ABSTRACT

Title of Dissertation:	ANTIBIOTIC ALTERNATIVES FOR TREATMENT OF MASTITIS IN DAIRY CATTLE
	Cynthia Madeleine Scholte, Doctor of Philosophy, 2019
Dissertation directed by:	Assistant Professor Kasey M. Moyes, Department of Animal and Avian Sciences

Mastitis is one of the most costly diseases to the dairy cow industry. Implementation of management protocols have decreased the incidence rate of contagious mastitis pathogens, while the incidence of cases caused by environmental pathogens, such as *Escherichia coli* (*E. coli*) and *Streptococcus uberis* (*Strep. uberis*), remains unchanged. Currently, antibiotics are the primary therapy utilized to control mastitis. However, growing concern for antibiotic overuse, improper use and bacterial resistance have led to the examination of alternative strategies. Two promising alternative mitigation strategies were explored in the research leading to this dissertation. The first is a potent endolysin, PlyC, which has demonstrated bactericidal activity against several streptococcal species in a variety of applications. However, it is unknown how PlyC interacts with the bovine immune system. Varying doses of PlyC were non-toxic and did not alter the oxidative burst response of bovine neutrophils, the first immune cells recruited to the intramammary infection. The

second alternative to an antimicrobial drug explored was citrus oil dissolved in ethanol. This was tested against *E. coli* strain P4. Prior antimicrobial investigations with citrus oil utilized dimethylsulfoxide as a carrier solvent, which is currently prohibited in the dairy industry. Citrus oil and its primary components were tested to determine minimum inhibitory, bactericidal and biofilm eradication concentrations. Citrus oil components, citral and linalool, demonstrated better antimicrobial activity than the native oil *in vitro.* In addition, citral and linalool were minimally toxic to bovine neutrophils and did not hinder their oxidative burst response *in vitro*. Because citral demonstrated greater antimicrobial activity than linalool, it was the logical candidate to test for the efficacy as an intramammary therapy against experimentally-induced *E. coli* mastitis. Unfortunately, citral treatment did not differ from a common intramammary antibiotic therapy or sterile buffered solution. In conclusion, the use of PlyC as an alternative therapy for *Strep. uberis* mastitis is promising as PlyC may not interfere with immune response during mastitis. The antimicrobial effects of citral may better serve as a disinfectant than a therapy for *E. coli* mastitis.

ANTIBIOTIC ALTERNATIVES FOR TREATMENT OF MASTITIS IN DAIRY CATTLE

by

Cynthia Madeleine Scholte

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Advisory Committee:
Professor Kasey M. Moyes, Chair
Dr. Theodore H. Elsasser
Professor Richard A. Erdman
Professor Daniel Nelson
Professor Robert R. Peters

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Dedication

for Patricia, Wilhelmina and Ronald

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List of Abbreviations

AB	antibiotic treatment, ceftiofur hydrochloride
ATP	adenosine triphosphate
BMBC	biofilm minimum bactericidal concentration
BMIC	biofilm minimum inhibitory concentration
CFU	colony forming unit
CMT	California Mastitis Test
CON	control treatment, sterile PBS
CV	coefficient of variance
d	day
DIM	days in milk
DMSO	dimethylsulfoxide
FDA	Food and Drug Administration
h	hour
LPS	lipopolysacharide
MBC	minimum bactericidal concentration
MBEC	minimum biofilm eradication concentration
MIC	minimum inhibitory concentration
NADPH	nicotinamide adenine dinucleotide phosphate
OO	orange oil treatment, 1.0% vol/vol (8,930 ug) citral
PBS	phosphate buffer solution
PlyCA	PlyC subunit A
PlyCB	PlyC subunit B

PMA	phorbol 12-myristate-13-acetate
PMN	polymorphonuclear leukocyte
ROS	reactive oxygen species
SCC	somatic cell count
SCS	somatic cell score
TSB	tryptic soy broth

Bacteria

E. coli	*Escherichia coli*
Staph. aureus	*Staphylococcus aureus*
Strep. agalactiae	*Streptococcus agalactiae*
Strep. dysgalactiae	*Streptococcus dysgalactiae*
Strep. equi	*Streptococcus equi*
Strep. pyogenes	*Streptococcus pyogenes*
Strep. uberis	*Streptococcus uberis*

Chapter 1: Introduction and Literature Review

Introduction

Bovine mastitis, an inflammation of the mammary gland, is the most costly disease impacting the dairy industry. Numerous pathogens are responsible for mastitis with the majority of infections caused by staphylococci, streptococci and coliforms. These pathogens are typically grouped by their transmission method into two groups: contagious and environmental. Current management practices have successfully decreased the prevalence of cases caused by contagious bacteria, but incidence rates caused by environmental bacteria remain steady. Due to the predominance of infectious mastitis and the importance of the use of antimicrobial agents in the treatment and control of it, this microbial infection continues to be a subject of investigation by several groups. However, there is a significant information gap concerning novel research into controlling environmental mastitis through nondrug strategies (i.e., alternatives to antimicrobial drugs).

Currently, antibiotic therapy is the main strategy for mastitis treatment and control. Detection of clinical mastitis is typically based on the observation of manifestations of inflammation. The majority of producers treat mastitis on these symptoms and not etiology. Antibiotic use has the potential to create drug residues in milk and meat for human consumption and presents a concern for consumers. Cost of use, emergence of antibiotic resistance, and governmental regulatory legislative changes have pressured the industry to reduce overall use of antibiotics. This has

necessitated and created opportunities for the search for, development and implementation of alternative strategies and therapies to manage mastitis and minimize antibiotic use in food-producing animals without the use of antibiotic drugs.

Several antimicrobial alternatives have recently been explored and two promising ones were investigated in this work. Recombinantly-derived endolysin PlyC is one of the most potent lysins to date as nanograms can eliminate billions of species-specific bacteria in seconds. In contrast, citrus oil and specifically its active components, have demonstrated many uses in the food industry as flavor compounds, anti-oxidants and preservatives. In human and animal health, the essential oils have useful antimicrobial activities, including anti-biofilm activity, inhibition of growth and the eradication of many bacterial and fungal species, and anti-inflammatory effects. Vastly different in origin and activity, both PlyC and citrus oil may be useful therapies and alternative to antibiotic treatment for bovine mastitis.

The chapters in this dissertation explore the possible use of PlyC and citrus oil as antimicrobial therapies for bovine mastitis.

Mastitis

Commonly defined as inflammation of the mammary gland, mastitis is the most prevalent and economically significant disease in the bovine dairy industry (US Department of Agriculture, 2009). The estimated losses to producers in the United States exceed $2 billion per year with each clinical case costing approximately $179 (Cha et al., 2011), and the majority of financial losses can be attributed to reduced milk production caused by asymptomatic, subclinical mastitis (Gill et al., 1990).

Additional costs are attributed to reduced milk quality, loss of milk quality premiums, treatment costs, veterinary care, increased labor costs, increased risk of removal from the herd, and discarded milk from antibiotic-treated cows during the Food and Drug Administration (FDA) mandated withdrawal period following antibiotic cessation. Since mastitis reduces the earning potential of affected cows and profitability of dairy operations, farmers seek to prevent, manage, diagnose and treat mastitis in their herd as effectively as possible.

Mastitis cases are characterized by the severity of the inflammatory response of the animal. Clinical cases are observable, recognized and easily defined through the appearance of symptoms. The clinical syndrome can present as mild, moderate or severe based on the severity of these symptoms. In some instances, the case may become chronic and persist in a subclinical form with occasional clinical appearances. Although clinical cases are easily defined and treated in the dairy industry, it is the subclinical cases that are of greatest concern to producers as subclinical case prevalence can range from 5-75% (Erskine, 2016). As subclinical cases are not observable or easily defined, they require further testing to detect their presence. Typically, subclinical mastitis cases are diagnosed through decreased milk yield, altered milk composition, and increased milk somatic cell count (SCC) and bacterial count (Blowey and Edmondson, 2010). Because the cow appears healthy and her milk normal, managers often overlook the diagnosis and treatment of these cases.

Regardless of presentation, diagnosis of mastitis is typically determined through observation of clinical symptoms and further testing of milk for composition changes, the number of somatic cells in the milk, and bacterial count and type. Initial

detection is based upon visualization and palpation of the mammary glands to detect the presence of an inflammatory response presumably to the infection. In clinical mastitis, the udder may turn red in color, and feel hard and hot to the touch as vascularity and blood flow change in the inflamed gland (Erskine, 2016). In some cases, palpation of the udder may even be painful to the cow. Additional visual symptoms will include abnormal milk from the infected quarters of the mammary gland at time of milking. Milk may appear serous, abnormal in color, or contain flakes or clots. Most milk abnormalities are observed when a milking technician strips milk from each quarter at the beginning of the milking protocol. Following milking, technicians may also note that mastitic cows have decreased total milk yield (Gill et al., 1990). If clinical symptoms are not present, further testing of milk for mastitis diagnosis is typically completed off-farm. Milk samples from the bulk tank or suspected infected animals can be sent to state or privately-owned laboratories to determine if milk contains pathogens, high SCC concentrations or abnormal composition. Elevated SCC (i.e., greater than 200,000 cells/mL for composite foremilk samples; 100,000 cells/mL for quarter foremilk samples) or somatic cell score (SCS; i.e., greater than 4) from either a composite or an individual quarter samples is one of the most common factors used to confirm the presence of mastitis (Pighetti et al., 2007).

Several factors contribute the nature of how mastitis is classified and therefore managed and treated. A wide variety of pathogens cause bovine mastitis including bacteria, yeast, viruses and algae. These pathogens may cause clinical or subclinical presentations of mastitis in the animal based on the species of pathogen, severity of

the infection and overall immune response from the animal (Wagner and Erskine, 2009). Mastitis pathogens are generally categorized into either environmental or contagious categories, based upon the organism's epidemiology. Historically, the most common contagious pathogens have been *Staphylococcus aureus* (*Staph. aureus*) and *Streptococcus agalactiae* (*Strep. agalactiae*); whereas, common environmental organisms include coagulase-negative *Streptococcus* species, *Streptococcus uberis* (*Strep. uberis*), *Streptococcus dysgalactiae* (*Strep. dysgalactiae*), *Klebsiella* species, and *Escherichia coli* (*E. coli*; Oliveira et al., 2013; Ruegg, 2015; National Mastitis Council, 2017). The adoption of modern milking practices, such as post-milking teat disinfection, dry cow therapy, milking and housing hygiene and culling chronic mastitic cows, have led to a notable decline in the prevalence of clinical mastitis caused by contagious pathogens (Blowey and Edmondson, 2010). However, the number of clinical cases caused by environmental pathogens remains relatively unchanged even with the addition of improved environmental hygiene, pre-dipping and use of teat sealants during the dry period (Bradley et al., 2007).

Environmental Mastitis

Environmental bacteria are an important cause of clinical mastitis on well-managed dairies and are most often isolated from acute clinical cases of mastitis (Barkema et al., 1998; Olde Riekerink et al., 2008). Gram-negative bacteria are typically considered environmental mastitis pathogens (National Mastitis Council, 2004). The term coliform mastitis is often incorrectly associated with Gram-negative

bacteria. *E. coli, Klebsiella* and *Enterobacter* are coliforms; however, other Gram-negative bacteria are also frequently isolated from intramammary infections (*Serratia, Pseudomonas,* and *Proteus*). Gram-negative bacteria occupy many habitats in the cow's environment and may be isolated from virtually any surface are of the cow or her surroundings. Entry of Gram-negative bacteria into the mammary gland is through the teat canal, and rapidly adjust and multiply in the lactose-rich and anaerobic conditions. Populations of coliforms can reach peak concentrations exceeding 10^8 colony forming units (CFU) per mL of milk in clinical cases in hours (Hogan et al., 1992). In subclinical cases and infections in later phases, concentrations are typically 10^2 CFU/mL milk. Low shedding rates and environmental pathogens contributing to the majority of sample contamination can make it difficult to diagnose intramammary infections caused by Gram-negative, environmental bacteria (Hogan and Smith, 2003).

Streptococcal (Gram-positive) species also originate in the environment and are responsible for high incidences of environmental mastitis. The majority of streptococcal infections originate during the dry and transition periods with *Strep. uberis* as the primary pathogen (Jayarao et al., 1999). *Strep. uberis* is one of the most common causes of clinical and subclinical mastitis in the United Kingdom and New Zealand (Zadoks, 2007; Petrovski et al., 2011). A ubiquitous microorganism, *Strep. uberis* has been localized on the cow and inside the cow, as well as the environment (Zadoks, 2007). Straw and other organic bedding material enhance the growth of *Strep. uberis* and increase the likelihood of intramammary infections (Blowey and Edmondson, 2010). Entry of Gram-positive bacteria into the mammary gland is

through the teat canal, however species like *Strep. uberis* can take longer to adjust and establish infections as compared to Gram-negative bacteria. *Strep. uberis* infections initiate the immune response within a d of post infection and peak CFU concentrations in milk 10^4 CFU/mL occur 5-6 d post infection (Pedersen et al., 2003; Rambeaud et al., 2003).

Clinical mastitis in most dairy herds is caused by environmental pathogens (Gruet et al., 2001). These cases, especially initial episodes, are typically transient; therefore, the epidemiology of clinical mastitis is typically assessed on incidence rate (the number of new cases per population at risk in a given time period) and not prevalence (the proportion of a population affected; Rothman et al., 2008). The methods for monitoring and diagnosing mastitis in individual cows are poor predictors of herd clinical mastitis episodes. Cows with chronic infections and increased SCC may occasionally display clinical mastitis. Cows with low SCC are also prone to developing clinical mastitis and may have a higher incidence rate of clinical cases caused by environmental organisms (30-50 cases/100 cows/year; Paape et al., 2002) than herds of cows with higher SCC. An inverse relationship exists between incidence and severity of clinical mastitis caused by Gram-negative bacteria, and milk SCC (Barkema et al., 1998). Incidence rate and probable risk factors (e.g., parity, milk production, pathogen, and bacteriological cure) should be recorded on well managed farms and can be utilized for predicting herd clinical mastitis episodes (Jamali et al., 2018). Ideally, well-managed herds should have an incidence rate of clinical mastitis of 1-2 cases/100/cows/month (Erskine, 2016).

Some scientists hypothesize that the persistence of some environmental intramammary infections may be caused by the formation of biofilms and not re-infection (Melchior, 2011). Of all the mastitis pathogens capable of producing biofilms in intramammary infections, contagious *Staph. aureus* is the best studied. However, more information regarding biofilms from environmental mastitis pathogens *E. coli* and *Strep. uberis* has become available. Costa et al. (2014) demonstrated that 27 strains of *E. coli* isolated from cases of bovine mastitis were capable of biofilm formation under conditions mimicking a mastitis-infected mammary gland. Four other bovine mastitis *E. coli* strains were able to form biofilms on mammary alveolar cells *in vitro* (Source Silva, 2014). *Strep. uberis* biofilms have formed under similar conditions of *Staph. aureus in vitro* and results suggest that they are composed of similar components and structure (Almeida et al., 2006; Varhimo et al., 2010). Although no histological samples have yet to be found in bovine udders, the ability of *E. coli* and *Strep. uberis* to form biofilms under similar environmental conditions may suggest that the physiological barrier of a biofilm helps contribute to the chronic infection.

Innate Immune Response to Mastitis

One third of all clinical cases of bovine mastitis are caused by Gram-negative bacteria and nearly 25% of these cases result in culling or death of the animal. These incidence rates are only expected to rise over the future as producers strive for lower herd SCC. Promptness and magnitude of recruitment of innate immune cells to the mammary gland has profound influence on the severity and outcome of mastitis (De

Cueninck, 1979; Hill, 1981). Neutrophils are the primary innate cell recruited (~90%) and have a prominent role in the defense of the mammary gland against bacterial infections (Paape et al., 2002). Cytokines, such as tumor necrosis factor-alpha, granulocyte-macrophage colony-stimulating factor and interleukins 1-beta, 6 and 8, from mammary epithelial cells and macrophages attract neutrophils in circulation (Jung et al., 1995; Boudjellab et al., 1998). Following extravasion into the lumen of the mammary gland, neutrophils utilize phagocytosis, reactive oxygen species (ROS) production, and extracellular traps to eradicate the pathogens. The bactericidal activity of neutrophils is inefficient in milk as compared to their activity in blood (Russell and Reiter, 1975; Paape et al., 2003). Milk lacks some compounds that favor neutrophil function, such as glucose for energy and oxygen for ROS production (Newbould, 1970; Goldberg et al., 1995; Mehrzad et al., 2001). In addition, the fat globules and casein present in milk are shown to interfere with neutrophil efficiency *in vitro* by reducing phagocytosis and oxidative burst activity (Russell and Reiter, 1975; Paape and Guidry, 1977; Paape et al., 2003).

Although these factors directly affect the main functions of neutrophils, bacteria can alter other factors of the neutrophil's ability to combat infection. The optimal concentration of neutrophils required to exert effective bactericidal activity is approximately 10^6 cell/mL milk (Paape et al., 1963; Herbelin et al., 1997). Increased concentrations beyond this assessment do not result in improved bactericidal activity and are linked to detrimental side effects caused by excessive pro-inflammatory and enzymatic activity of the neutrophils (Leijh et al., 1979; Paape et al., 2003). Nevertheless, the efficiency of neutrophils is largely dependent on the infecting

bacteria species and stage of infection. Neutrophils mobilized against *E. coli, Staph. aureus* and *Strep. agalactiae*-caused mastitis are fairly efficient even several hours post infection. However, neutrophils are less efficient toward mastitis caused by *Strep. uberis* and *Mycoplasma* species (Brownlie et al., 1979; Leigh and Field, 1994). Severity of *E. coli* mastitis is highly dependent upon the speed at which neutrophils are mobilized. A delayed appearance following infection correlates with high bacterial concentrations in the milk and severe mastitis (Vandeputte-van Messom et al., 1993). In comparison¸ *Strep. uberis* induces a delayed response for neutrophil recruitment via interleukin-8 from mammary epithelial cells *in vitro* (Wellnitz et al., 2006). Following the neutrophils' primary defense of the mammary gland, macrophages that are normally present in the healthy mammary gland assist with the bactericidal activity through phagocytosis of the pathogen and processing of the antigen for presentation to lymphocytes to begin the adaptive portion of the immune response (Politis et al., 1991; Fitzpatrick et al., 1992).

Mastitis Treatment with Antibiotics

Following the emergence of mastitis symptoms or lactation cessation, antibiotics serve as the conventional mastitis treatment (Oliver et al., 2011). Currently, there are only two classes of antibiotics approved by the FDA for the treatment of bovine mastitis: 1) new generation β-lactams (cell wall disruptors based on penicillin as the parent drug) such as cephalosporin, ceftiofur and cephapirin, and 2) licosamides (transpeptidation protein synthesis inhibitors based on clindamysin) such as pirlimycin. Depending on the type of mastitis and FDA labeling (where

information on the package label authorizes the approved usage), the drug may either be delivered via intramammary or systemic routes. Intramammary delivery is the most common method and accounts for the greatest use of antibiotics on dairy operations (US Department of Agriculture, 2007) as most farmers treat clinical mastitis cases based on symptoms (Hoe and Ruegg, 2006). However, across the stated spectrum of organisms that cause mastitis, bacterial pathogens are only isolated from ~50% of clinical mastitis cases (Olde Riekerink et al., 2008; Oliveira and Ruegg, 2014), resulting in the administration of antibiotics to cases that may not be of husbandry or clinical benefit, and therefore contributing to the problem of inappropriate antibiotic use and possible development of emerging drug resistant bacterial strains.

Growing concerns regarding antibiotic use and the potential for antimicrobial resistance from their use have led to the need to develop alternative strategies to antibiotics for controlling and perhaps even preventing mastitis, a measure which should reduce the use of antibiotics on-farm (Bannantine et al., 2013). Although no compelling evidence exists that the use of antibiotics to treat mastitis has resulted in increased prevalence of resistant pathogens on dairy farms in the US (Erskine et al., 2002; Rajala-Schultz et al., 2004; Pol and Ruegg, 2007), the White House released the *National Action Plan for Combating Antibiotic Resistant Bacteria* (2015) which calls for improved antibiotic stewardship, increased resistance monitoring, and accelerated antimicrobial therapy research and development in human and animal healthcare. Appropriate use of antibiotics is a public health priority and opportunity

exists for improvements and development in the use of new antimicrobials for treatment of bovine mastitis.

Alternative Antimicrobials

Many old and novel anti-mastitis strategies have been explored recently to help treat and control mastitis. Although many therapies were explored decades ago, the call for reduced antibiotic use in veterinary applications has renewed exploration. Some success has been found in vaccinations for bovine mastitis. Although the "miraculous" vaccine to prevent all mastitis remains elusive, the *E. coli* J5 vaccine has been successful at reducing the incidence rate and severity of coliform mastitis by 70-80% (Hogan et al., 1992). Some vaccines for *S. aureus* have also shown promise, but their effectiveness is dependent upon several factors, such as age of cow and environmental conditions (Hoedemaker et al., 2001; Kazemi et al., 2014). Nanoparticles have demonstrated to be useful in biomedical applications as delivery vehicles for antimicrobial agents, such as silver and nitric oxide nanoparticles to inhibit *S. aureus* and *E. coli* mastitis pathogens (Dehkordi et al., 2011; Cardozo et al., 2014). Photodynamic therapies also utilize nanoparticles to cause pathogen death. Administered light activates photosensitive compounds in the nanoparticles that convert oxygen to ROS, such as oxygen and hydroxide radicals. Both of these ROS are capable of killing a broad spectrum of pathogens and have shown to effectively inhibit growth of coagulase negative *Staphylococcus* and *Strep. dysgalactiae* (Hamblin, 2016; Parra Sellera et al., 2016; Moreira et al., 2018). Of the many potential alternative antimicrobials, the potential of lysin proteins from

bacteriophages and natural compound citrus oil appear very promising as a potential new therapy for bovine mastitis.

Lysins

An antimicrobial alternative currently being developed is the bacteriolytic cell wall hydrolases known as lysins (or endolysins). Bacteriophages evolved a system to allow progeny phages to exit the bacterium by utilizing proteins that weaken the bacterial cell wall and results in bacteria lysis. These enzymes, lysins, target the integrity of the cell wall by attacking one of the 4 major peptidoglycan bonds. Most lysins lack signal sequences and require additional help to pass the cytoplasmic membrane and access the cell wall. Secondary molecules, called holins, are inserted into the cytoplasmic membrane and form holes that lead to general membrane disruption (Wang et al., 2003). This allows the lysin enzymes in the cytoplasm to access the peptidoglycan and cause cell lysis.

For nearly a century, scientists have been aware of the lytic activity of phages. Recently, whole phages have been used to control staphylococcal strains known to cause bovine mastitis (Kwiatek et al., 2012). Although the phage therapy does not irritate the cow nor cause an increase is milk SCC (O'Flaherty et al., 2005), phage therapy was unsuccessful at curing experimentally-induced *Staph. aureus* mastitis (Gill et al., 2006), as preformed antiphage immunoglobulins likely inactivated the phage. Lysin enzymes can be inactivated by antibodies similarly to whole phage; however, repeated exposure to lysin enzymes in hyperimmune rabbit serum raised against the specific enzyme did not affect the lytic activity *in vitro* (Loeffler et al.,

2003; Rashel et al., 2007; Fischetti, 2010). Repeated dosing of lysin enzymes in mice raised their IgG antibody titer against the enzyme to low, but measurable levels; however, enzyme lysis activity against the target pathogen remained constant (Loeffler et al., 2003; Rashel et al., 2007). Given the high binding affinity of the enzyme for its substrate in the bacterial cell wall (Loessner et al., 2002), it is likely that this affinity is higher than the antibody's affinity for the enzyme. It may be possible for lysin enzymes to be used repeatedly to control bacterial infections.

In many instances, lysins only target one species of bacteria. Most lysins are active against Gram-positive bacteria as exogenous application of the lysin must be able to make direct contact with the cell wall carbohydrates and peptidoglycan. The outer membrane of the Gram-negative bacteria prevents this interaction. One of the most potent endolysins to date is the streptococcal-specific lysin, PlyC, which was characterized and purified from the virulent C_1 bacteriophage. Many bacteriophage-derived enzymes require microgram or milligram quantities to cause a multiple-log reduction of target bacteria over several minutes; whereas, PlyC requires only nanogram quantities and seconds to neutralize 10^7 CFU (Nelson et al., 2001).

Previous research spanning over 50 years has shown that PlyC can rapidly lyse streptococci groups A, C, and E, in addition to *Strep. uberis* and *Streptococcus equi* (*Strep. equi;* Krause, 1957; Nelson et al., 2001). Based on biochemical, biophysical and genetic data, PlyC is a multimeric holoenzyme composed of two subunits, PlyC subunit A (PlyCA) and PlyC subunit B (PlyCB). PlyCB is an octamer that is responsible for binding to the bacterial cell wall. PlyCA is responsible for the lysis activity by cleaving two bonds in the streptococcal peptidoglycan: 1) cysteine,

histidine-dependent aminohydrolases/peptidases catalytic domain that cleaves between *N*-acetyl muramic acid and L-alanine (Nelson et al., 2006), and 2) glycoside hydrolase catalytic domain that cleaves between N-acetyl glucosamine and N-acetyl muramic acid (McGowan et al., 2012). The unique structure of PlyC may be linked to its enhanced activity as compared to other lytic enzymes.

Over the past 20 years, PlyC has been utilized in numerous applications with no reported adverse effects. In mice, PlyC was administered orally as a prophylaxis to prevent colonization of *Streptococcus pyogenes* (*Strep. pyogenes*) and as a treatment for the elimination of a *Strep. pyogenes* infection in heavily colonized mice (Nelson et al., 2001). PlyC has demonstrated further bactericidal activity against *Strep. pyogenes* by eradicating biofilms (Shen et al., 2013), and targeting and controlling intracellular infections in human epithelial cells *in vitro* (Shen et al., 2016). As a germicidal spray disinfectant, PlyC was able to significantly reduce or eradicate *Strep. equi,* the causative agent of equine strangles, from a variety of materials and under conditions that mimic a horse stable (Hoopes et al., 2009). The variety of applications and the retention of lysin activity suggests that PlyC may be useful for treatment of bovine mastitis caused by streptococcal species.

Citrus Oil

Citrus oil is one of many well-known essential oils in the world with noted antimicrobial properties. The oil is produced by cells within the rind of lemon and orange fruit and is typically extracted as a by-product of juice production. Centrifugation produces a cold-pressed oil that is primarily terpenes with a varying

composition that occurs as a result of regional and seasonal changes (Shaw and Coleman, 1974). Regardless of variation, limonene accounts for ~90% of citrus oil's composition with various alcohols, esters, aldehydes, hydrocarbons, ketones and oxides accounting for the remaining portion (Bauer et al., 2001; Moufida and Marzouk, 2003). Limonene has demonstrated antimicrobial activity against food-borne pathogens; however, citral and linalool have been identified as the active compounds contributing to the bactericidal activity of citrus oil (Fisher and Phillips, 2006).

A potential alternative to traditional antimicrobial pharmaceutical drugs may be found in orange/citrus oil obtainable from by-products that otherwise might be discarded. Citrus oil has been used in the food industry for decades to create citrus flavor. In the past decade, citrus oil use has expanded to meat preservations as the compounds in the oils act as natural antioxidants and deter the growth of bacteria and other pathogens that cause spoilage and disease (Mahato et al., 2017). When applied directly to meat, citrus oil and it components have displayed bacteriostatic and bactericidal characteristics towards *E. coli, S. aureus*, and *Mycobacterium bovis* (Fisher and Phillips, 2006; Crandall et al., 2012; Muthaiyan et al., 2012; Federman et al., 2016). The antimicrobial properties of citrus oil are not limited to surface applications of meat but are active within milk *in vitro*, as well. A relatively low concentration (0.1% vol/vol) inhibited and reduced bacterial growth of *E. coli* and *S. aureus* in pasteurized milk (Dabbah et al., 1970). As many of the pathogens that cause food spoilage are also pathogenic species for bovine mastitis, these

characteristics of citrus oil may also prove useful for deterring and eliminating these species from the mammary gland.

Citrus oil components have shown some success as an alternative antimicrobial treatment for bovine mastitis. Relatively low concentrations of citrus oil components dissolved in dimethylsulfoxide (DMSO) have successfully inhibited and eradicated *S. aureus* planktonic and biofilm bacterial growth, and did not demonstrate long term cytotoxic effects on cultured bovine mammary cells (Aiemsaard et al., 2011; Garcia et al., 2015; Federman et al., 2016). However, the use of DMSO in dairy cattle is prohibited by the FDA (US Department of Health and Human Service and Public Health Service, 2015). A suitable, alternative solvent for citrus oil components is ethanol as it can maintain oil solubility at low concentrations and its use is not prohibited.

One of the active components contributing to citrus oil antimicrobial activity is citral, an ethanol-soluble chemical known as 3,7-dimethyl-2,6-octadienal. As a monoterpenoid aldehyde, citral has two isomers, geranial and neral, that are also commonly identified in the leaves and fruits of several plant species, including basil, lemon, lime, lemongrass, orange and bergamot (Hyldgaard et al., 2012). The strong, lemon odor of geranial and sweeter, more subtle lemon aroma of neral make citral a valuable compound that is widely used as a flavoring agent in food and drink and scent in perfumery (Nannapaneni et al., 2009; Choi et al., 2010). Beyond a scent and flavor, citral has favorable anti-inflammatory (Ortiz et al., 2010) and anti-corrosive effects (Korenblum et al., 2013), in addition to the increasing evidence to its

antibacterial (Onawunmi, 1989; Lu et al., 2016; Shi et al., 2016) and antifungal (De Bona Da Silva et al., 2008) properties.

Another active component of citrus oil is linalool, 3,7-dimethl-1,6-dien-3-ol. Also ethanol-soluble and a monoterpenoid, it has been isolated from over 200 different species of fruits, flowers and spice plants for many commercial applications, mainly for its pleasant scent (floral, with a touch of spiciness; Furia, 1980). Linalool has anti-oxidant (Mitic-Culafic et al., 2009; Mehri et al., 2015) and analgesic properties (Peana et al., 2004; Linck et al., 2009), in addition to low cytotoxic effects on human endothelial cells and fibroblasts (Prashar et al., 2004). Although linalool has not been directly tested on immune cells, restrained rats exposed to aerosolized linalool had decreased neutrophils and lymphocytes in their blood, suggesting possible stress reduction and anti-inflammatory effects (Nakamura et al., 2009).

Antimicrobial Assessment of Citrus Oil

Assessment of new antimicrobials, such as citrus oil, against pathogenic species typically begins with *in vitro* analyses to determine minimum concentrations to inhibit and reduce growth. Minimum inhibitory concentration (MIC) is defined as the lowest concentration of antimicrobial that prevents a clear suspension of bacteria (10^5 CFU/mL) from becoming turbid after overnight incubation (Levison and Levison, 2009). Minimum bactericidal concentration (MBC) of an antimicrobial product is the lowest concentration that reduces bacterial density by 99.9% (3-$\log_{10}$). For bacteriostatic antimicrobials, the MIC and MBC will be the same; whereas, the MBC of bacteriolytic drugs are many time greater than their MIC. Although both are

subjective measures, they illustrate the effective concentrations of the antimicrobial that deter and stop exponential bacterial growth.

MIC and MBC are useful tools for assessing new antimicrobial pharmacokinetics; however, they only assess antimicrobial susceptibility for planktonic and rapid bacterial growth. The most common known mastitis-causing bacterial pathogens (*Staph. aureus*, *Strep. uberis*, *E. coli* and *Strep. agalactiae*) can also create biofilms, structured bacterial communities that adhere to an inert or living surface (Melchior et al., 2006b). Biofilms can constitute a protected mode of growth for bacteria in hostile environments as a self-produced extracellular polysaccharide matrix allows bacterial cells to adhere to one another and create a multi-layer community. Bacterial cells within the biofilm structure are protected from antibiotics and the host-immune system, therefore promoting survival of the bacteria, recurrence of the infection and contributing to antibiotic resistance (Olson et al., 2002). Similar to planktonic bacterial growth, biofilm susceptibility to antimicrobials is measured through biofilm minimum inhibitory concentration (BMIC) and bactericidal concentration (BMBC), as well as minimum biofilm eradication concentration (MBEC). Susceptibility of the planktonic bacteria released from the biofilm during the overnight incubation is used to determine the BMIC and BMBC; whereas, the susceptibility of the biofilm bacteria after dislodging them from the biofilm through sonication and recovering them in media is used to determine the MBEC (Melchior et al., 2006a).

The objectives of this dissertation are: 1) determine the effects of PlyC on bovine blood neutrophils, 2) determine the MIC, MBC, MBEC against isolated mastitis pathogen *E. coli* strain P4, and effects on bovine blood neutrophils for citrus oil and its primary components, and 3) determine the effects of primary citrus oil, citral, as an intramammary therapy for experimentally-induced *E. coli* strain P4 mastitis.

Chapter 2: Short communication: Recombinant bacteriophage endolysin PlyC is nontoxic and does not alter blood neutrophil oxidative response in lactating dairy cows.

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Abstract

Mastitis is the leading cause of antimicrobial use on dairy farms. The potential for antimicrobial resistance has led to the examination of alternative strategies for controlling mastitis. One such alternative is PlyC, a potent peptidoglycan hydrolase derived from the streptococcal C1 bacteriophage that causes targeted lysis of the cell wall of *Streptococcus uberis*. At a concentration of 1.0 µg/mL, recombinant PlyC can induce lytic activity, suggesting that a low dose may successfully eliminate infection. We evaluated the dose effect of PlyC (1-50 µg/mL) on cytotoxicity and oxidative response on bovine blood polymorphonuclear leukocytes (PMN) obtained from twelve healthy, mid-lactation primiparous dairy cows. Following incubation at 0.5 and 2 h, cytotoxicity was characterized by measuring lactate dehydrogenase release from isolated cells. Oxidative burst response was characterized as the intensity of chemiluminescence produced in the interaction of ROS generated in response to 0 or 1.6 µg/mL phorbol 12-myristate-13-acetate (PMA) with a luminescent substrate with and without addition to PlyC to the incubation matrix. Data were analyzed as a complete randomized block design using mixed model procedures. Cytotoxicity of PlyC was not affected by concentrations up to 50 µg/mL. As expected, PlyC cytotoxicity on PMN varied across incubation time with greater cell toxicity measured at 2 h incubation as compared to 0.5 h and is primarily attributed to the short life of PMN *ex vivo*. Concentrations of PlyC up to 50 µg/mL did not affect oxidative response; however, oxidative response was affected by incubation time and PMA concentration. In summary, varying doses of PlyC are non-toxic as estimated by lactate dehydrogenase release from cells and do not appear to alter PMA-stimulated

ROS production in bovine PMN. These early observations support continued work on the potential for application of this novel agent in combating mastitis.

Short Communication

In regard to dairy production, mastitis is the most common and economically significant disease. The estimated losses to producers in the United States exceed $2 billion per year with each clinical case costing approximately $179 (Cha et al., 2011). A wide variety of pathogens cause bovine mastitis including bacteria, yeast and algae. Pathogens arising from the environment, such as *Strep. uberis* and *E. coli*, are consistently being detected and reported as a leading cause of subclinical and clinical bovine mastitis throughout the world (National Mastitis Council, 2015). *Strep. uberis* is the most prevalent environmental streptococci mastitis pathogen (Jayarao et al., 1999). The number of cases caused by *Strep. uberis* continues to rise even in well-managed herds as typical mastitis control practices, such as teat disinfection and sanitary housing and milking environments, are less effective at preventing *Strep. uberis*-associated mastitis (Kromker et al., 2014).

The most common treatment strategy for clinical cases of *Strep. uberis* is the administration of intramammary antibiotics, such as cephalosporin β-lactams or penicillin G (Oliver et al., 2011). Growing concerns regarding antibiotic use and the potential for antimicrobial resistance from their use have led to the need to develop alternative strategies to antibiotics for controlling mastitis, a measure which should reduce the use of antibiotics on-farm (Bannantine et al., 2013). An antimicrobial alternative currently being developed is the bacteriolytic cell wall hydrolases known

as lysins (or endolysins). These bacteriophage-derived enzymes rapidly and specifically lyse bacteria when applied exogenously (Fischetti, 2006). One of the most potent endolysins to date is the streptococcal-specific PlyC. Previous research spanning over 50 years has shown that PlyC can rapidly lyse streptococci groups A, C, and E in addition to *Strep. uberis* and *Strep. equi* and has been shown to protect mice from streptococcal infection challenge (Krause, 1957; Nelson et al., 2001). Therefore, the use of recombinant PlyC is an appealing choice for use against select streptococcal species in bovine mastitis, such as *Strep. uberis*.

Following bacteria entry into the mammary gland, circulating PMN, i.e. neutrophils, are the first cells recruited and then represent the most predominant somatic cell type (Burvenich et al., 1994). The initial magnitude of the PMN response will often dictate the overall effect of mastitis on the cow, as increased recruitment of PMN and greater overall PMN activity will decrease the mastitis duration and severity (Rainard and Riollet, 2003a). Stimuli from mammary cells and invading pathogens activate PMN to eliminate the pathogens through the production of chemical mediators, such as ROS, and phagocytosis (Miller et al., 1993). As an alternative to pathogenic bacteria, PMA is commonly used to stimulate cellular aspects of oxidative metabolism as PMA structure stimulates PMN similarly normally associated with bactericidal activity *in vitro* (Rinaldi et al., 2007; Freitas et al., 2009).

Although intramammary administration of PlyC is a promising new management strategy to treat *Strep. uberis* mastitis, the effects of PlyC on the bovine immune response remain unknown. Delayed appearance or decreased immunological activity of PMN can discourage the occurrence of a bacteriological cure. Potential

antibiotic alternatives should not interfere with or harm the host immune response if they are to be considered useful. The objective of this study was to evaluate any cytotoxicity effects of PlyC on bovine PMN and on the PMN oxidative response to activation. We hypothesized that various concentrations of PlyC would be non-toxic and not alter the inflammatory response of bovine PMN *in vitro*.

All procedures involving the use of live animals were approved in accordance with the regulations and guidelines set forth by the USDA-Beltsville Animal Care and Use Committee. Twelve, healthy primiparous Holstein cows were used for this study. To be eligible, cows must have had a composite milk SCC <100,000 cells/mL and cows must not have been treated for clinical signs of mastitis or any other disease during early lactation. Eligible cows were housed in tie-stalls and individually fed a standard TMR to meet the requirement of lactating dairy cows at the USDA-Beltsville Agricultural Research Center. Free choice water was available and cows were fed ad-libitum twice daily to ensure 5-10% refusals. All cows were milked twice daily at 0730 and 1630 h (hour) in a double-8 herring bone parlor with milk records recorded monthly. Cows averaged 178 ± 4 days in milk (DIM) and 40.7 ± 1.3 kg milk per day (d) at time of sampling.

Recombinant PlyC was expressed and purified as previously described (Nelson et al., 2001, 2006). Briefly, *E. coli* were grown at 37°C in baffled flasks to an optical density 600 = 1 in LB-Miller medium (Luria broth containing 10 g/L sodium chloride) supplemented with 100 µg/mL ampicillin. Expression of PlyC was induced by the addition of arabinose (0.25%). After growth for an additional 4 h at 37°C, cells were harvested, lysed by sonication, and crude phage extracts were purified by

ceramic hydroxyapatite resin (Bio-Rad Laboratories Inc., Hercules, CA) to remove cellular debris. As approximately 80-90% pure protein was eluted by 1 *M* sodium phosphate pH 7.2, a second round of purification by S200 size exclusion chromatography was performed to remove endotoxin. All PlyC used in experiments was produced in a single batch.

Approximately 200 mL of blood was collected via jugular venipuncture from each cow into 15 mL centrifuge tubes with conical bottoms (VWR, Radnor, PA) containing 2.5 mL of acid-citrate dextrose solution (Sigma-Aldrich Inc., St. Louis, MO) and stored on ice. Blood samples were processed within 1 h of collection. Isolation of PMN, viability and cell differentiation were performed using endotoxin free materials and reagents as previously described (Garcia et al., 2015). Viability of PMN was 96% with a cell differential of 91% PMN. Cells were counted by a TC20 Automated Cell Counter (Bio-Rad Laboratories) then diluted to concentrations appropriate for each assay. From each cow, 800 µL of PMN were placed in 24-well cell culture plates and treated with 200 µL of 0, 1, 10 or 50 µg of PlyC in 1X phosphate buffer solution (PBS). Plates were incubated for 0.5 or 2 h at 37°C, 95% relative humidity and 5% CO_2.

The cytotoxicity of PlyC on PMN was determined *in vitro*. Prior to incubation with PlyC, PMN were diluted to ~3.5×10^5 per mL for analysis of PMN cytotoxicity using the CytoTox 96 non-radioactive, colorimetric assay (Promega, Madison, WI) to quantify lactate dehydrogenase production. Relative cytotoxicity was calculated per instructions provided by the manufacturer (experimental divided by maximum lysis).

Within incubation period and cow, each PlyC treatment was performed in triplicate (coefficient of variance [CV] = 2.66%).

The oxidative response of PMN was determined by luminol chemiluminesence assay to quantify ROS production. Neutrophils were diluted to ~3.0×10^6 per mL prior to incubation with PlyC. Following incubation with PlyC for 0.5 or 2 h, 0.5 mL of media was transferred into round-bottomed, glass 7 mL tubes. Tubes were placed into a heating block and incubated at 37°C for 10 min. In a separate reaction tube (2 per each media tube), 900 µL of assay buffer (0.5 m*M* $CaCl_2$ in Hank's balanced salt solution) and 100 µL of a 5 m*M* luminol solution in dimethyl sulfoxide (Sigma-Aldrich) were added and placed in the heating block. At the end of the incubation, either 40 µL of sterile water or 1.6 µg/mL PMA (Sigma-Aldrich) solution were added to each media tube. Tubes were gently vortexed and returned to the heating block. After heating, 40 µL from the media tube was transferred to the reaction tube. Tubes were again lightly vortexed and immediately placed in the luminometer (AutoLumat LB 953, Berthold Technologies, Germany) to measure relative chemiluminescence for 20 minutes. Within incubation period, PMA concentration and cow, each PlyC treatment was performed in duplicate (CV = 11.96%).

Experimental units and the corresponding sample size of cows that were used in this study have been determined using the POWER procedure of SAS (SAS/STAT version 9.3; SAS Institute Inc., Cary, NC) for a one-way analysis of variance. Means and average standard deviation values for PMN oxidative response and PlyC cytotoxicity were calculated from a previously conducted pilot study. The following

are parameters included in the procedure: 1) an alpha set at 0.05 to declare significance, 2) an 80% probability of detecting a difference in treatment means, 3) a 15,000 luminescence unit difference between treatment means, and 4) an average standard deviation value of 6,400. The procedure was repeated for PlyC cytotoxicity with a 15 unit difference between means and an average standard deviation value of 7. Based on the results of the power analyses, it was concluded that at least 10 cows are required for this study.

Response variables were analyzed as a complete randomized block design using the PROC MIXED procedure of SAS. Sources of variation within the model included effects of PlyC concentration, PMA concentration (oxidative response model only), and incubation time as well as 2- and 3-way interaction among the main effects. Cow was designated as a random effect in the model. Separation of least square means for significant effects was accomplished using the PDIFF option in the MIXED procedure. Significance was declared at $P \leq 0.05$. Data are presented as least squares mean ± standard errors of the mean.

In the current study, concentrations of purified PlyC at 1, 10 and 50 µg/mL did not have a cytotoxic effect on PMN relative to cells incubated in buffered solution ($P = 0.41$) as cytotoxicity remained constant across concentrations (47-55%). These results indicate that PlyC, even at high doses, is not toxic to bovine PMN *in vitro*. Incubation time increased PlyC cytotoxicity by 25% from 0.5 h to 2 h (44.6 vs. 55.0 ± 2.8%; $P = 0.01$) as expected, given the relatively short half-life of circulating PMN is 6-8 h (Summers et al., 2010). However, incubation time across PlyC concentrations did not affect cytotoxicity ($P = 0.78$; Figure 2.1).

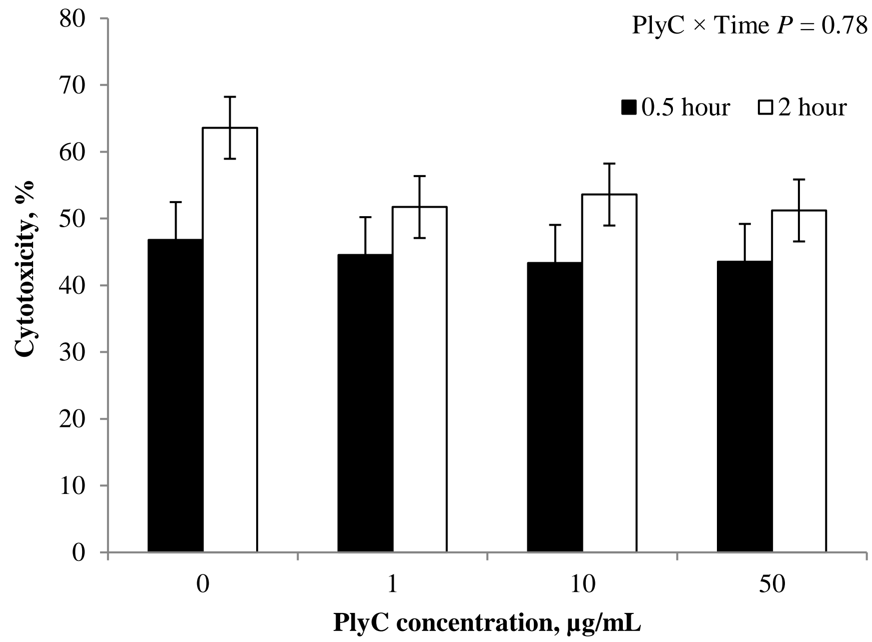

Figure 2.1: Least square means and standard errors for bovine neutrophil cytotoxicity for neutrophils incubated in vitro for 0.5 and 2 hours in various concentrations of recombinant streptococcal C1 phage lysin (PlyC).

Respiratory oxidative burst activity of PMN incubated with purified PlyC was determined by luminol chemiluminescence following 0.5 or 2 h of incubation and stimulation by 0 or 1.6 µg/mL PMA. Similar to cytotoxicity, respiratory burst was not affected by PlyC concentrations up to 50 µg/mL ($P = 0.52$) and relative luminescence remained constant (29,838 ± 3,315 relative luminescence units), indicating that PlyC may not interfere with the function of PMN during the inflammatory response *in vivo*. As expected, increasing PMA concentration from 0 to 1.6 µg/mL increased PMN respiratory burst response (21,514 vs. 38,162 ± 4,585 relative luminescence units; $P < 0.01$) reflecting the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and production of ROS, superoxide anions and hydrogen peroxide, similarly to that of an invading pathogen (White et al., 1984; Kobayashi et al., 2005). However, PMN respiratory response to PMA was dependent upon incubation time with longer incubation decreasing respiratory burst of PMN incubated with 1.6 µg/mL PMA ($P < 0.01$; Figure 2.2A). The overall decreased respiratory burst response across incubation time reflects the increased cell deterioration and death observed in the PMN cytotoxicity analysis. The relative oxidative burst of PMN was not affected by PlyC concentration across incubation time ($P = 0.45$) although relative luminescence was numerically lower following 2 h incubation (Figure 2.2B).

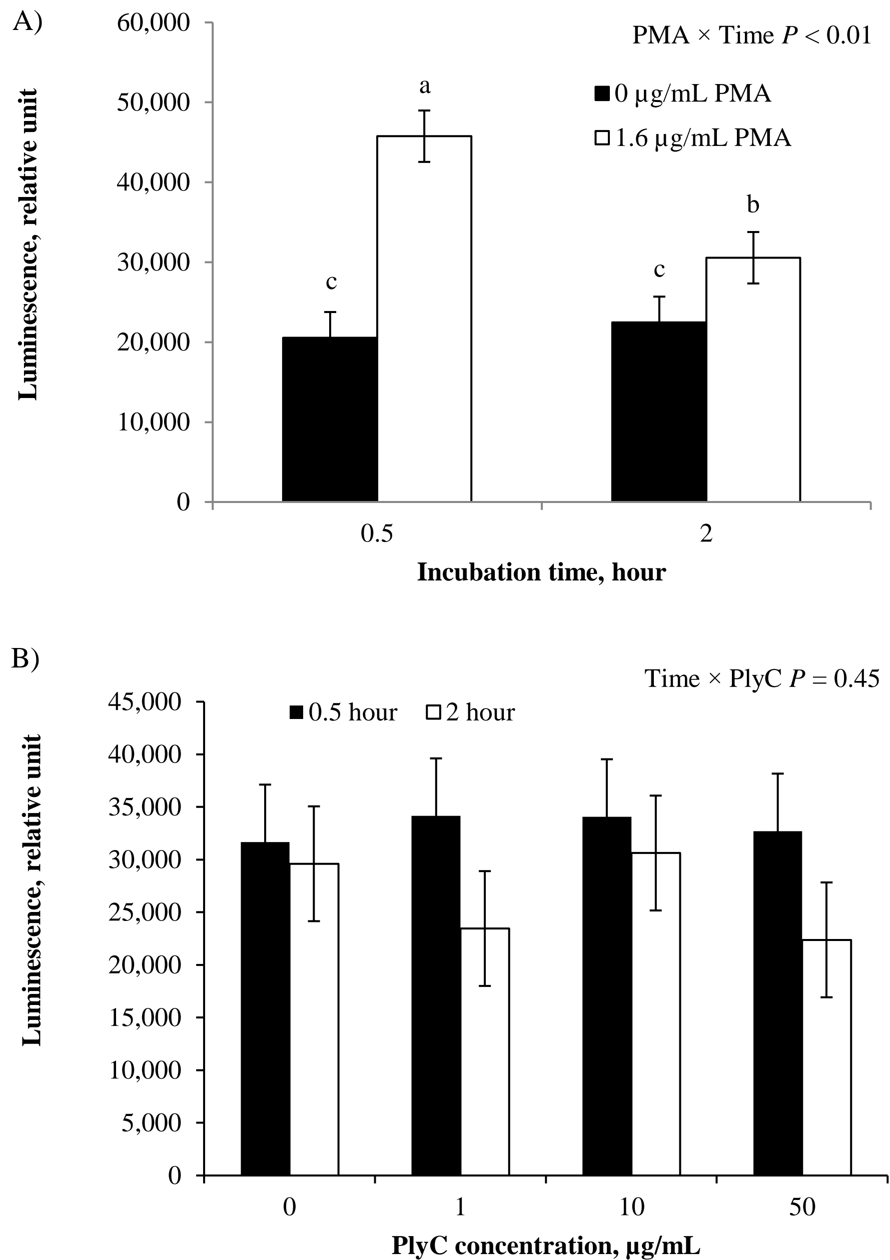

Figure 2.2: Least square means and standard errors for the bovine neutrophil oxidative response as determined by luminol chemiluminescence at 0.5 or 2 h of incubation and stimulated by 0 or 1.6 µg/mL phorbol 12-myristate-13-acetate (PMA) solution in various concentrations of recombinant streptococcal C1 phage lysin (PlyC). A) Oxidative response and incubation time across PMA concentration; B) oxidative response and incubation time across PlyC concentration. [a-c] Means with different subscripts differ ($P < 0.05$).

The growing concern for antibiotic resistance and improved stewardship has fueled the drive to find antibiotic and antimicrobial alternatives. Bacteriophage lysins have been used successfully in a variety of models to control pathogenic antibiotic resistant and tolerant bacteria found on mucosal surfaces and infected tissues. The streptococcal-specific endolysin PlyC is unique among the lysins and has rapid lethal effect *in vitro* (McGowan et al., 2012) and *in vivo* (Nelson et al., 2001). Relatively low concentrations of PlyC have successfully been used for disinfection of *Strep. equi* (1 µg/mL; Hoopes et al., 2009) and degradation of *Streptococcus pyogenes* biofilms (5 µg/mL; Shen et al., 2013) in addition to the prevention and elimination of *Strep. pyogenes* in mice (0.01 µg; Nelson et al., 2001). These effective concentrations were substantially lower than those examined in the current study. Concentrations of PlyC up to 50 µg/mL were nontoxic on bovine PMN. These results demonstrate that the lytic activity of PlyC does not target bovine PMN and that effective concentrations of PlyC would likely be nontoxic to recruited neutrophils during mastitis.

Recruited neutrophils use many techniques to destroy pathogenic bacteria that have entered the mammary gland, such as phagocytosis, extracellular traps and degranulation (Summers et al., 2010). ROS produced and released during the respiratory burst response aid in bacterial destruction and clearance of the infection. Again, high concentrations of PlyC (up to 50 µg/mL) did not affect the respiratory burst response of bovine PMN. Effective, lower concentrations of PlyC would likely not affect the respiratory response of PMN during the immunological response to infection.

To our knowledge, this study is the first to characterize the effects of bacteriophage-based lytic enzyme PlyC on bovine PMN. Results show that high concentrations of PlyC are non-toxic and do not impede the oxidative response of bovine PMN. Future studies investigating the use of PlyC as an alternative therapy for treatment of mastitis are warranted.

Acknowledgements

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Chapter 3: Citrus Oil and its components antimicrobial activity and effects on bovine blood neutrophils.

Introduction

Mastitis is one of the most important diseases in dairy cattle of which *E. coli* is a major pathogen (Shpigel et al., 1998). Neutrophils are the primary innate cell recruited (~90%) and have a prominent role in the defense of the mammary gland against bacterial infections (Paape et al., 2002). Many studies have demonstrated that the promptness and magnitude of neutrophil recruitment can influence the severity and outcome of mastitis caused by *E. coli* mastitis (Hill, 1981; Vandeputte-van Messom et al., 1993). Disruption of neutrophil abilities and efficiency to address the mastitis infection can have severe consequences on mastitis outcome (Rainard, 2003).

Despite an apparently good antimicrobial susceptibility *in vitro*, the cure of diseased animals from *E. coli* mastitis is often disappointing. Current available antibiotics have minimal effect on shortening the duration of the intramammary infection as some cases will spontaneously cure, while others result in recurrent clinical infections (Smith et al., 1985). Decreased antibiotic responsiveness, antibiotic overuse and misuse, and increased awareness of antibiotic resistant bacteria entering the human food chain prompted further exploration into alternative antimicrobial therapies. Natural compounds, such as those derived from plants, have biologically active agents with antimicrobial action. Plant-derived compounds have the potential of not inducing resistance after prolonged exposure (Chandra et al., 2017) or creating potentially harmful residues in the meat or milk (Gomes and Henriques, 2016).

A potential alternative to traditional antimicrobial pharmaceutical drugs used to treat bovine mastitis may be found in orange/citrus oil obtained from by-products that otherwise might be discarded. Citrus oil has been used in the food industry for decades to create citrus flavor (Hyldgaard et al., 2012). In the past decade, citrus oil use has expanded to meat preservations as the compounds in the oil act as natural antioxidants and deter the growth of bacteria and other pathogens that cause spoilage and disease (Mahato et al., 2017). When applied directly to meat, citrus oil and it components have displayed bacteriostatic and bactericidal characteristics towards *E. coli, Staph. aureus*, and *Mycobacterium bovis* (Fisher and Phillips, 2006; Crandall et al., 2012; Muthaiyan et al., 2012; Federman et al., 2016). The antimicrobial properties of citrus oil are not limited to surface applications of meat but are active within milk *in vitro*, as well. A relatively low concentration (0.1% vol/vol) inhibited and reduced bacterial growth of *E. coli* and *Staph. aureus* in pasteurized milk (Dabbah et al., 1970). As many of the pathogens that cause food spoilage are also pathogenic species for bovine mastitis, these characteristics of citrus oil may also prove useful for deterring and eliminating these species from the mammary gland.

MIC and MBC are useful tools for assessing new antimicrobial pharmacokinetics; however, they only assess antimicrobial susceptibility for planktonic and rapid bacterial growth. *E. coli* can also create biofilms, structured bacterial communities that adhere to an inert or living surface (Melchior et al., 2006b). Biofilms can constitute a protected mode of growth for bacteria in hostile environments as a self-produced extracellular polysaccharide matrix allows bacterial cells to adhere to one another and create a multi-layer community. Bacterial cells

within the biofilm structure are protected from antibiotics and the host-immune system, therefore promoting survival of the bacteria, recurrence of the infection and contributing to antibiotic resistance (Olson et al., 2002). Similar to planktonic bacterial growth, biofilm susceptibility to antimicrobials is measured through BMBC and MBEC.

Citrus oil components have shown some success as an alternative antimicrobial treatment for bovine mastitis. Relatively low concentrations of citrus oil components dissolved in DMSO have successfully inhibited and eradicated *Staph. aureus* planktonic and biofilm bacterial growth, and did not demonstrate cytotoxic effects on cultured bovine mammary cells or neutrophils (Aiemsaard et al., 2011; Garcia et al., 2015; Federman et al., 2016). However, the use of DMSO in dairy cattle is prohibited by the FDA (US Department of Health and Human Service and Public Health Service, 2015). A suitable, alternative solvent for citrus oil components is ethanol as it can maintain oil solubility at low concentrations and its use is not prohibited. It is prudent to reassess the effects of citrus oil dissolved in ethanol as the antioxidant effects and synergy of DMSO with antimicrobials may have altered the effects of citrus oil (Ansel et al., 1969; Mi et al., 2016).

Therefore, the objectives of this study are to determine the effects of citrus oil components on 1) the susceptibility of environmental pathogen, *E. coli*, and 2) bovine blood neutrophil viability and oxidative burst activity *in vitro*. We hypothesize that citrus oil components in ethanol will demonstrate inhibitory and eradication concentrations towards planktonic and biofilm bacterial growth as those previously

demonstrated in past studies, and will not be cytotoxic or inhibit neutrophil oxidative burst response.

Methods and Materials

Animals and Sampling Procedure

All procedures involving the use of live animals were approved in accordance with the regulations and guide-lines set forth by the University of Maryland Animal Care and Use Committee. Twelve healthy, mid-lactation multiparous Holstein cows were used for this study. To be eligible, cows must have had a composite milk SCC < 200,000 cells/mL, culture negative milk, and cows must not have been treated for clinical signs of mastitis or any other disease during their current lactation. Cows averaged 141 ± 17 DIM and 37.9 ± 1.3 kg milk/d at time of sampling. Cows were housed and fed in free stalls at the Central Maryland Regional Extension Center dairy unit in Clarksville, MD and milked twice daily at 0700 and 1900 h. Cows were fed daily a standard total mixed ration formulated to meet nutritional requirements (National Research Council, 2001) and 10% refusals.

Before milk sampling during the morning milking, a California Mastitis Test (CMT) was performed to ensure low SCC in each quarter of the cow. Teats were dipped in foam germicide containing hydrogen peroxide, stripped and wiped dry with single service, cloth towels. A teat end from a negative CMT quarter was rigorously cleaned with cotton balls containing 70% ethanol and approximately 50 mL of foremilk was aseptically collected. Milk samples were immediately refrigerated after collection. Following milking, approximately 150 mL of blood was collected via

jugular venipuncture from each cow into 15 mL centrifuge tubes with conical bottoms containing 2.5 mL of acid-citrate dextrose solution and stored on ice. Blood samples were processed within 1 h of collection.

Bacteria Growth

The strain used in this study was the *E. coli* strain P4, serotype O32:H37 (graciously provided by Dr. Jo Ann Van Kessel, US Department of Agriculture), which was isolated from a clinical mastitis case (Bramley, 1976) and is widely used to induce experimental mastitis (Blum et al., 2017).The strain is capable of causing clinical mastitis in cows and extracellular biofilms on murine mammary epithelial cells (Shpigel et al., 2008). The strain was maintained in a stock on lyophilization medium at -20°C. A stock of bacteria was sub-cultured at 37°C in brain-heart infusion broth (Sigma Aldrich) for 6-8 h then transferred and cultured in tryptic soy broth (TSB) for 18-24 h. Following incubation, inoculated TSB were stored at 4°C. Sub-cultures of TSB were diluted in pyrogen-free PBS and plated on MacConkey agar and 5% sheep blood agar plates to determine CFU/mL and detect possible contamination before performing antimicrobial susceptibility assays.

Antimicrobials

Citrus oils (Sigma-Aldrich Inc., St. Louis, MO) and citrus oil components citral (Sigma-Aldrich), linalool (Sigma-Aldrich), limonene (Sigma-Aldrich) and valencene (Sigma-Aldrich) were diluted in ethanol and sterile PBS on a percentage volume-by-volume basis. Results were converted to µg/mL for oils, post hoc. Ethanol

to oil concentrations were maintained at 7:1 (vol/vol) for oil solubility. An ethanol-only treatment was also examined to determine the effects of ethanol on *E. coli* growth with ethanol concentrations mirroring those used to maintain the oils in solution.

Citrus Oil Minimum Inhibitory and Bactericidal Concentration

Citrus oil and its various functional components were tested to determine the MIC required for *E. coli* P4. The inoculated TSB was diluted to 8 x 10^6 CFU/mL using pyrogen-free PBS and 100 µL of broth was transferred to sterile, 24-well flat bottom plates. All oil and ethanol concentrations were added (100 µL) in triplicate. On each plate, single well controls included broth, broth plus *E. coli* (no treatment), PBS and ethanol. Plates (n = 4) were incubated at 37°C for 24 h. MIC was defined as the amount of antimicrobial that inhibited visible growth in the well as measured by optical density at 595 nm (Pankey and Sabath, 2004). A subculture of 100 µL was taken from the wells displaying inhibited growth and plated on sheep's blood 5% agar and MacConkey, in duplicate. The lowest concentration of the treatment that prevented growth on the agar plates was taken as the MBC (Baskaran et al., 2009).

MIC and MBC were also obtained in bovine milk for citral, linalool, and ethanol using the same concentrations and plating procedure in 24-well, flat bottomed plates. All bovine milk was cultured on MacConkey agar and 5% sheep blood agar at 37°C for 24 h to confirm the absence of pathogenic bacteria prior to use of the milk as a culture medium. Following incubation at 37°C for 24 h, 100 µL from all

concentrations and controls were plated in duplicated on MacConkey agar and 5% sheep blood agar plates to determine MIC and MBC.

Minimum Biofilm Eradication Concentration

Measurements of the antimicrobial susceptibility of *E. coli* P4 biofilms was performed in the MBEC biofilm assay (Innovotech, Edmonton, AB, Canada) according to the manufacturer's instructions. This method facilitates formation of biofilms on the surface of pegs that can be exposed to an antimicrobial challenge for determination of the susceptibility of *E. coli* P4 biofilms for citrus oil components (Ceri et al., 1999).

Following establishment of the *E. coli* P4 inoculum, the TSB was diluted further in sterile TSB and culture-negative milk. In a sterile 96-well plate, biofilms were established by adding 150 µL of inoculated, diluted media to each well (~5.0×10^5 CFU) and covered with a lid containing 1 peg per well. The plate was incubated at 37°C for 16 h at approximately 95% humidity on a plate shaker at 110 revolutions per minute (VWR Scientific, Buffalo Grove, IL) to obtain ~10^5 CFU biofilm growth on each peg. Biofilm formation was determined by removing 3 pegs from the lid with sterilized pliers and placing each peg in TSB in a new 96-well plate. The biofilm was disrupted from the peg by sonicating the plate on high for 30 minutes, serial diluting the TSB in sterile PBS and spot plating 20 µL from each dilution on MacConkey agar and 5% sheep blood agar plates (Ceri et al., 1999; Fisher Scientific).

Once biofilms are established on the MBEC pegs, the pegged-lid was transferred to another 96-well plate containing 2-fold dilutions of the antimicrobial treatments citral, linalool, and corresponding ethanol-only. The plate was incubated at 37°C for 12 h to correspond with the average time between treatments when cows are milked twice daily. After incubation, the lid with pegs was removed, rinsed in PBS and placed in a second 96-well plate containing TSB. Planktonic bacteria released from the biofilm in the first 96-well plate with the antimicrobial treatments (20 μL) was transferred to a third plate containing 180 μL of TSB to determine BMBC. The second 96-well plate was sonicated, as previously described, to dislodge the biofilm to measure antimicrobial susceptibility of the *E. coli* bacteria (MBEC). The BMBC and MBEC plates were incubated at 37°C for 24 h and visible growth in the plates was measured at 650 nm in a 96-well plate reader (Bio-Rad Laboratories, Hercules, CA). All treatment dilutions were performed in duplicate on the 96-well plates and the experiment were repeated 3 times, similarly to previous studies (Ceri et al., 1999; Olson et al., 2002).

Blood Neutrophil Isolation and Incubation

Isolation of blood neutrophils, viability and cell differentiation were performed using endotoxin free materials and reagents as previously described (Garcia et al., 2015). Cells were counted by a TC20 Automated Cell Counter (Bio-Rad Laboratories) then diluted to concentrations appropriate for each assay. Isolated cells were 94.4 ± 1.3% neutrophils with 92.2 ± 0.8% being viable. From each cow, 1 mL of neutrophils were placed in 24-well cell culture plates and treated with 1 mL of

citral or linalool dissolved in ethanol and PBS with a 7:1 ethanol to oil ratio (vol/vol) to maintain oil solubility. A control treatment of ethanol-only was also tested to discern the effects of ethanol on bovine neutrophils. Plates were incubated for 2 h at 39°C, 95% relative humidity and 5% CO_2.

Cytotoxic Effects on Bovine Blood Neutrophils

The cytotoxic effects of citral, linalool and ethanol on neutrophils was determined in vitro. Prior to incubation with oils and ethanol, neutrophils were diluted to ~3.5×10^5 cells/mL for analysis of oil and ethanol cytotoxicity using the CytoTox 96 non-radioactive, colorimetric assay (Promega, Madison, WI) to quantify lactate dehydrogenase release from cells. Relative cytotoxicity was calculated per instructions provided by the manufacturer.

$$\%\ \text{Cytotoxicity} = \frac{\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous}}{\text{Target Maximum} - \text{Target Spontaneous}} \times 100$$

Neutrophil Oxidative Burst Response

The oxidative response of neutrophils was determined by luminol chemiluminesence assay to quantify ROS production. Neutrophils were diluted to ~3.0×10^6 per mL prior to incubation with citral and linalool. Following incubation with citrus oil fractions for 2 h, 0.5 mL of media was transferred into round-bottomed, glass 7 mL tubes. Tubes were placed into a heating block and incubated at 37°C for 10 min. In a separate reaction tube (2 per each media tube), 900 µL of assay

buffer (0.5 *mM* $CaCl_2$ in Hank's balanced salt solution) and 100 µL of a 5 *mM* luminol solution in DMSO; Sigma-Aldrich) were added and placed in the heating block. At the end of the incubation, either 40 µL of sterile water or 1.6 µg/mL PMA (Sigma-Aldrich) solution were added to each media tube. Tubes were gently vortexed and returned to the heating block. After heating, 40 µL from the media tube was transferred to the reaction tube. Tubes were again lightly vortexed and immediately placed in the luminometer (AutoLumat LB 953, Berthold Technologies, Germany) to measure relative chemiluminescence for 20 min. Each oil and ethanol treatment concentration was performed in duplicate within PMA concentration and cow.

Statistical Analysis

The number of animals required to observe significant changes in neutrophil oxidative burst response and cytotoxicity of citrus oil components dissolved in ethanol was determined using the POWER procedure for a one-way analysis of variance in SAS version 9.4 (SAS/STAT version 9.4; SAS Institute Inc., Cary, NC). Experimental response means and standard deviation values were based on a previous study where the effects of citrus oil components dissolved in DMSO on bovine blood neutrophils were examined. It was concluded that 10 animals would produce 85% probability to detect a difference between treatment means (alpha = 0.05) in neutrophil response and citrus oil component cytotoxicity. The number of biological and experimental replicates for MIC, MBC, BMBC and MBEC was determined from previous literature (Costa et al., 2014; Silva et al., 2014).

Response variables, cytotoxicity and oxidative burst chemiluminescence, were analyzed as a complete randomized block design using the PROC MIXED procedure of SAS (SAS/STAT version 9.4). Sources of variation within the model included oil concentration and a separate ANOVA was run for each oil treatment type. Cow was designated as a random effect in the model. Separation of least square means for significant effects was accomplished using the TUKEY option in the MIXED procedure. Significance was declared at $P \leq 0.05$. Data are presented as least squares mean ± standard error of the mean.

Results

Citrus Oil Minimum Inhibitory and Bactericidal Concentrations

In TSB, citrus oil, and citrus oil fractions limonene and valencene concentrations up to 4,000 µg/mL did not inhibit *E. coli* P4 growth (Figure 3.1). The ethanol-only treatment performed similarly, as concentrations required to maintain the citrus oil and citrus oil fractions in solution (up to 7% vol/vol ethanol) did not affect bacterial growth. Fractions of citral and linalool inhibited bacterial growth at 1,786 µg/mL and 1,740 µg/mL, respectively. These concentrations were slightly greater than the MIC observed when citrus oils and its fractions were dissolved in DMSO. Limonene, valencene and citrus oils performed similarly with much higher MIC values than those of citral (447 µg/mL) and linalool (870 µg/mL) when previously dissolved in DMSO.

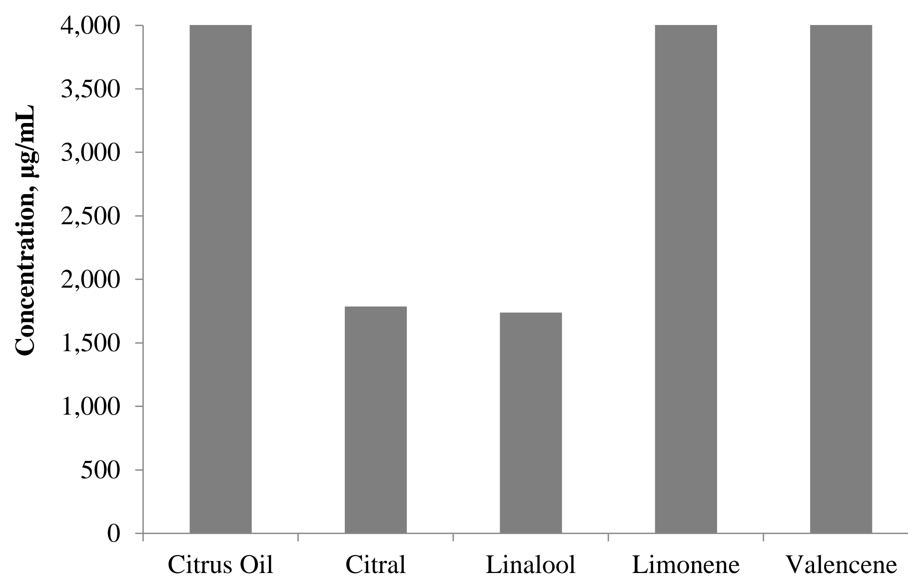

Figure 3.1: Minimum inhibitory concentrations of citrus oil and citrus oil fractions when dissolved in ethanol against *Escherichia coli* strain P4 when cultured in tryptic soy agar broth.

Similar MIC and MBC values were determined for citral and linalool when *E. coli* was cultured in bovine milk (Table 3.1). Citral demonstrated a similar concentration for MIC (1786 µg/mL) and a slightly greater concentration for MBC (3,572 µg/mL) as compared to TSB. Linalool required much higher concentrations than citral to inhibit growth (3,480 µg/mL) and kill the *E. coli* (3,480 µg/mL). The concentration of ethanol required to maintain citral and linalool solubility did not appear to influence MIC and MBC as the concentration of ethanol required to inhibit growth and neutralize *E. coli* (70% vol/vol) was much greater than the concentrations required to maintain the citral and linalool in solution (maximum required ethanol 11.2% vol/vol).

Table 3.1: Minimum inhibitory (MIC) and bactericidal (MBC) concentrations (μg/mL) for citral, linalool and ethanol-only (% vol/vol) against *Escherichia coli* P4 cultured in bovine milk.

	Citral (μg/mL)	**Linalool** (μg/mL)	**Ethanol-only** (% vol/vol)
MIC	1,786	3,480	70
MBC	3,572	3,480	70

Citrus Oil Fractions Minimum Biofilm Eradication Concentration

Escherichia coli biofilms were successfully established and averaged 2.945×10^5 CFU/mL when grown in TSB and 2.994×10^5 CFU/mL in bovine milk. Lower concentrations of citral were required to eradicate the established *E. coli* biofilms and planktonic growth as compared to linalool in both milk and TSB (Table 3.2).

Table 3.2. Minimum concentrations (µg/mL) for citrus oil fractions and ethanol-only against *Escherichia coli* P4 for biofilm eradication, and eradication and inhibition of biofilm planktonic growth

	Citral (µg/mL)	**Linalool** (µg/mL)	**Ethanol-only** (% vol/vol)
Biofilm Minimum Eradication Concentration			
Milk	2,775	5,440	8.75
TSB	1,390	2,720	8.75
Minimum Bactericidal Concentration			
Milk	5,550	5,440	8.75
TSB	695	2,720	8.75

Citrus Oil Fractions Cytotoxicity

The CytoTox 96 colorimetric assay did not accurately measure bovine neutrophil cytotoxicity in treatments containing high concentrations of ethanol (greater than 11.2%; Figure 3.2). It is likely that the high concentrations of ethanol inhibited or interfered with the lactate dehydrogenase and therefore it could not be accurately assessed. Ethanol concentrations up to 11.2% vol/vol did not exhibit cytotoxic effects on bovine neutrophils. Citral and linalool had minimal cytotoxic effects, as well. Citral concentrations (ranging from 447 to 3,573 μg/mL) were slightly more toxic to neutrophils than PBS; the buffered saline; however, the increased cytotoxicity is biologically negligible. Similar results were observed for linalool concentrations ranging from 870 to 6,960 μg/mL.

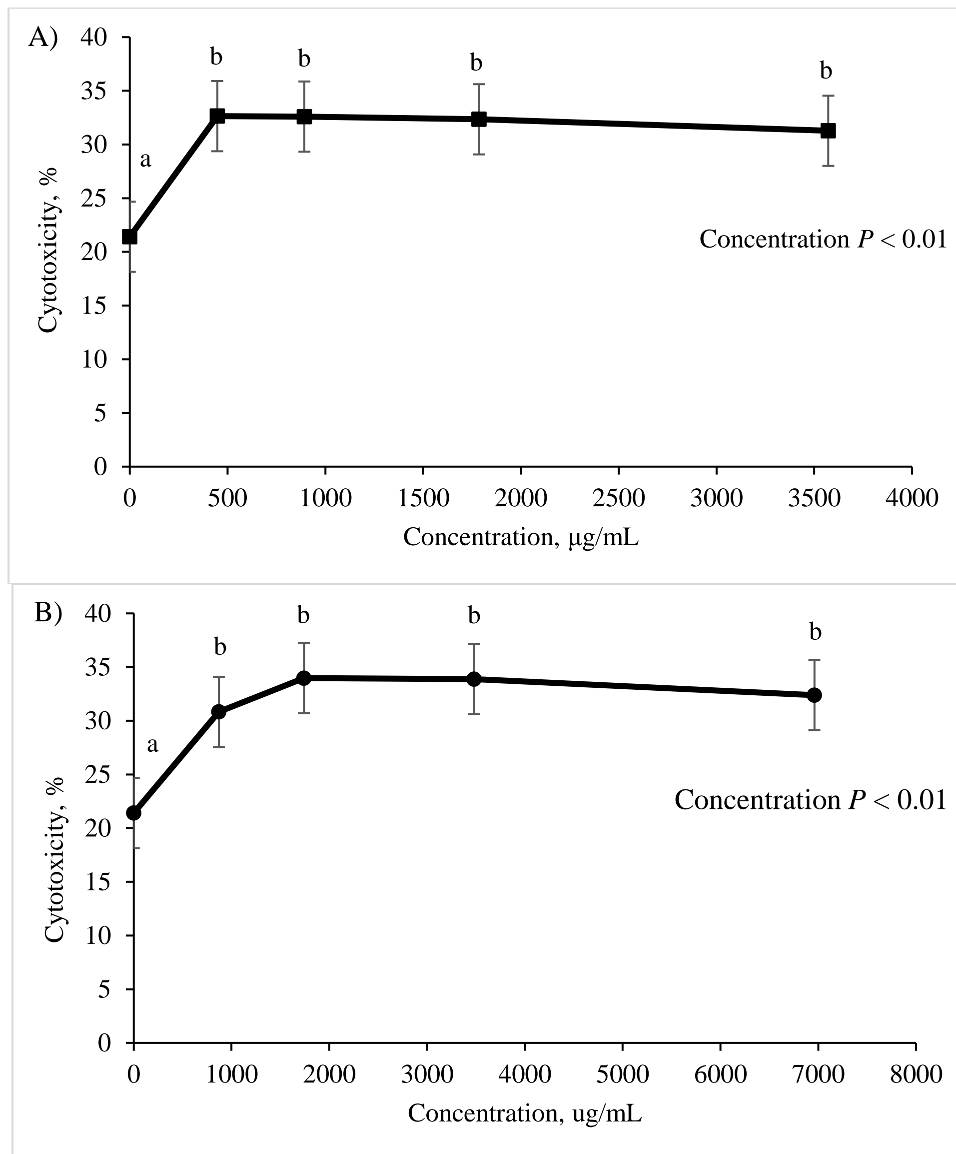

Figure 3.2: Least square means and standard errors for the cytotoxicity of bovine neutrophils incubated in various concentrations of citrus oil fractions, citral (A) and linalool (B). a-b Means with different subscripts differ ($P < 0.05$).

Bovine Neutrophil Oxidative Burst Response

Overall oxidative burst response (i.e., respiratory burst) was measured through relative luminescence as ROS reacted with luminol (Figure 3.3). Citral concentrations ranging from 893 to 1,786 μg/mL and linalool concentration 1,740 μg/mL increased relative oxidative response as compared to neutrophils in PBS. Relative ethanol concentrations required to maintain citral and linalool in solution did not impact oxidative response, indicating that citral and linalool may be aiding overall neutrophil respiratory burst. High concentrations of citral (44,600 μg/mL) and linalool (43,500 μg/mL) did not affect oxidative burst, as compared to neutrophils incubated with PBS; however, the relative ethanol content required to maintain the oil solubility (70% vol/vol) increased oxidative burst.

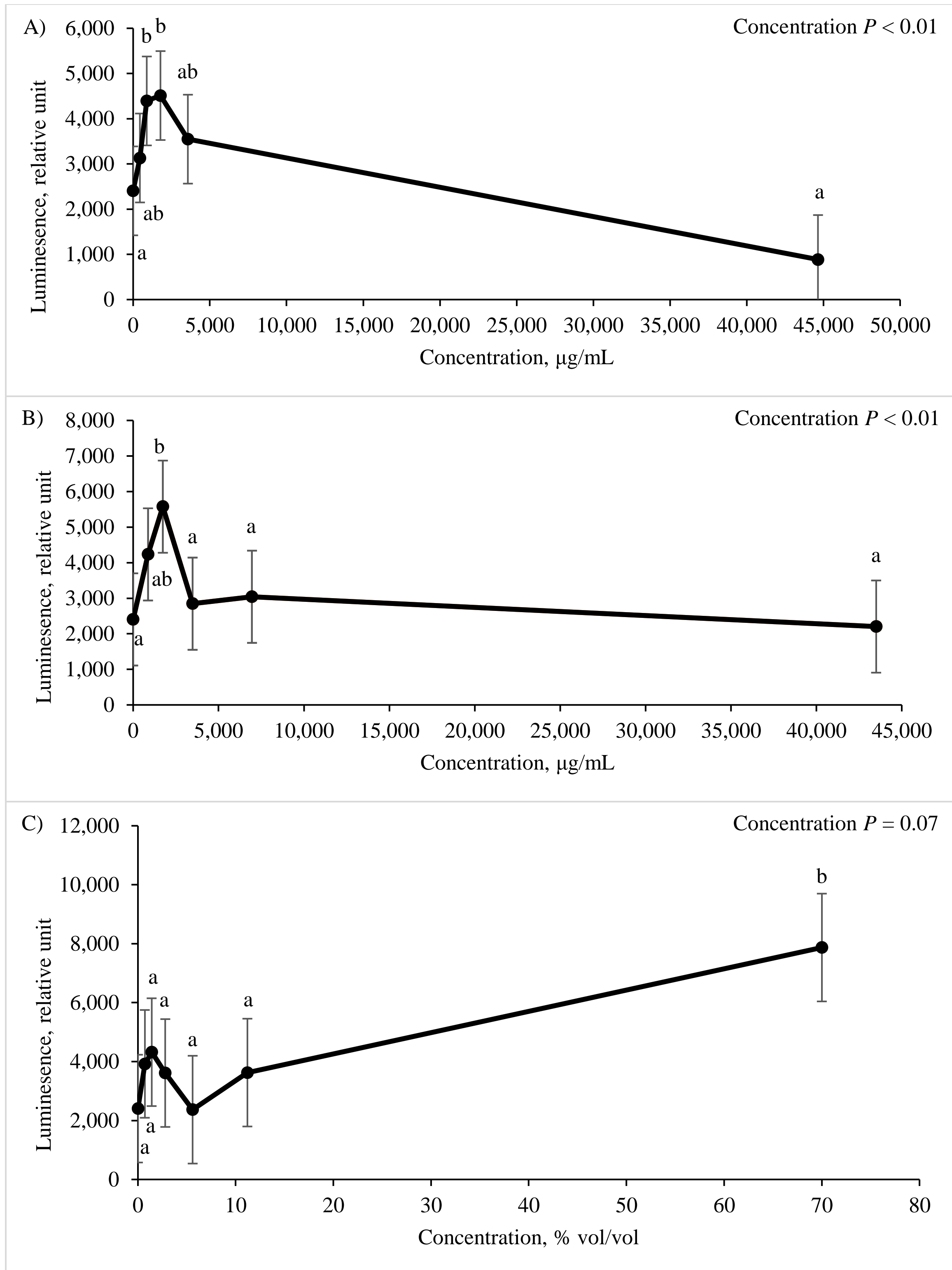

Figure 3.3: Least square means and standard errors for the bovine neutrophil oxidative response as determined by luminol chemiluminescence following incubation in various concentrations of citral (A), linalool (B), and ethanol (C). a-b Means with different subscripts differ ($P < 0.05$).

Discussion

Citrus oil has demonstrated bacteriostatic and bactericidal effects across many applications and pathogens in food science and as a possible antimicrobial for treatment of infectious disease. Because of their complexity and variation, no general statement can be made as to citrus oil's antimicrobial properties. The degree of growth inhibition of bacterial growth varies considerably with the pathogen and system tested. Regardless of variation, citrus oil is composed primarily of limonene (~90%) and various alcohols, esters, aldehydes, hydrocarbons, ketones and oxides (Bauer et al., 2001; Moufida and Marzouk, 2003). This study has demonstrated that, using the MIC method, that components of citrus oil have greater bacteriostatic and bactericidal properties against *E. coli* strain P4 than the oil alone. In TSB, citral and linalool required lower concentrations than citrus oil and other major components to inhibit *E. coli* growth. Fisher and Phillips (2006) observed similar results as limonene and citrus oil had limited antimicrobial activity against *E. coli* (O157:H7) and other food-borne pathogens; whereas, linalool and citral inhibited growth at lower concentrations in broth.

Many essential oils, including citrus oil, citral and linalool, have demonstrated anti-biofilm activity. Several in vitro studies have shown that bacteria growing in a biofilm can become more resistant to the effects of antimicrobial agents as compared to planktonic growing bacteria of the same strain (Ceri et al., 1999; Olson et al., 2002). Mastitis associated with bacterial mammary infections may be caused by biofilms due to the observed shift in antimicrobial susceptibility (Melchior, 2011). Biofilm formation and antibiotic resistance are regulated in bacteria through

intracellular communication mechanism like quorum sensing. Inhibition or dysregulation of quorum sensing can directly affect biofilm activity. Linalool and essential oils with high concentrations of linalool, such as coriander, display anti-quorum sensing activity in *Campylobacter* species biofilms (Duarte et al., 2016). Concentrations required to disrupt formed biofilms in the current study were 1-2× greater than those required to inhibit growth and eradicate planktonic bacteria. Similar results were observed for oregano, thyme and cinnamon essential oils against several different bacteria, as concentrations similar to MIC and MBC values were able to eradicate biofilms (Szczepanski and Lipski, 2014).

Few have tested the antimicrobial effects of citrus oil in milk. Dabbah et al. (1970) observed that orange oil was an effective antimicrobial in skim milk with a 1-2 log reduction of food borne pathogens. In pasteurized milk, the effects of the citrus oils on bacteria growth varied with the fat content of the milk. Increased fat content resulted in decreased antimicrobial effectiveness of the oils (Fisher and Phillips, 2008). Holley and Patel (2005) speculate that the higher concentrations of lipids in the milk created a layer around the bacteria and prevented the oils from interacting with the bacteria. Similar results were observed in the current study as citral and linalool MIC, MBC and MBEC values for *E. coli* were greater in milk as compared to broth.

The action mechanism for essential oils' antimicrobial activity is not fully understood. Evidence of changes in intracellular adenosine triphosphate (ATP) concentration, reduced cellular pH and cell membrane hyperpolarization, in addition to membrane microstructure damage witnessed through electron microscopy of

Cronobacter sakazakii (Gram-negative rod related to *Enterobacter* species) following exposure to citral suggest that it damages the cell membrane by binding to the surface and interacting with cell target molecules (Shi et al., 2016). However, this action was not observed when *Candida* species were incubated with citral (Clerya et al., 2014). The antimicrobial activity of linalool is speculated to involve penetration and disruption of the lipid fraction of the cellular membrane of *Staph. aureus* and *E. coli* (Trombetta et al., 2005). In addition, there is limited knowledge regarding the mechanisms that lead to tolerance against essential oils and their components and the extent the tolerance can occur in microorganisms. There is some evidence that strains of *E. coli* can become less susceptible to essential oils from repeated use, but MIC values increased (2-14×) only to a limited extent (Walsh et al., 2003). Gomes Neto et al. (2015) observed the involvement of gene regulation in increasing tolerance of *E. coli* following repeated exposure of two essential oils at sub-inhibitory concentrations.

Ethanol and DMSO have known antimicrobial properties that inhibit bacterial growth of many pathogens at low concentrations. The soluble nature of ethanol is effective at dissolving surface lipids and denaturing proteins of pathogens, including most bacteria, fungi and many viruses. The most effective concentration is 70% vol/vol ethanol because of osmotic pressure; pathogens become dehydrated as the osmotic pressure across the cell membrane is disrupted, leading to cell death (Pohorecky and Brick, 1988). Similarly, Ansel et al. (1969) noted that 12% vol/vol DMSO inhibited over 90% growth of *E. coli.* As a solvent, DMSO affects the antimicrobial abilities of essential oils and their components against pathogens by

affecting the nature of the compound. With cinnamon oil, DMSO partitions the oil between two different phases and ultimately limits is microbial effects (Hili et al., 1997). In contrast, limonene dissolved in DMSO has an increased droplet size that results in higher antimicrobial activity, likely due to higher contact between the droplet and microbial membrane (Van de Vel et al., 2017). This effect on droplet size of the oil in the DMSO emulsion may account for the increased antimicrobial activity observed in citrus oils and its components as compared with those dissolved in ethanol. The concentration of ethanol required to maintain effective concentrations of citral and linalool in solution did not appear to impact the overall bacteriostatic and bactericidal effects of the oil on *E. coli.*

Citral and linalool are generally recognized as a safe food additive and are approved by the Food and Drug Administration for use in foods (Food and Drug Administration, 2018). In addition, they have been registered by the European Commission for use a flavoring in foodstuffs because its use does not present risk to the health of the consumer (Burt, 2004). Given the possibility of utilizing the oils as an intramammary therapy for *E. coli* mastitis, there may not be a need for milk withdrawal as there are few risks associated with ingestion of the oils. In regard to the cow, the oils do not appear to have any severe negative impacts on bovine immune cells either. In the current study, citral and linalool and the concentration of ethanol required to maintain them in solution were mildly cytotoxic toward bovine blood neutrophils, but did not negatively impact neutrophil oxidative burst response. Others have noted that citrus oils have little to no effect on mammary epithelial cells *in vitro*

either (Federman et al., 2016), suggesting that citrus oils and their components are not likely to harm nor interfere with cells in the bovine mammary gland.

Conclusion

Citral and linalool maintained much of their antimicrobial activity when dissolved in ethanol as compared to DMSO and were effective in milk as well as broth. In addition, citral and linalool did not cause severe harm or interference with bovine neutrophils. The ability of citral and linalool to inhibit proliferation of *E. coli* P4 planktonic cells and biofilms highlight their potential as alternative antimicrobial therapies for bovine mastitis.

Acknowledgements

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Chapter 4: Citral is an ineffective intramammary therapy for experimentally-induced *Escherichia coli* mastitis in dairy cows.

Introduction

Citrus oil is one of many well-known essential oils in the world with noted antimicrobial properties (Gomes and Henriques, 2016). The oil is produced by cells within the rind of lemon and orange fruit and is typically extracted as a by-product of juice production. Centrifugation produces a cold-pressed oil that is primarily terpenes with a varying composition that occurs as a result of regional and seasonal changes (Shaw and Coleman, 1974). Regardless of variation, limonene accounts for ~90% of citrus oil's composition with various alcohols, esters, aldehydes, hydrocarbons, ketones and oxides accounting for the remaining portion (Bauer et al., 2001; Moufida and Marzouk, 2003). Limonene has demonstrated antimicrobial activity against food-borne pathogens; however, citral and linalool have been identified as the active compounds contributing to the bactericidal activity of citrus oil (Fisher and Phillips, 2006).

Citral is chemically known as 3,7-dimethyl-2,6-octadienal. As a cyclic unsaturated monoterpene aldehyde, citral has two isomers, geranial and neral, that are commonly identified in the leaves and fruits of several plant species, including basil, lemon, lime, lemongrass, orange and bergamot (Hyldgaard et al., 2012). The strong, lemon odor of geranial and sweeter, more subtle lemon aroma of neral make citral a valuable compound that is widely used as a flavoring agent in food and drink and scent in perfumery (Nannapaneni et al., 2009; Choi et al., 2010). Beyond a scent and

flavor, citral has favorable anti-inflammatory (Ortiz et al., 2010) and anti-corrosive effects (Korenblum et al., 2013) in addition to the increasing evidence to its antibacterial (Onawunmi, 1989; Lu et al., 2016; Shi et al., 2016) and antifungal (De Bona Da Silva et al., 2008) properties.

Bovine mastitis, an inflammatory condition of the mammary gland, is often caused by bacterial intramammary infection and treated with antibiotic therapies directly infused into the mammary gland. Current available antibiotics have minimal effect on shortening the duration of many intramammary infections as some cases will spontaneously cure, while others result in recurrent clinical infections (Smith et al., 1985). With the growing national concern of antibiotic overuse, misuse and resistance, many have sought alternative approaches for controlling and treating mastitis in dairy cows. The bacteriostatic and bacteriocidal activity of citrus oils and its active component, citral, are natural antimicrobials that may serve as an alternative to antibiotic therapy. Citrus oils have demonstrated bacteriostatic and bactericidal activity toward mastitis pathogens, such as *Staph. aureus*, *E. coli,* and *Mycoplasma bovis* (Fisher and Phillips, 2006; Crandall et al., 2012; Muthaiyan et al., 2012; Federman et al., 2016) and effective concentrations do not appear to disrupt the bovine innate immune response (Garcia et al., 2015), nor harm bovine mammary epithelial cells (Federman et al., 2016). Our experiments with low concentrations of citral dissolved in ethanol have demonstrated similar results as compared to citrus oils emulsified in DMSO. Low concentrations of citral were nontoxic for bovine neutrophils and did not disrupt their oxidative burst response *in vitro*. *E. coli* P4 (O32:H37; EDL993), a strain isolated from a clinical mastitis case, was susceptible to

citral as low concentrations inhibited growth and eradicated the bacteria in broth and milk. Citral is also generally recognized as safe food additive and is approved by the Food and Drug Administration for use in foods (Food and Drug Administration, 2018). In addition, citral has been registered by the European Commission for use a flavoring in foodstuffs because its use does not present risk to the health of the consumer (Burt, 2004). Therefore, it is prudent to test citral as an antimicrobial therapy for bovine mastitis *in vivo*.

The objectives for the current study are to 1) determine the effects of citral as an intramammary therapy in healthy, multiparous dairy cows challenged with *E. coli* mastitis, and 2) compare the effectiveness of citral to a common intramammary antibiotic therapy (ceftiofur hydrochloride) for *E. coli* mastitis and no treatment. We hypothesize that citral will be an effective intramammary treatment for *E. coli* mastitis that will not impede the dairy cow.

Materials and Methods

Animals

All procedures involving the use of live animals were approved in accordance with the regulations and guidelines set forth by the University of Maryland Animal Care and Use Committee. Eighteen, healthy multiparous Holstein cows in mid-lactation (>100 DIM) were used for this study. All eligible cows displayed no signs of clinical disease, had no previous history of mastitis during the current lactation, and were free of sub-clinical mastitis (quarter SCC < 200,000 cells/mL) and had no

detectable bacterial growth (i.e. bacteriologically negative for culturable pathogens) in their milk prior to enrollment in the study.

Once eligible, cows were blocked based on parity and milk production, removed from the herd and housed in individual tie-stalls. Cows were fed twice daily a standard TMR formulated to meet requirements and 10% refusals. Cows had ad libitum access to water and were milked twice daily at 0700 and 1900 h. Eligible and enrolled study cows were quarantined from the rest of the herd and milked last in order to prevent the transfer of *E. coli* mastitis. After a one-week adjustment period, aseptic quarter milk samples were collected and analyzed again for SCC and bacteriological status to re-confirm eligibility.

Bacteria and Intramammary Inoculation

To reproduce clinical signs of mastitis, the strain used in this study was the *E. coli* strain P4, serotype O32:H37. The strain was maintained in a stock on lyophilization medium at -20°C. A stock of bacteria was sub-cultured at 37°C in brain-heart infusion broth (Sigma Aldrich, St. Louis, MO) for 6-8 h then transferred and cultured in TSB (Sigma Aldrich) for 18-24 h. Following incubation, inoculated TSB was stored at 4°C. Sub-cultures of TSB were diluted in pyrogen-free PBS and plated on MacConkey and 5% sheep blood agar plates to determine CFU per milliliter and detect possible contamination. Immediately before intramammary inoculation, a final concentration of 200 CFU/mL bacterial suspension was prepared in pyrogen-free PBS. The total volume of inoculant was 4 mL (~800 CFU total).

Intramammary inoculation with the bacterial suspension was performed immediately following morning milking (d 0). One rear quarter was randomly chosen

per eligible cow to be inoculated. The teat end from this quarter was rigorously cleaned with cotton balls wetted with 70% ethanol. The bacterial suspension was infused into the rear quarter via a sterile, disposable syringe fitted with a sterile teat cannula using the partial insertion method as recommended by the National Mastitis Council (National Mastitis Council, 2004). Immediately following inoculation, teats were immersed in a teat dip cup post-milking teat disinfectant containing 1% iodine with lanolin.

Intramammary Treatment

Infected quarters were randomly assigned to an intramammary experimental therapy. After infection was established (24 h post-inoculation, d 1), each inoculated rear quarter (n = 6 quarters/treatment) was randomly assigned to receive 1 of 3 treatments following recommendations of the National Mastitis Council (National Mastitis Council, 2004) for 4 consecutive d that included either:

1) 0% citral (100% sterile PBS; CON) in 10 mL administered 2X/d,
2) 1.0% citral (90% PBS, 9% ethanol, 1% citral vol/vol; 8,930 µg citral; OO) in 10 mL administered 2X/d, or
3) ceftiofur hydrochloride (125 mg; Spectramast LC, Zoetis, Parsippany-Troy Hills, NJ; AB) in 10 mL administered 1X/d, as per label instruction (morning milking only).

Ceftiofur hydrochloride is an antibiotic commonly used to treat *E. coli* bovine mastitis in lactating dairy cows. The concentration of citral used in the current study was determined in experiments conducted in Chapter 4 that analyzed MIC, MBC, and MBEC *in vitro*, in addition to effects on isolated bovine neutrophils.

Prior to administration of the treatments, teats were rigorously cleaned with cotton balls wetted with 70% ethanol. All treatments were administered using the partial insertion method. Immediately following treatment, teats were immersed in a post-milking teat disinfectant containing 1% iodine with lanolin.

Health Examinations

Physical exams (including heart rate, respiration rate, rectal temperature, appetite, dehydration, lethargy, posture, and diarrhea), mammary palpation exams (including udder swelling, redness, and soreness) and clinical score were performed daily for 7 consecutive d post-inoculation, then once per week until 35 d. Clinical score was recorded on a 5-point scale (National Mastitis Council, 2004):

1 = normal milk and normal quarter,

2 = normal quarter but milk was questionable,

3 = normal quarter but abnormal milk,

4 = a swollen quarter and abnormal milk, and

5 = swollen quarter, abnormal milk, and systemic signs of infection.

Sampling Procedure

Feed intake, feed refusals, and milk production were recorded daily. Quarter foremilk samples were collected from all quarters during the morning milking on d -7, -4 and 0 prior to inoculation and d 7 and 35 post inoculation to identify bacterial status. Milk samples were collected twice daily (prior to morning and afternoon milkings) from the infected rear quarter for 7 consecutive d following inoculation (d 0), then once weekly until d 35 post inoculation for assessment of bacteriological

cure. Half units for d indicate samples taken during afternoon milkings. Milk samples were analyzed for concentrations of fat, protein, other solids and somatic cells by Lancaster DHIA (Manheim, PA). SCS was calculated by $\log_2(SCC/100) + 3$ where SCC is in units of 1,000 cells/mL.

Milk Culture

Quarter foremilk samples for culture were collected aseptically according to National Mastitis Council (2004) recommendations. These samples (100 µL) were plated using the swirl plate method onto tryptic soy agar containing 5% sheep's blood and MacConkey agar plates using a sterile, disposable L-shaped cell spreader. If needed, the samples were serially diluted with sterile, endotoxin-free PBS to facilitate colony counting. Milk was allowed to dry before inverting the plates. Inoculated plates were incubated at 37°C for a maximum of 48 h and analyzed for the presence of mastitis-causing pathogens (National Mastitis Council, 2004). Colonies of *E. coli* were counted and bacterial concentrations in milk were calculated. A minimum of 3 CFU will be used as the criteria to identify a positive isolation for any one pathogen; three or more types of pathogens isolated from the same culture were identified as contaminated. An infection was considered to be present if the sample contained greater than 1 CFU/mL of *E. coli* in 2 of 3 consecutive samples.

Statistical Analysis

Experimental units and the corresponding sample size of cows that were used in this study have been determined using the POWER procedure of SAS (SAS/STAT version 9.4; SAS Institute Inc., Cary, NC) for a one-way analysis of variance. Means

values for log10 SCC data were calculated from a previously conducted pilot study. The following are parameters included in the procedure: 1) an alpha set at 0.05 to declare significance, 2) an 85% probability of detecting a difference in treatment means, and 3) a projected standard deviation value of 0.3. Based on the results of the power analyses, it was concluded that at least 5 cows per treatment are required for this study. Studies with similar objectives have reported a similar number of biological replicates (Oldham and Daley, 1991). To be conservative and considerate of the high variance between cow responses during a mastitis challenge, at least 6 cows were assigned to each treatment.

Response variables were analyzed as a complete randomized design using the MIXED procedure of SAS (SAS/STAT version 9.4; SAS Institute Inc., Cary, NC) with the repeated measure of d relative to intramammary *E. coli* challenge (d 0). Milk CFU of *E. coli* were transformed to log10 values for statistical analysis. Sources of variation within the model included treatment, time relative to the intramammary challenge and the interaction of treatment across time. Separation of least square means for significant effects was accomplished using the PDIFF option in the MIXED procedure. Chi-square analysis was used to test the significance of differences in the number of quarters cured in each treatment group at d 35 following inoculation by utilizing the GENMOD procedure and LOGIT model of SAS (SAS/STAT version 9.3; Oldham and Daley, 1991). Statistical differences were declared as significant at $P < 0.05$ and trends toward significance are discussed at $0.05 < P < 0.10$.

Results and Discussion

All eighteen cows were successfully infected with *E. coli* P4 and displayed clinical symptoms within 12 h following inoculation. Incubation of the prepared inoculum revealed that cows were inoculated with an average of 837 CFU total. Milk CFU was log10 transformed and not affected by treatment over the 35 d sampling period (P = 0.56), or first week post challenge (P = 0.36). Milk CFU concentrations were similar to previous studies utilizing *E. coli* strain P4 as an experimental mastitis challenge (Herry et al., 2017). Differences between the AB treatment (ceftiofur hydrochloride), OO treatment (citral) and CON treatment were expected, but not observed in the current study. The antibiotic treated cows had numerically lower milk bacteria count as compared to the cows receiving the control treatment (Figure 4.1). This suggests that the experimental mastitis challenge model was likely successful and would have revealed a treatment effect with a greater number of cows assigned to the study. Many *E. coli* mastitis challenge trials utilized more than 10 cows per treatment and observed treatment effects between the experimental treatment and control (Shpigel et al., 1997; Vangroenweghe et al., 2004a; b). With greater availability to resources, a similar occurrence may have been observed in the present study.

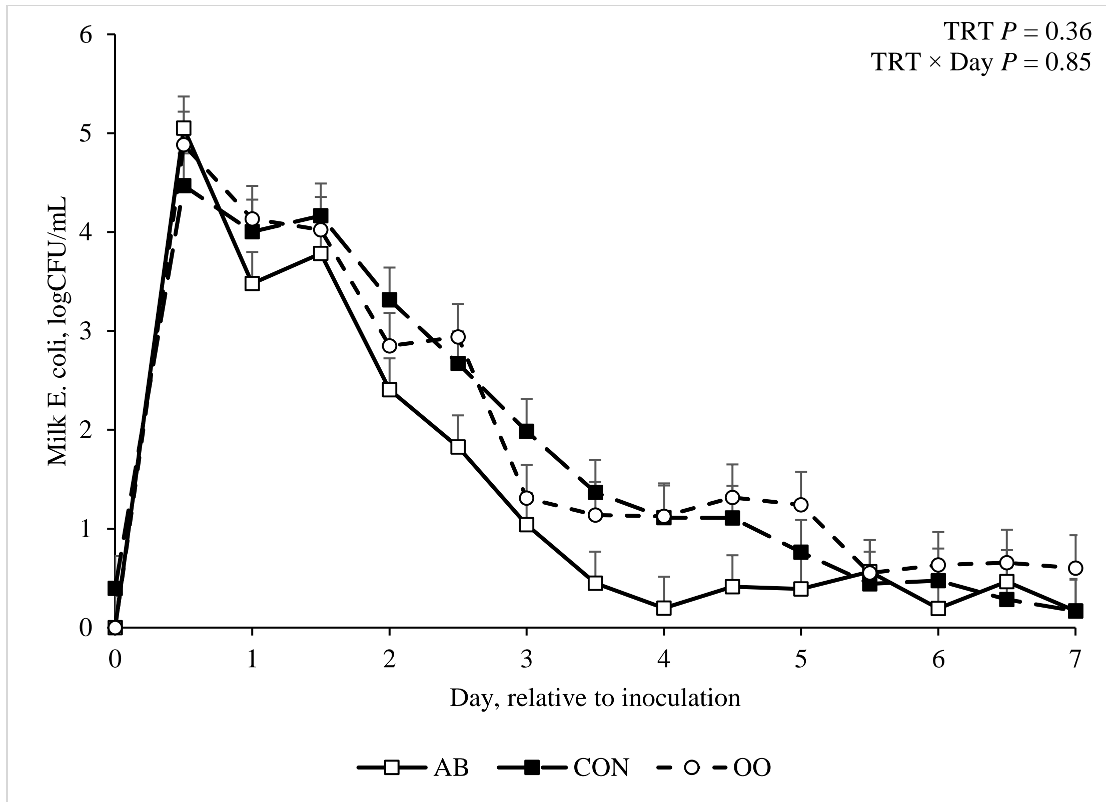

Figure 4.1: Least square means and standard errors for log10-transformed CFU/mL of *Escherichia coli* strain P4 in milk from infected rear quarters following inoculation (d 0). Treatment was implemented 24 h post inoculation with one of three treatments for 4 consecutive days: 125 mg ceftiofur hydrochloride daily (AB), sterile saline twice daily (CON), or 8,930 µg citral (1% vol/vol) twice daily (OO).

Observed local and systemic signs in the present study were similar to those described by others using the same model and could not be differentiated from natural cases of clinical *E. coli* mastitis (Hill, 1981; Lohuis et al., 1990; Pyorala et al., 1994; Shpigel et al., 1997). Somatic cell count quickly rose and peaked between d 1 and 2 following inoculation. Following the onset of treatments on d 1 post inoculation, SCC differed by treatment across time (P = 0.06; Figure 4.2). Afternoon milking samples (0.5 d units) during the treatment period, d 1-4, tended to vary by treatment (P = 0.01; Table 4.2). The AB treatment tended to decrease SCC as compared to OO and CON on d 1.5. In addition, OO tended to decrease SCC as compared to AB and CON on d 2.5 and 3.5.

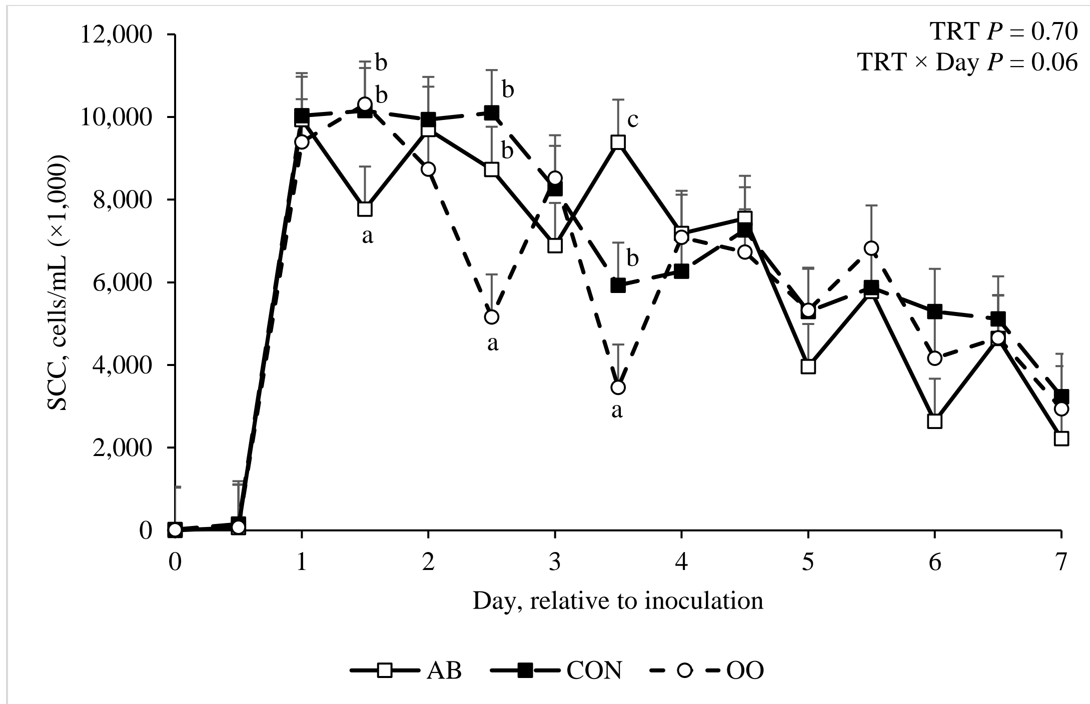

Figure 4.2: Least square means and standard errors for milk somatic cell count (SCC, cells/mL ×1,000) from infected rear quarters following inoculation (d 0; ~800 CFU *Escherichia coli* P4). Treatment was implemented 24 h post inoculation with one of three treatments for 4 consecutive days: 125 mg ceftiofur hydrochloride daily (AB), sterile saline twice daily (CON), or 8,930 µg citral (1% vol/vol) twice daily (OO). a-c Means with different superscripts tend to differ ($P < 0.10$).

The anti-inflammatory properties of citral may have decreased SCC in milk following the administration of the treatment following the morning milking on d 2 and 3. Systemic administration of citral in rats decreased inflammation and edema in paws irritated by a carrageenan injection (Ortiz et al., 2010) and decreased body temperature following an injection of LPS (Carlini et al., 1986). A potential mechanism may be through a reduced inflammatory state for cells. Macrophages are resident immune cells in the bovine mammary gland that, when activated, are essential for neutrophil recruitment (Rainard and Riollet, 2003b). In LPS stimulated murine macrophages, citral reduced nitric oxide and tumor necrosis factor α production and inhibited inducible nitric oxide synthase, cyclooxygenase 2 and nuclear factor kappa B protein expression (Lin et al., 2008). The activation of nuclear factor kappa B is a major signaling pathway for cytokine and chemokine production that drive the onset of the mammary inflammatory cascade (Boulanger et al., 2003; Bannerman, 2009). Reduced inflammation and immune cell recruitment and activity is not necessarily wanted in *E. coli* mastitis as cure rate and animal survival is highly correlated with the rate of neutrophil recruitment and inflammatory response (Burvenich et al., 2003). However, the anti-inflammatory effects of citral may be useful when cows are in chronic inflammatory states if they can be sustained. The inflammatory effects on SCC in the present study were not sustained and only observed in the afternoon milking. The short period of time (10 h) between treatment administration following the morning milking and afternoon sampling suggest that the anti-inflammatory effects are transient. Citral degrades rapidly in acidic environments, such as mastitic milk, and is quickly metabolized (PubChem, 2004).

The anti-inflammatory effects may have also been diluted as milk production between afternoon to morning milk sampling was numerically greater (5-7 kg) than the period between morning and afternoon milk sampling (Table 4.1).

Table 4.1: Dry matter intake, and milk production and characteristics among treatment groups for week 1 following inoculation. Cows were infected with ~800 CFU of *Escherichia coli* P4 into one rear mammary quarter and treated 24 h later for 4 consecutive days.

	Treatment[1]				*P*-value	
Item	AB	CON	OO	SEM	TRT	TRT × Day
Dry Matter Intake, kg/d[2]	23.5	21.8	20.9	1.3	0.37	0.29
Milk Production, kg/d						
Morning	17.7	16.8	16.2	1.5	0.76	0.02
Afternoon	11.2	11.7	9.8	0.9	0.27	0.01
Daily Total	30.0	27.0	26.4	2.3	0.52	0.78
Milk Components, %						
Fat	4.04	4.48	4.02	0.53	0.79	0.82
Protein	3.28	3.51	3.59	0.15	0.35	0.63
OS	5.09	5.04	5.00	0.10	0.82	0.84

[1]Treatments: AB = 125 mg ceftiofur hydrochloride, 1×/day; CON = sterile, endotoxin-free buffered solution, 2×/day; OO = 8,930 μg citral (1% vol/vol), 2×/day

Other intramammary characteristics did not differ across treatments. Clinical score, SCS and the ratio of logCFU and SCC did not vary by treatment and treatment across time (Table 4.2). The severity of infection, quantified through the ratio of logCFU and SCS, varied by treatment across time ($P = 0.01$; Table 4.2) for the first week following inoculation; however, there were no discernable trends of the ratio over time by treatment. Mammary and milk characteristics were similar in related *E. coli* intramammary challenges utilizing strain P4 (Blum et al., 2017).

Table 4.2: Intramammary infection characteristics among treatment groups for week 1 following inoculation. Cows were infected with ~800 CFU of *Escherichia coli* P4 into one rear mammary quarter and treated 24 h later for 4 days.

	Treatment[1]				*P*-value	
Item	AB	CON	OO	SEM	TRT	TRT × Day
Clinical Score[2]	2.75	2.85	3.00	0.15	0.46	0.73
logCFU[3]	1.36	1.78	1.83	0.25	0.36	0.85
SCC[4]	5,800	6,380	5,620	670	0.70	0.06
SCS[5]	7.84	7.98	7.69	0.24	0.69	0.98
SCC ratio[6]	0.007	0.012	0.010	0.004	0.68	0.99
SCS ratio[7]	0.289	0.307	0.300	0.044	0.95	0.01

[1]Treatments: AB = 125 mg ceftiofur hydrochloride, 1×/day; CON = sterile, endotoxin-free buffered solution, 2×/day; OO = 8,930 µg citral (1% vol/vol), 2×/day

[2]Score Range 1-5 (National Mastitis Council, 2004):

1 = normal milk and normal quarter,
2 = normal quarter but milk was questionable,
3 = normal quarter but abnormal milk,
4 = a swollen quarter and abnormal milk, and
5 = swollen quarter, abnormal milk, and systemic signs of infection.

[3]*Escherichia coli* $\log_{10}$(CFU/mL)

[4]Somatic Cell Count, cells/mL (×1,000)

[5]Somatic Cell Score

[6]SCC ratio = $\log_{10}$(CFU/mL) / SCC

[7]SCS ratio = $\log_{10}$(CFU/mL) / SCS

Dry matter intake, milk production and milk components were not affected by treatments. Cows treated with AB numerically ate more feed across the sample period. Subsequently, cows also produced more milk as compared to CON and OO treated cows in the first week and across the 35 d sampling period (Table 4.1; Figure 4.3). Similar results regarding milk production, milk components and DMI were observed in related experimental *E. coli* mastitis challenge utilizing strain P4 (Blum et al., 2017)

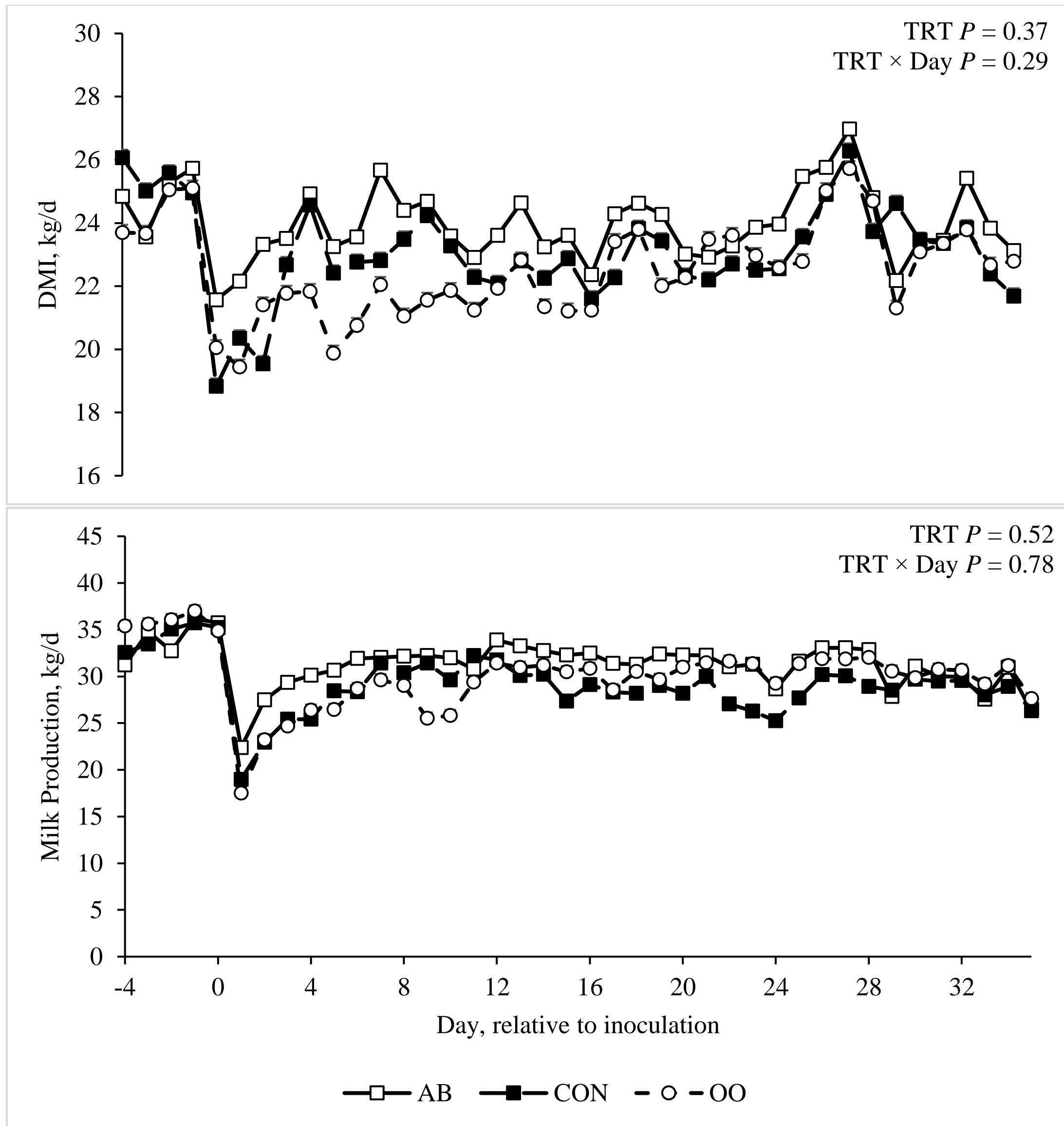

Figure 4.3: Least square means and standard errors for A) dry matter intake (DMI), kg/d, and B) milk production, kg/d, from cows with an infected rear quarter following inoculation (d 0; ~800 CFU *Escherichia coli* P4). Treatment was implemented 24 h post inoculation with one of three treatments for 4 consecutive days: 125 mg ceftiofur hydrochloride daily (AB), sterile saline twice daily (CON), or 8,930 µg citral (1% vol/vol) twice daily (OO).

Clinical characteristics of infected cows tended to vary for heart rate across treatment ($P = 0.09$; Table 4.3; Appendix 1). Heart rate (beats/minute) for AB treated cows was slower than the rate for cows receiving CON treatment. Biologically speaking, the difference in heart rate between AB and CON treatments is negligible; however, increases in heart rate are positively correlated with severity of the intramammary infection when cows are experimentally challenged with *E. coli* (Lohuis et al., 1990). Fever (i.e., rectal temperature > 39.4°C) is typically observed within the first 24 hours of cows challenged with *E. coli* mastitis (Bannerman et al., 2004). Blum and associates (2017) observed similar results to the current study as cows challenged with strain P4 did not have elevated rectal temperatures or fever following inoculation (Appendix 2). Respiration rate did not vary across treatment or time (Appendix 3).

Table 4.3: Clinical characteristics among treatment groups following inoculation through day 35 post inoculation. Cows were infected with ~800 CFU of *Escherichia coli* P4 into one rear mammary quarter and treated 24 h later for 4 consecutive days.

	Treatment[1]				*P*-value	
Item	AB	CON	OO	SEM	TRT	TRT × Day
Heart Rate, beats/minute	70.3[b]	73.7[a]	71.8[ab]	1.0	0.09	0.23
Respiration Rate, breaths/minute	47.9	53.3	49.6	2.3	0.27	0.43
Rectal Temperature, °C	38.4	38.4	38.4	0.7	0.69	0.82

[a-b]Means within a row with different superscripts differ ($P < 0.05$).

[1]Treatments: AB = 125 mg ceftiofur hydrochloride, 1×/day; CON = sterile, endotoxin-free buffered solution, 2×/day; OO = 8,930 µg citral (1% vol/vol), 2×/day

Mastitis cure has been defined many ways over the years and a variety of parameters and measurements can be taken into consideration. The National Mastitis Council (2004) defines healthy as milk samples that have SCC < 200,000 cells/mL, test negative for cultureable, pathogenic bacteria and have normal milk and udder (clinical score = 0). Days to reach these parameters following inoculation did not vary by treatment. Again, AB treatment was numerically lower than CON and OO and OO was similar to CON treatment (Table 4.4). Another common cure parameter for comparing antimicrobial therapy effectiveness assesses infected quarter bacteriological status post inoculation. An inoculated quarter is typically considered cured when at least two subsequent samples test negative for the inoculated bacterium at the end of the milk sampling period, typically 14-35 d post inoculation (Oldham and Daley, 1991; Shpigel et al., 1997). Re-occurrence of infection during this period is typically considered to be caused by the bacterium from the initial inoculation (National Mastitis Council, 2004). In the current study, no differences between treatments for bacteriological cure at d 14 ($\chi^2 = 0.97$) and d 35 ($\chi^2 = 0.32$) were detected.

Table 4.4: Number of days post inoculation to reach cure parameters for two consecutive samples. Cows were infected with ~800 CFU of *Escherichia coli* P4 into one rear mammary quarter and treated 24 h later for 4 consecutive days.

	Treatment[1]				
	AB	CON	OO	SEM	*P*-value TRT
SCC < 200,000 cells/mL[2]	21.0	22.8	21.0	4.9	0.92
logCFU = 0 [3]	3.3	4.4	5.5	1.1	0.38
Clinical Score = 0	8.8	12.8	12.8	2.7	0.50

[1]Treatments: AB = 125 mg ceftiofur hydrochloride, 1×/day; CON = sterile, endotoxin-free buffered solution, 2×/day; OO = 8,930 µg citral (1% vol/vol), 2×/day
[2]Infected quarter somatic cell count
[3]*Escherichia coli* $\log_{10}$(CFU/mL)

Conclusion

In conclusion, citral did not differ from antibiotic treatment or sterile, buffered solution. Although citral exhibits low MIC and MBC *in vitro* and effective antimicrobial activity against *E. coli* in milk, it was ineffective as a therapy to cure experimentally-induced *E. coli* mastitis. Citral exhibited some anti-inflammatory effects in the udder through reduced SCC in milk. However, reduced SCC during *E. coli* mastitis may not be desirable. The antimicrobial and anti-inflammatory effects of citral may be better suited for use as a topical disinfectant within the dairy industry.

Acknowledgements

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Summary and Conclusion

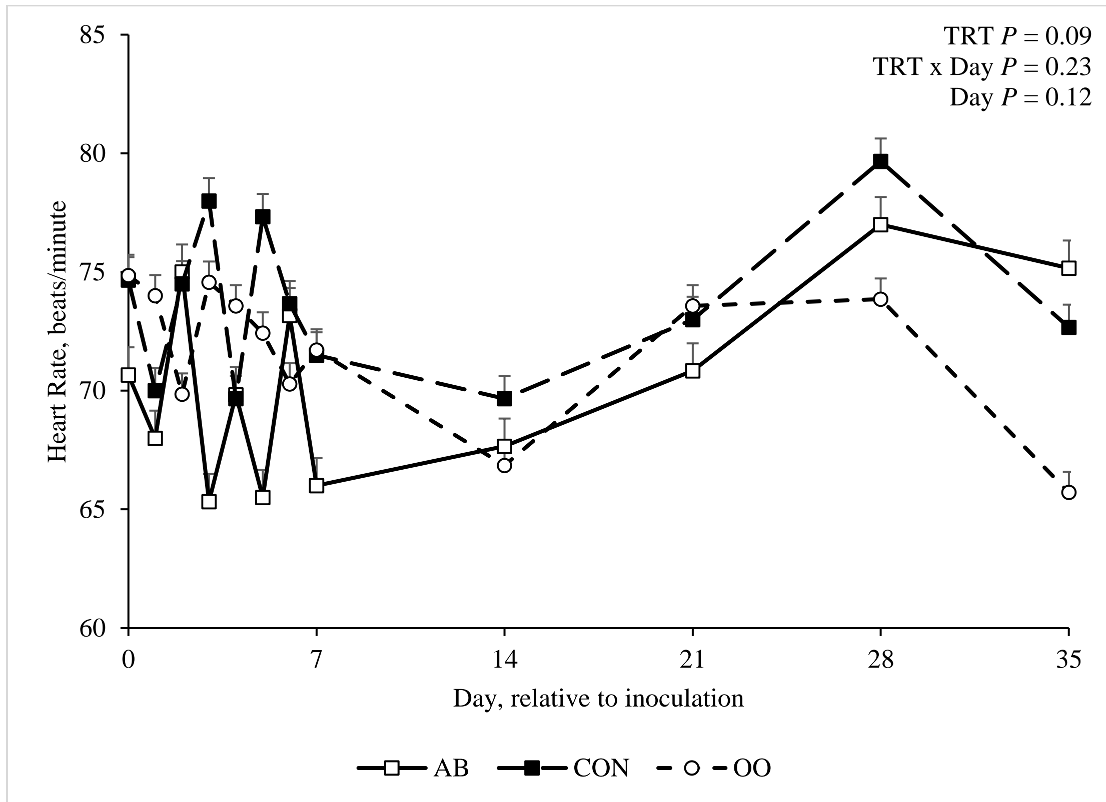
This dissertation explored the possible use of PlyC and citrus oils as alternatives to antibiotic therapies for bovine mastitis. Varying doses of PlyC are non-toxic and do not alter ROS production in bovine neutrophils. The use of PlyC as an alternative therapy for *Strep. uberis* mastitis is promising where PlyC may not interfere with immune response during mastitis as neutrophils are the primary cell initially recruited during the onset of mastitis. Citrus oils demonstrated as equally promising results. The essential oil and its components were dissolved in ethanol to maintain the oils in solution. Previously, similar research had been conducted with the oils dissolved in the industrial solvent, DMSO; however, use of DMSO is forbidden in the U.S. dairy industry. Components of citrus oils, citral and linalool, demonstrated greater antimicrobial activity against isolated, environmental bovine mastitis pathogen *E. coli* strain P4 than the essential oil in both broth and bovine milk for planktonic growth and established biofilm eradication. In addition, citral and linalool will likely not interfere with the immune response of the mammary gland during mastitis as both did not inhibit neutrophil oxidative burst response and were negligibly toxic. The concentrations required to maintain the oils in solution were also tested and did not impact the antimicrobial or immune results.

Citral looked the most promising of the components of citrus oils as it required lower doses to induce antimicrobial effects and demonstrated equal effects toward bovine neutrophils as the other active component, linalool. It was prudent to test the effectiveness of citral against *E. coli* in an experimentally-induced, mastitis challenge. Unfortunately, citral did not differ from the antibiotic treatment (ceftiofur

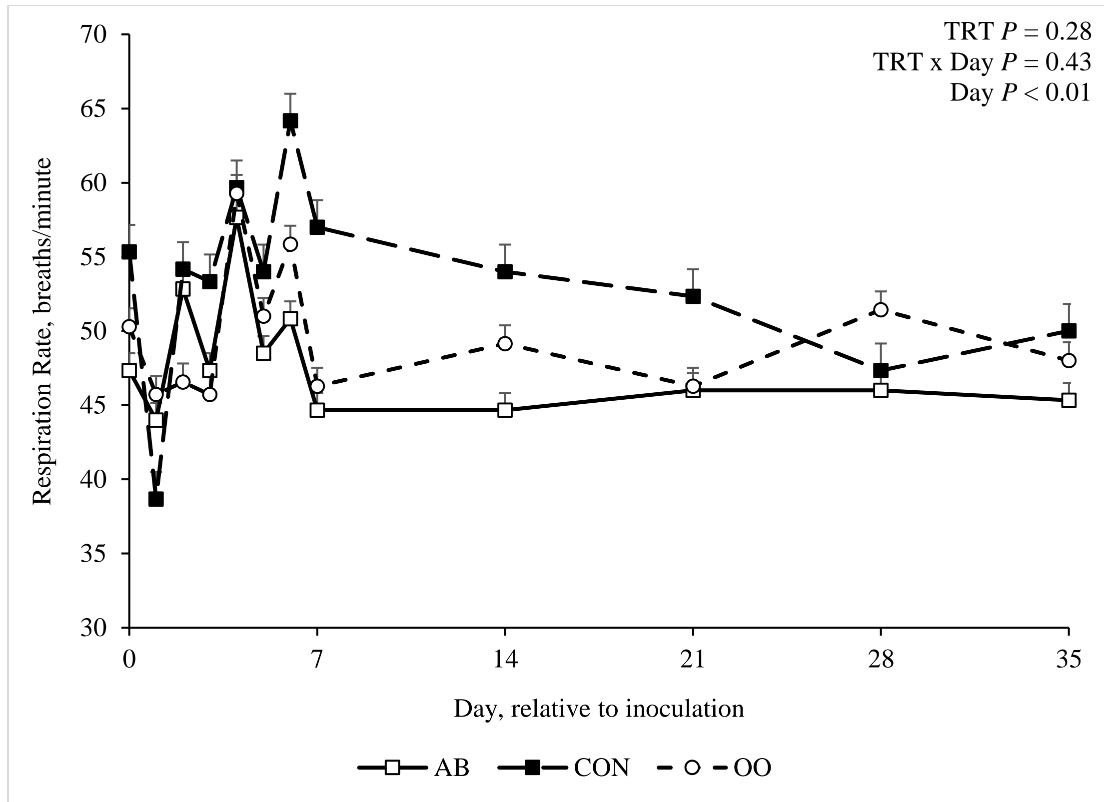
hydrochloride), or control (sterile, endotoxin-free buffered solution). Citral did demonstrate some anti-inflammatory effects through reduced SCC during the treatment period; however, effects were transient and unlikely to benefit as a therapeutic for *E. coli* bovine mastitis.

In conclusion, lysin PlyC is a promising alternative to antibiotics for treatment and management of bovine mastitis caused by streptococcal species. The future directions for PlyC will likely involve testing the curative effects of PlyC against experimentally-induced bovine mastitis. On the other hand, citrus oils and specifically citral appear to have greater antimicrobial effects against bacteria *in vitro* and in the food safety industry when compared to an *in vivo* bovine mammary challenge with *E. coli*.

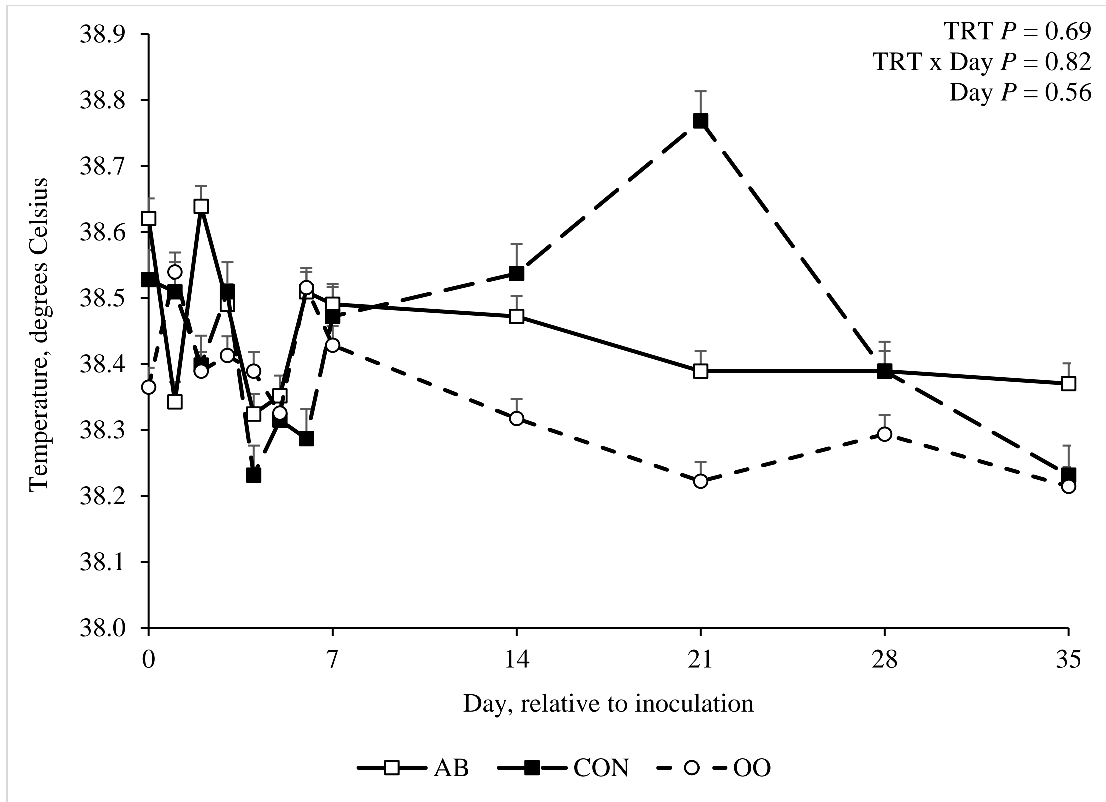
Appendices



Appendix 1: Least square means and standard errors for heart rate (beats/minute) from cows with an infected rear quarter following inoculation (d 0; ~800 CFU *Escherichia coli* P4). Treatment was implemented 24 h post inoculation with one of three treatments for 4 consecutive days: 125 mg ceftiofur hydrochloride daily (AB), sterile saline twice daily (CON), or 8,930 µg citral (1% vol/vol) twice daily (OO).



Appendix 2: Least square means and standard errors for respiration rate (breaths/minute) from cows with an infected rear quarter following inoculation (d 0; ~800 CFU *Escherichia coli* P4). Treatment was implemented 24 h post inoculation with one of three treatments for 4 consecutive days: 125 mg ceftiofur hydrochloride daily (AB), sterile saline twice daily (CON), or 8,930 µg citral (1% vol/vol) twice daily (OO).



Appendix 3: Least square means and standard errors for rectal temperature (degrees Celsius) from cows with an infected rear quarter following inoculation (d 0; ~800 CFU *Escherichia coli* P4). Treatment was implemented 24 h post inoculation with one of three treatments for 4 consecutive days: 125 mg ceftiofur hydrochloride daily (AB), sterile saline twice daily (CON), or 8,930 µg citral (1% vol/vol) twice daily (OO).

Bibliography

Aiemsaard, J., S. Aiumlamai, C. Aromdee, S. Taweechaisupapong, and W. Khunkitti. 2011. The effect of lemongrass oil and its major components on clinical isolate mastitis pathogens and their mechanisms of action on Staphylococcus aureus DMST 4745. Res. Vet. Sci.. doi:10.1016/j.rvsc.2011.01.012.

Almeida, R.A., D.A. Luther, H.-M. Park, and S.P. Oliver. 2006. Identification, isolation, and partial characterization of a novel Streptococcus uberis adhesion molecule (SUAM). Vet. Microbiol. 115:183–191. doi:10.1016/j.vetmic.2006.02.005.

Ansel, H.C., W.P. Norred, and I.L. Roth. 1969. Antimicrobial Activity of Dimethyl Sulfoxide Against Escherichia coli, Pseudomonas aeruginosa, and Bacillus megaterium. J. Pharm. Sci. 58:836–839.

Bannantine, J.P., S.C. Olsen, M.E. Kehrli, T.B. Stanton, E. Casas, D.L. Whipple, and K.A. Zuelke. 2013. High-impact animal health research conducted at the USDA's National Animal Disease Center. doi:10.1016/j.vetmic.2013.04.010.

Bannerman, D.D. 2009. Pathogen-dependent induction of cytokines and other soluble inflammatory mediators during intramammary infection of dairy cows. J. Anim. Sci. 87:10–25. doi:10.2527/jas.2008-1187.

Bannerman, D.D., M.J. Paape, J. Lee, X. Zhao, J.C. Hope, and P. Rainard. 2004. Escherichia coli and Staphylococcus aureus elicit differential innate immune responses following intramammary infection. Clin. Diagn. Lab. Immunol. 11:463–472. doi:10.1128/CDLI.11.3.463–472.2004.

Barkema, H.W., Y.H. Schukken, T.J.G.M. Lam, M.L. Beiboer, H. Wilmink, , G Benedictus, and A. Brand. 1998. Incidence of Clinical Mastitis in Dairy Herds Grouped in Three Categories by Bulk Milk Somatic Cell Counts.

Baskaran, S.A., G.W. Kazmer, L. Hinckley, S.M. Andrew, and K. Venkitanarayanan. 2009. Antibacterial effect of plant-derived antimicrobials on major bacterial mastitis pathogens in vitro. J. Dairy Sci. 92:1423–1429. doi:10.3168/jds.2008-1384.

Bauer, K., D. Garbe, and H. Surburg. 2001. Common Fragrance and Flavor Materials. 4th ed. VCH.

Blowey, R., and P. Edmondson. 2010. Mastitis Control in Dairy Herds. 2nd ed. Butler Tanner & Dennis, Oxfordshire, OX, UK.

Blum, S.E., E.D. Heller, S. Jacoby, O. Krifucks, and G. Leitner. 2017. Comparison of the immune responses associated with experimental bovine mastitis caused by different strains of Escherichia coli. J. Dairy Res. 84:190–197. doi:10.1017/S0022029917000206.

De Bona Da Silva, C., S.S. Guterres, V. Weisheimer, and E.E.S. Schapoval. 2008.

Antifungal Activity of the Lemongrass Oil and Citral Against Candida spp. BJID Brazilian J. Infect. Dis. 1212:63–66.

Boudjellab, N., H.S. Chan-Tang, X. Li, and X. Zhao. 1998. Interleukin 8 response by bovine mammary epithelial cells to lipopolysaccharide stimulation. Am. J. Vet. Res. 59:1563–1567.

Boulanger, D., F. Bureau, D. Mé, J. Mainil, P. Lekeux, and D. Boulanger. 2003. Increased Nuclear Factor κB Activity in Milk Cells of Mastitis-Affected Cows. J. Dairy Sci 86:1259–1267. doi:10.3168/jds.S0022-0302(03)73710-2.

Bradley, A.J., K.A. Leach, J.E. Breen, L.E. Green, and M.J. Green. 2007. Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales. Vet. Rec. 253–258.

Bramley, A.J. 1976. Variations in the susceptibility of lactating and non-lactating bovine udders to infection when infused with Escherichia coli. J. Dairy Res.. doi:10.1017/S0022029900015752.

Brownlie, J., C.J. Howard, and R.N. Gourlay. 1979. The Effect of an Intramammary Infusion of Endotoxin on Experimentally Induced Mycoplasmal Mastitis.

Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods—a review. Int. J. Food Microbiol. 94:223–253. doi:10.1016/j.ijfoodmicro.2004.03.022.

Burvenich, C., V. Van Merris, J. Mehrzad, A. Diez-Fraile, and L. Duchateau. 2003. Severity of E. coli mastitis is mainly determined by cow factors. Vet. Res 34:521–564. doi:10.1051/vetres:2003023.

Burvenich, C., M.J. Paape, A.W. Hill, A.J. Guidry, R.H. Miller, R. Heyneman, W.D. Kremer, and A. Brand. 1994. Role of the neutrophil leucocyte in the local and systemic reactions during experimentally induced E. coli mastitis in cows immediately after calving.. Vet. Q. 16:45–50. doi:10.1080/01652176.1994.9694416.

Cardozo, V.F., C.A.C. Lancheros, A.M. Narciso, E.C.S. Valereto, R.K.T. Kobayashi, A.B. Seabra, and G. Nakazato. 2014. Pharmaceutical nanotechnology Evaluation of antibacterial activity of nitric oxide-releasing polymeric particles against Staphylococcus aureus and Escherichia coli from bovine mastitis. Int. J. Pharm. 473:20–29. doi:10.1016/j.ijpharm.2014.06.051.

Carlini, E.A., J. De D.P. Contar, A.R. Silva-Filho, N.G. Da Silveira-Filho, M.L. Frochtengarten, and O.F.A. Bueno. 1986. Pharmacology of lemongrass (Cymbopogon citratus Stapf). I. Effects of teas prepared from the leaves on laboratory animals. J. Ethnopharmacol. 17:37–64. doi:10.1016/0378-8741(86)90072-3.

Ceri, H., M.E. Olson, C. Stremick, R.R. Read, D. Morck, and A.A. Buret. 1999. The Calgary Biofilm Device: New Technology for Rapid Determination of Antibiotic Susceptibilities of Bacterial Biofilms. J. Clin. Microbiol. 37:1771–

1776.

Cha, E., D. Bar, J.A. Hertl, L.W. Tauer, G. Bennett, R.N. González, Y.H. Schukken, F.L. Welcome, and Y.T. Gröhn. 2011. The cost and management of different types of clinical mastitis in dairy cows estimated by dynamic programming. J. Dairy Sci.. doi:10.3168/jds.2010-4123.

Chandra, H., P. Bishnoi, A. Yadav, B. Patni, A. Mishra, and A. Nautiyal. 2017. Antimicrobial Resistance and the Alternative Resources with Special Emphasis on Plant-Based Antimicrobials—A Review. Plants. doi:10.3390/plants6020016.

Choi, S.J., E.A. Decker, L. Henson, L.M. Popplewell, and D.J. Mcclements. 2010. Inhibition of citral degradation in model beverage emulsions using micelles and reverse micelles. Food Chem. 122:111–116. doi:10.1016/j.foodchem.2010.02.025.

Clerya, M., A. Leite, A. Parente De Brito Bezerra, J. Pereira De Sousa, F. Queiroga, S. Guerra, E. De, and O. Lima. 2014. Evaluation of Antifungal Activity and Mechanism of Action of Citral against Candida albicans. doi:10.1155/2014/378280.

Costa, J.C.M., I. de F. Espeschit, F.A. Pieri, L.A. Benjamin, and M.A.S. Moreira. 2014. Increase in biofilm formation by Escherichia coli under conditions that mimic the mastitic mammary gland. Ciência Rural 44:666–671. doi:10.1590/S0103-84782014000400015.

Crandall, P.G., S.C. Ricke, C. O'Bryan, and N. Parrish. 2012. In vitro effects of citrus oils against Mycobacterium tuberculosis and non-tuberculous Mycobacteria of clinical importance.. J Env. Sci Heal. B 47:736–741.

De Cueninck, B.J. 1979. Immune-mediated inflammation in the lumen of the bovine mammary gland.. Int. Arch. Allergy Appl. Immunol. 59:394–402.

Dabbah, R., V.M. Edwards, and A.W.A. Moats. 1970. Antimicrobial Action of Some Citrus Fruit Oils on Selected Food-Borne Bacteria. APPLED Microbiol. 19:27–31.

Dehkordi, S.H., F. Hosseinpour, and A.E. Kahrizangi. 2011. An in vitro evaluation of antibacterial effect of silver nanoparticles on Staphylococcus aureus isolated from bovine subclinical mastitis. African J. Biotechnol. 10:10795–10797. doi:10.5897/AJB11.1499.

Duarte, A., Â. Luís, M. Oleastro, and F.C. Domingues. 2016. Antioxidant properties of coriander essential oil and linalool and their potential to control Campylobacter spp.. Food Control 61:115–122. doi:10.1016/j.foodcont.2015.09.033.

Erskine, R.J. 2016. Mastitis in Cattle. Accessed. http://www.merckvetmanual.com/reproductive-system/mastitis-in-large-animals/mastitis-in-cattle.

Erskine, R.J., R.D. Walker, C.A. Bolin, P.C. Bartlett, and D.G. White. 2002. Trends in Antibacterial Susceptibility of Mastitis Pathogens During a Seven-Year Period. J. Dairy Sci. 85:1111–1118. doi:10.3168/jds.S0022-0302(02)74172-6.

Federman, C., J. Joo, J.A. Almario, S. Salaheen, and D. Biswas. 2016. Citrus-derived oil inhibits Staphylococcus aureus growth and alters its interactions with bovine mammary cells. J. Dairy Sci. 99:3667–3674. doi:10.3168/jds.2015-10538.

Fischetti, V.A. 2006. Using phage lytic enzymes to control pathogenic bacteria. Page Suppl 1:S16 in BMC Oral Health.

Fischetti, V.A. 2010. Bacteriophage endolysins: A novel anti-infective to control Gram-positive pathogens. Int. J. Med. Microbiol. 300:357–362. doi:10.1016/j.ijmm.2010.04.002.

Fisher, K., and C. Phillips. 2008. Potential antimicrobial uses of essential oils in food: is citrus the answer?. Trends Food Sci. Technol. 19:156–164. doi:10.1016/j.tifs.2007.11.006.

Fisher, K., and C.A. Phillips. 2006. The effect of lemon, orange and bergamot essential oils and their components on the survival of Campylobacter jejuni, Escherichia coli O157, Listeria monocytogenes, Bacillus cereus and Staphylococcus aureus in vitro and in food systems. J. Appl. Microbiol. 101:1232–1240. doi:10.1111/j.1365-2672.2006.03035.x.

Fitzpatrick, J.L., P.J. Cripps, A.W. Hill, P.W. Bland, and C.R. Stokes. 1992. MHC class II expression in the bovine mammary gland. Vet. Immunol. Immunopathol. TA - TT - 32:13–23. doi:10.1016/0165-2427(92)90065-X LK - https://umaryland.on.worldcat.org/oclc/4930169689.

Food and Drug Administration, U.S.D.A. 2018. Synthetic flavoring substances and adjuvants. 21CFR 182.60 Fed. Regist.

Freitas, M., G. Porto, J.L.F.C. Lima, and E. Fernandes. 2009. Optimization of experimental settings for the analysis of human neutrophils oxidative burst in vitro. Talanta. doi:10.1016/j.talanta.2009.02.050.

Furia, T.E. 1980. CRC Handbook of Food Additives. 2nd ed. CRC Press, Inc., Boca Raton, Florida.

Garcia, M., T. Elsasser, D. Biswas, and K. Moyes. 2015. The effect of citrus-derived oil on bovine blood neutrophil function and gene expression in vitro. J. Dairy Sci 98:918–916. doi:10.3168/jds.2014-8450.

Gill, J.J., J.C. Pacan, M.E. Carson, K.E. Leslie, M.W. Griffiths, and P.M. Sabour. 2006. Efficacy and pharmacokinetics of bacteriophage therapy in treatment of subclinical Staphylococcus aureus mastitis in lactating dairy cattle. Antimicrob. Agents Chemother. 50:2912–2918. doi:10.1128/AAC.01630-05.

Gill, R., W.H. Howard, K.E. Leslie, and K. Lissemore. 1990. Economics of Mastitis Control. J Dairy Sci 73:3340–3348. doi:10.3168/jds.S0022-0302(90)79029-7.

Goldberg, J.J., J.W. Pankey, I. Politis, B. Zavizion, and A.J. Bramley. 1995. Effect of oxygen tension on killing of Escherichia coli by bovine polymorphonuclear neutrophil leucocytes in vitro. J. Dairy Res. 62:331. doi:10.1017/S0022029900031022.

Gomes, F., and M. Henriques. 2016. Control of Bovine Mastitis: Old and Recent Therapeutic Approaches. Curr. Microbiol. 72:377–382. doi:10.1007/s00284-015-0958-8.

Gomes Neto, N.J., M. Magnani, B. Chueca, D. García-Gonzalo, R. Pagán, and E. Leite De Souza. 2015. Influence of general stress-response alternative sigma factors σS (RpoS) and σB (SigB) on bacterial tolerance to the essential oils from Origanum vulgare L. and Rosmarinus officinalis L. and pulsed electric fields. Int. J. Food Microbiol. 211:32–37. doi:10.1016/j.ijfoodmicro.2015.06.030.

Gruet, P., P. Maincent, X. Berthelot, and V. Kaltsatos. 2001. Bovine mastitis and intramammary drug delivery: review and perspectives. Adv. Drug Deliv. Rev. 50:245–259.

Hamblin, M.R. 2016. Antimicrobial photodynamic inactivation: a bright new technique to kill resistant microbes. Curr. Opin. Microbiol.. doi:10.1016/j.mib.2016.06.008.

Herbelin, C., B. Poutrel, F.B. Gilbert, and P. Rainard. 1997. Immune Recruitment and Bactericidal Activity of Neutrophils in Milk of Cows Vaccinated with Staphylococcal a-Toxin. J. Dairy Sci. 80:2025–2034. doi:10.3168/jds.S0022-0302(97)76147-2.

Herry, V., C. Gitton, G. Tabouret, M. Répérant, L. Forge, C. Tasca, F.B. Gilbert, E. Guitton, C. Barc, C. Staub, D.G.E. Smith, P. Germon, G. Foucras, and P. Rainard. 2017. Local immunization impacts the response of dairy cows to Escherichia coli mastitis OPEN. Sci. Rep. 7:3441–3457. doi:10.1038/s41598-017-03724-7.

Hili, P., E. CS, and V. RG. 1997. Antimicrobial action of essential oils: the effect of dimethylsulphoxide on the activity of cinnamon oil.. Lett. Appl. Microbiol. TA - TT - 24:269–275.

Hill, A.W. 1981. Factors influencing the outcome of Escherichia coli mastitis in the dairy cow.. Res. Vet. Sci. 31:107–112.

Hoe, F.G.H., and P.L. Ruegg. 2006. Opinions and Practices of Wisconsin Dairy Producers About Biosecurity and Animal Well-Being. J. Dairy Sci 89:2297–2308. doi:10.3168/jds.S0022-0302(06)72301-3.

Hoedemaker, M., B. Korff, B. Edler, M. Emmert, and E. Bleckmann. 2001. Dynamics of Staphylococcus aureus infections during vaccination with an autogenous bacterin in dairy cattle. J. Vet. Med. Ser. B 48:373–383. doi:10.1046/j.1439-0450.2001.00465.x.

Hogan, J., and K.L. Smith. 2003. Coliform mastitis. Vet. Res. 34:507–519.

doi:10.1051/vetres:2003022.

Hogan, J.S., K.L. Smith, D.A. Todhunter, and P.S. Schoenberger. 1992. Field Trial to Determine Efficacy of an Escherichia coli J5 Mastitis Vaccine. J. Dairy Sci. 75:78–84. doi:10.3168/jds.S0022-0302(92)77741-8.

Holley, R.A., and D. Patel. 2005. Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. FOOD Microbiol. 22:273–292. doi:10.1016/j.fm.2004.08.006.

Hoopes, J.T., C.J. Stark, H.A. Kim, D.J. Sussman, D.M. Donovan, and D.C. Nelson. 2009. Use of a bacteriophage lysin, PlyC, as an enzyme disinfectant against Streptococcus equi. Appl. Environ. Microbiol.. doi:10.1128/AEM.02195-08.

Hyldgaard, M., T. Mygind, R.L. Meyer, and C. Knapp. 2012. Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. Front. Microbiol. eCollection 3. doi:10.3389/fmicb.2012.00012.

Jamali, H., H.W. Barkema, M. Jacques, E.-M. Lavallée-Bourget, F. Malouin, V. Saini, H. Stryhn, and S. Dufour. 2018. Invited review: Incidence, risk factors, and effects of clinical mastitis recurrence in dairy cows. doi:10.3168/jds.2017-13730.

Jayarao, B.M., B.E. Gillespie, M.J. Lewis, H.H. Dowlen, and S.P. Oliver. 1999. Epidemiology of Streptococcus uberis Intramammary Infections in a Dairy Herd. J. Vet. Med. B 46:433–442.

Jung, H.C., L. Eckmann, S.-K. Yang, A. Panja, J. Fierer, E. Morzycka-Wroblewska, and M.F. Kagnoff. 1995. A Distinct Array of Proinflammatory Cytokines Is Expressed in Human Colon Epithelial Cells in Response to Bacterial Invasion. J. Clin. Invest. 95:55–65.

Kazemi, J., M. Ahmadi, H. Dastmalchi Saei, and M. Adib hesami. 2014. Antibacterial effect of silver nanoparticles along with protein synthesis-inhibiting antibiotics on Staphylococcus aureus isolated from cattle mastitis.

Kobayashi, S.D., J.M. Voyich, C. Burlak, and F.R. Deleo. 2005. Neutrophils in the innate immune response. ORG Arch Immunol Ther Exp 53:505–517.

Korenblum, E., F. Regina de Vasconcelos Goulart, I. de Almeida Rodrigues, F. Abreu, U. Lins, P. Barreto Alves, A. Fitzgerald Blank, É. Valoni, G. V Sebastián, D. Sales Alviano, C. Sales Alviano, and L. Seldin. 2013. Antimicrobial action and anti-corrosion effect against sulfate reducing bacteria by lemongrass (Cymbopogon citratus) essential oil and its major component, the citral. AMB Express 3:1. doi:10.1186/2191-0855-3-44.

Krause, R.M. 1957. Studies on bacteriophages of hemolytic streptococci. J Exp Med 106:365–384.

Kromker, V., R. Reinecke, J. Paduch, and N. Grabowski. 2014. Bovine Streptococcus uberis Intramammary Infections and Mastitis. Clin. Microbiol. Open Access 03.

doi:10.4172/2327-5073.1000157.

Kwiatek, M., S. Parasion, L. Mizak, R. Gryko, M. Bartoszcze, and J. Kocik. 2012. Characterization of a bacteriophage, isolated from a cow with mastitis, that is lytic against Staphylococcus aureus strains. Arch. Virol. 157:225–234. doi:10.1007/s00705-011-1160-3.

Leigh, J.A., and T.R. Field. 1994. Streptococcus uberis Resists the Bactericidal Action of Bovine Neutrophils Despite the Presence of Bound Immunoglobulin.

Leijh, P.C.J., M.T. Van Den Barselaar, T.L. Van Zwet, I. Dubbeldeman-Rempt, and R. Van Furth. 1979. Kinetics of phagocytosis of Staphylococcus aureus and Escherichia coli by human granulocytes. Immunology 37:453–465.

Levison, M.E., and J.H. Levison. 2009. Pharmacokinetics and Pharmacodynamics of Antibacterial Agents. Infect Dis Clin North Am 23:791–819. doi:10.1016/j.idc.2009.06.008.Pharmacokinetics.

Lin, C.-T., C.-J. Chen, T.-Y. Lin, J.C. Tung, and S.-Y. Wang. 2008. Anti-inflammation activity of fruit essential oil from Cinnamomum insularimontanum Hayata. Bioresour. Technol. 99:8783–8787. doi:10.1016/j.biortech.2008.04.041.

Linck, V. de M., A.L. da Silva, M. Figueiró, Â. Luis Piato, A. Paula Herrmann, F. Dupont Birck, E. Bastos Caramão, D. Sávio Nunes, P.R.H. Moreno, and E. Elisabetsky. 2009. Inhaled linalool-induced sedation in mice. Phytomedicine 16:303–307. doi:10.1016/J.PHYMED.2008.08.001.

Loeffler, J.M., S. Djurkovic, and V.A. Fischetti. 2003. Phage Lytic Enzyme Cpl-1 as a Novel Antimicrobial for Pneumococcal Bacteremia. Infect. Immun. 71:6199–6204. doi:10.1128/IAI.71.11.6199-6204.2003.

Loessner, M.J., K. Kramer, F. Ebel, and S. Scherer. 2002. C-terminal domains of 44:335–349. doi:10.1046/j.1365-2958.2002.02889.x.

Lohuis, J.A.C.M., Y.H. Schukken, J.H.M. Verheijden, A. Brand, and A.S.J.P.A.M. Van Miert. 1990. Effect of severity of systemic signs during the acute phase of experimentally induced Escherichia coli mastitis on milk production losses. J. Dairy Sci. 73:333–341. doi:10.3168/jds.S0022-0302(90)78678-X.

Lu, W.-C., D.-W. Huang, C.-C.R. Wang, C.-H. Yeh, J.-C. Tsai, Y.-T. Huang, and P.-H. Li. 2016. Preparation, characterization, and antimicrobial activity of nanoemulsions incorporating citral essential oil ScienceDirect. J. Food Drug Anal.. doi:10.1016/j.jfda.2016.12.018.

Mahato, N., K. Sharma, R. Koteswararao, M. Sinha, E. Baral, and M. Hwan Cho. 2017. Critical Reviews in Food Science and Nutrition Citrus essential oils: Extraction, authentication and application in food preservation Citrus essential oils: Extraction, authentication and application in food preservation. Food Sci. Nutr.. doi:10.1080/10408398.2017.1384716.

McGowan, S., A.M. Buckle, M.S. Mitchell, J.T. Hoopes, D.T. Gallagher, R.D.

Heselpoth, Y. Shen, C.F. Reboul, R.H.P. Law, V.A. Fischetti, J.C. Whisstock, and D.C. Nelson. 2012. X-ray crystal structure of the streptococcal specific phage lysin PlyC. Proc. Natl. Acad. Sci. 109:12752–12757. doi:10.1073/pnas.1208424109.

Mehri, S., M.A. Meshki, and H. Hosseinzadeh. 2015. Linalool as a neuroprotective agent against acrylamide-induced neurotoxicity in Wistar rats. Drug Chem. Toxicol. 38:162–166. doi:10.3109/01480545.2014.919585.

Mehrzad, J., H. Dosogne, E. Meyer, R. Heyneman, and C. Burvenich. 2001. Respiratory burst activity of blood and milk neutrophils in dairy cows during different stages of lactation. J. Dairy Res. 85:3268–3276. doi:10.1017/S0022029901005039.

Melchior, M.B. 2011. Bovine Mastitis and Biofilms. S.L. Percival, ed. Springer-Verlage Berlin Heidelberg, Heeten, The Netherlands.

Melchior, M.B., J. Fink-Gremmels, and W. Gaastra. 2006a. Comparative assessment of the antimicrobial susceptibility of Staphylococcus aureus isolates from bovine mastitis in biofilm versus planktonic culture. J. Vet. Med. Ser. B Infect. Dis. Vet. Public Heal. 53:326–332. doi:10.1111/j.1439-0450.2006.00962.x.

Melchior, M.B., H. Vaarkamp, and J. Fink-Gremmels. 2006b. Biofilms: A role in recurrent mastitis infections?. Vet. J. 171:398–407. doi:10.1016/j.tvjl.2005.01.006.

Mi, H., D. Wang, Y. Xue, Z. Zhang, J. Niu, Y. Hong, K. Drlica, X. Zhao, and O. Xilin Zhao. 2016. Dimethyl sulfoxide protects Escherichia coli from rapid antimicrobial-mediated killing. Antimicrob. Agents Chemother. 60:5054–5058. doi:10.1128/AAC.03003-15.

Miller, J.K., E. Brzezinska-Slebodzinska, and F.C. Madsen. 1993. Oxidative Stress, Antioxidants, and Animal Function. J. Dairy Sci.. doi:10.3168/jds.S0022-0302(93)77620-1.

Mitic-Culafic, D., B. Zegura, B. Nikolic, B. Vukovic-Gacic, J. Knezevic-Vukcevic, and M. Filipic. 2009. Protective effect of linalool, myrcene and eucalyptol against t-butyl hydroperoxide induced genotoxicity in bacteria and cultured human cells. Food Chem. Toxicol. 47:260–266. doi:10.1016/j.fct.2008.11.015.

Moreira, L.H., J.C.P. de Souza, C.J. de Lima, M.A.C. Salgado, A.B. Fernandes, D.I.K. Andreani, A.B. Villaverde, and R.A. Zângaro. 2018. Use of photodynamic therapy in the treatment of bovine subclinical mastitis. Photodiagnosis Photodyn. Ther. 21:246–251. doi:10.1016/j.pdpdt.2017.12.009.

Moufida, S., and B. Marzouk. 2003. Biochemical characterization of blood orange, sweet orange, lemon, bergamot and bitter orange. Phytochemistry 62:1283–1289. doi:10.1016/S0031-9422(02)00631-3.

Muthaiyan, A., E.M. Martin, S. Natesan, P.G. Crandall, B.J. Wilkinson, and S.C. Ricke. 2012. Antimicrobial Effect and Mode of Action of Terpeneless Cold

Pressed Valencia Orange Essential Oil on Methicillin-Resistant Staphylococcus aureus. J Appl Microbiol 112:1020–1033. doi:10.1111/j.1365-2672.2012.05270.x.

Nakamura, A., S. Fujiwara, I. Matsumoto, and K. Abe. 2009. Stress Repression in Restrained Rats by (R)-(-)-Linalool Inhalation and Gene Expression Profiling of Their Whole Blood Cells. J. Agric. Food Chem. 57:5480–5485. doi:10.1021/jf900420g.

Nannapaneni, R., V.I. Chalova, P.G. Crandall, S.C. Ricke, M.G. Johnson, and C.A. O 'bryan. 2009. Campylobacter and Arcobacter species sensitivity to commercial orange oil fractions. Int. J. Food Microbiol. 129:43–49. doi:10.1016/j.ijfoodmicro.2008.11.008.

National Mastitis Council. 2004. Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality. 4th ed. S. Oliver, R. Gonzalez, and J. Hogan, ed. National Mastitis Council, Verona, WI.

National Mastitis Council. 2015. A Practical Look at Environmental Mastitis. Verona, WI.

National Mastitis Council. 2017. Laboratory Handbook on Bovine Mastitis. 3rd ed. National Mastitis Council, Verona, WI.

National Research Council. 2001. Nutrient Requirements of Dairy Cattle. 7th ed. National Academies Press, Washington, D.C.

Nelson, D., L. Loomis, and V.A. Fischetti. 2001. Prevention and elimination of upper respiratory colonization of mice by group A streptococci by using a bacteriophage lytic enzyme. Proc. Natl. Acad. Sci. 98:4107–4112. doi:10.1073/pnas.061038398.

Nelson, D., R. Schuch, P. Chahales, S. Zhu, and V.A. Fischetti. 2006. PlyC: A multimeric bacteriophage lysin. Proc. Natl. Acad. Sci.. doi:10.1073/pnas.0604521103.

Newbould, F.H.S. 1970. The Effect of Addled Serum and Glucose, and Some Inherent Factors, on Phagocytosis in vitro by Milk Leukocytes from Several Cows. Can. J. Vet. Res. 37:189–194.

O'Flaherty, S., R.P. Ross, J. Flynn, W.J. Meaney, G.F. Fitzgerald, and A. Coffey. 2005. Isolation and characterization of two anti-staphylococcal bacteriophages specific for pathogenic Staphylococcus aureus associated with bovine infections. Lett. Appl. Microbiol. 41:482–486. doi:10.1111/j.1472-765X.2005.01781.x.

Olde Riekerink, R.G.M., H.W. Barkema, D.F. Kelton, and D.T. Scholl. 2008. Incidence Rate of Clinical Mastitis on Canadian Dairy Farms. J. Dairy Sci.. doi:10.3168/jds.2007-0757.

Oldham, E.R., and M.J. Daley. 1991. Lysostaphin: Use of a Recombinant Bactericidal Enzyme as a Mastitis Therapeutic. J. Dairy Sci. 74:4175–4182.

doi:10.3168/jds.S0022-0302(91)78612-8.

Oliveira, L., C. Hulland, and P. Ruegg. 2013. Characterization of clinical mastitis occurring in cows on 50 large dairy herds in Wisconsin. J. Dairy Sci. 96:7538–7549. doi:10.3168/jds.2012-6078.

Oliveira, L., and P. Ruegg. 2014. Treatments of clinical mastitis occurring in cows on 51 large dairy herds in Wisconsin. J. Dairy Sci. 97:5426–5436. doi:10.3168/jds.2013-7756.

Oliver, S.P., S.E. Murinda, and B.M. Jayarao. 2011. Impact of Antibiotic Use in Adult Dairy Cows on Antimicrobial Resistance of Veterinary and Human Pathogens: A Comprehensive Review. Foodborne Pathog. Dis.. doi:10.1089/fpd.2010.0730.

Olson, M.E., H. Ceri, D.W. Morck, A.G. Buret, and R.R. Read. 2002. Biofilm bacteria: Formation and comparative susceptibility to antibiotics. Can. J. Vet. Res. 66:86–92.

Onawunmi, G.O. 1989. Evaluation of the antimicrobial activity of citral. Lett. Appl. Microbiol. 9:105–108.

Ortiz, M.I., M.P. González-García, H.A. Ponce-Monter, G. Castañeda-Hernández, and P. Aguilar-Robles. 2010. Synergistic effect of the interaction between naproxen and citral on inflammation in rats. Phytomedicine 18:74–79. doi:10.1016/j.phymed.2010.05.009.

Paape, M., J. Mehrzad, X. Zhao, J. Detilleux, and C. Burvenich. 2002. Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. J. Mammary Gland Biol. Neoplasia. doi:10.1023/A:1020343717817.

Paape, M.J., D.D. Bannerman, X. Zhao, and J.W. Lee. 2003. The bovine neutrophil: Structure and function in blood and milk. Vet. Res.. doi:10.1051/vetres:2003024.

Paape, M.J., and A.J. Guidry. 1977. Effect of fat and casein on intracellular killing of Staphylococcus aureus by milk leukocytes.. Proc. Soc. Exp. Biol. Med. 155:588–593.

Paape, M.J., H.D. Ha, and A.W. W Snyder. 1963. Variation of Estimated Numbers of Milk Somatic Cells Stained with Wright's Stain or Pyronin Y - Methyl Green Stain1. J. Dairy Sci. 46:1211–1216. doi:10.3168/jds.S0022-0302(63)89248-6.

Pankey, G.A., and L.D. Sabath. 2004. Clinical Relevance of Bacteriostatic versus Bactericidal Mechanisms of Action in the Treatment of Gram-Positive Bacterial Infections. Clin. Infect. Dis. 38:864–870. doi:10.1086/381972.

Parra Sellera, F., C.P. Sabino, M.S. Ribeiro, R. Gomes Gargano, N.R. Benites, P.A. Melville, and F.C. Pogliani. 2016. In vitro photoinactivation of bovine mastitis related pathogens. Photodiagnosis Photodyn. Ther. 13:276–281. doi:10.1016/j.pdpdt.2015.08.007.

Peana, A.T., M. Graziella De Montis, S. Sechi, G. Sircana, P.S. D'aquila, and P.

Pippia. 2004. Effects of (-)-linalool in the acute hyperalgesia induced by carrageenan, L-glutamate and prostaglandin E 2. Eur. J. Pharmacol. 497:279–284. doi:10.1016/j.ejphar.2004.06.006.

Pedersen, L.H., B. Aalbæk, C.M. Røntved, K.L. Ingvartsen, N.S. Sorensen, P.M.H. Heegaard, and H.E. Jensen. 2003. Early pathogenesis and inflammatory response in experimental bovine mastitis due to Streptococcus uberis. J. Comp. Pathol.. doi:10.1053/jcpa.2002.0620.

Petrovski, K., N. Williamson, N. Lopez-Villalobos, T. Parkinson, and I. G Tucker. 2011. Culture results from milk samples submitted to veterinary diagnostic laboratories from August 2003 to December 2006 in New Zealand. N. Z. Vet. J. 59:317–322. doi:10.1080/00480169.2011.610286.

Pighetti, G.M., S. Piper, and K.H. Campbell. 2007. Using DHI Reports to Troubleshoot Mastitis.

Pohorecky, L., and J. Brick. 1988. Pharmacology of ethanol.. Pharmacol. Ther. 36:2–3.

Pol, M., and P.L. Ruegg. 2007. Relationship Between Antimicrobial Drug Usage and Antimicrobial Susceptibility of Gram-Positive Mastitis Pathogens. J. Dairy Sci. 90:262–273. doi:10.3168/jds.S0022-0302(07)72627-9.

Politis, I., X. Zhao, B.W. Mcbride, and J.H. Burton. 1991. The Effect of Lipopolysaccharide on Bovine Mammary Macrophage Function. Can. J. Vet. Res. 55:220–223.

Prashar, A., I.C. Locke, and C.S. Evans. 2004. Cytotoxicity of lavender oil and its major components to human skin cells. Cell Prolif. 37:221–229. doi:10.1111/j.1365-2184.2004.00307.x.

PubChem. 2004. Citral OECD CAS 5392-40-5.

Pyorala, S., L. Kaartinen, H. Kack, and V. Rainio. 1994. Efficacy of Two Therapy Regimens for Treatment of Experimentally Induced Escherichia coli Mastitis in Cows. J. Dairy Sci. 77:453–461. doi:10.3168/jds.S0022-0302(94)76973-3.

Rainard, P. 2003. The complement in milk and defense of the bovine mammary gland against infections. Vet. Res.. doi:10.1051/vetres:2003025.

Rainard, P., and C. Riollet. 2003a. Mobilization of neutrophils and defense of the bovine mammary gland. Reprod. Nutr. Dev. EDP Sci. 43:439–457.

Rainard, P., and C. Riollet. 2003b. Mobilization of neutrophils and defense of the bovine mammary gland. Reprod. Nutr. Dev. EDP Sci. 43:439–457. doi:10.1051/rnd:2003031>.

Rajala-Schultz, P.J., K.L. Smith, J.S. Hogan, and B.C. Love. 2004. Antimicrobial susceptibility of mastitis pathogens from first lactation and older cows. Vet. Microbiol. 102:33–42. doi:10.1016/j.vetmic.2004.04.010.

Rambeaud, M., R.A. Almeida, G.M. Pighetti, and S.P. Oliver. 2003. Dynamics of leukocytes and cytokines during experimentally induced Streptococcus uberis mastitis. Vet. Immunol. Immunopathol.. doi:10.1016/j.vetimm.2003.08.008.

Rashel, M., J. Uchiyama, T. Ujihara, Y. Uehara, S. Kuramoto, S. Sugihara, K.-I. Yagyu, A. Muraoka, M. Sugai, K. Hiramatsu, K. Honke, and S. Matsuzaki. 2007. Efficient Elimination of Multidrug-Resistant Staphylococcus aureus by Cloned Lysin Derived from Bacteriophage fMR11. J. Infect. Dis. 196:1237–1284. doi:10.1086/521305.

Rinaldi, M., P. Moroni, M.J. Paape, and D.D. Bannerman. 2007. Evaluation of assays for the measurement of bovine neutrophil reactive oxygen species. Vet. Immunol. Immunopathol.. doi:10.1016/j.vetimm.2006.09.009.

Rothman, K.J., S. Greenland, and T.T. Lash. 2008. Modern Epidemiology. 3rd Editio. Wolters Kluwer Health/Lippincott Williams & Wilkins, Piladelphia, PA.

Ruegg, P.L. 2015. ON-FARM CULTURE AND TREATMENT DECISIONS. Madison, WI.

Russell, M.W., and B. Reiter. 1975. Phagocytic deficiency of bovine milk leucocytes: an effect of casein.. J. Reticuloendothel. Soc. 18:1–13.

Shaw, P.E., and R.L. Coleman. 1974. Quantitative Composition of Cold-Pressed Orange Oils. J Agr Food Chem 22:785–787.

Shen, Y., M. Barros, T. Vennemann, D.T. Gallagher, Y. Yin, S.B. Linden, R.D. Heselpoth, D.J. Spencer, D.M. Donovan, J. Moult, V.A. Fischetti, F. Heinrich, M. Lösche, and D.C. Nelson. 2016. A bacteriophage endolysin that eliminates intracellular streptococci. Elife. doi:10.7554/eLife.13152.

Shen, Y., T. Köller, B. Kreikemeyer, and D.C. Nelson. 2013. Rapid degradation of Streptococcus pyogenes biofilms by PlyC, a bacteriophage-encoded endolysin. J. Antimicrob. Chemother.. doi:10.1093/jac/dkt104.

Shi, C., K. Song, X. Zhang, Y. Sun, Y. Sui, Y. Chen, Z. Jia, H. Sun, Z. Sun, and X.X. Xia. 2016. Antimicrobial activity and possible mechanism of action of citral against cronobacter sakazakii. PLoS One. doi:10.1371/journal.pone.0159006.

Shpigel, N.Y., S. Elazar, and I. Rosenshine. 2008. Mammary pathogenic Escherichia coli. Curr. Opin. Microbiol. 11:60–65. doi:10.1016/j.mib.2008.01.004.

Shpigel, N.Y., D. Levin, M. Winkler, A. Saran, G. Ziv, and A. Böttner. 1997. Efficacy of Cefquinome for Treatment of Cows with Mastitis Experimentally Induced Using Escherichia coli. J. Dairy Sci. 80:318–323. doi:10.3168/jds.S0022-0302(97)75941-1.

Shpigel, N.Y., M. Winkler, G. Ziv, and A. Saran. 1998. Clinical, bacteriological and epidemiological aspects of clinical mastitis in Israeli dairy herds. Prev. Vet. Med. 35:1–9.

Silva, V.O., L.O. Soares, A.S. Júnior, H.C. Mantovani, Y.-F. Chang, M. Aparecida,

and S. Moreira. 2014. Biofilm Formation on Biotic and Abiotic Surfaces in the Presence of Antimicrobials by Escherichia coli Isolates from Cases of Bovine Mastitis. Appl. Environ. Microbiol. 80:6136–6145. doi:10.1128/AEM.01953-14.

Smith, K., D. Todhunter, and P. Schoenberger. 1985. Environmental mastitis: cause, prevalence, prevention.. J. Dairy Sci. 66:1531–1553.

Summers, C., S.M. Rankin, A.M. Condliffe, N. Singh, A.M. Peters, and E.R. Chilvers. 2010. Neutrophil kinetics in health and disease. Trends Immunol.. doi:10.1016/j.it.2010.05.006.

Szczepanski, S., and A. Lipski. 2014. Essential oils show specific inhibiting effects on bacterial biofilm formation. Food Control 36:224–229. doi:10.1016/j.foodcont.2013.08.023.

The White House. 2015. National Action Plan for Combating Antibiotic-Resistant Bacteria.

Trombetta, D., F. Castelli, M.G. Sarpietro, V. Venuti, M. Cristani, C. Daniele, A. Saija, G. Mazzanti, and G. Bisignano. 2005. Mechanisms of Antibacterial Action of Three Monoterpenes. Antimicrob. Agents Chemother. 49:2474–2478. doi:10.1128/AAC.49.6.2474-2478.2005.

US Department of Agriculture. 2007. Dairy 2007, Part I: Reference of Dairy Cattle Health and Management Practices in the United States. Fort Collins, CO.

US Department of Agriculture. 2009. Dairy 2007, Part V: Changes in Dairy Cattle Health and Management Practices in the United States, 1996-2007. Fort Collins, CO.

US Department of Health and Human Service, and Public Health Service. 2015. Grade A Pasteuraized Milk Ordinance 57–58.

Vandeputte-van Messom, G., C. Burvenich, E. Roets, A. Massart-leen, R. Heyneman, W.D. J KREMERt, and A. BRANDf. 1993. Classification of newly calved cows into moderate and severe responders to experimentally induced Escherichia coli mastitis. J. Dairy Res. 60:19–29. doi:10.1017/S002202990002731X.

Vangroenweghe, F., L. Duchateau, and C. Burvenich. 2004a. Moderate Inflammatory Reaction During Experimental Escherichia coli Mastitis in Primiparous Cows. J. Dairy Sci. 87:886–895. doi:10.3168/jds.S0022-0302(04)73233-6.

Vangroenweghe, F., P. Rainard, M. Paape, L. Duchateau, and C. Burvenich. 2004b. Increase of Escherichia coli Inoculum Doses Induces Faster Innate Immune Response in Primiparous Cows. J. Dairy Sci 87:4132–4144. doi:10.3168/jds.S0022-0302(04)73556-0.

Varhimo, E., P. Varmanen, A. Fallarero, M. Skogman, S. Pyö Rä Lä C, A. Iivanainen, A. Sukura, P. Vuorela, and K. Savijoki. 2010. Alpha- and Beta-casein components of host milk induce biofilm formation in the mastitis bacterium Streptococcus uberis. Vet. Microbiol. 149:381–389.

doi:10.1016/j.vetmic.2010.11.010.

Van de Vel, E., I. Sampers, and K. Raes. 2017. A review on influencing factors on the minimum inhibitory concentration of essential oils. Crit. Rev. Food Sci. Nutr.. doi:10.1080/10408398.2017.1371112.

Wagner, S.A., and R.J. Erskine. 2009. Decision Making in Mastitis Therapy. 5th editio. D. Anderson, ed. Saunders Elsevier.

Walsh, S.E., J.-Y. Maillard, A.D. Russell, C.E. Catrenich, D.L. Charbonneau, and R.G. Bartolo. 2003. Development of bacterial resistance to several biocides and effects on antibiotic susceptibility. J. Hosp. Infect. 55:98–107. doi:10.1016/S0195-6701(03)00240-8.

Wang, I.-N., J. Deaton, and R. Young. 2003. Sizing the Holin Lesion with an Endolysin-Galactosidase Fusion. J. Bacteriol. 185:779–787. doi:10.1128/JB.185.3.779-787.2003.

Wellnitz, O., P. Reith, S.C. Haas, and H.H.D. Meyer. 2006. Immune relevant gene expression of mammary epithelial cells and their influence on leukocyte chemotaxis in response to different mastitis pathogens. Vet. Med. (Praha). 51:125–312.

White, J.R., C.K. Huang, and J.M. Hill. 1984. Effect of phorbol 12-myristate 13-acetate and its analogue 4α-phorbol 12,13-didecanoate on protein phosphorylation and lysosomal enzyme release in rabbit neutrophils. J. Biol. Chem. 259:8605–8611.

Zadoks, R.N. 2007. Sources and epidemiology of Streptococcus uberis, with special emphasis on mastitis in dairy cattle.. CAB Rev. Perspect. Agric. Vet. Sci. Nutr. Nat. Resour. 2:15 pp. doi:10.1079/PAVSNNR20072030.