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EVALUATION OF CASEIN HYDROLYSATE AS AN ALTERNATIVE DRY-OFF
TREATMENT AND MILK QUALITY MANAGEMENT TOOL IN DAIRY COWS

by

Justine Elena Britten

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Animal Health and Disease

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2019

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ABSTRACT

Evaluation of Casein Hydrolysate as an Alternative Dry-off Treatment and Milk Quality
Management Tool in Dairy Cows

by

Justine E. Britten, Doctor of Philosophy

Utah State University, 2019

Major Professor: Dr. David J. Wilson
Department: Animal, Dairy and Veterinary Sciences

The focus of our research was the effect of intramammary casein hydrolysate administered to dairy cows on involution of either a single mammary quarter mid-lactation or multiple quarters at the time of dry-off before the next calving. Three studies were conducted. The primary objectives of the first study were to investigate whether intramammary casein hydrolysate treatment of single infected quarters was followed by cessation of quarter milk production for the remainder of lactation, decrease in cow-level somatic cell count and resumption of milk production following calving. The second study compared intramammary infusion of casein hydrolysate alone or with standard dry cow treatment and/or an internal teat sealant for their effects on the rate of mammary involution, as assessed by changes to biochemical markers in milk at different time points of mammary involution. The third study compared quantitative histological changes in mammary tissues following intramammary infusion of casein hydrolysate alone or with

standard dry cow treatment and/or an internal teat sealant at two time points after dry-off. Differences were observed in all of the studies between cows and quarters that received intramammary treatment of casein hydrolysate and those that did not. At no time did any animals treated with casein hydrolysate display signs of pain or discomfort. The overall results of these studies indicated that intramammary infusion of casein hydrolysate was safe for dairy cows, had some efficacy against mastitis, and may be a useful tool for reducing mastitis in lactating and dry cows.

(208 pages)

PUBLIC ABSTRACT

Evaluation of Casein Hydrolysate as an Alternative Dry-off Treatment and Milk Quality
Management Tool in Dairy Cows

Justine E. Britten

Mastitis, an infection of the mammary gland, is the most common and expensive animal health problem for the dairy industry and affects every dairy farm to some degree. This disease complex is painful for dairy cows, increases the on-farm use of antibiotics, presents a threat to milk quality and is a waste of time, money and milk production. Each year, the dairy industry loses as much as a billion dollars to mastitis.

Many cows will experience mastitis at least once during a lactation cycle and some animals will develop recurring mastitis episodes in a single mammary quarter. These mastitic quarters can be difficult to manage during the lactation cycle. Cessation of production in the quarter while continuing to milk the other three can be a beneficial management decision in this scenario. However, the current methods available for cessation of lactation in a single quarter are limited. This study investigated the use of casein hydrolysate as a non-antibiotic option for causing cessation of lactation in a quarter.

From this preliminary study we were able to apply our results to another aspect of mastitis prevention, which is the routine use of intramammary antibiotics at the end of the lactation cycle. This management practice is known as dry treatment and is a standard

practice in the dairy industry with many years of proven efficacy against clearing infections present at the end of the lactation cycle. Increasing pressure from consumers to decrease antibiotic use in food production animals has caused this practice to come under scrutiny. This secondary study investigated the use of casein hydrolysates as a non-antibiotic alternative to standard antibiotic dry cow treatment.

Overall, these studies demonstrated that casein hydrolysate has some efficacy in inducing mammary involution of a single quarter mid-lactation and also potentially as an alternative dry cow treatment. None of the animals treated in these studies displayed any symptoms of pain or discomfort, and all treated quarters resumed milk production after the next calving. Additionally, all antimicrobial milk tests on treated animals were negative. Casein hydrolysates may be a useful management tool for milk quality and animal health within the dairy industry.

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To my parents....I could not have done this without your unwavering support over the years as I stumbled through life figuring out what I wanted to do. Also my friends who kept cheering me on when I couldn't see the end of the process.

Justine E. Britten

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CHAPTER 1

INTRODUCTION

Mastitis is an inflammatory disease of the mammary gland that impacts all food production animals that are reared for milk harvest but is most noticeably a problem in dairy cows. It is estimated that intramammary infections are present in 48.5% of cows in the United States dairy industry and prevalence may be higher if subclinical mastitis is being routinely missed (Wilson et al., 1997b, Piepers et al., 2007, De Vliegher et al., 2012). Even with the continuing advancement of mastitis knowledge and increased sophistication of mastitis detection tools, management of the disease continues to pose a challenge to many producers. At least \$1 billion is lost each year to the U.S. dairy industry in treatment costs, production losses and animal losses because of mastitis (Ott, 1999, Hogeveen et al., 2011, Rollin et al., 2015). Awareness of mastitis management and overall milk quality have undergone substantial changes since the birth of modern milking. National and international standards for milk quality have evolved in the last 50 years with increasing stringency of requirements for dairy producers to meet (Smith and Hogan, 1998, Ruegg, 2011, APHIS and USDA, 2014). The most recent development of note is the European Union's (E.U.) decision to lower the legally required somatic cell count limit (SCC) from 750,000/ml to 400,000/ml, which puts them in the same category as New Zealand, Australia, Switzerland and Norway (Smith and Hogan, 1998). The legal upper limit in the U.S. remains at 750,000/ml and is the highest of all developed, major dairy producing countries in the world (Smith and Hogan, 1998, Barbano et al., 2006, APHIS and USDA, 2014). Only four states have chosen to implement lower SCC

standards: California (600,000/ml), Oregon (500,000/ml), Idaho and Washington (both 400,000/ml) (APHIS, 2017). Additionally, the consumer now plays a more active role in how milk is produced. Consumer concern over animal health and welfare, treatment practices and potential chemical residues in milk has helped to steer the direction of the modern dairy industry. The ultimate result of all this is that some dairy practices have been abandoned, while the need for developing new management tools remains strong.

Mastitis may be a “one and done” disease occurrence in a dairy cow in the instance of clinical mastitis, which is most commonly identified by swelling, heat and/or redness of the udder and the presence of clots and/or flakes in the milk (Hogan et al., 1989, Schukken et al., 2011b), but can also be severe enough to warrant culling or even cause death (Steenneveld et al., 2008). Frequently however, dairy producers will face the challenging scenario of cows which experience repeated or persistent mammary infections (Bar et al., 2007). According to a previous study, 9% of cows experience repeat episodes of clinical mastitis within the same lactation and same quarter of the udder, caused by the same bacterial group (Hogan et al., 1989), but the full range of potential repeat cases is from 6% up to 31% (Hogan et al., 1989, Bar et al., 2007). There is no universal industry standard on how to handle these animals, but one option is the creation of the “three-quartered” cow. A three-quartered cow is a cow that has one quarter no longer producing milk, either from injury or intentional cessation of milking and is therefore only being milked from the remaining three quarters. Removing a damaged or persistently infected quarter from production has been used by dairy

producers for some time but the industry lacks a standardized method for doing so. In our first study, we used intramammary infusion of casein hydrolysate to initiate involution in individual mastitic quarters as a strategy for managing milk quality. In our second study, we studied intramammary infusion of casein hydrolysate in combination with or without dry cow antibiotics and/or internal teat sealant, as a potential alternative to traditional dry cow therapy.

This dissertation covers an overview of mastitis management, milk quality and how it has evolved until the present time. This includes how milk quality is evaluated, some of the primary pathogens responsible for chronic mastitis and a review of past and current tools and treatment practices for mastitis. The primary focus is the effects of intramammary casein hydrolysate on involution of either a single quarter mid-lactation or multiple quarters at the time of dry-off. Casein is believed to be one of the milk-borne factors responsible for initiating mammary involution in the udder (Shamay et al., 2002, Leitner et al., 2007, Wang et al., 2013) and the goal was to explore whether intramammary infusion of casein hydrolysate into a single mammary quarter, or at dry-off was advantageous in cessation of milk production (Shamay et al., 2003, tho Seeth et al., 2016, Britten et al., 2018).

CHAPTER II

LITERATURE REVIEW

Rise of the U.S. Dairy Industry

Humans all across the globe have been harvesting milk from animals for consumption, dating back thousands of years, as early as 7000 B.C. (Fuquay et al., 2011). It was not always collected primarily from cows as is common today; goats, sheep, camels and even horses were the more common suppliers in ancient times (Fuquay et al., 2011). Harvesting milk from cows developed along with civilization and population growth in Europe, as families began to farm and raise livestock for food. Dairy wasn't born in the English colonies until the 1600s (USDA, 2002) when European immigrants brought cattle with them as a source of meat and fresh dairy products. However, animals were not bred and raised specifically for the purpose of milk production until the late 1800s, as part of the industrial revolution (USDA, 2002). Until that time, fluid milk and other fresh dairy products were mainly only consumed locally by the families that raised the animals and perhaps within the local community. The Industrial Revolution drove the growth of urban populations and the need for rural farming families to increase their production of food, so it could be distributed and sold within the cities (USDA, 2002). The first designated dairy cow breeds were Jerseys, Ayrshires and Guernseys, and they were imported into the U.S. during the early 1800s (Foreman et al., 1965) but it wasn't until the mid to late 1800s that they were kept as purebreds and not allowed to mix with

local breeds. The rapidly growing cities needed daily deliveries of fresh milk to sustain their populations but because milk was highly perishable, and transportation limited, it still had little use as a commercial product. Pasteurization of milk developed in the late 1800s, following Louis Pasteur's experiments with heating wine to prevent abnormal fermentation (Fuquay et al., 2011). The application of pasteurization to milk was not executed by Pasteur himself but first by an American pediatrician, Henry Koplik, and later by philanthropist Nathan Strauss (Steele, 2000). The morbidity and mortality rate resulting from drinking contaminated and/or spoiled dairy products dropped remarkably and distributors quickly recognized the merit of heat treating milk (McCullough, 1928). In addition to pasteurization of milk, individual bottling, refrigeration and the invention of the first cream separator in 1878 by G.P. de Laval were implemented as tools for extending the shelf life of milk (McCullough, 1928). During this early history of commercial dairying, most of the farming and milk production was concentrated in the Midwest, which made transportation a major challenge. With the development of these preservation methods, milk and cheese could now be moved by train to urban areas (Fuquay et al., 2011).

Food Safety and Dairy Production Regulation

Despite advancements in the dairy industry, there was still a need for quality regulations. In the early 1900s, of all food-borne disease cases caused by contaminated food or water, 25% resulted from the consumption of milk products (McCullough, 1928, Steele, 2000). This proportion has decreased but dairy is still linked to 20% of foodborne

illnesses and 15% of deaths related to food poisoning (Headrick et al., 1998, Painter JA, 2013, CDC, 2016). Young children and elderly citizens are especially vulnerable to disease or even death from milk-borne food poisoning (USHHS, 2009, Lucey, 2015). In an attempt to address this problem, the United States Public Health Service (USPHS) developed a set of regulations originally known as the Standardized Milk Ordinance, which led to the first version of the Pasteurized Milk Ordinance, intended as a model ordinance for cities (USHHS, 2009). The goal of the original Pasteurized Milk Ordinance (PMO) was to initiate programs for the prevention and control of milk-borne diseases and was created in conjunction with the Food and Drug Administration (FDA). This first version was put out for voluntary implementation by state and local milk control agencies in 1924 (Steele, 2000, USHHS, 2009). It has undergone many revisions since its origin, as new knowledge and technology is continually expanded, and is currently in its 41st revision and known as the Grade A PMO (USHHS, 2017). All levels of local, state and federal government and all segments of the dairy industry, including milk processors, producers, equipment manufacturers, educational institutions and dairy associations have been involved in shaping the PMO into what it is today (USHHS, 2009). The PMO is an extensive document but the mission statement of what it embodies is as follows:

“An ordinance to regulate the production, transportation, processing, handling, sampling, examination, labeling, and sale of Grade A milk and milk products; the inspection of dairy farms, milk plants, receiving stations, transfer stations, milk tank truck cleaning facilities, milk tank trucks and bulk milk haulers/samplers; the issuing and revocation of permits to milk producers, bulk milk haulers/samplers, milk tank trucks, milk transportation companies, milk plants, receiving stations, transfer stations, milk tank truck cleaning facilities, haulers, and distributors; and the fixing of penalties.” (USHHS, 2009).”

The USPHS and the FDA do not have the legal jurisdiction to enforce the milk sanitation standards listed within the PMO except for the instances of interstate carriers or commerce (USHHS, 2011). However, interstate shipment is common for most milk procurement and marketing companies today. Actual enforcement of regulations is done at the state level and the PMO is the standard used by the Certification of Interstate Milk Shippers program and the FDA and is encouraged to be used by adoption into law in all 50 states, the District of Columbia and the U.S. Territories (USHHS, 2009). Additionally, the FDA made pasteurization of all fluid milk and milk products mandatory if designated for human consumption, which effectively banned any shipping of raw milk between states (NCSL, 2016). Raw milk and/or raw milk products may still be sold at the state level and there are 29 states which currently allow this but each state has their own regulations for the production and sale of raw milk (NCSL, 2016).

Part of the PMO specifies the standards for qualification as Grade A milk, for both raw milk slated for pasteurization and for milk that has already undergone pasteurization or ultra-pasteurization. Grade A raw milk or milk products must be handled to conform to the physical, chemical, bacteriological and temperature standards as listed in the PMO, during all parts of the production process (USHHS, 2011, 2017). This is measured by submitting samples to approved milk testing laboratories, which are certified by a Milk Laboratory Evaluation Officer (LEO) of the FDA, within each state (Sanitation Compliance and Enforcement Ratings of Interstate Milk Shippers List, 2016). Laboratories need to pass recertification tests annually for the procedures which they are approved to perform (Sanitation Compliance and Enforcement Ratings of Interstate Milk

Shippers List, 2016). Some laboratories may be approved to perform more procedures than others but all certified milk laboratories need to be approved for the following required standardized milk tests: SCC, standard plate count (SPC), and the bacterial growth inhibitor (primarily antibiotics) test of choice the lab uses (USHHS, 2011). An SPC test and an SCC test are both quantitative tests used to determine the raw bacterial count and the leukocyte count, respectively present per 1 ml of milk (Wehr et al., 2004, Barbano et al., 2006). These counts are indicative of the estimated proportion of infected quarters present within the herd at a given time; for example the U.S. legal upper limit for SCC of 750,000/ml of milk estimates an approximate 25% prevalence of infected quarters (Voelker, 1981). These tests are standardized at certified milk testing laboratories across the country by staying in compliance with the Standard Methods for the Examination of Dairy Products (Wehr et al., 2004). The minimum standards as set forth in the PMO for Grade A raw milk are: the milk must be chilled to 10° C (50° F) within four hours or less of harvest from the udder, have an SPC of no more than 100,000 cfu/ml, an SCC of no more than 750,000/ml and be negative for any antibiotic residue detection (USHHS, 2011, 2017). After milk is pasteurized, ultra-pasteurized, concentrated or dried, it is subject to additional bacterial and coliform count limits.

Machine Milking

During the late 18th and early 19th century, the transition from milking cows by hand to the use of milking machines took place. Various types of mechanized devices designed for milking cows began appearing as early as the mid to late 1800s (Bramley,

1992, Holloway and Bear, 2017) and included many creative new methods. Some inserted metal cannulas into the teat orifice to allow the milk to flow freely, pulled by gravity, into an attached pail (Holloway and Bear, 2017). Others applied pressure to the outside of the teat and were powered mechanically, pneumatically or hydraulically (Bramley, 1992). All in all, more than eighty patents were awarded in the 1800s for over thirty different milking machine designs but the first milking machine to become commercially available was the Murchland machine in 1889 (Bramley, 1992). The Murchland machine was designed by a plumber in Scotland and had faint but distinct ties to modern milking systems in that it was designed to be operated with a central vacuum system and multiple milking units (Bramley, 1992). At this time however, the idea of using pulsation to relieve force on the ends of the teats and to ensure blood flow within the teats had not yet been developed and the continuous suction applied to the teats caused mammary tissue damage and physical discomfort to the cows (Bramley, 1992, Besier et al., 2016). The idea of pulsation did not come until 1895 and was developed by Dr. Shiels of Glasgow, who was the developer of the Thistle milking machine (Bramley, 1992). He introduced a mechanically operated valve, which allowed air into the vacuum line at regular intervals and allowed the vacuum at the teat end to pulsate between 4 and 15 inches of mercury (Bramley, 1992). These early milking machine and pulsation inventions continued to be developed, mostly by European inventors, until 1922 when the U.S. became actively involved with the invention of the “Surge” milker by the Babson Brothers of Chicago, Illinois (Fuquay et al., 2011). The Surge milker employed the already proven pulsation idea and remained the predominantly used machine by U.S. and

Canadian dairy producers for almost 25 years after that (Bramley, 1992). Milking parlors began appearing in the 1920s but even as late as 1950, only about half of the cows in the United States were milked in a parlor by a milking machine (Fuquay et al., 2011). Improvements continued and major breakthroughs in milking technology took place in the 1950s (Bramley, 1992). It was during this period that researchers began to pay closer attention to mastitis and the relationship between mastitis and the use of milking machines. Many dairy producers and their veterinarians had already held the opinion that milking machines played an important role as a fomite for transferring infections between cows and scientific research began to confirm this (Bramley, 1992). Studies have demonstrated that fluctuations in vacuum levels exerted on the ends of teats leads to increased prevalence of intramammary infection (Baxter et al., 1992, Besier et al., 2016). One reason for this is believed to be the movement of any bacterial pathogens present in the streak canal or on the teat ends upward into the teat canal as a result of reverse pressure movement of air from vacuum flux, thus allowing the bacteria access to the mammary gland (Baxter et al., 1992, Bramley, 1992, Besier et al., 2016). The highest period of risk occurs near the end of milking, when teat cisterns are no longer refilling quickly and vacuum force is exerted on empty canals (Besier et al., 2016). This can cause injury to epithelial and sub-epithelial tissues of the teat canal and cause hyperkeratosis of the teat ends (Barkema et al., 1999, Francesca Neijenhuis et al., 2004, Besier et al., 2016). Rough, thickened and cracked teat ends are difficult to sanitize and allow pathogens to avoid remain hidden from disinfectants and therefore remain present on teat ends (Francesca Neijenhuis et al., 2004, Besier et al., 2016). Milking liners can also act as a

fomite to transfer contagious mastitis pathogens from one infected animal to another and facilitate spread within the herd (Wilson et al., 1995, Barkema et al., 1999). The transition from hand milking of cows to use of modern milking machines presents different challenges that must be addressed through careful management.

Mastitis Classification

The very broad definition of mastitis is an inflammation of the mammary gland (Bradley, 2002, Contreras and Rodríguez, 2011, Aiello, 2012, Hughes and Watson, 2018). Between 1950 and 1980, the field of milk quality developed rapidly, and substantial advances were made in understanding the causes of mastitis (Bradley, 2002, Barkema et al., 2009, Royster and Wagner, 2015). For the first time, instead of simply making the association that bacteria were responsible for souring milk or causing milk-borne illness, specific bacterial pathogens began to be identified (Jain, 1979, Zadoks and Fitzpatrick, 2009b, Zadoks et al., 2011). These pathogens could be divided into two broad categories based on their common sources and modes of transmission: contagious spread via direct contact from cow to cow or environmental, coming from bedding and other cow contact surfaces (Fox and Gay, 1993, Bradley, 2002, Zadoks and Fitzpatrick, 2009b). Within each category, pathogens can be grouped by other characteristics such as Gram reaction, antibiotic sensitivity, ideal growth environment and whether the mastitis caused is primarily clinical or subclinical.

Mastitis can further be categorized as clinical, subclinical, acute or chronic (Jain, 1979, Bradley, 2002, Contreras and Rodríguez, 2011). Clinical mastitis is commonly identified by the presence of abnormal appearance of the milk and/or heat, swelling and redness of the udder (Schukken et al., 2011b, Aiello, 2012). In severe cases, the animal may become systemically ill and present with fever, dehydration, anorexia, recumbency or death (Aiello, 2012, Hogan and Smith, 2012a). Abnormal milk is defined as flakes, clots, blood, watery consistency, discoloration or any other deviation from normal fluid milk and it is a violation of the PMO for this milk to be offered for sale (USHHS, 2011). There are three common ways to divert this milk from the bulk tank: milk the cow into a bucket, remove her from the main milking string to a separate milking facility, often an older parlor, or move her to a group of cows which are milked last (Erskine et al., 1987, Oliver, 2012, Schroeder, 2012). Milking these cows with abnormal milk last minimizes the risk of contaminating milking equipment surfaces, and if they were treated with antibiotics or other treatments with a milk withdrawal, keeps drug violative residues out of the saleable milk supply. The milk collected is either discarded before it reaches the bulk tank or directed into a separate bulk tank, trailer tank or some other type of collection vessel besides the bulk tank(s) with saleable milk (Erskine et al., 1987, Schewe et al., 2015). If abnormal milk from clinically mastitic cows is accidentally milked into the bulk tank(s) containing saleable milk, it will have a degrading effect on the overall quality of the tank milk and if cows with violative drug residues still present in milk are accidentally milked into the tank there is the risk of the entire load being rejected (Hillerton et al., 1999, Oliver, 2012, USHHS, 2017). Clinical mastitis cases may last only a few

days before the cow is healthy again and able to return the milking string or the infection may establish itself within the gland and persist for the remainder of the lactation (Jain, 1979, Aiello, 2012). These animals with recurring infections may experience repeated episodes of clinical “flare-ups”, where the abnormal milk must then be diverted from the rest of the saleable bulk tank milk (Reinemann et al., 2002, Bar et al., 2007). Repeat treatments for non-responsive clinical mastitis cases are expensive, labor intensive and create a problem for milking staff and farm management (Cha et al., 2011, Hogeveen et al., 2011, Rollin et al., 2015). It is repeated treatment episodes, more than any other single reason, that frequently result in culling of the animal from the herd (Hutton et al., 1990, Barkema et al., 1999, Schukken et al., 2009b).

Three important and common contagious mastitis pathogens which many dairy producers and veterinarians focus on controlling are *S. aureus*, *S. agalactiae*, and *Mycoplasma* spp. (Oliver and Mitchell, 1984, Fox and Gay, 1993, Fox et al., 2005, Barkema et al., 2009). These three pathogens, alone or in combination, were responsible for inflicting substantial damage to milk quality and udder health during the major growth of dairy farms in the 1950s through the 1980s (Gonzalez et al., 1986, Britten, 2012). Lack of understanding of the pathogens and the non-existence of disease control or prevention programs on dairies, combined with highly contagious transfer from cow to cow during milking allowed these bacteria to spread aggressively (Fox and Gay, 1993, Barkema et al., 2009, Britten, 2012). These pathogens continue to be present and a threat to udder health on modern dairies today (Punyapornwithaya et al., 2010, Middleton, 2013, Levison et al., 2016, Nicholas et al., 2016, Tomazi et al., 2018). Depending on

which of these pathogens or which combination of these pathogens a dairy herd is afflicted with, substantial increases in SCC, SPC, decreased milk production, abnormal milk, swollen quarters, systemic illness or even fatalities are seen (Jain, 1979, Smith and Hogan, 1998, Barkema et al., 2009, Schukken et al., 2011b).

The most common environmental mastitis pathogens can be generally sorted into three primary groups: coagulase-negative staphylococci (CNS), non-agalactiae streptococci and coliforms (Hogan, 1999, Pyorala and Taponen, 2009, Hogan and Smith, 2012a). Within the latter two groups, primary organisms of interest are *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Enterococcus* spp. and *Escherichia coli*, *Klebsiella* spp., *Citrobacter* spp., and *Enterobacter* spp., respectively (Jain, 1979, Oliver and Mitchell, 1984, Zadoks and Fitzpatrick, 2009a, Hogan and Smith, 2012a). The third group, CNS, is the most common mastitis pathogen in most dairy herds and often causes subclinical mastitis (Pyorala and Taponen, 2009, Schukken et al., 2009a, Pinzon-Sanchez et al., 2011, Bludau et al., 2014). Historically, CNS has been believed to be a common opportunistic bacteria found on teat skin that is responsible for mild and often subclinical infections, that may or may not cause elevated SCC counts (Taponen et al., 2007, Pinzon-Sanchez et al., 2011, Fry et al., 2014). While this still holds true, recent studies using molecular methods to differentiate species of CNS have found variation between species (Supré et al., 2011, Fry et al., 2014, Nyman et al., 2018). The species of CNS most frequently isolated from milk samples differ from those most frequently found on teat skin (Pyorala and Taponen, 2009, Taponen and Pyorala, 2009). *Staphylococcus chromogenes* is the most common species to be isolated from intramammary infections,

but *S. epidermidis* and *S. simulans* are frequently isolated from milk samples also (White et al., 1989, Pyorala and Taponen, 2009, Schukken et al., 2009a, Taponen and Pyorala, 2009). The epidemiology of CNS mastitis can be compared to that of *S. aureus*; both pathogens are responsible for mostly subclinical mastitis, prolonged elevated SCC in infected quarters and variable response to antibiotic treatment (Chaffer et al., 1999, Dego et al., 2002, Taponen et al., 2007, Taponen and Pyorala, 2009). Other mastitis organisms of interest are *Pseudomonas aeruginosa*, *Serratia* spp., *Prototheca* spp., *Corynebacterium* spp., Yeast, *Trueperella pyogenes*, *Pasteurella* spp. and *Nocardia* spp. (Wilson et al., 1997b, Zadoks and Fitzpatrick, 2009b, Dohoo et al., 2011). Most of the pathogens in this latter group above are rare in most herds most of the time and may not be seen for years at a time but can cause substantial damage in uncommon outbreaks. All of these pathogens survive in the environment but rarely spread contagiously from cow to cow during milking (Smith et al., 1985a, Zadoks and Fitzpatrick, 2009a). These bacteria are frequently responsible for recurrent clinical mastitis episodes, repeat treatment regimens and may have a higher proportion of cases resulting in systemic illness or death of cows (Smith et al., 1985a, Hogan and Smith, 2012a). While the contagious pathogens can be so damaging that they are capable of eliminating a dairy's Grade A milk status, environmental pathogens play the biggest role in affecting milk quality on a daily basis on most dairy farms and must be controlled by careful management (Smith and Hogan, 1993, Smith and Hogan, 2008, Hogan and Smith, 2012b). The mastitis organisms listed here will be covered in further detail in subsequent sections.

Contagious Mastitis Pathogens

Staphylococcus aureus is the most frequent of the contagious mastitis pathogens and comprises about 10% of all clinical mastitis samples, based on a 2015 study done of conventional and organic Canadian dairy farms (Levison et al., 2016). *Staphylococcus aureus* spreads via cow to cow transmission but often remains subclinical for months after the initial infection, making it difficult to detect without the use of culture (Wilson et al., 1995, Zadoks et al., 2001, Barkema et al., 2006). Additionally, heifer calves may become infected with the organism very early in life (Middleton, 2013) and harbor the infection in the undeveloped mammary gland until their first lactation (Britten, 2012, Bludau et al., 2014, Levison et al., 2016). Unless a dairy is routinely screening all of their postpartum animals for *S. aureus* infections, these cases in cows beginning after their first calving will be missed and will continue to spread the disease to other animals (Barkema et al., 2009, Britten, 2012, Keefe, 2012). This may be one reason why *S. aureus* has maintained an almost 10% prevalence (Oliver and Mitchell, 1984, Piepers et al., 2007) within the industry and most dairy producers are unable to eradicate it completely from their herds. However, the pathogenesis of how *S. aureus* mastitis is acquired and progresses over time is more likely the key reason for its persistence. *Staphylococcus aureus* may come into contact with the teat orifice from contaminated milking equipment and/or poor milking time hygiene and once there, can easily persist and multiply (Wilson et al., 1995, Kerro Dego et al., 2002). Intramammary infection occurs when *S. aureus* enters the teat orifice, breaching the streak canal, colonizing the distal part of the mammary gland and adhering to ductular epithelial cells (Frost, 1975, Dego et al., 2002,

Middleton, 2013). *Staphylococcus aureus* are able to adhere to receptors on the surface of mammary epithelial cells and survive host defense mechanisms by intracellular survival within macrophages and the epithelial cells (Almeida et al., 1996, Dego et al., 2002). The means by which the organism is able to gain access to the gland is often a reverse pressure gradient caused by liner slip during milking time (Baxter et al., 1992, Bramley, 1992) but careless intramammary infusion practices may also serve as a vector (Erskine et al., 1987). During an episode of mastitis the primary host defense mechanism is phagocytic killing of the invading organism (Dego et al., 2002, Schukken et al., 2011b), however the bacteria have been found enclosed in membrane vacuoles in the cytoplasm of mammary epithelial cells, thus enabling them to escape host defense mechanisms (Dego et al., 2002, Oviedo-Boyso et al., 2007, Schukken et al., 2011b). *Staphylococcus aureus* not only invades but can survive macrophage activity by the production of an antiphagocytic exopolysaccharide capsule that covers the cell wall and protein A, which is distributed throughout the cell wall and also believed to have antiphagocytic properties (Dego et al., 2002, Schukken et al., 2011b). The production of the exopolysaccharide capsule varies between strains of *S. aureus* and is usually absent from environmental strains but is found commonly in strains isolated directly from intramammary infection (Dego et al., 2002, Zadoks et al., 2011). The development of a capsule and the intracellular location of *S. aureus* make it largely inaccessible to antibiotics; cure rates have an extremely wide range of 3-70%, including dry period cures, depending on product, treatment protocol, age of the animal and at what point in the lactation the treatment is administered (Dego et al., 2002, Barkema et al., 2006, Middleton, 2013).

Cows positive for *S. aureus* during their first lactation that may have remained undetected will usually be detected by their second lactation if they remain infected after the dry period (Petersson-Wolfe et al., 2010, Schukken et al., 2011b, Middleton, 2013). This is a result of chronic infection with *S. aureus*, which causes damage to alveolar ductal cells and the mammary secretory epithelial cells and over time causes a sustained elevated SCC, which facilitates detection of the infection (Petersson-Wolfe et al., 2010, Schukken et al., 2011b, Middleton, 2013). *Staphylococcus aureus* is primarily associated with subclinical mastitis but can also cause clinical mastitis, generally in cows that are at least third lactation and older (Waage et al., 1999, Piepers et al., 2007, Petersson-Wolfe et al., 2010). If this pathogen is uncontrolled and allowed to spread, the herd level SCC will frequently rise until it exceeds the federal legal limit of 750,000/ml, at which point the milk is not legally saleable (USHHS, 2011). This point comes sooner in the instances of individual states or processors which have put a lower SCC limit into place, such as Idaho and Washington or Oregon where the legal SCC limits are 400,000/ml and 500,000/ml, respectively (Hoard's, 2012, IDA, 2013, APHIS, 2017). A control measure that is often employed to prevent reaching this point is placing *S. aureus* positive animals into a separate group and milking them last before the milking system is cleaned and sanitized, to reduce the spread of disease (Zadoks et al., 2001, Petersson-Wolfe et al., 2010, Keefe, 2012). *Staphylococcus aureus* and other contagious pathogens can be transmitted from cow to cow at milking time from milk residues left on the surface of milking inflations (Wilson et al., 1995, Barkema et al., 2009, Keefe, 2012). Another possible control measure for *S. aureus* is to stop milk production in the infected quarter of

cows that have only a single quarter infected, effectively making them “three-quartered” cows (Middleton and Fox, 1999, Middleton et al., 2001). Frequently, it is not economically feasible or practical to cull every animal infected with *S. aureus* mastitis, especially with high producing younger animals or in an outbreak situation where $\geq 50\%$ of the herd is infected (Wilson et al., 1995, Barkema et al., 2006). Using management techniques such as segregation of known *S. aureus*-positive animals and milking them last or with separate milking units (Wilson et al., 1995) or therapeutic cessation of milking in *S. aureus* positive quarters (Middleton and Fox, 2001, Middleton et al., 2001), reduces the financial losses a dairy producer may experience from operating with this disease.

Staphylococcus aureus was not always the dominant contagious pathogen. This picture has changed significantly in the last thirty to fifty years from when *S. agalactiae* was the most highly prevalent and damaging mastitis organism in the industry and responsible for the majority of mastitis outbreaks (Keefe, 1997, Zadoks and Fitzpatrick, 2009b). As soon as penicillin became widely available and its use as an intramammary antibiotic treatment was widely adopted, aggressive detection and treatment of *S. agalactiae* nearly eradicated it from the industry (Jain, 1979, Keefe, 1997). However, in some parts of the world *S. agalactiae* has been described as reemerging as a mastitis pathogen of concern, with prevalence as high as 28-35% in some South American countries (Zadoks et al., 2011, Mahmmoud et al., 2015, Tomazi et al., 2018) or an increase to 6% from approximately 2% in Scandinavia (Mahmmoud et al., 2015). *Streptococcus agalactiae* is highly contagious but has poor survival outside the mammary gland (Keefe,

1997, Tomazi et al., 2018). However, once the organism has invaded the mammary gland, it multiplies rapidly in milk and on the surface of mammary epithelial cells, often leading to a high prevalence within an infected herd (Keefe, 1997, Keefe, 2012).

Streptococcus agalactiae may occasionally result in clinical mastitis but most frequently causes a persistent subclinical response in the udder (Jain, 1979, Keefe, 2012) and elevated bulk tank milk SPC and SCC counts (Keefe, 1997, Wilson et al., 1997a, Keefe, 2012). One of the most unique features of a *S. agalactiae* mastitis outbreak is how fast both the SCC and bacteria counts in bulk tank milk can increase simultaneously (Keefe, 1997, Keefe, 2012). In contrast to *S. aureus*, in which a dairy can still produce legally saleable milk for some time if the disease is carefully managed, *S. agalactiae* can threaten to put a dairy out of business within months if not dealt with (Erskine and Eberhart, 1990, Olde Riekerink et al., 2006, Britten, 2012). Antibiotic treatment is highly effective at controlling and subsequently stopping an outbreak, by method of blitz treating all four quarters of infected animals with penicillin, but consequently makes *S. agalactiae* outbreaks very expensive (Erskine and Eberhart, 1990, Keefe, 2012). *Streptococcus agalactiae* can be easily and fairly quickly identified on culture, which combined with as high as an 87% cure rate depending on which antibiotic is used for treatment (Wilson et al., 1999) are factors contributing to keeping the overall prevalence of the disease low in the industry (Keefe, 1997, Barkema et al., 1998, Keefe, 2012). Most dairy herds are entirely free of *S. agalactiae* mastitis; results of a large study detected it in 4% of all milk samples tested over a 7 year period (Makovec and Ruegg, 2003, Tomazi et al., 2018).

Although *Mycoplasma* spp. are only present at between a 1-8% herd level prevalence in the industry (Fox et al., 2005, Olde Riekerink et al., 2006), they are still one of the most highly damaging organisms responsible for clinical mastitis that is non-responsive to therapy and can cause severe milk loss or death in cows (Wilson et al., 1997b, González and Wilson, 2003). *Mycoplasma* spp. undergoes bacterial shedding cycles in the animal, with concentrations ranging from 10^2 to $>10^6$ colony forming units (cfu)/ml in milk (Bennett and Jasper, 1977a, Nicholas et al., 2016). When a cow is in a low shedding cycle ($<10^2$ cfu/ml) the infection is usually undetectable on culture (if the inoculum volume is 0.01 ml of milk, commonly used) whereas when she is in a high shedding cycle ($>10^6$ cfu/ml) she will nearly always be culture-positive because there will be a mean of 10,000 cfu in the inoculum (Bennett and Jasper, 1977a, Fox et al., 2005, Britten, 2012). These shedding cycles, combined with the fact that mycoplasma grow slowly on culture media (some positive cultures are only evident as long as 10 days later), can make the disease difficult, time consuming and expensive to track down and eradicate in a dairy herd (Fox et al., 2005, Nicholas et al., 2016). Pathogenically, mycoplasmas are arguably one of the most damaging mastitis pathogens, in terms of milk loss, cost per case and contagious spread within the herd (Wilson et al., 1997b, Fox et al., 2005). Money lost per lactation due to a specific mastitis pathogen has been estimated by two methods: DHIA projections of milk production using linear score and milk value (Wilson et al., 1997b) and comparison of mean DHIA fat-corrected milk production between infected and uninfected cows for each pathogen (Wilson et al., 1997b). Only

Pasteurella spp. has been shown to have higher dollar loss per lactation per infected cow than *Mycoplasma* spp., based on these assessment methods (Wilson et al., 1997b).

Cows were inoculated in one quarter via intramammary infusion with 70 cfu/ml of *Mycoplasma bovis* (Bennett and Jasper, 1977a). Histopathologic examination of tissues retrieved from all four quarters of each animal at the conclusion of the study revealed microscopic lesions of the alveolar epithelium, in both inoculated and uninoculated quarters, indicating hematogenous spread between quarters (Bennett and Jasper, 1977a). Disturbance of the alveoli and alveolar cells is considered to be a general characteristic of a mycoplasma infection (Bennett and Jasper, 1977a, González and Wilson, 2003), and will eventually result in partial involution of the alveoli in the udder (Bennett and Jasper, 1977b). The distension of the alveoli by large, vacuole-like structures (Bennett and Jasper, 1977a), is consistent with tissue changes seen during mammary involution (Holst et al., 1987, Sordillo and Nickerson, 1988) and explains the marked drop in milk production sometimes associated with mycoplasma mastitis (González and Wilson, 2003). Further examination of histologic samples revealed numerous lymphocytes and macrophages in close association with the alveolar cells and portions of the tissue were completely void of functional alveoli altogether (Bennett and Jasper, 1977b). Mycoplasma lack a cell wall, which means the beta-lactam class of antibiotics has no effect on them (Fox et al., 2005, Tolboom et al., 2008, Romaniuk and Cegelski, 2015). Previous studies have investigated *Mycoplasma* spp. sensitivity to macrolides and aminoglycosides (Jasper, 1981) but antibiotic therapy has not been effective as a control method (Jasper, 1981, Fox et al., 2005, Nicholas et al., 2016).

Animals that are positive on culture should be removed from the herd, even in the absence of clinical signs of mastitis (Fox et al., 2005, Nicholas et al., 2016). Its reputation within the industry is an interesting mix of fear and disregard; some dairy producers use diagnostic screening practices for mycoplasma but no other pathogens, while others' herds have been mycoplasma-negative for their entire farming career and are simply unconcerned about the possibility of the disease entering their herd (Wilson et al., 2007). A sharp rise in cases of clinical mastitis can be seen with a mycoplasma outbreak, especially into a previously naïve herd (Wilson et al., 2007). When this occurs, the owners or management team on the affected dairy are usually prompted to act (González and Wilson, 2003, Fox et al., 2005, Wilson et al., 2007, Punyapornwithaya et al., 2010). *Mycoplasma* spp. can remain hidden in an affected herd for a long period of time; even if the farm is not currently experiencing mycoplasma mastitis the potential always exists of a later major outbreak of respiratory disease, arthritis, mastitis, recumbency requiring euthanasia, and high death loss of cows infected with mycoplasma (Jasper, 1981, González and Wilson, 2003, Punyapornwithaya et al., 2010). Mycoplasma is distinct from other mastitis pathogens in that it has been demonstrated to reside at other body sites on the animal without causing disease (Biddle et al., 2005, Punyapornwithaya et al., 2010). Mucosal surfaces of the eyes, nasal cavities, ears and urogenital tract are known colonization sites for mycoplasma (Biddle et al., 2005, Fox et al., 2005) and studies comparing strains isolated from these body sites to those from the mammary gland showed that 90% of them matched, which suggests the potential for systemic transmission to the udder and that at least some of these other body sites may serve as a

reservoir (Biddle et al., 2005, Fox et al., 2005). In addition to the damage that mycoplasma causes with regards to severe clinical mastitis, milk loss (González and Wilson, 2003) and the health of affected animals, it is a dangerous pathogen to the young animals on the farm as well (González and Wilson, 2003, Wilson et al., 2007). Calves may become exposed at birth via direct contact with the urogenital tract, from the nasal discharge of the dam or from bacteria shed into the milk fed to them (Woldehiwet et al., 1990, González and Wilson, 2003). Between 80-92% of herds contain calves with at least one nasal swab positive for *Mycoplasma* spp., often detected in 30-50% of pre-weaned calves with *Mycoplasma bovis* being the primary strain responsible for respiratory disease, otitis and arthritis (Woldehiwet et al., 1990, Wilson et al., 2007, Maunsell and Donovan, 2009). An interesting feature of the mycoplasmal disease complex in dairy cattle is that infection is widespread in young calves, but absent in the adult cows in the vast majority of dairy herds at any given time (Woldehiwet et al., 1990, Wilson et al., 2007, Maunsell and Donovan, 2009, Zadoks et al., 2011).

Subclinical Mastitis

Subclinical mastitis, by definition, does not result in visibly abnormal milk, such as clots or watery milk, and is therefore much more difficult to detect. However, there is a specific organism commonly associated with the majority of subclinical mastitis in most herds, coagulase-negative staphylococci (CNS) (Thorberg et al., 2009). The prevalence most commonly seen in the United States, Canada and European countries is 3-30% of quarters and 27-55% of cows culture positive with CNS (Oliver et al., 1990, Gillespie et

al., 2009, Pyorala and Taponen, 2009). Historically, CNS has been associated with causing only subclinical or mild clinical mastitis (Pyorala and Taponen, 2009). While this still holds true, the estimated 15-28% prevalence amongst mastitic quarters (Pyorala and Taponen, 2009) and more recent studies demonstrating the organisms' ability to persist (Taponen and Pyorala, 2009), have increased focus on it as an important mastitis pathogen. Routine use of a milk quality diagnostic lab culture (sometimes followed by additional testing on colonies isolated such as MALDI-TOF, PCR, etc.) on individual cow samples is the best and most likely method of detection of this organism (Pyorala and Taponen, 2009, Britten, 2012). The milk from these cows is normal in appearance but is associated with elevated SCC counts in bulk tank milk (Wilson et al., 1997b, Pyorala and Taponen, 2009). A study in which cows were followed for an entire lactation has shown that quarters with a persistent CNS infection had a mean SCC of greater than 600,000/ml, compared to a mean SCC of about 60,000/ml in healthy quarters (Taponen et al., 2007, Pyorala and Taponen, 2009). Subclinical CNS infections may be transient or they may develop into persistent infections, lasting in the udder for months or perhaps the entire duration of the lactation (Taponen et al., 2006). Most CNS cases respond well to antimicrobial therapy (Taponen et al., 2003, Pyorala and Taponen, 2009) if detected, with bacterial cure rates as high as 81% (Wilson et al., 1999). Because CNS is an opportunistic pathogen that is a part of the cow's environment and found readily on teat skin, reinfection within a lactation is always a risk (Chaffer et al., 1999). However, differentiating between persistent and new infections is difficult (Taponen et al., 2006). Primiparous heifers are especially susceptible to CNS infections, which can colonize in

the mammary gland of pregnant and even immature animals and develop into IMI at the time of calving (Matthews et al., 1992, Pyorala and Taponen, 2009).

Coagulase-negative staphylococci are not associated with the more severe ramifications of significantly elevated SCC, bacterial counts or cases of clinical mastitis that are seen with major mastitis pathogens or contagious mastitis pathogens (Barkema et al., 1998). However, in herds aiming for excellent milk quality and low bulk tank milk SCC, CNS is a relatively important pathogen (Hutton et al., 1990, Wilson et al., 1997a, Schewe et al., 2015). Additionally, while CNS IMI are often transient infections, more recent studies have shown that some CNS species are responsible for infections that linger throughout the lactation (Taponen and Pyorala, 2009), with as many as half persisting until cessation of milking (Taponen et al., 2007).

Environmental Mastitis Pathogens

Environmental pathogens are broadly defined as opportunistic organisms, whose primary reservoir is the cows' environment, whether that be soil, bedding or water (Smith et al., 1985a, Smith and Hogan, 1993). These organisms vary in their responsiveness to antibiotic treatment and though they are defined as non-contagious mastitis organisms, cow-to-cow transmission may be possible and they are all capable of causing elevated SCC and/or persistent infections that may last in the udder for an extended period of time (Smith et al., 1985a, Reyher et al., 2012). Development of chronic IMI is defined by an infection lasting for 2 months or more (Aiello, 2012) or the same organism being isolated

from samples from two episodes of clinical mastitis no longer than 14 days apart (Barkema et al., 1998, Hertl et al., 2011). Chronic IMI may be expressed as recurrent episodes of clinical mastitis but often will remain largely asymptomatic (Aiello, 2012). Recurrent infections may take place in the same quarter of the udder each time or in different quarters (Berry and Meaney, 2006, Dohoo et al., 2011, Nyman et al., 2018). The environmental pathogens commonly associated with chronic mastitis are composed of both Gram-positive and Gram-negative bacteria (Smith et al., 1985a). This is an important point, as traditionally Gram-positive infections have been cured at a higher rate following antibiotic therapy than Gram-negatives (Erskine et al., 2002, Erskine et al., 2003). Many culture based udder health programs recommend refraining from treating Gram-negative bacteria because of little or no susceptibility to approved intramammary antibiotics (Lago et al., 2011, Royster and Wagner, 2015), or the ability of the cow's immune system to clear the infection on her own (Erskine et al., 2003, Lago et al., 2011). Most studies have shown no significant differences for bacteriological cure, risk of new IMI and treatment failure risk, between treated and non-treated cows with environmental mastitis (Guterbock et al., 1993, Lago et al., 2011). These findings make an economic argument for making culture-based treatment decisions, which reduce overall use of antibiotics by nearly half and shorten the total time milk is withheld from the bulk tank (Lago et al., 2011, Royster and Wagner, 2015).

While the organisms addressed above are the most common causes of mastitis and the focus of most treatment protocols, there are other environmental pathogens that while rare have the potential to be extremely damaging (Wilson et al., 1997b, Rajala-Schultz et

al., 2004). *Serratia* spp., *Proteus* spp., *Trueperella pyogenes*, *Prototheca* spp., *Bacillus* spp., *Pseudomonas aeruginosa*, *Pseudomonas* spp., *Candida* spp., *Nocardia* spp. and yeasts are all pathogenic mastitis organisms (Smith et al., 1985a, Smith and Hogan, 2008, Zadoks et al., 2011). These pathogens are present in many parts of the cows' environment: bedding, standing water, manure, or feed (Smith et al., 1985a, Smith and Hogan, 2008, Hogan and Smith, 2012b). The pervasive nature of these organisms allows for opportunistic infections and also many IMI caused by these pathogens originate during the dry period (Smith and Hogan, 2008). Antibiotic therapy is not recommended for mastitis cases caused by any of these organisms (Pinzon-Sanchez et al., 2011), rather prevention is believed to be the most successful strategy for control (Smith et al., 1985a, Smith and Hogan, 2008). Environmental infections are characteristically of short duration and a higher proportion of them result in clinical mastitis compared with the contagious pathogens (Smith and Hogan, 2008) but they can also persist longer or become chronic, specifically *Serratia* spp. and *Prototheca* spp. (Todhunter et al., 1991, Pieper et al., 2012).

Intramammary Antibiotics

The intramammary use of antibiotics to treat mastitis has undergone many changes throughout the last 70 years. Mastitis is the leading cause of antimicrobial use on dairy farms, with an estimated 16% of all dairy cows being treated for the disease at least once in their productive life (APHIS, 2008). The introduction of penicillin to the industry 70 years ago was a massive breakthrough in treating mastitis and reducing outbreaks

(Wilson et al., 1970). Studies have shown that 20-35% of dairy producers use intramammary antibiotics on all clinical mastitis cases, regardless of knowing the causative pathogen or not (Schewe et al., 2015, Kayitsinga et al., 2017). More recently, concerns about emerging antimicrobial resistance have shifted the focus of treatment protocols to preventative management and selective treatment of mastitis cases (Pinzon-Sanchez et al., 2011, Royster and Wagner, 2015, Kayitsinga et al., 2017). The intramammary antibiotics which are FDA approved for use in lactating cows are limited to three antibiotic classes: beta-lactams, macrolides and lincosamides (NMPF, 2012, Ruegg, 2015). From these classes, a limited number of intramammary treatments are available (NMPF, 2012, Royster and Wagner, 2015). The beta-lactam class of antibiotics, which includes cephalosporins and penicillin, is by far the most commonly used class of drugs for intramammary therapy in cattle (Oliver and Mitchell, 1984, APHIS, 2008). A report published in 2014 by the National Animal Health Monitoring and Surveillance, under the United States Department of Agriculture (USDA) found that 97% of dairy farm operations used some type of antimicrobial protocol for mastitis, of which 89% were intramammary (APHIS and USDA, 2014). Mastitis is most common reason for on-farm antibiotic use and 99% of operations reported to having at least one case of mastitis per year (Hill et al., 2009, APHIS and USDA, 2014). An increasingly negative consumer perception of livestock management practices and concerns over antibiotic resistance have driven farms to focus management practices more toward prevention rather than treatment. Mastitis is nearly always caused by bacteria, which as previously mentioned are classified as Gram-negative or Gram-positive. Bacteria are assigned to one of these

classifications by their physical structure. Gram-positive bacteria have two cellular walls, with a thick layer of peptidoglycan in between them. When stained, this peptidoglycan layer binds the stain and turns purple, which identifies it as Gram-positive (Beveridge, 2001, Romaniuk and Cegelski, 2015). Gram-negative bacteria also have the inner cellular membrane that Gram-positive bacteria have but additionally they have a unique outer cellular membrane and only a thin layer of peptidoglycan (Romaniuk and Cegelski, 2015). The thin layer of peptidoglycan does not bind the stain to the degree that Gram-positive bacteria do and therefore stains red, which differentiates it. The outer cell membrane of Gram-negative bacteria also provides protection against toxins, including antibiotics (Beveridge, 2001, Miller, 2016). Previous research has shown that as much as 50% of single-quarter clinical mastitis cases are negative for bacterial growth on culture, so in these cases there is no organism to target for the use of antibiotic treatment (Lago et al., 2011, Pinzon-Sanchez et al., 2011). An additional 20-30% of clinical mastitis cases are Gram-negative pathogens that are unlikely to respond to antibiotic therapy (Lago et al., 2011, Britten, 2012). Which leaves roughly 20-30% of mastitis cases as likely being Gram-positive and susceptible to antibiotic treatment (Lago et al., 2011, Pinzon-Sanchez et al., 2011). This research has supported selective antibiotic treatment based on the identification of the causative bacteria of mastitis (Roberson, 2003, Lago et al., 2011, Pinzon-Sanchez et al., 2011).

Mammary Physiology

The increased amount of information available on mastitis and the shift towards selective use of antibiotics has also changed the role of dairy management in terms of mastitis detection and management protocols for how to handle affected cows. Some cows will develop a chronic infection in a single quarter resulting in recurrent clinical episodes of mastitis and/or a chronically high SCC, which negatively impacts overall bulk tank milk (Middleton and Fox, 2001, Seeth et al., 2016, Britten et al., 2018). One management solution to this problem is to stop milking the affected quarter, resulting in mammary involution of just that quarter (Nickerson, 1993, Britten et al., 2018). The bovine udder is comprised of four mammary glands drained by four individual teats (Capuco and Akers, 1999, Akers and Akers, 2016b). The four glands are structurally separated and function independently of each other, with no internal connection. Previous studies have shown that if one quarter is removed from production, the three remaining quarters will compensate in their production by an average of 4% per quarter (Hamann and Reichmuth, 1990, Hamann and Gyodi, 2009). Currently, there is no standardized method within the dairy industry for creating three-quartered cows. Most commonly, individual quarters are dried off mid-lactation by intramammary infusion of iodine or chlorhexidine (Middleton and Fox, 1999, 2001) or simply ceasing to milk the quarter (Natzke et al., 1975, Zobel et al., 2013). Infusing iodine into the quarter successfully stops production but is also irreversible and permanently removes the quarter from production (Middleton and Fox, 2001). Chlorhexidine infusion is also effective, and some animals may regain function in the treated quarter in the subsequent lactation, but many

of them will also lose the quarter permanently (Middleton and Fox, 1999, 2001). Additionally, both methods carry the risk of antimicrobial residues ending up in the bulk tank milk if accidentally milked, which violates the Pasteurized Milk Ordinance (Middleton et al., 2003, USHHS, 2011, 2017). Abrupt cessation of milking the quarter is less risky than infusion of iodine or chlorhexidine from a residue standpoint, however this can be difficult in high-producing animals. While milk stasis from cessation of milking does initiate mammary involution, high-producing cows may develop severe distension of the quarter, mastitis and show signs of discomfort (Oliver and Sordillo, 1989, Shamay et al., 2003). Mammary involution is natural part of a dairy cow's lactation cycle and occurs late in gestation, when daily milking is stopped at approximately 60 days prepartum and she enters the "dry period" until the beginning of the next lactation following calving (Natzke et al., 1975, Stefanon et al., 2002, Cermakova et al., 2014). The primary goals of the dry period are to use antibiotic treatment to cure existing subclinical mastitis infections (Bradley and Green, 2004, van Knegsel et al., 2013) and maximize milk production in the next lactation (van Knegsel et al., 2013).

Hormonal Regulation of Lactation and Mammary Involution

The dairy cow lactation cycle has distinct differences between lactating and non-lactating phases, as described previously. The hormones that are associated with mammary development (mammogenesis), lactation production (lactogenesis) and cessation of milk production (involution) can be divided into three categories: reproductive hormones, metabolic hormones and mammary hormones (Chakriyarat et al.,

1978, Neville et al., 2002). The reproductive hormones include prolactin, oxytocin, estrogen, progesterone and placental lactogen and are primarily responsible for preparing the mammary gland for delivery of milk (Neville et al., 2002). The key metabolic hormones are corticosteroids, thyroid hormone and insulin and they regulate the body's responses to metabolic needs and stress associated with pregnancy and milk production (Neville et al., 2002, Svennersten-Sjaunja and Olsson, 2005). The mammary hormones include growth hormone (GH), prolactin, leptin and parathyroid hormone-related protein (PTHrP). It has been recognized relatively recently that this latter group of hormones is produced by the mammary gland itself and secreted into the milk (Neville et al., 2002). Prolactin and GH are required for transitioning the mammary gland into a lactating state, however it is not clear whether GH acts directly upon the mammary gland or indirectly via insulin growth factor-1 (IGF-1) (Svennersten-Sjaunja and Olsson, 2005). Lactation is marked by an increase in IGF-1, which is believed to be optimized by prolactin suppression of IGF binding protein and GH stimulus of IGF synthesis (Svennersten-Sjaunja and Olsson, 2005). The prolactin, GH and IGF-1 hormone levels decrease during involution in non-pregnant animals, which is associated with the loss of epithelial cells through apoptosis (Svennersten-Sjaunja and Olsson, 2005). Dairy animals are unique in that they are lactating and then undergo mammary involution while concurrently pregnant. Hormones that increase during pregnancy and inhibit lactation in many species, such as progesterone do not appear to inhibit milk synthesis in dairy cattle which are routinely milked up until 8-9 weeks prior to parturition (Tucker, 2000, Svennersten-Sjaunja and Olsson, 2005). Additionally, the elevated blood concentrations of estrogen

and progesterone seen in late pregnancy stimulate extreme parenchymal growth which is occurring during the dry period when the cow is not producing milk (Hurley and Loor, 2011, Akers and Akers, 2016a). Because of these opposing hormonal influences, dairy animals do not experience the degree of mammary epithelial cell turnover that is observed in rodents or other species that are not pregnant during mammary involution (Zarzynska and Motyl, 2008, Hurley and Loor, 2011, Akers and Akers, 2016a).

Mammary Involution: Physiological and Histological Changes

The mammary gland undergoes distinct physiological changes between an active lactating state and a fully involuted gland, which is comprised of mostly non-secretory tissue. These changes include both physiological changes to tissues within the gland and changes to immune cell populations present. While the morphologic changes that occur during involution in dairy animals are less pronounced than those seen in nonpregnant mice (Strange et al., 1992), there were distinct morphologic changes observed between lactating and involuting bovine mammary tissues (Capuco and Akers, 1999). Briefly, there is an inverse relationship between luminal area and thickness of intra-alveolar stroma (Capuco and Akers, 1999). Lumen diameters and total luminal area decrease to less than 10% of total mammary tissue at 35 days dry, compared with approximately 21% of the gland in lactating cattle (Capuco and Akers, 1999, Akers et al., 2006). In contrast, proportion of stromal tissue was previously described as maximized at 35 days dry and then decreased shortly before calving (Capuco and Akers, 1999). Interstitial stroma is a continuous tissue type between the lumina and throughout the

mammary gland, as opposed to a homogeneous well-defined structure, which makes it difficult to precisely quantify changes. Alveolar structure is maintained throughout the dry period but the height of the epithelial cells increases throughout the process of involution (Hurley, 1989). As lumen diameters decrease, the height of the alveolar epithelial cells surrounding them increases (Sordillo and Nickerson, 1988). These morphologic changes begin immediately following cessation of milking and continue for 14-21 days post dry-off (Oliver and Sordillo, 1989).

Increased epithelial height, decreased secretory luminal diameter and increased interstitial stromal thickness are the primary mammary tissue changes observed in the transition from lactation to the non-lactating dry period. Mammary tissue during lactation is primarily composed of large secretory alveolar lumina which are lined with well-differentiated secretory epithelial cells and surrounded by minimal amounts of interstitial stroma (Akers et al., 1990). After cessation of milking, the rapid increase in hydrostatic pressure and effect of milk stasis on these tissues begins to initiate change (Akers and Akers, 2016d). Alveolar epithelial cells are initially well-defined and cuboidal in shape and then become non-secretory, less well-differentiated and mostly columnar (Akers et al., 1990, Capuco and Akers, 1999). The thickness of interstitial stroma increases as luminal diameters decrease (Sordillo and Nickerson, 1988, Hurley, 1989).

The predominant immune cell types found in healthy mammary tissue are lymphocytes and macrophages, with a very low population of neutrophils (Stelwagen et al., 2009, Sordillo, 2018). Macrophages and neutrophils are integral for recognition and phagocytosis of foreign elements and lymphocytes regulate the sensitization or

suppression of the immune response (Paape et al., 1979, Nickerson, 1989). During episodes of mastitis, the innate immunity of the mammary gland dominates the early recognition and response to infection by bacteria and the cell populations change. The overall leukocyte count is considerably elevated and large numbers of neutrophils are recruited to the site, becoming the predominant cell type over macrophages and lymphocytes (Oviedo-Boyso et al., 2007, Sordillo, 2018). The process of bovine mammary involution elicits a similar immune response to that of mastitis, as it is also an inflammatory process. Leukocyte counts increase within seven days of cessation of milking and remain elevated for several weeks into the dry period (Nickerson, 1989). In uninfected dry mammary glands, neutrophil populations remain proportionally lower than macrophages and lymphocytes, similar to healthy lactating tissue even though all cell types are elevated during involution. However, if a bacterial organism is present at dry-off the neutrophil count becomes the dominant white cell type, again similar to within lactating glands (Nickerson, 1989, Atabai et al., 2007). Neutrophil activity is inhibited during involution by the increased presence of casein micelles and fat globules, which are indiscriminately phagocytized by neutrophils (Nickerson, 1989). The reduction in neutrophilic antibacterial activity following phagocytosis of casein and fat increases the susceptibility for infection of the mammary gland during the nonlactating period.

Some other changing components of milk secretions during mammary involution include lactoferrin, bovine serum albumin (BSA) and pH. Bovine lactoferrin and BSA have frequently been used as measures of involution in previous research (Sordillo et al., 1987, Kutila et al., 2003, Boutinaud et al., 2016, Lanctôt et al., 2017). Bovine lactoferrin

is found in low concentrations during lactation and increases significantly during involution (Hurley, 1989, Nickerson, 1989). Lactoferrin plays an immunologically important role by binding available iron and making it unavailable for iron-dependent bacteria to grow (Nonnecke and Smith, 1984, Galfi et al., 2016). Serum albumin is a blood protein that, like lactoferrin, is low in normal milk but increases substantially during involution or episodes of mastitis, reflecting the leakage of the tight junctions which allows an influx of interstitial fluid (Nonnecke and Smith, 1984, Stelwagen and Singh, 2014) that becomes mixed with milk during involution or mastitis episodes from an influx of interstitial fluid which occurs as a result of leaky tight junctions between the mammary epithelial cells (Nguyen and Neville, 1998, Stelwagen and Singh, 2014). During lactation, the mammary epithelia form an impermeable barrier between the apical (milk) side of the barrier and the basal (blood) side, by tight junctions between the cells (Hurley, 1989, Stelwagen and Singh, 2014). The increases in lactoferrin and bovine serum albumin cause the overall milk protein concentration to rise with involution, which is in contrast to the decrease in casein that takes place during involution. During involution, there is a slight increase in the pH of the milk secretions but the reasons behind this are not fully understood. The disruption of the blood-membrane barrier and enzymatic activity are believed to contribute to changes in pH (Sordillo et al., 1987, Hurley, 1989).

Lactation Cycle and the Dry Period

Involution of the mammary gland is not unique to cows and has in fact been studied more thoroughly in rats, mice and rabbits than in the bovine species (Strange et al., 1992, Capuco and Akers, 1999, Zarzynska and Motyl, 2008). The mammary gland is an intricate organ and mammary involution is a complex, multi-step process (Stein et al., 2007). Mouse mammary glands have been used most commonly to study the remodeling which occurs during involution (Strange et al., 1992, Zarzynska and Motyl, 2008), but key differences have been identified between involution in the bovine udder and that of a rodent mammary gland (Akers et al., 1990, Akers and Akers, 2016b). Bovine involution occurs at a much slower rate than in rodents and is believed to be a regenerative process, with approximately 50% of the original epithelial cells undergoing apoptosis (Zarzynska and Motyl, 2008). However, dairy cows are typically in their final trimester of pregnancy at the time of dry-off and during the dry period, which is an important difference to note when comparing to studies in other species (Akers et al., 1990, Hughes and Watson, 2018). Recent research has determined that the amount of mammary tissue regression that takes place in pregnant cattle during involution compared to lactating cattle is unremarkable; dry cows have been shown to only have 10% less mammary epithelial cells than lactating cows (Capuco et al., 1997, Hughes and Watson, 2018). Another study comparing apoptosis in bovine mammary involution to lactating tissue, showed that the proportion of apoptotic cells in lactating tissue was only approximately 2% less than involuting tissue (Wilde et al., 1997). This suggests that while the dry period may be important for optimizing milk production in the following lactation, substantial gland

remodeling is not a significant contributor to curing existing mammary infections (Holst et al., 1987, Atabai et al., 2007, Hughes and Watson, 2018). Cessation of daily udder emptying is the initiator of mammary involution, which begins within 24-48 hours after milking stops (Akers et al., 1990, Zarzynska and Motyl, 2008). Milk stasis results in udder engorgement and causes alveolar lumens to stretch and lose their structural integrity (Holst et al., 1987, Capuco and Akers, 1999). During lactation, tight junctions which surround each mammary epithelial cell form an impermeable barrier between the basolateral and the apical sides of the gland and serve as prevention against transportation of substances across the epithelium by means of moving through intercellular spaces (Nguyen and Neville, 1998, Capuco and Akers, 1999, Shamay et al., 2002). Tight junctions are regulated by two major protein families, occludins and claudins, which are linked to epithelial cells' cytoskeletons (Stelwagen and Singh, 2014). These proteins become compromised by mammary inflammation, which results from mastitis or mammary involution, and tight junctions are no longer impermeable and become "leaky", allowing blood serum proteins to diffuse from the apical to basolateral side into the secretory lumen of the alveoli and then into the milk, consequentially raising the concentration of BSA (Hurley, 1989, Stein et al., 2007, Stelwagen and Singh, 2014). Plasmin is the dominant protease in milk, found mainly in its inactive form, plasminogen (Politis et al., 1989, Silanikove et al., 2000). The conversion of plasminogen into its active form, plasmin, is controlled by plasminogen activator (PA) in milk (Politis et al., 1989, Politics, 1996, Ponchon et al., 2014). The mechanism by which plasminogen is converted into plasmin is known as the PA-plasminogen-plasmin system (PPS) and is

suggested to be part of control of milk secretion and tissue remodeling during involution (Politis et al., 1989, Silanikove et al., 2000, Shamay et al., 2002). It has been established that increased PA content in milk is associated with reduced milk yield and induction of involution; involution also activates the PPS system, which naturally fragments β -casein proteins in the milk (Politis et al., 1989, Politis, 1996, Shamay et al., 2002, Shamay et al., 2003, Ponchon et al., 2014). Intramammary infusion of a pure β -casein fraction into the udder lumens of goats was investigated and demonstrated a transient decrease in milk reduction following a single dose, with a more sustained decrease in milk production following multiple treatments (Shamay et al., 2002).

While the benefits of the dry period have been recognized, the transition states of lactating to nonlactating and vice versa are vulnerable periods and pose a high risk of new IMI (Godden et al., 2003, Schukken et al., 2011a). However, once the mammary gland is fully involuted, it is quite robust against new intramammary infections (Oliver and Sordillo, 1989, Atabai et al., 2007). Infections present at the time of dry-off or new IMI acquired during the dry period often persist into the subsequent lactation and can be responsible for mastitis episodes in the early part of the next lactation (Oliver and Sordillo, 1989, Anderson et al., 2010). It has been demonstrated that quarters which became infected during the dry period produced significantly less milk during the following lactation, compared to other quarters (Natzke et al., 1975, Anderson et al., 2010). Quarters which were infected at dry-off and at parturition produced 48% less milk, and quarters which were infected at dry-off but not at parturition produced 11% less milk than uninfected contralateral quarters of the same cow (Smith et al., 1968, Oliver and

Sordillo, 1989). The chemical composition of milk during lactation is not optimized to defend the gland against pathogens. Concentration of leukocytes and IgG antibodies is relatively low during lactation and phagocytes arbitrarily ingest fat and casein, which alters the morphology of neutrophils that are present and reduces their phagocytosis of mastitis pathogens (Oliver and Sordillo, 1989, Paape et al., 2003). The chemical composition of secretions from fully involuted mammary glands is quite different than from milk; concentrations of fat and casein are low and there are more phagocytic cells, which allows for more efficient phagocytosis (Oliver and Sordillo, 1989, Oviedo-Boyso et al., 2007).

There are two common methods to dry off cows: intermittent milking or abrupt cessation of milking (Natzke et al., 1975, Zobel et al., 2013). While both methods are effective, the latter may be associated with extreme udder engorgement, signs of discomfort, or mastitis, especially in high-producing animals that may still be yielding \geq 60 lbs./day at dry-off (Leitner et al., 2007, Zobel et al., 2013). Along with cessation of milking, a traditional management protocol is the administration of an intramammary dry cow antibiotic into each quarter (Godden et al., 2003, NMC, 2006). The efficacy of using dry cow antibiotic therapy to cure infections present at dry-off and prophylactically against new IMI has been well documented (Hassan et al., 1999, NMC, 2006, Golder et al., 2016, Bonsaglia et al., 2017). The dry period allows for increased retention time of the antibiotic in the udder and a higher dosage (NMC, 2006).

Currently, controversy exists about the “blanket” use of dry cow therapy, where every quarter of every cow is infused, versus selective dry cow therapy which only

targets high risk animals (Scherpenzeel et al., 2016, Vanhoeij et al., 2016). While selective dry cow therapy may reduce use of antibiotics, treatment may fail to reach 20% to 40% of infected quarters in the herd (NMC, 2006, Scherpenzeel et al., 2016). Current research substantiates the belief that blanket dry cow therapy better protects cows against new IMI than selective treatment (Halasa et al., 2009, Scherpenzeel et al., 2016). However, selective use of dry cow treatment results in a decrease of antimicrobial use and when compared to blanket use of dry cow treatment, has not been found to be associated with an increase in clinical mastitis (Cameron et al., 2015, Scherpenzeel et al., 2016, Vanhoeij et al., 2016).

Economic Effects of Mastitis

Mastitis is the most common and expensive disease the dairy industry faces (Ott, 1999, Losinger, 2005, Cha et al., 2011, Hogeveen et al., 2011, Ruegg, 2011). Mastitis effects milk production, antibiotic usage, veterinary fees, labor, fertility rates, cull rates and replacement animal costs (Schroeder, 2012, Tiwari et al., 2013, Schewe et al., 2015, Akers and Akers, 2016e, Scherpenzeel et al., 2016, Kayitsinga et al., 2017). The estimated annual cost of mastitis to the United States dairy industry exceeds 1 billion dollars (Ott, 1999, Ruegg, 2011). Much of this loss comes from clinical mastitis, which averages \$444 per case, and is responsible for production losses, antibiotic usage and is the most common reason for morbidity in dairy cows (NAHMS, 2007, Cha et al., 2011, Rollin et al., 2015). Direct costs associated with this number are diagnostic tests, veterinary services, treatment costs, discarded milk and death loss (Ruegg, 2011, Rollin

et al., 2015). Indirect costs are more difficult to estimate but include replacement animals, future production loss and reproductive loss (Hogeveen et al., 2011, Rollin et al., 2015).

The lost revenue from mastitis can include high bulk tank milk SCC. Earlier, the legal limits that producers are required to maintain with their SCC and standard bacteria counts to maintain Grade A status were discussed briefly (USHHS, 2011, 2017). These SCC legal limits do not account for reduced milk production and the consequent loss associated with increased bulk tank SCCs (Wilson et al., 1997a, Losinger, 2005). The association between bulk tank SCCs and the prevalence of mastitis cows in the herd is not a linear relationship but estimates can be made of the proportion of mastitic and high SCC cows that are degrading overall bulk tank milk quality (Barkema et al., 1998, Barkema et al., 2013). For example, on average a herd with a bulk tank SCC of $\geq 350,000/\text{ml}$ will have 40% of cows with an individual SCC of $\geq 200,000/\text{ml}$ (Green et al., 2006, Barkema et al., 2013). Cows with an individual SCC of $>$ than $200,000/\text{ml}$ experience up to a 200 lb. production loss per lactation for first lactation cows and 400 lb. loss per lactation for second lactation and older animals, compared with cows having an SCC below that threshold (Losinger, 2005, Anderson et al., 2010, Hogeveen et al., 2011, Ruegg, 2011). This loss is most pronounced in second parity and older animals, which lose 45% more milk in the first 24 hours of mastitis than first parity cows (Hand et al., 2012). Bulk tank SCC greater $500,000/\text{ml}$ has an association with approximately 6% lost milk production at the herd level, resulting in economic loss (Losinger, 2005, Campbell, 2010, De Vliegher et al., 2012, Rollin et al., 2015).

Consumer Impact on the Dairy Industry

In the last 50 years, the outward appearance of the dairy industry and how milk is produced has changed dramatically. The industry, which used to be predominantly comprised of smaller dairy herds continues to consolidate into fewer and larger herds (NASS, 2010). In 1987, half of all dairy cows in the U.S. were on farms with an average herd size of 80 or fewer cows, whereas 25 years later, that median herd size had increased to 900 cows (NASS, 2010, Welshans, 2016). While this newer model of dairy farming has proven to be more economically efficient while still maintaining excellent milk quality standards, the perception of dairy farms and dairy production from the consumer viewpoint has been overwhelmingly negative (Grunert et al., 2000, Muirhead, 2016). Large dairies are viewed as “factory farms” and concerns regarding animal welfare, antibiotic use, growth hormone use and management practices of raising young stock and milk harvesting are of concern to consumers (Grunert et al., 2000, Vanhonacker et al., 2008). As a result, consumption of fluid milk and some other dairy products has steadily fallen and continues to decline (Hayden Stewart, 2013). Much of the problem lies in the knowledge gap between the consumer and how their food is produced (Grunert et al., 2000, Muirhead, 2016). Larger and fewer dairy farms also means fewer farming families and most consumers are now several generations removed from the farm (Te Velde et al., 2002, Vanhonacker et al., 2008, Muirhead, 2016). This lack of education in food production, combined with widespread access to internet and ease of sharing negative imagery, has led to negativity and distrust of many farming and livestock raising

practices by much of the public (Te Velde et al., 2002, Marie, 2006, Vanhonacker et al., 2008).

Primary concerns expressed by consumers towards food animals and food animal production are the prophylactic use of antibiotics, the suspected use of growth hormones and animal welfare (Grunert et al., 2000, Marie, 2006). Part of the regulations covered in the PMO, as discussed previously, are that every load of milk must be tested and declared free of antibiotics, antimicrobial residues or any other adulteration of the milk (USHHS, 2011, 2017). A 2015 survey by the FDA of nearly 2,000 dairy farms revealed only 0.7% of loads of milk shipped from farms positive for antibiotic residues and no antibiotics were detected in retail-ready, finished products (FDA, 2015). In 1993, the FDA approved the use of a formulation of recombinant bovine somatotropin growth hormone (rBST) to increase milk production in dairy cows (FDA, 2009), which also received approval from the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) in 1998 (FDA, 2015). During the pre- and post-approval data monitoring period of rBST, no increase in residue levels of any kind was detected (FDA, 2009). Despite the documented evidence that there is no danger or impact on human health by consuming milk from cows treated with rBST, the condemning response from consumers led many milk processing plants to ban the use of rBST by their dairy producers (Hayden Stewart, 2013). The use of rBST is virtually non-existent in the U.S. now (DHM, 2017).

Animal welfare of food animals is the other mainstream area of concern (Grunert et al., 2000). Unfortunately, much of the belief that food animals are abused and/or raised

under inhumane conditions comes from lack of understanding of livestock and misinformation (Trevisi et al., 2006). Regardless, the push from consumers for food that is produced under humane conditions and with minimal antibiotic usage is unlikely to change if food is plentiful and relatively inexpensive in the U.S. It is the responsibility of the dairy industry to not only educate the consumer on how dairy cows are raised and milk is produced, but also to hold themselves to the highest standards possible of farm practices and animal care. With the public eye focused on food production and trust already compromised, only continued evidence of antibiotic-free and residue-negative milk and humanely raised food animals will alleviate consumer fears over time.

Summary

Mastitis is an expensive and damaging disease that affects many dairy cows and all dairy herds. Mastitis in dairy cows impacts milk quality, antibiotic usage, animal welfare and causes economic loss. Careful management during lactation is critical to reducing the frequency of both clinical and subclinical mastitis episodes. One management strategy is cessation of milking of single unhealthy quarters within a cow, thus diverting poor quality milk from the bulk tank and allowing the cow to safely continue a useful production life. Although this strategy is employed on many farms, a need for an improved method of achieving this still exists within the industry. It is also clear that the nonlactating (dry) period of the lactation cycle is a critical phase for milk quality and udder health. While the current strategies of using antibiotic dry cow therapy and/or internal teat sealant at dry-off are effective, there is pressure to reduce

antimicrobial usage in food livestock. Potential alternative dry-off treatments should be investigated. Intramammary infusion of casein hydrolysate appears to be an interesting and effective non-antibiotic alternative to products previously used for involution of single mastitic quarters during lactation, or of all quarters at the end of lactation.

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CHAPTER III

Intramammary infusion of casein hydrolysate for involution of single mastitic mammary quarters elevating cow-level somatic cell count**Justine E. Britten ¹, Kerry A. Rood, and David J. Wilson**

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Abstract

Mastitis in a single quarter can cause high somatic cell counts (SCC), clinical mastitis, and death in dairy cows. Currently, management of these mastitic quarters presents a problem for the dairy industry. Casein hydrolysate (CH) is an intramammary (IMM) infusion treatment reported to induce mammary involution. The primary objectives of this study were to investigate whether IMM CH treatment of single high SCC quarters, followed by cessation of quarter milk production for the remainder of

lactation, was effective in reducing cow-level SCC and whether that quarter resumed milk production following calving. Three treatment groups were used: CH, non-hydrolyzed casein (NHC), and cessation of milking only (negative; N). Treatments were assigned in a 2:2:1 ratio for 40 cows enrolled in the study; 27 cows completed the entire protocol. Following IMM infusion and involution of the single mastitic quarter, decreases in cow-level SCC ($-966,000/\text{ml}$) and milk production (-11 lb (5 kg) , -14%) with 3 remaining lactating quarters were significant for all 28 cows combined. Cows treated with CH ($n = 17$) had a significant decrease in cow-level SCC ($-1,150,000/\text{ml}$) during remaining lactation. All treated quarters returned to milk production after calving, and their proportion of total-cow milk production (24%) was not different than before treatment (28%). After calving, treated quarters' decrease in SCC was significant for CH ($-2,763,000/\text{ml}$; $n = 14$) and N ($-5,324,000/\text{ml}$; $n = 5$). Of 16 quarters with positive milk culture before treatment that completed the protocol, 88% ($14/16$) were cured (no isolation of the same bacteria for 3 weeks following calving). A new intramammary infection (IMI) was detected in 67% ($18/27$) of previously treated quarters post-calving. Infusing single mastitic quarters with casein hydrolysate to induce involution for the remainder of lactation may be a promising alternative to current methods.

Keywords: Dairy; Mastitis; Management; SCC; Milk Quality

Abbreviations

SCC: Somatic Cell Count; IMM: Intramammary; CH: Casein Hydrolysate, NHC: Non-hydrolyzed Casein, N: cessation of milking only, negative; IMI: Intramammary Infection;

BTSCC: Bulk Tank Somatic Cell Count; DHIA: Dairy Herd Improvement Association; DIM: Days in Milk, CMT: California Mastitis Test.

Introduction

Mastitis is the most expensive disease complex in the dairy industry. Costs include lost milk production, antibiotic treatment, discarded milk because of antibiotic therapy, and death loss. Reduced bulk tank milk quality and milk price also result from increased white blood cells, reported as somatic cell count (SCC), being shed into milk by affected quarters. The goal is to maintain a bulk tank SCC < 200,000/ml (Wilson et al., 1997a, Barbano et al., 2006, Troendle et al., 2017). A single mastitic quarter may have an individual SCC of millions per milliliter of milk, impacting the cow-level and bulk tank milk SCC. Previous studies have documented the negative impact that single quarters with extremely elevated SCC can have on the overall quality of bulk tank milk (Rysanek et al., 2007). Most dairy producers receive economic benefit from maintaining a low bulk tank somatic cell count (BTSCC) and understand the advantage of diverting high SCC milk from chronically inflamed quarters from entering the bulk tank (Losinger, 2005). If cows with a single quarter causing high SCC in their composite milk are undesirable for other health or production reasons, the decision may be made to remove them from the herd altogether. However, many times these animals are pregnant and/or otherwise productive dairy cows, so producers simply cease milk production in the high SCC quarter. In modern high-producing dairy cows this can sometimes be difficult to achieve without causing permanent damage to the quarter or other adverse effects on the animal (Middleton and Fox, 2001, Shamay et al., 2003). High-yielding cows with high

SCC milk from one quarter or recurring mastitis episodes in a single quarter, commonly lead producers to the management practice of attempting to remove the affected quarter by intramammary infusion (IMM) of caustic substances such as strong iodine (e.g. 2% to 7% iodine) or 2% chlorhexidine (Middleton and Fox, 1999). Previous studies have shown efficacy of these methods for cessation of milk production in a single mammary quarter (Middleton and Fox, 1999, 2001) but both iodine and chlorhexidine were reported to induce undesired consequences (Middleton et al., 2003). Use of strong iodine was associated with no return to production, essentially creating a permanently “three-quartered” cow (Middleton and Fox, 1999). Intramammary chlorhexidine resulted in some cows regaining full use of the treated quarter in the subsequent lactation (Middleton and Fox, 1999), but antimicrobial residue was detected 35 to 42 days post infusion by Delvotest[†]¹ (Hillerton et al., 1999, Middleton et al., 2003) validating the concern that accidental milking of an infused “dry” quarter could lead to antimicrobial residue violations in bulk milk. Therefore, the off-label IMM of chlorhexidine is not recommended as a method for ceasing milk production in a quarter (Middleton et al., 2003).

Earlier studies found IMM of casein hydrolysate effective in inducing involution of the mammary gland without systemic disease or causing permanent quarter damage (Shamay et al., 2003, Leitner et al., 2012). Casein hydrolysates are milk-borne factors believed to be part of the biological pathway which causes involution in the bovine mammary gland, 40–70 days prior to expected parturition (Shamay et al., 2003).

¹ Delvotest-NT, DSM Company, Heerlen, Netherlands

Intramammary infusion of casein hydrolysates has been demonstrated to locally induce involution within a single quarter, as a management strategy for cessation of lactation in quarters with a persistently elevated SCC or repeated episodes of mastitis (Leitner et al., 2012), without the consequences of antimicrobial residues and/or permanent mammary gland destruction.

The basis for this study was to address the need for managing mastitic quarters mid-lactation in otherwise healthy dairy cows. Currently, there is no widely accepted method available for producers to use on these animals. This shortfall has resulted in unsatisfactory outcomes. The primary objective was to evaluate cessation of milking in individual mastitic quarters using intramammary infusion of CH, in comparison with intramammary infusion of a placebo or no infusion.

Material and Methods

Selection of study cows

Study cows were selected from 6 commercial Idaho dairy farms. All cows were housed in outdoor, shaded dry lot pens typical of dairy farms in that region. Participating farms were on a twice per day milking schedule and following a regular monthly dairy herd improvement association (DHIA) testing schedule (Voelker, 1981). Lactation number, SCC, pregnancy status, days until expected dry off date, days until expected calving and daily milk production data were obtained from DHIA records. To be eligible, cow-level $\text{SCC} \geq 500,000/\text{ml}$, confirmed pregnant, ≥ 35 days before scheduled dry-off date,

estimated 95–220 days until expected calving, and daily milk production ≥ 50 lb (22.7 kg) were required.

Cows meeting eligibility requirements were then screened for quarter-level IMI using the California Mastitis Test (CMT) (Schalm and Noorlander, 1957). For trial inclusion, CMT scores of 2–plus in a single quarter and Negative or Trace in the other 3 quarters were required. Using aseptic sampling technique, an individual milk sample was collected from the quarter with an elevated CMT score, along with a pooled milk sample of the other three quarters. An aliquot of the individual quarter sample was used for microbial culture, while the remainder of the quarter sample and the pooled sample of the other quarters were both tested for SCC. Somatic cell count was measured by use of the FossomaticTM automatic cell counter² (Miller et al., 1986). An SCC of $\geq 1,000,000/\text{ml}$ in the single mastitic quarter, $\text{SCC} \leq 400,000/\text{ml}$ in the 3 non-mastitic quarters, and *Mycoplasma* spp.–negative culture results finalized enrollment in the study.

Using a completely randomized block design, cows were blocked by lactation number (1st, 2nd–plus) and mastitic quarter culture result (growth, no growth), for a total of 4 blocks. There were 3 treatment groups: casein hydrolysate (CH), non-hydrolyzed casein (NHC), and cessation of milking only (negative; N). Cows were randomly assigned to treatment groups within each block, in a 2:2:1 proportion due to the challenges of obtaining a large sample size and in a purposeful determination to allot most of the animals to the CH and NHC treatment groups.

² FossomaticTM 7, FOSS, Eden Prairie, MN

Milk microbiology

Milk sample bacterial cultures were completed according to standard methods (Hogan, 1999, Britten, 2012). In brief, an inoculum of 10 µl of milk was plated on washed cow blood agar and placed in a standard, non-CO₂ incubator at 37° C for 48 h. Plates were examined by laboratory technicians at 24 h and 48 h for bacterial growth; organism identification was determined by colony morphology and biochemical secondary tests on any isolates found.

Preparation of casein hydrolysate

Two batches of casein hydrolysate were prepared as per Shamay et al. (2003) using aseptic technique. Each batch was prepared using 100 g of commercially purchased bovine casein powder³ dissolved in 1 L of autoclaved deionized water containing 3 g of TRIS buffer and enzymatically digested with Trypsin. After digestion, remaining particulate material was removed via two centrifugation cycles of 15 minutes at 3000 x g. Solution was boiled for 15 minutes between centrifugation cycles to denature any remaining enzyme and kill possible environmental contaminant bacteria, followed by sterilization using vacuum membrane filtration. The final product was dispensed (15 ml) into sterile syringes and stored frozen at – 20 °C. Two batches of NHC solution were produced following the same methods as above but omitting enzymatic digestion, thus preventing hydrolysis.

³ Sigma Aldrich, St. Louis, MO

Each batch of casein hydrolysate or NHC was screened for bacterial contamination by inoculating tryptic soy broth with 1 ml of solution, incubating for 24 h and inoculating blood agar. Blood agar plates were incubated for a total of 48 h at 37 °C and read at 24 and 48 h for bacterial growth. A separate blood agar plate was plated directly with 100 µl of non-enriched solution and then incubated and examined in the same way. The definition of an uncontaminated batch was no growth of any bacterial colonies on either direct or enriched cultures. Protein concentration of each batch was quantified using a bicinchoninic acid (BCA) assay⁴, according to the manufacturer's instructions. The BCA assay is commonly used for protein quantification (Smith et al., 1985b).

Milk weight collection

Mastitic quarter contribution to total-cow milk production was measured by bucket milking on Day 1 of the 48 h treatment protocol. Animals were milked into a clear, graduated, 80 lb (36.3 kg) capacity bucket in two steps: the three healthy quarters were milked together first, followed by the single mastitic quarter. This allowed measurement of milk production from the total cow for that milking and the proportional contribution of the mastitic quarter. This bucket milking process was repeated at a single milking, between 10–21 days in milk (DIM) in the subsequent lactation, to evaluate the milk production and proportion of total-cow milk from the mastitic quarter.

Treatment administration

⁴ ThermoFisher Scientific, Waltham, MA

All mastitic quarters were milked once per day for the 3-day treatment period. Each animal had all 4 quarters milked in the morning, followed by infusion of the mastitic quarter with the assigned treatment (CH or NHC) or no infusion (N), followed by skipping the evening milking of that mastitic quarter. This process was repeated at 24 h (day 2) and again at 48 h (day 3) for the third and final treatment. This gradual cessation of milking was intended to cease milk production in the target quarter with minimal discomfort and risk of adverse effects to the animal. The mastitic quarter was not milked again for the remainder of the lactation. Somatic cell count data from the 3 remaining quarters was obtained from the next DHIA test following treatment.

Evaluation of intramammary infections following dry period

Bacteriological cure of previous IMI and new infection rates were not primary objects of this study. However, involution is a mechanism of clearing existing IMI during the dry period (Nonnecke and Smith, 1984, Hassan et al., 1999) and this is an important outcome for any treatment during the dry period in dairy cattle. After calving the previously treated quarters were resampled 3 times, once per week at 1–7 DIM, 8–14 DIM and 15–21 DIM. Resulting case definitions were: Cure = all 3 post-calving cultures negative for any bacteria isolated from the pre-treatment sample; Chronic IMI = any bacteria isolated from the pre-treatment sample, followed by isolation of the same bacteria from at least one post-calving culture. New IMI = one or more bacteria not isolated from the pre-treatment sample, followed by isolation from at least one post-calving culture sample (multiple bacterial species only count as one IMI).

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 7.01⁵ and SAS Studio⁶. Descriptive statistics were calculated. To evaluate for possible confounding, pre-treatment variables were compared among the 3 treatment groups. The continuous variables DIM, days until expected dry-off, cow-level SCC, mastitic quarter SCC, total-cow milk production, and proportion of treated quarter contribution to total-cow milk weight were evaluated for possible differences between treatment groups using analysis of variance (ANOVA). The grand means of cow-level SCC, mastitic quarter SCC, total-cow milk production, and mastitic quarter milk production (including comparison between all front and rear quarters) were compared pre- and post-treatment using a t-test. Means of the continuous variables cow-level SCC, mastitic quarter SCC, total-cow milk production and quarter proportion of total-cow milk were compared pre- and post-treatment between treatment groups using ANOVA. The change in each of the above outcome variables from pre- to post-treatment was tested for significance within each group, also using ANOVA. For the continuous variables DIM, DCC, pre-treatment SCC of total cow and individual quarter, pre-treatment milk weight of total cow and individual quarter and contribution of the individual quarter to the total cow production, association with post-treatment SCC in the remaining three quarters was evaluated using multiple regression (PROC REG). Input variables that might logically be associated with the outcome variable – log of post-treatment SCC in the remaining 3 lactating quarters – were evaluated in both a linear mixed model (PROC GLIMMIX, SAS Studio) and a general linear model (PROC GLM, SAS

⁵ GraphPad Software, Inc. La Jolla, CA, USA

⁶ SAS Institute Inc., Cary, NC, USA

Studio). Initial models included all logical potential input variables followed by backward elimination until the final model included only input variables with $P < \alpha$. The categorical outcomes of Cure, Chronic IMI, and New IMI were compared for significant differences between the categorical variable treatment group using Chi-square. Alpha was 0.05 for all statistical analyses.

Results

Forty cows (Holstein $n=38$, Jersey $n=2$) were initially enrolled in the study. Their single mastitic quarters (5 right front, 8 left front, 14 right rear, 13 left rear), were randomly assigned among the 3 treatment groups (2:2:1 ratio). There were 18, 15 and 7 cows in the CH, NHC and N treatment groups, respectively. Six cows were in 1st lactation and 34 were in 2nd-plus lactation, 23 treated quarters had bacteria isolated pre-treatment, while 17 quarters had no growth on culture. All 40 mastitic quarters were successfully dried off, without any reports of adverse effects to the animal (e.g., swelling, edema, milk leakage, etc.). Before they could calve again, 12 cows were sold (4 CH, 6 NHC, 2 N treated cows) because of mastitis ($n = 3$), abortion ($n = 5$), infertility ($n = 2$) or died from displaced abomasum complications ($n = 2$). One cow died before her third post-calving culture sample could be collected; cause of death was unknown. Therefore, 27 cows finished the entire study protocol. However, the last cow who died contributed data for all other outcome variables, so for most outcomes, there were 28 cows, with the final distribution of animals per treatment group: CH ($n = 14$), NHC ($n = 9$) and N ($n = 5$). All treated quarters of the cows that remained in the study resumed milk production following the next calving.

Bacterial contamination checks for all batches produced, both hydrolyzed and non-hydrolyzed casein, were negative for any microbial growth. Final protein concentration of CH solutions was 1.5 mg/ml, which resulted in 22.5 mg per 15 ml dose. Final concentration of NHC was 0.2 mg/ml, which resulted in 3 mg per 15 ml dose. Each batch was also assessed for purity by running a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gel⁷. Heavy bands were seen at approximately 13-15 kD, 23 kD and 35 kD in the non-hydrolyzed casein batches. In the hydrolyzed batches, bands were seen primarily at 13-14 kD and 20 kD but also some at 30-35 kD. Visible fragments with these molecular weights was consistent with previous studies of enzymatically hydrolyzed casein, indicating that only casein was present in the sample and no other compounds were available for enzymatic breakdown (Wang et al., 2013). Obvious differences between hydrolyzed and non-hydrolyzed casein were present in the appearance of the gel (Figure 1).

There were no statistically significant differences between cows assigned to treatment groups for the following parameters: DIM (CH = 264, NHC = 219, N = 222), days carried calf (DCC) (CH = 115, NHC = 102, N = 86), pre-treatment cow-level SCC (CH = 1,792,000/ml, NHC = 1,464,000/ml, N=1,590,000/ml), mastitic quarter SCC (CH = 4,363,000/ml, NHC = 3,745,000/ml, N=5,852,000/ml), total-cow milk production before treatment (CH = 79 lb [36 kg], NHC = 74 lb [34 kg], N = 86 lb [39 kg]) and proportion of mastitic quarters' contribution to total-cow milk production (CH = 26%, NHC = 28%, N=32%) (all $P > 0.5$ ANOVA, Table 1). The average length of the dry

⁷ Bio-Rad Laboratories, Hercules, CA

period for the treated quarters was 181, 177 and 151 days for CH, NHC and N, respectively ($P > 0.14$, ANOVA).

Following treatment (leaving 3 remaining lactating quarters), mean cow-level SCC was 668,000/ml and mean milk production was 67 lb (30 kg) for all 39 cows who survived to the next monthly DHIA test (Table 1). For those 39 cows, the decreases in cow-level SCC ($-966,000/\text{ml}$; $P = 0.0002$) and in total-cow milk production, -11 lb (-5 kg , -14% ; $P = 0.02$) were statistically significant, as tested by students t -test. (Table 1). No statistical significance was found for the effects of DIM, pre-treatment cow-level SCC, pre-treatment mastitic quarter SCC, total-cow milk production or individual quarter contribution on post-treatment SCC, as tested in the multiple regression model.

The final general linear model was significant ($P \leq 0.001$) with $R^2 = 0.65$. The input (explanatory) variables pre-treatment cow-level SCC, bacterial agent, lactation number (1^{st} , 2^{nd} -plus), treatment, and interaction of treatment and lactation number were all significantly associated with the outcome variable, log of post-treatment SCC in the remaining 3 lactating quarters (all $P \leq .02$). The same variables with the same P values were also detected as significant in the final mixed model. Higher pre-treatment cow-level SCC was associated with lower post-treatment SCC in the 3 remaining quarters, and this was particularly evident among cows infected with several bacterial agents. For the following agents, pre-treatment cow-level SCC and post-treatment SCC in the 3 remaining quarters, respectively were: *E. coli*, 1,780,000/ml, 1,436,000/ml; no growth, 1,731,000/ml, 841,000/ml; *Staphylococcus* spp., 1,089,000/ml, 436,000/ml. The

treatment effect and its interaction with lactation number were driven by cows in first lactation; their post-treatment means of SCC in the 3 remaining quarters by treatment were: CH = 1,020,000/ml, NHC = 90,000/ml, N = 1,000/ml. In contrast, for cows in 2nd-plus lactation, the post-treatment means of SCC in the 3 remaining quarters were: CH = 561,000/ml, NHC = 857,000/ml, N = 637,000/ml.

Within treatment groups, the decrease in total-cow milk production following involution of the mastitic quarter was not significant except for the decrease from 74 lb (34 kg) to 59 lb (27 kg) in the 15 NHC treated cows ($P = 0.04$, ANOVA, Table 1). All 28 treated quarters in the cows who calved again returned to milk production after calving; mean SCC of the 28 quarters was 1,414,000/ml, significantly decreased ($-2,978,000/\text{ml}$; $P = <0.0001$, Table 1) and their contribution to total-cow milk production was 24%, not different from their 28% contribution during the previous lactation before treatment ($P = 0.46$, student's t -test). Front or rear quarters did not differ significantly in outcomes (all $P > 0.6$, ANOVA).

Cow-level SCC during the remainder of lactation decreased significantly following treatment with CH in the mastitic quarter ($-1,150,000/\text{ml}$ [$n = 17$]; $P = 0.003$, Table 1). Following calving and resumption of milk production, significant decreases in SCC in the previously treated mastitic quarters were observed within all three treatment groups (CH: $-2,763,000/\text{ml}$ [$n = 14$], $P = 0.0002$; NHC: $-2,129,000/\text{ml}$ [$n = 9$], $P = 0.01$; N: $-5,324,000/\text{ml}$ [$n = 5$]; $P < 0.0001$, all ANOVA, Table 1). All other pre-versus post-treatment comparisons by treatment group were not statistically significant.

Bacterial isolation, cures and new intramammary infections

The pre-enrollment milk culture screening found a pathogenic bacterial organism in 23/40 (58%) mastitic quarters. The predominant organisms isolated were *Streptococcus* spp. (n = 10) and coagulase-negative *Staphylococcus* spp. (n = 5). Also isolated were *Escherichia coli* (n = 3), *Staphylococcus aureus* (n = 3) and *Pseudomonas* spp. (n = 1), unknown pathogen (n = 1). Of the 23 cows whose mastitic quarters had bacteria isolated, 16 finished the study and were available for evaluation of bacterial cure or persistence; 14/16 (88%) quarters had a bacteriological Cure (Table 2). Of the 27 cows who calved again and survived to have all 3 post-calving milk cultures, 18/27 (67%) contracted a New IMI in the previously involuted quarter. Treatment groups were not significantly different in their outcomes of Cure or Chronic IMI, (all $P \geq 0.35$, Fisher's exact test). There were significantly fewer new IMI among cows treated with CH ($P=0.046$, Fisher's exact test). For cows in the NHC and N treatment groups, the proportion of New IMI was not significantly different from that for all cows ($P \geq 0.19$).

Discussion

After involution of a single mastitic quarter with high SCC, cows produced a mean of 86% of their previous total production from the 3 remaining lactating quarters at the time of their next monthly DHIA test. Postpartum, all cows resumed milk production with the treated quarters contributing a mean of one-fourth of total-cow production. The average decrease of 1,100,000/ml in cow-level SCC following involution of a single

mastitic quarter, and the reduction of SCC in mastitic quarters by over 2.5 million/ml when their milk production resumed after calving provides support for IMM casein hydrolysate as a management option to create three-quartered cows to enhance milk quality. Interestingly, our analysis identified that first lactation cows varied considerably in post-treatment SCC among the treatment groups, resulting in a highly significant interaction between treatment and lactation. Among older cows, which comprised majority of cows in the dataset, there were no significant differences in cow-level SCC between treatment groups. In many cases, mammary involution is believed to be a contributor to the spontaneous cure of previous IMIs from one lactation to the next (Atabai et al., 2007). By design, the mastitic quarters in our study were dry for longer than the typical dry period of approximately 60 days (Cermakova et al., 2014), with more than 200 days in some cases. Bacterial cure was observed in over 85% of the treated and involuted quarters in this study, while two-thirds contracted a new IMI while dry. Average IMI prevalence reported in early lactation cows ranges from 10–29% (Dingwell et al., 2003, Godden et al., 2003, Petzer et al., 2009). A limitation of this study was a relatively small sample size, which was due to logistics and expense.

Casein is the primary protein component of milk and accounts for approximately 80% of total protein found in fluid milk (Wang et al., 2013). The molecular structure is composed of four different constituents: α s1-, α s2-, β - and κ -casein. These four casein types differ in their structure and molecular weights but range between 19-25 kD. Previous studies have discovered that enzymatic digestion of casein with Trypsin results in an increase of hydrolysates at molecular weight of 20kD and less and a decrease in peptides

over 50 kD (Wang et al., 2013). These trends continue with longer hydrolyzation periods and this is consistent with our results. It should be noted that the molecular weight of Trypsin is 23.3 kD and while the enzyme was inactivated by heat, its presence may still be reflected in the final product. Because the molecular weight of Trypsin falls in the same range as casein, it cannot be determined if all the bands observed are from casein only or also contain Trypsin. Investigation of Trypsin presence falls outside the scope of this study.

The results of this study indicate that IMM use of CH may be a promising alternative to traditional methods of treating mastitis. Some disadvantages of using conventional intramammary antibiotic treatment for mastitis include the potential for antibiotic residues, the milk lost due to required withhold times and the increasingly negative consumer perception of antibiotic use in food animal management. Additionally, many bacterial organisms that are responsible for causing mastitis are not susceptible to antibiotics and for that reason, do not warrant treatment with such. Milk cessation in individual quarters using IMM CH is a novel approach to mastitis management, which utilizes the natural process of tissue rebuilding that occurs during mammary involution, without any use of intramammary antibiotics. This method could potentially extend the productive life of many dairy animals, without jeopardizing milk quality or causing adverse physical effects.

Conclusion

This study evaluated three methods for cessation of milking of a single mastitic quarter mid-lactation to attempt to improve cow-level milk quality. None of the animals showed any signs of pain or physical distress in response to drying off the quarter. All

treated quarters returned to adequate milk production, with the favorable outcome of prolonged life of lactating cows. Cessation of production in the chronic mastitic quarters resulted in a decrease in cow-level SCC for all treated animals, regardless of treatment, which indicates this is an appropriate milk quality management tool.

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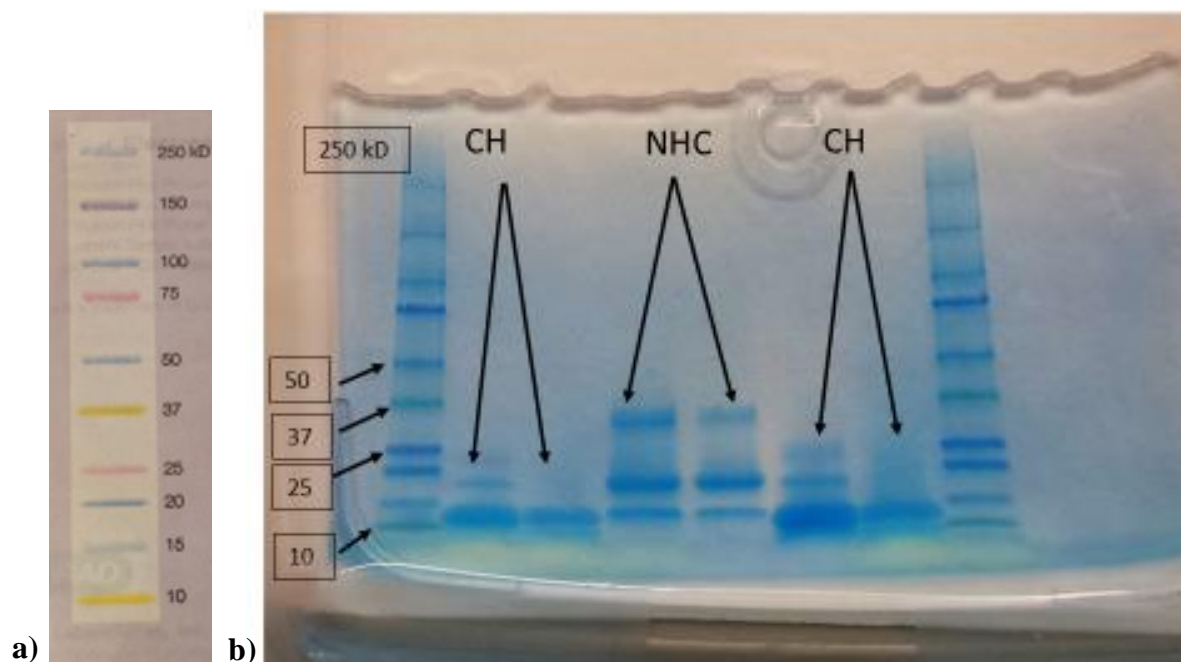


Figure 1. SDS-PAGE gel of digested and undigested casein. **a)** Molecular ladder **b)** From left to right, Columns 1 and 8 are molecular ladders, columns 2-3 are hydrolyzed casein, columns 4-5 are undigested casein and columns 6-7 are hydrolyzed casein with a larger sample volume.

Table 1. Pre- and post-treatment comparisons of cow-level SCC, quarter SCC, total-cow milk and quarter percentage of total-cow milk by treatment group and overall

Measurement/treatment group	Pre-	Post-	P-value [‡]	Change [£]
Cow SCC–Casein (x1000 cells/ml)	^a 1792 (n=18)	^e 642 (n=17)	0.003	ⁱ –1150
Cow SCC–Non-hydrolyzed casein (x1000 cells/ml)	^a 1464 (n=15)	^e 755 (n=15)	0.08	ⁱ –709
Cow SCC–Negative (x1000 cells/ml)	^a 1590 (n=7)	^e 546 (n=7)	0.08	ⁱ –1044
Qtr. SCC–Casein (x1000 cells/ml)	^b 4363 (n=18)	^f 1600(n=14)	0.0002	^j –2763
Qtr. SCC– Non-hydrolyzed casein (x1000 cells/ml)	^b 3745 (n=15)	^f 1616 (n=9)	0.01	^j –2129
Qtr. SCC–Negative (x1000 cells/ml)	^b 5852 (n=7)	^f 528 (n=5)	<0.0001	^j –5324
Total milk – Casein (kg)	^c 36 (n=18)	^g 32 (n=17)	0.20	^k –4
Total milk – Non-hydrolyzed casein (kg)	^c 34 (n=15)	^g 27 (n=15)	0.04	^k –7
Total milk – Negative (kg)	^c 39 (n=7)	^g 36 (n=7)	0.57	^k –3
Qtr.% of total Cow – Casein	^d 26 (n=18)	^h 24.5 (n=14)	0.60	^l –1.5
Qtr.% of total Cow – Non-hydrolyzed casein	^d 28 (n=15)	^h 22 (n=9)	0.17	^l –6
Qtr. % of total Cow– Negative	^d 32 (n=7)	^h 27 (n=5)	0.41	^l –5
Cow SCC (x1000 cells/ml)–All cows	1634 (n=40)	668 (n=39)	0.0002	–966
Qtr. SCC (x1000 cells/ml)–All cows	4392 (n=40)	1414 (n=28)	<0.0001	–2978
Cow Milk Yield (lb)–All cows	78 (n=40)	67 (n=39)	0.02	–11

[‡] For test of significance of difference from pre- to post-treatment, within each treatment group

[£] Change in measurement (post minus pre), post-infusion

Means with same letter were not significantly different between treatment groups (p >0.05, ANOVA)

Table 2. Bacteriological cures and new infections by treatment group

	Pre-Treatment Culture (n= 40)		Post-Treatment Culture (n = 27)				
Treatment Group	Culture – positive	Culture– negative	Previously positive	Previously negative	Chronic IMI ¹	IMI Cured ²	New IMI ³
CH [†]	10	8	7	6	(0/7) 0%	(7/7) 100%	(6/13) 46%
NHC [†]	9	6	6	3	(1/6) 17%	(5/6) 83%	(8/9) 88%
N [†]	4	3	3	2	(1/3) 33%	(2/3) 67%	(4/5) 80%
Totals	23 [£]	17	16 [§]	11	(2/16) 12%	(14/16) 88%	(18/27) 67%

¹Chronic IMI = any bacteria isolated from the pre-treatment sample, followed by isolation of the same bacteria from at least one post-calving culture

²Cure = all 3 post-calving cultures negative for any bacteria isolated from the pre-treatment sample

³New IMI = one or more bacteria not isolated from the pre-treatment sample, followed by isolation from at least one post-calving culture sample (multiple bacterial species only count as one IMI)

[†]CH=casein hydrolysate; NHC=Non-hydrolyzed casein; N=cessation of milking only, Negative.

[£]23/40 Cows had a mastitis organism present pre-treatment; 16 remained in the study long enough for 3 post-treatment sample collection

[§]27/40 cows completed the study with 3 post-treatment cultures; 11 of these did not have a mastitis organism present pre-treatment, which left 16 cows eligible to be chronic IMI or cured

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CHAPTER IV

Intramammary infusion of casein hydrolysate compared with other treatments at dry-off and time until bovine mammary involution as assessed by compositional changes of biomarkers in milk

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ABSTRACT

The objective of this study was to compare the intramammary (IMM) use of casein hydrolysate (CH) in combination with or without standard dry cow treatment (DCT) and/or an internal teat sealant (TS), at different time points of bovine mammary involution. Four treatment groups were studied; one udder half was assigned a treatment

and the contralateral half was administered DCT + TS (Control). Treatment groups were: CH, CH + DCT (CHDC), CH + TS (CHTS) and all three combined (CHDCTS). Cows were blocked by number of intramammary infections (IMI) per udder half (0 or 1+) and randomized to treatments. Fifteen of 16 enrolled cows completed the entire study. Cure rates of IMI during dry period were: CH=0% (0/2), CHDC=100% (1/1), CHTS=50% (1/2), CHDCTS=N/A (no eligible quarters), Control=50% (1/2). Milk production was not different between Control or treated udder halves post-calving (Treated:Control = 6.5 kg:6.0 kg, all $P > 0.20$). A generalized linear mixed model tested for differences between treatment groups and against the Control group in mammary involution indicators pH, SCC, bovine lactoferrin (bLf) and bovine serum albumin (BSA) in milk. Statistically significant differences were: CH treated cows were higher in bLf than CHDC, CHTS and Controls at 7 days dry, CH cows were higher in BSA than CHDC, CHDCTS and Controls at 10 days dry. Across all time points, CH cows were higher in bLf compared to the Controls and CHDCTS cows were higher in BSA compared to Controls. Following IMM CH at dry-off, the proportion of total-cow milk production from treated udder halves was unchanged (51%) early in the subsequent lactation. Cows did not exhibit signs of discomfort following any of the dry treatments containing CH. Intramammary infusion of CH at the end of lactation may be an alternative or possible adjunct to antibiotic dry cow therapy.

Key words: dry cow therapy, casein hydrolysate, intramammary infection, udder health, mastitis

INTRODUCTION

The importance of the nonlactating (dry) period in dairy cows has long been established within the dairy industry (Natzke et al., 1975, Cermakova et al., 2014). Increased somatic cell count and rates of intramammary infections (IMI), and decreased milk production in subsequent lactations have been documented from mismanagement of the dry period (Hutton et al., 1990, Hassan et al., 1999, Cameron et al., 2014). Cows may carry existing IMI into the dry period that remain present into the next lactation and are at an increased risk for acquiring new IMI immediately following cessation of milking (dry-off), prior to mammary involution and then again as the gland undergoes changes from nonlactating tissue back to lactating, immediately before calving (Holst et al., 1987, Oliver and Sordillo, 1989). Currently, the standard protocol in North America for mitigating IMI risk at dry-off is the blanket use of intramammary dry cow antibiotic therapy (Dodd et al., 1969, NMC, 2006). Previous studies have reliably demonstrated that blanket use of dry cow therapy (BDCT) has been effective in eliminating many existing IMI present at dry-off and preventing new IMI in the early dry period (Hassan et al., 1999, Bradley and Green, 2004). Additionally, the routine use of dry cow therapy has been shown to effectively lower bulk tank milk somatic cell counts (Wilson et al., 1997a) and nearly eradicate certain pathogens in some countries (Olde Riekerink et al., 2006). Recently however, the practice of BDCT has come under scrutiny on an international scale (Scherpenzeel et al., 2016).

Among consumers, scientists and health professionals, there is increasing concern over the widespread use of antibiotics in food-producing animals and the potential contribution to antimicrobial resistance, both in veterinary pathogens and in human

medicine (Goff et al., 2017). Intensive livestock raising practices in most developed countries have supported subtherapeutic concentrations of antibiotics used prophylactically to reduce disease and promote growth (Goff et al., 2017). In the world of human medicine, especially in hospitals, the overuse of antibiotics has bred multidrug-resistant bacteria, also known as superbugs (Goff et al., 2017). The concern is that overuse of antibiotics in food-producing animals will also increase antimicrobial resistance in the human population in general and especially in those who are in direct contact with livestock (Tang et al., 2017). The data remains indeterminate on whether antibiotic use in livestock definitively causes antimicrobial resistance in humans, however correlations between the use of these drugs in adult dairy cows and other food-producing livestock do exist (Tang et al., 2017). Within the dairy industry, BDCT has become a major focus as an area which could potentially reduce use of antibiotics. At present, over 90% of U.S. dairy operations use an antibiotic to manage udder health and control new infections during the dry period (Sneeringer, 2017). These are long-acting intramammary antibiotics and have been the recommendation of the National Mastitis Council as a key point of a successful mastitis prevention program for many years (NMC, 2006, Bonsaglia et al., 2017). Recently, there has been interest in selective use of dry cow therapy (SDCT), as a possible method of reducing antimicrobial use, while still maintaining the benefits of reducing incidence rate of clinical mastitis and lowering bulk tank somatic cell counts (BTSCC) (Cameron et al., 2014, Scherpenzeel et al., 2014). Studies investigating the efficacy of SDCT has produced variable results (Cameron et al., 2014, Scherpenzeel et al., 2016). Cows dried-off without antibiotics experience higher rates of both clinical and subclinical mastitis in the following lactation and any economic

benefit gained from reducing antimicrobial use at dry-off is lost by the increase in mastitis cases (Scherpenzeel et al., 2016). Conclusively, there are disadvantages to both BDCT and SDCT and a need for an alternative dry cow treatment still exists within the dairy industry.

Previous studies have explored the use of casein hydrolysate (CH) as an intramammary infusion to manage mastitic quarters mid-lactation by inducing involution (tho Seeth et al., 2016, Britten et al., 2018), in combination with dry cow antibiotics to increase bacterial cures and milk yield (Leitner et al., 2011) and as a non-antibiotic alternative to standard dry cow therapy (Leitner et al., 2007, Leitner et al., 2011). Casein hydrolysates are milk-borne factors believed to be part of the biological pathway which causes involution in the bovine mammary gland, 40–70 days prior to expected parturition (Shamay et al., 2003). Intramammary infusion of CH has been demonstrated to locally induce involution within a single quarter, as a management strategy for cessation of lactation in quarters with a persistently elevated SCC or repeated episodes of mastitis, without the consequences of antimicrobial residues and/or permanent mammary gland destruction (tho Seeth et al., 2016, Britten et al., 2018). Based on earlier research, we hypothesized that intramammary infusion of CH alone, in combination with dry cow therapy, internal teat sealant, or both, might affect the time until completion of the process of mammary involution. Comparisons of the treatment regimens that included CH with the widely used practice of dry cow therapy with internal teat sealant for the outcomes of bacteriological cure or existing IMI and proportion of NI during the dry period were also performed. The present study aimed to evaluate the effects of four

different treatment combinations, all containing CH, against a non-CH control treatment during the dry period.

MATERIALS AND METHODS

Animals and Management

Study cows were sourced from 6 commercial Utah dairy farms. All farms followed a twice daily milking schedule and cows were housed on shaded dry lots that are typical of the region. Lactation number, days in milk (DIM), and estimated calving date were obtained from dairy records. To be eligible for the study, cows were pregnant and within 40-70 days before expected parturition and scheduled for dry-off. Inclusion criteria required four lactating quarters; cows with blind quarters or presenting with clinical mastitis were excluded. All animal handling and treatments in this study were performed in compliance with an Institutional Animal Care and Use Committee (IACUC) approved protocol #2739. Casein hydrolysate is not labeled for use in food animals; this study was conducted with FDA permission and followed typical guidelines for milk and meat withhold times for commercial treatments, and FDA-suggested withhold times for CH.

Experimental Design

A completely randomized block design was used for this study. All cows were blocked into two groups by number of quarters containing a subclinical IMI (0 or 1+)

before drying off. Infection status was determined by the results of a pre-treatment sample, taken 4 days before time of treatment administration. There were 4 treatment groups and one control: (1) Casein hydrolysate only (CH), (2) Casein hydrolysate + dry cow antibiotic¹ (CHDC), (3) Casein hydrolysate + teat sealant² (CHTS), (4) Casein hydrolysate + dry cow antibiotic¹ + teat sealant² (CHDCTS) and DCT+TS (DCTS) as a control. One half of each udder, front and rear quarter, was randomized to a treatment group, while the other half received Control, allowing every cow to serve as her own control and eliminate variability between animals (Foret et al., 2003, Fitzpatrick et al., 2018). Control halves were treated with 500 mg cloxacillin benzathine (Dry-Clox®⁸), followed by an internal teat sealant (Orbeseal®⁹). Study cows were marked with colored leg bands on both hind legs, along with an additional leg band used to identify the Control side. Treatments were preassigned using a random number generator to assign first which udder half would be treated versus control, followed by the treatment group assignment. Assignment of treatment sides and groups was forced only for the last cow within each block to ensure blocks were balanced with an equal number of animals per treatment group and equal distribution of treated and control sides.

A second sample for culture was taken immediately prior to the time of treatment, for evaluation of bacteriological cure. Bacteriological cure of previous IMI and new infection rates were not primary objects of this study. However, involution is a mechanism of curing existing IMI during the dry period and this is an important outcome for any treatment during the dry period in dairy cattle (Schukken et al., 2011a, Collier et al., 2012).

⁸ Dry-Clox DC, Boehringer Ingelheim

⁹ Orbeseal, Zoetis Animal Health

Quarters which had the same organism isolated from the same quarter from both pre-treatment samples (“+,+”), were eligible for bacterial cure evaluation post-calving. Postpartum, the previously treated quarters were resampled 3 times, once per week at 1-7 DIM, 8-14 DIM and 15-21 DIM. Resulting case definitions were: Cure = all 3 post-calving cultures negative for any bacteria isolated from both pre-treatment samples; Fail = any bacteria isolated from both pre-treatment samples that was also isolated from at least one post-calving culture. New IMI = one or more bacteria not isolated from either pre-treatment sample (“-,-”), being isolated from at least one post-calving culture sample (multiple bacterial species isolated from the same quarter only counted as one IMI). Cows whose pre-treatment cultures resulted in no growth from one sample but growth in another (“+, - “or “-, +”) and/or different organisms isolated from each sample, were ineligible for bacterial cure evaluation post-calving. All cows were eligible for new infections, including with a new pathogen if they had an IMI before dry-off.

Treatment Administration

Cows meeting inclusion criteria were administered treatment at their scheduled dry-off time, Time 0. Prior to receiving treatment, all cows were bucket milked simultaneously by udder half for their final milking, to obtain udder half milk weights and determine proportional contributions to total-cow milk production. Individual quarter milk samples were also collected again at Time 0. Following milking and sample collection, the front and rear quarter of each udder half was infused aseptically with the pre-assigned treatment or control. Teats were dipped with disinfectant post-treatment and cows were immediately moved to the nonlactating pen. Individual quarter milk samples

were collected again at d 2, d 4, d 7 and d 10 post dry-off, for a total of 6 sampling dates and 24 quarter samples per cow. In order to be minimally disruptive to the involution process, no more than 10 ml of milk was removed from each quarter per sample. If teat sealant was part of the treatment, it was administered following the final sampling. Collected milk samples were analyzed for direct microscopic somatic cell count (DMSCC), pH, bovine lactoferrin (bLf) and bovine serum albumin (BSA) as established biomarkers of involution (Boutinaud et al., 2016, Lanctôt et al., 2017). Following calving, milk from treated cows was withheld from the bulk tank milk for 72 h and pending a negative antibiotic residue sample (Delvotest-NT)¹⁰. At 72 h, bucket milking by udder half was repeated and individual quarter samples collected for bacterial culture. Quarter sampling for bacterial culture was repeated once between 7-14 DIM and again once between 15-21 DIM.

Bacteriology

Bacterial culture of milk samples was performed according to standard methods approved by the National Mastitis Council (Hogan, 1999, Britten, 2012). A 10 µl inoculum of milk was streaked onto washed cow blood agar and placed in a standard, non-CO₂ incubator at 37°C for 24 h. Plates were examined by laboratory analysts for bacterial growth at 24 and 48 h; organism identification was determined primarily by colony morphology and confirmed by secondary biochemical tests where appropriate.

¹⁰ Delvotest-NTR, DSM Food Specialties, Delft, The Netherlands

Presence of one colony was considered positive for identification; therefore, detection limit was 100 colony forming units (CFU/ml).

Milk Testing Assays

Concentrations of BSA and bLf in milk were measured using the lactoferrin quantitative ELISA and bovine serum albumin quantitative ELISA kits for identifying those proteins in milk, from Bethyl Laboratories¹¹. Procedures were performed according to manufacturer's instructions. Plates (96 wells) were pre-coated with goat anti-bovine bLf antibody or sheep anti-bovine BSA antibody, respectively. Briefly, 100 µl of diluted sample was added to each well and incubated at room temperature. Following a wash cycle to remove unbound proteins, a biotinylated detection anti-antibody was added to bind to the antibody-tagged bLf or BSA. After a final incubation and washing step, a streptavidin-conjugated horseradish peroxidase enzyme was added to catalyze a colorimetric reaction with the chromogenic substrate on the anti-antibody. This reaction was read at 450 nm with an automatic plate reader and generated a standard curve from which the actual sample concentrations of bLf or BSA were calculated. Samples assayed for bLf were diluted 1:10,000 for d 0 and d 2 post dry-off and 1: 100,000 for d 4, d 7 and d 10 after dry-off. Milk samples assayed for BSA were diluted 1:1000 for samples taken at d 0, d 2 and d 4 post dry-off and 1:5000 for d 7 and d 10 post dry-off. This variable dilution scheme was necessary to accommodate the increasing concentration of BSA and bLf in the milk as involution progressed and to remain in range of the assay standards.

¹¹ Bethyl Laboratories, INC, Montgomery, TX

The Form 2400 FDA approved method was used to determine DMSCC as an approved method under the National Conference on Interstate Milk Shippers (NCIMS, 2016).

Preparation of Casein Hydrolysate

Two batches of casein hydrolysate were prepared as previously described (Shamay et al., 2003) using aseptic technique. Each batch was prepared using 100 g of commercially purchased >99.9% pure bovine casein powder¹² dissolved in 1 L of autoclaved deionized water, adjusted to pH 8.8 with sodium hydroxide, containing 3 g of TRIS buffer and enzymatically digested with trypsin. After digestion, remaining particulate material was removed via two centrifugation cycles of 15 minutes at 3000 x g. Solution was boiled for 15 minutes between centrifugation cycles to denature any remaining enzyme and kill possible environmental contaminant bacteria, followed by sterilization using vacuum membrane filtration. The final product was dispensed (15 ml) into sterile syringes and stored frozen at - 20° C. Each batch of CH was screened for bacterial contamination by inoculating tryptic soy broth with 1 ml of the solution, incubating for 24 h and inoculating 100 µl onto blood agar. Blood agar plates were incubated for a total of 48 h at 37° C and read at 24 and 48 h for bacterial growth. A separate blood agar plate was plated directly with 100 µl of non-enriched casein hydrolysate and then incubated and examined in the same way. The definition of an uncontaminated batch was when no growth of any bacterial colonies was observed on

¹² Sigma-Aldrich Chemicals company, St. Louis, MO

either direct or enriched cultures. Protein concentration for each batch was quantified via bicinchoninic acid assay (BCA¹³), according to manufacturer's instructions. The BCA assay is commonly used for protein quantification (Smith et al., 1985b). Final concentration of CH solutions was 5.6 mg/ml, for a total dose of 84 mg of hydrolyzed casein per 15 ml dose. Purity was assessed using sodium dodecyl sulfate polyacrylamide gel electrophoresis gel.

Milk Weight Collection

All cows were bucket milked at their final milking before treatment and again at 72 h post-calving, to determine the proportion of total-cow milk production from each udder half. Two clear graduated 80 lb (36.3 kg) milking buckets, two milking units with teat plugs to seal the two empty teat cups and two sources of vacuum were used to allow for simultaneous milking of each udder half.

Statistical Analyses

Statistical analyses were performed using SAS Studio¹⁴, using the generalized linear mixed model procedure (GLIMMIX). Milk production by udder half were compared and analyzed by student's t-test. The predictor variables time and treatment were treated as fixed effects, individual cow ID was treated as a random effect and the interaction between time and treatment were analyzed for each outcome variable. Means

¹³ Pierce BCA Assay, ThermoFisher Scientific

¹⁴ SAS Institute Inc., Cary, NC, USA

of the continuous outcome variables were compared pairwise against every other treatment group, using the Tukey-Kramer adjustment for multiple comparisons (E.g. the means of bLf between CH treated cows and CHDC treated cows, CH and CHTS, etc.). The Dunnett's test was used to compare all treatment groups' outcome variable means to the Control group's means across all time points. Natural log transformations were taken of all continuous outcome variables to correct for the wide range of numerical values resulting in non-homogenous variances. Categorical outcomes of Cure, Chronic and New IMI were compared for significant differences, using Fisher's Exact test. Differences were considered statistically significant when $P \leq .05$ for all analyses.

RESULTS

A total of 16 commercial dairy cows, comprised of Holsteins (n=12) and Jerseys (n=4) were enrolled in the study. One animal died of unknown causes shortly after calving and was not replaced, therefore 15 cows finished with complete datasets. Post-calving milk weight and bacterial cultures were the only data points unable to be collected from the cow that died, so n=16 for all other outcomes. Cows ranged in parity from first through seventh lactation, with an average of 3.1 lactations and 337 DIM. Culture information and milk production data was gathered from the 15 cows which completed the entire study. There were no statistically significant differences between each treatment group of 4 cows in DIM (CH = 359, CHDC = 352, CHTS = 331, CHDCTS = 306), average lactation number (CH = 3.0, CHDC = 3.0, CHTS = 3.3, CHDCTS = 3.3) or udder half percentages of total-cow milk between any of the treatment groups or the Control group before treatment (all ≥ 0.20 , ANOVA). There were

also no significant differences between pre- and post-treatment contribution of udder halves to total-cow milk production within all treatment groups (NSD) (all $P \geq 0.06$, ANOVA).

Bacterial contamination checks for all batches produced, both hydrolyzed and non-hydrolyzed casein, were negative for any microbial growth. Final protein concentration of CH solutions was 1.5 mg/ml, which resulted in 22.5 mg per 15 ml dose. Final concentration of NHC was 0.2 mg/ml, which resulted in 3 mg per 15 ml dose. Each batch was also assessed for purity by running a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gel. Heavy bands were seen at approximately 13-15 kD, 23 kD and 35 kD in the non-hydrolyzed casein batches. In the hydrolyzed batches, bands were seen primarily at 13-14 kD and 20 kD but also some at 30-35 kD. Visible fragments with these molecular weights was consistent with previous studies of enzymatically hydrolyzed casein, indicating that only casein was present in the sample and no other compounds were available for enzymatic breakdown (Wang et al., 2013). Obvious differences between hydrolyzed and non-hydrolyzed casein were present in the appearance of the gel (Figure 1).

The largest proportional difference in milk production between udder halves of any cow was 75% on one side and 25% on the contralateral side (3 lb [6.6 kg]. and 1 lb [2.2 kg] in early lactation, respectively); most cows' proportions of total milk were no more divergent than 46% on one side and 54% on the contralateral side. All treated cows completed the dry period and calved near their estimated due date without complication. One cow died within 36 hours of calving for unknown reasons; mastitis was ruled out as

a possible cause. All cows tested negative for antimicrobial residues at 72 h (Delvotest-NT).

All cows (15 cows; 60 quarters) were quarter sampled twice before treatment, at 4 days prior to treatment and again at dry-off. Twenty-seven quarters (45%) grew a bacterial organism but only 7 quarters grew the same bacterial isolate in both pre-treatment samples and were eligible for a bacterial cure evaluation. The other 20 quarters had bacterial growth in at least one of their pre-treatment samples but were ineligible for cure rate data either because one sample produced no growth or there were different isolates in the 2 samples. Quarters eligible for bacterial cure by treatment group were: CH (n=2), CHDC (n=1), CHTS (n=2), CHDCTS (n=0; no infections at dry-off), and Control (n=2). Bacterial cures were as follows: CH 0/2 (0%), CHDC 1/1 (100%), CHTS 1/2 (50%), CHDCTS N/A, Control 1/2 (50%). Chronic (failed cure) cases were: CH 2/2 (100%), CHDC 0/1 (0%), CHTS 1/2 (50%), Control, 1/2 (50%). New infection rates were: CH 7/8 (88%), CHDC 2/8 (25%), CHTS 4/6 (67%), CHDCTS 5/8 (63%) and Control 15/30 (50%) (Table 1). Cure rate was not significantly different between treatment groups (Fisher's Exact Test, all $P \geq 0.33$). There was one significant difference in new infection rates during the dry period; CH treated cows' new infection rate (88%) was higher than that of CHTS treated cows (25%) (Fisher's Exact Test, $P = 0.04$; all other $P \geq 0.11$).

All measured outcome variables (indicators of involution) increased significantly from the day of drying-off (d 0) to d 10 ($P < 0.0001$, GLIMMIX), which is to be expected as a natural process of involution (Figures 1-5). However, only the outcome variables bLf and BSA showed a significant time \times treatment interaction ($P = 0.01$, GLIMMIX).

(Figures 1-2). Cows in the CH group also had a higher concentration of bLf at d 7 than cows in the CHDC, CHTS treatment groups and Controls (all $P \leq .02$, GLIMMIX) (Table 2). The cows dry treated with CH had a higher concentration of BSA than CHDC, CHDCTS and Control at d 10 (all $P \leq 0.04$, GLIMMIX) (Table 2). Across all time points, cows dry treated with CH had higher concentrations of bLf than Control cows ($P = 0.02$, Dunnett's), and CHDCTS dry treated cows had higher concentrations of BSA than Control cows ($P = 0.04$, Dunnett's).

DISCUSSION

This is the first study conducted in North America to investigate the efficacy of using intramammary CH to supplement or possibly replace standard dry cow therapy. The results of this study demonstrated an association between the use of CH and a faster increase in concentration of mammary involution markers. Fully involuted glands are quite robust against new IMI (Sordillo et al., 1987, Hurley, 1989), which suggests that intramammary use of CH may be beneficial to cow comfort and udder health at the time of dry-off, with or without the addition of an antibiotic. All markers of involution increased from the time of dry-off and treatment to the final sampling 10 days later, consistent with previously published studies (Boutinaud et al., 2016, Lanctôt et al., 2017). While at several time points there were statistically significant differences in markers of involution between treatments and control, no single treatment was consistently associated with faster involution.

Casein is the primary protein component of milk and accounts for approximately 80% of total protein found in fluid milk (Wang et al., 2013). The molecular structure is composed of four different constituents: α s1-, α s2-, β - and κ -casein. These four casein types differ in their structure and molecular weights but range between 19-25 kD. Previous studies have discovered that enzymatic digestion of casein with Trypsin results in an increase of hydrolysates at molecular weight of 20kD and a decrease in peptides over 50 kD (Wang et al., 2013). These trends continue with longer hydrolyzation periods and this is consistent with our results. It should be noted that the molecular weight of Trypsin is 23.3 kD and while the enzyme was inactivated by heat, its presence may still be reflected in the final product. Because the molecular weight of Trypsin falls in the same range as casein, it cannot be determined if all the bands observed are from casein only or also contain Trypsin. Investigation of Trypsin presence falls outside the scope of this study.

Bovine lactoferrin and BSA have frequently been used as measures of involution in previous research (Sordillo et al., 1987, Kutila et al., 2003, Boutinaud et al., 2016, Lanctôt et al., 2017). In this study these two predictors of involution increased faster for two of the treatment groups than the control group of animals across all time points, and between several treatment groups at specific time points. Bovine lactoferrin plays an immunologically important role during mammary involution, by binding available iron and making it unavailable for iron-dependent bacteria to grow (Nonnecke and Smith, 1984, Galfi et al., 2016). The concentration of lactoferrin was higher in cows that were treated only with CH compared with the controls and some other treatments at some time points, therefore it is possible these animals experienced an udder health benefit. This is a potentially interesting outcome when considering the use of alternatives to antimicrobial

dry treatment. Cows are at an increased risk for acquiring a new IMI in the early dry period, primarily because of the milk stasis that occurs from cessation of milking. This provides a rich growth environment for any bacteria that are present at the time of dry-off. Additionally, the increase in mammary pressure from milk stasis may cause leakage from the teats and facilitate further microbial entry (Natzke et al., 1975, Oliver and Sordillo, 1989). Prophylactic use of intramammary antibiotics is the current method of addressing this risk and has been proven to be relatively successful but carries the risk of residues in milk (Hassan et al., 1999, Petzer et al., 2009). Results of the present study showed that quarters treated only with CH failed to cure IMI that were present pre-treatment and experienced a significantly higher percentage of new IMI during the dry period than the other treatment groups, despite seeing some increased indicators of involution at 7 days dry. This suggests that lactoferrin may not present as robust a defense as previously suggested. Quarters treated as part of the CHTS group which also had no antimicrobial agent, only the internal teat sealant added, did no worse than the control quarters in cure of established IMI. This suggests that a physical barrier against entry of new bacterial organisms is still a helpful contributor in preventing new IMI, and potentially more important than either increasing the rate of involution or prophylactic use of antibiotics.

CONCLUSIONS

Cows experienced no milk production loss and did not exhibit any signs of pain or discomfort following any of the dry treatments containing casein hydrolysate. Restricting blanket use of dry cow therapy in the U.S. dairy industry may become a

reality in the future and the results of this study show that intramammary use of casein hydrolysate was associated with faster increases in some measures of bovine mammary involution during the early dry period. Use of casein hydrolysate combined with internal teat sealant may be an alternative to antibiotic dry cow therapy.

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Table 1. Bacteriological cures and chronic cases by treatment group (P < 0.05, Fisher's Exact Test)

Treatment group	Pre-Treatment Culture Results (# of Qtrs. Infected)			Post-Treatment Culture Results		
	IMI ²	Mixed ³	NG	Cure ⁴	Chronic ⁵	New IMI ⁶
CH ¹	2	5	1	0% (0/2) ^a	100% (2/2) ^b	88% (7/8) ^c
CHDC ¹	1	1	6	100% (1/1) ^a	0% (0/1) ^b	25% (2/8) ^d
CHTS ¹	2	1	3	50% (1/2) ^a	50% (1/2) ^b	67% (4/6) ^{cd}
CHDCTS ¹	0	1	7	N/A	N/A	63% (5/8) ^{cd}
Control	2	12	16	50% (1/2) ^a	50% (1/2) ^b	50% (15/30) ^{cd}
Totals	7	20	33	43% (3/7)	57% (4/7)	55% (33/60)

¹CH = casein hydrolysate; CHDC = casein hydrolysate + dry cow antibiotic; CHTS = casein hydrolysate + teat sealant; CHDCTS = casein hydrolysate + dry cow antibiotic + teat sealant

²Same mastitis organism isolated for both pre-treatment cultures; IMI are eligible to be either Cure or Chronic following dry cow treatment and subsequent calving

³Different mastitis organisms isolated from pre-treatment cultures; quarters are ineligible for Cure or Chronic evaluation

⁴Cure = All 3 post-treatment cultures negative for pre-treatment IMI

⁵Chronic = At least 1 post-treatment culture positive for pre-treatment IMI

⁶New IMI = Any organism isolated from any of the three post-calving cultures that was not present in either pre-treatment culture

^{a-d}Values with the same letter superscript within the same column were not significantly different, all P > 0.05

^{a-d}Values with different letter superscripts within the same column were significantly different, all P < 0.05

Table 2. Raw means of indicators of mammary involution, for each time and treatment group (P<0.05, GLIMMIX)

BSA	CH ¹	CHDC ¹	CHTS ¹	CHDCTS ¹	Control
0 days dry	² 0.20 (n=8) ^a	1.30 (n=8) ^a	0.18 (n=8) ^a	0.24 (n=8) ^{a£}	0.20 (n=32) ^a
2 days dry	0.28 (n=8) ^b	0.56 (n=8) ^b	0.74 (n=8) ^b	0.55 (n=8) ^{b£}	0.54 (n=32) ^b
4 days dry	0.72 (n=8) ^c	0.88 (n=8) ^c	0.93 (n=8)^c	0.80 (n=8) ^{c£}	0.57 (n=32)^c
7 days dry	2.20 (n=8) ^d	0.92 (n=8) ^d	0.55 (n=8)^d	0.81 (n=8) ^{d£}	0.71 (n=32)^d
10 days dry	3.93 (n=8)^e	0.97 (n=8) ^f	0.80 (n=8)^e	0.84 (n=8) ^{f£}	0.95 (n=32)^f
bLf					
0 days dry	0.96 (n=8) ^{g£}	1.40 (n=8) ^g	0.72 (n=8) ^g	0.95 (n=8) ^g	0.81 (n=32) ^g
2 days dry	1.92 (n=8) ^{h£}	4.55 (n=8) ^h	4.35 (n=8) ^h	1.94 (n=8) ^h	2.90 (n=32)^h
4 days dry	6.44 (n=8) ^{i£}	10.8 (n=8)ⁱ	8.90 (n=8)ⁱ	5.40 (n=8)ⁱ	10.5 (n=32)ⁱ
7 days dry	23.8 (n=8)^{j£}	14.4 (n=8)^k	6.50 (n=8)^k	21.2 (n=8)^j	15.1 (n=32)^k
10 days dry	14.0 (n=8)^{l£}	15.1 (n=8)^l	10.5 (n=8)^l	13.2 (n=8)^l	22.1 (n=32)^l
pH					
0 days dry	6.8 (n=8) ^m	6.8 (n=8) ^m	6.7 (n=8) ^m	6.8 (n=8) ^m	6.8 (n=32) ^m
2 days dry	6.9 (n=8) ⁿ	7.0 (n=8) ⁿ	6.9 (n=8) ⁿ	7.0 (n=8) ⁿ	6.9 (n=32) ⁿ
4 days dry	7.2 (n=8)^o	7.2 (n=8)^o	7.1 (n=8)^o	7.1 (n=8)^o	7.1 (n=32)^o
7 days dry	7.4 (n=8)^p	7.1 (n=8) ^p	7.1 (n=8)^p	7.2 (n=8)^p	7.2 (n=32)^p
10 days dry	7.4 (n=8)^q	7.0 (n=8) ^q	7.1 (n=8)^q	7.2 (n=8)^q	7.2 (n=32)^q
DMSCC (x1000)					
0 days dry	628 (n=8) ^w	380 (n=8) ^w	505 (n=8) ^w	378 (n=8) ^w	264 (n=32) ^w
2 days dry	1476 (n=8) ^x	2274 (n=8) ^x	1892 (n=8) ^x	1811 (n=8) ^x	1280 (n=32)^x
4 days dry	5171 (n=8) ^y	1841 (n=8) ^y	4296 (n=8)^y	2929 (n=8)^y	2012 (n=32)^y
7 days dry	6236 (n=8)^z	3607 (n=8)^z	3449 (n=8)^z	3601 (n=8)^z	3993 (n=32)^z
10 days dry	7001 (n=8)^{aa}	4167 (n=8)^{aa}	5622 (n=8)^{aa}	3014 (n=8)^{aa}	5407 (n=32)^{aa}

¹CH = casein hydrolysate; CHDC = casein hydrolysate + dry cow antibiotic; CHTS = casein hydrolysate + teat sealant; CHDCTS = casein hydrolysate + dry cow antibiotic + teat sealant

²Means that were significantly different from Controls, across all time points, are denoted with £ (all P < 0.05)

³Means that are significantly different from 0 days dry, within a treatment, are in **bold**

^{a-aa}Means with the same letter superscript within the same row were not significantly different, all P ≥ 0.05

^{a-aa}Means with different letter superscripts within the same row were significantly different, all P < 0.05

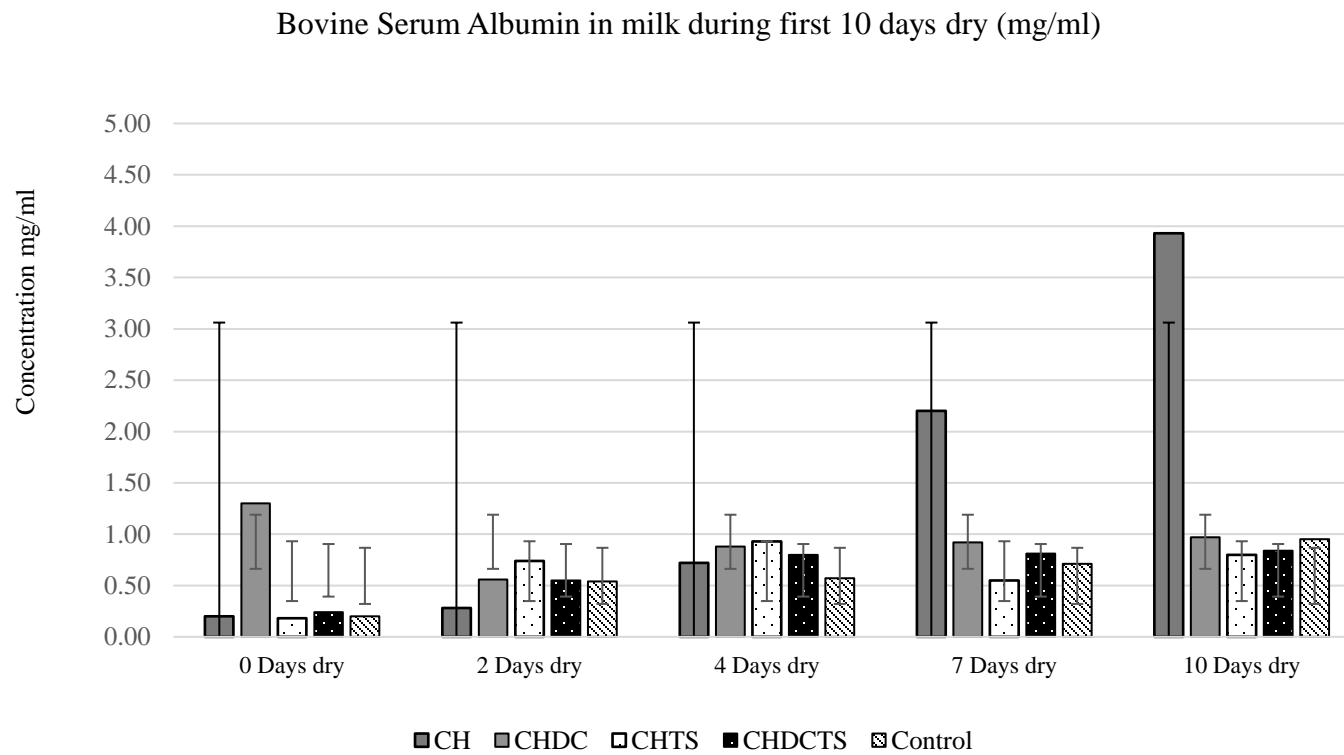


Figure 1. Increase in BSA by days dry and dry treatment group. CH= Casein hydrolysate only; CHDC =Casein hydrolysate + dry cow treatment; CHTS = Casein hydrolysate + teat sealant; CHDCTS = Casein hydrolysate + dry cow treatment + teat sealant; Control = dry cow treatment + teat sealant

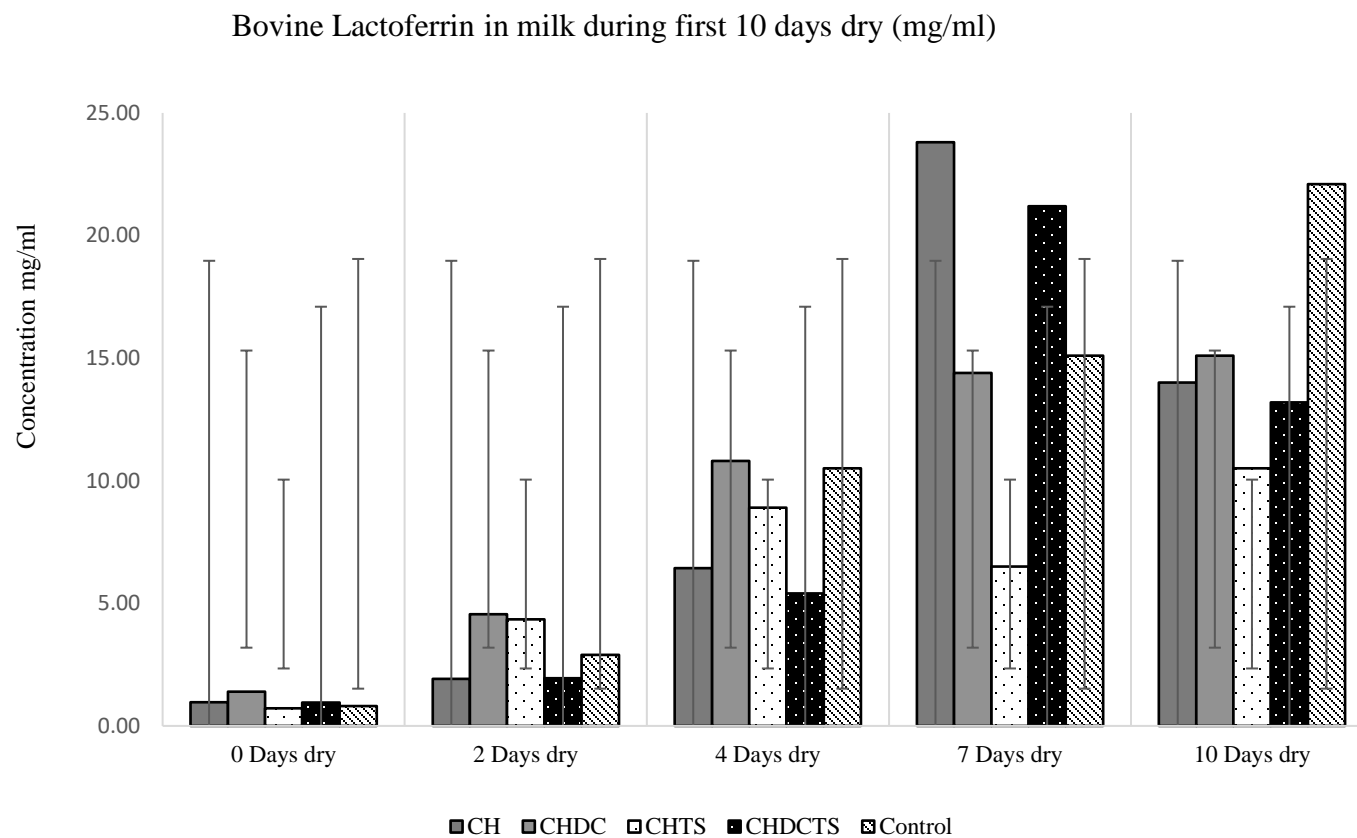


Figure 2. Increase in bovine lactoferrin by days dry and dry treatment group. **CH**= Casein hydrolysate only; **CHDC** =Casein hydrolysate + dry cow treatment; **CHTS** = Casein hydrolysate + teat sealant; **CHDCTS** = Casein hydrolysate + dry cow treatment + teat sealant; **Control** = dry cow treatment + teat sealant

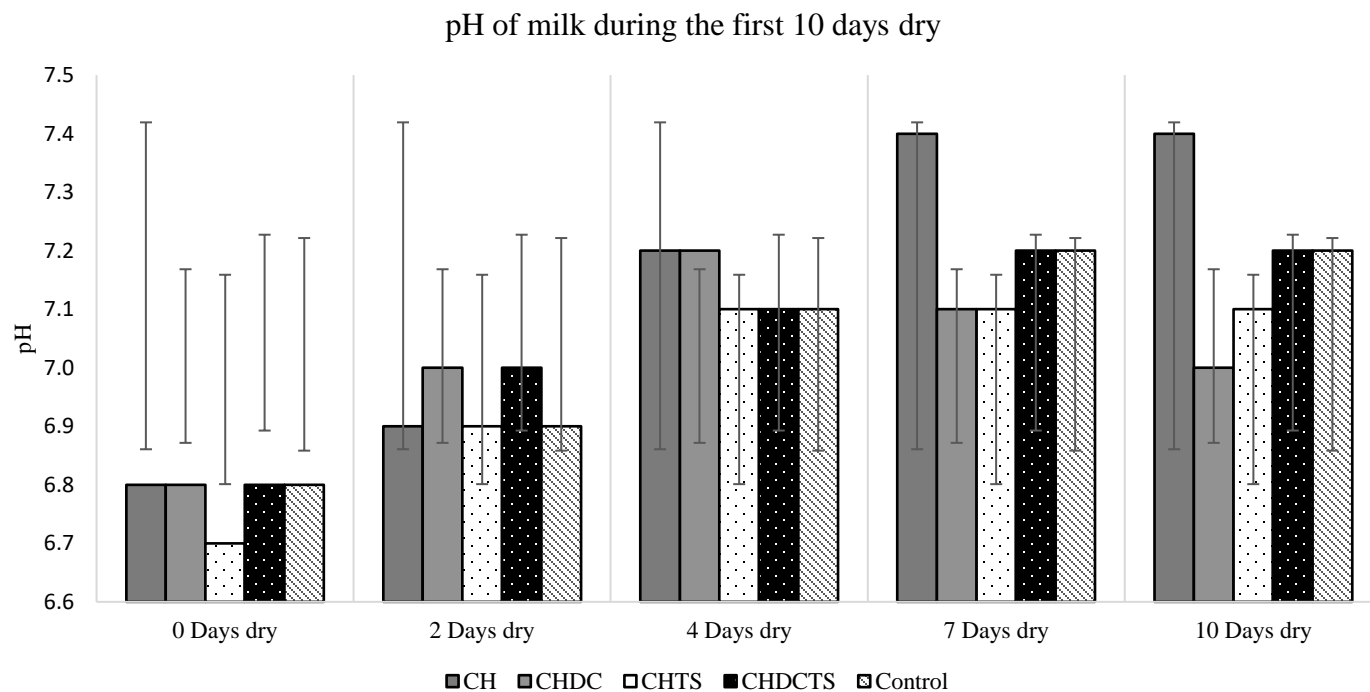


Figure 3. Increase in SCC by days dry and dry treatment group; no significant differences between treatments found. **CH**= Casein hydrolysate only; **CHDC** =Casein hydrolysate + dry cow treatment; **CHTS** = Casein hydrolysate + teat sealant; **CHDCTS** = Casein hydrolysate + dry cow treatment + teat sealant; **Control** = dry cow treatment + teat sealant

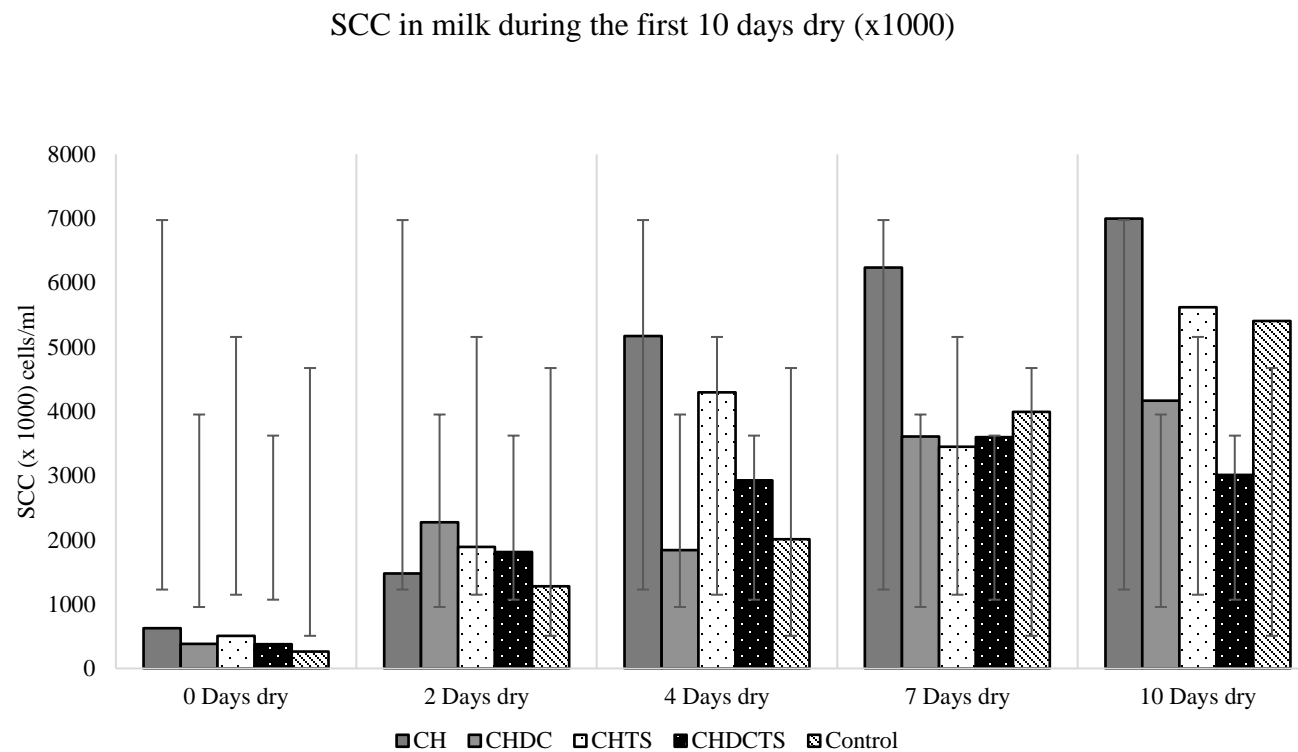


Figure 4. Increase in pH by days dry and dry treatment group; no significant differences between treatments found. CH= Casein hydrolysate only; CHDC =Casein hydrolysate + dry cow treatment; CHTS = Casein hydrolysate + teat sealant; CHDCTS = Casein hydrolysate + dry cow treatment + teat sealant; Control = dry cow treatment + teat sealant

CHAPTER V

Morphometric and histopathologic analysis of mammary gland involution following intramammary administration of casein hydrolysate at dry-off**Justine E. Britten¹, Chad S. Clancy², Kerry A. Rood, and David J. Wilson**

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ABSTRACT

The objective of this study was to compare quantitative histological changes of bovine mammary involution following intramammary infusion of casein hydrolysate

(CH) alone or with standard dry cow treatment (DCT) and/or an internal teat sealant (TS) when cows were dried off. A split udder design was used; one udder half of each cow received a treatment and the contralateral half was administered DCT + TS as a Control. Treatment groups were: CH, CH + DCT (CHDC), CH + TS (CHTS) and CH+DCT+TS (CHDCTS). Cows were randomized to be euthanized at 2 days dry (d 2) or 7 days dry (d 7), and then randomized to treatments within assigned day of euthanasia. A generalized linear mixed model tested for significant differences between treatment groups in changes to alveolar epithelial height, lumen diameter and interstitial stromal thickness as indicators of mammary involution in ventral (V), mid (M) and dorsal (D) anatomic regions of each mammary quarter. Compared to Control udder halves, epithelial height was greater in CHDC cows at d 2, M region; d 7, V region; and decreased in CH cows at d 7, V region. Within treatments, CH cows had smaller epithelial heights in V region than CHDC and CHTS cows at d 2. Lumen diameters were smaller and thickness of interstitial stroma was greater at d 2 in cows within all treatment groups compared to Control udder halves within all anatomic regions. Clinical chemistry and CBC with differential blood cell count tests were performed on all cows approximately 14 days before dry-off. Intramammary administration of CH at dry-off was associated with earlier histological indications of mammary involution than Controls at d 2 and d 7. Intramammary infusion of CH was associated with faster ultrastructural involutionary changes in bovine mammary gland tissues. These changes in biological indicators of mammary involution suggest that intramammary infusion of CH may increase the speed of involution early in the dry period.

Key words: dry period, casein hydrolysate, udder involution, histology, morphometry

INTRODUCTION

The dry period is an important part of a dairy cow's lactation cycle and requires most dairy farms to carefully manage the transition from lactating to dry and then back to lactating again (Natzke et al., 1975, Vanhoeij et al., 2016). Previous studies have demonstrated that a dry period before the next calving is beneficial for milk production in the following lactation and facilitates cell turnover in the mammary gland (Watters et al., 2008, Collier et al., 2012). Milk production per cow has increased dramatically in the last 100 years, from an average of less than 6,000 pounds/year in the early 1920s to nearly 23,000 pounds/year in 2017, with 12% of that increase just in the last 10 years (USDA, 2018). This trend has made the transition into the nonlactating state more difficult. Dairy cows are highly susceptible to acquiring new intramammary infection (IMI) at the time of dry-off and during the dry period, primarily due to milk stasis following abrupt cessation of milking (Natzke et al., 1975, Zobel et al., 2013) and the changes to the mammary cell populations (Oliver and Sordillo, 1989). Fully involuted mammary glands are more robust against new infections and while this process can take up to 21 days, much of bovine mammary involution takes place within the first 7 days after cows are dried off (Sordillo and Nickerson, 1988, Oliver and Sordillo, 1989). Cows with low production at the time of dry-off involute faster than higher producing animals (Sordillo et al., 1987), which has prompted research into possible mechanisms of stimulating faster bovine mammary involution, as many modern dairy cows are still producing a high volume (e.g. > 60 lb [27 kg]) of daily milk late in their lactation cycle (Zobel et al., 2013).

Epithelial height, secretory luminal diameter and interstitial stromal thickness undergo distinct changes when transitioning from lactating tissue to nonlactating tissue. Mammary tissue during lactation is mostly composed of large secretory alveolar lumina which are lined with well-differentiated secretory epithelial cells and surrounded by minimal amounts of interstitial stroma (Akers et al., 1990). After cessation of milking, the pressure and effect of milk stasis on these tissues begins to initiate change (Akers and Akers, 2016d). Lumen diameter decreases as milk production decreases and alveolar epithelial cells become non-secretory, less well-differentiated and mostly columnar in shape (Akers et al., 1990, Capuco and Akers, 1999). The thickness of interstitial stroma increases as luminal diameters decrease (Sordillo and Nickerson, 1988, Hurley, 1989). These changes are normal parts of mammary involution and will occur regardless of whether dry cow therapy is used or not. There are several factors that contribute to the risk of acquiring new IMI at dry-off and during the dry period. While mammary tissue is undergoing these physiologic changes, it is highly susceptible to new infection. This susceptibility combined with milk stasis, which both provides a growth environment for bacteria and is no longer serving to flush the teat canal daily, facilitates any bacteria present to colonize, rapidly increase their numbers and gain access to the mammary gland (Sordillo et al., 1987). After mammary tissues are fully involuted however, the udder is quite robust against new infection (Oliver and Sordillo, 1989). This suggests that an alternative treatment at dry-off that might increase the rate of mammary involution would shorten the period of risk for new IMI and benefit udder health of the animal.

The predominant immune cell types found in healthy mammary tissue are lymphocytes and macrophages, with a very low population of neutrophils (Stelwagen et

al., 2009, Sordillo, 2018). Macrophages and neutrophils are integral for recognition and phagocytosis of foreign elements and lymphocytes regulate the sensitization or suppression of the immune response (Paape et al., 1979, Nickerson, 1989). During episodes of mastitis, the innate immunity of the mammary gland dominates the early recognition and response to infection by bacteria and the cell populations change. The overall leukocyte count is considerably elevated and large numbers of neutrophils are recruited to the site, becoming the predominant cell type over macrophages and lymphocytes (Oviedo-Boyso et al., 2007, Sordillo, 2018). The process of bovine mammary involution elicits a similar immune response to that of mastitis, as it is also an inflammatory process. Leukocyte counts increase within 7 days of cessation of milking and remain elevated for several weeks into the dry period (Nickerson, 1989). In uninfected dry mammary glands, neutrophil populations remain proportionally lower than macrophages and lymphocytes, similar to healthy lactating tissue even though all cell types are elevated during involution. However, if a bacterial organism is present at dry-off the neutrophil count becomes the dominant white cell type, again similar to within lactating glands (Nickerson, 1989, Atabai et al., 2007). Neutrophil activity is inhibited during involution by the increased presence of casein micelles and fat globules, which are indiscriminately phagocytized by neutrophils (Nickerson, 1989). The reduction in neutrophilic antibacterial activity following phagocytosis of casein and fat increases the susceptibility for infection of the mammary gland during the nonlactating period.

Abrupt cessation of milking is currently the standard management practice used at the time of dry-off on many dairies (Stefanon et al., 2002). This may lead to elevated udder pressure and signs of discomfort in some high-producing animals (Leitner et al.,

2007). Cessation of milking is an important part of initiating the mammary involution process, caused by the pressure that results from milk stasis and udder engorgement (Capuco and Akers, 1999). There are constituents in the milk itself that are hypothesized to provide inhibitory chemical feedback as part of the involution process (Collier et al., 2012). This has been explored in prior studies where intramammary (IMM) infusions of casein hydrolysate (CH) were infused to involute individual quarters (Shamay et al., 2003, Britten et al., 2018) or at the time of dry-off to increase cow comfort (Silanikove et al., 2005, Leitner et al., 2007). Intramammary infusions of CH contain fractionated β -casein peptides that are believed to be responsible for the induction of plasmin activity and consequentially triggering active mammary involution (Shamay et al., 2002, Shamay et al., 2003). More recently, IMM chitosan hydrogels (Lancôt et al., 2017) or intramuscular administration of cabergoline, an inhibitor of the hormone prolactin, which is required for maintaining lactation (Boutinaud et al., 2016) were explored as alternative approaches to manipulation of mammary involution. Histopathologic studies of the structural changes that take place in bovine mammary tissue during involution have been conducted previously (Akers et al., 1990, Capuco and Akers, 1999) for the purposes of understanding mammary gland remodeling during the dry period. There is a lack of histologic data available regarding the effects of different intramammary treatments on mammary involution. While the morphologic changes that occur during involution in dairy animals are less pronounced than those seen in nonpregnant mice (Strange et al., 1992), there were distinct morphologic changes observed between lactating and involuting bovine mammary tissues (Capuco and Akers, 1999). Briefly, there is an inverse relationship between luminal area and thickness of intra-alveolar stroma (Capuco

and Akers, 1999). Lumen diameters and total luminal area decrease to less than 10% of total mammary tissue at 35 days dry, compared with approximately 21% of the gland in lactating cattle (Capuco and Akers, 1999, Akers et al., 2006). In contrast, proportion of stromal tissue was previously described as maximized at 35 days dry and then decreased shortly before calving (Capuco and Akers, 1999). Alveolar structure is maintained throughout the dry period but the height of the epithelial cells increases throughout the process of involution (Hurley, 1989). As lumen diameters decrease, the height of the alveolar epithelial cells surrounding them increase (Sordillo and Nickerson, 1988). These morphologic changes begin immediately following cessation of milking and continue for 14-21 days post dry-off (Oliver and Sordillo, 1989).

The objective of this study was to evaluate the effect of 4 different treatment combinations, all containing CH, against a non-CH control treatment as possible method of hastening mammary involution, using quantitative morphometry as a method of assessment. Based on previous studies (Shamay et al., 2003, Leitner et al., 2007, Seeth et al., 2016, Britten et al., 2018), we hypothesize that intramammary infusion of CH may be used to induce faster involution as detected histologically. Measurements of alveolar epithelial height, alveolar lumen diameter and interstitial stroma thickness between tissues from treated and control udder halves were used to assess changes.

MATERIALS AND METHODS

Animals and Management

Study cows were sourced from 6 commercial Utah dairy farms. All farms followed a twice daily milking schedule and cows were housed on shaded dry lots that are typical of the region. Days in milk (DIM) were obtained from dairy records. To be eligible for the study, cows were non-pregnant, ≥ 300 days in milk (DIM) with 4 normally lactating quarters.

Prior to enrollment, 2 blood samples were collected from each cow approximately 14 days before dry-off, and analyzed for complete blood count (CBC) and large animal clinical chemistry panel using the laboratory normal ranges of the Utah Veterinary Diagnostic Laboratory (UVDL), Logan, UT. Any cows with hematologic or serum chemical abnormalities indicative of infectious or inflammatory disease were excluded.

All animal handling and treatments in this study were performed in compliance with an Institutional Animal Care and Use Committee (IACUC) approved Utah State University protocol. Casein hydrolysate is not labeled for use in food animals; this study was conducted with FDA permission and followed FDA-approved guidelines for withhold times. Additionally, these animals were euthanized as part of the study and no meat or milk from treated animals entered the food chain.

Experimental Design

Cows were randomly assigned to days dry when they were to be euthanized (d 2 or d 7), then randomly assigned to a treatment group, then randomized for which udder half would be treated versus Control, all using a random number generator. There were 4 treatment groups and one control: (1) Casein hydrolysate (CH), (2) Casein hydrolysate + dry cow antibiotic (500 mg cloxacillin benzathine)¹⁵ (CHDC), (3) Casein hydrolysate + internal teat sealant¹⁶ (CHTS), (4) Casein hydrolysate + dry cow antibiotic + internal teat sealant (CHDCTS) and DCT+TS (Control). Assignment of treatment group and udder half was forced only for the last cow to ensure an equal number of animals (4) per treatment group and equal distribution of treated and control left or right halves of the udder within each treatment group. Study cows were marked with colored leg bands on both hind legs (colors were not associated with any treatment group in order to preserve blinding to farm personnel).

Treatment and Sample Collection

Cows were administered their assigned treatment at time of dry-off, which was defined as Time 0. After the final milking, teats were cleaned with alcohol wipes and the assigned treatment and Control were each administered IMM to their assigned udder half using aseptic technique. Teats were dipped with a 0.5% titratable iodine teat disinfectant after treatment. All cows were transported from the dairy to the UVDL on their pre-

¹⁵Dry-Clox DC, Boehringer Ingelheim

¹⁶ Orbeseal, Zoetis Animal Health

determined euthanasia days (2 d or 7 d dry) and humanely euthanized according to the IACUC protocol. Immediately after euthanasia each animal was exsanguinated and prepared for tissue sample collection. All mammary quarters were bisected cranially to caudally along the midline, resulting in a cross-section of each quarter. Biopsy tissue samples were collected from 3 anatomic regions of each quarter, along a vertical axis from the center of the teat end to the parenchymal tissue next to the ventral body wall of the cow as described by (Akers et al., 2006), for a total of 12 biopsies per cow. The anatomic regions were: 1. Immediately above the teat canal (ventral [V]), 2. Near the edge of the ventral body wall of the cow (dorsal [D]) and approximately halfway between V and D (in the middle of the quarter from ventral to dorsal) (mid [M]) (Figure 1). Tissue samples were fixed in formalin for 48 h, followed by fixation in 70% ethanol for 24 h and processed for histology (Bancroft and Gamble, 2008). One slide was made from each of the 12 tissue samples per cow.

Preparation of Casein Hydrolysate

Two batches of casein hydrolysate were prepared as previously described (Shamay et al., 2003) using aseptic technique. Each batch was prepared using 100 g of commercially purchased >99.9% pure bovine casein powder¹⁷ dissolved in 1 L of autoclaved deionized water, adjusted to pH 8.8 with sodium hydroxide, containing 3 g of TRIS buffer and enzymatically digested with trypsin. After digestion, remaining particulate material was removed via two centrifugation cycles of 15 minutes at 3000 x g.

¹⁷ Sigma-Aldrich Chemicals company, St. Louis, MO

Solution was boiled for 15 minutes between centrifugation cycles to denature any remaining enzyme and kill possible environmental contaminant bacteria, followed by sterilization using vacuum membrane filtration. The final product was dispensed (15 ml) into sterile syringes and stored frozen at - 20° C. Each batch of CH was screened for bacterial contamination by inoculating tryptic soy broth with 1 ml of the solution, incubating for 24 h and inoculating 100 µl onto blood agar. Blood agar plates were incubated for a total of 48 h at 37° C and read at 24 and 48 h for bacterial growth. A separate blood agar plate was plated directly with 100 µl of non-enriched casein hydrolysate and then incubated and examined in the same way. The definition of an uncontaminated batch was when no growth of any bacterial colonies was observed on either direct or enriched cultures. Protein concentration for each batch was quantified via bicinchoninic acid assay (BCA¹⁸), according to manufacturer's instructions. The BCA assay is commonly used for protein quantification (Smith et al., 1985b). Final concentration of CH solutions was 5.6 mg/ml, for a total dose of 84 mg of hydrolyzed casein per 15 ml dose. Purity was assessed using sodium dodecyl sulfate polyacrylamide gel electrophoresis gel.

Morphometric Analysis

Quantitative morphologic analysis was used to evaluate changes in 3 mammary tissue structures: interalveolar stroma, epithelium and alveolar secretory lumina. This method has been described in previous studies (Sordillo and Nickerson, 1988, Akers et

¹⁸ Pierce BCA Assay, ThermoFisher Scientific

al., 2006), using a gridded field in an ocular microscope lens. For our study, we used morphometric image analysis. Ten random photographic images were taken of each slide, using a microscope lens camera at magnification power 200X, for a total of 120 images per cow. Measurements were taken using cellSens¹⁹ software package and recorded categorically, within each image, for three tissue types: interalveolar stroma thickness, epithelial diameter and alveolar lumen diameter. The actual number of measurements taken of each tissue structure varied with the appearance of structures in each image, but no less than 25 alveolar lumina were measured per slide.

Inflammatory Cell Type Evaluation

The histology slides were analyzed by a veterinary anatomic pathologist (CSC), who was blinded to the treatment groups, cows, etc. Each anatomic region was analyzed for extent of inflammation (severity), distribution of inflammation and inflammatory infiltrate cell type. Severity was graded according to non-parametric analysis using the pathologist's expertise from absent (no inflammation) to severe. Histologic changes of secretory lumina and interstitial tissues were evaluated and scored for inflammation and cell population distribution, at 2 and 7 days post-treatment. Primary cell types were polymorphonuclear cells (neutrophils; PMNs) and lymphocytes. Statistical analysis was not performed on these observations as per the current recommendations of the International Harmonization of Toxicologic Pathology Nomenclature (Gad and Rousseaux, 2002, Mann et al., 2012)

¹⁹ Olympus Corporation, Life Science Solutions, USA

Statistical Analysis

Statistical analyses were performed in SAS Studio²⁰, using the generalized linear mixed model procedure (GLIMMIX). Time, treatment and anatomic region were treated as fixed effects and cow ID as a random effect, with cow nested within time because each cow could only be euthanized and evaluated at one time point post-dryoff. Presence or absence of elevated liver enzymes (categorical variable with levels of yes or no) was also tested as a covariate and for interaction with time, treatment and anatomic region. The full model tested for individual and interaction effects of the above predictors; separate models were made to test for significant individual effects or interactions within each mammary tissue type, as specified by the full model. The model emphasized differences between each treatment and the Control within each cow to account for variability between animals. Where statistical significance existed between two-way or three-way interactions of time, treatment and anatomic region, the LSMEANS procedure in SAS and Tukey-Kramer adjustment were used to compare means within each level of time and anatomic region to all other levels. Natural log transformations were taken of all continuous outcome variables to correct for the wide range of numerical values resulting in non-homogenous variances. Measurements were averaged across all 10 images per slide, for each tissue type, within each anatomic region of the gland (D, M, or V) for all udder halves within a given treatment group (or Control). Differences were considered statistically significant when $P < 0.05$ for all analyses.

²⁰ SAS Institute Inc., Cary, NC, USA

RESULTS

Seventeen cows were enrolled in the study. One animal died of unknown cause, for a final sample size of 16 cows comprised of Holsteins (n=12) and Jerseys (n=4). Blood chemistry showed 7 cows with elevated liver enzyme levels; no cows were excluded because there was no abnormal bloodwork indicating infectious or inflammatory disease. Cows ranged in parity from first through fifth lactation, with overall means of lactation number (parity) 2.1 and 439 DIM. There were no statistically significant differences between each treatment group of 4 cows for mean DIM (CH = 437, CHDC = 433, CHTS = 422, CHDCTS = 471) or mean lactation number (CH = 2.3, CHDC = 2.3, CHTS = 2.0, CHDCTS = 2.3) (All $P \geq 0.99$, ANOVA). Measurements taken of epithelial height, luminal diameters and interstitial stroma thickness totaled 52,914 individual observations.

Bacterial contamination checks for all batches produced, both hydrolyzed and non-hydrolyzed casein, were negative for any microbial growth. Final protein concentration of CH solutions was 1.5 mg/ml, which resulted in 22.5 mg per 15 ml dose. Final concentration of NHC was 0.2 mg/ml, which resulted in 3 mg per 15 ml dose. Each batch was also assessed for purity by running a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gel. Heavy bands were seen at approximately 13-15 kD, 23 kD and 35 kD in the non-hydrolyzed casein batches. In the hydrolyzed batches, bands were seen primarily at 13-14 kD and 20 kD but also some at 30-35 kD. Visible fragments with these molecular weights was consistent with previous studies of enzymatically hydrolyzed casein, indicating that only casein was present in the sample and no other compounds were

available for enzymatic breakdown (Wang et al., 2013). Obvious differences between hydrolyzed and non-hydrolyzed casein were present in the appearance of the gel (Figure 1).

Analysis of Quantitative Morphological Changes

No significant interactions existed between time, treatment, anatomic region of the mammary gland or liver enzymes for luminal or stromal tissues, however there was a significant time \times treatment \times anatomic region interaction in epithelia, as tested in the full model ($P = 0.04$, PROC GLIMMIX)(Table 3). Epithelial heights in CH treated cows were less than CHDC and CHTS cows in region V at d 7 ($P < 0.02$, GLIMMIX)(Table 3). Within the CH treated cows, their epithelial heights in region V were greater at d 2 than d 7 ($P = 0.001$, GLIMMIX)(Table 3). In comparison to Controls, CHDC cows had greater epithelial heights at d 2 in region M and d 7 in region V (both $P = 0.02$, GLIMMIX), while epithelial heights in CH treated cows were less at d 7 in region V ($P = 0.004$, GLIMMIX)(Table 3).

Luminal diameters in the CHDC treatment group were smaller than Controls within all anatomic regions at d 2 and d 7 ($P = 0.003$, GLIMMIX)(Table 3). Luminal diameters in region V for cows in all treatment groups were less than in Controls at d 2 and d 7 ($P = 0.0004$)(Table 3). However, in contrast to the smaller secretory lumina in all treatment groups in region V, luminal size was not different between groups in region M or region D at d 2 and d 7. Involutionary decreases in luminal diameters were observed sooner in the V region of the udder than in the M and D regions. The thickness of

interstitial stroma was significantly larger than Controls for cows in all treatment groups and within all regions of the gland at d 2 ($P = 0.003$, GLIMMIX); stromal thickness was not different between treatment groups on d 2 or d 7 (Table 3).

Necrotic tissue was almost entirely absent from histology slides and few macrophages were seen (Table 2, Figure 3). All treatment groups saw an increase of inflammatory infiltrates within the lumina and interstitial tissues at 7 d post-treatment relative to 2 d post-treatment (Table 1, Figure 2). At 2 d after dry treatment 56% and 71% of lumina and interstitial tissues, respectively, showed signs of inflammation. At d 7 dry, 94% and 97% of lumina and interstitial tissues, respectively showed at least mild or moderate inflammation, with no apparent association with any treatment group. All groups increased in the number of neutrophils present in the lumina, from d 2 after dry-off to d 7 (Figure 3). At d 2, 22 of 48 slides showed neutrophil infiltration of lumina, in comparison with 43 of 48 slides at d 7, with no association with treatment group. Cellular infiltrates of interstitial tissues included both neutrophils and lymphocytes at d 2 and d 7 post-treatment (Figure 3). Inflammatory cell infiltration into mammary gland tissue increased from d 2 to d 7 in all treatment groups except for CH treated cows. At d 7, the CH group showed predominantly neutrophils and macrophages but almost no lymphocytes (Figure 3).

DISCUSSION

It is well-documented that dairy cattle require a dry period for optimal milk production and udder health but the transition from the lactating to non-lactating (dry)

state and then back to lactating again is a time of increased risk to cows' health. Previous studies have investigated various methods of managing the dry period, including procedures for cessation of milking, different forms of dry cow treatment and dry period lengths. It is difficult to measure the changes taking place in the udder during mammary involution to determine if one best practice exists. Histologic examination of tissue changes presents a more precise method of assessment than do changes in milk composition, but for practical reasons, this is usually not an available option. The primary objective of this study was to quantify the associations of alternative dry-off treatments with morphologic changes which occur in 3 primary tissue structures within bovine mammary tissue.

Because we used a split udder design for this study, every cow served as her own control. This design proved to be useful in observing differences between treated quarters and controls, which is where most of the significant differences were observed. Measurements within each tissue type were markedly variable between different animals, as much as 40 times different in some cases. All significant differences in histological evidence of involution between treatments and controls were observed in the ventral and mid anatomic regions, mainly in the ventral part of the udder. This suggests that any treatment effects were taking place in an upward vertical process, beginning in the teat cistern and moving dorsally towards the body wall. This upward progress of mammary involution is substantiated by similar findings in previous studies (Akers et al., 1990, Akers and Akers, 2016c). Differences in the 3 mammary tissue types between treatments or compared to Controls were less clear. Significant differences included increases in epithelial height and decreases in luminal diameters during the early dry period. An

interesting outcome was that significant increases in stromal thickness were observed in treated quarters across all treatments, compared with control quarters in the cows euthanized 2 d after dry-off. All treatments were apparently associated with faster evolution within 2 d after intramammary infusion at dry-off in comparison to the control treatment of dry cow antibiotic and internal teat sealant. There were no clear trends observed between treatment group, time or anatomic region for differences in inflammatory infiltrate or severity of inflammation. This supports previous studies and suggests recruitment of neutrophils and lymphocytes within both the lumina and interstitial spaces serves a physiologic role in mammary gland involution and are not indicative of a pathologic process during the dry period (Oviedo-Boyso et al., 2007, Sordillo, 2018). While recruitment of inflammatory infiltrates was observed in the study, there was no histologic evidence of tissue necrosis, epithelial metaplasia or stromal abnormalities in any treatment group. Lack of significant histopathologic changes in association with intramammary casein administration supports the commercial use of this product.

At present, “blanket” use of antibiotic therapy for all cows at the end of lactation is still the standard recommendation and primary method of preventing new infections (Schukken et al., 2011a). The fear among much of the public regarding possible propagation of antibiotic resistance by use of antibiotics in food producing animals and the increasingly negative perception of consumers towards livestock raising practices is a concern for the dairy industry. Although use of dry cow therapy has been shown to be highly effective in preventing against new IMI, the mammary gland does have natural

defense mechanisms which aid in preventing infection (Rainard and Riollet, 2006, Schukken et al., 2011b).

The results of this study demonstrated that intramammary administration of casein hydrolysate (whether combined with an antibiotic, an internal sealant or alone) at dry-off was associated with changes in bovine mammary gland involution. No single treatment group was found to consistently exhibit signs of faster or slower involution from the other treatments; however, differences comparing all treatment groups to the controls indicated that quarters which received casein hydrolysate involuted faster, as indicated in epithelial heights and luminal diameters. Furthermore, no results indicated that casein hydrolysate ever slowed involution compared to the control treatment of dry cow antibiotic and internal teat sealant; involution in treated quarters was either faster or no different. Faster mammary involution may potentially decrease udder engorgement associated with abrupt cessation of milking and enhance cow comfort, which would make intramammary use of casein hydrolysate at dry-off a useful management tool for commercial dairies, without the use of antibiotics.

Casein is the primary protein component of milk and accounts for approximately 80% of total protein found in fluid milk (Wang et al., 2013). The molecular structure is composed of four different constituents: α s1-, α s2-, β - and κ -casein. These four casein types differ in their structure and molecular weights but range between 19-25 kD. Previous studies have discovered that enzymatic digestion of casein with Trypsin results in an increase of hydrolysates at molecular weight of 20kD and a decrease in peptides over 50 kD (Wang et al., 2013). These trends continue with longer hydrolyzation periods and this

is consistent with our results. It should be noted that the molecular weight of Trypsin is 23.3 kD and while the enzyme was inactivated by heat, its presence may still be reflected in the final product. Because the molecular weight of Trypsin falls in the same range as casein, it cannot be determined if all the bands observed are from casein only or also contain Trypsin. Investigation of Trypsin presence falls outside the scope of this study.

CONCLUSION

Restricting “blanket” use of dry cow therapy in the U.S. dairy industry may become a reality in the future. Results of this study showed that intramammary infusion of casein hydrolysate was associated with faster ultrastructural involutionary changes in bovine mammary gland tissues. In particular, secretory luminal diameters decreased, interstitial stroma became thicker and epithelial cells increased in height, all indicators of involution, in comparison to a standard industry treatment of dry cow antibiotic and internal teat sealant. These changes in biological indicators of mammary involution suggest that intramammary infusion of casein hydrolysate may increase the speed of involution early in the dry period.

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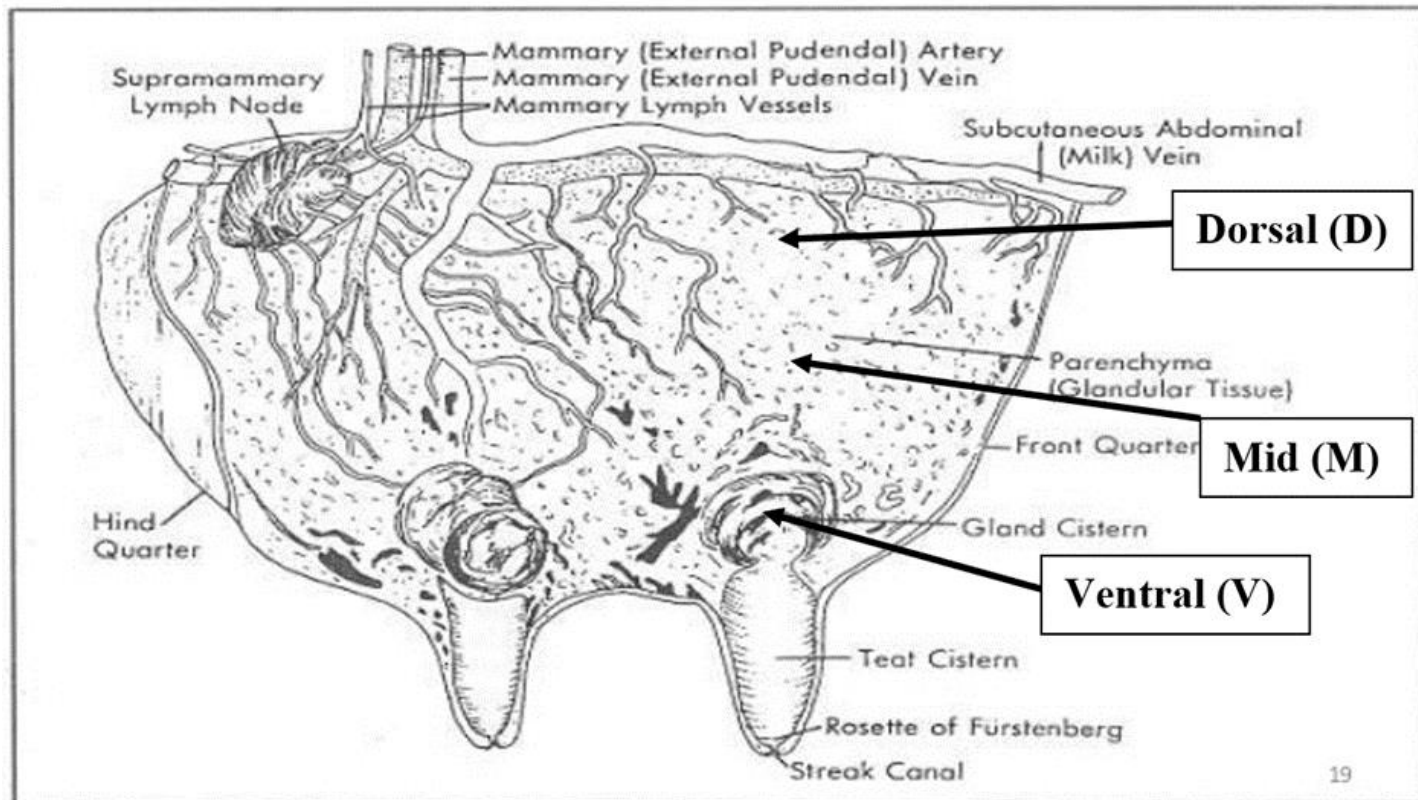


Figure 1. Three dissection regions used for tissue collection in each mammary quarter; Ventral (V), Mid (M), Dorsal (D)

Table 1. Inflammatory Score of the Interstitial and Secretory Lumen Tissues at 2 and 7 Days after Dry-off as determined by pathologic evaluation

Treatment	Time	Lumen Inflammatory Scores ¹				Interstitial Inflammatory Scores ¹			
		Negative	Mild	Moderate	Severe	Negative	Mild	Moderate	Severe
CH ²	2 d	33%	67%	0%	0%	42%	33%	25%	0%
		(4/12)	(8/12)	(0/12)	(0/12)	(5/12)	(4/12)	(3/12)	(0/12)
CH ²	7 d	0%	75%	25%	0%	8%	67%	25%	0%
		(0/12)	(9/12)	(3/12)	(0/12)	(1/12)	(8/12)	(3/12)	(0/12)
CHDC ²	2 d	50%	50%	0%	0%	8%	75%	17%	0%
		(6/12)	(6/12)	(0/12)	(0/12)	(1/12)	(9/12)	(2/12)	(0/12)
CHDC ²	7 d	17%	58%	25%	0%	0%	83%	17%	0%
		(2/12)	(7/12)	(3/12)	(0/12)	(0/12)	(10/12)	(2/12)	(0/12)
CHTS ²	2 d	42%	33%	0%	25%	25%	75%	0%	0%
		(5/12)	(4/12)	(0/12)	(3/12)	(3/12)	(9/12)	(0/12)	(0/12)
CHTS ²	7 d	0%	75%	25%	0%	0%	83%	17%	0%
		(0/12)	(9/12)	(3/12)	(0/12)	(0/12)	(10/12)	(2/12)	(0/12)
CHDCTS ²	2 d	50%	50%	0%	0%	8%	50%	33%	8%
		(6/12)	(6/12)	(0/12)	(0/12)	(1/12)	(6/12)	(4/12)	(1/12)
CHDCTS ²	7 d	0%	92%	8%	0%	0%	92%	8%	0%
		(0/12)	(11/12)	(1/12)	(0/12)	(0/12)	(11/12)	(1/12)	(0/12)
Control	2 d	54%	42%	4%	0%	38%	58%	4%	0%
		(26/48)	(20/48)	(2/48)	(0/48)	(18/48)	(28/48)	(2/48)	(0/48)
Control	7 d	8%	65%	27%	0%	4%	83%	13%	0%
		(4/48)	(31/48)	(13/48)	(0/48)	(2/48)	(40/48)	(6/48)	(0/48)

¹Scores given as percentage first (number of slides with given score/total slides in same treatment and time group); raw data shown after percentage²CH = Casein hydrolysate, CHDC = Casein hydrolysate + dry cow treatment, CHTS = Casein hydrolysate + teat sealant, CHDCTS = Casein hydrolysate + dry cow treatment + teat sealant

Table 2. Distribution of Lumen and Interstitial Cell Types Observed Histologically at 2 and 7 Days after Dry-off as determined by pathologic evaluation

Treatment	Time	Lumen Cell Types ¹²					Time	Interstitial Cell Types ¹²				
		None	PMN	Lymphocyte	Macrophage	Necrosis		None	PMN	Lymphocyte	Macrophage	Necrosis
CH ³	2 d	(33%) 4/12	(58%) 7/12	(0%) 0/12	(8%) 1/12	(0%) 0/12	2 d	(42%) 5/12	(25%) 3/12	(50%) 6/12	(0%) 0/12	(0%) 0/12
CH ³	7 d	(0%) 0/12	(75%) 9/12	(0%) 0/12	(75%) 9/12	(0%) 0/12	7 d	(0%) 0/12	(92%) 11/12	(8%) 1/12	(25%) 3/12	(0%) 0/12
CHDC ³	2 d	(50%) 6/12	(33%) 4/12	(0%) 0/12	(17%) 2/12	(0%) 0/12	2 d	(8%) 1/12	(17%) 2/12	(92%) 11/12	(0%) 0/12	(0%) 0/12
CHDC ³	7 d	(17%) 2/12	(83%) 10/12	(0%) 0/12	(25%) 3/12	(0%) 0/12	7 d	(0%) 0/12	(25%) 3/12	(100%) 12/12	(0%) 0/12	(0%) 0/12
CHTS ³	2 d	(42%) 5/12	(50%) 6/12	(0%) 0/12	(17%) 2/12	(0%) 0/12	2 d	(25%) 3/12	(42%) 5/12	(42%) 5/12	(0%) 0/12	(0%) 0/12
CHTS ³	7 d	(0%) 0/12	(100%) 12/12	(0%) 0/12	(8%) 1/12	(0%) 0/12	7 d	(0%) 0/12	(8%) 1/12	(100%) 12/12	(0%) 0/12	(0%) 0/12
CHDCTS ³	2 d	(50%) 6/12	(42%) 5/12	(0%) 0/12	(8%) 1/12	(8%) 1/12	2 d	(8%) 1/12	(17%) 2/12	(83%) 10/12	(0%) 0/12	(0%) 0/12
CHDCTS ³	7 d	(0%) 0/12	(100%) 12/12	(0%) 0/12	(17%) 2/12	(8%) 1/12	7 d	(0%) 0/12	(0%) 0/12	(100%) 12/12	(0%) 0/12	(0%) 0/12
Control	2 d	(54%) 26/48	(31%) 15/48	(0%) 0/48	(15%) 7/48	(0%) 0/48	2 d	(38%) 18/48	(6%) 3/48	(60%) 29/48	(0%) 0/48	(0%) 0/48
Control	7 d	(8%) 4/48	(85%) 41/48	(0%) 0/48	(25%) 12/48	(10%) 5/48	7 d	(4%) 2/48	(40%) 19/48	(90%) 43/48	(0%) 0/48	(0%) 0/48

¹More than one cell type may be present within each evaluated space²Scores given as percentage first, followed by number of slides with that cell type observed/total slides in same treatment and time group³CH = Casein hydrolysate, CHDC = Casein hydrolysate + dry cow treatment, CHTS = Casein hydrolysate + teat sealant, CHDCTS = Casein hydrolysate + dry cow treatment + teat sealant

Table 3. Summary of raw means of continuous measurements by treatment group and tissue type. All measurements are in micrometers (μm)

Group	n	Time	Tissue Type								
			Epithelia Height (V)	Epithelia Height (M)	Epithelia Height (D)	Lumen Diameter (V)	Lumen Diameter (M)	Lumen Diameter (D)	Stromal Width (V)	Stromal Width (M)	Stromal Width (D)
CH ¹	2 Cows	2 d	12 ^a	10 ^{ab}	10 ^{ab}	42 ^{eγβ}	64 ^e	54 ^e	17 ^{gγ}	12 ^{gγ}	13 ^{gγ}
CH ¹	2 Cows	7 d	244 ^{bcdβ}	236 ^{ab}	226 ^{ab}	688 ^{eγβ}	874 ^e	897 ^e	413 ^g	419 ^g	404 ^g
CHDC ¹	2 Cows	2 d	437 ^{acd}	400 ^{abγ}	423 ^{ab}	1524 ^{eγβ}	1701 ^{eγβ}	1977 ^{eγβ}	565 ^{gγ}	557 ^{gγ}	530 ^{gγ}
CHDC ¹	2 Cows	7 d	277 ^a	241 ^{ab}	310 ^{ab}	480 ^{eγβ}	1019 ^{eγβ}	1108 ^{eγβ}	330 ^{gγβ}	273 ^{gγβ}	303 ^{gγβ}
CHTS ¹	2 Cows	2 d	9 ^{ad}	8 ^{ab}	9 ^{ab}	51 ^{eγβ}	63 ^e	64 ^e	13 ^{gγ}	10 ^{gγ}	12 ^{gγ}
CHTS ¹	2 Cows	7 d	247 ^a	214 ^{ab}	207 ^{ab}	839 ^{eγβ}	894 ^e	983 ^e	368 ^g	339 ^g	252 ^g
CHDCTS ¹	2 Cows	2 d	11 ^{ad}	10 ^{ab}	9 ^{ab}	39 ^{eγβ}	47 ^e	65 ^e	18 ^{gγ}	15 ^{gγ}	11 ^{gγ}
CHDCTS ¹	2 Cows	7 d	16 ^a	14 ^{ab}	14 ^{ab}	28 ^{eγβ}	44 ^e	45 ^e	20 ^g	16 ^g	16 ^g
Control	8 Cows	2 d	98 ^{ab}	100 ^{abγ}	118 ^{ab}	624 ^γ	749 ^{eγ}	731 ^{eγ}	103 ^{gγ}	104 ^{gγ}	108 ^{gγ}
Control	8 Cows	7 d	215 ^{aβ}	210 ^{ab}	175 ^{ab}	661 ^β	858 ^{eβ}	779 ^{eβ}	261 ^{gβ}	229 ^{gβ}	228 ^{gβ}

¹CH = Casein hydrolysate, CHDC = Casein hydrolysate + dry cow treatment, CHTS = Casein hydrolysate + teat sealant, CHDCTS = Casein hydrolysate + dry cow treatment + teat sealant

²Means that are significantly different from the Control at 2 d are marked with γ , within same tissue type (all $P < 0.05$, GLIMMIX)

³Means that are significantly different from the Control at 7 d are marked with β , within same tissue type (all $P < 0.05$, GLIMMIX)

^{a-g} Means that are significantly different between treatment groups or time points within the same tissue type are denoted by different letter superscripts (all $P < 0.05$, GLIMMIX).

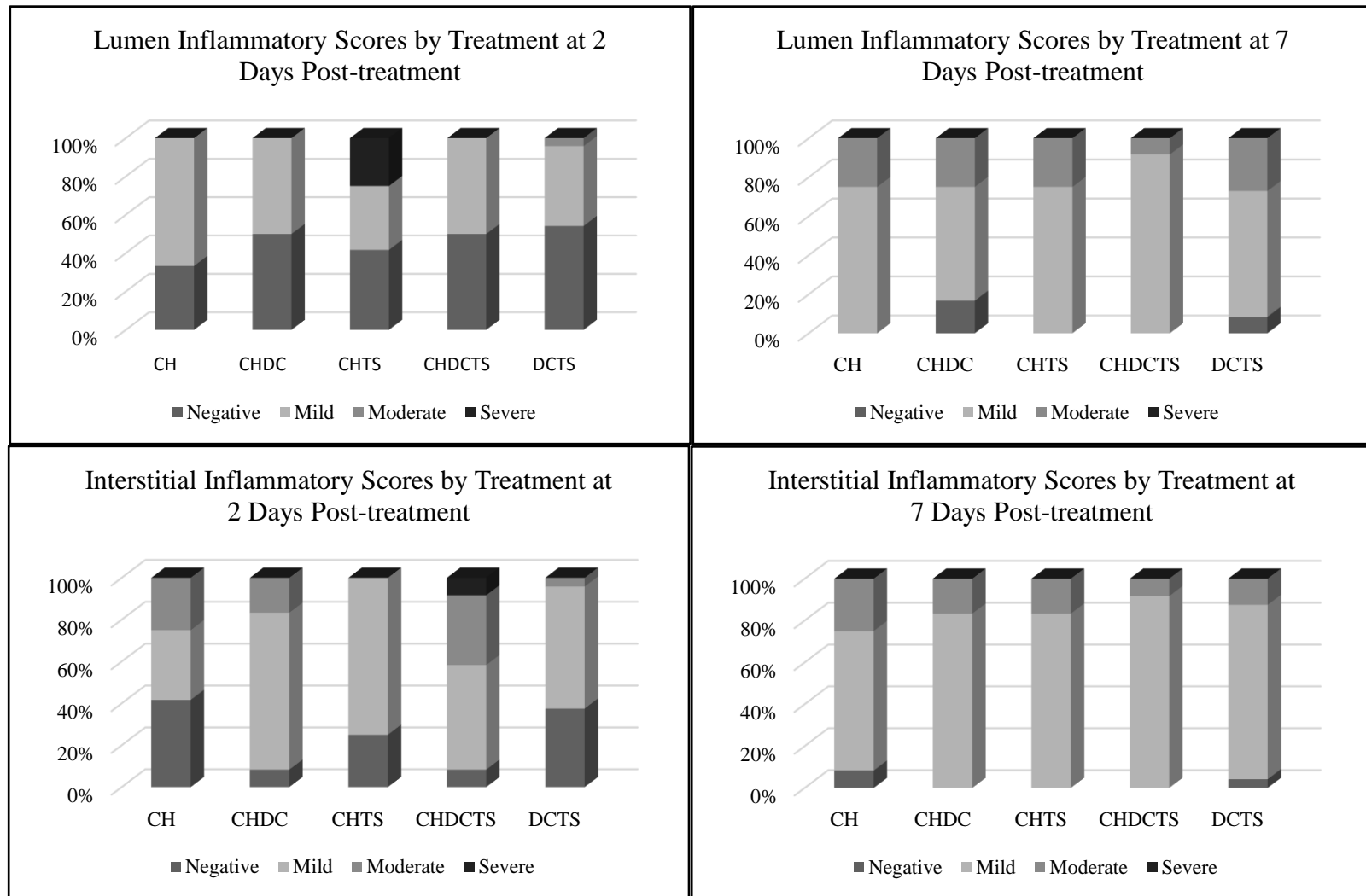


Figure 2. Inflammatory scores of interstitial and lumen tissues at 2 and 7 days after dry-off. CH = casein hydrolysate; CHDC = casein hydrolysate + dry cow treatment; CHTS = casein hydrolysate + teat sealant; CHDCTS = casein hydrolysate + dry cow treatment + teat sealant

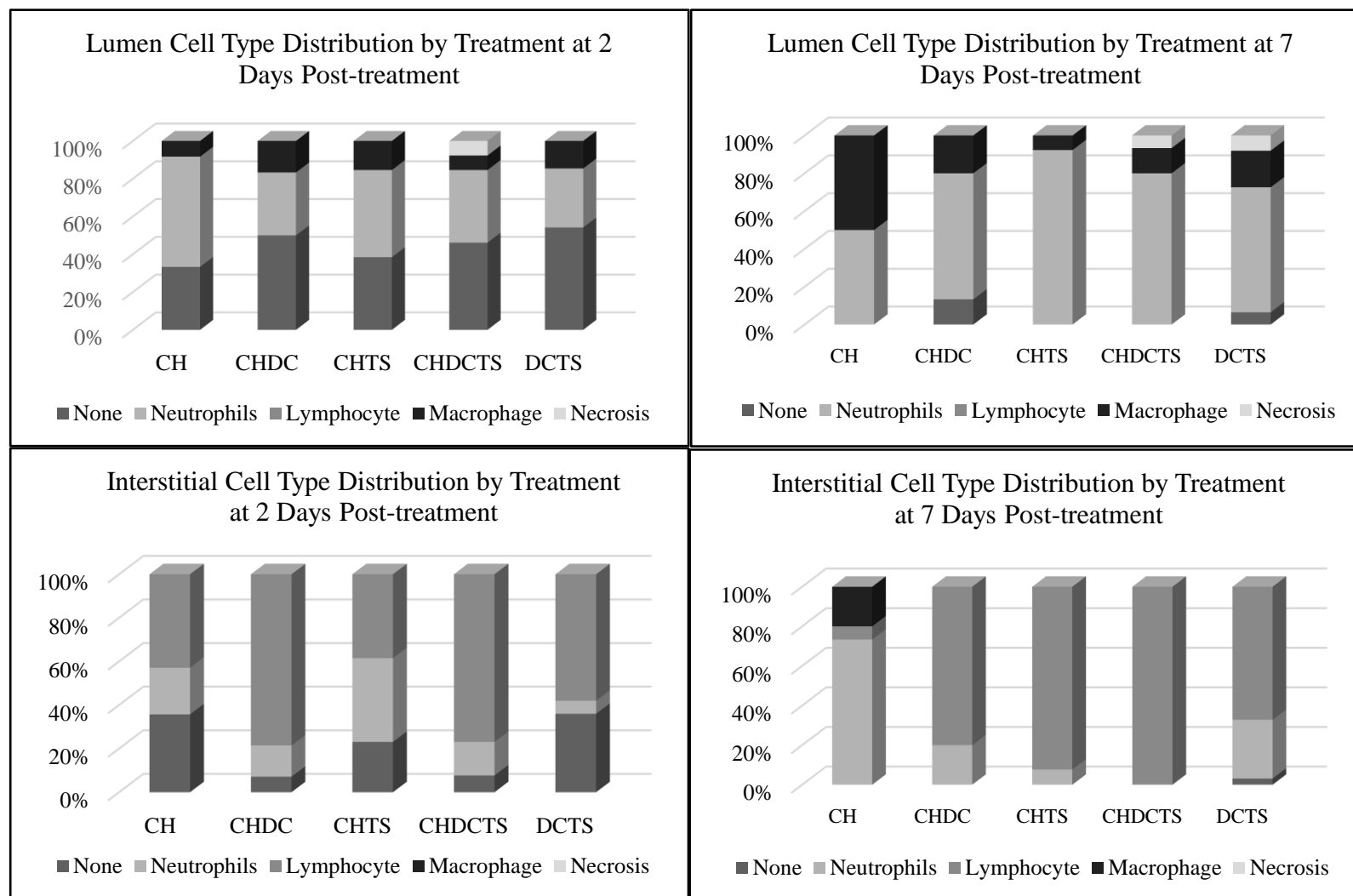


Figure 3. Lumen and interstitial cell population distributions at 2 and 7 days after dry-off. CH = casein hydrolysate; CHDC = casein hydrolysate + dry cow treatment; CHTS = casein hydrolysate + teat sealant; CHDCTS = casein hydrolysate + dry cow treatment + teat sealant

Figure 4. Comparison of treated versus Control quarters of two treatment groups at 2 days post dry-off. **(Upper left)** CHDC Treated quarter, Mid region, Day 2; **(Upper right)** Control quarter, Mid region, Day 2; **(Lower left)** Control Treated quarter, Ventral region, Day 2; **(Lower right)** CH Treated quarter; Ventral region, Day 2

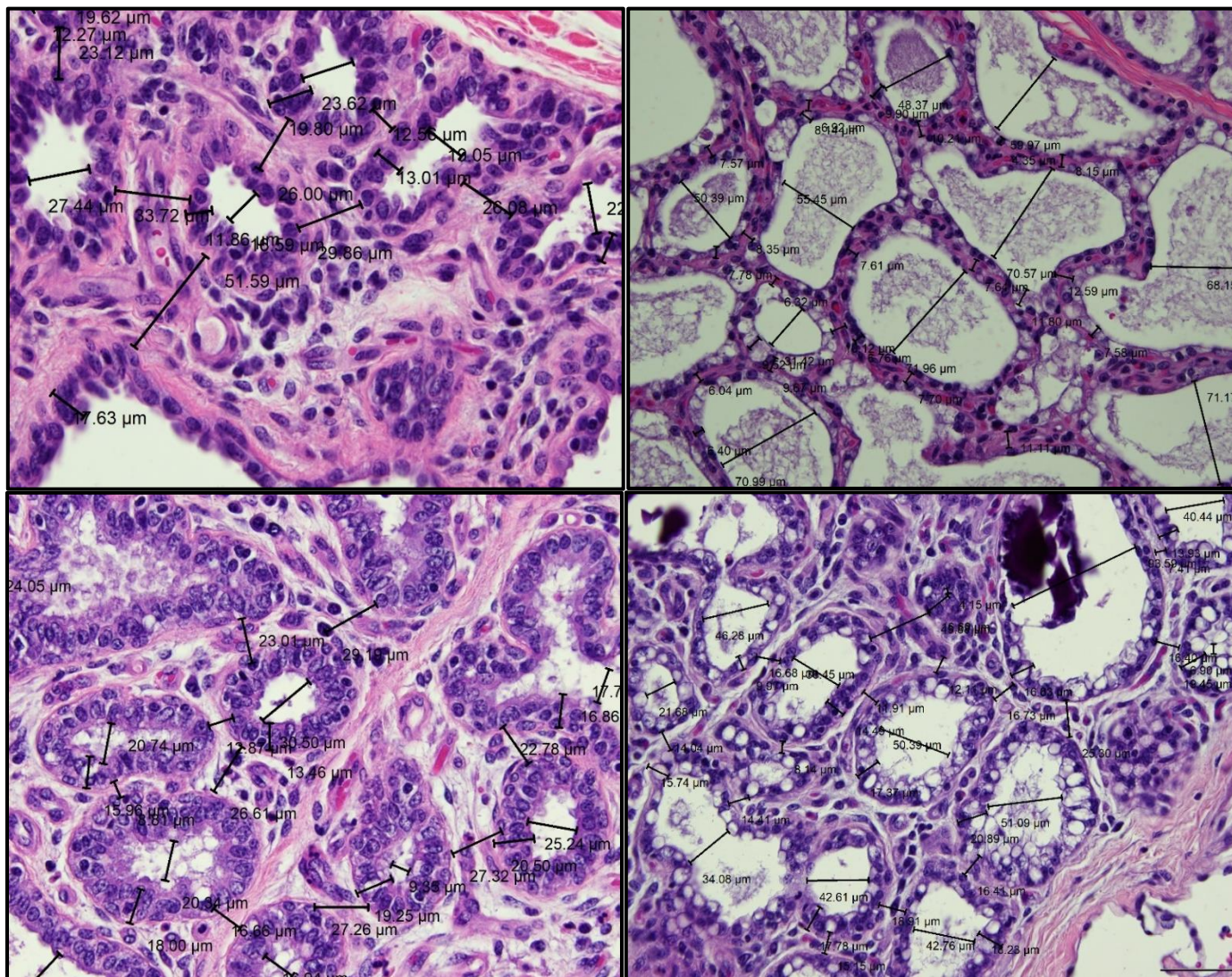


Figure 5. Comparison of treated versus Control quarters of two treatment groups at 7 days post dry-off. **(Upper left)** CHTS Treated quarter, Ventral, Day 7; **(Upper right)** Control quarter, Ventral region, Day 7; **(Lower left)** CHDCTS Treated quarter, Mid region, Day 7; **(Lower right)** Control quarter, Mid region, Day 7

CONCLUSIONS

Mastitis continues to be the most expensive disease that dairy farms encounter and extends to farms across the globe. The United States produces some of the highest quality milk in the world but intramammary infections are still a problem for every dairy herd. Control and prevention of mastitis goes beyond simply achieving good udder health; it is part of the much larger umbrella of food safety and public health. Concern over antibiotic resistance in humans as a possible result of use in livestock raising practices is a growing fear among consumers and health professionals. Additionally, apprehensions regarding the health and welfare of how food production animals are raised has influenced consumer choices and shaped the direction of the modern dairy industry. Looking forward to the future, there is clearly a need for development of new management tools and alternatives to any treatments that may result in chemical or antibiotic residues in food products. This dissertation addresses the problem of cessation of milk production in a single mastitic quarter within a cow for the remainder of lactation without the risk of chemical residues (Chapter 2), reports on the effects of alternative treatments used at the time of dry-off on cows that are followed through calving and the beginning of the subsequent lactation (Chapter 3) and describes the histological changes to three types of mammary tissue following administration of alternative treatments at the time of dry-off (Chapter 4).

In Chapter 2, we focused on a potential solution for dealing with single mammary quarters within one cow with high enough somatic cell counts that they elevated total-cow somatic cell counts. These animals present a risk to the overall quality of the milk shipped from the farm. Many times, these are cows that are already pregnant and

otherwise valuable contributions to the herd, apart from the unhealthy quarter. Ceasing production in problematic quarters mid-lactation, creating a “three-quartered” cow, is not a novel idea within the dairy industry but a safe and universally used method for doing so does not exist. Previously studied methods such as intramammary infusion of iodine or chlorhexidine were found to be efficacious in ceasing milk production but came with the risk of chemical residues ending up in the bulk tank if the quarter were accidentally milked and/or permanently destroying the quarter. Our objective was to compare intramammary infusion of casein hydrolysate to a placebo or abrupt cessation of milking as an inducer of mammary involution in a single quarter without the risk of residues or permanent damage. Results demonstrated that this was an efficacious method for managing mastitic quarters mid-lactation by lowering total-cow somatic cell count, having minimal impact on total-cow production and not causing any visible signs of pain or distress. All treated quarters returned to adequate production (approximately 25% of total-cow milk) in the following lactation and no milk samples tested positive for any antimicrobial residue.

In Chapter 3, the objective was to compare the intramammary use of casein hydrolysate in combination with or without standard dry cow treatment and/or an internal teat sealant as an alternative treatment at the time of dry-off. “Blanket” use of antibiotic therapy administered to all cows at the time of dry-off has been the industry standard for many years and has repeatedly been shown to be efficacious at bacteriologically curing infections already present in the gland at that time. However, the pressure from consumers to decrease antibiotic use in food livestock has created a need for investigation of alternative treatments at the time of dry-off. We tested for differences

between treatment groups and against the control group using five indicators of mammary involution. Milk production of treated and control udder halves was assessed pre- and post-treatment for any potential production loss and bacterial cures and new infection prevalence was calculated. Cows did not exhibit any signs of pain or discomfort with casein hydrolysate treatments and did not experience milk production loss in the following lactation. Casein hydrolysate dry treated cows had higher concentrations of the markers of involution bovine lactoferrin and bovine serum albumin than most other treatment groups or the control treatment of dry cow treatment and internal teat sealant at either 7 days, 10 days or both time intervals post-dryoff. Cows dry treated with casein hydrolysate in combination with dry cow treatment or internal teat sealant were not significantly different in cure rates of mastitis during the dry period than those treated with the control treatment of dry cow treatment and internal teat sealant. All cows tested negative for any antimicrobial residues after calving, which is consistent with findings reported in Chapter 2.

In Chapter 4, treatment groups including casein hydrolysate were the same as reported on in Chapter 3. The primary objective was to compare histological changes indicative of involution in bovine mammary tissue between dry cow treatment groups at 2 days and 7 days after dry-off. Specifically, quantitative morphometric changes to alveolar epithelial cell height, lumen diameter and interstitial stromal thickness in the mammary glands were measured. Differences in involution were observed between treatment groups and some measures of involution were also faster with treatments including casein hydrolysate in comparison to controls, predominantly in epithelial tissues. However, the differences were not consistent for any one treatment for epithelial

cell height, stromal thickness, or luminal diameter or between 2 days after dry-off and 7 days after dry-off.

In all of the studies there were some differences observed between cows and quarters that received intramammary treatment of casein hydrolysate and those that did not. At no time did any animals treated with casein hydrolysate display signs of pain or discomfort. Treated quarters did not decrease in the proportion of total-cow milk that they produced, remaining at approximately 25% of the cows' milk. Administration of casein hydrolysate at dry-off was associated with faster increases in some measures of bovine mammary involution during the early dry period. Cure rates during the dry period of intramammary infections found at the time of dry-off were not significantly different in cows that received casein hydrolysate treatments from those that did not. The overall results of these studies indicated that intramammary infusion of casein hydrolysate was safe for dairy cows, had some efficacy against mastitis, and may be a useful tool for reducing mastitis in lactating and dry cows.

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Career Objective

I have proven that I am committed to promoting and teaching milk quality in the dairy industry to producers, veterinarians, laboratory technicians and fellow milk quality specialists by the work experience to date. As Laboratory Director of Udder Health Systems, I will provide technical consulting services as part of the interface of laboratory results and client support. Additionally, I have a special interest in food safety and public health and hope to grow that part of the business. I absolutely believe in a continuing need to promote education and knowledge of sustainable practices that will deliver the maximum production of excellent quality milk.

Education

Utah State University 2014-2019

- Doctoral candidate-Animal Health and Disease
- Defended December 2018; Anticipated Graduation Date: May 2019
- GPA: 3.85

Boise State University 2008-2010

- Post-baccalaureate coursework
- GPA: 3.6

Washington State University 2000-2004

- Bachelor of Science in Animal Science
- Minor in Spanish
- GPA: 2.9

Presentations/Awards

- 2019 University Doctoral Researcher of the Year nomination, Utah State University (Results pending)
- 2018 College of Agriculture and Applied Sciences Doctoral Researcher of the Year, Utah State University
- 2018 Department of Animal, Dairy and Veterinary Sciences Doctoral Researcher of the Year, Utah State University
- 2018 American Association of Extension Veterinarians Annual Public Health Symposium, Invited speaker
- 2018 National Mastitis Council Annual Meeting Technology Transfer session presenter
- 2018 USU Animal, Dairy and Veterinary Sciences departmental research symposium, 2nd Place Oral/Poster Presenter
- 2017 American Association of Extension Veterinarians Annual Public Health Symposium, Invited speaker

- 2016 American Association of Extension Veterinarians Annual Public Health Symposium, Invited Speaker
- 2016 USU Animal, Dairy and Veterinary Sciences department research symposium, 2nd Place Oral Presenter
- 2015 USU Animal, Dairy and Veterinary Sciences department research symposium, Best Oral Presenter
- 2015 American Association of Extension Veterinarians Annual Public Health Symposium, Invited speaker
- 2015 Presidential Service Award, Bronze level
- 2015 Utah State University Ignite Speaker
- USAID volunteer with Winrock International to Nigeria; agricultural curriculum assessment and development
- National Mastitis Council Regional Meeting 2013 Short Course Instructor
- Certificate of Achievement Dairy Science and Sanitation Course; Cornell University Dairy Foods Extension and Oregon State Dairy Processing Program
- National Mastitis Council Annual Meeting 2009— “The Detection of *Acholeplasma sp.* in Individual Cow Milk Samples as ‘False Positive’ Growth on Mycoplasma Culture”
- National Mastitis Council Annual Meeting 2008— “Noncompliance of the Pasteurized Milk Ordinance Due to Blood in Milk”
- Presenter at National Dairy Herd Information Association, Western Idaho region meeting on Milk Quality—2008

Refereed Publications

- Britten, J., Rood, K. A., Wilson, D. J. (2018) Intramammary Infusion of Casein Hydrolysate for Involution of Single Mastitic Mammary Quarters Elevating Cow-Level Somatic Cell Count. J Adv Dairy Res, 6(3). DOI: 10.4172/2329-888X.1000211

Teaching

- 2017 National Mastitis Council Regional Meeting Short Course Instructor
- Epidemiology guest lecturer, Utah State School of Veterinary Medicine (USU-SVM), 2016-2018
- Udder Health Systems Mastitis Microbiology School Instructor— November 2011, July and October 2012, January, May, September 2013, January 2014

Funding

- 2017 Utah State University Extension Grant

- 2017 Joseph C. Memorial Scholarship
- 2017 William Claypool Scholarship
- 2015 John Seymour Memorial Scholarship
- 2015 School of Research and Graduate Studies Project Grant
- 2015 Utah State University Extension Grant

Professional Memberships

- National Mastitis Council Board of Directors
- American Dairy Science Association

Non-academic Experience

- Udder Health Systems, Laboratory Director, Boise Idaho (January 2019-Present)
- Udder Health Systems, Customer Relations Manager, Boise Idaho (May 2011-2014)
- Therapeutic Associates, Physical Therapy Aide, Boise Idaho (June 2010-April)
- MWI Veterinary Supply, Regional Dairy Specialist, Meridian Idaho (May-October 2009)
- Udder Health Systems Idaho, Field Operations Manager, Boise Idaho (July 2004-April 2009)