Effect of intramammary infusion of chitosan hydrogels on bovine mammary gland involution after drying-off

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ABSTRACT

The transition from lactation to the dry period in dairy cows is a period of high risk for acquiring new intramammary infections. This risk is reduced when the involution of the mammary gland is completed. Accordingly, approaches that speed up the involution process after drying-off could reduce the incidence of mastitis. The research presented in this thesis aimed at developing a biological response modifier that could be injected into cow teats to promote immune cell migration and speed up mammary gland involution. Chitosan, a natural polysaccharide derived from chitin, is able to triggers host innate immunity. We developed 2 formulations, made from either high- or low- viscosity chitosan. Both are liquid at room temperature but form a hydrogel at the body temperature. In the first experiment, each udder quarter of 7 Holstein cows in late lactation was randomly assigned at drying-off to receive one of the following intra-mammary infusions: 2.5 or 5 mL of low-viscosity chitosan hydrogel, 5 mL of high-viscosity chitosan hydrogel, or 5 mL of water. Milk (mammary secretion) samples of each quarter were collected on days before and after drying-off to measure different immune and involution markers. The chitosan hydrogel infusions significantly hastened the increases in somatic cell count, serum albumin and lactoferrin concentrations, and the lactate dehydrogenase activity in mammary secretions. No major differences between sources or volumes of chitosan were observed for the measured parameters. These results suggest that chitosan hydrogel infusion hastened mammary gland involution, which may reduce the risk of acquiring new intra-mammary infection during the dry period. The compatibility of chitosan hydrogel with an internal teat sealant was verified in the second experiment. Each udder quarter of 8 Holstein cows in late lactation was randomly assigned at drying-off to administration of an intra-mammary infusion of 5 mL of low viscosity chitosan hydrogel, 4 g of an internal teat sealant, combination of sealant and chitosan, or 5 mL of water. Milk (mammary secretion) samples of each quarter were collected on days before and after drying-off to measure different involution markers. As in the first experiment, chitosan induced changes in involution and immune responses markers. Those parameters were not affected by the presence of the teat sealant, showing that both could be used in combination. Further studies are needed to determine whether administration of chitosan hydrogel could also reduce the incidence of new cases of intra mammary infections during the dry period. Ultimately, this approach could be used as an alternative to dry cow antibiotic therapy for non-infected cows.

RESUME

La transition entre la lactation et le tarissement est une période à risque élevé pour la vache laitière de contracter de nouvelles infections intra-mammaires. Ce risque est réduit lorsque l'involution de la glande mammaire est terminée. Par conséquent, une approche permettant d'accélérer le processus d'involution après le tarissement pourrait réduire l'incidence de la mammite. Notre programme de recherche vise à développer un modificateur de la réponse biologique pouvant être injecté dans les trayons de la vache afin de promouvoir la migration rapide des cellules immunitaires, accélérant ainsi l'involution mammaire. Le chitosan est un polysaccharide naturel dérivé de la chitine capable de stimuler l'immunité innée de l'hôte. Nous avons développé deux formulations; fabriqué à partir de chitosane ayant une viscosité faible ou élevé. Ces formulations sont liquides à température ambiante mais forment un hydrogel à la température du corps. Dans une première expérience, Chaque quartier de 7 vaches Holstein en fin de lactation a été assigné de manière aléatoire à une infusion intra-mammaire de 2.5ml ou 5ml de chitosan ayant une viscosité faible, 5ml de chitosan ayant une viscosité élevée ou 5ml d'eau. Des échantillons de lait (sécrétions mammaires) de chaque quartier ont été recueillis lors des jours précédents et suivants le tarissement afin de mesurer plusieurs marqueurs de l'involution mammaire. Les infusions d'hydrogel de chitosan ont toutes accéléré l'augmentation des teneurs des sécrétions mammaires en cellules somatiques, albumine sérique et lactoferrine, ainsi que de l'activité de la lactate déshydrogénase. Aucune différence notable n'a été observée entre les différents traitements de chitosan. Ces résultats indiquent que la perfusion d'hydrogel de chitosan accélère le processus d'involution de la glande mammaire. La compatibilité de cette approche avec un scellant à trayon interne a été vérifiée dans une seconde expérience. Chaque quartier de 8 vaches Holstein en fin de lactation a été assigné de manière aléatoire à une infusion intra-mammaire de 5 ml de chitosan ayant une viscosité faible, 4 g de scellant, une combinaison de sellant et de chitosan, ou 5 ml de l'eau. Des échantillons de lait (sécrétions mammaires) de chaque quartier ont été recueillis lors des jours précédents et suivants le tarissement. Les effets du chitosan sur les marqueurs d'involution et les réponses immunitaires ont été similaires à la première expérience. Ces effets n'ont pas été affectés par la présence du scellant à trayon, ce qui montre que les deux approches sont entièrement compatibles et peuvent être utilisés en combinaison. Bien que cela devra être évalué, ces résultats suggèrent que l'administration d'un hydrogel de chitosan au tarissement pourrait réduire l'incidence des nouveaux cas de infection intra mammaire durant la période de tarissement. Finalement, cette approche pourrait être utilisée comme une alternative au traitement antibiotique au tarissement pour les vaches non infectées.

CONTRIBUTION OF AUTHORS

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Pierre Lacasse and Xin Zhao designed experiments, analyzed data, reviewed the manuscript and supervised the primary author. Samuel Lanctôt designed and conducted all of the experiments, analyzed the data and wrote the manuscript. Patrick Fustier, Ali Tahérian and Barbara Bisalowski contributed in development of the chitosan hydrogel.

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TABLE OF CONTENTS

LIST OF ABBREVIATION

ACTB:	Beta Actin
ADD:	Animal defined-daily dose
ADUR:	Antimicrobial drug use rate
BRM:	Biological response modifier
BSA:	Bovine serum albumin
CCL2:	Chemokine (C-C motif) ligand 2
CD14:	Cluster of differentiation 14
cDNA:	Complementary deoxyribonucleic acid
CSF:	Colony-stimulating factors
CXCL8:	Chemokine (C-X-C motif) ligand 8
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
IFN-γ:	Interferon gamma
IL1-β:	Interleukin 1 beta
IMI:	Intramammary infection
LDH:	Lactate dehydrogenase
LPS:	Lipopolysaccharides
LTA:	Lipoteichoic acids
MAC:	Menbrane attack complex
NAGase:	N-acetyl-β-d-glucosaminidase
NF-κB:	Nuclear factor-kappaB
PAMP:	Pathogen-associated molecular patterns
PBS:	Phosphate buffered saline
PMN:	Polymorphonuclear neutrophils
PPIA:	Peptidylprolyl isomerase
PRR:	Pattern recognition receptor
RNA:	Ribonucleic acid

SCC: Somatic cell count

TLR: Toll-like receptor

TNF-α: Tumor necrosis factor alpha

YWHAZ: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta

CHAPTER 1. INTRODUCTION

The lactation cycle of a dairy cow must include a dry period for optimal milk production in the following lactation (Andersen et al., 2005). Although milking cessation is essential for proper cell renewal, during early involution, the cow mammary gland is vulnerable to new intramammary infection (**IMI**) (Smith et al., 1985; Leelahapongsathon et al., 2016).

When early involution of the mammary gland is completed, the risk of acquiring a new IMI is minimalized (Tatarczuch et al., 2002). Consequently, Oliver and Smith (1982) have proposed that accelerating the involution process after drying-off could enhance the resistance of the mammary gland to new IMI during early involution. This acceleration can be achieved by using a biological response modifier (BRM) (Dallard et al., 2010). However, the BRMs tested so far are of short duration (Shamay et al., 2003; Dallard et al., 2010). Ideally, the immunostimulation should last for the whole period of active involution. Therefore, developing a BRM which hasten involution as well as induce and sustain a moderate recruitment of immune cells is important.

Chitosan is a natural biocompatible polysaccharide produced from chitin (Rinaudo, 2006). Chitosan can be formulated to be injectable at room temperature but forms a biodegradable hydrogel at body temperature (Chenite et al., 2000). Moreover, chitosan has bacteriostatic, bioadhesive and bioactive properties (Şenel and McClure, 2004). Our research program aimed to develop a chitosan-based BRM formulation that could be injected into the cow teat to promote sustained immune cell migration and hasten involution at drying-off. In addition, the effect of combining the chitosan-based BRM with an internal teat sealant was verified.

The present thesis includes information on bovine dry-periods and mastitis as well as an article which covers the experiments design, results and discussion.

CHAPTER 2. REVIEW OF THE LITTERATURE

2.1 The Dairy industry

The Canadian's dairy industry is a major part of the agricultural sector. In 2015, its net farm receipts represented 6.02 billion. Based on farm cash receipts, the dairy industry ranks the third most important agriculture sector in Canada. At that time, there were 11,683 farms milking 959,600 dairy cows in Canada. The Canadian dairy industry is world renowned for its superior animal genetic and excellence in dairy products. In 2015, the Canadian average milk production for all dairy cow breeds was 10, 043 kg per 305-days lactation period with an average of 3.95% fat and 3.23% protein (Agriculture and Agri-Food Canada, 2015).

Genetic selection and better management led to the improvement of milk production performance. However, selection for milk production has increased metabolic stress, reproduction problems, lameness and mastitis cases throughout the lactation period (Oltenacu and Broom, 2010).

2.2 Bovine mammary gland

2.2.1 Lactation

Although lactation begins at calving, synthesis of milk components is initiated a few weeks before, during a period called lactogenesis. Once lactation is established, milk production increases drastically and peaks between two and eight weeks after parturition. After the lactation peak, milk production decreases gradually until complete dry-off (Craplet, 1960).

The activity and number of mammary secretory cells change over the lactation cycle. During gestation and early lactation, the volume of secretory tissue increases exponentially (Knight and Wilde, 1993). After parturition, milking stimulus causes activation of secretory cells, hence leading to milk production increase before the lactation peak (Tucker, 1980). Throughout the lactation period, apoptosis and proliferation of secretory cells occur simultaneousy, where half of original secretory cells are replaced over that period (Capuco et al., 2001). After the milk production peak, an amplified apoptosis death and a reduced cell proliferation result in a gradual mammary epithelial cell loss. Consequently, it is the decline of mammary cell number and not a reduced cellular activity that causes observed milk yield decrease from peak until the end of lactation (Capuco et al., 2001).

2.2.2 Dry period

In nature, when mammals' offsprings stop suckling, the mother's milk production ceases. When milk is not being produced during the dry period, the mammary gland regenerates itself for the next lactation. In the dairy industry, this period is crucial for a better performance during the next lactation. Cows continuously milked between calving produce less milk during the following early lactation period (Andersen et al., 2005). Moreover, continuous milking does not allow for colostrum production and would reduce offspring survival chances (Santschi and Lefebvre, 2014). Studies showed that cows should stop being milked 8 weeks before calving, in order to allow renewal of epithelial cells and optimize milk production for next lactation (Capuco et al., 1997).

In most mammals, involution is induced quickly. For instance, rats and mice initiate it within 24h. However, this process is slower for cows, and milk production can completely reinitiated within 7 days of milk stasis (Singh et al., 2015). Afterward, normal milk secretion cannot be resumed without loss of milk production (Noble and Hurley, 1999). Lack of milking more than 7 days induces mammary gland morphological changes in mammals. As opposed to other type of mammals, such as mice and rats, bovine mammary glands undergo less tissue regression. This difference probably results from the fact that the cow is already in late pregnancy during the dry period (Capuco and Akers, 1999). The involution process in bovine is mostly characterized by cell turnover and changes in their secretory state and volume. The dry period can be divided into three different phases: active involution, steady state involution and redevelopment/colostrogenesis (Hurley, 1989).

2.2.2.1 Active involution

The active involution can be divided in two stages. The first stage, named acute involution, begins when milk removal from the mammary gland is ceased. Since milk secretion continues for some time, milk accumulates in the mammary gland during two to four days (Hurley, 2010). After two days, formation of large intracellular vacuoles of lipid droplets and secretory vesicles can be observed (Holst et al., 1987). Gradually, tight junctions between epithelial cells become more permeable. This can be assessed by measuring concentrations of serum albumin and immunoglobulin in milk secretion (Hurley, 2010).

The second stage begins around 7 days without milking. After this period, mammary secretory state and volume change and lactation cannot be completely recovered (Singh et al., 2015). As the second stage progress, secretory epithelial cells' cytoplasmic organelles implicated in milk synthesis such as rough endoplasmic reticulum, Golgi and secretory vesicles regress. As a result, concentrations of milk components decrease. For example, citrate, an indicator of mammary gland activity, decreases as involution progresses (Hyvönen et al., 2010). However, cells still preserve viable and intact organelles to maintain metabolic and other secretory functions (Holst et al., 1987). For instance, they produce lactoferrin, a protein which binds iron; hence, making it unavailable for iron-dependent bacteria (Capuco and Akers, 1999). Another natural protection molecule produced in the mammary gland is keratin. As involution progresses, keratin accumulates in the teat canal and acts as a natural sealant (Capuco et al., 1992).

Also, antimicrobial proteins and leukocytes enter into the mammary gland to protect it from potential invading pathogens. In fact, most of the cells found in milk secretion are immune cells. Epithelial cells contribution is less than 2% of the somatic cells (Hurley, 1989). During the first 3-7 days, polymorphonuclear neutrophils (PMN) are the main type of leukocytes entering into the mammary gland. After 7 days, macrophages become the major type of leukocytes in the mammary gland (Hurley, 1989). Both are phagocytic cell types which eat most of the remaining milk components such as lipid droplets casein and cellular debris (Sordillo and Nickerson, 1988; Tatarczuch, 2002). As for lymphocytes, they are always present. However, their concentration rises as macrophage concentration increases (Hurley, 1989). Since neutrophils and macrophages engulf milk components, their capacity to phagocyte bacteria is diminished. This contributes to cows' high vulnerability to new mammary gland infection during early involution (Tatarczuch, 2002)

2.2.2.2 Steady state involution

During the steady state involution, macrophages regain phagocytic capacity, and vulnerability to infection decreases (Tatarczuch, 2002). During this period, most teats are sealed and the mammary gland contains small volume of fluid (Hurley, 2010). Epithelial cells are in a non-secretory state and contain no secretory vesicles or fat droplets. Moreover, the number of mammary cells does not change throughout this period (Capuco et al., 1997).

2.2.2.3 Redevelopment/colostrogenesis

During the redevelopment and colostrogenesis, the mammary gland undergoes drastic changes to resume milk secretion. This period, referred as lactogenesis, can be divided into two stages. The first stage (2-3 weeks before parturition) comprises a gradual cytological and enzymatic differentiation of the epithelial cells (Gao, 2013). Moreover, a limited milk secretion of milk proteins and pre-colostrum can be observed during this stage (Finucane, 2008). The second stage occurs near parturition. Throughout this stage, the integrity of tight junctions between the alveolar cells is re-established, secretory organelles are restored, secretory cells undergoes histological structures changes, colostrum secretion accumulates in the alveolar lumen; and finally, abundant secretion of milk is produced (Gao, 2013). During the fluid accumulation, teats can leak; as a result, vulnerability to new infection of the mammary gland increases during this period (Hurley, 2010).

2.3 Intramammary infection

Intramammary gland infections **(IMI)** result from pathogens invading the mammary gland via the teat canal. Once the pathogens overcome the host's natural defences, they establish in the mammary gland and start to proliferate. After pathogen recognition, the host innate immune response causes inflammation and substantial increase of milk somatic cells. At this point, clinical symptoms begin to appear (Wellnitz and Bruckmaier, 2012). In any cases, mastitis causes a substantial milk loss. Rajala-Schultz et al. (1999) evaluated the milk daily losses to be 1.0 to 2.5 kg during 2 weeks after mastitis diagnosis. They assessed the whole lactation milk losses between 110 and 552 kg depending on days in milk and parity.

Cows are vulnerable to IMI at any time point during the lactation cycle. However, new infections mostly occur during critical periods, including early involution and peripartum (Hurley, 2010). During the early involution, high fat, casein and lactose concentrations favor bacterial growth and interfere with the phagocytic capacity of immune cells (Sordillo and Nickerson, 1988). Moreover, slow involution process of cow delays milk secretion's increase of antibacterial components and concentrations of immune cells (Sordillo et al., 1987). Similarly, during peripartum, colostrum accumulation dilutes protective immune factors (Bradley, and Green, 2004). During both periods, cows secrete a significant amount of milk; however, there is no milking. As a result, a significant amount of milk accumulates in the mammary gland. The resulting pressure increase risk of milk leak (Tucker et al., 2009) and impairs keratin plug (Dingwell et al. 2004). Once the teat canal is open, microorganisms gain access to the mammary

gland and can cause infection (Cousins et al., 1980). Therefore, it is of great importance to improve the mammary gland defence to prevent new IMI.

2.3.1 Mammary gland defence mechanisms

2.3.1.1 Anatomical protection

The primary defence mechanism of the udder is the teat canal. The first physical barrier is the teat sphincter. The teat sphincter muscle contracts between milking to seal the orifice tightly. Overmilking or vacuum fluctuation during milking can damage the teat end and increase incidence of mastitis (Bhutto et al., 2010).The second obstacle against invasion of pathogens is the keratin lining in the teat canal. Keratin is a waxy substance which accumulates in the teat canal, creating a physical obstruction against invading pathogens. Moreover, esterified and non-esterified fatty acids in keratine, including palmitoleic, myristic and linoleic acid, have bacteriostatic properties. Additionally, cationic proteins related to keratin can bind bacteria and affect their cell wall integrity (Oviedo-Boyso et al., 2007). The rate of closure differs between cows and some teat canals can remain open during the dry period (Dingwell et al., 2004). During peripartum and early involution, milk accumulates in the mammary gland and internal pressure causes teat leakage. When there is a milk leakage, the teat canal defence mechanisms are disrupted. Accordingly, Schukken et al., (1993) reported that cow leaking during dry-off had 4 fold greater risk of acquiring a new intramammary infection.

2.3.1.2 Cellular protection

The second line of defence involves immune cells, which are major players of the innate immunity (Table 1). A major point of entry of immune cells is the Furstenberg's Rosette, a structure strategically located at the internal end of the streak canal. At this location, immune cells leave the teat wall, and enter into the teat cistern to intercept bacteria before they reach the mammary gland (Nickerson and Pankey, 1983).

Neutrophils and macrophages are important components of the innate immunity and constitute a large proportion of leukocytes. In healthy udders, neutrophil number is low. However, during an infection, the neutrophil number increases drastically and can reach near 100% of leukocytes (Paape et al., 2000). Inflammatory mediators attract them from the blood stream to the mammary gland, where they display non-specific phagocytosis and bacteria killing

abilities. Neutrophils can generate reactive oxygen species and small antibacterial peptides such as defensins, which destroy invading pathogens (Selsted et al., 1993).

As for macrophages, they are the main type of milk somatic cells in healthy quarters (Paape et al., 2000). Like neutrophils, they have a non-specific capacity to phagocyte bacteria. However, their greatest contribution to the mammary gland protection is their ability to release pro-inflammatory cytokines that recruit and activate neutrophils (Oviedo-Boyso et al., 2007). Similarly to the macrophages, bacterial adhesion or toxins trigger epithelial cell response, hence resulting in the production of immune cells recruiting cytokines (Sordillo and Streicher, 2002).

After acute neutrophil cell migration, if the infection persists, T and B lymphocytes initiate specific immune responses. Lymphocytes express membrane receptors capable of recognizing different antigenic structure which triggers a specific immune response (Carroll and Forsberg, 2007). During an IMI, B lymphocytes and macrophages present antigen-MHC class II complexes, which activate CD4+ T lymphocytes. In turn CD4+ T cells produce specific immunoregulatory cytokines. This includes IL-2, an interleukin that promote B cell proliferation and differentiation into mature B cells (memory cells) or plasma cells. In turn, plasma cells produce antigen specific antibodies. The CD8+ T lymphocytes have the ability to downregulate immune expression and eliminate host cell which present foreign antigen. Gamma-delta T cells can also destroy altered epithelial cells. However, their biological role is not well characterized (Oviedo-Boyso et al., 2007).

Finally, natural killer cells are antigen independent immune cells. They bind to infected and abnormal cells, degranulate and release perforin which disrupt cell membrane leading to cell destruction. Natural killer cells also activate immune response by releasing large amount of cytokines (Carroll and Forsberg, 2007). Moreover, they have an independent cytotoxic effect against gram-positive and gram-negative bacteria (Garcia-Penarrubia et al., 1989). Therefore, they could play an important role for mammary gland defenses (Shafer-Weaver and Sordillo, 1996).

Table 1. Summary of Mammary Gland Cellular Defenses

Factor	Biological function
Neutrophils	Phagocytosis and intracellular killing of bacteria; secretion of antibacterial factors
Macrophages	Phagocytosis and intracellular killing of bacteria; antigen presentation in conjunction with MHC
Natural killer cells	Nonimmune lymphocytes that secrete antibacterial proteins upon activation
T lymphocytes CD4+ (T helper)	Production of immunoregulatory cytokines following antigen recognition with MHC class II molecules; memory cells following antigen recognition
CD8+ (T cytotoxic)	Lysis of altered or damaged host cells when complexed with MHC class I molecules; production of cytokines that can down-regulate certain leukocyte functions
γδ T lymphocytes	Biological role in the mammary gland is speculative
B lymphocytes Mature B cells	Display membrane-bound antibody molecules to facilitate antigen presentation; memory cells following antigen interactions
Plasma cell	Terminally differentiated B lymphocytes that synthesize and secrete antibody against a specific antigen

(Sordillo and Streicher, 2002)

2.3.1.3 Soluble immune factors

Different innate and specific soluble immune factors protect the mammary gland. Soluble factors assist immune cells to identify pathogens and modulate immune response. For example, antibodies produced by antigen-activated B lymphocytes such as IgG1, IgG2, and IgM have the ability to target (opsonize) bacteria to enhance neutrophils and macrophages phagocytosis. Other antibodies such as IgA have the ability to neutralize toxins and cause bacteria agglutination, hence reducing the spread of the infection (Paape et al., 2000).

Complement proteins are other soluble factor able to opsonize bacteria and attract neutrophils. Complement proteins such as C3 is a key factor which binds covalently to pathogens and promotes phagocytosis. Similarly, C5a has also the ability to increase bactericidal activity of neutrophils. The complement system can directly disrupt bacteria's membrane by assembling of C5b, C6, C7,C8 and several copies of C9 to form a membrane attack complex (MAC) (Rainard,

2003). Complement concentrations are low in milk of healthy lactating udders, but high in colostrum and infected or involuted mammary glands (Sordillo and Streicher, 2002).

Other soluble factors disrupting bacteria's cell wall include lysozyme and lactoferrin. Lysozyme is a bactericidal protein which cleaves peptidoglycan of gram positive bacteria's cell walls. It can also disrupt outer cell membrane's peptidoglycan of gram negative bacteria. Lysozyme has low effectiveness against intramammary infecting pathogens. Nevertheless, it has synergic effects on other bactericidal soluble factors (Ezzat Alnakip et al., 2014). Lactoferrin is a bacteriostatic and bactericidal iron-binding protein. Lactoferrin sequesters iron; therefore, it is unavailable for iron dependent bacteria such as *Klebsiella pneumoniae* and *Escherichia coli*. Moreover, lactoferrin bactericidal activity results from its ability to bind and disrupt the gram-negative bacteria outer membrane (Ellison, et al., 1988). Finally, lactoferrin is ineffective against some bacteria; as an example, *Streptococcus agalactiae* is able to use it as an iron source (Sordillo and Streicher, 2002). The lactoferrin concentration is low in lactating mammary gland, but increases drastically during involution. Its concentration is maximal after 3-4 weeks of involution. During this period, it is nearly 100-fold greater than in the course of lactation (Ezzat Alnakip et al., 2014). Its concentration also increases during an infection. However, elevated concentrations of citrate during lactation chelate iron, and reduce effectiveness of lactoferrin against pathogens (Bishop et al., 1976; Sordillo and Streicher, 2002).

2.3.1.4 Cytokines

Cytokine are small proteins produced and released by cells that help them interact and communicate together. Different cells can produce the same cytokine and one cytokine can affect different cell types. When released, cytokines can affect distant cells, nearby cells and also may have an effect on the cell that has produced the cytokines (autocrine action). Cytokines usually produce a cascade response by inducing cells to produce more cytokines (Zhang and An, 2007). During an immune response, intercellular communication is achieved by cytokine signaling (Alluwaimi, 2004). Once activated, immune cells and mammary epithelial cells produce and release cytokines. Immunomodulation consists of a complex network of pro-inflammatory and anti-inflammatory cytokines (Gunther, et al., 2011). Mammary gland common pro-inflammatory cytokines include TNF-α, interferon gamma (IFN-γ), colony-stimulating factors (CSF), and several interleukins (IL-1β, IL-2, IL-6, IL-8, IL-12). When pro-inflammatory cytokines binds to

macrophage and neutrophil receptors, they increase their bactericidal capacity. Moreover, cytokines termed chemokines attract neutrophils towards the site of infection (Oviedo-Boyso et al., 2007). Each kind of cytokines has a different type of control on the immune response (Table 2).

Table 2. Cytokine Effects on Mammary Immune and Inflammatory Responses

Cytokine	Observations
IL-1	Mediates acute phase inflammatory response Increases neutrophil numbers Enhances neutrophil phagocytosis and bactericidal activity Triggers neutrophil migration into infected mammary gland
IL-2	Enhances mammary mononuclear cell proliferation Enhances cytotoxic and bactericidal activities of lymphocytes Increases plasma cell numbers Activates NK cells
IL-8	Induces inflammation Mediates IL-1 induced neutrophil migration Potent chemoattractant
Granulocyte-CSF	Increases numbers of blood and milk neutrophils Increases milk SCC Increases phagocytosis and bactericidal activity Decreases neutrophil migration
granulocyte-monocyte-CSF	Enhances chemotactic and bactericidal activities of neutrophils Enhances cytotoxic activity Increases number of phagocytic cells
Macrophage-CSF	Regulates proliferation and differentiation of macrophages Potent macrophage chemoattractant
IFN-γ	Enhances neutrophil phagocytosis and bactericidal activity Reverses suppressive effects of mammary gland secretions
TNF-α	Enhances acute phase inflammatory response Enhances neutrophil phagocytosis and bactericidal activity Enhances endothelial adhesion molecule expression

(Sordillo and Streicher, 2002)

2.3.2 Pathogen types and clinical symptoms

In a dairy herd, the most common infecting pathogens can be either contagious or environmental. Often associated with chronic or subclinical mastitis, contagious bacteria survive in the udder. They can be transmitted from quarter to quarter or cow to cow by contaminated milking equipment, towels, or milker's hands. Examples of common contagious bacteria are

Staphylococcus aureus, Streptococcus agalactiae, as well as various mycoplasma and *Arcanobacterium spp* (Oviedo-Boyso et al., 2007). Environmental bacteria mainly shed in ground, manure, and bedding. Milk leakage between milking and dirty udder is the main causes of infections by these pathogens. Major pathogens in this category are *Escherichia coli, Streptococcus dysgalactiae, Streptococcus uberis, Klebsiella pneumoniae,* and *Bacillus spp.* (Oviedo-Boyso et al., 2007). The mammary gland innate immune response is similar among different type of pathogens. However, severity of the response depends on the pathogen type (Table 3).

Table 3: Immune response of bovine mammary gland to different bacteria

Bacterium	Type of mastitis	Innate immune response
Staphylococcus aureus	Clinic or Subclinic /chronic	Increase in SCC Transitory increase in TNF-a, IL-1b, and C5a concentration Increase in IL-12 concentration Increase in CD8þ lymphocytes recruitment Increase in IgG2 concentration
Escherichia coli	Clinic	Increase in SCC Increase in TNF-a, IL-1b, IL-8, IL-12, IFN-g, and C5a concentration Increase in LBP, BSA, and sCD14 concentration
Streptococcus uberis	Clinic	Increase in SCC Increase in TNF-a, IL-1b, IL-8, IL-12, IFN-g, pmCD14, and LBP concentration
Serratia marcescens	Clinic	Low concentrations of IL-12, IFN-g, pmCD14, and LBP
Klebsiella pneumoniae	Clinic	Increase in SCC Increase in TNF-a, IL-1b, and IL-12 concentration Increase in IL-8, C5a, LBP, and pmCD14 concentration
Pseudomonas aeruginosa	Clinic	Increase in TNF-a, IL-8, IL-12, IL-10, C5a, and LBP concentration

(Oviedo-Boyso et al., 2007)

2.3.2.1 Clinical symptoms

Clinical symptoms caused by mammary gland infection vary from subclinical and chronic to acute and severe. Essentially, clinical outcomes depend on host immune status and infecting pathogen type. Cow's immune status is affected by presence of other diseases, nutritional status, stress, lactation stage, parity and genetic resistance (Goff and Kimura, 2004; Carroll and Forsberg, 2007; Schukken et al., 2011). Even if innate immune response is non-

specific, it is activated by specific cellular pattern recognition receptors (PRR) on leucocytes and epithelial cells. Therefore, PRR bind to pathogen specific molecules named pathogen-associated molecular patterns (PAMPs) which are released during pathogen duplication or degradation (Wellnitz and Bruckmaier, 2012). In cattle, PRR includes ten toll-like receptors (TLR), which bind to different PAMPs (Rinaldi, et al., 2010). When bound to PAMPs, TLRs activate a downstream signaling cascade, leading to the nuclear factor-kappaB (NF-kB) activation. Subsequently, it induces production of proinflammatory cytokines and chemokines (Kawai and Akira, 2007).

Variability between immune response depends upon infecting pathogen's PAMPs. Most of the IMI result from gram-negative and gram-positive bacterial infections with distinct host responses. Common diagnosed bacteria are *Escherichia coli* (gram-negative), which normally causes acute/clinical response, and *Staphylococcus aureus* (gram-positive) leading to chronic/sub-clinical infection (Wellnitz and Bruckmaier, 2012).

2.3.2.2 Escherichia coli

Escherichia coli, like other Gram-negative bacteria, possess lipopolysaccharides (LPS) on their cell membrane. This endotoxin provoke acute immune response characterized by drastic increase in somatic cell counts, fever, pain, milk coagulation, and lower milk production (Werner-Misof et al., 2007). Inflammation is initiated when LPS binds to the CD14/TLR4 complex on the macrophage membrane. Cytokines such as TNF-α, IL-1B, and IL8 induce the inflammatory response. The IL-1 and TNF-α amount released will directly affect clinical symptoms amplitude. In fact, the immune response causes more damage than the bacterium itself. During acute mastitis, the inflammatory cascade can lead to permanent tissue damage and even death by septic shock (Gunther, et al., 2011). The response to LPS is dose dependent; Werner-Misof et al. (2007) concluded that an intra-mammary injection of 1 μg of LPS does not cause clinical signs whereas a dose of 3 μg of LPS results in clinical signs. When the inflammation response of the host is sufficient to destroy the invading bacteria, inflammation resolves by anti-inflammatory cytokines release including IL10 and TGFβ1 (Rinaldi, et al., 2010).

2.3.2.3 Staphylococcus aureus

Staphylococcus aureus, a Gram-positive bacterium, usually induces minimal clinical signs, but infects cows chronically. It possesses different PAMP, which includes peptidoglycans, lipoproteins, and lipoteichoic acids (LTA) (Wellnitz and Bruckmaier, 2012). Yang et al (2008) suggested that chronic *S. aureus* infection is caused by compromised immune response pathways, which increases the vulnerability of the host. They demonstrated that *S. aureus* bacteria, and particularly LTA, activate mammary epithelial cells' TLR2 receptors. However, further signals are partially blocked, leading to impaired NF-kB activation. Impair NF-kB activation results in reduced cytokine (TNF-α, IL-1β) secretion, explaining the lower immune response, inferior systemic inflammation, and minor clinical symptoms observed during *S. aureus* mastitis. Therefore, *S. aureus*' ability to inhibit the immune response allows its survival in the host (Gunther et al., 2011).

Most of the acute mastitis symptoms caused by *S. aureus* result from its ability to produce toxins those damages epithelial cells membranes. Moreover, damaging tissue is beneficial for the pathogen, since it enables its adhesion and migration into epithelial cells. Once in the host's cells, *S. aureus* is protected from the immune response, but also from conventional antibiotic therapy (Oviedo-Boyso et al., 2007). A bulk of research aims to efficiently treat chronic subclinical *S. aureus* mammary infection.

2.3.2.4 Other bacteria

Another common bacterium named *Streptococcus uberis* triggers immune responses such as cytokines production, leukocytes recruitment and soluble immune factors release. It also induces clinical symptoms, comprising fever and swelling (Rambeaud et al., 2003). Similarly, other mastitis causing pathogen such as *Mycoplasma bovis, Streptococcus uberis, Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Serratia marcescens* activate host's immune response (Bannerman, 2009).

The ultimate consequences of mammary gland infection are similar among pathogens. Intramammary infections results in production losses, lower product quality, higher drug usage, discarded milk, veterinary services, extra labor, more diagnostic tests, other related diseases (Halasa et al., 2007). With this regard and in any case, developing methods to prevent the disease is more than crucial.

2.3.3 Mastitis detection

There are different management tools available to detect mastitis. During an IMI, pathogens and host immune components are found in milk. Milk analysis is used by producers to identify potentially infected animals. Somatic cell count (SCC), lactate dehydrogenase (LDH), and N-acetyl-β-d-glucosaminidase (NAGase) can be used as diagnostic metabolites. However, Nyman et al., (2016) reported that SCC has the greatest ability to detect IMI.

Once an animal is recognized as being infected, the principal method to identify infecting pathogens is conventional microbiology culture. The main weaknesses associated with this diagnostic tool are the high costs and the extended time period between sampling and results. Mansion-de Vries et al. (2014) compared traditional bacterial culture with on-farm 3M Petrifilm^{TM} 24h culture. They used two different Petrifilm^{TM} to differentiate between gram-positive or gram-negative bacteria infection, therefore allowing for rapid and proper treatment procedures. They concluded that it was a proper alternative method to determinate the most appropriate treatment. Other diagnostic tests including biosensors, immunoassays, enzymatic assays and PCRs are new alternatives that can be used for rapid identification of infecting pathogens (Viguier et al., 2009).

2.3.4 Mastitis treatment and prevention

Once identified, IMI can be cured using antibiotics. Saini et al., (2012a) assessed the use of antimicrobial in Canadian dairy herd and estimated that the national intramammary antimicrobial drug use rate (ADUR) was 5.07 animal defined-daily doses (ADD)/1,000 cow-days. The ADUR for clinical mastitis was 3.52 ADD/1,000 cow-days compared to 1.55 for dry off therapy.

Since cow's mammary gland is highly vulnerable to new infection during early involution (Neave et al., 1950, Smith et al., 1985; Leelahapongsathon et al., 2016), many producers adopted blanket dry cow therapy. It is a common mastitis control program which consists of treating all cows at the end of lactation, regardless of infection status. This method aims at curing existing infection, and preventing new IMI during early involution critical period.

The prophylactic use of antibiotic treatment could explain the result of another Canadian national study, where 20% of *S. aureus*, 18% of *E. coli* and 37% of *Klebsiella* isolates obtained from mastitis cases were resistant to at least one antibiotic and many of them were resistant to

two or more antibiotics (Saini et al., 2012b). According to Oliver et al. (2011), antimicrobial resistance caused by this prevention technique is minimal. However, they did mention the fact that blanket dry cow therapy has low efficiency against coliform bacteria, which are the main pathogens associated with early involution infection (Oliver et al., 2011).

Another downside of this prevention method is the risk of antibiotic contamination in early lactation milk. This is of great importance for short dry period management and/or after premature calving (Santschi and Lefebvre, 2014). Another major concern is probably the consumer perception. Some European countries are severely limiting prophylactic use of antibiotics. Producers can only use selective dry cow treatments; therefore, treating only infected quarters (Swinkels et al., 2015). However, Berry and Hillerton (2002) estimated the reduction of new IMI by 80% when a dry cow therapy is used. Therefore, without a blanket dry cow therapy, non-infected quarters are more vulnerable to new IMI. Consequently, there is an increasing need for effective non-antibiotic IMI prevention treatments.

2.3.5 Alternative treatments for prevention of IMI at dry-off

Internal teat sealants may provide an alternative to protect healthy quarters from invading pathogens during the dry period. Teat sealing is a substitute to prophylactic dry cow therapy and contributes to reduced drug use in dairy herds. Long lasting biocompatible bismuth-based preparation can remain stable in the teat canal during the entire dry period and protect from invading pathogens. Krömker et al. (2014) showed a decrease infection rate to a third of the untreated group, when quarters were treated only with sealant during the dry period. Therefore, the use of teat sealant is beneficial but not fully effective.

Other protection methods against mastitis are external teat sealant and routine iodine based teat dipping. However, Whist et al. (2006) did not observed any effect on infection rate from applying external teat sealant during dry off, while, a significant reduced risk of acquiring clinical mastitis was demonstrated when tie-stall cows were iodine-dipped during early involution. Routine iodine based teat dipping requires extra labor since dry cows teats need to be dipped for several days. As free stall herd proportion increases, it is not a viable long term solution. As a result, there is an interest in developing new antibiotic free dry-off IMI prevention treatments.

2.4 Biological response modifier

Immunomodulation is commonly used to prevent diseases. Various compounds referred as biological response modifier (BRM) or immunomodulators are able to interact with the host immune system. As a result, use of such compounds has beneficial prophylactic or therapeutic effects (Tzianabos, 2000).

The best known BRM are vaccines, which act by stimulating the adaptive immunity. Many researchers evaluated the effect of vaccines on mastitis pathogens. Although some vaccines have the ability to reduce clinical symptoms caused by mastitis; they have limited effects on new IMI prevention (Talbot and Lacasse, 2005). As of today, commercially available vaccines show marginal effects in preventing neither infections of *E.coli* (Tomita et al., 2000) nor *S. aureus* (Tenhagen et al., 2001). Although vaccination is an attractive method to protect against IMI, the perspective of having a vaccine protecting efficiently the mammary gland against most mastitis pathogen is very remoted.

Stimulation of innate immunity may help to protect the mammary gland during critical periods and the use of BRMs that stimulate immunity during early involution has been tested. Oliver and Smith (1982) showed that endotoxin and colchicine infusion at dry-off increase of cow's natural defense components such as phagocytic cells, lactoferrin, and IgG. Shamay et al. (2003) showed similar results with casein hydrolyzates. Dallard et al. (2010) injected a single dose of lipopolysaccharide and cellular fractions of Escherichia coli into the mammary gland at dry-off. As a result, they detected a massive leukocyte migration without severe clinical symptoms, suggesting an increase protection against new IMI. However, in this case, mammary gland involution was not accelerated. The drawbacks of most BRM tested so far is that stimulation of the immune system only last for a short period of time. Ideally, the immunostimulation should last for the whole period of active involution. In addition, a BRM should induce a moderate recruitment of immune cells and be limited to the teat area. Nevertheless, the BRM should resist milk pressure, and not be rejected if milk leakage occurs in the days following drying-off.

2.5 Chitosan

Chitosan is a natural biopolymer made from chitin. It is used in various fields including agriculture, water and waste treatment, food and beverage, cosmetic and toiletries as well as

biopharmaceutics (Rinaudo, 2006). Chitosan has interesting properties such as biocompatibility, biodegradability and bioactivity which enable its usage for medical application (Şenel and McClure, 2004). Moreover, chitosan can be formulated to form a biodegradable hydrogel when injected at body temperature (Han et al., 2004).

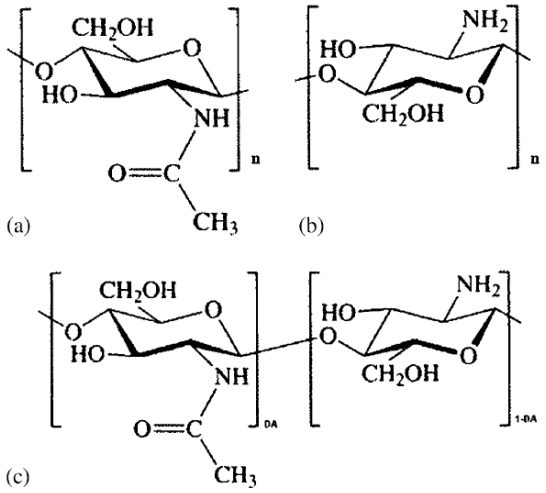
2.5.1 Sources and processing

Chitosan is derived by the deacetylation of chitin, which is the second most abundant polysaccharide in nature after cellulose. The main sources of chitin are crustacean shells, insect exoskeletons and cell walls of fungi. Related organisms produce roughly 10 billion tons of chitin every year (Zargar et al., 2015). The main commercial sources of chitin to produce chitosan are the crab and shrimp canning industry (Rinaudo, 2006). It is an abundant by-product that can be processed at low cost (Han et al., 2004). To extract chitin from crustacean shells, demineralization, deproteination and decolorization are required. At this stage, chitin has poor solubility and low biological activity (Goy et al., 2009). Therefore, a concentrated alkaline solution is added to chitin. As a result, it undergoes partial deacetylation (Zargar et al., 2015). When more than 50% of the acetyl groups are removed from chitin, the compound becomes chitosan (Brugnerotto, 2001).

2.5.2 Structure and formulation

Chitosan is a glucose-based unbranched polysaccharide, consisting in a combination of glucosamine and N-acetyl glucosamine copolymers. Proportion of each copolymer depends on the degree of deacetylation, which represents the amount of amine groups formed from the original number of acytyl groups on chitin (Figure 1) (Martínez-Ruvalcaba, et al., 2007). Chain length varies according to total number of polymers. Consequently, the molecular weight fluctuates between 10,000 and 2 million Dalton (Şenel and McClure, 2004). Like chitin, chitosan is insoluble at alkaline and neutral pH. However, chitosan is soluble in diluted acid with pH below 6.5. After deacetylation, removal of the acetyl groups from the amine radicals (-NH2), amines can be protonated (-NH+3 in acidic condition) and chitosan become a water-soluble cationic polyelectrolyte (Goy et al., 2009; Zargar et al., 2015). Many distinctive properties of chitosan arise from its capacity to be positively charged (Argin-Soysal et al., 2009; Zargar et al., 2015).

Fig. 1 Chemical structure of chitin and chitosan



Chemical structure (a) of chitin poly(N-acetyl-b-Dglucosamine) and
(b) of chitosan (poly(D-glucosamine) repeat units.
(c) Structure of partially acetylated chitosan, a copolymer characterized by its average degree of acetylation DA.

(Rinaudo, 2006)

2.5.3 Properties and applications

Chitosan possesses various physicochemical properties. Because chitosan' solubility is pH dependent, it becomes liquid at pH values below 6.2. However, when pH is increased by a weak base, interchain electrostatic repulsion is reduced and chitosan precipitate into a hydrated gel-like substance (Han et al., 2004). Chenite et al. (2000) were able to prevent immediate precipitation by using β-glycerophosphate as a weak base. Moreover, because of multiple interactions between chitosan, β-glycerophosphate and water, this preparation had thermosensitive gelling properties. Therefore, it can be formulated to be liquid at room temperature and form a hydrogel at body temperature (≈37°C) (Zhou et al., 2015). Han et al. (2004) demonstrated that after subcutaneous injections, this preparation forms a biocompatible and biodegradable hydrogel. They also mentioned chitosan's ability to remain at the site of application due to its bioadhesive properties, as a result of its positive charge.

Chitosan exhibits various biological properties. It has been used for drug formulation over the past 20 years (Şenel and McClure, 2004). Important biological properties include; biocompatibility, bioadhesivity, bioactivity, nontoxicity, biodegradability, absorbability and antimicrobial activity (Zargar et al., 2015). Chitosan has the distinctive ability to enhance local drug delivery by opening epithelial tight junctions (Han et al., 2004).

Although chitosan is biocompatible, it has immuno-stimulating properties (Otterlei et al., 1994). The processes, by which chitosan triggers host immune responses, are still partly understood. One possible explanation is the recognition of chitin and chitosan polymers as foreign molecules by the mammalian innate immune system (Bueter et al., 2013). Chitosan activates both humoral and innate immunity; therefore making it appealing as a vaccine adjuvant (Wen et al., 2011). Moreover, chitosan's ability to stimulate inflammation has enabled its usage to accelerate wound healing (Şenel and McClure, 2004). Ueno et al., (2001) reported that chitosan activates immune cells such as polymorphonuclear leukocytes, macrophage and fibroblasts. Similarly, Otterlei et al. (1994) observed TNF-α production upon activation of monocytes by chitosan. Because chitosan structure is similar to LPS, they suggested that chitosan is involved in CD14 activation. However, response varies according to particle size and degree of acetylation. For example, Bueter et al. (2011) demonstrated a negative correlation between chitosan particle size and macrophage activation. They suggested that phagocytosis of enzyme degraded chitosan particles activates NLRP3 inflammasome, which in turn induce IL-1β production. Activation is also dependent on degree of acetylation. They observed an inhibition of the effect on macrophages after acetylation of chitosan. Chitosan' immune cell activation and proinflammatory cytokine production accelerate wound healing but also increase protection against infections (Şenel and McClure, 2004).

Moreover, chitosan is a good candidate for wound protection because it has antibacterial properties. Since chitosan is positively charged, it disrupts the negatively charged microbial cell wall. Hence, it results in cell leakages (Bégin and Van Calsteren, 1999). Goy et al. (2009) proposed that chitosan can also penetrate in microorganism's cytoplast to prevent mRNA and protein synthesis. Furthermore, they suggested that chitosan can form an external barrier and eliminate microbial access to essential nutrients. Moreover, the lower the molecular weight and degree of acetylation are, the greater is the inhibition of microbial growth and multiplication

(Goy et al., 2009). Finally, chitosan‘s antibacterial activity is pH dependent (Şenel and McClure, 2004). In acidic conditions, protonation of chitosan increases its charge; therefore, increasing its activity (Zargar et al., 2015). Because chitosan provides antibacterial protection and increases immune defense, it has an enormous potential as an alternative to antibiotics (Şenel and McClure, 2004).

CHAPTER 3. MAIN OBJECTIVE, HYPOTHESIS AND SPECIFIC OBJECTIVES

3.1 Main objective

To develop a chitosan-based BRM that could remain in the teat, stimulate an inflow of immune cells and hasten involution to reduce new IMI susceptibility at dry-off.

3.2 Hypotheses of this thesis

- Chitosan hydrogel can be formulated to be liquid at acidic pH and room temperature but to form a gel at body temperature and biological pH

- Chitosan hydrogel will remain in teat cistern during early involution

- The presence of chitosan in the teat cistern will induce a sustained migration of immune cells in the teat via Furstenberg's rosette

- The Chitosan preparations will cause opening of tight junctions and result in hasten involution.

- The combination of chitosan hydrogel and an internal teat sealant does not reduce effects of chitosan on the immune response.

3.3 Specific objectives

Objective 1: to evaluate mammary gland immune response and involution rate when using different chitosan hydrogel formulations at dry-off.

Objective 2: to evaluate the compatibility of most promising chitosan hydrogels with an internal teat sealant

CHAPTER 4. PRELIMINARY EXPERIMENTS

First, chitosan formulations required for our objectives were developed at Agriculture and Agri-Food Canada's St-Hyacinthe Research and Development Centre by Patrick Fustier, Ali Tahérian and Barbara Bisalowski. They developed different chitosan hydrogel formulations which were injectable at acidic pH and room temperature and formed a gel at body temperature and pH

The most promising formulations were selected for in vivo experimentation. An assessment of the acute effect of each selected BRM was carried out in lactating cows. After morning milking, each teat was randomly assigned to a selected chitosan formulation or water (control). Signs of inflammation were monitored for the following days. The afternoon milking was skipped but a sample of milk was collected through a plastic needle. At the following morning, foremilk samples of milk were collected. Cows were milked with a quarter milking machine and additional samples were collected for a total of 7 days. Somatic cell count was determined on the collected milk samples. Chitosan formulations which induced a moderate recruitment of somatic cell in the teat without causing acute symptoms of inflammation on the rest of the mammary gland were selected for subsequent studies.

Table 4: Preliminary experiments resume

Experiment	Cow number	Treatments	Conclusion
1	4	- High viscosity chitosan 1.5% - High viscosity chitosan 2% - Water (Control)	- Chitosan increased somatic cell count for 4 days - No inflammation - Fast degradation of hydrogels - No major differences between concentrations
2	4	- High viscosity chitosan 5% - Complex chitosan:xanthan 3% - Water (Control)	- Complex caused major inflammation - Chitosan increased somatic cell count for 4 days
3	2	- High viscosity chitosan 5% - Low viscosity chitosan 5% - Complex chitosan:PVA - Water (Control)	- Complex caused major inflammation - Chitosan increased somatic cell count for 4 days - No major differences between low and high viscosity chitosan

CHAPTER 5. ARTICLE

Interpretive Summary

Effect of intramammary infusion of chitosan hydrogels on bovine mammary gland involution after drying-off. *By Lanctôt et al.* The transition from lactation to the dry period in dairy cows is a period of high risk for acquiring new intramammary infections. This study aimed to develop a biological response modifier that could be injected into cow teats to speed up involution. Chitosan is natural polysaccharide able to trigger the innate immunity of the host. Our results show that teat infusion with chitosan hydrogel promoted immune cell migration and hastened mammary gland involution. This approach could be used as an alternative to dry-cow antibiotic therapy for uninfected cows.

Effect of intramammary infusion of chitosan hydrogels at drying-off on bovine mammary gland involution

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5.1 Abstract

The transition from lactation to the dry period in dairy cows is a period of high risk for acquiring new intramammary infections. This risk is reduced when the involution of the mammary gland is completed. Accordingly, approaches that speed up the involution process after drying-off could reduce the incidence of mastitis. The current study aimed to develop a biological response modifier that could be injected into cow teats to promote immune cell migration and speed up involution. Chitosan, a natural polysaccharide derived from chitin, is able to trigger host innate immunity. We developed 2 formulations, made from either high- or low-viscosity chitosan. Both are liquid at room temperature but form a hydrogel at body temperature. In the first experiment, each udder quarter of 7 Holstein cows in late lactation was randomly assigned at drying-off to receive one of the following intramammary infusions: 2.5 or 5 mL of 5% (w/v) low-viscosity chitosan hydrogel, 5 mL of 5% high-viscosity chitosan hydrogel, or 5 mL of water. Milk (mammary secretion) samples were collected from each quarter on d −4, −1 (drying-off), 1, 3, 5, 7, and 10. Milk somatic cell counts and the concentrations of involution markers such as BSA, lactate dehydrogenase, and lactoferrin were measured in each sample. In comparison with the control, the chitosan hydrogel infusions significantly hastened the increases in somatic cell counts, BSA and lactoferrin concentrations, and lactate dehydrogenase activity in mammary secretions. No major differences between sources or volumes of chitosan were observed for the measured parameters. The compatibility of this approach with an internal teat sealant was verified in the second experiment. Each udder quarter of 8 Holstein cows was randomly assigned at drying-off to receive one of the following intramammary infusions: 5 mL of 2% low-viscosity chitosan hydrogel, 4 g of an internal teat sealant, a combination of sealant and chitosan, or 5 mL of water. Milk (mammary secretion) samples were collected from each quarter on d −4, −1 (drying-off), 5, and 10 to measure involution markers. These results suggest that chitosan hydrogel infusion hastened mammary gland involution and activate immune response, which may reduce the risk of acquiring new intramammary infections during the drying-off period. Those results were not affected by the presence of the teat sealant, showing that both approaches are fully compatible and could be used in combination.

Key words: mastitis, involution, immunity, chitosan, dairy cow

5.2 Introduction

The lactation cycle of a dairy cow must include a dry period for optimal milk production in the following lactation (Andersen et al., 2005). Although milking cessation is essential for proper cell renewal, during early involution the cow mammary gland is vulnerable to new IMI (Smith et al., 1985; Leelahapongsathon et al., 2016). Even though milking is stopped, high-yielding cows still secrete a significant amount of milk. The pressure buildup causes milk to leak and impairs keratin formation (Dingwell et al., 2004). Once the teat canal is open, microorganisms gain access to the mammary gland and cause infection (Cousins et al., 1980). Moreover, during early involution, the level of antibacterial components and concentration of immune cells in the milk secretions are minimal (Sordillo et al., 1987). Finally, high fat, casein, and lactose concentrations favor bacterial growth and interfere with the phagocytosis capacity of immune cells (Sordillo and Nickerson, 1988). Therefore, implementing an efficient mastitis prevention program during this period is crucial.

An important element of many mastitis control programs is the treatment of all cows with antibiotics at the end of lactation, regardless of the cows' infection status (Berry and Hillerton, 2002). Although this method aims to cure existing infections and prevent new IMI during the dry period, it is not equally effective against all pathogens (Oliver et al., 2011). The major concern, perhaps, is consumer perception. As a result, Germany and the Netherlands have prohibited the prophylactic use of antibiotics in livestock, such that only cows with IMI can be treated (Swinkels et al., 2015). Consequently, there is an increasing need for effective nonantibiotic IMI prevention treatments. Internal teat sealants could provide alternatives to dry-cow therapy. However, despite their benefits, the inert bismuth-based preparations are not totally effective (Krömker et al., 2014). Another proposed alternative to blanket dry-cow therapy is external teat-dipping regimens using iodine-based teat dips at drying-off. Despite their effectiveness in tie-stall herds, these regimens are labor intensive and difficult to implement in free-stall operations (Whist et al., 2006).

When early involution of the mammary gland is completed, the risk of acquiring a new IMI is minimal (Tatarczuch et al., 2002). Consequently, Oliver and Smith (1982) proposed that accelerating the involution process after drying-off could enhance the resistance of the mammary gland to new IMI during early involution. This acceleration can be achieved by using a biological response modifier (BRM) (Tzianabos, 2000). Biological response modifier (BRM) or

immunomodulators are able to interact with the host immune system. As a result, use of such compounds has beneficial prophylactic or therapeutic effects (Tzianabos, 2000).

However, the effect of the BRMs tested so far are of short duration (Shamay et al., 2003; Dallard et al., 2010). Chitosan is a natural biocompatible polysaccharide derived by the partial deacetylation of chitin, which is the second most abundant polysaccharide in nature after cellulose (Rinaudo, 2006). Chitosan can be formulated to be injectable at room temperature but form a biodegradable hydrogel at body temperature (Chenite et al., 2000). Chitosan exhibits various biological properties. It has been used for drug formulation over the past 20 years. Moreover, chitosan has bacteriostatic, bioadhesive, and bioactive properties (Şenel and McClure, 2004).

After drying-off, the permeability of tight junctions between epithelial cells increases, which allows paracellular transport between the interstitial space and milk (Nguyen and Neville, 1998). This transport can be assessed by measuring concentration of serum albumin and immunoglobulin in milk secretions (Hurley, 1989). Furthermore, the regression of mammary secretory tissue is accompanied by changes in milk secretion composition that occur gradually during early involution (Oliver and Sordillo, 1989). For instance, epithelial cells produce more lactoferrin as involution progresses (Capuco and Akers, 1999). Accordingly, milk secretion concentration of those markers is used to assess mammary gland involution progress.

The present study aimed to develop a chitosan-based BRM formulation that could be injected into the cow teat to promote sustained immune cell migration and hasten involution at drying-off. In addition, the effect of combining the chitosan-based BRM with an internal teat sealant was evaluated.

5.3 Materials and methods

5.3.1 Preparation of treatments

All treatments were prepared with aseptic, nonpyrogenic products and materials under a laminar flow hood. For each concentration of chitosan (2% and 5% wt/vol), a 200-mL solution was made by adding 120 mL of nonpyrogenic water (<0.005 endotoxin units/mL; Lonza, Walkersville, MD) to preweighed chitosan. The solution was agitated at 200 rpm with a metal mixing rod. The pH of the solution was reduced to 3 via the addition of 0.1 *M* HCl (Sigma-Aldrich Co., St. Louis, MO). The sample was kept overnight at room temperature for complete

hydration. The following day, the sample pH was brought up to 6.8 using a 50% (wt/vol) β-glycerophosphate disodium salt hydrate (Sigma-Aldrich Co.) solution. Then, the volume was adjusted to 200 mL by the addition of nonpyrogenic water (Lonza). Finally, plastic syringes were filled with the desired volume, sealed with a cap, and stored at room temperature. We developed 2 formulations, using either high-viscosity (130-cP) or low-viscosity (90-cP) chitosan provided by Qingdao Yuda Century Economy and Trade Co. (Shibei District, Qingdao, China). The chitosan supplier company provided a certificate of analysis for each chitosan we used. This document confirmed that chitosan is free of salmonella or coliform, that it contains less than 1 ppm of heavy metals and less than 1% of ash and of insoluble.

5.3.2 Animals and experimental design

The experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care (1993). The cows were housed in individual tie stalls at Agriculture and Agri-Food Canada's Sherbrooke Research and Development Centre (Sherbrooke, QC, Canada).

Experiment 1. Seven Holstein cows in late lactation (319 ± 29 DIM at drying off) producing more than 15 kg (average 22.6 ± 1.9) of milk per day were used. Cows were milked twice a day and projected or real 305 days milk production was 9312 ± 749 kg. The group of cow was dryied off at the same time, 90 ± 17 days before expected calving date. Prior to dry-off (d –4), quarter SCC averaged 122693 ± 34520 cell/mL. Until dry-off, the cows were fed ad libitum a late-lactation diet. After drying off, the cows were fed ad libitum a dry period diet and dry hay. Water was available ad libitum during the whole experiment.

At drying-off, each udder quarter was randomly assigned to 1 of 4 intramammary infusions, as follows: 5 mL of nonpyrogenic water (Lonza) (**CTRL**; n = 7), 2.5 mL of 5% (w/v) low-viscosity chitosan solution (**LV2.5**; n = 7), 5 mL of 5% low-viscosity chitosan solution (**LV5**; n = 7), or 5 mL of 5% high-viscosity chitosan solution (**HV5**; n = 7). Before the infusions, the teats were compressed at the top to keep the infused preparation in the teat.

Milk samples (200 mL) were manually collected from each quarter just before the morning milking on d −4 relative to drying-off as well as just before the last milking before drying-off (d −1). Mammary secretions from each quarter (100 mL) were manually collected aseptically (National Mastitis Council, 1996) on d 1, 3, 5, 7, and 10 after the last milking. The samples were used to measure SCC, the proportion of somatic cell types, and bacterial counts. Skim milk and somatic cells were separated by centrifugation (1,000 × *g*, 4°C, 20 min). Skim

milk aliquots were stored at −20°C until determination of BSA concentration, lactate dehydrogenase (**LDH**) activity, and lactoferrin concentration. Pelleted somatic cells were washed with 10 mL of PBS (Mediatech, Manassas, VA) and centrifuged (500 × *g*, 4°C, 10 min). The PBS was discarded, and the cell pellet was suspended in 250 µL of PBS. The samples were then stabilized in 1 mL of RNAlater (Sigma-Aldrich Co.) and stored at −80°C until RNA extraction

Experiment 2. Eight Holstein cows in late lactation (328 ± 17 DIM at drying off) producing more than 15 kg (average 20.5 ± 1.1) of milk per day were used. Cows were milked twice a day and projected or real 305 days milk production was 10881 ± 1359 kg. The group of cow was dryied off at the same time, 62 ± 4 days before expected calving date. Prior to dry-off (d –4), quarter SCC averaged 87 654 ± 23 287cell/mL.

At drying-off, each udder quarter was randomly assigned to 1 of 4 intramammary infusions, as follows: 5 mL of nonpyrogenic water (Lonza) (n = 8), 5 mL of 2% (wt/vol) low-viscosity chitosan solution (n = 8), 4 g of Orbeseal teat sealant solution (Zoetis, Kirkland, QC, Canada) (n = 8), or 4 g of Orbeseal teat sealant solution followed by 5 mL of 2% low-viscosity chitosan solution (n = 8).

Milk samples (200 mL) on d −4 and −1 and mammary secretions on d 5 and 10 were collected, prepared, and stored as described for experiment 1.

5.3.3 Animal evaluation

In both experiments, the quarters were assessed for inflammation symptoms every 2 h for the first 12 h after the infusions and then 3 times per day (0900, 1300, and 1900 h) for the following 7 d. Inflammation was scored from 1 to 6 according to the mammary gland chart created by Rambeaud et al. (2003), as follows: 1 = normal; 2 = slight swelling; 3 = moderate swelling; 4 = severe swelling; 5 = scar tissue; and 6 = edema. Rectal temperature was determined at the same time.

5.3.4 SCC in milk and mammary secretions

Somatic cell counts were determined from fresh whole milk samples and mammary secretion samples using an automatic cell counter (DeLaval International AB, Tumba, Sweden). Samples of mammary secretions were diluted with commercial microfiltered skim milk until the SCC obtained was between 100 and 200 cells/µL.

5.3.5 Identification of somatic cells by flow cytometry

Seven-color immunophenotyping of somatic cells was performed on samples collected on d −1 and 1 of experiment 1 and on d −1 and 5 of experiment 2. Milk samples (20 mL) were diluted with 20 mL of PBS 1X and centrifuged (1,000 × *g*, 23°C, 15 min). The supernatant was removed, and the pellet was resuspended in 15 mL of washing buffer consisting of PBS 1X + 1% BSA (Sigma-Aldrich Co.) + 2% normal goat serum (Meridian Life Sciences, Memphis, TN). The mixture was then centrifuged (500 × *g*, 4°C, 10 min). Cell washing was repeated with 25 mL of washing buffer until no more fat could be observed. Washing buffer was added to the cell pellet to reach a concentration of approximately 1×10^7 somatic cells/mL. A control pool was made with 100 µL from each sample. A 100-µL volume from each sample and the pool were transferred into a 96-well round bottom plate. The plate was centrifuged (300 × *g*, 4°C, 5 min), and the supernatant was removed. The cells were suspended with 100 µL of washing buffer containing the primary antibodies listed in Table 5. The plate was incubated on ice in the dark for 25 min. The cells were then washed 3 times with washing buffer. The plate was centrifuged (300 × *g*, 4°C, 3 min), and the cells were resuspended with 100 µL of washing buffer containing the secondary antibodies mix (Table 5). The plate was incubated again on ice in the dark for 25 min, and the cells were washed 3 times. The cells were resuspended in 200 µL of washing buffer.

The samples were analyzed immediately on a BD FACSCanto II flow cytometer (BD Biosciences, Mississauga, ON, Canada) equipped with 3 lasers in a 4-2-2 configuration. The BD FACSDiva version 8.0.1 operating software (BD Biosciences) was used for data acquisition and data analysis. The proportion of each somatic cell type was determined using distinctive receptors found on granulocytes, monocytes, and lymphocytes (Table 5). Then, subclasses of T-lymphocytes and non-T-lymphocytes were assessed by using other specific receptors. During the design of the experiment, fluorescent probes were selected to minimize the amount of fluorescence compensation to be done inside the different types of cells analyzed. Before the beginning of the experiment, each primary antibody was titered and tested for cross-reactivity with secondary antibodies. None of the primary antibodies showed cross-reactions or unspecific binding of secondary antibodies. Finally, a single-stain marker and FMO (Full Minus One cocktail) were used to determine all gates.

5.3.6 Bacterial count determination in milk and mammary secretions

For bacterial count determination, milk and mammary secretion samples were plated just after collection on tryptic soy agar, mannitol salt agar, and MacConkey II agar (Becton, Dickinson and Company, Mississauga, ON, Canada). The plates were then incubated at 37°C for 24 h before colonies were counted. Cow udder quarters infected with pathogens were excluded from the experiment. Accordingly, on experiment 1, data from one quarter treated with 2.5 mL of 5% low-viscosity chitosan solution were not used on d 5, 7 and 10.

5.3.7 BSA concentration in milk and mammary secretions

The concentration of BSA in milk and mammary secretion samples was evaluated by a colorimetric assay as previously described by Bouchard et al. (1999), with some modifications. Briefly, 200 µL of a skimmed milk sample was mixed with 450 µL of water and 450 µL of a solution containing 1 volume of 1.2 m*M* bromocresol green dissolved in 5 m*M* NaOH, 3 volumes of 0.2 *M* succinic acid (pH 4.0), and 0.8% Brij-35 detergent. The sample was then mixed by inversion and centrifuged at room temperature (1,900 × *g*, 10 min). The optical density of the supernatant was read at 640 nm using a SpectraMax 250 microplate reader (Molecular Devices, Sunnydale, CA). For experiment 1, the intra- and interassay coefficients of variation were 4.6% and 7.6%, respectively. For experiment 2, the intra- and interassay coefficients of variation were 1.1% and 3.1%, respectively.

5.3.8 LDH activity in milk and mammary Secretions

The LDH assay was performed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI) according to the manufacturer's instructions. For experiment 1, the intra- and interassay coefficients of variation were 1.8% and 3.5%, respectively. For experiment 2, the intra- and interassay coefficients of variation were 2.3% and 4.8%, respectively.

5.3.9 Lactoferrin concentration in milk and mammary secretions

The concentration of lactoferrin in the skim milk and mammary secretions was measured by ELISA using a commercial bovine lactoferrin ELISA quantitation set (Bethyl Laboratories Inc., Montgomery, TX). For experiment 1, the intra- and interassay coefficients of variation were 4.6 and 5.9%, respectively. For experiment 2, intra- and interassay coefficients of variation were 4.5 and 6.3%, respectively.

5.3.10 Real-Time PCR

Total RNA was extracted from somatic cells (−80°C samples) using the PureLink RNA Mini Kit and TRIzol RNA isolation reagents (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The extraction process included on-column digestion with PureLink DNase (Life Technologies) to eliminate possible DNA contamination. The concentration and purity of the RNA were evaluated by spectrophotometric analysis using an ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE), and RNA integrity was assessed with an Agilent 2100 Bioanalyzer system (Agilent Technologies, Waldbronn, Germany) using an Agilent RNA 600 Nano kit (Agilent Technologies) according to the manufacturer's instructions. Samples containing less than 31.25 ng/µL were concentrated with RNA Clean & Concentrator-5 (Zymo Research, Irvine, CA) according to the manufacturer's protocol. Reverse transcription was performed using TransScript First-Strand cDNA Synthesis Super Mix (TransGen Biotech, Beijing, China) according to the manufacturer's protocol. From the resulting cDNA, 3.5 µL of each sample was used to make a pool (116 samples from this study). The remaining cDNA was diluted 1:15 in water. A mixture of 3 µL of cDNA, 5 µL of Fast SYBR Green PCR Master Mix (Applied Biosystems Inc., Foster City, CA), and 2 µL of primers (Applied Biosystems Inc.) was used for amplification and quantification. The primer concentrations are presented in Table 6. The PCR conditions consisted of denaturation at 95 °C for 20 s and then 40 cycles of amplification at 95°C for 3 s and 60°C for 30 s. The samples were quantified with standard curve experiments run on a StepOnePlus real-time PCR system (Applied Biosystems Inc.) using a standard curve derived from a serial dilution of the pool. The genes *ACTB* (actin, beta), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *PPIA* (peptidylprolyl isomerase A), and *YWHAZ* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta) were tested as potential housekeeping genes for the normalization of gene expression using NormFinder software (Andersen et al., 2004). For experiment 1, expression of the *PPIA* and *YWHAZ* genes showed less variability between treatments, and those genes were therefore selected as the housekeeping genes. For experiment 2, *GAPDH* and *YWHAZ* were selected as the housekeeping genes. The normalized values were obtained from the ratio of the expression of the gene of interest to the geometric mean of the respective housekeeping genes.

5.3.11 Statistical analysis

Data were analyzed by ANOVA using the MIXED procedure of SAS software (SAS Institute Inc., Cary, NC). For experiment 1, time was used as a repeated effect, and treatment(cow) was used as the subject. Orthogonal contrasts were performed to compare the effect of each treatment to that of the control. Other treatment comparisons were performed using the Tukey–Kramer adjustment. For experiment 2, data were analyzed as a factorial design with chitosan and teat sealant as main factors. Time was used as a repeated effect, and sealant*chitosan(cow) was used as the subject. When variances were not homogeneous, data were $\log_{10}$-transformed prior to analyses. Differences were considered statistically significant when $P \leq 0.05$ and considered a trend when $P < 0.1$.

5.4 Results

5.4.1 Experiment 1

The udder quarter inflammation scores for the periods from 0 to 24 h, 25 to 48 h, and 49 to 170 h after the infusions are presented in Table 7. In the period from 0 to 24 h, the average inflammation scores were slightly greater in the quarters treated with LV2.5 ($P < 0.01$) and LV5 ($P < 0.01$) than in the control quarters. Conversely, the quarters treated with HV5 were not significantly ($P > 0.1$) different from the control quarters in terms of inflammation scores. No differences in inflammation scores were observed after 24 h ($P > 0.05$).

The SCC, LDH activity, and BSA and lactoferrin concentrations measured in milk and mammary secretions are presented in Figure 1. No differences were observed between the quarters for all these parameters during the pretreatment period (on d −4 and −1) ($P > 0.1$). All measured markers increased from the day of drying-off (d −1) to d 10 ($P < 0.001$), regardless of the treatment. However, all parameters showed a treatment × time interaction ($P < 0.001$). The concentration of BSA was greater ($P < 0.001$) on d 1 to 5 in the quarters treated with chitosan than in the control quarters. Similarly, lactoferrin concentration in all the chitosan-treated quarters was greater on d 3 ($P < 0.001$) and 5 ($P < 0.01$) in comparison with the control quarters. On d 1 to 7, LDH activity was greater in the quarters treated with chitosan ($P < 0.01$) than in the control quarters. Somatic cell count was greater ($P < 0.001$) on d 1 to 5 in all the chitosan-treated quarters than in the control quarters. Except on d 1, when the SCC in the LV-treated quarters was greater in comparison with that of the HV5-treated quarters ($P < 0.01$), there were no differences between the chitosan-treated quarters for all parameters.

The proportions of monocytes, granulocytes, and lymphocytes plus other cell types in milk are presented in Table 8. The proportion of monocytes decreased ($P < 0.001$) and that of granulocytes increased ($P < 0.001$) after drying-off. There was no effect of treatments on the proportion of these cell populations.

The expression of key immune regulators—CXCL8 [chemokine (C X C motif) ligand 8], CCL2 [chemokine (C C motif) ligand 2], TNF (tumor necrosis factor), CD14 (CD14 molecule), and IL1β (Bos taurus interleukin 1 beta)—by somatic cells was determined on d 1, 3, and 5 after the treatments, and the results are presented in Table 5. We observed a treatment × day interaction ($P < 0.01$) for the expression of all genes. Gene expression was greater on d 1 than on d 3 for CXCL8 ($P < 0.001$), TNF ($P = 0.09$), and IL1β ($P = 0.04$). In comparison with d 5, gene expression on d 1 was greater ($P < 0.01$) for CXCL8, CCL2, TNF, CD14, and IL1β. On d 1, the quarters treated with chitosan had greater expression of CXCL8 ($P < 0.001$), CCL2 ($P < 0.01$), TNF ($P < 0.001$) CD14 ($P < 0.001$), and IL1β ($P < 0.01$) than the control quarters. On d 3, the quarters treated with chitosan had greater expression of TNF ($P < 0.01$) than the control quarters. The quarters treated with chitosan had greater expression of CXCL8 ($P = 0.02$), and tend to have greater expression of TNF ($P = 0.09$), and CD14 ($P = 0.08$) on d 5 in comparison with the control quarters. The quarters treated with HV5 had greater expression of CXCL8 ($P < 0.01$), CCL2 ($P < 0.01$), TNF ($P < 0.01$), CD14 ($P = 0.09$), and IL1β ($P = 0.06$) on d 1, of TNF ($P < 0.01$) and CD14 ($P < 0.001$) on d 3, and IL1β ($P < 0.01$) on d 5 in comparison with the low viscosity-treated quarters. The quarters treated with LV5 had greater expression of IL1β ($P < 0.01$) on d 5 than those treated with LV2.5.

5.4.2 Experiment 2

The udder quarter inflammation scores were increased ($P < 0.01$) by chitosan during the first period, from 0 to 24 h after the treatments (Table 10). Sealant did not have an effect or interact ($P > 0.1$) with chitosan on inflammation scores. Sealant and chitosan did not affect ($P < 0.1$) inflammation scores after the first day.

The immune and involution markers measured in milk and mammary secretions are presented in Figure 2. During the pretreatment period (on d −4 and −1), no differences were observed between the udder quarters for all these parameters. Additionally, in every quarter, all measured markers increased from the day of drying-off (d −1) to d 10 ($P < 0.001$). Nevertheless, we observed a chitosan × time interaction for BSA concentration ($P < 0.01$), lactoferrin

concentration (P = 0.06), LDH activity (P < 0.001), and SCC (P < 0.001). On d 5, milk from the chitosan-treated quarters had greater BSA (P < 0.01), lactoferrin (P = 0.001), LDH (P < 0.0001), and SCC (P < 0.001) values than the quarters without chitosan. The infusion of sealant did not have an effect (P > 0.1) or interact with chitosan on any of these markers.

The expression of key immune regulators (*CXCL8*, *CCL2*, *TNF*, *CD14*, and *IL1β*) by somatic cells was determined on d 5 after the treatments, and the results are presented in Table 8. Chitosan increased expression of the somatic cell genes *CXCL8* (*P* < 0.001), *CCL2* (*P* < 0.001), and *IL1β* (*P* < 0.01). However, gene expression of *TNF* and *CD14* was not affected by chitosan. The infusion of sealant did not have any effect (*P* > 0.1) or interact with chitosan on any of these genes.

5.5 Discussion

In both of our experiments, the chitosan hydrogel infusions significantly hastened the increase in both BSA and lactoferrin concentrations in mammary secretions after drying-off. Our results demonstrate that the intramammary infusion of chitosan hydrogel at drying-off disrupted tight junction integrity and changed the cells' secretory state, therefore hastening the involution process. These results are consistent with a previous report suggesting that chitosan has the ability to alter tight junctions (Yeh et al., 2011).

Lactoferrin also acts as an immune factor that protects the mammary gland. Lactoferrin disrupts the outer membrane of gram-negative bacteria but also binds iron, making it unavailable for iron-dependent bacteria (Ellison et al., 1988). Given that the concentration of lactoferrin increased faster in the mammary secretions of the chitosan-treated cows, mammary gland defense may be improved by the infusion of chitosan hydrogel.

In this study, we also assessed mammary epithelium integrity by measuring the activity in mammary secretions of LDH released by damaged cells. In both experiments, we observed a transient increase in LDH activity as involution progressed. Moreover, all chitosan hydrogel infusions significantly hastened the increase in LDH activity after drying-off. In experiment 1, the 3 chitosan formulations resulted in similar effects on LDH activity. The increase in milk LDH activity suggests a cytotoxic effect of chitosan on mammary epithelium. Symons and Wright (1974) reported that elevated milk LDH above serum levels found in mastitis milk was released from damaged mammary epithelium caused by endotoxin. However, according to Kato

et al. (1989), leukocytes also participate in the increase in milk LDH activity during mastitis. This effect can be explain by the high LDH activity, 1,000 U/mg of protein, of leukocytes.

The number of somatic cells increases in mammary secretions during the early involution period (Jensen and Eberhart, 1981). There is evidence that immune cells play an important role in mammary gland defense, particularly at the Furstenberg's rosette, which is a structure strategically located at the internal end of the streak canal. At this location, immune cells leave the teat wall and enter the teat cistern to intercept bacteria before they reach the mammary gland (Nickerson and Pankey, 1983). The intramammary administration of proinflammatory agents increases the number of somatic cells in the gland (Oliver and Sordillo, 1989; Wedlock et al., 2004). In both of our experiments, SCC in mammary secretions increased faster in all the chitosan-treated quarters than in the control quarters. In experiment 1, we observed a significant increase in SCC in the chitosan-treated quarters only 24 h after the treatment was administered. Moreover, in both experiments, the elevated SCC in the mammary secretions from the chitosan-treated quarters were maintained throughout the experimental period. These results are consistent with the literature reports of chitosan's immunostimulating properties (Otterlei et al., 1994; Wen et al., 2011).

The SCC in mammary secretions increases during an immune response as well as during involution. In both cases, the proportions of somatic cell types also change (Hurley, 1989; Sordillo and Streicher, 2002). In both of our experiments, the proportion of polymorphonuclear neutrophils increased after drying-off. During the first 3 to 7 d of drying-off, polymorphonuclear neutrophils are the main types of leukocytes entering the mammary gland (Hurley, 1989). Similarly, during an infection, the proportion of polymorphonuclear neutrophils increases drastically and can reach 90% of leukocytes (Sordillo and Streicher, 2002). However, there was no treatment effect on somatic cell types for both experiments. These results confirm that chitosan has the ability to activate immune pathways that accelerate the infiltration of multiple types of inflammatory cells simultaneously (Ueno et al., 1999).

To provide optimal protection against pathogens, the mammary gland immune system needs to be activated (Sordillo and Streicher, 2002). Once that activation has occurred, the expression of immunoregulatory genes by mammary immune and epithelial cells increases. As a result, those cells produce and release proinflammatory cytokines that bind to macrophage and neutrophil receptors and increase their bactericidal capacity (Oviedo-Boyso et al., 2007). In both

of our experiments, the chitosan hydrogel infusions increased the expression of immunoregulatory genes by somatic cells. In experiment 1, gene expression was measured on the first samples harvested 24 h after the treatments were administered. At this time, we observed elevated expression of the genes CXCL8, CCL2, TNF, CD14, and IL1β caused by the chitosan hydrogels. On the following days (d 3 and 5), the treatment effect on immune gene expression was decreased. In experiment 2, gene expression was measured on samples harvested 5 d after the treatments were administered. At this time, expression of the genes CXCL8, CCL2, and IL1β was still greater in the chitosan-treated udder quarters.

The processes by which chitosan triggers host immune responses are still only partly understood. One possible explanation is the recognition of chitin and chitosan polymers as foreign molecules by the mammalian innate immune system (Bueter et al., 2013). Otterlei et al. (1994) observed TNF α production upon activation of monocytes by chitosan. Because chitosan structure is similar to that of LPS, those authors suggested that chitosan is involved in CD14 activation. Bueter et al. (2011) suggested that the phagocytosis of enzyme-degraded chitosan particles activates the NLRP3 inflammasome, which in turn induces IL1β production. Overall, chitosan's immunomodulatory properties activate immune cells and accelerate proinflammatory cytokine production and, therefore, should increase protection against infections (Şenel and McClure, 2004).

Ideally, a BRM should induce the moderate recruitment of immune cells into the teat without causing acute inflammation symptoms. In experiment 1, quarters treated with low-viscosity chitosan showed transient signs of inflammation during the first 24 h following the administration of the treatments. Therefore, for experiment 2 we used the same chitosan but at a lower concentration. Similarly, during that experiment, the inflammation scores during the period from 0 to 24 h of the quarters treated with chitosan were greater than those scores of the quarters without chitosan. The injected chitosan dosage that was used in our study resulted in transient signs of inflammation similar to those observed during the infusion of other BRMs (Oliver and Smith, 1982; Dallard et al., 2010). Nevertheless, in all cases in our study, inflammation symptoms were mild and transient.

Teat sealing is a substitute for prophylactic antibiotic dry-cow therapy. Long-lasting, biocompatible, bismuth-based preparations can remain stable in the teat canal throughout the dry period and act as a physical barrier against invading pathogens. Krömker et al. (2014) showed a

decrease in infection rate to a third of that of the control quarters when quarters were treated with sealant only during the dry period. Therefore, the use of teat sealant is beneficial but not fully effective. In experiment 2, the sealant did not affect involution and immune response markers but also did not alter the effect of the chitosan hydrogel when both substances were injected into the same quarter. Thus, both approaches are fully compatible and could be used in combination.

The results of the present study suggest that a chitosan hydrogel infusion activates innate immune response and hastens the involution process of the mammary gland. Ollier et al. (2014, 2015) demonstrated that prolactin-release inhibition hastens mammary involution, resulting in reduced susceptibility to IMI. Although this effect needs to be evaluated, the administration of chitosan hydrogel at drying-off could also reduce the incidence of new cases of IMI during the dry period. Ultimately, this approach could be used as an alternative to dry-cow antibiotic therapy for uninfected cows.

5.6 Acknowledgments

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5.7 Tables

Table 5. Antibodies used for somatic cell identification

Targeted cells	Receptor/ marker	Secondary marker	Clone	Isotype	Secondary antibody	Fluorochrome	Concentration (µg/mL)	Company
Granulocytes	Pan-granulocyte		CH138 A	IgM			10	WSU Monoclonal Antibody Center, Pullman, WA
					Rat anti-mouse-IgM	PE/Cy7	0.2	SouthernBiotech, Birmingham, AL
Monocytes/ macrophages	$CD14^+$		M5E2	IgG2a		PE/Cy5.5	15	BioLegend, San Diego, CA
T-lymphocytes	$CD3^+$		MM1A	IgG1			10	WSU Monoclonal Antibody Center, Pullman, WA
					Rat anti-mouse-IgG1	rPE	0.2	SouthernBiotech, Birmingham, AL
T-lymphocytes (subpopulation)	$CD3^+$	$CD4^+$	CC8	IgG2a		FITC	7.5	AbD Serotec, Raleigh, NC
		$CD8^+$	CC63	IgG2a		AlexaFluor 647	3.75	AbD Serotec, Raleigh, NC
		Gamma-delta	GB21A	IgG2b			5	WSU Monoclonal Antibody Center, Pullman, WA
					Goat anti-mouse IgG2b	APC/Cy7	0.5	SouthernBiotech, Birmingham, AL
Non-T-lymphocytes (gamma-delta)	$CD3^-$, $CD14^-$	Gamma-delta	GB21A				5	WSU Monoclonal Antibody Center, Pullman, WA
					Goat anti-mouse IgG2b	APC/Cy7	0.5	SouthernBiotech, Birmingham, AL
Non-T-lymphocytes (B-lymphocytes)	$CD3^-$, $CD14^-$	B-cell receptor			Goat anti-bovine IgG (H+L)	Dylight405	3	Jackson ImmunoResearch, West Grove, PA

Table 6. Primers used during real-time PCR

Gene[1]	GenBank number[2]	Hybridization	Primers (5′–3′)	Primers[3] (n*M*)	Amplicon length (bp)
ACTB	NM_173979[a]	F 1051	TGGCACCCAGCACAATGA	300/300	123
		R 1173	CCTGCTTGCTGATCCACATCT		
CCL2	NM_174006[b]	F 222	CCTAAAGAGGCTGTGATTTTCAAGACC	300/50	142
		R 363	TGGGTTGTGGAGTGAGTGCTC		
CD14	NM_174008[b]	F 20	AAAGAATCCACAGTCCAGCCGA	300/50	145
		R 164	GCTCGCAGGGTTCTGTTGTG		
CXCL8	NM_173925[a]	F 239	GAGAGTGGGCCACACTGTGAA	300/300	116
		R 354	TTCACAAATACCTGCACAACCTTCT		
GAPDH	NM_001034034[b]	F 513	GCCTCCTGCACCACCAACT	300/50	113
		R 625	TCTTCTGGGTGGCAGTGATG		
IL1β	NM_174093[a]	F 437	AAACTCCAGGACAGAGAGCAAAA	300/300	126
		R 562	CTCTCCTTGCACAAAGCTCATG		
PPIA	NM_178320[a]	F 317	ATGCTGGCCCCAACACAA	300/300	101
		R 417	CCCTCTTTCACCTTGCCAAA		
TNF	NM_173966[a]	F 408	GCCCTCTGGTTCAAACACTCA	300/50	127
		R 534	TGAGGGCATTGGCATACGA		
YWHAZ	NM_174814[a]	F 530	AATGCAACCAACACATCCTATCAG	300/300	131
		R 660	GTTCAGCAATGGCTTCATCAAAT		

[1]*ACTB* = actin, beta; *CCL2* = chemokine (C-C motif) ligand 2; *CD14* = CD14 molecule; *CXCL8* = chemokine (C-X-C motif) ligand 8; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *IL1β* = *Bos taurus* interleukin 1 beta; *PPIA* = peptidylprolyl isomerase A; *TNF* = tumor necrosis factor; *YWHAZ* = tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta.

[2]Primers were either a) taken from Dudemaine et al. (2014) or b) designed using the Ensembl gene browser (Yates et al., 2016) following the primer design of Brosseau et al. (2010) (website: http://test.lgfus.ca/cgi-bin/designs/index.pl).

[3]Primer concentrations ranging from 50 to 900 n*M* were tested during optimization reactions.

Table 7. Average inflammation scores for 3 periods (0–24 h, 25–48 h, and 49–170 h) after the infusion of dairy cow udder quarters at drying-off with 5 mL of 5% low-viscosity chitosan solution (LV5; n = 7), 2.5 mL of 5% low-viscosity chitosan solution (LV2.5; n = 7), 5 mL of 5% high-viscosity chitosan solution (HV5; n = 7), or water (control, CTRL; n = 7). Data are presented as least squares means

	Treatment					*P*-value		
Period	LV2.5	LV5	HV5	CTRL	SEM	LV2.5 vs. CTRL	LV5 vs. CTRL	HV5 vs. CTRL
0–24 h	1.39	1.39	1.13	1.02	0.07	<0.01	<0.01	0.31
25–48 h	1.28	1.09	1.11	1.24	0.05	0.63	0.06	0.09
49–170 h	1.11	1.11	1.05	1.16	0.06	0.52	0.51	0.21

Table 8. Percentages of somatic cell types (monocytes, granulocytes, lymphocytes + others) before (d −1) and after (d 1) the infusion of dairy cow udder quarters at drying-off with 5 mL of 5% low-viscosity chitosan solution (LV5; n = 6), 2.5 mL of 5% low-viscosity chitosan solution (LV2.5; n = 6), 5 mL of 5% high-viscosity chitosan solution (HV5; n = 6), or water (control, CTRL; n = 6). Data are presented as least squares means

	Day −1				Day 1					*P*-value		
Cell type	LV2.5	LV5	HV5	CTRL	LV2.5	LV5	HV5	CTRL	SEM	TRT[1]	Day	TRT*Day
Monocytes	32.4	30.6	26.9	33.8	25.8	23.6	18.9	16.4	3.7	0.46	<0.001	0.34
Granulocytes	44.3	45.6	52.7	43.9	50.2	62.4	60.6	67.0	6.3	0.50	<0.001	0.46
Lymphocytes + others	23.3	23.9	20.3	22.4	24.1	14.0	20.5	16.6	3.3	0.53	0.12	0.31

[1]TRT = treatment.

Table 9. Normalized expression in milk of the somatic cell genes *CXCL8* [chemokine (C-X-C motif) ligand 8], *IL1β* (*Bos taurus* interleukin 1 beta), *TNF* (tumor necrosis factor), *CCL2* [chemokine (C-C motif) ligand 2], and *CD14* (CD14 molecule) on d 1, 3, and 5 after the infusion of dairy cow udder quarters at drying-off with 5 mL of 5% low-viscosity chitosan solution (LV5; n = 7), 2.5 mL of 5% low-viscosity chitosan solution (LV2.5; n = 7), 5 mL of 5% high-viscosity chitosan solution (HV5; n = 7), or water (control, CTRL; n = 7). Data are presented as least squares means of log_{10}-transformed values. Different letters indicate significant differences ($P < 0.05$) among treatments

		Treatments					*P*-value		
	Genes	LV2.5	LV5	HV5	CTRL	SEM	CHI[1] vs. CTRL	HV[2] vs. LV	2.5 mL[3] vs. 5 mL
Day 1	*CXCL8*	0.61^{a}	0.69^{a}	1.03^{a}	0.07^{b}	0.10	<0.001	<0.01	0.61
	IL1β	0.57^{a}	0.35^{ab}	0.75^{a}	0.27^{b}	0.11	<0.01	0.06	0.21
	TNF	0.34^{b}	0.48^{a}	0.74^{a}	0.12^{b}	0.09	<0.001	<0.01	0.25
	CCL2	0.31^{b}	0.43^{b}	0.79^{a}	0.17^{b}	0.08	<0.01	<0.01	0.41
	CD14	0.42^{a}	0.47^{a}	0.52^{a}	0.18^{b}	0.04	<0.001	0.09	0.41
Day 3	*CXCL8*	0.34	0.29	0.32	0.3	0.10	0.88	0.98	0.75
	IL1β	0.24	0.41	0.28	0.27	0.06	0.67	0.62	0.13
	TNF	0.31^{ab}	0.29^{ab}	0.51^{a}	0.16^{b}	0.06	<0.01	<0.01	0.78
	CCL2	0.44	0.27	0.46	0.19	0.09	0.13	0.41	0.28
	CD14	0.26^{b}	0.37^{ab}	0.53^{a}	0.35^{b}	0.04	0.48	<0.001	0.10
Day 5	*CXCL8*	0.19^{b}	0.32^{a}	0.25^{b}	0.08^{b}	0.07	0.02	0.96	0.14
	IL1β	0.25^{b}	0.46^{a}	0.16^{b}	0.22^{b}	0.04	0.24	<0.01	<0.01
	TNF	0.23	0.25	0.26	0.18	0.03	0.09	0.58	0.59
	CCL2	0.16	0.14	0.21	0.21	0.03	0.38	0.23	0.68
	CD14	0.23	0.27	0.23	0.32	0.03	0.08	0.61	0.42

[1]LV2.5+LV5+HV5 vs CTRL

[2]HV5 vs LV2.5+LV5

[3] LV2.5 vs LV5

Table 10. Average inflammation scores for 3 periods (0–24 h, 25–48 h, and 49–170 h) after the infusion of dairy cow udder quarters at drying-off with 5 mL of 2% low-viscosity chitosan solution (CHI; n = 8), 4 g of teat sealant solution followed by 5 mL of 2% low-viscosity chitosan solution (CHI + SEAL; n = 8), 4 g of teat sealant solution (SEAL; n = 8), or water (control, CTRL; n = 8). Data are presented as least squares means ± standard error of the LS means.

	Treatment				*P*-value		
Period	CHI	CHI + SEAL	SEAL	CTRL	SEAL	CHI	SEAL*CHI
0–24 h	1.76±0.14	1.55±0.14	1.19±0.18	1.25±0.18	0.47	<0.01	0.67
25–48 h	1.30±0.10	1.30±0.10	1.45±0.13	1.45±0.13	1.00	0.14	1.00
49–170 h	1.06±0.06	1.11±0.06	1.18±0.07	1.08±0.07	0.30	0.40	0.73

Table 11. Percentages of somatic cell types (monocytes, granulocytes, lymphocytes + others) before (d −1) and after (d 5) the infusion of dairy cow udder quarters at drying-off with 5 mL of 2% low-viscosity chitosan solution (CHI; n = 8), 4 g of teat sealant solution followed by 5 mL of 2% low-viscosity chitosan solution (CHI + SEAL; n = 8), 4 g of teat sealant solution (SEAL; n = 8), or water (control, CTRL; n = 8). Data are presented as least squares means

	Day −1					Day 5					*P*-value			
Cell type	CHI	CHI + SEAL	SEAL	CTRL	SEM	CHI	CHI + SEAL	SEAL	CTRL	SEM	DAY	SEAL	CHI	SEAL*CHI
Monocytes	27.9	32.0	35.9	30.3	4.4	17.1	17.3	26.2	17.5	3.3	<0.001	0.07	0.12	0.31
Granulocytes	48.8	46.4	37.6	45.5	5.4	65.6	65.2	55.7	61.8	3.6	<0.001	0.17	0.04	0.35
Lymphocytes + others	23.4	21.6	26.6	24.1	2.9	17.4	17.5	18.2	20.8	1.7	<0.01	0.75	0.09	0.79

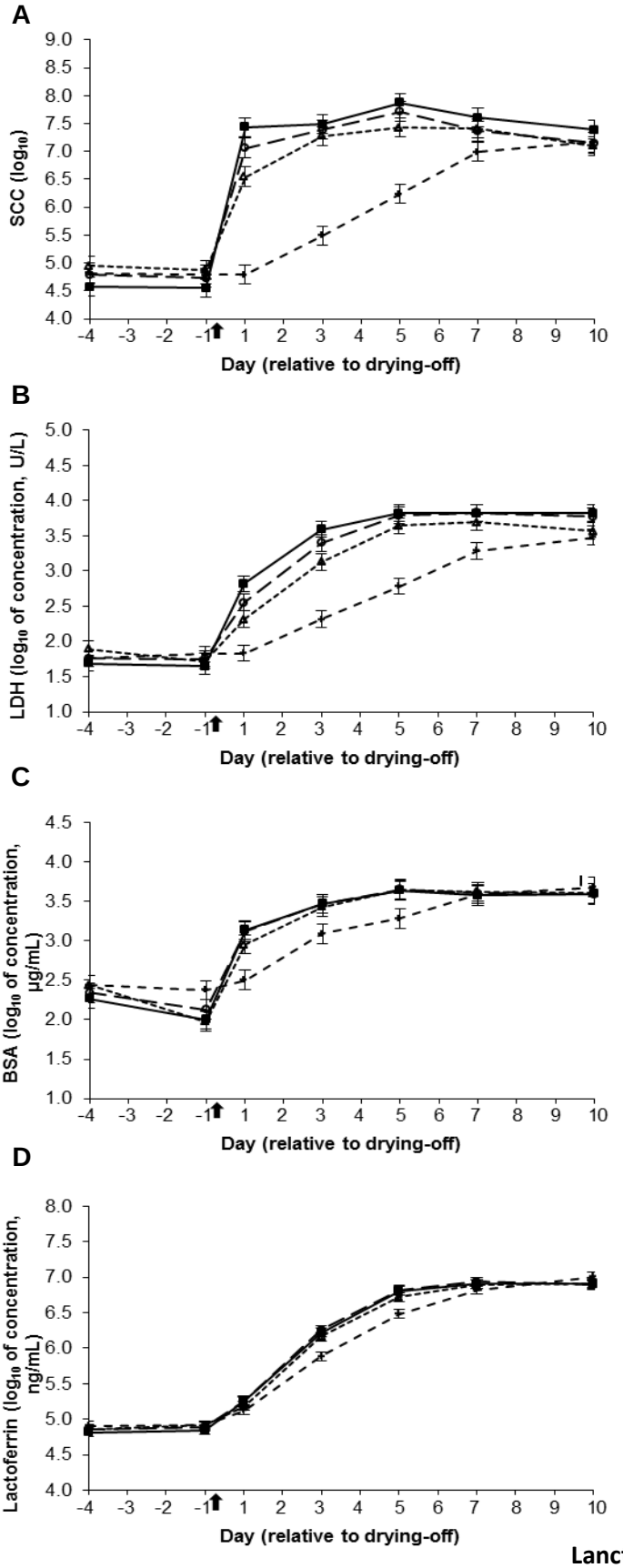
Table 12. Normalized milk somatic cell gene expression of *CXCL8* [chemokine (C-X-C motif) ligand 8], *IL1β* (*Bos taurus* interleukin 1 beta), *TNF* (tumor necrosis factor), *CCL2* [chemokine (C-C motif) ligand 2],; and *CD14* (CD14 molecule) on d 5 after the infusion of dairy cow udder quarters at drying-off with 5 mL of 2% low-viscosity chitosan solution (CHI; n = 8), 4 g of teat sealant solution followed by 5 mL of 2% low-viscosity chitosan solution (CHI + SEAL; n = 8), 4 g of teat sealant solution (SEAL; n = 8), or water (control, CTRL; n = 8). Data are presented as least squares means of log_{10}-transformed values. Different letters indicate significant differences ($P < 0.05$) among treatments

		Treatments					*P*-value		
	Genes	CHI	CHI + SEAL	SEAL	CTRL	SEM	SEAL	CHI	SEAL*CHI
Day 5	*CXCL8*	0.37[a]	0.42[a]	0.16[b]	0.14[b]	0.06	0.53	<0.001	0.82
	IL1β	0.35[a]	0.35[a]	0.26[b]	0.20[b]	0.04	0.50	<0.01	0.46
	TNF	0.22	0.21	0.21	0.13	0.04	0.28	0.17	0.21
	CCL2	0.20[a]	0.26[a]	0.10[b]	0.09[b]	0.03	0.23	<0.001	0.32
	CD14	0.24	0.25	0.28	0.28	0.03	0.73	0.20	0.85

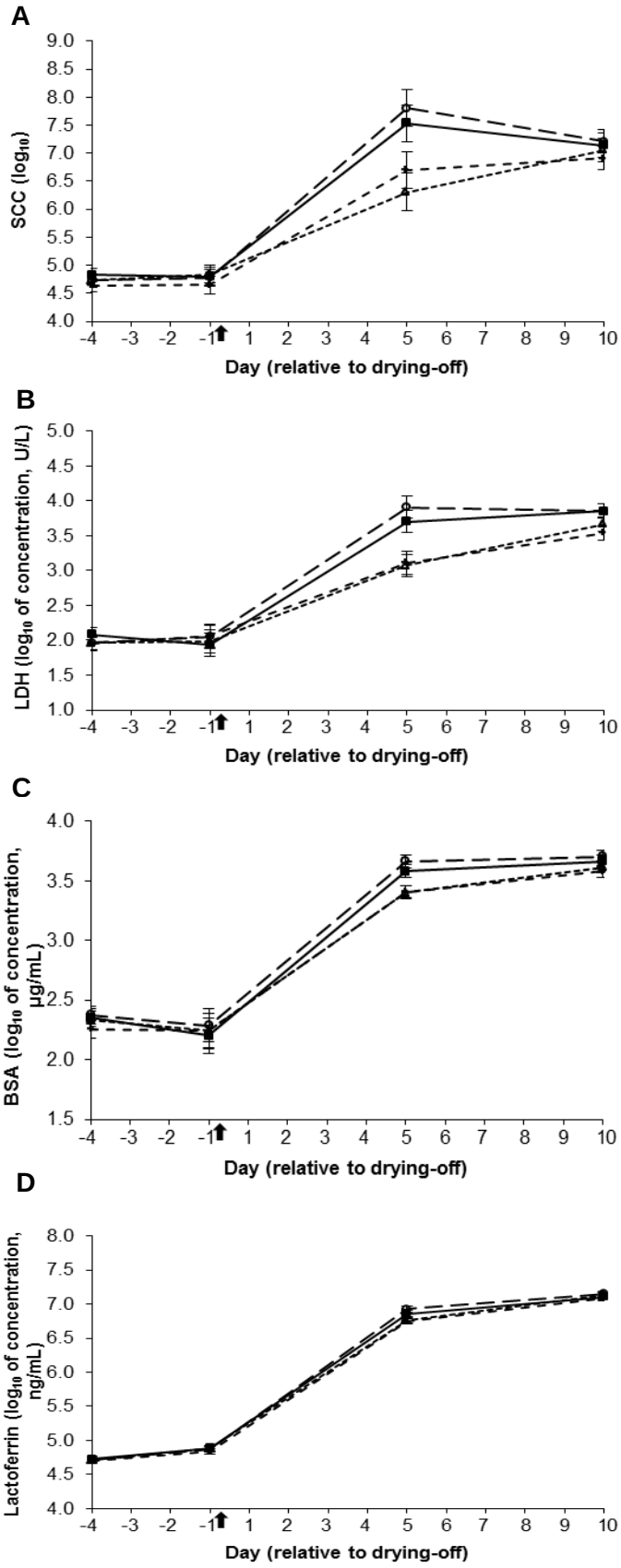
5.8 Figures and figure legends

Figure 2. Somatic cell count (A), lactate dehydrogenase (LDH) activity (B), BSA concentration (C), and lactoferrin concentration (D) in milk and mammary secretions from dairy cow udder quarters infused at drying-off with 5 mL of 5% low-viscosity chitosan solution (■, solid line; n = 7), 2.5 mL of 5% low-viscosity chitosan (○, long-dashed line; n = 7), 5 mL of 5% high-viscosity chitosan solution (Δ, short-dashed line; n = 7), or 5 mL of water (control; +, medium-dashed line; n = 7). Treatment time is indicated by an arrow. Data are presented as least squares means ± standard error of the means of $\log_{10}$-transformed values.

Figure 3. Somatic cell count (A), lactate dehydrogenase (LDH) activity (B), BSA concentration (C), and lactoferrin concentration (D) in milk and mammary secretions from dairy cow udder quarters infused at drying-off with 5 mL of 2% low-viscosity chitosan solution (■, solid line; n = 8), 4 g of teat sealant solution followed by 5 mL of 2% low-viscosity chitosan solution (○, long-dashed line; n = 8), 4 g of teat sealant solution (Δ, short-dashed line; n = 8), or 5 mL of water (control; +, medium-dashed line; n = 8). Treatment time is indicated by an arrow. Data are presented as least squares means ± standard error of the means of $\log_{10}$-transformed values.



Lanctôt et al., Figure 2



Lanctôt et al., Figure 3

CHAPTER 6. CONCLUSION

In summary, we have tested the effect of different types, volumes and concentrations of chitosan infusion, on dairy cow mammary gland. They are different reasons which may explain the lack of differences in quarters treated with different chitosan concentrations and viscosities. The active immune system is really sensitive. Once activated, it induces a cascade of immune reactions. As a result, relation between immunostimulants and immune response is not linear. It is plausible that the lowest chitosan concentration used was large enough to provide an important immune response. Therefore higher concentration of chitosan did not result in a higher immune stimulation. Moreover, sampling of milk caused partial removal of chitosan hydrogel which may have affected the differences between chitosan treatments. We tested different viscosities because it is the characteristic which differ the most between batches of chitosan. However, once prepared, both hydrogels showed similar physical characteristics. Chitosan viscosity did not change hydrogel properties and therefore did not affect its biological properties.

During experiment 1, we observed a similar effect with 2.5 mL of 5% chitosan and 5mL of 5% chitosan. Therefore, for experiment 2, we decided to use 5 mL with a concentration of 2%. We wanted to minimise used of chitosan but used 5ml to fill the teat canal and aim for a long period of degradation of the hydrogel. Accordingly, we observed a similar effect with 5mL of 2% chitosan on the mammary gland involution and immune response. During experiment 2, we also used an internal teat sealant. The presence of the teat sealant did not affect involution or immune response. We think the chitosan effect on the mammary gland is clearly biological and not physical. Moreover, we did not observe a volume effect after the infusion of 2.5mL or 5mL of chitosan, which go in the same sense. Infusion of 5ml of 2% low viscosity chitosan seems to have the desired effect on mammary gland involution and immune responses. However, after the infusion of this treatment, we observed a transient inflammation of the quarter for 24 hours. Still, cows maintained normal behaviors and no anaphylactic choc or animal in distress was observed in those studies.

As public concern about animal welfare increases, it is important to assess the effect of this treatment on animal wellbeing. Therefore, an experiment with a large quantity of cow will be required to determinate if this treatment causes any other side effects. Additionally, it will be important to study shelf life as well as influence of storage conditions on chitosan solution effect.

We have also demonstrated that, when injected in the same quarter, internal teat sealant do not affect chitosan hydrogel effect on those parameters. Because fast involution and increased immune activation would increase protection against mastitis causing pathogens, the hydrogel of chitosan could reduce the incidence of new cases of intramammary infection during the dry period. Moreover, since internal teat sealant and chitosan are compatible, they can be used in combination, which could increase protection. However, effect of the administration of a chitosan hydrogel on the incidence of new cases of IMI during the dry period will need to be assessed in another study. Ultimately, this approach could be used as an alternative to dry cow antibiotic therapy for non-infected cows.This would partially address the public concerns in regard with antibiotic use in the dairy industry.

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