Meeting the Challenges of Improving Health in Periparturient Dairy Cows

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Take Home Messages

- Peripartum diseases of dairy cows are increasing in numbers and the culling rates reached a record high during 2010.
- Present reductionist methodology used by veterinary science for studying and treating peripartum diseases is not able to contribute any further in the understanding of the etiopathogenesis and treatment of peripartum diseases and has to be replaced by a new recently introduced methodology known as the systems biology approach.
- The present approach to treatment of peripartum diseases known as reactive medicine that uses palliative approaches in the treatment of diseases needs to be substituted with a better way of treatment known as the proactive, and prophylactic approach.
- As part of the prophylactic intervention our team developed a new barley grain technology that is able to reduce incidence of development of sub-acute rumen acidosis, improve health status, and milk composition in dairy cows.
- Application of probiotics directly in the reproductive tract of transition dairy cows can improve uterine health, reproductive performance, and their productivity.
- An oral vaccine against cell wall components of Gram-negative and Gram-positive bacteria, lipopolysaccharide and lipoteichoic acid, lowers the incidences of laminitis, uterine infection, and retained placenta in periparturient dairy cows.

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▪ Periparturient Health Issues and Culling Rates in Dairy Herds in Canada

Maintaining production efficiency and controlling the cost of dairy operations are the two most important challenges of today's dairy industry. Both of them are affected by the length of productive life of dairy cows, which directly affects herd replacement rate and profitability of dairy farms. According to CanWest Dairy Herd Improvement (CanWest DHI, 2010) statistics almost 38 percent of dairy cows in Canadian dairy herds registered with DHI, were culled during 2010. This is a 13% increase in the culling rate of cows, compared with 2001 and the highest during the last decade (i.e., 2001-2010). Total replacement rate, including culling for reasons besides sickness, was increased at the record high of ~ 50% (CanWest DHI, 2010). As a result, the productive lifespan of a cow in dairy herds across Canada averages no longer than 2 lactations. As per DHI statistics the productive longevity of a cow is affected mainly by her health status and fertility performance. In fact one or multiple peripartum diseases affect every other cow in a dairy herd during the transition period (Drackley and Dann, 2005). The major periparturient diseases that affect dairy cows are metritis, mastitis, laminitis, ruminal acidosis, displaced abomasum, fatty liver, ketosis, milk fever, retained placenta, downer cow syndrome, liver abscesses, udder edema, infertility, milk fat depression syndrome, and bloat (Ametaj et al., 2010a).

The DHI report for the last 10 years (i.e., 2001-2010) also indicates that the number one reason for culling of cows from a dairy herd has been and continues to be their failure to remain pregnant. During 2010 the number of cows culled for reproductive problems was 54,230 cows. The second peripartum disease that inflicts heavy financial losses to the dairy industry is mastitis or inflammation of the mammary gland. Mastitis was responsible for culling of 33,141 cows, whereas feet and leg problems, low milk production, overall sickness, milk fever, and displaced abomasum were responsible for culling of 22,855, 18,571, 16,079, 1,653, and 1,587 cows, respectively. Since DHI data are based on producers' records, many of the sick cows have not been properly diagnosed and most of the cows culled from the dairy herds throughout Canada have been classified as culled for unknown reasons. The number of cows culled under this grouping, during 2010, was 77,701 cows, a higher number than all other diagnosed diseases. As a consequence, the financial losses to dairy industry in Canada, based on the value of the cows culled from all diseases during 2010, is $667,275,000 (CanWest DHI, 2010). DHI data also indicated that the number of cows culled for various diseases during the last decade has been increasing and has reached a record high value during 2010. The reason for this increase in the number of sick cows is not known. Although much is known about the etiopathology of some of the periparturient diseases of dairy cows like milk fever, fatty liver, ketosis, and

milk fat depression there is a lack of understanding for diseases like laminitis, displaced abomasum, retained placenta, and downer cow syndrome.

■ Grain Feeding, Gut Microbiota, Inflammation, and Periparturient Diseases

Several investigators have reported that periparturient diseases are interrelated with each other; however, the reason why they are related has not been clarified yet (Correa et al., 1993). Different teams across the world are putting much effort to better understand the causes of periparturient diseases. One common finding is that feeding high-grain diets initiates a chain of alterations in the gastrointestinal tract that causes major changes in the composition of microbiota and the release of large amounts of endotoxin and several other not very well identified bacterial toxins in the rumen fluid like lipoteichoic acid and peptidoglycans (Andersen et al., 1994;

Emmanuel et al., 2008). Other sources of bacterial toxic products in transition dairy cows are the uterus and mammary gland, during infections from Gram-negative and Gram-positive bacteria (Wenz et al., 2001). Lipopolysacharide (LPS), also known as endotoxin, is a cell wall component of all Gram-negative bacteria and is released during their rapid growth or death (Beveridge, 1999). During feeding of high grain diets, because of major changes in the rumen environment (i.e., lower pH), some of the Gram-negatives cannot survive the low pH and end-up dying and releasing large amounts of free endotoxin into the rumen fluid; some others adjust quickly to the new environment and grow rapidly, releasing additional LPS during their growth phase (Beveridge, 1999). Gram-negative bacteria have a characteristic that is not observed in Gram-positive bacteria; they shed constantly outer membrane vesicles from the surface of the cell during their growth. As the vesicles are being shed from the surface, they contain some of the underlying periplasm, so that they are actually small particles of Gram-negative cell wall. The vesicles contain LPS, phospholipids, and periplasmic constituents; all located as they usually are in a bacterium, but on a smaller scale (Beveridge, 1999). These 50- to 250-nm-diameter spherical, bilayered, membranous structures are released from the surfaces of almost all Gram-negative bacteria (Beveridge and Kadurugamuwa, 1996). All these sources make up the free endotoxin that is found in the rumen fluid. What happens to this free endotoxin in the rumen? Part of it is degraded and neutralized, or eliminated with the feces. However, part of the endotoxin has been reported to translocate through intestinal wall barriers of various species, including humans, rodents, and ruminants, into the systemic circulation causing metabolic endotoxemia and initiating a non-specific immune response known as acute phase response (Emmanuel et al., 2007; Ametaj et al., 2010, 2011).

Mounting evidence by various teams, including ours, is demonstrating that the acute phase response is present in various diseases that affect cows during the transition period (Emmanuel et al., 2008; Ametaj et al., 2009, 2011; Trevisi et al., 2011). In a recent review article we summarized reports from different labs suggesting the potential involvement of endotoxin in multiple diseases of dairy cows like laminitis, fatty liver, milk fever, displaced abomasum, and retained placenta (Ametaj et al., 2010). Additionally, in several recent publications we reported strong correlations among rumen endotoxin and perturbations of various blood metabolites related to carbohydrate and lipid metabolism and minerals as well as a strong relationship between rumen endotoxin and milk fat depression syndrome in dairy cows (Zebeli and Ametaj, 2009; Zebeli et al., 2010a; 2011a). In conclusion, an increasing line of evidence pinpoints the potential role that endotoxin and other bacterial toxic compounds might play in the etiology and pathogenesis of multiple diseases of the transition period in dairy cows.

■ Shifting Paradigms from Reductionism to Systems Biology Approach

Recently, an increasing number of researchers are involved in linking feeding and dietary components with the immune status and pathogenesis of disease, a science known as nutritional immunology. Furthermore, there is an increasing interest in using high-throughput technologies like genomics, transcriptomics, proteomics, and metabolomics to study the etiopathology of diseases during the transition period. These new approaches hold promise to deepen our understanding of the causal agent(s) involved in the initiation of the peripartum disease(s) and open new perspectives in development of novel strategies for improving health and productivity of dairy cows.

These novel technologies also are influencing the philosophy of how to treat diseases in veterinary medicine. In fact, the approach that veterinary science has used, during the last century, to tackle the etiopathology of periparturient diseases of dairy cows, has been to separate diseases from each other and study them as isolated entities, although most of them occur during the transition period and are interrelated with each other. This methodology split the whole into smaller and smaller units and identified perturbation of one single metabolite with one disease state. In fact researchers were focused more in studying the metabolism in a way that a disturbance of one metabolite was related to a certain metabolic disorder instead of looking at how metabolites interacted with their network of other metabolic pathways or the whole system. Based on these assumptions, hypotheses were drawn whereby a specific disease state was the result of perturbation of one single metabolite and had nothing to do with the whole or other networks. Focusing on the single-metabolite approach and leaving aside all the other perturbed metabolites in the network(s) has affected our in-depth understanding of the

disease process and our success in the treatment of disease state. Because the cow's health status is "reduced" into studying one disease at a time and a few variables from various body fluids, this approach has been known as the "reductionism" approach. In fact this methodology has influenced the way veterinary science uses to diagnose, treat, and tackle periparturient diseases of dairy cows. While this type of approach contributed to incredible successes in harnessing health issues in dairy cows, there are limitations that need to be addressed in a completely different way as pointed out below.

An example of a reductionist approach to dairy cow health issues was the Compton metabolic profile test developed during the late 60s and early 70s in UK (Payne, 1972). This approach consisted in the analysis of blood samples from cows in the dry off period as well as cows with middle- and high-milk yield. Blood analyses were used to evaluate the metabolic status and define whether the cows in a dairy herd had perturbations of blood metabolites. This methodology, and the research work conducted during the last century, established the concept of one key perturbed metabolite to one disease (Payne, 1972). Thus, milk fever was related to calcium deficiency, ketosis to lower glucose and increased ketone bodies, fatty liver to increased concentrations of non-esterified fatty acids and their storage in the liver as triacylglycerols, and grass tetany to magnesium deficiency (Payne, 1972). This approach looked at isolation of one or a few metabolites most responsible for the disease state and treatment of that particular disease by 'fixing' the perturbed variable. However, this approach was not able to assign an altered metabolite to diseases like displaced abomasum, downer cow syndrome, retained placenta, rumen acidosis, udder edema, or infertility. According to reductionist approach these diseases should be considered 'orphan' diseases, waiting to be assigned a metabolite that is disturbed during the disease state. As a consequence, there is no recommended treatment for the aforementioned orphan diseases, except for palliative interventions. The drawback of the reductionist methodology is that even for the diseases that have been assigned a perturbed metabolite there are not yet well-established treatments. For example, cows with milk fever are treated subcutaneously or intravenously with solutions containing calcium borogluconate; however, a large number of cows relapse and do not respond to this type of treatment indicating that the approach lacks in-depth understanding of the cause of the disease. Lack of success in prevention of peripartum diseases during the last 100 years of research and clinical treatments suggest that the reductionist approach is not the best approach to understand the etiopathology and solve the problem of periparturient diseases in dairy cows and emphasizes the need for a completely different and new methodology.

Recent developments in the field of physical chemistry have made possible the designing and manufacturing of new high throughput analytical instruments like nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), liquid chromatography (LC)-MS, direct infusion

(DI)-MS, electrospray ionization (EI) and the emergence of a new science known as metabolomics. What is metabolomics? Metabolomics is the science that looks at identification and measurement of all metabolites present in various body fluids and uses that to better understand a process, a system or a condition (Roessner and Bowne, 2009). Furthermore, combination of metabolomics with genomics, transcriptomics, and proteomics holds promise to give further insight into the functioning or malfunctioning of the whole organism and is known as the systems biology approach (Kitano, 2002). The new emerging science of systems biology studies the interactions among different components in a biological system as a whole. Its approaches offer advantages that far surpass the traditional reductionist approach. Systems biology is built within a cross-disciplinary environment, and requires close collaboration among biologists, chemists, computer scientists, engineers, mathematicians, and physicists to tackle a certain biological problem.

■ Switching Gears from a Palliative to a Prophylactic Medicine

Another drawback of the reductionist approach to treatment of disease is that it is palliative in nature, focuses more on relieving the symptoms and the sufferings of an animal, without having a curative effect on the underlying disease or the cause of the disease. In fact, this is known as reactive medicine that deals with an already established disease state and aims at easing the symptoms of the disease; however, this type of intervention does not deal with elimination of the cause(s) of the disease and does not deal with eradication of the cause(s) of the disease. Unfortunately, some diseases are deadly because symptoms typically develop after the disease has progressed beyond the point that it can be treated by medication. As a consequence the reductionist approach has not been able to resolve the problem with the peripartum diseases of dairy cows.

This suggests that it is time to look for a new way of dealing with periparturient diseases. The newly developed science of systems biology proposes a completely different way of dealing with diseases. Systems biology as applied to disease state and wellbeing of dairy cows promises to start an important shift from reactive to proactive veterinary medicine, a medicine that is prognostic and prophylactic in nature. Systems biology looks at different systems in the body as connected and interacting with each other and a whole variety of networks interrelated to each other. Also, this approach looks at various diseases that affect cows immediately after calving as interrelated and part of a continuous diseased state.

Another drawback of the reductionist approach is that cows are considered as part of a herd and not on an individual basis. Each cow has its own characteristics, specific conditions, and an individual disease state. If we like

to extend the productive life of a cow then we have to monitor each cow individually during the most critical period(s) of her life (i.e., transition period), 3 wk before up to 8 wk after calving, and then during the period of insemination(s). Therefore, we have to take care of individual cows to prevent development of disease state and prolong their productive life span.

As part of the new prophylactic medical intervention is also the identification of biomarkers of disease state far ahead the appearance of symptoms of the disease. The intent is to use metabolomics to screen for hundreds or thousands of metabolites in various body fluids including urine, milk or plasma by high-throughput technologies and identify early indicators of the disease state. Our team has been working on a similar project and data will become available in the near future. This new technology is able to provide producers simple dipsticks measurements to monitor and test quickly the health status of each cow during the most critical stages of transition period. These novel technologies will make possible prolongation of productive life of a cow by preventing and eradicating the cause(s) of the disease.

In the next three sections we will discuss three new and very encouraging technologies developed by our team as prophylactic tools against peripartum diseases of dairy cows. More specifically we will discuss about development of a new barley grain processing technology, preparation of a mixture of Lactic acid bacteria to prevent uterine infection and improve fertility and productivity of dairy cows, and an oral vaccine to lower the incidence of the diseases of the transition period in dairy cows.

▪ A New Barley Grain Processing Technology to Improve Health and Productivity of Dairy Cows

Barley grain is a cereal rich in starch, digestible fibers, and most importantly crude protein. Due to these characteristics barley grain is considered an appropriate cereal used in the feeding of livestock and an attractive alternative to corn for dairy and beef cattle diets. Canada is the second largest barley producer in the world with a production of close to 13 million tons annually, where Alberta produces almost 50% of Canada's barley grain. Indeed, most of the barley grain grown in Alberta goes toward the feeding of cattle. In the feeding of cows during early lactation, which often experience a negative energy balance, one approach to maximize energy intake is to increase the energy density of the diets by feeding large amounts of readily fermentable grains. In Western Canada, these energy requirements are often met by including high proportions of barley grain as a cost-effective metabolizable energy source in the diet of lactating dairy cows.

However, feeding cattle high barley grain-based diets to support high milk production has been linked to an increased incidence of digestive and

metabolic disturbances. The high degradation rate of barley starch results in rapid accumulation of short-chain fatty acids (SCFA), which subsequently lower the rumen pH to acidotic and pathologic values (Yang et al., 1997; Emmanuel et al., 2008). The duration of time at which rumen pH remains below 5.8, on a daily basis, is critical in the development of subacute ruminal acidosis (SARA; Owens et al., 1998; Zebeli et al., 2008). Among others, the detrimental effects of SARA includes decreased dry matter (DM) intake, fiber digestion, milk production, and milk fat content (Nocek, 1997). SARA also has been implicated in the high incidence rates of metabolic diseases such as laminitis, displaced abomasum, bloat, ruminitis, liver abscesses, and fatty liver (Nocek, 1997; Ametaj et al., 2005). Moreover, impairment of rumen conditions and a rapid decrease of ruminal pH are often associated with lowered milk fat content (Zebeli and Ametaj, 2009), which inflicts important economic losses to dairy producers. All these drawbacks lower the feeding value of barley grain in ruminants.

Although the exact mechanism(s) behind the side effects of feeding diets rich in fermentable carbohydrates (like barley grain) on metabolic health and productivity of dairy cattle is not understood yet, recent reports suggest that the rapid fermentation rate of starch contributes to those nutritional and health problems (Nocek, 1997; Zebeli et al., 2008). In fact, between 80 to 90% of the barley starch is digested in the rumen, whereas the corresponding values for corn range between 55 to 70%, making corn the first feed choice for ruminant nutrition (Nocek and Tamminga, 1991).

During the last decade, a number of studies have examined various ways to amend rumen degradability of barley grain. Those researchers have employed physical and thermal processing techniques such as pelleting, steam rolling, steam flaking, and toasting which utilize moisture, heat, or pressure to gelatinize starch granules (Svihus et al., 2005). Other researchers have attempted treatment of grains with different chemical compounds, including formaldehyde or NaOH, to improve feed efficiency of cattle by altering the nature and amount of the starch available to rumen microbiota and by shifting part of starch digestion into the hindgut (Campling, 1991; Noziere et al., 2005). Given the high cost and the corrosive nature of some of the aforementioned chemicals (i.e., posing health risks to the laborers), those processing technologies have not been embraced by the dairy industry.

Interestingly food industry researchers were involved in studies trying to increase the amount of starch from grain kernels and improve the quality of grain for diabetic people and they reported that steeping of various grains into a low concentration of lactic acid (LA) solution was increasing starch yield and lowering its degradation rate (Östman et al., 2002). We also hypothesized that by steeping barley grain in LA would modulate its degradation characteristics in the rumen of dairy cows. As a result we designed several pioneer studies to evaluate the effects of feeding barley grain steeped in mild

solutions of LA on rumen fermentation profiling, health, and productivity of dairy cows. We steeped rolled barley grain in 0.5% LA for 48 h and measured its degradation rate in the rumen by *in situ* techniques. Furthermore, the effect of feeding mid-lactation dairy cows the LA-treated barley grain, as part of a total mixed ration (TMR), on the capacity to modulate rumen fermentation patterns and maintain high productivity and metabolic health status was tested in an in vivo trial (Iqbal et al., 2009; 2010). Data from this research indicated that steeping barley grain in 0.5% LA resulted in a slight reduction in the degradation rate of barley DM, alterations in the rumen SCFA profiles, and lowering of the concentration of total SCFA in the rumen fluid (Iqbal et al., 2009). As a consequence there was a decrease in the time that rumen pH remained below the SARA breakpoint of 5.8, during the most intensive phases of fermentation (6-12 h post-feeding), decreasing the risk of SARA. In addition cows fed the LA-treated diet showed a better metabolic and health status as evidenced by greater concentrations of glucose in the plasma and lower concentrations of acute phase proteins like serum amyloid A (SAA) and haptoglobin in the plasma (Iqbal et al., 2010).

Recent research showed that heat treatment of grain modulates starch degradation characteristics by modulating its gelatinization and retrogradation reactions (Svihus et al., 2005) and helps in generation of resistant starch (Östman et al., 2002). Therefore, we hypothesized that treating rolled barley grain with 1% LA and heat at 55°C for 48 h (LAH) may slow down its degradation rate in the rumen, improve rumen fermentation profiles, and lower the risk of SARA in dairy cows.

The *in situ* data of this experiment are shown in Figure 3. These results showed that mid- to late lactation cows fed the LAH diet had lower DM disappearance rate of barley grain substrate from 0h (19.4 vs. 36.5%; $P < 0.001$) up to 24h (73.4 vs. 80.7%; $P = 0.02$) post-incubation (Figure 3). However, DM disappearance rates were equal in both treatments starting at 48h (86.5 and 87.2% for treatment and control diet, respectively; $P = 0.59$) until 72h ($P = 0.47$) post-incubation. Moreover, cows fed the LAH-treated diet showed greater *in situ* lag time than controls (9.2 vs. 4.2h; $P < 0.001$). In addition, results showed greater ($P = 0.01$) rumen pH for cows fed the treated diet. Cows fed the diet treated with LAH maintained greater rumen pH (5.92 vs. 5.67) at the nadir (8h post-feeding), as well as at 10h (6.02 vs. 5.75) and 12h (6.10 vs. 5.78) post-feeding, thus clearly indicating that the delayed *in situ* DM disappearance due to the feeding of barley grain treated with LAH prevented the decline of rumen pH at SARA levels post-feeding.

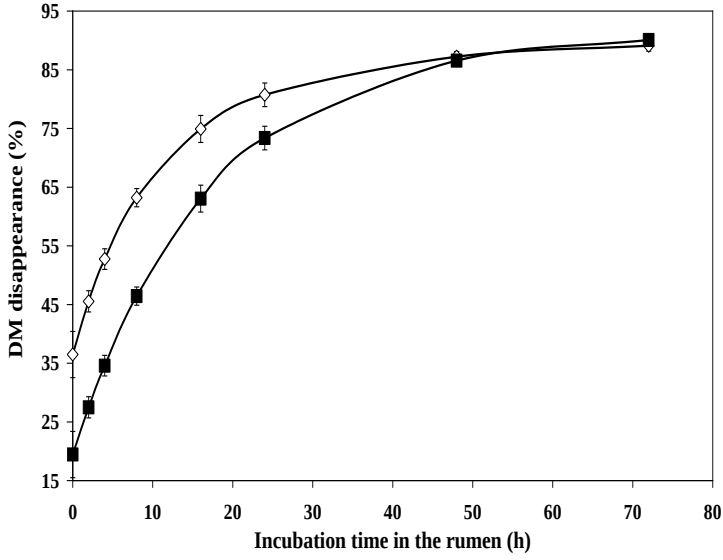

Figure 3. *In situ* dry matter disappearance of untreated rolled barley grain substrate (i.e., not steeped in water) measured in rumen fluid of lactating Holstein cows fed diets based on rolled barley grain steeped in tap water (control; ◇) or in 1.0% lactic acid (LA) solution and heat (treatment; ■).

Besides *in situ* degradation and rumen pH, a crossover trial with 8 mid- to late-lactating dairy cows was conducted to determine the effect of feeding barley grain treated with 1% LA and heat on rumen fermentation profile, plasma metabolites, acute phase proteins, and milk production and composition. A principal component analysis, which is an unsupervised pattern recognition method, was performed to examine the intrinsic variation in this large data set, and to lower the dimensionality of the data (Figure 4). As determined by score plots, strong differences among the data coming from control cows and cows fed the LA-treated diet were observed. Score plots of the data of control cows were clustered together and those of treated cows are different and placed further apart, indicating that each of the 2 diets fed could be distinguished on the basis of the measured rumen and plasma variables (Figure 4).

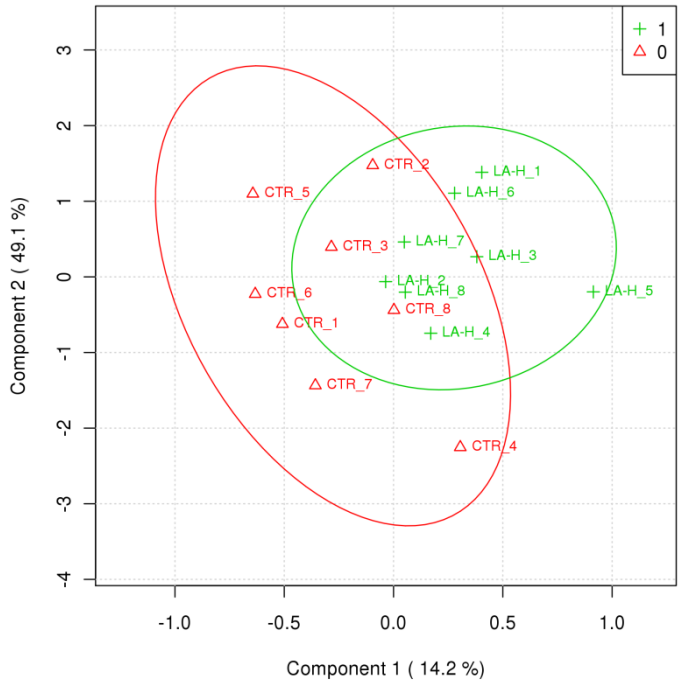

Figure 4. Principal component analysis (PCA) of the rumen and plasma metabolic profile. The PCA score plot distinguishes the metabolic profiles in cows fed diets based on barley grain steeped in tap water (CTR; 0) or 1% lactic acid and heat (LAH; 1). The two first most important components were displayed. The number in the data points represents the cow number (1 to 8 cows).

Interestingly, rumen fermentation data demonstrated that cows fed the treated diet had lower concentrations of total VFA (98 vs. 109 mmol/L; $P = 0.04$), and propionate (19.7 vs. 22.0 mmol/L; $P = 0.04$), but greater concentration of acetate (68.4 vs. 63.3 mmol/L; $P = 0.06$) and butyrate (15.4 vs. 13.3%; $P < 0.01$) in the rumen fluid (Iqbal et al., 2012). These results were reflected in the data of milk composition and feed efficiency of the cows. For example, data showed that cows fed the LA and heat-treated diet had greater milk fat content (3.6 vs. 3.1%; $P < 0.05$). In addition, milk fat yield increased when cows were fed the LAH versus the control diet (0.83 vs. 0.97 kg/d; $P < 0.01$). Consequently, there was a tendency for 4% fat-corrected milk (25.0 vs. 27.4 kg/d; $P = 0.06$) and energy-corrected milk (25.5 vs. 27.6 kg/d; $P = 0.07$) to be greater for cows fed the LAH diet compared with the control group (Iqbal et al., 2012). Moreover, results showed that cows fed the LAH diet had lower preprandial concentrations of rumen endotoxin (472 vs. 793 ng/mL; $P < 0.01$), and cholesterol, and greater lactate in the plasma. Postprandial responses in

the plasma showed that LAH diet tended to lower concentration of SAA (4.67 vs. 8.50 μg/mL; *P* = 0.06).

In conclusion, results of our experiments indicated that the novel processing technologies of barley grain using LA alone or LA plus heat have the potential to modulate rumen fermentation profiles toward lowering the risk of SARA, improve rumen health, and productivity of mid- to late-lactation dairy cows. When comparing the two processing methods (with or without heat) results indicated that treatment of barley grain with LAH further improved rumen fermentation patterns and lowered the risk of SARA versus the LA only diet. Improvements were also observed in milk composition, with greater milk fat, better milk energy efficiency, and immune status of the cows fed the LAH treated diet. In order to bring this exciting technology to dairy producers further research is warranted to explore the benefits of this novel processing technology in transition dairy cows and commercialize the technology as quickly as possible.

■ Intravaginal Probiotics to Improve Uterine Health, Reproductive Performance, and Productivity of Transition Dairy Cows

Transition from pregnancy to lactation is a very delicate stage for the dairy cow because of three main events: parturition, initiation of lactation, and suppression of immune responses. The cows going through parturition besides the high risk for physical damage of the uterine tissue during the birth process and the potential failure to release the placenta within 24 h after calving have also an increased risk of microbial infections both before and after calving. Because of a suppressed immune response around parturition, uterine tissues and mammary glands of some of the cows are infected by pathogenic bacteria even during late pregnancy, which is associated with premature calving and health problems in the offspring. However, the major risk for the health and productivity of the cows is the bacterial contamination of the uterus shortly after calving. Almost 80-100% of postpartal cows have presence of pathogenic bacteria in their uterine environment within the first 2 weeks after calving, while there are studies reporting the presence of bacteria in the genital tract in more than 60% of animals 3 weeks after parturition (Williams et al., 2008). In fact, most of the cows are able to clean bacterial infections efficiently; however, up to 40% of them persist to have bacterial infections at or after 3 weeks postpartum (Gilbert et al., 2005; Sheldon et al., 2008).

It has been reported that cell mediated and humoral immune functions are suppressed in dairy cows from 1 to 2 weeks prepartum until 2-3 weeks postpartum (Kehrli, 1989, Mallard, 1998). Moreover, cows with uterine infections have a more profound impairment of innate immunity, starting

several weeks before the disease appears clinically (Hammon, 2006). The precise causes of impaired immune functions in the transition cows are unclear and deserve further investigation. In fact, the normal uterus is able to clear bacterial infections and it is difficult to experimentally produce chronic uterine infections, even by intrauterine infusion of pathogenic bacteria like *Arcanobacterium pyogenes* (Gilbert and Schwark, 1992). The most important component of uterine defense is non-specific phagocytosis by neutrophils (Hussain, 1989; Bondurant, 1999). Recent reports have provided evidence that the functions of neutrophils are impaired in dairy cows that develop uterine infections. For example, Sheldon (2004) showed that *Escherichia coli*, as well as their products, in addition to *A. pyogenes*, inhibit the phagocytic activity of neutrophils. Studies have shown a relationship between suppressed functions of neutrophils during the periparturient period and retained placenta (Kimura et al., 2002), and metritis (Cai et al., 1994) in dairy cows. In addition, neutrophils from cows with retained placenta have decreased migration ability (Gunnink, 1984) and decreased myeloperoxidase activity (Kimura et al., 2002).

The presence of fluids rich in nutrients in the uterine environment postpartum supports the growth of a variety of bacteria. Most of these bacteria are not part of the normal microbiota of the uterine tissue and are removed by a variety of local immune mechanisms. Maintaining the balance of microbiological organisms in the vagina is a key element in the protection of the reproductive health (William et al., 2007). Subtle shifts in the vaginal environment may allow organisms with pathological potential to proliferate, causing infectious symptoms (Sheldon et al., 2004). A healthy vaginal microflora is characterized by dominance of lactobacilli, which coexist with other bacterial species (Földi, 2006). During bacterial infections of the vagina, this bacterial balance is disrupted by the overgrowth of pathogens. Uterine disease is commonly associated with growth of bacteria such as *E. coli*, *A. pyogenes*, *Fusobacterium necrophorum*, and *Prevotella* species. Indeed, *A. pyogenes*, *F. necrophorum*, and *Prevotella* species have been shown to act synergistically to enhance the likelihood of uterine disease, and increase the risk of clinical endometritis and its severity (Ruder et al., 1981; Olson et al., 1984). Numerically the most prevalent pathogens are *E. coli* (37% of pathogenic bacteria isolated) and *A. pyogenes* (49%) (Williams et al., 2005). Furthermore, the *E. coli* infections appear to precede and pave the way for the *A. pyogenes* infection (Williams et al., 2007).

Parturition also is associated with major changes in concentration of steroid hormones. For example, concentration of progesteron decreases to basal values, and there is an increase in the concentration of the follicle-stimulating hormone (FSH) in the plasma within days of calving that stimulates the emergence of the first postpartum follicular wave. At around 10-12 days after calving the first dominant follicle is selected (Savio et al., 1990; Beam and Butler, 1997). These events occur in all postpartum cows irrespective of

periparturient disease, environment or dietary deficiencies. Beam and Butler (1997) indicated that the first dominant follicle has three possible fates: 1) to ovulate and form the first corpus luteum, 2) to degrade and give way to one or more follicular waves without ovulation, or 3) to establish an ovarian follicular cyst. In fact, early return of ovarian cyclical activity is accepted as a beneficial physiologic event for subsequent fertility (Darwash et al., 1997). However, Olson et al. (1984) suggested that an early postpartum first ovulation in the presence of uterine infection could lead to pyometra with persistence of a corpus luteum in the presence of pus within the uterine lumen.

The expelling of placenta is expected to occur within 6 h from calving; however, if it is still present by 24 h postcalving it is defined as a retained placenta. The occurrence of retained placenta ranges between 2 and 5% of animals in a herd, but can be increased in cows with twins, after dystocia and where infectious agents are present. The expression of clinical uterine infection depends on the balance between factors such as the animal, immunity, the number and pathogenicity of the microbes, and the uterine environment. Typically, 25-40% of animals have clinical metritis in the first 2 weeks after calving, and disease persists in up to 20% of animals as clinical endometritis (Sheldon et al., 2009).

Although the clinical signs of uterine disease such as purulent material discharging from the uterus into the vagina are readily detected, the role of subclinical uterine disease is less well characterized but is an emerging issue. Up to 50% of cows 40-60 days after calving have neutrophils in the uterine lumen or endometrium, concomitant with inflammation of the tissues, and subclinical endometritis reduces conception rates (Kasimanickam et al., 2004; Gilbert et al., 2005). Chronic endometrial scarring, obstruction of the uterine fallopian tubes and adhesions between the ovary and the bursa are other consequences of uterine bacterial infection. However, these are less of a problem in cattle than other mammals including humans, with the incidence of ovaro-bursal adhesions affecting about 2% of cows.

Uterine infection and the associated inflammatory and immune responses compromise animal welfare, causing subfertility and infertility (Sheldon and Dobson, 2004). Uterine infections cause lower first service per conception rate (29.8 vs 37.9%), longer calving to conception interval (151 vs 119 days), lower submission and pregnancy rates, higher infertility rate, higher culling rate (6.7 vs 3.8%), lower feed intake, lower body condition score and milk production (McDougall, 2001; LeBlanc, 2002). CanWest DHI & Valacta (2007) reported that about 24% (or 237,480 cows) of dairy cows in Canada, registered with DHI, were culled in 2007 for a number of reasons. Almost 80% of all those cows were culled for metabolic-related conditions and infertility was the number one culling reason, accounting for 30% of all metabolic-related conditions. In 2010, 50,230 cows were culled in Canada for infertility

reasons, incurring > $135 million in financial losses to the dairy industry (CanWest DHI & Valacta, 2010).

Besides direct losses due to the market value of a cow other financial losses from uterine infections come from the cost of treatment of uterine diseases, lowering of milk production in affected cows, and expenses related to dealing with the infertile cow. For example, Esslemont et al. (2001) reported that the financial losses from the direct cost and the loss of milk production (at 300L/lactation/cow) was at €91 or CDN$138. Sheldon et al. (2008) also indicated losses from direct cost from 100 cows at €1,059 or CDN$1,600. The same authors also reported indirect cost losses from expenses related to longer calving intervals, increased culling rate, extra inseminations, and lower estrus expression rate at €192/cow or CDN$290/cow. Kasimanickam et al. (2004) estimated a loss of $285 per lactation due to uterine diseases. Gilbert et al. (1998) estimated that subclinical endometritis is likely to cost the dairy industry in North America over $1 billion annually in days open alone.

Currently there is no strategy to prevent uterine infections in dairy cows (LeBlanc, 2008). In fact, uterine diseases are treated with different antimicrobials such as antibiotics including tetracycline, penicillin, cephapirin, chloramphenicol, gentamycin, spectinomycin; iodine solutions such as iodine and diluted Lugol's iodine; sulfa drugs such as nitrofurasone, and sulfonamide as well as chemical antimicrobials such as chlorhexidine. Utilization of antimicrobials directed at Gram-negative or Gram-positive bacteria besides killing the pathogenic bacteria cause multiple harmful effects on uterine tissue and local immune responses. In fact, some authors indicate that infusion of irritants (i.e., antimicrobials) into an already inflamed uterus may actually be detrimental to subsequent fertility of the cow (LeBlanc, 2002). Furthermore, one large study demonstrated that when cows with acute uterine infections were treated with antibiotics and/or flunixin meglumine, did not result in beneficial effects on clinical cure, milk yield within 6 d after the first treatment, or reproductive performance (Drillich et al., 2007).

Several studies conducted in human subjects have shown that lactobacilli have been able to prevent vaginal or urinary tract infections (Reid, 2006). Although the precise mechanism of action of probiotic *Lactobacillus* spp. is not fully understood several hypothesis have been put forward during the years such as lowering of vaginal pH through production of lactic acid. High vaginal pH supports growth of pathogenic bacteria and their enzymatic activity. *Lactobacillus* spp. also produces H_2O_2 (i.e., hydrogen peroxide) and bacteriocin that have been demonstrated to have bactericidal activity.

Interestingly, there are no previous research activities related to prevention of uterine infections in humans or animals alike. We hypothesized that by applying probiotic bacteria [i.e., isolated by healthy cows at Dairy Research and Technology Centre, University of Alberta (DRTC)] in the vagina of

transition dairy cows several times before and after parturition would prevent development of uterine infections and improve general health and productivity of dairy cows postpartum. The main objectives of this project were to: 1) identify and isolate *Lactobacillus* spp. bacteria from the reproductive tract of healthy dairy cows; 2) develop probiotic bacteria from the commensal isolates; and 3) evaluate effects of probiotic bacteria for prevention of uterine infections, overall general health, and productivity in dairy cows.

The experiment was conducted at DRTC, University of Alberta. A total of 80 primiparous and multiparous Holstein dairy cows were randomly allocated to 2 groups (40 cows per each group), with 1 control and 1 treatment group in a completely randomized block design. Cows were blocked by the expected day of calving, parity, body condition score at 2 weeks before the expected day of calving and milk production on previous lactation. Cows of the first treatment group (i.e., probiotics) were administered intravaginally once per week on week -2 and -1 before the expected day of calving and on week +1, +2, +3, and +4 after calving with 10 to 12 cfu of probiotic/wk/cow. Cows of the control group received an intravaginal carrier (i.e., 1 mL skimmed milk) once per week for 6 weeks. A dried mixture of 3 probiotic bacteria prepared by our team and stored at -20°C in 3 mL vials was reconstituted in 1 mL sterile 0.9% saline and administered within 2 h intravaginally to the cows. Probiotics were administered with plastic syringes and insemination plastic pipettes.

Bacterial microbiota of Holstein healthy pregnant cows and cows with uterine infections postpartum were characterized with culture dependent and culture independent methods (Wang et al., 2008). Isolates were characterized by random amplification of polymorphic DNA (RAPD) analysis and partial 16S ribosomal RNA gene sequencing. Polymerase chain reaction (PCR) based denaturing gradient gel electrophoresis (DGGE) was carried out to verify that all bacteria present in the samples were cultured. Populations of staphylococci and lactic acid bacteria of the genera *Enterococcus*, *Lactobacillus*, and *Pediococcus* were present in both healthy and infected cows (Wang et al., 2008). However, infected cows had substantially greater vaginal bacteria counts besides an increase in the enteric bacteria population, which consisted mainly of *Escherichia* species. A total of three *E. coli* isolates harbored the gene coding for Shiga-Like-Toxin I or II. Several isolates of the genus *Pediococcus* were found to produce the bacteriocin pediocin PA-1 (Wang et al., 2008).

Clinical data showed that intravaginal administration of the probiotic mixture improved all variables of the uterine health and the involution rate of the reproductive tract and the reproductive performance as indicated by the data below. For example, administration of probiotics lowered the incidence of purulent discharges (16.5 vs 53%; $P < 0.01$) in the treated cows, tended to shorten the interval from calving to conception (93 vs 145 d; $P < 0.10$), and tended to increase the pregnancy rate at first insemination ($P < 0.10$). Cows

receiving probiotics also had lower incidence of foul-smelling ($P < 0.05$) discharges at 3 weeks after calving. Additionally, the treated cows had lower rate of uterine horn fluctuations ($P < 0.05$), smaller cervix size on week +3 ($P < 0.001$) and +5 ($P < 0.06$), and lower uterine horn asymmetry on week +3 ($P < 0.01$) and +5 ($P < 0.01$) postpartum. Survival analysis indicated that cows treated with probiotics had a better overall pregnancy rate ($P < 0.01$; Figure 5) compared to the control group.

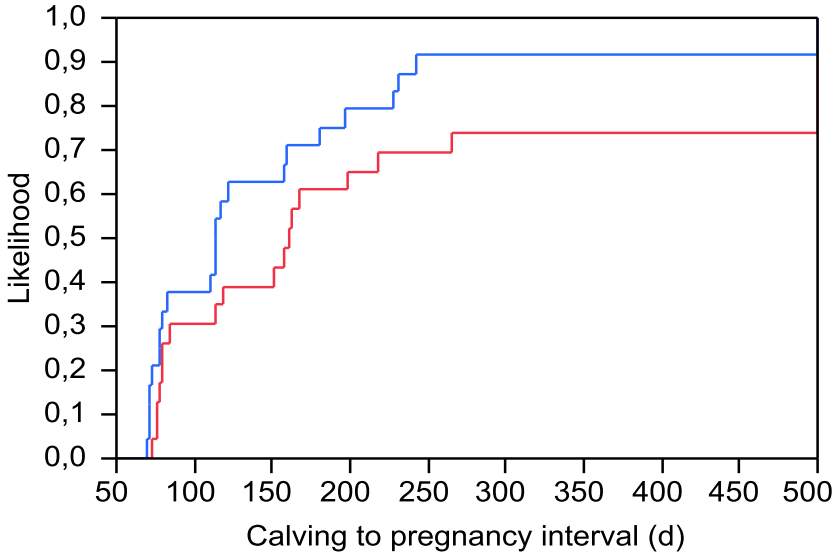

Figure 5. Survival analyses of the likelihood of pregnancy for cows treated intravaginally with 1 mL of a mixture of lactic acid bacteria at 10^{10} cfu/cow/treatment (upper line) or with 1 mL of carrier alone (i.e., skim milk; lower line) during wk -2, -1, +1, +2, +3, and + 4 relative to the date of parturition.

Data also showed that improvement of uterine health was associated with improvement of the immune status and overall health of the cows. Thus, cows treated with probiotics had lowered concentration of haptoglobin in the plasma than in control cows ($P < 0.01$; Iqbal et al., 2010a), suggesting that treatment with probiotics has the potential to improve the innate immunity in cows around calving. In addition, the treated cows had lower incidence of lameness (56.1 vs. 34.2%; $P = 0.04$) and mastitis (27.5 vs. 15.9%; $P < 0.01$; Zebeli et al., 2010b). Also, the probiotic treatment tended to lower the number of medications (7.3 vs. 4.5 medications/cow; $P = 0.09$) per cow throughout the experiment. Treated cows also showed tendencies for lower somatic cell counts (73,000 vs. 235,000 cells/mL; $P < 0.10$) in the milk indicating that uterine health is important to maintain health in other organs in the body including mammary gland (Iqbal et al., 2010b). Hansen et al. (2004) reviewed

various publications supporting the line of thought that mammary gland infections affect reproductive performance of dairy cows. Data of our research and others suggest that maintaining uterine health postpartum is also important to prevent occurrence of other peripartum diseases and improve overall health status of dairy cows during the transition period.

Uterine infections are typically associated with lowered feed intake and performance of the cows (Sheldon et al., 2008). Figure 6 shows that feed intake was greater in cows of the probiotics group compared to those of the control group. Moreover, cows developing metritis showed lower feed intake starting at immediately after parturition. Overall, the treated cows consumed up to 5 kg feed (ca. 2.5 kg DM) per day more than the control cows. During the time when cows were affected by metritis this difference was even greater (Figure 6). The low feed intake of the cows affected by metritis can be explained by the negative effects of inflammation on the eating behavior. It is well established that both Gram-negative and Gram-positive bacterial infections are associated with the release of cytokines like tumor necrosis factor-alpha, interleukin(IL)-1 or IL-6 (Van Deuren et al., 1992). The latter three cytokines affect eating behavior, suppressing feed intake in animals (Weingarten, 1996).

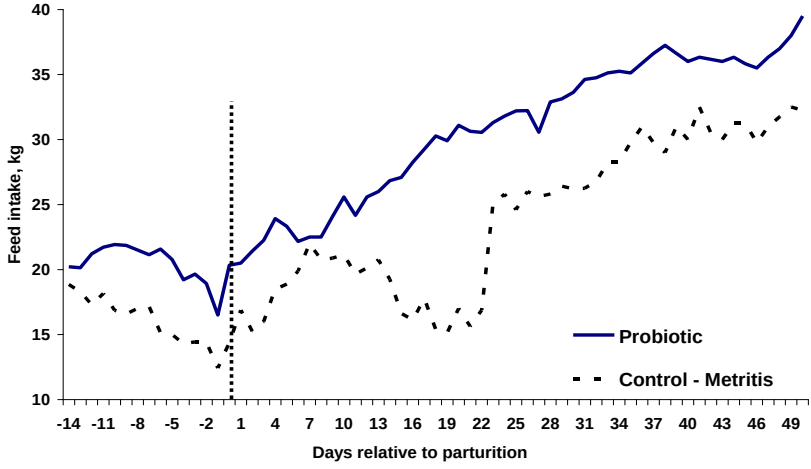

Figure 6. Daily feed intake during the transition period in healthy cows treated intravaginally with probiotics and control cows developing metritis at two weeks postpartum (the sick cows did not reach the level of feed intake of the healthy cows despite recovering from metritis).

In conclusion, data obtained by this experiment are very encouraging and suggest that intravaginal probiotics can be used as a means to lower the incidence of uterine infections, improve reproductive and productive

performance as well as the overall health status of dairy cows. More research is warranted to determine the best dose of probiotics and the frequency of application to make this treatment practical and cost-efficient.

▪ An Oral Vaccine as a Prophylactic Tool for Periparturient Diseases of Dairy Cows

As already described in the introduction section of this article there is an increasing amount of indirect and direct evidence that indicates that endotoxin is involved in the etiology and pathogenesis of multiple diseases of dairy cows like fatty liver, laminitis, displaced abomasum, milk fever, retained placenta, and downer cow syndrome (Ametaj et al., 2010). The indirect evidence comes from several studies demonstrating that a non-specific inflammatory condition is present in the systemic circulation of dairy cows suffering from endotoxin-related diseases, known as acute phase response (Ametaj et al., 2011; Emmanuel et al., 2008). For example, our team showed a strong relationship among concentration of endotoxin in the rumen fluid and plasma metabolites and minerals in dairy cows fed high grain diets, implicating endotoxin in perturbations of the intermediary metabolism of dairy cows (Zebeli et al., 2010a; 2011a). Rumen endotoxin also was related to depression of milk fat content suggesting that this bacterial compound induces also changes in the lipid metabolism of the cows (Zebeli and Ametaj, 2009). Several other investigators have shown that intravenous (Steiger et al., 1999; Waldron et al., 2003) or intramammary (Waldron et al., 2006) administration of LPS in dairy cows influences concentration of various plasma minerals and metabolites related to lipid and carbohydrate metabolism.

Direct evidence for involvement of endotoxin or its mediators of inflammation (i.e., cytokines) in multiple peripartum diseases of dairy cows comes from studies investigating effects of endotoxin or cytokine administration through various routes. Thus, Boosman et al. (1991) administered endotoxin systemically or locally in the hoof area and was able to develop laminitis in dairy cows. Bradford et al. (2009) injected subcutaneously recombinant bovine tumor necrosis factor-alpha (TNF-α; a cytokine released by macrophages during endotoxemia) for 7 consecutive days and reported accumulation of triglyceride in the liver and development of fatty liver in dairy cows. Recently we also proved that intermittently induced endotoxemia, by infusing LPS into the blood circulation of dairy cows, was associated with increased incidence of displaced abomasum and retained placenta (Zebeli et al., 2011). Furthermore infusion of endotoxin in the mammary gland is associated with development of mastitis in dairy cattle (Vels et al., 2009).

Besides the role of endotoxin in development of peripartum diseases in dairy cows there are also a few indications that lipoteichoic acid (LTA), a cell-wall

component of Gram-positive bacteria, might also be another agent of disease in dairy cows (Bone, 1994; Card et al., 1994; Timmerman et al., 2003). Recently it was shown that infusion of purified LTA, from *Staphylococcus aureus*, in the lumen of mammary gland was reported to induce a subclinical inflammatory response at 10 µg/quarter and development of clinical mastitis at 100 µg/quarter. In another study Daly et al. (2009) demonstrated differences in how mammary epithelial cells, challenged with LPS derived from *Escherichia coli* and LTA derived from *Staphilococcus aureus,* responded to these toxic compounds (Daly et al., 2009). Strandberg et al. (2005) also showed that bovine mammary epithelia cells responded to LPS with production of IL-1□, IL-8, TNF-□, CXL6, and □-defensin. Stimulation of the same cells with LTA did not alter expression of these genes. However, the mRNA levels of all genes increased rapidly after stimulation for 2-4 h with both LPS and LTA but only LPS treatment resulted in sustained responses. In contrast, the increased gene expression for LTA stimulated cells returned to resting levels after 8-16 h with the exception of β-defensin. These authors concluded that the limited and unsustained cytokine response to LTA might explain why mastitis caused by Gram-positive bacteria has greater potential for chronic intra-mammary infection than Gram-negative bacterial infections (Strandberg et al., 2005).

Although the evidence for involvement of endotoxin in dairy cows sickness and diseases around calving is mounting, there is a lack of efforts for developing prevention strategies against deleterious effects of endotoxin. In fact most of the studies conducted are observational studies and they do not address the question of how to prevent deleterious effects of endotoxin in cows. Since the major source of bacterial toxins in dairy cows is believed to be the gastrointestinal tract and mucosal surfaces like uterus and mammary gland (Linder et al., 1988; Emmanuel et al., 2008; Dohmen et al., 2000; Wenz et al., 2001) it would be of great interest to develop a strategy for prevention of LPS and LTA translocation into the host's systemic circulation by immunizing or making the mucosal layers tolerant to both these bacterial toxic compounds. Also, finding ways to ease the host responses to LPS and LTA is very desirable.

Previous research findings conducted in mice indicated that oral administration LTA from *S. pyogenes* and LPS from *Klebsilella pneumonia* was associated with enhanced IgA and IgG antibody synthesis in the lungs (Kofler et al., 1996). We hypothesized that oral application of vaccines containing LPS alone or a combination of LPS and LTA would establish an efficient host mucosal immune status against those bacterial toxins, mediated principally by immunoglobulin A (IgA) in co-operation with adaptive immunity. Furthermore, the immune response would be different depending on the site of priming, and inducing immune responses at oral and nasal mucosal tissues seemed to be the most effective approach for inducing immunity in the

gastrointestinal tract and other mucosal layers because of the interconnectivity of mucosal inductive and effector sites.

Keeping in mind all the above factors, our team adopted a proactive approach and conducted two in vivo experiments. The first experiment included utilization of oronasal LPS as a vaccine around parturition in dairy cows. Thus, 100 pregnant Holstein cows were blocked by parity and day of calving, and were randomly allocated to 1 of 2 different treatment groups. Fifty cows were treated oronasally with 3 mL of sterile saline (2 mL in the mouth and 1 mL in the nasal cavity) and served as the control group. The other 50 cows were treated oronasally with 3 mL of sterile saline (2 mL in the mouth and 1 mL in the nasal cavity) containing 3 increasing doses of LPS from *E. coli* 0111:B4 (Sigma-Aldrich Canada Ltd. Oakville, ON) for 3 consecutive weeks during the 2 weeks before and 1 week after parturition as follows: 1) 0.01 μg of LPS/kg of body weight (BW) on d -14 and -10; 2) 0.05 μg of LPS/kg of BW on d -7 and -3; and 3) 0.1 μg of LPS/kg of BW on d 3 and 7 postpartum.

In the second experiment both LPS and LTA were included in the vaccine and were administered orally during the transition period. Thus, 30 cows were randomly assigned to two groups (n = 15 each) and treated for 3 consecutive weeks orally either with carrier alone (sterile saline at 2 mL, controls) or 2 mL of sterile saline containing the same strain and amount of LPS (i.e., *E. coli* 0111:B4), as described for experiment 1, and a flat dose of LTA from *Bacillus subtilis* (Sigma-Aldrich Canada Ltd. Oakville, ON) at 120 μg/cow/treatment. The dose of LTA used was determined previously by our team based on a dose study conducted before the start of the experiment (data not published). This dose of LTA was selected because it had no effects on cow's temperature, respiration rate, rumen contraction rate, and feed intake. Administration of treatment was conducted twice per week during 2 weeks before and 1 week after calving.

The most interesting finding of the first study was that repeated oronasal administration of LPS in dairy cows around parturition lowered the incidence of udder edema in primiparous cows ($P < 0.05$; Hosseini et al., 2010). Although there was a decrease in the number of cases with laminitis (30 vs 18 cases; $P > 0.5$) the difference did not reach significance. The number of cows affected by mastitis was not different between the two treatment groups (20 vs 18 cases, $P > 0.5$). Interestingly another group of researchers reported that pretreatment of the mammary gland of dairy cows with LPS prevented development of *E. coli* induced mastitis (Petzl et al., 2011). Our data suggest that endotoxin might play a role in development of udder edema. It should be pointed out that the doses of LPS used were very low and they were split between the mouth and the nasal cavity.

Results of the second study with the oral vaccine containing both LPS and LTA showed that the oral treatment lowered the number of cases with

laminitis (9 vs 3 cases, $P < 0.01$) and the severity of the disease as measured by the number of days (11 vs 4 d; $P < 0.001$) that cows were sick from laminitis (Iqbal et al., 2010b). The data suggest that both LPS and LTA might be involved in the etiopathology of laminitis. This is the first time that repeated treatment with oral LPS and LTA is able to lower the incidence of one of the top 3 diseases that significantly affect the number of cows culled from a dairy herd. It has been known for a long time that endotoxin released in the rumen fluid during feeding of high grain diets might be one of the causal agents of laminitis and it was even demonstrated experimentally that systemic or local administration of LPS in the hoof area leads to the development of laminitis (Boosman et al., 1991). In fact results of our first study demonstrated that oral LPS was able to lower the incidence and severity of laminitis without reaching significance. However, inclusion of both LPS and LTA in the vaccine lowered significantly the cases of laminitis in transition dairy cows.

Another finding of interest of our experiments was that repeated oronasal application of LPS in periparturient cows had no effect on the incidence of uterine infections, whereas oral administration of both LPS and LTA was associated with lowering of the number of cases with severe and mild metritis (8 vs 2 cases; $P < 0.05$) after parturition. These data suggest that uterine lumen of the cows after parturition might be contaminated by both Gram-negative and Gram-positive pathogenic bacteria. Sheldon et al. (2008) observed that 25-40% of cows are affected by clinical metritis during the first 2 weeks after calving and that the disease continues in the form of clinical endometritis in the 20% of the cows. Interestingly, our findings showed that oral treatment with both LPS-LTA lowered also the severity of vaginal purulent discharges as indicated by the lower amounts of discharges from the treated cows.

Previous research work suggested that endotoxin might play a role in the etipathology of retained placenta (Dohmen et al., 2000). Moreover, our team proved directly that experimental intermittent endotoxemia is associated with increased incidence of retained placenta in postpartum dairy cows (Zebeli et al., 2011). Involvement of LPS in the pathogenesis of retained placenta has been suggested to be related to induction of reactive oxygen species like nitric oxide (Kankofer et al., 2005). Data from our research demonstrated that oronasal LPS had a tendency to lower the incidence of retained placenta (26 vs 12 cases, $P < 0.10$); however, administration of both LPS and LTA into the oral cavity significantly lowered the incidence rate of retained placenta (4 vs 0 cases; $P < 0.05$). The underlying mechanistic details of how LPS and LTA are involved in the decreased incidence of retained placenta needs to be studied further. It might involve the increased extravasations of neutrophils whose infiltration is decreased due to reduced expression of cell adhesion molecule L-selectin following exposure to LPS (Lynam et al., 1994). The presence of Gram-negative bacteria like *E. coli* in cows with retained placenta has been evidenced previously (Dohmen et al., 2000); however further studies need to

be conducted to address the role of LTA in the disease process. Taken together, our data showed that oral vaccination against both LPS and LTA holds the promise to be used as a preventive treatment against retained placenta and opens new avenues in the etiopathology of retained placenta.

Most importantly, we found that cows (i.e., including both primiparous and multiparous) treated with both LPS and LTA had less number of days with more than one disease (16 vs 6 d; $P < 0.001$; Figure 7), which indicated that involvement of both LPS and LTA in peripartum disease is more likely to explain the etiopathogenesis of overall sickness of dairy cows during transition period than LPS alone.

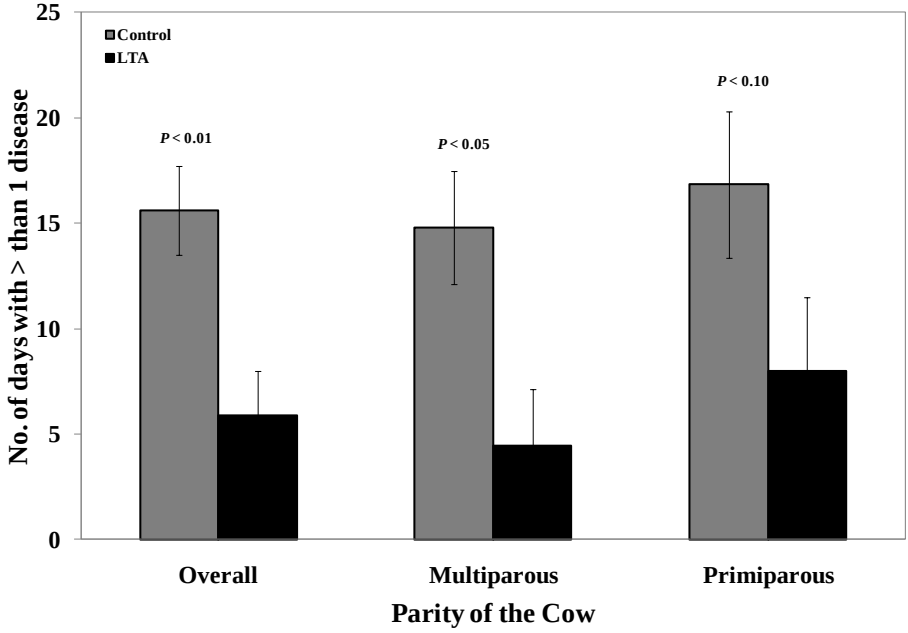

Figure 7. Number of days that cows (overall or including primiparous and multiparous) were affected with more than one disease during the 2 weeks before and 4 weeks after calving in dairy cows treated orally with lipopolysaccharide (LPS) and lipoteichoic acid (LTA). Lipopolysaccharide from *E. coli* 0111:B4 (Sigma-Aldrich Canada Ltd. Oakville, ON) was applied in the mouth cavity twice per week during 2 weeks before and 1 week after calving at three increasing doses as follows: (1) 0.01 µg of LPS/kg of body weight (BW) on d -14 and -10; (2) 0.05 µg of LPS/kg of BW on d -7 and -3; and (3) 0.1 µg of LPS/kg of BW on d 3 and 7 postpartum; whereas a flat dose of LTA from *Bacillus subtilis* (Sigma-Aldrich Canada Ltd. Oakville, ON) at 120 µg/cow/treatment was applied during the same times as LPS.

In conclusion, oronasal vaccination with LPS alone lowered the incidence of udder edema and had a tendency to lower retained placenta; however, combination of both LPS and LTA administered in the oral cavity during 3 weeks around calving lowered the incidence rates and severity of three most important peripartum diseases of dairy cows including laminitis, uterine infections, and retained placenta. Moreover, the number of days that cows were affected by multiple diseases was decreased. Given that endotoxin has been implicated in the etipathology of several other metabolic diseases of transition dairy cows including fatty liver, milk fever, displaced abomasum, and downer cow syndrome (Ametaj et al., 2010a) it is possible that those diseases also might be tackle by the same approach. Because combination of LPS with LTA as an oral treatment gave better results in lowering the incidence of peripartum diseases compared with LPS alone, our data suggest that LTA from Gram-positive bacteria might be another major player in the etiopathology of multiple diseases of dairy cows. More research is warranted to elucidate the involvement of both LPS and LTA, and other potential bacterial compounds, in the etiopathology of peripartum diseases of dairy cows to better understand the mechanism(s) behind disease process and develop a novel strategy for their prevention in the future.

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