols (TAG), and lower synthesis of lipids in the mammary gland. It is assumed that the treated cows, having better humoral responses against LPS and LTA, lower the amount of those toxins entering into the host systemic circulation and their deleterious effects resulting in greater fat synthesis in the mammary gland. Another potential mechanism explaining the effects observed might be that bacterial toxins present into the systemic circulation indirectly affect milk-fat synthesis by triggering secretion of

proinflammatory cytokines from local immune cells (Sweet and Hume, 1996), such as TNF-α, which inhibits LPL activity (Sweet and Hume, 1996; Merkel et al., 2002; Khovidhunkit et al., 2004). Lipopolysaccharide also has been demonstrated to suppress key enzymes for the de-novo synthesis of milk fat such as fatty acid synthetase and acetyl-CoA carboxylase (Pekala et al., 1983; López-Soriano and Williamson, 1994). The lower fat to protein ratio in the control animals might also be related to fluctuations of the activity of the protein related enzymes and increased concentrations of proteins in the milk during inflammatory processes (Oliver and Calvinho, 1995).

Results of this study showed that treatment with LPS and LTA lowered milk protein yield in multiparous cows, in contrast to primiparous cows. The greater protein yield in the multiparous control cows compared with their treated counterparts is in line with a few studies that reported elevated milk protein concentration in LPS-infused mammary glands, particularly lactoferrin, synthesized primarily by MEC (Harmon and Newbould, 1980; Shuster et al., 1991a, b). Lactoferrin is an anti-inflammatory protein that sequesters iron, essential for bacterial growth, and kills directly bacteria (Velliyagounder et al., 2003). The stage of lactation and cow parity are considered to be an important physiological factor that influences severity of inflammation and ultimately the composition of milk. Most inflammatory markers following LPS challenge vary between second to sixth lactation (Kremer et al., 1993a, b; van Werven et al., 1997). Furthermore, it is evident that immune cells function better in primiparous cows than multiparous cows during the periparturient period (Gilbert et al., 1993;

van Werven et al., 1997; Mehrzad et al., 2002). Vangroenweghe et al. (2004) observed that primiparous cows deal better with experimental infection with *E. coli* compared to multiparous cows, as shown by a more moderate inflammatory response, faster influx of immune cells into the infected quarters, more efficient bacterial clearance, and faster recovery of milk production in both infected and non-infected glands. Thus, one probable explanation for higher milk protein and greater fat yield in the treated primiparous cows might be the different responses of the cows related to age.

Conversely to protein yield, MUN was greater in the treated multiparous cows compared to the control animals, with no differences in primiparous cows. It should be pointed out that although MUN was greater in the treated cows the amount of MUN was within the normal ranges for dairy cows in both groups (i.e., 10-14 mg/dL). It is known that the amount of MUN is proportional to the blood urea nitrogen (BUN). Elsasser et al. (1996) reported increased BUN in calves challenged i.v. with 0.2 μg/kg of LPS from *E. coli* 055:B5. Endotoxin lowers the capacity for nitrogen retention, decreases protein synthesis, and mobilizes amino acids from muscle tissue (Zamir et al., 1992). Regarding age differences (i.e., greater MUN in multiparous) our data are in line with Wattiaux et al. (2005) who observed a drastic difference between primiparous and multiparous cows regarding MUN, which is mainly influenced by FCM in multiparous cows, but this association was not evident for primiparous cows. Furthermore, Li et al. (1993) determined the influence of age on metabolic responses to endotoxin infusion and they observed that the older piglets died earlier with more severe

metabolic acidosis compared to younger animals who lived longer after endotoxin infusion.

Data indicated that milk lactose yield was greater in primiparous cows challenged orally with LPS and LTA versus the controls. It is assumed that animals treated orally with LPS and LTA were in a better humoral immune status and did not allow endotoxin insults in the mammary gland. This line of thinking is supported by research involving intrammary LPS challenge in cows. For example, Lengemann and Pitzrick (1986) demonstrated that intra-mammary infusion of 1 ug of LPS in dairy cows lowered lactose content of the milk. For example, the amount of lactose leaving the alveoli after LPS infusion was only 22 mg/mL versus 50 mg/mL before LPS infusion. Recent findings also support earlier reports demonstrating that lactose content of the milk decreases following intra-mammary infusion of LPS in cows at doses ranging between 12.5 to 100 μg (Werner-Misof et al., 2007).

Treatment had no effect on milk SCC. However, the week of observation showed an influence on milk SCC being lower in the treated cows following parturition. These findings are in agreement with an earlier study by Lengemann and Pitzrick (1986) who observed that SCC in milk increase after intrammamary infusion of very small amounts of LPS (i.e., 0.10 μg) in lactating cows. Because oral treatment with LPS and LTA enhanced salivary IgA secretion, it is hypothesized that this might be the case with the mammary gland. Our observations are in accordance with another report where oral immunization with the DNP-conjugated pneumococcus antigen produced high levels of colostral

antibodies. It indicates that precursors of antibody-forming cells initially are sensitized in the oral mucosa and then home to other mucosal sites including the mammary gland (Montgomery et al., 1974; Ogra and Dayton, 1979).

**8.5 Conclusions**

In conclusion, repeated oral administration of LPS from *E. coli* 0111:B4 and LTA from *B. subtilis* modulated milk composition and increased the overall productivity of dairy cows postpartum. Overall analysis of the data indicated that cows administered orally with LPS and LTA had a tendency for lower feed intake with greater MEE and MFE, enhanced fat to protein ratio, and decreased milk SCC. Results revealed a strong influence of the parity class on different milk components, with primiparous cows in the treatment group producing more milk, along with greater $NE_L$ intake, milk energy, milk lactose, protein yield, and ECM. The treated primiparous cows also showed tendencies for greater milk-fat yield and FCM. On the other hand, the treated multiparous cows had lower $NE_L$ intake, protein yield, and greater MUN. Thus, oral administration of LPS from *E. coli* 0111:B4 and LTA from *B. subtilis* around parturition increased milk production and improved the overall productive performance of dairy cows. Of note, cows at different age groups responded differently to the treatment.

**8.6 Acknowledgement**

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Research and Technology Centre, University of Alberta for their help and care to the cows.

**Table 8-1.** Ingredients and chemical composition of the diet for dry cows

| Ingredients, % of DM | Prepartum diet |
|---|---|
| Alfalfa hay | 10.0 |
| Barley silage | 63.0 |
| Alfalfa silage | 00.0 |
| CUD[1] grain | 27.0 |
| Nutrient composition of cud grain (dietary supplement) % amount in 100 kg mix | |
| Barley grain, rolled | 55.0 |
| Canola meal | 7.5 |
| Dairy dry cow micro-premix | 6.2 |
| Limestone | 8.7 |
| Animate | 15.7 |
| Molasses | 0.9 |
| Canola oil | 4.1 |
| Yeast | 1.7 |

[1]CUD = giving more mineral especially Ca to avoid milk fever in early lactating cows

**Table 8-2.** Ingredients and chemical composition of the diet for early lactating cows

| Ingredients, % of DM | Early lactation diet |
|---|---|
| Alfalfa hay | 10.0 |
| Barley silage | 40.8 |
| Dairy supplement | 49.2 |
| Nutrient composition of dairy supplement, % amount in 100 kg mix | |
| ADE Vit Pak-30 Natural E | 0.056 |
| Ruminant TM Pak | 0.1025 |
| Selenium 1000 mg/kg (UNscr Fine Cr) | 0.065 |
| Custom TM Complex pmx | 0.060 |
| Di-calcium phosphate 21% | 1.25 |
| Co-op alantic Corn Dist | 10.0 |
| Corn ground | 25.0 |
| Corn rolled | 30.105 |
| Vit D- 10,000 KIU/kg | 0.015 |
| Diamond V XPC | 0.14 |
| Magalac/Enertia | 1.00 |
| Fermenten | 2.00 |
| Limestone | 1.50 |
| Mag Ox -56% | 0.37 |
| Canola meal | 15.5 |
| Hi bypass soy (Amino plus) | 2.75 |
| Soy bean meal-47.5% | 6.50 |
| Sodium bicarbonate | 1.00 |
| Salt | 0.113 |
| Pork-Tallow | 2.45 |
| Biotin 2%-Rovimix H-2 | 0.007 |
| ADM Vit E 405 Vegetable source | 0.015 |

**Table 8-3.** Data of dry matter intake, milk production and composition, as well as production efficiency of lactating primiparous cows administered orally with saline (CTR) or saline containing increasing doses of LPS and a flat dose of LTA

| Variable | Treatment groups[1] | | | Overall Effect,[2] *P*-value | | |
|---|---|---|---|---|---|---|
| | Multiparous cows | | | | | |
| | TRT | CTR | SEM | Trt | wk | Trt x wk |
| Protein yield, kg/d | 1.15 | 1.26 | 0.04 | 0.04 | 0.75 | 0.41 |
| Milk urea N, mg/dL | 13.2 | 10.4 | 0.52 | <0.01 | 0.36 | 0.87 |
| $NE_L$ intake, Mcal/d | 34.2 | 40.7 | 1.07 | <0.01 | <0.01 | 0.59 |
| **Primiparous** | | | | | | |
| Feed intake, kg/d | 30.3 | 27.1 | 0.91 | 0.02 | <0.01 | 0.54 |
| Milk yield,[3] kg/d | 30.8 | 24.7 | 1.91 | 0.04 | <0.01 | 0.15 |
| FCM[4] | 20.8 | 17.4 | 1.21 | 0.07 | 0.76 | 0.20 |
| ECM[5] | 22.9 | 19.3 | 1.09 | 0.03 | 0.83 | 0.09 |
| Fat, kg/d | 1.23 | 1.03 | 0.06 | 0.07 | 0.77 | 0.20 |
| Protein yield, kg/d | 0.92 | 0.79 | 0.04 | 0.02 | 0.87 | 0.71 |
| Lactose yield, kg/d | 1.41 | 1.19 | 0.05 | 0.01 | <0.01 | 0.09 |
| $NE_L$ intake, Mcal/d | 31.5 | 28.2 | 0.94 | 0.02 | <0.01 | 0.54 |
| Milk energy,[6] Mcal/d | 22.0 | 18.8 | 0.89 | 0.02 | 0.65 | 0.04 |

[1]CTR = cows administered orally with saline solution; TRT = cows administered orally with increasing doses of lipopolysaccharide and flat dose of lipoteichoic acid.
[2]Effect of treatment (Trt), measurement week (wk), and treatment by week interaction (Trt x wk).
[3]Data across wk 1, 2, 3, and 4 (n = 15).
[4]FCM = Milk amount (kg) * (0.4255 + 16.425 * % fat/100).
[5]ECM = Milk amount (kg) * (0.327 + 7.2 * % protein/100 + 12.96 * % fat/100).
[6]Milk energy (Mcal/kg milk) = 0.0929 * % fat + 0.0547 * % CP + 0.0359 * % lactose.

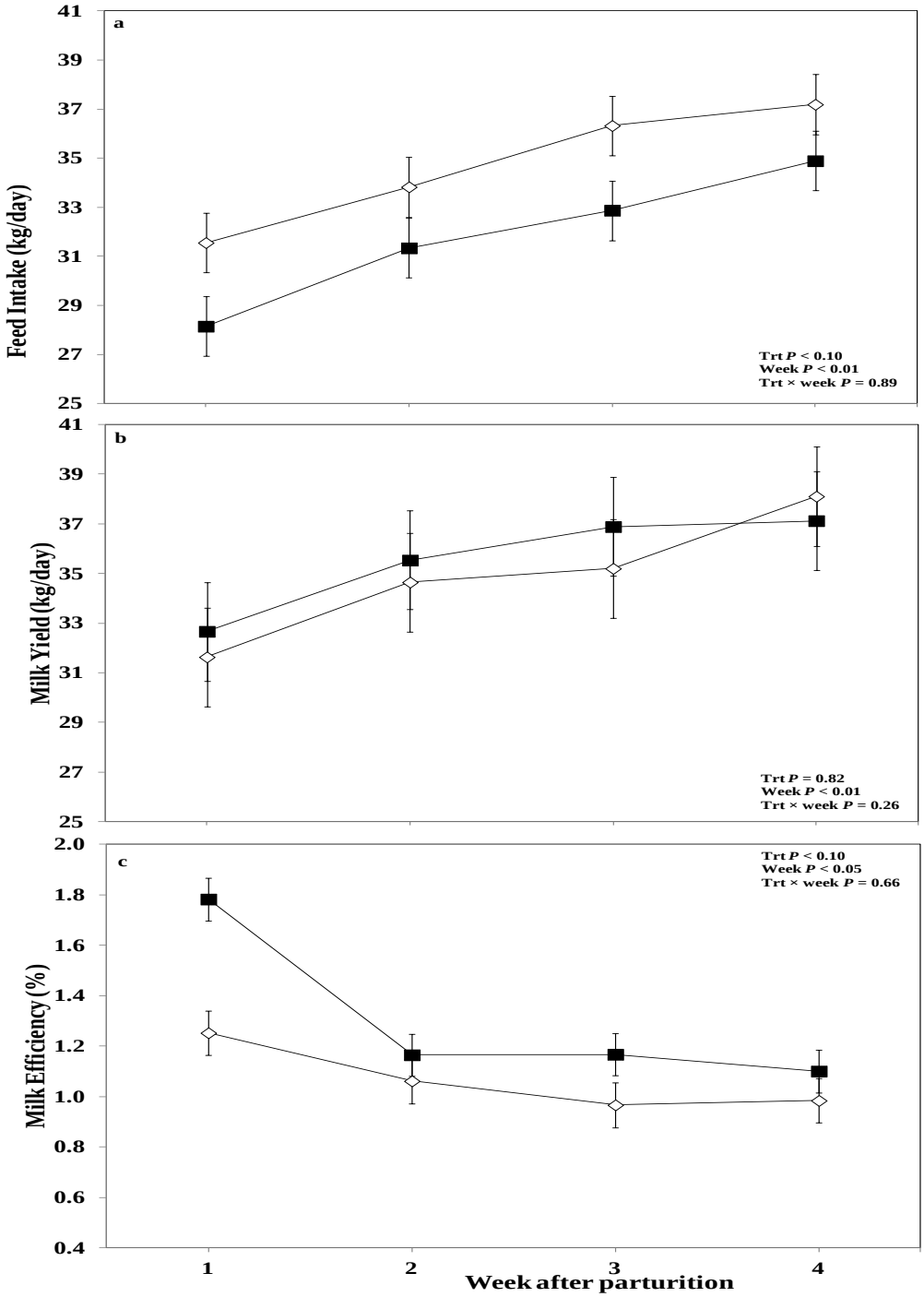


**Figure 8-1.** Weekly variations in (a) feed intake, (b) milk yield, and (c) milk efficiency of multiparous and primiparous Holstein cows challenged with oral treatment of LPS-LTA (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 16; Trt = effect of treatment; Week = effect of sampling week, Trt x week = effect of treatment by sampling week).

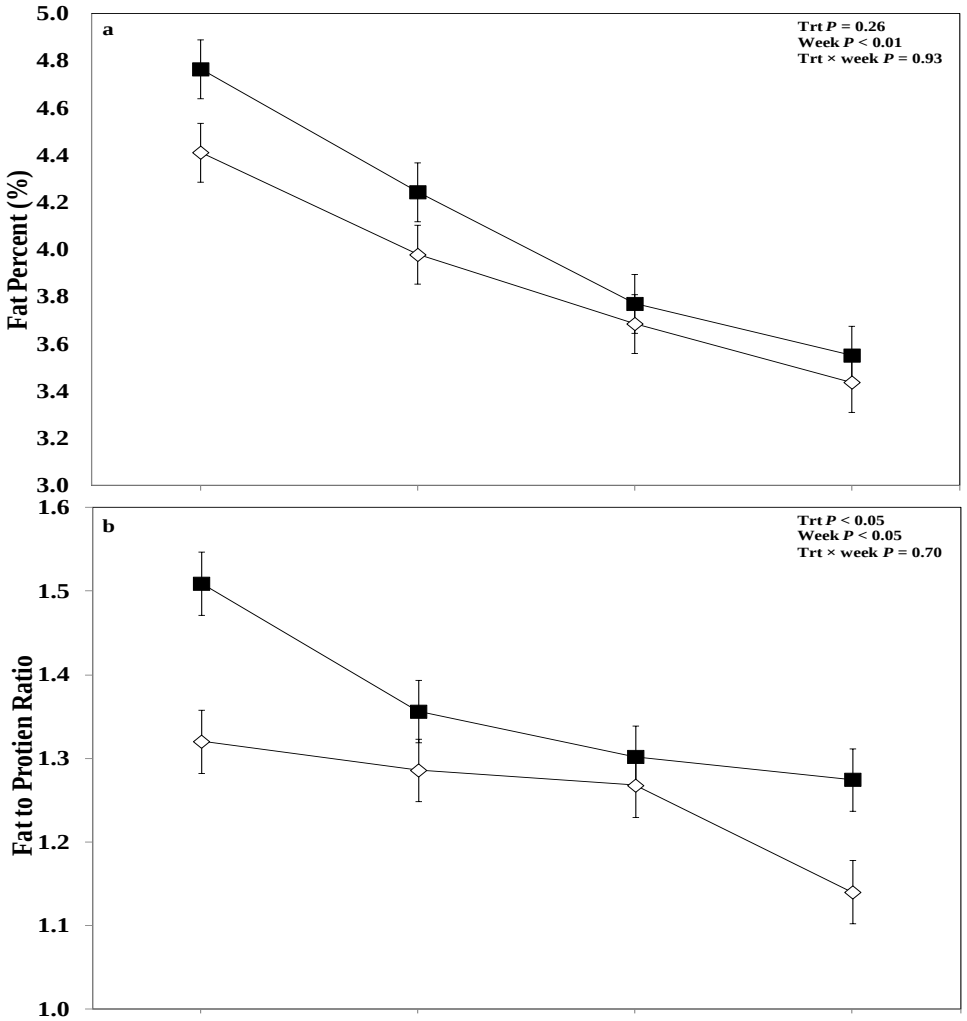


**Figure 8-2.** Weekly variations in (a) milk fat content, and (b) fat to protein ratio of multiparous and primiparous Holstein cows challenged with oral treatment of LPS-LTA (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 16; Trt = effect of treatment; Week = effect of sampling week, Trt x week = effect of treatment by sampling week).

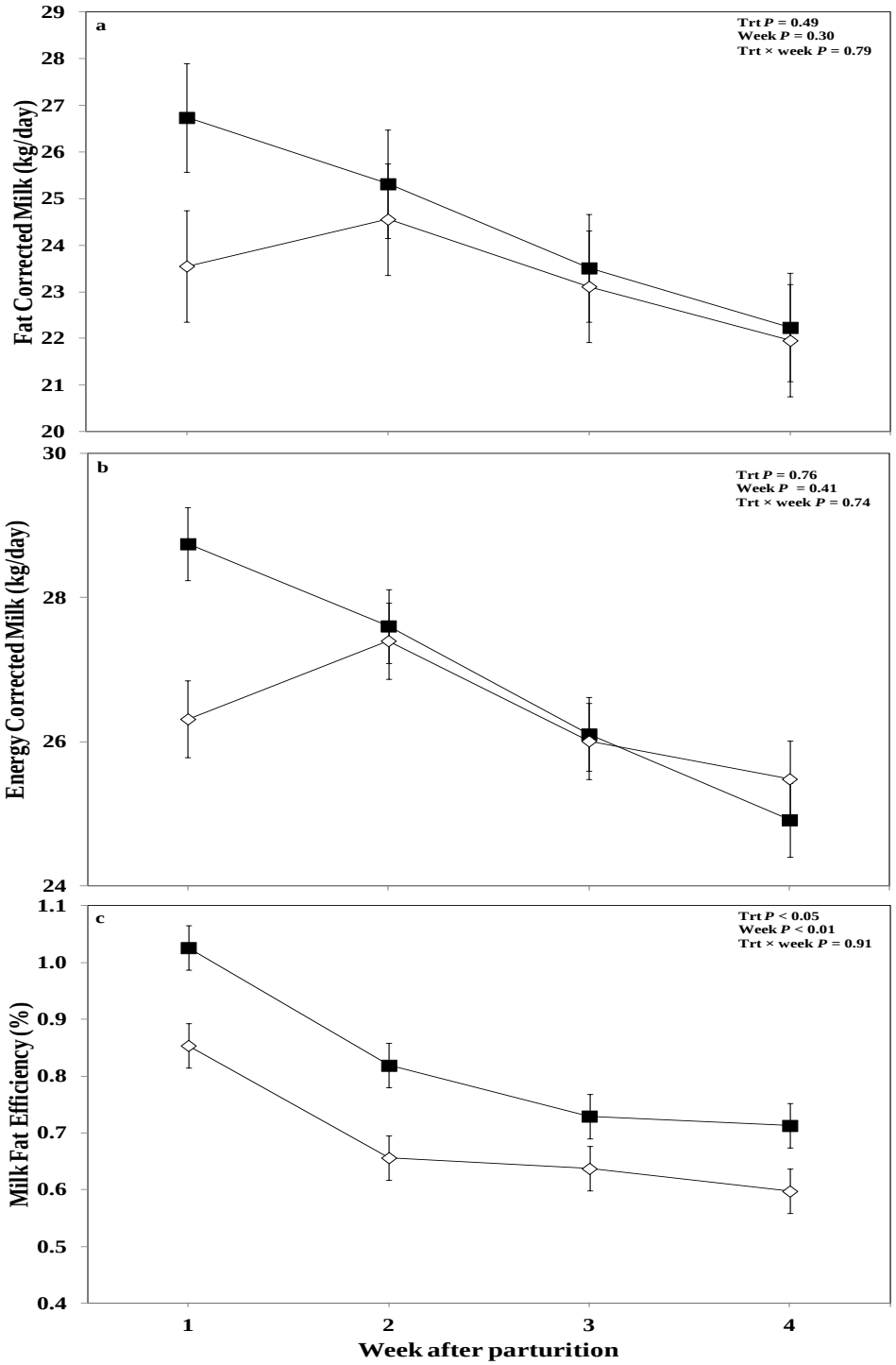


**Figure 8-3.** Weekly variations in (a) fat corrected milk, (b) energy corrected milk, and (c) milk fat efficiency of multiparous and primiparous Holstein cows challenged with oral treatment of LPS-LTA (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 16; Trt = effect of treatment; Week = effect of sampling week, Trt x week = effect of treatment by sampling week).

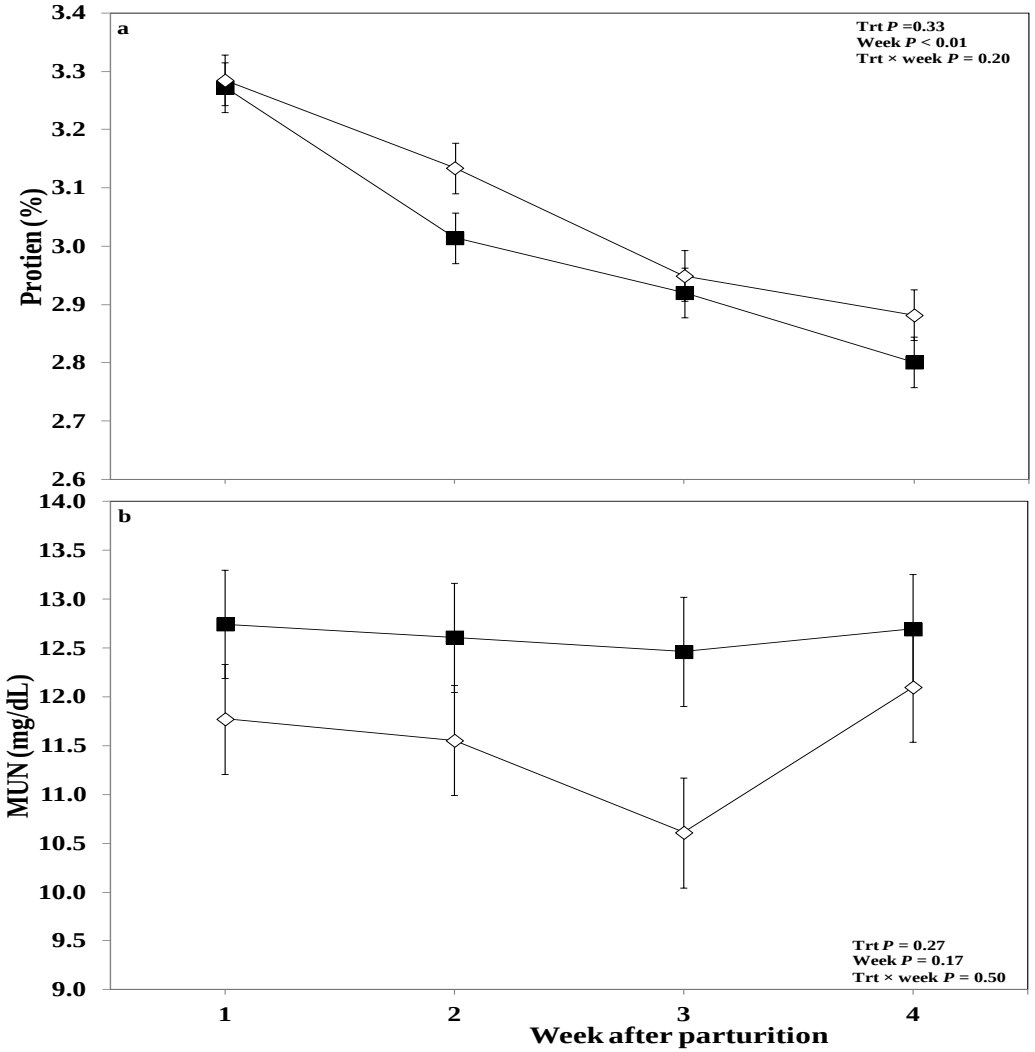


**Figure 8-4.** Weekly variations in (a) milk protein content, and (b) milk urea nitrogen of multiparous and primiparous Holstein cows challenged with oral treatment of LPS-LTA (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 16; Trt = effect of treatment; Week = effect of sampling week, Trt x week = effect of treatment by sampling week).

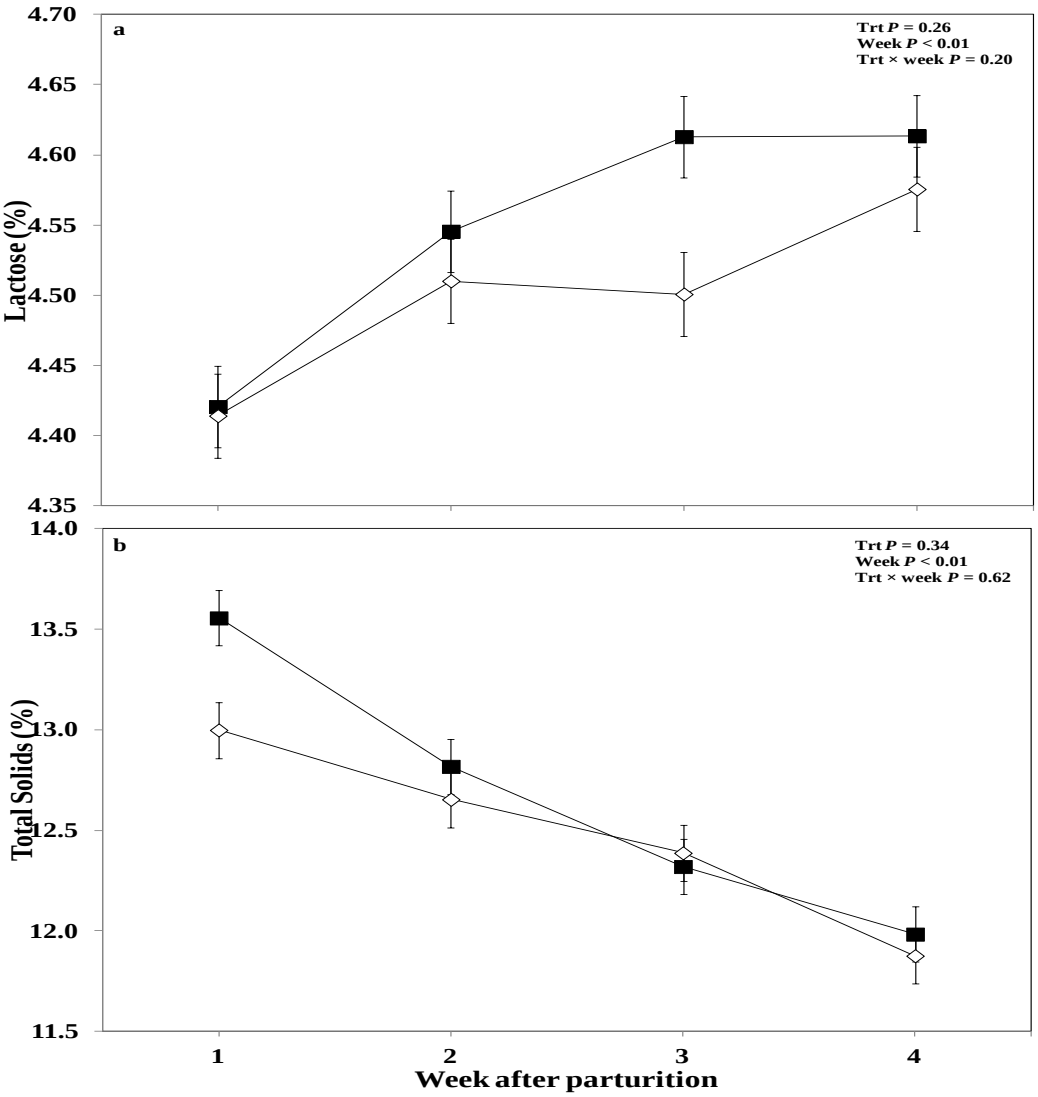


**Figure 8-5.** Weekly variations in (a) milk lactose content, and (b) milk total solids content of multiparous and primiparous Holstein cows challenged with oral treatment of LPS-LTA (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 16; Trt = effect of treatment; Week = effect of sampling week, Trt x week = effect of treatment by sampling week).

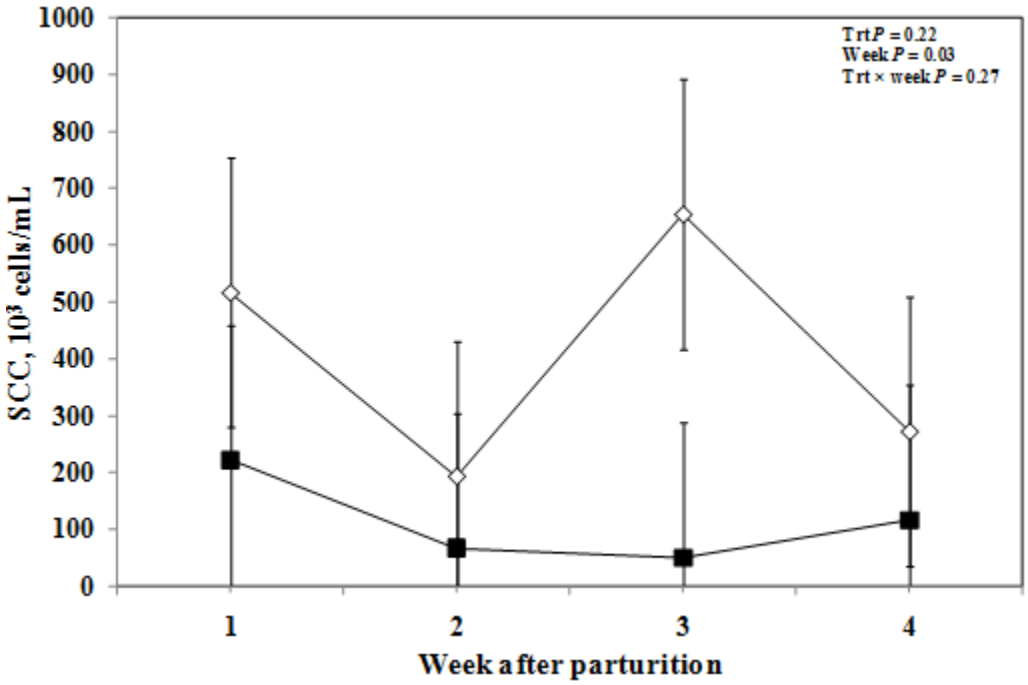


**Figure 8-6.** Weekly variations in milk somatic cell count of multiparous and primiparous Holstein cows challenged with oral treatment of LPS-LTA (TRT; ■) or saline (Control; ◇) (LSM ± SEM; n = 16; Trt = effect of treatment; Week = effect of sampling week, Trt x week = effect of treatment by sampling week).

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# Chapter 9

# Overall discussion

Two studies were investigated during this doctoral research work including oronasal application of LPS and oral administration of LPS and LTA in transition dairy cows during the prepartal period to lower the incidence of multiple periparturient diseases and improve their immune status and productivity. Results of these studies are interesting and will be discussed in more detail below:

**9.1 Study 1:**

In this study we hypothesized that oronasal application of three increasing doses of LPS, for a total of five times during wk -4 to -2 prepartum, would lower the incidence of periparturient diseases in transition dairy cows and improve their metabolic and immune status as well as overall productivity. The reason for applying LPS for five times is related to the fact that repeated exposure to LPS induces tolerance (Bellmann et al., 1997). Furthermore, increasing consecutive doses of LPS were applied because administration of large doses might induce fever and sickness if lower sensitizing doses are not administered first. We used 0.01, 0.05, and 0.1 µg/kg of body weight of LPS per each cow in the treatment group. The aforementioned doses of LPS were split between nasal (1/3) and oral cavities (2/3). The LPS also was administered only during the prepartal period. The reason for that was to avoid discarding of milk and to prepare the immune response of the cows against LPS ahead of endotoxin assault that occurs mainly during the postpartal period when cows are fed high amounts of grains and when

uterus and mammary gland are infected by Gram-negative bacteria (GNB). It is known that feeding high amounts of grain is associated with the release of very large amounts of endotoxin (Ametaj et al., 2012a). Also, infection of the uterus and mammary gland postpartum by GNB is associated with great release of endotoxin in those organs. Free LPS passes mucosal barriers and enters the host systemic or lymphatic circulations and induces a whole variety of responses and pathologies in dairy cows (Ametaj et al., 2010).

Using LPS as a mucosal vaccine is a new concept that only recently has been gaining importance. For example, Marquez-Velasco et al. (2007) demonstrated that oral LPS was able to protect mice against experimental sepsis induced by cecal ligation and puncture. In a recent review Inagawa et al (2011) summarized results showing that oral LPS has preventative and curative properties against various diseases such as allergic and lifestyle-related diseases. The concept of a vaccine against LPS in transition dairy cows was developed, for the first time, by our group several years back based on the mounting evidence that GN bacterial endotoxins play an important etiopathogenetic role in multiple periparturient diseases of transition dairy cows including laminitis, retained placenta, fatty liver, milk fever, and displaced abomasum (reviewed in Ametaj et al., 2010). Based on this evidence our group conducted a small-scale study during 2006, which involved treating transition dairy cows with oral LPS during -2 wk before and +1 wk after parturition. Results of this study were encouraging because they showed that oral administration of LPS was able to lower the incidence of periparturient diseases and affect multiple plasma metabolites and humoral

immune responses in transition dairy cows (Ametaj et al., 2012b; Zebeli et al., 2013).

The most interesting finding of the present research was that the oronasal LPS induced sIgA secretion in both the saliva and vaginal mucus. Until recently it was not clear whether polysaccharide antigens were able to induce humoral immune responses and memory B cells in mammals. However, recent evidence has shown that humans and other mammals are able to confer long-lasting humoral immunity and generate memory B-cells to polysaccharide antigens (Obhukanych and Nussenzweig, 2006). Results of this study confirm that LPS, a GN lipopolysaccharide, is able to induce humoral immune responses in dairy cows. We cannot say with certainty whether the oronasal LPS was able to induce memory B cells in dairy cows. It requires other studies in the near future to address this issue. Results of this study also are very encouraging because they suggest that application of bacterial polysaccharides on the oronasal mucosae are able to induce humoral immunity in other mucosal layers such as GI and reproductive tracts.

Another interesting finding of this study was that oronasal LPS lowered the number of SCC. Somatic cells are neutrophils that migrate from systemic circulation into the mammary gland in cases of inflammation or infection (Mehrzad et al., 2010). The fact that the number of SCC was lowered in the treated cows suggests that oronasal application of LPS improved mammary gland immunity against GNB. It is known that *E. coli* infections are one of the main causes of mastitis in dairy cows (Kremer et al., 1993). It is not clear how oronasal

LPS has improved mammary gland immunity. However, induction of memory B cells and homing might be one potential explanation. More research is warranted to prove these findings and validate them in a larger cohort of animals.

Results of the present study also showed that oronasal LPS affected various components of milk especially milk-fat content and fat-corrected milk (FCM). This is an interesting finding that confirms a previous suggestion from our group that endotoxins might play a key role in milk-fat depression syndrome. In a recent publication we reported strong relationships between rumen LPS and the drop in milk-fat content in dairy cows. We suggested that during grain feeding-induced endotoxemia blood lipids are diverged from mammary gland to the liver and this is associated with a drop in milk-fat content (Zebeli and Ametaj, 2009). The fact that oronasal application of LPS was associated with greater milk fat content supports the hypothesis that prevention of endotoxin translocation into the host circulation by vaccination might be a good approach to address the syndrome of milk-fat depression in dairy cows.

Oronasal vaccination with LPS, in the present study, had no effect on most of the blood variables measured and did not lower the incidence of periparturient diseases in transition dairy cows. These results are not in agreement with our previous study that demonstrated that oral LPS was able to lower the incidence rates of several periparturient diseases in the treated cows. For example, in our previous study we showed that oral LPS lowered both NEFA and BHBA in the plasma, especially after calving, suggesting that it might be a potential vaccine against fatty liver and ketosis (Zebeli et al., 2013). The reason for this discrepancy

might be that in the present study we split the same doses of LPS, used in our previous study, between the nasal and oral cavities versus the whole dose applied to the oral cavity in our previous study.

Given the multiple benefits and encouraging results of oronasal LPS in transition dairy cows it would be of interest to continue this type of research in the future to address dose responses to LPS, development of memory B cells, and validation of results in a larger cohort of animals.

**9.2 Study 2:**

Since treatment of transition dairy cows with oronasal LPS only was unable to lower significantly the incidence of periparturient diseases, especially those related to Gram-positive bacteria (GPB), we hypothesized that treating transition cows with both LPS and LTA might be a more efficient vaccination program. Indeed results showed that this type of vaccination also influenced various plasma metabolites, humoral immune responses, and the overall productive performance of dairy cows.

A total of 5 consecutive administrations of increasing doses of LPS and a flat dose of LTA were applied. Again repeated exposure to both these bacterial toxins was done to induce tolerance and humoral immunity before parturition when cows are exposed to bacterial toxins because of high-grain diets and infection of the uterus, mammary gland, or hoofs by both GN and GP bacteria. The amount of LTA administered was based on a preliminary dose study which showed that 120 µg of LTA per cow had no clinical effects on rectal temperature,

respiration and rumination rates as well as eating and drinking behaviors of the treated cows.

The most important finding of this investigation was that oral application of LPS and LTA lowered the incidence rates of uterine infections, lameness, and retained placenta. The treatment also lowered numerically the number of SCC, or the incidence of subclinical mastitis. Retained placenta and uterine infections are associated with infertility and are the main reason for culling of dairy cows in Canada (Ametaj et al., 2012a). Lameness also ranks among the top three reasons for culling of cows in Canada. These diseases are associated with significant financial losses to the dairy industry. The fact that oral LPS and LTA lowered the incidence of three major diseases in dairy cows indicates that both GN and GP bacterial toxins play a significant role in the etiology and pathogenesis of multiple periparturient diseases of transition dairy cows. However, the mechanism(s) of how bacterial toxins affect health of dairy cows warrants further investigation. Treated cows also had less no of days with more than one disease, which again suggests a multi-causal factor for periparturient diseases of dairy cows.

Another important finding of this study was enhanced saliva and vaginal mucus sIgA in the treated cows. Secretory IgA serves as the first line of defense in protecting the mucosal epithelium from bacterial toxins and pathogenic microorganisms through a process known as immune exclusion, which is mediated primarily by blocking their access to epithelial receptors, entrapping them in mucus, and facilitating their removal by peristaltic and mucociliary activities (Mantis et al., 2011). It should be pointed out that the concentrations of

sIgA in the saliva and vaginal mucus of cows treated with LPS and LTA were greater than those treated with oronasal LPS. Also, another difference between the two treatments was that sIgA remained greater in the cows treated with both LPS and LTA during the 4 wk postpartum versus those treated with LPS only where salivary sIgA decreased to the control levels at 4 wk after calving. This suggests that combination of LPS with LTA is a stronger stimulus for induction of mucosal humoral immune responses in transition dairy cows. Induction of mucosal immunity throughout the mucosal tissues just by application of antigens in the oral cavity is an interesting concept that deserves further exploration.

Both plasma IgA and IgG were lowered in the treated cows which is in line with our recently published data indicating that dairy cows challenged with increasing oral doses of LPS lowered concentrations of plasma anti-LPS IgA and IgG (Ametaj et al., 2012b). The fact that specific immunoglobulins against LPS were lowered in the treated cows indicates that the oral treatment of cows has lowered the translocation of endotoxin into the host systemic circulation. This is another support for the efficiency of the oral vaccination. In support of this finding are also data indicating that plasma acute phase proteins (APP) such as lipopolysaccharide binding protein (LBP) and haptoglobin and to a certain degree serum-amyloid-A (SAA) as well as plasma proinflammatory cytokines including tumor necrosis factor(TNF)-α and interleukin(IL)-1 were lower in the cows treated with LPS and LTA.

Cows treated with LPS and LTA also showed better milk energy efficiency (MEE) which is indicative from lower feed intake relative to greater

milk production in this group. Also treated cows had better milk fat efficiency (MFE), with greater fat to protein ratio. These data again support our previous hypothesis that bacterial toxins modulate lipid metabolism which influence milk fat content as well as overall milk production in dairy cows.

**9.3 Conclusions**

Altogether results of this study showed that cows treated oronasally with LPS only showed improvements in humoral immunity and numerical lowering of certain periparturient diseases in transition dairy cows. Also, they had better milk-fat content and FCM compared to control animals. Compared with cows treated oronasally with LPS, cows treated orally with both LPS and LTA had far better clinical results with significantly lower incidence rates of several perparturient diseases and improved mucosal and blood humoral immunity as well as lower APP and proinflammatory cytokines. Oral LPS and LTA also improved milk production and MEE as well as MFE. Results of these studies suggest utilization of oral LPS and LTA as a vaccine to lower the incidence rates of multiple periparturient diseases in transition dairy cows and as a tool to improve their immune status and productivity. However, because the number of experimental animals in the LPS and LTA study was only 15 cows per group these data suggest further studies to validate the results in a greater cohort of animals.

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