ABSTRACT

Title of Document: *In Vitro* Study of an Orange Oil Derived
                            Alternative to Antibiotics in the Treatment of
                            Bovine Mastitis

                            Cassandra Skye Federman, Masters of Animal
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Directed By: Dr. Debabrata Biswas, Animal Science

Bovine mastitis is a costly disease in the U.S. dairy industry. Its major causative

agent *Staphylococcus aureus* is often unresponsive to antibiotic therapy. Our first

study examined terpeneless, cold-pressed Valencia (CPV) orange oil as a possible

alternative to antibiotic therapy in the treatment of *S. aureus* associated bovine

mastitis. Orange oil showed significant inhibition of *S. aureus* growth and invasion

of bovine epithelial mammary cells, but only modest reductions in pre-formed

biofilms, which contribute to persistence of *S. aureus* infections. Our second study

examined major components of terpeneless, CPV orange oil. Of four major

compounds tested, only citral and linalool had significant inhibitory effects on *S.

aureus* growth. In addition, they were capable of reducing pre-formed biofilms as

well as association and invasion to bovine epithelial mammary cells. Part of this

inhibition was due to downregulation of virulence and biofilm genes.

In Vitro Study of an Orange Oil Derived Alternative to Antibiotics in the Treatment
of Bovine Mastitis

By

Cassandra Skye Federman

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Advisory Committee:
Dr. Debabrata Biswas, Chair
Dr. Zhengguo Xiao
Dr. Robert Peters

Dedication

For my fiancé, Robert, who always provided me with strength and encouragement,

and for my friends and family for all of their support.

Acknowledgements

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

Bovine Mastitis

Infection in Dairy Cattle

Bovine mastitis is described as inflammation of the udder tissue. It can affect one or more quarters of the udder. Clinical symptoms may only include changes in the milk such as watery appearance, pus, flakes, clots, and blood. Symptoms of increased severity often include changes in the milk and hardness, heat, pain, redness, and swelling of the udder. Cows may also suffer systemic symptoms of illness from mastitis, such as marked reductions in milk production, lack of appetite, fever, lethargy, weakness, dehydration, and diarrhea. Most cases of mastitis, however, are asymptomatic or subclinical in nature and require a test to evaluate the number of leucocytes or somatic cell count (SCC) present in the milk. Somatic cell counts of milk from normal or uninfected quarters has less than or equal to 100,000 cells. A SCC of 200,000 or greater is defined as being subclinically infected (Smith, 2001). A California Mastitis Test (CMT) may be performed cow side to evaluate the SCC in each quarter and the probability of infection. This involves stripping milk from each quarter into a separate well of a CMT paddle, adding CMT reagent to the milk, mixing the regent and milk, and visually scoring the degree of gelling displayed by the milk-regent mixture within 20 seconds. CMT scores of Negative, Trace, 1, 2, and 3 correspond with SCC of 100,000 or less, 300,000, 900,000, 2.7 million, and 8.1 million (University of Wisconsin Milk Quality, 2011). . Prognosis for eliminating the infection varies depending on the pathogen, length of infection, stage of lactation, and

age of the cow and other host and environmental factors. Mastitis may permanently damage the udder, reduce milk synthesis and quality, decrease fertility, and in severe cases, cause death (Harmon, 1994).

Causative Agents

Bovine mastitis is primarily caused by bacterial pathogens. Bacterial species capable of causing mastitis are varied. *Staphylococcus* spp., in particular *S. aureus*, *Streptococcus* spp., especially *Streptococcus agalactiae*, and *Escherichia coli* are among the most common major bacterial pathogens (Contreras et al., 2011; Wilson et al., 1997). However, non-bacterial causative agents exist, such as viruses like herpesvirus, certain species of algae like *Prototheca* spp., and yeasts (Contreras et al., 2011). Incidences of non-bacterial associated mastitis are relatively low (Contreras et al., 2011; Wilson et al., 1997).

Various Types of Mastitis

Bovine mastitis is classified based on symptoms, source of infection, and course of infection. In terms of symptoms, there is both clinical and subclinical mastitis. Clinical mastitis tends to be acute and have obvious clinical symptoms, such as inflammation of the udder and altered milk appearance. Subclinical mastitis cases are asymptomatic. Despite the lack of symptoms, milk production and quality are reduced. As for sources of infection, mastitis-causing agents are classified as either contagious or environmental. Contagious pathogens are those that are directly passed from cow to cow, usually during the milking process by equipment, towels, and

hands. Environmental pathogens are those that exist in the cow's housing and environment and are transferred to the cow as it interacts with its environment.

Treatments/Therapeutics

Appropriate treatment of mastitis varies based on the mastitis causing agent, the form of infection, and whether the cow is lactating or not. Generally, most antibiotic drugs will be administered through an intramammary route except in cases of acute, severe infection or coliform bacterial pathogens such as *E. coli*, in which parenteral administration is utilized to deliver fluids and anti-inflammatory drugs (du Preez, 2000; Barlow, 2011). Depending on the route of administration, some antibiotics will better distribute across the udder than others (du Preez, 2000). The most effective antibiotics will differ based on causative agent (du Preez, 2000). Some antibiotics will only display activity against gram-positive or gram-negative bacteria. As mastitis has a variety of causative agents, it is important to tailor treatment to each case.

As compared with antibiotic therapy, during lactation, treatment of subclinical infections at dry-off with antibiotic products designed for dry cow therapy are more efficacious. During lactation, organisms such as *Staphylococcus* spp., have low cure rates (du Preez, 2000). Nevertheless, (J.E. Hillerton and E.A. Berry) recommend prompt treatment of all cases of clinical mastitis. Antibiotics should be given 3 times in 12 hour intervals (du Preez, 2000).   In subacute mastitis infections, which have mild clinical symptoms and are often localized, the course of treatment is often determined by the causative agent (du Preez, 2000). However, treatment should

always continue for 24 hours after clinical symptoms disappear (du Preez, 2000). For

acute mastitis infections, which have clinical symptoms with rapid onset and

resolution, parental administration is recommended as antibiotics delivered through

intramammary administration may fail to disperse throughout the udder tissue (du

Preez, 2000). Treatment usually lasts 3-5 days (du Preez, 2000). Peracute infections

are the most severe clinical infections and often result in the cow's death. They have

largely the same treatments, but require greater monitoring of the cow's condition to

avoid shock and death (du Preez, 2000). If a quarter becomes gangrenous, teat

amputation is recommended (du Preez, 2000). Permanent organ damage is a

possibility after infection is resolved, so tying off the mammary vein is suggested to

prevent bacterial toxins entering the bloodstream (du Preez, 2000). If the cow

develops a chronic mastitis infection, it is best to destroy infected quarters or cull the

cow as cure rates are very low (du Preez, 2000).

Cost to Dairy Industry

Losses resulting from bovine mastitis cost the U.S. dairy industry $2 billion

annually. Economic losses for mastitis per a cow are $184.40 per a cow annually

(National Mastitis Council, 1996). Reduced production accounts for $121 of these

losses. The average production loss is 1,600 pounds per a quarter (National Mastitis

Council, 1996). However, losses are better estimated by SCC. An increase from

50,000 SCC per ml to lactation average of 100,000 SCC per mL^{-1} in the first lactation

results in a 200-pound decrease in annual milk production (National Mastitis Council,

1996; Seegers et al., 2003). Thereafter, each two-fold increase in SCC increases

production loss by an additional 200 pounds. For example, production losses for

cows with SCC averaging 100,000, 200,000, 400,000, and 800,000 are associated

with production losses of 200, 400, 600, and 800 pounds per lactation, respectively.

Production losses at each respective lactation average SCC of second and greater

lactation cows are two-fold (National Mastitis Council, 1996) those of primiparous

cows.   After a mastitis case, future lactations can also suffer from a reduced milk

yield (Halasa et al., 2007; Hortet et al., 1998). The remainder of economic losses are

from discarded milk ($10.45), replacement cost ($41.73), extra labor ($1.14),

treatment ($7.36), and veterinary services ($2.72) (National Mastitis Council, 1996).

S. aureus

General Information

S. aureus is gram positive bacteria, which grows in cocci. It can cause a wide

range of diseases, depending on where in the host body it colonizes, but it is best

known for skin infections. Discovered in 1800, Alexander Ogston, a Scottish

surgeon, found it in pus taken from a knee joint surgical abscess. However, the

species was not named and differentiated from others in the *Staphylococcus* genera

until 1884 by Julius Rosenbach, a physician from Germany.

S. aureus is a fairly ubiquitous bacterium, living on the skin and in the noses

of 25% of people. However, in addition, to being a pathogen of humans and cattle, it

can infect a wide variety of animals, including most pet and livestock species (Weese,

2010).   Infection normally begins when there is some kind of injury to the skin or

mucosa, allowing *S. aureus* entry into deeper tissues or into the bloodstream (Lowy, 1998; Lui, 2009). *S. aureus* can infect a variety of locations, such as the skin, bones, joints, blood vessels, respiratory tract, and gastrointestinal tract (Lowy, 1998). Severity of the infection is variable, but it can be responsible for life-threatening diseases, such as bacteremia, endocarditis, sepsis, and toxic shock syndrome (Lowy, 1998).

S. aureus Associated Mastitis

S. aureus is a contagious mastitis pathogen. It is normally transferred to cattle from infected cows to uninfected cows through fomites that have been in contact with *S. aureus* infected milk. These include the milking machine, human handlers, sponges, and towels. *S. aureus* is a major mastitis pathogen, which is a pathogen that invades the entire mammary gland, as opposed to a minor pathogen, which colonizes only the teat end (Reyher et al., 2012). Infection begins with *S. aureus* colonizing the teat orifice (Dego et al., 2002). From there, *S. aureus* can continue colonization into the teat canal or be carried into the teat canal from pressure changes in the udder after the milking process (Dego et al., 2002). When *S. aureus* contacts the milk, it can spread further into the udder, particularly during pressure changes in the udder (Dego et al., 2002). *S. aureus* can then adhere to the surface of mammary epithelial cells and invade into them (Dego et al., 2002). The invasion into cells allows *S. aureus* to evade the host immune response (Dego et al., 2002).

In the 1960s and 1970s, *S. aureus* was the predominant mastitis-causing pathogen (Neave et al., 1969; Philpot, 1978). To combat *S. aureus* and other

contagious mastitis pathogens, the United Kingdom began recommending the five-point plan to farmers in the 1960s, which consisted of "1) post-milking teat disinfection; 2) total dry cow therapy; 3) therapy of clinical cases during lactation; 4) proper maintenance of milking machine; and 5) culling problem cows" (National Mastitis Council, 2006). These practices were successful at reducing *S. aureus* associated mastitis infections in dairy herds as well as other gram-positive contagious mastitis pathogens (Neave et al., 1969; Philpot, 1978). This was further expanded to a ten-point plan by the National Mastitis Council (2006), which included "1) Establishment of goals for udder health. 2)Maintenance of a clean, dry, comfortable environment. 3) Proper milking procedures. 4) Proper maintenance and us of milking equipment. 5) Good record keeping. 6) Appropriate management of clinical mastitis during lactation. 7) Effective dry cow management. 8) Maintenance of biosecurity for contagious pathogens and marketing of chronically infected cows. 9) Regular monitoring of udder health status. 10) Periodic review of mastitis control program". Currently, the prevalence of contagious pathogens tends to be low (Shum et al., 2009). However, of the contagious pathogens, *S. aureus* is the most prevalent (Animal and Plant Health Inspection Service, 2008).

Treatment of *S. aureus* Associated Mastitis

S. aureus associated mastitis is generally treated with intramammary infusions of antibiotics for 3 days during lactation therapy. Dry cow therapy, which involves treatment of antibiotics when the cow is no longer lactating, is often used in cases of subclinical mastitis infection. However, despite antibiotic therapy being available, *S.*

aureus associated mastitis has a highly variable cure rate. However, a study on heifers from the Netherlands exhibiting clinical symptoms shows a cure rate of about 52% (Sol et al., 2000). Subclinical mastitis cases suffer from a variable cure rate, ranging from 4% to 92% (Barkema et al., 2006). When therapy was administered during lactation, the average cure rate was 34.4% for penicillin-sensitive *S. aureus* (Sol et al., 1997). The average cure rate for *S. aureus* increased when dry cow therapy when utilized, but was still only 65.8% (Sol et al., 1994). However, these cure rates are largely dependent on a combination of host, pathogen, and treatment factors.

Host factors that reduce cure rate include higher parity, rear quarter infection, more affected quarters, higher colony forming units (CFU), and higher SCC (Barkema et al., 2006; Deluyker et al., 2005; Dingwall et al., 2003; Osteras et al., 1999; Sol et al., 1994; Sol et al., 1997). Higher parity may reduce cure rate for a variety of reasons. First, older animals are more prone to disease and infection in general (Barkema et al., 2006). Second, older cows have larger mammary glands, making it more difficult for antibiotics to diffuse within them (Barkema et al., 2006). Lastly, older cows tend to have more penicillin resistant mastitis infections (Barkema et al., 2006, Sol et al., 2000). The infection of multiple quarters poses the risk of reinfection if not all quarters are cured (Barkema et al., 2006; Zadoks et al., 2001). In addition, rear quarters often yielded higher SCC and more frequent isolation of pathogens, making them more difficult to successfully treat (Barkema et al., 1997; Barkema et al., 2006).

The main bacterial factor to antibiotic susceptibility is strain. Different strains have different transmission rates and manifestations, which is attributable to different genes controlling antibiotic resistance, intracellular survivability, biofilm formation, and host immune system evasion (Barkema et al., 2006). Treatment can be complicated further by the fact that while infected herds may have one main strain, there are often other strains present (Barkema et al., 2006; Zadoks et al., 2000). Antibiotic resistance rates for *S. aureus* strains involved in mastitis tend to be highly variable between countries and geographical regions, but the presence of antibiotic resistance makes some mastitis strains more difficult to treat than others (Barkema et al., 2006). In one study on dairy herds in New York, ampicillin, erythromycin, and penicillin resistance was found on both conventional and organic farms (Tikofsky et al., 2003). Erythromycin resistance was the most common with it being found in 50.4% of isolates in conventional farms and 44.4% of isolates in organic farms (Tikofsky et al., 2003). However, both ampicillin and penicillin resistance were found at high rates as well. Rates of ampicillin resistance were found to be 38.5% on conventional farms and 19.4% on organic farms (Tikofsky et al., 2003). This is similar to the rates of penicillin resistance, which were 34.2% on conventional farms and 20.1% on organic farms (Tikosky et al., 2003). Interestingly, resistance to one antibiotic seems to be correlated to antibiotic therapy resistance *in vivo*. For example, penicillin-resistant strains of *S. aureus* have lower cure rates even when treating with other classes of antibiotics (Barkema et al., 2006; Sol et al., 2000). Biofilm production also plays a larger part in therapy resistance and chronic infection. *S. aureus* isolates taken from mastitic milk samples of cows produced more biofilm than

isolates obtained from teat skin or milking unit liners (Fox et al., 2005). It has been

suggested that biofilm formation plays important part in establishing infection

through greater adhesion and attachment to tissues (Fox et al., 2005). These biofilms

can also serve to maintain infection as biofilms have been shown to protect bacteria

from the host immune response and be highly resistant to antimicrobials due to the

exopolysaccharide slime covering the bacteria within a biofilm (Melchior et al.,

2006).

Treatment factors for cure rates are the types of antibiotics used, duration of

use, method of administration, and point in lactation cycle that therapy is

administered (Barkema et al., 2006). Generally, higher cure rates were seen when

drug combinations were used and therapy was extended to 5 or 8 days (Barkema et

al., 2006).

Whether it is economically justified to treat *S. aureus* associated mastitis

depends on all these host, bacterial, and treatment factors (Barkema et al., 2006;

Swinkels et al., 2005).

Alternative Therapies

As *S. aureus* associated mastitis is difficult to treat, there have been many

studies that have looked at alternatives to antibiotics. The most studied alternative

therapies are bacteriocins, bacteriophages, vaccines, and genetic modification.

Bacteriocins are antimicrobial peptides produced by other bacteria. However, these

generally run into similar issues to antibiotics, such as low cure rates (Barkema et al.,

2006) and the possibility of developing resistance. Bacteriophages are viruses that

infect bacteria. When used for mastitis, they have low efficacy (Gill et al., 2006) and extreme host specificity. Vaccinations are commercially available and many more have been studied. However, none provide complete protection against infection or disease (Pereira et al., 2011). They only increase efficacy of antibiotic treatment and decrease SCC (Barkema et al., 2006). In addition, vaccines may be strain specific and fail to protect against a broad range of *S. aureus* strains (Barkema et al., 2006). Lastly, genetically modification has the potential to produce cattle that are resistant to mastitis and *S. aureus* infection (Wall et al., 2005). However, public opinion on genetically modified organisms (GMO) is controversial and often polarized into pro- and anti-GMO interest groups, so commercial production of GMO animals is not currently a viable solution.

Orange Oil

Composition

The exact composition of orange oil is difficult to pinpoint (Shaw et al., 1974). There is a wide variety of components, each making up small fractions of the oil. In addition, composition tends to change depending on when the orange was harvested in the year (Shaw et al., 1974). Terpenes, however, make up the majority of the essential oil. Of these, limonene is the most abundant terpene in orange oil, making up 93-96% of the oil (Azer et al., 2011; Shaw et al., 1974; Verzera et al., 2004). The abundances of the rest of the terpenes are normally below 1%, but they are numerous (Azer et al., 2011; Shaw et al., 1974; Verzera et al., 2004). For

11

example, linalool is said to make up 0.3-0.8% of orange oil (Azer et al., 2011; Shaw et al., 1974; Verzera et al., 2004), and citral is said to make up another 0.25-0.65% (Shaw et al., 1974; Verzera et al., 2004). There are other kinds of compounds present, but in much lower abundance, including alcohols, esters, aldehydes, hydrocarbons, ketones, and oxides (Azer et al., 2011; Verzera et al., 2004). However, many microbiological studies use terpeneless, cold-pressed Valencia orange oil. The composition of terpeneless orange oil is quite different. One study found the composition of terpeneless, CPV orange oil to be 20.2% linalool, 18.0% decanal, 9.1% geranial (which is a form of citral), 5.8% α-terpineol, 5.2% valencene, 5.0% neral (which is another form of citral), 4.1% dodecanal, 3.9% citronellal, and 0.3% limonene (Nannapaneni et al., 2009).

Antimicrobial Activity

Many essential oils have been studied for their antimicrobial properties, normally in relation to food safety. Orange oil, whether whole or terpeneless, has been shown to strongly inhibit *Salmonalla enterica* (Dabbah et al., 1970; O'Bryan, 2008), *Escherichia coli* (Dabbah et al., 1970), *S. aureus* (Dabbah et al., 1970; Muthaiyan et al., 2012), and *Pseudomonas* species (Dabbah et al., 1970), *Campylobacter jejuni* (Nannapaneni et al., 2009), *Campylobacter coli* (Nannapaneni et al., 2009), *Arcobacter* species (Nannapaneni et al., 2009). The addition of orange oil in milk has been shown to increase shelf life, illustrating its antimicrobial ability in different media (Dabbah et al., 1970). In addition, the individual components of

orange oil, particularly citral and linalool, have demonstrated antimicrobial activity as well (Fisher et al., 2008; Kim et al., 1995).

The mechanism for this antimicrobial action is not well understood. The prevailing theory is that essential oils and their components can penetrate the bacterial cell wall (Burt, 2004; Fisher et al., 2008). This leads to an increase in cell permeability, resulting in leakage of cytoplasmic components, ions, and nutrients, which leads to cell death (Bakkali et al., 2008; Burt, 2004; Fisher et al., 2008). Tea tree oil has been demonstrated to cause potassium ion leakage in *S. aureus*, which interferes with critical cellular processes (Fisher et al., 2008). Through electron microscopy, one study was able to illustrate how cold-pressed Valencia orange oil caused cell wall damage in methicillin-resistant *S. aureus* (Muthaiyan et al., 2012).

As an Alternative to Antibiotics

In 2012, the U.S. Food and Drug Administration (FDA) issues guidelines for the use of antimicrobial drugs in food-producing animals. This guidance mainly targets production uses of antimicrobials, such as increasing feed efficiency or weight gain (FDA, 2012). The concern is that the mostly unregulated use of antimicrobial drugs in food-producing animals could lead to antibiotic resistance for drugs used in both animals and people (FDA, 2012). The FDA claims that antimicrobial use be limited to what is necessary for assuring animal health (FDA, 2012). Unfortunately, antibiotic use for subclinical mastitis is mostly ineffective, so it may do more to contribute to antimicrobial resistance rather than the animals' well beings. The use of

alternative products would mean that medically-important antimicrobial drugs would not be at risk for developing resistance.

In addition, organic farming is more limited that conventional farming in how they can use antibiotics. Organic dairy farmers are not allowed to withhold antibiotic treatment from cattle, but cattle cannot have their milk sold as organic for 12 months after treatment (U.S. Department of Agriculture [USDA], 2015). This can result in a substantial loss due to milk unable to be sold. An antibiotic alternative would allow organic dairy producers to continue selling organic milk as well as treat their cattle.

Orange Oil is listed as generally recognized as safe (FDA, 2014). Any generally recognized as safe (GRAS) product can be used for human consumption, meaning it presents a very low risk to human health. However, the FDA also allows use of GRAS products in animal feed as well (FDA, 2010). Due to the low risk to health, orange oil should be a safe product to use for cattle. In addition, the USDA allows any GRAS product to be used in the organic livestock production (2015).

Goals and Objectives

Due to rising rates of antibiotic resistance and low success rates of mastitis treatments, the goal study aimed to determine whether cheap natural plant products could be used as a treatment option. There were two objectives to this study:

1. Examine the effects of terpeneless, cold-pressed, Valencia (CPV) orange oil on *Staphylococcus aureus*, bovine mammary cells, and the interaction between the two.

2. Determine the effects of the major components found in terpeneless, CPV orange oil on *S. aureus*, bovine mammary cells, and the interaction between the two.

Chapter 2: Terpeneless, Cold-pressed Valencia Orange Oil and its Effects on *Staphylococcus aureus*, Bovine Epithelial Mammary Cells, and Infection

Abstract

This experiment examines the effects of cold-pressed, terpeneless Valencia orange oil (CPV) on growth of *Staphylococcus aureus*, a major cause of contagious bovine mastitis, and invasion of bovine mammary cells (MAC-T).   To determine minimum inhibitory concentration, we utilized the broth dilution method, using CPV concentrations range from 0.0125% to 0.4% with two-fold dilutions. Growth inhibition was examined by adding 0.00%, 0.05%, 0.025%, 0.0125%, and 0.00625% CPV to 10^5 CFU ml^{-1} *S. aureus* in nutrient broth and enumerating colonies after serial dilution. In a 96 well plate, *S. aureus* (10^7 CFU ml^{-1}) was allowed to form a biofilm, treated with 0%, 0.025%, 0.5%, or 1% CPV and then was measured using a spectrometer. Cytotoxic effect on immortalized bovine mammary epithelial cells (MAC-T) was also examined at various concentrations of CPV using an MTT assay. We observed that the minimum inhibitory concentration of CPV to inhibit the growth of *S. aureus in vitro* was 0.025% CPV. A time kill curve for CPV's action on *S. aureus* over 4 h was generated. CPV completely eliminated *S. aureus* after 3 h incubation at concentration of 0.25% or after 2 h incubation at concentrations of 0.05%. It was also observed that CPV had no effect on pre-formed biofilms except at a concentration of 0.05%, in which there was a modest reduction in the measured absorbance. In addition, CPV was capable of inhibiting association to and invasion

of MAC-T cells by *S. aureus* after a 1 h treatment with CPV. CPV was able to

increase cellular proliferation of MAC-T cells at concentrations up 0.05% and had no

effect at a concentration of 0.1%. Our data suggests that CPV should be considered

for further research as a preventative measure against or treatment of bovine mastitis.

Introduction

Mastitis, an inflammation of the mammary gland, is one of the costliest

diseases impacting dairy farming, exceeding $2 billion annually, with economic

losses resulting from premature culling, additional labor, management and veterinary

costs, and reduced milk production and quality (Seegers et al., 2003; Bar et al., 2008).

This cost excludes irreversible damage to the mammary gland that can lead to future

reduced milk production, milk composition changes, and reproductive inefficiency

from cows with mastitis (Harmon, 1994).

Mastitis is characterized by an increase in milk somatic cell count (SCC) and

may be accompanied by the presence of an intramammary pathogen (Paape et al.,

2003). *Staphylococcus aureus* is a major mastitis pathogen, meaning it infects the

entire udder rather than solely the teat canal (Reyher et al., 2012). Major mastitis

pathogens, such as *S. aureus*, are considered as more virulent and damaging causative

agents than minor mastitis pathogens, which only affect the teat canal (Reyher et al.,

2012). Current management practices have greatly decreased prevalence of *S. aureus*

and other contagious pathogens. However, it is still one of the more common

causative mastitis agents after environmental pathogens (Pitkälä et al., 2004;

Tenhagen et al., 2006). In addition, *S. aureus* is one of the most common contagious mastitis-causing agent (APHIS, 2008).

Treatment of the causative bacteria using antibiotics is coming under increasing public scrutiny due to the possible development of resistant pathogens (Craven, 1987; Guterbock et al., 1993) and risk of residues appearing in the milk (De Vliegher et al., 2010). One study, which included conventional dairy farms in New York, *S. aureus* resistance to ampicillin, erythromycin, penicillin, and tetracycline was found among isolates obtained from mastitic milk (Tikosky et al., 2003). In addition, cure rates using antibiotics for *S. aureus* mastitis vary considerably ranging from 4 to 92% (Barkema et al., 2006).   A study on clinical mastitis cases in the Netherlands found a cure rate of only 52% (Sol et al., 2000). For subclinical mastitis cases, cure rates were 34.4% for penicillin-sensitive *S. aureus* during antibiotic therapy during lactation (Sol et al., 1997) and 65.8% during dry cow therapy (Sol et al., 1994). Thus, alternative strategies for control and treatment for *S. aureus* is essential for improving animal health and economic outcome for the farmer (Barkema et al., 2006).

Many plant derivatives and essential oils derived from the citrus fruits and other plants contain secondary metabolites that can inhibit bacterial growth (Burt and Reinders, 2003; Burt, 2004). Essential oils, or fractions thereof, have been traditionally used as flavoring agents in foods, and it has frequently been noted that many possess antimicrobial properties (Kim et al., 1995; Lis-Balchin and Deans, 1997; Smith-Palmer et al., 1998; Alzoreky and Nakahara, 2003). Cold-pressed, terpeneless Valencia orange oil (CPV) has recently been shown to inhibit growth of

methicillin resistant *S. aureus* (MRSA) (Muthaiyan et al., 2012). Therefore, examining its use as an alternative to antibiotic therapy for mastitis is warranted. To our knowledge, no studies have examined the toxicity of CPV on mammary epithelial cells for use as an alternative therapy for mastitis. Our objective was to determine the inhibitory effect of CPV on the growth of bovine pathogen *S. aureus* and its role in host cell, bovine mammary epithelial cells (MAC-T), - *S. aureus* interactions to explore the potential strategy to control bovine mastitis.

Methods and Materials

Bacterial Strains and Growth Conditions

S. aureus (ATCC 29740) strain was used in this study. This strain has been isolated from bovine mastitic milk. Response time and infection rate of this *S. aureus* strain has been documented previously with consistent changes in onset and duration of the host response beginning as early as 56 h and continuing through 240 h. (Bannerman, 2009). Bacteria were maintained in nutrient broth or nutrient agar (Gibco, USA) and were grown for 18-24 h before use.

Preparation of Orange Oil

Terpeneless, cold-pressed Valencia orange oil was provided by Firmenich Citrus Center (Safety Harbor, USA). A stock solution was prepared by dissolving

CPV in dimethylsulfoxide (DMSO, Sigma, USA) to a final concentration of 40% CPV. However, for the pre-formed biofilm experiment, a 10% CPV in DMSO stock solution was needed due to orange oil evaporation. The 10% stock solution was also used for detection of cytotoxicity.

Determination of Minimum Inhibitory Concentration (MIC)

The broth dilution method was used to determine the MIC of *S. aureus* (Muthaiyan et al., 2012b). Concentrations of CPV ranging from 0.0125% to 0.4% CPV with half step dilutions were added to 24 well plates containing 10^5 CFU ml^{-1} of *S. aureus*. Plates were incubated overnight and MIC was determined as the lowest concentration of CPV inhibiting visual growth of bacteria in the wells of 24-well plate (Greinerbio-one, USA). This was confirmed by colony forming unit (CFU) assay.

Growth Inhibition Assay

Using a spectrometer, *S. aureus* was diluted 10^8 CFU ml^-1 in nutrient broth (Difco, USA) by adjusting it to OD_{600} ~0.08-0.12. This was further diluted to 10^5 CFU ml^{-1}, and placed in cell culture tubes. *S. aureus* was treated with CPV at concentrations of 0.00%, 0.05%, 0.025%, 0.0125%, and 0.00625%. For 4 h, the cell culture tubes were kept in a shaking incubator at 37°C. We took 100 µl of *S. aureus* every 15 m for 1 h, then every 30 m for 1 h, and then every 60 m for a total of 4 h of incubation. This was serially diluted and plated on to nutrient agar (Difco, USA). Viability was determined by colonies count after overnight culture at 37°C.

Inhibition of Pre-Formed Biofilms

The ability of CPV to affect preformed *S. aureus* biofilms was performed, using methods adapted from Karaolis et al. (2005), O'Toole (2011), and Ma et al. (2012). Briefly, bacterial cells were inoculated into nutrient broth and incubated for 18 h. The OD_{600} of the bacterial suspension was adjusted to ~0.08-0.12 (10^8 CFU ml^{-1}) and then further diluted with nutrient broth to 10^7 CFU ml^{-1}. Diluted cultures were aliquoted (200 µL) into a 96-well polystyrene flat-bottom microtiter plate (Greinerbio-one, USA) and incubated statically at 37°C for 24 h. Following incubation, the supernatant was removed and fresh nutrient broth (200 µL) containing 0%, 0.025%, 0.05%, or 0.1% CPV was placed in the wells and wrapped with Parafilm (Sigma-Aldrich, USA) for an incubation interval of 24 h at 37°C. After incubation, the plate rinsed thrice with distilled water. Afterwards, 0.1% crystal violet (200 µL) was added, and the plate was incubated at room temperature for 10-15 m. The plates were rinsed thrice and allowed to air dry overnight. Aliquots of 200 µL 30% acetic acid in distilled water was used to dissolve the crystal violet, and plates were shaken for 1 h before being the solution in each well was transferred to a new 96-well plate. To determine biofilm growth, the absorbance was measured by a spectrophotometer (Thermo Scientific, USA) at 540 nm.

Mammalian Cell Culture and Monolayer Formation

The immortalized bovine mammary gland epithelial cell line (MAC-T) was maintained in 75 cm^2 cell culture flasks in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 µg of gentamicin mL^{-1} at 37°C, 5% CO_2.

Inhibition Assay for *S. aureus* Association to and Invasion of MAC-T cells

The adherence and invasion assay was modified from Biswas et al. (2000) and Salaheen et al. (2014). In a 24 well plate, 10^5 MAC-T cells in 500 µL in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) were seeded into each well and incubated for 24 h at 37°C, 5% CO_2. *S. aureus* was cultured and incubated for 18 h at 37°C. *S. aureus* was then diluted to 10^8 CFU ml^{-1} in nutrient broth by adjusting it to OD_{600} ~0.08-0.12 before being further diluted to 10^7 CFU mL^{-1}. The wells containing MAC-T cells were then washed three times before being infected with 10 µL of 10^7 CFU mL^{-1} *S. aureus* and 1mL of DMEM supplemented with 10% FBS. The plates were incubated for 1 h at 37°C with 5% CO_2 to allow time for infection. Wells are then washed thrice with DMEM, and then treated with DMEM supplemented 10% FBS and 0%, 0.025%, 0.05%, 0.1%, or 0.2% CPV. The plates were incubated again for 1 h at 37°C, 5% CO_2. To measure association to MAC-T cells, the cells were washed three times with DMEM, treated with 0.1% Triton X-100, and incubated for 15 m at 37°C, 5% CO_2. Associated bacterial cells were then serial diluted and plated for enumeration. To measure bacterial invasion of MAC-T cells, cells were first treated with DMEM supplemented

22

with 10% FBS and 100 µg of gentamicin/mL for 1 h at 37°C, 5% CO_2 before the following the same procedure to measure association.

Detection of Cytotoxicity of Orange Oil on MAC-T cells

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay methods were adapted from Murbarak et al. (2015) and Yousefzadi et al. (2011). From an initial cell suspension of $2x10^5$ MAC-T cells, 200 µL of this suspension was added into each well, for a final cell population of ~ $4x10^4$ cells/well into a round bottom 96 well plate and incubated for 24 h at 32°C, 5% CO_2. The media was removed, and cells were washed three times with DMEM before media was replaced with 200 µL DMEM supplemented with 10% FBS, 100 µg of gentamicin mL^{-1}, and 0%, 0.025%, 0.05%, or 0.1% CPV. This plate was incubated for 1 h at 37°C, 5% CO_2. Media was removed again, and the cells were washed three times with DMEM before 200 µL of DMEM supplemented with 10% FBS and 100 µg mL^{-1} gentamicin was added to each well with 20 µL of 5 mg mL^{-1} MTT, reconstituted in 1x PBS. The cells were incubated for 2 h to allow reduction of MTT at 37C, 5% CO_2. Lysed cells will not react with MTT. Afterwards, media was removed and 200 µL of DMSO was added to each well. The 96 well-plate was incubated for 10-15 m with shaking before reading at an absorbance of 570 nm. A blank of 100% DMSO was used.

Statistical Analysis

Two-way ANOVA analysis and Tukey's HSD was carried out using the

general linear model in Statistical Analysis System® (SAS, Cary, North Carolina)

statistical significance between trials. Results were considered statistically significant

if mean differences were $p < 0.05$.

Results

Minimum Inhibitory Concentration of CPV

The growth of *S. aureus* was inspected after treatment of CPV at

concentrations ranging from 0.0125% to 0.4% for 18 to 24 h. For *S. aureus*, the

minimum inhibitory concentration was 0.025% CPV during an 18-24 h period as

determined by visual inspection.

Growth Inhibition of *S. aureus* by CPV

S. aureus growth was reduced most of CPV concentration and time point

except incubated in medium containing 0.00625 % and 0.0125% CPV for 15 min

(Figure 1). All CPV trials were capable of significant reductions in *S. aureus* bacterial

cell count, but the magnitude and speed of this inhibitory action depended on

concentration. After only 15 min, the 0.05% CPV trial and 0.025% CPV trial created

statistically significant reductions compared to the DMSO trial with an average of

4.39 ± 0.12 log CFU ml^{-1} and 4.61 ± 0.21 log CFU ml^{-1}, respectively, compared to

4.78 ± 0.35 log CFU ml^{-1} in the DMSO control. After 30 min, the bacterial counts

0.0125% CPV reduces to at 4.1126 ± 0.62 log CFU ml^{-1}, which is statistically

significant decrease in bacterial count the DMSO control at 4.82 ± 0.26 log CFU ml^{-1}.

At the lowest concentration used of 0.00625% CPV, the numbers of *S. aureus* were

reduced from 4.98 ± 0.14 log CFU ml^{-1} to 4.15 ± 0.21 log CFU ml^{-1} over the 4 h time

period. This reduction begins around 60 m of exposure, when numbers of *S. aureus*

are 4.78 ± 0.05 log CFU ml^{-1}, which is statistically significant from the DMSO

control trial at 5.02 ± 0.22 log CFU ml^{-1}. All trials show further reduction at every

following interval. Some concentrations were able to effectively eliminate *S. aureus*

below detectable limits. At a concentration of 0.025% CPV, no *S. aureus* was

recovered after 180 m and at a higher concentration of 0.05% CPV, no *S. aureus* was

recovered after 120 m. However, *S. aureus* was only reduced to 2.70 ± 0.12 log CFU

ml^{-1} at a concentration of 0.0125% after 4 h. Lower concentrations also failed to

eliminate *S. aureus* below detectable limits.

Effect of CPV on Pre-Formed Biofilms

CPV's effect on preformed *S. aureus* biofilms is shown in Figure 2. These

biofilms were formed in 96 well plates over 24 h before being treated with CPV for

another 24 h. Concentrations of CPV used were 0%, 0.025%, 0.05%, and 0.1% in

nutrient broth. Absorbance in each well was measured at 540 nm. There was no

statistical difference in absorbance in biofilm growth between 0% CPV, the control,

0.025% CPV, and 0.1% CPV. However, there was a statistically significant

difference in biofilm growth between 0.05% CPV (A_{540} 0.056) and the control (A_{540} 0.061).

Cytotoxicity of CPV on MAC-T cells

CPV has no cytotoxic effect on MAC-T cells, which is displayed in Figure 3. This was measured after 1 h of treatment. CPV concentrations start at 0.025% with half step dilutions up to 0.1%. At the lowest concentrations, CPV was able to increase proliferation. Relative cytotoxicity was measured -38.0% for 0.025% CPV and -42.2% for 0.05% CPV. However, at the highest concentration, 0.1%, there was no effect on MAC-T cells. The relative cytotoxicity measured for 0.1% CPV was -13.5%, which was not significant difference from the baseline.

Inhibition of *S. aureus* Association to and Invasion of MAC-T cells

Figure 4 displays quantities of *S. aureus* recovered from MAC-T cells after CPV treatments. *S. aureus* with MAC-T cells were treated for 1 h with range of CPV conditions starting at 0.2% with half step dilutions down to 0.025% and a control using DMSO. Associated bacteria are those adhered to and invaded in host cells. CPV had an effect on association of *S. aureus* to MAC-T cells at the highest concentrations tested. No bacterial cells were recovered after treating with 0.1% and 0.2% CPV for 1 h. These results were statistically significant at $p<0.05$. Adhesiveness of *S. aureus* to MAC-T cells was 46% and 70% reduced in 0.025 and 0.05% CPV treated group compared with the non-treated control, respectively (Figure 3). However, these results were not statistically significant.

CPV was shown to inhibit invasion of *S. aureus* specifically as well. A bar graph

depicting this data is displayed in Figure 5. The concentrations of CPV tested were

identical to those used in the association assay. Concentrations as low as 0.025%

CPV were capable of inhibiting invasion by *S. aureus*. There was significant

difference between the control of 0% CPV and all the CPV treatments. For *S. aureus*

in 0.025% CPV, bacterial count decreased from 2.34×10^4 CFU mL^{-1} to 6.17×10^2

CFU mL^{-1}. At a concentration of 0.5% CPV, recovered *S. aureus* decreased to $1.00 \times$

10^2 CFU mL^{-1}. At concentrations of 0.1% CPV and above, *S. aureus* was no longer

detectable.

Discussion

The effects antimicrobial effects of citrus derived oil have been well-

documented, but most studies have focused on its effects on foodborne pathogens.

This study aimed to test the antimicrobial properties of CPV on a common mastitis-

causing pathogen, *S. aureus*. The strain of *S. aureus* used was isolated from the milk

of cows with mastitis as opposed to strains found in foodborne infection. However,

studies done on foodborne strains of *S. aureus* suggest CPV will be effective against

S. aureus strains associated with mastitis as CPV acts on the cell wall of the bacteria

(Muthaiyan et al., 2012b).

In this study, we investigated antimicrobial properties of CPV on *S. aureus*

and it was fast and effective. We identified that MIC of CPV was 0.025% on growth

of *S. aureus* after 18 to 24 h incubation. This is far lower than an MIC of 0.18% that

has been shown for other strains of *S. aureus* (Muthaiyan et al., 2012b). However, the MIC for *S. aureus* is variable depending on strain with another study reporting an MIC of 0.5% (Espina et al., 2011). Even low concentrations of CPV, such as 0.00625%, also inhibited *S. aureus* growth in a time kill curve assay. CPV inhibited *S. aureus* growth in all CPV treatments compared with a 1% DMSO treatment. *S. aureus* was eliminated below detectable limits in medium containing 0.025% and 0.05% CPV after 180 min and 120 min incubation, respectively (Figure 1). This is consistent with findings in another study that showed a 0.1% concentration of orange oil and orange terpeneless oil was effective at *S. aureus* growth by 100% (Dabbah et al. 1970) as elimination of *S. aureus* below detectable limits occurs at 0.025% CPV or higher. There are various proposed mechanisms of action. The first is that the essential oils cause damage to the cell well (Burt, 2003; Helander et al., 1998; Muthaiyan et al., 2012b). The second is the essential oils cause damage to the cell membrane and mitochondrial membrane (Burt, 2004). This is due to the fact the essential oils tend to be hydrophobic, so they disrupt the membrane, increasing membrane permeability (Burt, 2004; Fisher et al., 2008). The third is that essential oils damage membrane proteins (Burt, 2004). All of these mechanisms can result in leakage of ions and nutrients out of the cell (Burt, 2004; Fisher et al., 2008).

The effect of CPV on preformed *S. aureus* biofilms was tested. *S. aureus* biofilms play a role in chronic and recurrent mastitis infections, allowing the infection to hide from antimicrobials (Melchior et al., 2006). To our knowledge, no other study has examined the effect of CPV alone on *S. aureus* biofilms. However, other essential oils have been documented to inhibit *S. aureus* and *Staphylococcus*

epidermis biofilms (Solórzano-Santos et al., 2012). In addition, one study had used a 1:1 blend of orange oil and bergamot oil to inhibit *S. aureus* (Laird et al., 2012). There were modest reductions in organisms after a 24 h exposure (Laird et al., 2012). After growing biofilms for 18-24 h, CPV treatment was applied, and then the absorbance at OD_{570} was measured. CPV was shown to only have a modest effect on biofilms at a concentration of 0.05%, but no effect at 0.025% and 0.1%. This is similar to the action of some antibiotics on biofilms (Kaplan, 2011). Induction of biofilms is often seen at low doses, and occasionally, at high ones as well, creating a u-shaped dip in which the antibiotic is most effective (Kaplan, 2011). Low doses of antibiotics are capable of inducing transcription with about 5% of bacterial promoters being upregulated during exposure (Kaplan, 2011). Notably, biofilm formation appears to be upregulated by some low doses of antibiotics in some bacteria, such as rifampin on *S. epidermis* (Kaplan, 2011). It is possible that orange oil is acting in a similar manner to antibiotics and is inducing transcription of biofilm genes at lower concentrations. High doses stimulation is also seen in other bacteria during high dose antibiotic exposure (Kaplan, 2011). As CPV is a modest inhibitor preformed biofilms, it may be unable to limit chronic cases of mastitis except when used in moderate doses.

We then used MAC-T cells for an *in vitro* model of CPV's effect on *S. aureus* in a mammary gland. Inhibition of association was tested. Association measures adherence as well as invasion. Infection by *S. aureus* begins with adhesion to bovine mammary gland cells, and adherence is the initial step in pathogenesis (Dego et al., 2002). A concentration of 0.1% CPV or higher was required to significantly inhibit

association by *S. aureus* to MAC-T cells (Figure 3). Inhibition of invasion was then

tested. Chronic mastitis by *S. aureus* is a large problem, caused by invasion into the

cell, since antimicrobials typically only affect extracellular bacteria (Barkema et al.,

2006). Therefore, treatments are needed which can prevent and inhibit intracellular

invasion. In MAC-T cells, it only took a concentration of 0.025% CPV to inhibit

invasion by *S. aureus* significantly from 2.34×10^4 CFU ml^{-1} to 6.17×10^2 CFU ml^{-1}.

Higher concentrations resulted in greater reductions in bacterial count. Other studies

have documented orange oil's ability to eliminate *S. aureus* from infected

keratinocytes after using a concentration of 0.2% to treat cells (Muthaiyan et al.,

2012b). Invasion of *S. aureus* into bovine mammary cells is thought to be caused by

S. aureus producing factors which mobilize F actin microfilaments in the bovine cell

cytoskeleton, allowing the bacteria to be internalized through endocytosis (Almeida et

al., 1996). Binding to fibronectin appears to be necessary and sufficient for adhesion

as well as invasion (Menzies, 2003; Dego, 2002). *S. aureus* has fibronectin binding

proteins on the surface of the cell membrane (Menzies, 2003). It is possible CPV

disrupts fibronectin binding proteins, preventing endocytosis of *S. aureus*, but further

study is required.

Determination of any cytotoxic effect of CPV on bovine mammary epithelial

cells is necessary to ensure safety if used *in vivo*. Cytotoxicity of CPV was tested on

MAC-T cells in order to model *in vivo* bovine mammary epithelial cells. CPV does

not have a cytotoxic effect on MAC-T cells. Its proliferation stimulating effect at

concentrations of 0.025% and 0.5% may even serve to reverse damage to udder tissue

following mastitis infection. To our knowledge, no studies have examined the effect

of CPV on bovine mammary cells. However, there is a study, which found that CPV has no effect on mice macrophage cells at concentrations of 0.025% and 0.05% (Scelza et al., 2006). From both of these studies, it does not appear that CPV negative impacts cell viability.

In this study, we provided evidence that CPV has an antimicrobial effect on *S. aureus*. Our data suggest that CPV has the potential to be a treatment for *S. aureus* mediated mastitis. Other studies can be done to see whether CPV's other effects on *S. aureus*, such as inhibition of the release of exotoxins, inhibition of autoaggregation, and hydrophobic action. In addition, more work should be done to examine its antibiofilm properties, including its effects on biofilm growth across a greater range of concentrations as well as how it effects gene expression of biofilm genes. To ensure its safety for cattle, more work must be done to determine CPV's effects on MAC-T cells, such as cytokine production and milk synthesis. Lastly, CPV should be researched on other causative agents of bovine mastitis to see its effects on other pathogens.

Chapter 3: Major Components of Terpeneless, Cold-pressed Valencia Orange Oil and its Effects on *Staphylococcus aureus*, Bovine Epithelial Mammary Cells, and Infection

Abstract

Bovine mastitis is a costly disease in the dairy industry that does not always respond to antibiotic treatment. The major components of terpeneless, cold-pressed Valencia orange oil, citral, linalool, decanal, and valencene, were examined as potential alternative treatments for *Staphylococcus aureus* associated mastitis. The minimum inhibitory concentration (MIC) of all four components against *S. aureus* was determined after 24 h incubation. Growth inhibition assay was performed for all effective components on *S. aureus* for either a 3 h or 72 h treatment. These components were tested for the ability to disrupt pre-formed *S. aureus* biofilms after 24 h of treatment by measuring absorbances at 540nm. Cytotoxicity against immortalized bovine mammary epithelial (MAC-T) cells was measured using MTT assay following a 1 h exposure. Only concentrations below the 50% cytostatic concentration (CC50) were used in an adherence and invasion assay of *S. aureus* on MAC-T cells and measurements of gene expression for virulence and biofilm genes via qPCR. The MICs of citral and linalool were 0.02% and 0.12%, respectively, but decanal and valencene were ineffective. Citral and linalool were capable of inhibiting growth of *S. aureus* after 24 h at their MIC values and inhibited pre-formed biofilms of *S. aureus*. The concentrations below CC50 were 0.02% for citral, and 0.12% for linalool. These concentrations inhibited the adhesion and invasion ability of *S.*

aureus and downregulated virulence genes. Only 0.12% linalool was enough to downregulate the expression of *S. aureus* biofilm forming genes. These components should be considered in further *in vivo* study.

Introduction

Bovine mastitis is inflammation of the udder tissue in at least one quarter and is usually caused by a bacterial pathogen (Contreras et al., 2011; Wilson et al., 1997). However, treatment for bovine mastitis can be expensive depending on the type of infection. On a national level, the US dairy industry loses $2 billion in costs associated with mastitis each year (National Mastitis Council, 1996). On a farm level, the cost per a cow can vary depending on severity and chronicity of infection. Symptoms of clinical mastitis may include localized swelling and redness of one or more udder quarters and in more severe cases, systemic signs of depression, anorexia, fever, and reduced milk quality and production. Clinical cases of mastitis cost $179 per a cow (Bar et al., 2008). Yet, subclinical cases are more prevalent (Barlow, 2011; Haveri et al., 2005). Subclinical cases may only present with reduced milk quality and production as well as an elevated somatic cell count (SCC). With a medium SCC, subclinical cases of mastitis cost $108 per a cow (Ott, 1999). However, subclinical cases with high SCC can cost dairy producers $295.24 per a cow (Ott, 1999). In addition to economic losses, mastitis can have a long term impact on the health of dairy cattle. The bacterial infections can cause permanent damage to the

mammary tissue, which reduces milk production and quality for a lifetime, and decreased reproductive efficiency (Harmon, 1994).

Staphylococcus aureus is an infectious bacterial pathogen, which is usually spread between cows during the milking process. The most common form of treatment is antibiotic therapy. However, antibiotics often fail to cure *S. aureus* associated mastitis, particularly in subclinical and chronic cases (Barkema et al., 2006). This combined with pressure from the FDA as well as consumers to reduce antibiotic usage makes it urgent to search for alternative in the treatment of mastitis.

Essential oils have been studied for their antimicrobial properties (Kim et al., 1995; Lis-Balchin and Deans, 1997; Smith-Palmer et al., 1998; Alzoreky and Nakahara, 2003). One commonly studied essential oil is orange/citrus oil. Orange oil has been demonstrated to inhibit methicillin resistant *S. aureus* (MRSA) growth (Muthaiyan et al., 2012). Since it is a generally recognized as safe product, the FDA allows its use in human and animal feed. In addition, it can be cheaply extracted, making it a cost effective antimicrobial product. However, orange oil contains many compounds of which do not have antimicrobial properties. The major components of terpeneless, cold-pressed Valencia orange oil as being 20.2% linalool, 18.0% decanal, 14.1% citral, 5.8% α-terpineol, 5.2% valencene, 4.1% dodecanal, 3.9% citronellal, and 0.3% limonene (Nannapaneni et al., 2009). Both linalool and decanal have demonstrated inhibition of *S. aureus* growth (Liu et al., 2012). Citral has been studied for its ability to inhibit a wide range of bacterial pathogens, such as *S. aureus*, as well pathogenic fungi (Saddiq et al., 2010). As a result, these compounds are of interest as antibiotic alternatives.

In this study, we aimed to investigate the potential role of the most prevalent components of cold-pressed, terpeneless Valencia orange oil, linalool, decanal, citral, and valencene as an antibiotic alternative in the treatment of *S. aureus* associated bovine mastitis. Their interaction with *S. aureus* and bovine mammary epithelial (MAC-T) cells was examined.

Methods and Materials

Bacterial Strains and Growth Conditions

The *S. aureus* (ATCC 29740) strain, isolated from mastitic milk, was used for this study. *S. aureus* was grown and maintained on nutrient agar or nutrient broth (Gibco, USA). Prior to each experiment, *S. aureus* was sub-cultured on nutrient agar plate from the freezing (-80°C) stock and grown for 18-24 h at 37°C.

Preparation of Orange Oil Components

Citral, linalool, decanal, and valencene (Sigma-Aldrich, USA) were used for this study. A 10% stock solution for these components in 1% Tween-80 in distilled water was used for all experiments.

Determination of Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) was determined using the broth dilution method described by Muthaiyan et al. (2012b). Concentrations of citral, linalool, decanal, and valencene ranging from 0.01% to 2.5% were tested for antimicrobial activity against *S. aureus*. These concentrations were added to 10^5 CFU ml^{-1} of *S. aureus* in a 24 well plate (Greinerbio-one, USA). *S. aureus* was incubated overnight at 37°C, and visually inspected for growth inhibition to determine the MIC.

Growth Inhibition Assay

S. aureus was diluted to 10^5 CFU ml^{-1} in LB or nutrient broth (Difco, USA) after being adjusted to OD$_{600}$ ~0.08-0.12, which is equal to 10^8 CFU ml^{-1}. The LB or nutrient broth was either supplemented with 0.02% citral, 0.04% citral, 0.12% linalool, or 0.24% linalool. A control was used, which contained unsupplemented LB or nutrient broth. *S. aureus* was incubated for 72 h at 37°C in cell culture tubes, and enumeration took place every 24 h. If no bacteria were detectable after 24 h for any concentration, the experiment was repeated except with incubation for 3 h and enumeration occurring every 1 h.

Inhibition of Pre-Formed Biofilms

Methods were adapted from literature previously described by Karaolis et al. (2005), O'Toole (2011), and Ma et al. (2012). *S. aureus* was adjusted to 10^8 CFU ml^{-1} by diluting to OD$_{600}$ ~0.08-0.12. This was then further diluted to 10^7 CFU ml^{-1}. Aliquots of 200 µL were added into each well of a 96 well polystyrene flat-bottom

microtiter plate (Greinerbio-one, USA). These plates were incubated statically for 24 h at 37°C. Supernatants of the 24-wells plate were the removed and discarded, and the biofilms were then treated with unsupplemented nutrient broth or nutrient broth supplemented with 0.02% citral, 0.04% citral, 0.08% citral, 0.12% linalool, 0.24% linalool, or 0.48% linalool. These plates were sealed with Parafilm (Sigma-Aldrich) to prevent evaporation of citral and linalool and incubated for 24 h at 37°C. Supernatant was shaken out, and 96 well plates were rinsed by being submerged in water. Plates were rinsed three times with water being shaken out after each rinse. Aliquots of 200 µL of 0.1% crystal violet was added to each biofilm and incubated for 10-15 min at room temperature. The plates were then rinsed again three times with water being shaken out after each rinse, and allowed to dry overnight. To each well, 200 µL of 30% acetic acid in distilled water was added. Plates were shaken at 1 h, and the acetic acid solution was added to a new plate. The absorbances of the solution were then read at 540 nm using a spectrometer (Thermo Scientific, USA).

Mammalian Cell Culture and Monolayer Formation

The MAC-T cell line is an immortalized bovine mammary gland epithelial cell line and was used for all cell culture work. These cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 µg of gentamicin mL^{-1} in 75 cm^2 cell culture flasks and incubated at 37°C for overnight in a 5% CO_2 incubator.

Detection of Cytotoxicity of Citral and Linalool on MAC-T cells

Methods were adapted from Murbarak et al. (2015) and Yousefzadi et al. (2011) and used a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for determining cytotoxicity. Aliquots of 200 μL of a 2×10^5 MAC-T cell suspension into a 96 well plate.   This plate was incubated for 24 h at 32°C in a 5% CO_2 incubator. The media was removed, and the cells were washed three times with DMEM. The cells were then treated with DMEM supplemented with 10% FBS, 100 μg mL^{-1} gentamicin, and either 0.02% citral, 0.04% citral, 0.08% citral, 0.12% linalool, 0.24% linalool, or 0.48% linalool. A control of only DMEM supplemented with 10% FBS, 100 μg mL^{-1} gentamicin was used as well. The cells were incubated again for 1 h at 32°C in a 5% CO_2 incubator. Afterwards, the media was removed and cells were washed three times with DMEM. Cells were then incubated with 200 μL of DMEM supplemented with 10% FBS and 100 μg mL^{-1} gentamicin and 20 μL of 5 mg mL^{-1} MTT, reconstituted in 1x PBS for 2 h at 37°C in a 5% CO_2 incubator. The media was then removed, and dimethylsulfoxide (DMSO, Sigma-Aldrich, USA). The 96 well-plate was shaken for 10-15 min. The absorbance was read at 492 nm on a spectrometer with a blank of 100% DMSO.

S. aureus Association and Invasion Assay for MAC-T cells

The methods from Biswas et al. (2000) and Salaheen et al. (2014b) were adapted for this assay. Aliquots of 500 μL of a suspension of 2×10^5 MAC-T cell mL^{-1} in DMEM supplemented with 10% FBS were added to a 24 well plate. The cells were allowed to incubate for 18-24 h at 37°C in a 5% CO_2 incubator. The cells

were washed three times with DMEM, 1 mL of DMEM supplemented with 10% FBS was added to the cells, and 10 μL of 10^8 CFU ml^{-1} *S. aureus*, for a final concentration of 10^6 CFU mL^{-1} *S. aureus*, was used to infect the cells. These cells were incubated at 37°C in a 5% CO_2 incubator for 1 h. Afterwards, cells were washed three times with DMEM and treated with DMEM supplemented with 10% FBS and either 0.02% citral, 0.04% citral, or 0.12% linalool. A control of only DMEM supplemented with 10% FBS was used again. The cells were incubated again for 1 h at 37°C in a 5% CO_2 incubator. Cells were then washed three times with DMEM. To enumerate associated bacteria, cells were incubated with 0.1% Triton X-100 (Sigma, USA) for 15 min at 37°C, in a 5% CO_2 incubator, and the associated bacteria was plated on LB agar to count colonies. To enumerate invaded bacteria, cells were first treated with DMEM supplemented with 10% FBS and 100 μg mL^{-1} gentamicin for 1 h at 37°C in a 5% CO_2 incubator. Afterwards, cells were incubated with 0.1% Triton X-100 (Sigma, USA) for 15 min at 37°C in a 5% CO_2 incubator, and the invaded bacteria was plated on LB agar to count colonies.

Effect on Gene Expression of *S. aureus* Virulence and Biofilm Genes

For gene expression of *S. aureus* virulence genes and MAC-T cells cytokine genes, MAC-T cells were infected and treated in a similar fashion to the association and invasion assay, but scaled up to 6 mL in 25cm^2 flasks. Afterwards, RNA was extracted using methods described in Ahn et al. (2014). A NanoDrop ND-1000 was used to measure the concentration of extracted RNA. A concentration of 1 μg of RNA was used to create cDNA using the instructions provided in the Verso cDNA

Synthesis Kit (Thermo Scientific, USA). All quantitative PCR used the methods described in Ahn et al. (2014) and Salaheen et al. (2014), except cDNA was diluted 10 fold. The cytokine genes measured were IL-1β, IL-6, IL-8, TNFα, IFNγ, IL-12, and IL-17A with RPLP0 being used as a reference gene. These cytokine genes and primers are listed in Table 1. The virulence genes measured were *agrA*, *sodA*, *sirA*, *hla*, *hlb*, *spa*, *sbi*, *sarA*, *sarR*, *sarS*, and *sigB*, and the reference gene used was 16sRNA. The virulence genes and primers used in this study are listed in Table 2. To determine expression of *S. aureus* biofilm genes, *S. aureus* was formed and treated in the same way as the inhibition of pre-formed biofilms assay. However, treatments only included a LB broth control, 0.02% citral, 0.04% citral, and 0.12% linalool. After 24 h of treatment, RNA extract, cDNA synthesis, and qPCR was carried out using the same methods described for the virulence genes above except cDNA synthesis used qScript cDNA SuperMix (Quanta Biosciences, USA). The biofilm genes measured were *icaA*, *icaB*, *icaC*, *icaD*, *fnbA*, *fnbB*, *clfA*, *clfB*, and *ebps* and 16sRNA was used a reference. All biofilm genes are primers used are listed in Table 3.

Statistical Analysis

Statistical significance between treatments was determined by two-way ANOVA analysis and Tukey's HSD, using the general linear model in Statistical Analysis System® (SAS, Cary, North Carolina). If p<0.05, mean differences were considered statistically significant.

Results

MICs of Citral, Linalool, Decanal, and Valencene Required to Inhibit *S. aureus*

The MICs of citral, linalool, decanal, and valencene are shown in Table 4. Only citral and linalool were found to have an inhibitory effect on *S. aureus*. The MICs for citral and linalool were 0.02% and 0.12%, respectively. A concentration of 2.5% was not even sufficient to inhibit growth of *S. aureus* for either decanal or valencene. Due to higher MIC values and cytotoxic effects, decanal and valencene were excluded from the remainder of the study.

Time Dependent Inhibitory Effects of Citral and Linalool on *S. aureus* Growth

Growth inhibition of *S. aureus* by various concentrations of citral and linalool over 72 h is shown in Figure 6. Concentrations of 0.04% citral and 0.24% linalool inhibited the growth of *S. aureus* below detectable limits after 24 h of treatment. The minimum concentration, 0.02% citral, inhibited growth by 3.76 log CFU mL^{-1}, and 0.12% linalool inhibited growth by 2.67 log CFU mL^{-1}. The difference in inhibitory effect between citral and linalool after 24 h of treatment was not statistically significant. This inhibitory effect was maintained for 72 h for both citral and linalool. However, the inhibitory effect was reduced after 48 h for both citral and linalool.

A shorter 3 h growth inhibition assay was used for 0.04% citral and 0.24% linalool, which is shown in Figure 7. A concentration of 0.04% citral decreased *S. aureus* concentration below that of the initial inocula after 1 h and eliminated *S. aureus* below detectable limits by 3 h. A concentration 0.24% linalool also demonstrated inhibition of *S. aureus* growth after 1 h. However, 0.24% linalool did

not decrease *S. aureus* concentration below that of the initial inocula until 3 h of
treatment.

Inhibition of Pre-formed *S. aureus* Biofilms with Citral and Linalool

Figure 8 illustrates the inhibition of *S. aureus* pre-formed biofilms by both
citral and linalool. All concentrations tested, which included the MIC values above,
were capable of inhibiting pre-formed biofilms. The most effective concentrations
were 0.08% citral, 0.24% linalool, and 0.48% linalool, which had absorbances of A_{540}
0.079, A_{540} 0.075 and A_{540} 0.067, respectively. The difference in absorbance between
these concentrations was not statistically significant. However, this was significantly
lower than the absorbances of the control and 0.02% citral, which were A_{540} 0.104
and A_{540} 0.090, respectively.

Cytotoxic Effects of Minimum Concentration of Citral and Linalool on MAC-T cells

Cytotoxicity of the minimum concentration of citral and linalool on MAC-T
cells are shown in Figure 9 and 10, respectively. Figure 9 represents the depicting
relative cytotoxicity for citral and Figure 10 represents the depicting relative
cytotoxicity for linalool. Both citral and linalool showed cytotoxicity for all
concentrations tested. For citral, relative cytotoxicities for 0.02%, 0.04%, and 0.08%
citral were 29.8%, 47.7%, and 59.6%, respectively. For linalool, the relative
cytotoxicities for 0.12%, 0.24%, and 0.48% linalool were 31.9%, 72.4%, and 74.7%,
respectively. Only concentrations with relative cytotoxicities below 50% cytostatic

concentration (CC50), which were 0.02% citral, 0.04% citral, and 0.12% linalool, were used in further experiments.

Role of Citral and Linalool in Adhesion to and Invasion into MAC-T cells by *S. aureus*

Citral and linalool's effect on *S. aureus* association to MAC-T cells are shown in Figure 11. All concentrations of citral and linalool tested reduced *S. aureus* adherence to MAC-T cells compared to the control. A concentration 0.02% citral had the lowest reduction at 4.5×10^3 CFU mL^{-1}. A concentration of 0.12% linalool reduced adhered *S. aureus* by 6.0×10^3 CFU mL^{-1}, which was not significant from 0.02% citral. A concentration 0.04% citral produced the lowest reduction at 7.2×10^3 CFU mL^{-1}, which was not significant from 0.12% linalool.

Citral and linalool's ability to inhibit *S. aureus* invasion of MAC-T cells is shown in Figure 12. Concentrations of 0.02% and 0.04% citral were able to reduce invaded *S. aureus* by 5.2×10^3 CFU mL^{-1} and 9.7×10^3 CFU mL^{-1}, respectively. There was a statistical difference between 0.02% citral and 0.04% citral. The lowest reduction made was by 0.12% linalool, which was 1.0×10^4 CFU mL^{-1}. This was not significant different from 0.04% citral.

Effects of Citral and Linalool on Expression of MAC-T Cytokine Genes and *S. aureus* Genes Involved in Virulence and Biofilms

Figure 13 illustrates the effects of citral and linalool on MAC-T cytokine expression during infection. The treatments were compared to an infected but

untreated control. All three treatments were capable of significantly upregulating IL-17A. A 1 h treatment of 0.12% linalool also significantly upregulated IFNγ. In addition, a 1 h treatment of 0.04% citral significantly downregulated IL-1β.

Figure 14 shows the impact of citral and linalool on *S. aureus* virulence gene expression. All concentrations, 0.02% citral, 0.04% citral, and 0.12% linalool, decreased the expression of *sirA*, *hla*, *spa*, and *sarS*. Both concentrations of citral also decreased expression of *agrA*, *sodA*, *hlb*, and *sarR*. In addition, a concentration of 0.04% citral reduced expression of *sbi*, *sarA*, and *sigB*.

Figure 15 illustrates citral and linalool's impact on biofilm gene expression. While citral had no impact on any of the biofilm genes examined, 0.12% linalool decreased expression of *icaA*, *icaB*, *fnbB*, *clfA*, *clfB*, and *ebps* by greater than 2 fold. The expression of *icaC*, *icaD*, and *fnbA* were not impacted by citral and linalool.

Discussion

The major components of terpeneless, cold-pressed Valencia orange oil, linalool, citral decanal, and valencene, were examined for their inhibitory effects on *S. aureus* growth, and alteration ability in *S. aureus*-MAC-T cell interaction. Of the four components, only citral and linalool were capable of inhibiting *S. aureus* growth, having MIC values of 0.02% and 0.12%, respectively. Another study has examined the MIC of these components on *S. aureus* as well, having previously determined to be 0.06% for citral and 0.125% for linalool (Fisher et al., 2006). These results are similar to our own findings. In addition, both citral and linalool were able to decrease *S. aureus* growth after 24 h of exposure at their MIC values and could completely

44

eliminate *S. aureus* below detectable limits at twice that value. Citral and linalool, in addition to many other terpenes, have been researched for their antimicrobial properties. Their mode of action is theorized to be due to damage to the cell membrane of bacteria, which leads to K^+ ion leakage and reduced membrane potential (Inoue et al., 2004).

Citral and linalool's effects on pre-formed biofilms were also examined. Linalool appeared to be stronger than citral at inhibiting biofilms. At linalool's MIC value 0.12%, it reduced pre-formed biofilms beyond that of the MIC value of citral, 0.02%. It should be noted that all concentrations tested resulted in statistically significant reductions of pre-formed biofilms compared to control. We were able to find no other studies that examined citral and linalool's impact on pre-formed biofilms. Yet, we can infer some information based on studies that examined other terpenes' ability to disrupt biofilms. Other terpenes have been found to possess anti-biofilm agents against *S. aureus* biofilms (Kuźma et al., 2007), meaning our results are consistent with past research. As for the mode of action, one study found that terpenes disrupted biofilms by promoting cell separation, caused by terpenes reducing the fatty acid composition of the cell membrane and increasing hydrophobicity (de Carvalho et al., 2007). It is possible that citral and linalool act in a similar manner, but further study is required.

Cytotoxicity was tested on MAC-T cells for both citral and linalool. All concentrations tested for both citral and linalool were found to be cytotoxic. There were only three concentrations found to be below CC50, 0.02% citral, 0.04% citral, and 0.12% linalool. As a result, only these concentrations were used for the

45

remainder of the study. In one past study, citral was determined to have a high

cytotoxicity in human skin cells (Hayes et al., 2002). Yet, it should be noted that *in

vivo* toxicity in rat models was found to be 100 fold lower than *in vitro* results (Hayes

et al., 2002). In addition, it was only at concentrations above 1% that citral caused

any skin sensitization (Hayes et al., 2002). Therefore, concentrations of 0.02% and

0.04% citral are probably safe for *in vivo* use. Linalool has also been tested for

cytotoxicity in a past study, but again on human skins cells (Prasher et al., 2004).

Previous results are consistent with our own, in which a large increase in cytotoxicity

occurs between 0.125% and 0.25% (Prasher et al., 2004). We found a large increase

in relative cytotoxicity between 0.12% linalool, which had a relative cytotoxicity of

31.9%, and 0.24% linalool, which had a relative cytotoxicity of 72.4%.

Concentrations of 0.12% linalool and lower should be safe for use, but it is not

advised to use higher concentrations. More studies would be needed to determine the

safe dosages for use *in vivo*.

The impact of citral and linalool on MAC-T cytokine expression was also

examined. Citral and linalool had no impact on the expression of IL-6, IL-8, IL-12,

and TNFα during infection. However, all three treatments upregulated IL-17A.

During *S. aureus* infection of bovine mammary epithelial cells, IL-17 is upregulated,

which in turn upregulates expression of chemokines that target both neutrophils and

mononuclear leucocytes (Bougarn et al., 2011). Increased expression IL-17A could

serve to better recruit neutrophils and leucocytes to the target area in order to combat

infection. In addition, 0.12% linalool also upregulated IFNγ. IFNγ is often

downregulated during *S. aureus* mastitis infection despite the cytokine's ability to

elicit the most effective immune response against the pathogen (Lee et al., 2005). It is thought that *S. aureus* may cause the suppression of IFNγ (Lee et al., 2005). By upregulating IFNγ, bovine mammary epithelial cells may be able to more effectively clear *S. aureus* infection. Lastly, 0.04% citral downregulated IL-1β. IL-1β is a pro-inflammatory cytokine that is often upregulated during *S. aureus* infection of the bovine mammary gland (Simojoki et al., 2011). Downregulation of this cytokine could create difficulties in clearing the infection.

The study then determined whether citral and linalool impacted *S. aureus*' association to and invasion of MAC-T cells. All of the concentrations below CC50 were capable of decreasing association and invasion. The most effective concentrations were 0.04% citral and 0.12% linalool with there being no statistically significant difference between the two. While the mechanism is unclear, our results on gene expression of virulence genes may offer some insight into the effects of citral and linalool. Both citral and linalool downregulated expression of *sirA*, *hla*, *spa*, and *sarS*. However, citral alone reduced expression of *agrA*, *sodA*, *hlb*, and *sarR*. In addition, only a concentration of 0.04% citral resulted in decreased expression of *sbi*, *sarA*, and *sigB*. *AgrA* is transcriptional regulator for virulence gene expression and is considered the sole quorum-sensing regulator (Lowy, 1998; Queck et al., 2008). It is possible that the downregulation of *agrA* could contribute to the downregulation of the other genes examined. The sar protein family, sarA, sarR, and sarS, are regulatory proteins for cell wall and extracellular proteins (Ster et al., 2005). Both sarA and sarS work antagonistically while sarR modulates the two (Ster et al., 2005). Reduced expression of all three may result in the reduced expression of other

proteins. *SigB* gene acts as another regulatory factor that impacts virulence as well as stress responses (Ster et al., 2005). Its downregulation may serve to increase susceptibility to stressful environmental conditions. *SirA* is involved iron acquisition for *S. aureus* (Heinrichs et al., 1999). As iron is a critical nutrient for infection and is often sequestered in cells, methods of iron acquisition are critical (Trivier et al., 1996). Due to the down-regulatory effects of citral and linalool on *sirA*, *S. aureus* may have problems obtaining iron for infection. Sbi is an immunoglobulin-binding protein (Burman et al., 2008). It plays a role in the evasion of adaptive immunity by binding to IgG as well as the evasion of innate immunity by binding complement (Burman et al., 2008). SpA, surface protein A, also binds IgG as well (Burman et al., 2008). While this would not affect the *in vitro* work done, it may serve to limit infections *in vivo*. Hla and hlb are both haemolysins (Ster et al., 2005). With lowered expression, cell damage may be reduced. This downregulation of major virulence genes may serve to inhibit *S. aureus* pathogenesis and infection. However, for biofilm genes, only linalool appeared to be able to downregulate expression. A concentration 0.12% linalool was capable of reducing expression of *icaA*, *icaB*, *fnbB*, *clfA*, *clfB*, and *ebps*. *IcaA* and *icaB* are part of a larger *ica* operon, which also includes *icaC* and *icaD*. The *ica* operon (*icaABCD*) facilitates cell to cell adhesion in biofilm formation (O'Gara, 2007). This is accomplished by the production of polysaccharide intercellular adhesin, which is translocated to the cell surface and deacetylated for attachment (O'Gara, 2007). The role of icaA in conjunction with icaD is to produce polysaccharide intercellular adhesion while the role of icaB is to deacetylate it to attach to surfaces and other cells (O'Gara, 2007). Downregulating

these two genes may interfere with cell to cell attachment and the growth of biofilms. *FnbA* and *fnbB* can also initiate biofilm development (Houston et al., 2010). However, they appear to play larger roles in the persistence of biofilms (Atshan et al., 2013; Houston et al., 2010). They also help facilitate adherence to surfaces as does *clfA* and *clfB* (Götz, 2002) and *ebps* (Downer et al., 2001). Downregulation of *fnbA* and *fnbB* would interfere with another mode of biofilm development as well as initial attachments. Adherence would be further reduced by the reduction of *clfA*, *clfB*, and *ebps* expression. Reduction of expression of these genes would all interfere with biofilm production and maintenance, which could serve to limit *S. aureus* colonization and infection.

Citral and linalool should be considered for further research in the treatment of *S. aureus* associated mastitis. Any issues with cytotoxicity should not present any problems *in vivo* according to past research in mice models. Concentrations with relative cytotoxicity below the CC50 may have greater benefits than risks. Both citral and linalool reduced *in vitro* growth and pre-formed biofilms. For linalool treatments, this decrease in pre-formed biofilms may be due in part to downregulation of genes that play a role in biofilm formation and development. Both citral and linalool also decreased *S. aureus* association and invasion, which may be due to their downregulation of virulence genes. Future directions include examining the impact of multiple treatments as well as safety and efficacy *in vivo*.

Chapter 4: Conclusion

The first study examined the potential of terpeneless, CPV orange oil as an alternative to antibiotics in the treatment of *S. aureus* associated bovine mastitis. In certain aspects, the compound was highly effective, such as reducing *S. aureus* growth and invasion of bovine epithelial mammary cells as well as increasing bovine epithelial mammary cell proliferation. However, it was only capable of reducing pre-formed biofilms at a single concentration tested, which was a modest reduction. For an effective treatment, reduction of pre-formed biofilms is necessary as most cases of mastitis are chronic, in which biofilm formation plays a role in persistence. In addition, the concentrations necessary to reduce association to epithelial mammary cells was very high and not the same concentration necessary to reduce pre-formed biofilms. This mismatch in dosages made it a poor choice in treatment. The second study examined individual components of terpeneless, CPV orange oil. While citral, linalool, decanal, and valencene were initially examined, only citral and linalool were found to be effective at inhibiting *S. aureus* growth. In addition, citral and linalool appeared to be more potent inhibitors of pre-formed biofilms, being effective at all concentrations tested. However, only linalool was capable of reducing expression of major biofilm genes. Both citral and linalool are partly cytotoxic and two concertation of citral (0.02% and 0.04%) and only concentration of linalool (0.12%), tested in this study, were deemed safe enough on bovine epithelial mammary cells. In addition, unpublished research suggests that citral and linalool have no effect on *Lactobacillus*, making it a safer than traditional antibiotics for beneficial microflora. Further experiments are required to evaluate the role purified extract of terpeneless

50

CPV in control mastitis in dairy cattle. These concentrations were also capable of

reducing interactions, both association and invasion, of bovine epithelial mammary

cells by *S. aureus*, showing the ability to inhibit infection. This inhibitory effect may

be due in part to their downregulation of various virulence genes. These studies

suggest that citral and linalool may be viable candidates as alternatives to antibiotics

in the treatment of *S. aureus* associated bovine mastitis.

Chapter 5: List of Tables

Table 1: Primers used for cytokine gene expression in this study.

Genes	Forward Primer (5' -> 3')	Reverse Primer (5' -> 3')	Reference
IL-1β	AAATGAACCGAGAAGTGGTGTT	TTCCATATTCCTCTTGGGGTAGA	(Strandberg Lutzow et al., 2008)
IL-6	CTGGGTTCAATCAGGCGAT	CAGCAGGTCAGTGTTTGTGG	(Strandberg Lutzow et al., 2008)
IL-8	CTGTGTGAAGCTGCAGTTCT	TAAGCAGACCTCGTTTCCAT	(Strandberg Lutzow et al., 2008)
TNFα	CTGGTTCAGACACTCAGGTCCT	GAGGTAAAGCCCGTCAGCA	(Strandberg Lutzow et al., 2008)
IFN-γ	TCTTCTCAAGCCTCAAGTAACAAGT	ACGTCATTCATCACTTTCATGAGTTC	(Strandberg Lutzow et al., 2008)
IL-12	TCATTAAGCGCATGGTCGACAAA	TCAGCTTATTTTCTGCCAGTGTCT	(Lee et al., 2006)
IL-17A	TGAGGACAAGAACTTCCCACAGCA	TAATCGGTGGGCCTTCTGGAGTTT	(Aranday-Cortes et al., 2013)
RPLPO	CAACCCTGAAGTGCTTGACAT	AGGCAGATGGATCAGCCA	(Strandberg Lutzow et al., 2008)

Table 2: Primers used for S. aureus virulence gene expression in this study.

Genes	Forward Primer (5' -> 3')	Reverse Primer (5' -> 3')	Reference
AgrA	CAAAGAGAAAACATGGTTACCAT TATTAA	CTCAAGCACCTCATAAGGATT ATCAG	(Garzoni et al., 2007)
sodA	TGTTGCTAATTTAGACAGTGTAC CAGCTA	TCCCAGAATAATGAATGGTTT AAATG	(Garzoni et al., 2007)
sirA	GGGAAACCAAAGCGTGTTGT	TGTCCATGATTCTACAGCACC TACA	(Garzoni et al., 2007)
hla	GCGAAGAAGGTGCTAACAAAAG TGG	CGCCAATTTTTCCTGTATCAT CACC	(Ster et al., 2005)
hlb	CGACCGTTTTGTATCCAAACTGG G	TTTGTCCCACCCTGATTGAGA ACG	(Ster et al., 2005)
spa	ATATCTGGTGGCGTAACACCTGC TG	CGCATCAGCTTTTGGAGCTTG AGAG	(Ster et al., 2005)
Sbi	AAGACAGCAAGAACCCAGACCG AC	CCAAACTTGTTGGCTTCTATC AGGG	(Ster et al., 2005)
SarA	GCTTTGAGTTGTTATCAATGGTCA C	CTCTTTGTTTTCGCTGATGTAT GTC	(Ster et al., 2005)
SarR	TCAACGCAACATTTCAAGTTAAG	TCTGAGCACTTAGCAATCTCT TTAG	(Ster et al., 2005)
SarS	AAAAGTCAAGCCTGAAGTCGATA TG	CTGCAATTTTCTCTCGTTGTTC TTC	(Ster et al., 2005)
SigB	GTCCTTTGAACGGAAGTTTGAAG CC	GAAGGTGAACGCTCTAATTCA GCGG	(Ster et al., 2005)
16sRNA	TATGGAGGAACACCAGTGGCGAA G	TCATCGTTTACGGCGTGGACT ACC	(Ster et al., 2005)

Table 3: Primers used for S. aureus biofilm gene expression in this study.

Genes	Forward Primer (5' -> 3')	Reverse Primer (5' -> 3')	Reference
icaA	GAGGTAAAGCCAACGCACTC	CCTGTAACCGCACCAAGTTT	(Atshan et al., 2013)
icaB	ATACCGGCGACTGGGTTTAT	TTGCAAATCGTGGGTATGTGT	(Atshan et al., 2013)
icaC	CTTGGGTATTTGCACGCATT	GCAATATCATGCCGACACCT	(Atshan et al., 2013)
icaD	ACCCAACGCTAAAATCATCG	GCGAAAATGCCCATAGTTTC	(Atshan et al., 2013)
fnbA	AAATTGGGAGCAGCATCAGT	GCAGCTGAATTCCCATTTTC	(Atshan et al., 2013)
fnbB	ACGCTCAAGGCGACGGCAAAG	ACCTTCTGCATGACCTTCTGCACCT	(Atshan et al., 2013)
clfA	ACCCAGGTTCAGATTCTGGCAGCG	TCGCTGAGTCGGAATCGCTTGCT	(Atshan et al., 2013)
clfB	AACTCCAGGGCCGCCGGTTG	CCTGAGTCGCTGTCTGAGCCTGAG	(Atshan et al., 2013)
ebps	GGTGCAGCTGGTGCAATGGGTGT	GCTGCGCCTCCAGCCAAACCT	(Atshan et al., 2013)
Cna	AATAGAGGCGCCACGACCGT	GTGCCTTCCCAAACCTTTTGAGCA	(Atshan et al., 2013)
16sRNA	TATGGAGGAACACCAGTGGCGAAG	TCATCGTTTACGGCGTGGACTACC	(Ster et al., 2005)

Table 4: Minimum Inhibitory Concentrations (MIC) of Citral, Linalool, Decanal, and
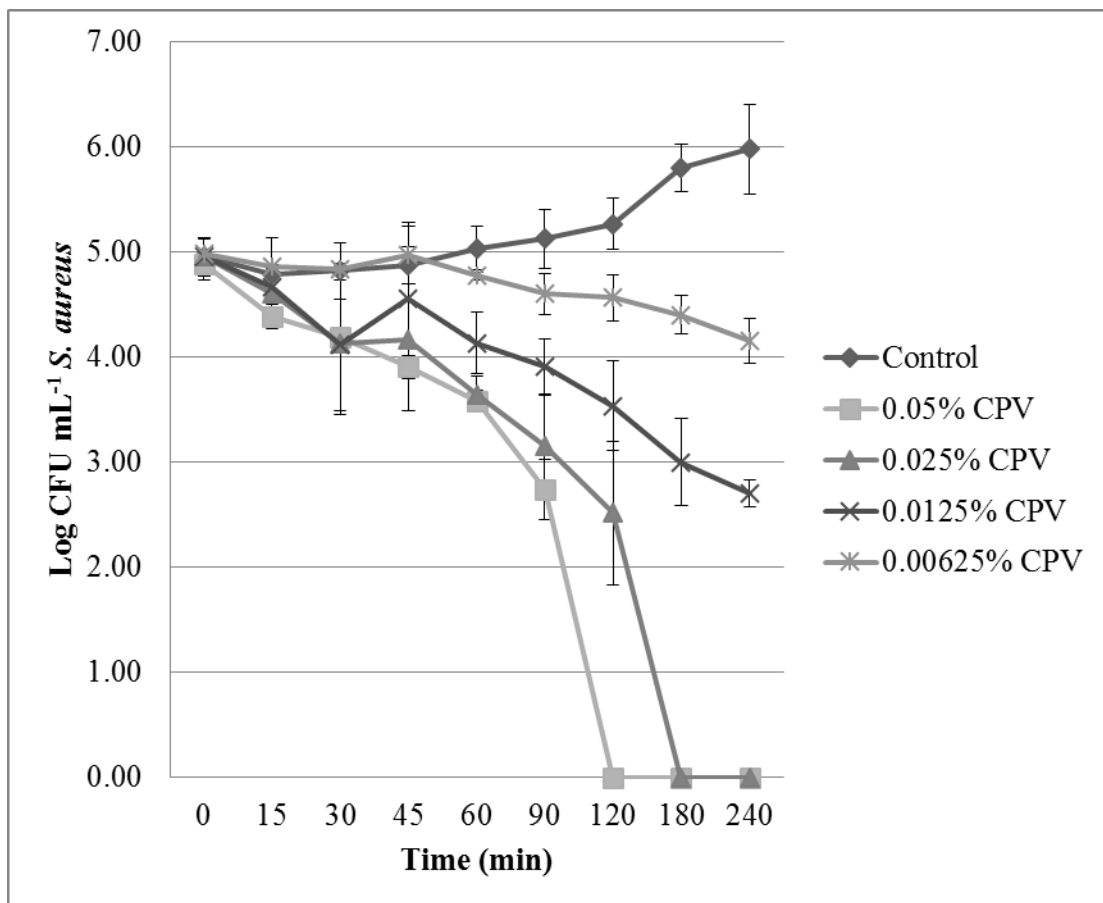
Valencene on S. aureus

	Citral[a]	Linalool	Decanal	Valencene
MIC Values	0.02%	0.12%	>2.5%	>2.5%

[a]A 10% stock solution of citral, linalool, decanal, and valencene was used with each

component dissolved in 1% Tween-80 in distilled water.
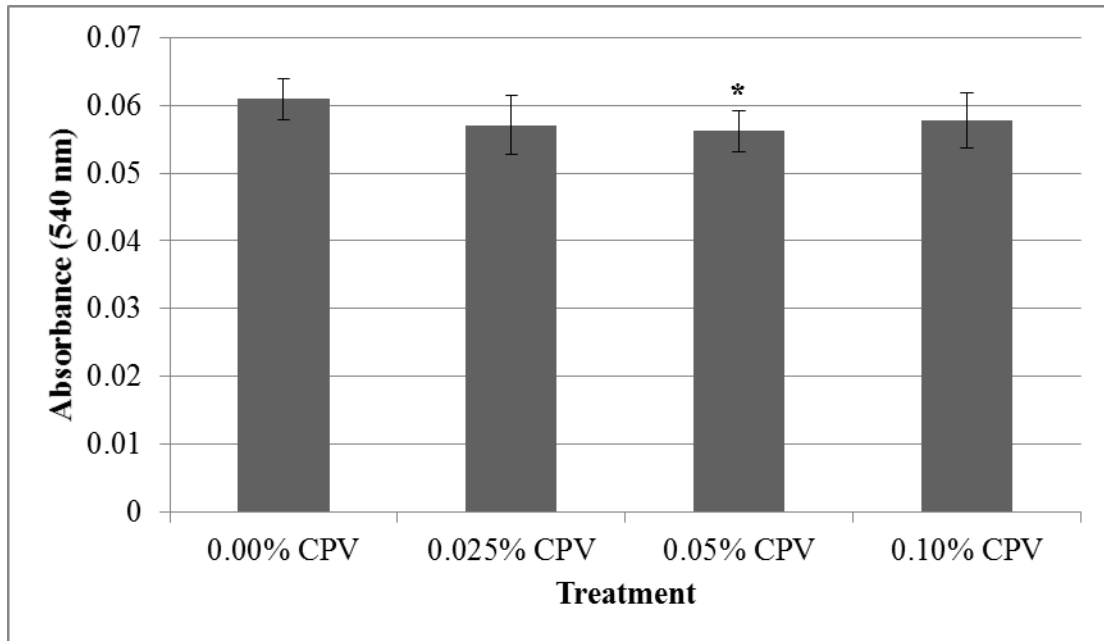
Chapter 6: List of Figures

Growth inhibition of *S. aureus* during a 4 h treatment with 0.00%, 0.05%, 0.025%, 0.0125%, and 0.00625% CPV. Log CFU mL^{-1} for both bacteria were measured after 0, 15, 30, 45, 60, 90, 120, 180, and 240 min. A starting concentration of 10^5 CFU mL^{-1} was used for all trials.

Figure 2: Inhibition of Pre-Formed S. aureus Biofilms by Cold-Pressed, Terpeneless

Valencia Orange Oil

The effect of citrus derived oil on preformed *S. aureus* biofilms. Absorbance at 540 nm was measured for the CPV concentrations of 0%, 0.025%, 0.05%, and 0.1%. Asterisks indicate significant difference ($p < 0.05$) from 0% control based on One Way ANOVA and Tukey's HSD.

*Figure 3: Relative Cytotoxicity of Orange Oil on MAC-T Cells*
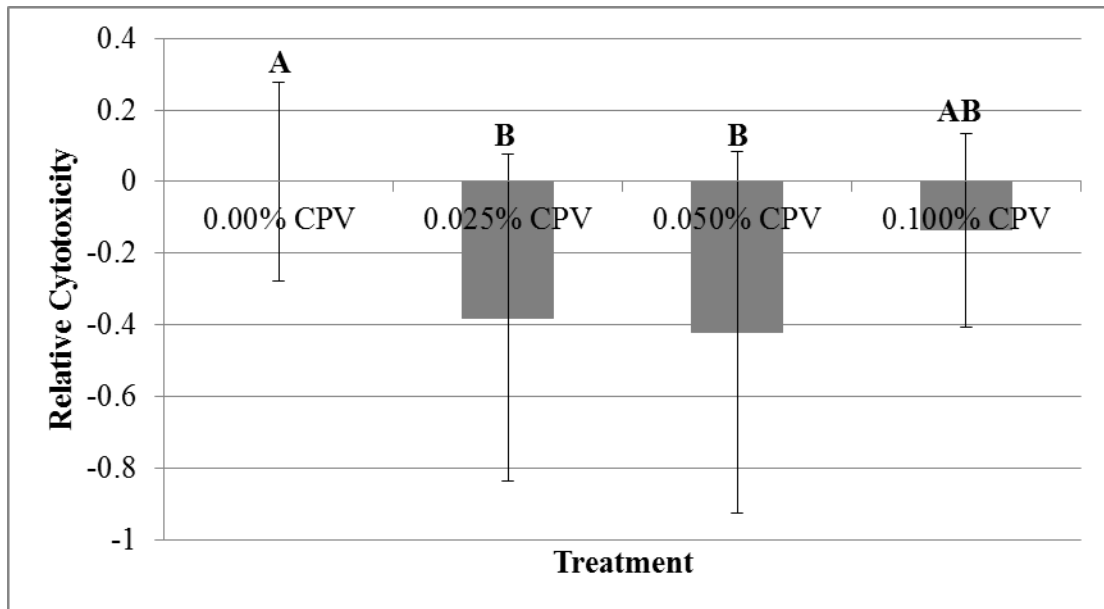
Relative Cytotoxicity of CPV on MAC-T cells measured after 1 hour of treatment with concentrations of CPV starting at 0.025% with half step dilutions up to 0.1%. A control of only DMEM supplemented with 10% FBS and 100 μg mL^{-1} gentamicin was used for the 0% CPV treatment.

Figure 4: Inhibition of S. aureus Association to MAC-T cells by Cold-Pressed,

Terpeneless Valencia Orange Oil
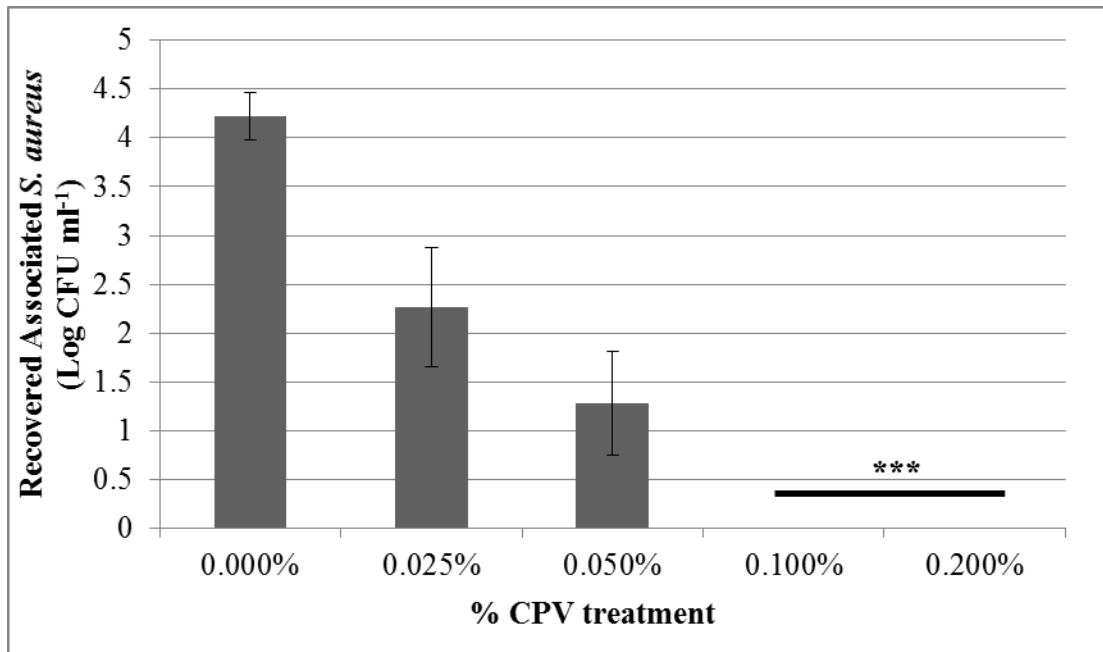
Recovered associated log CFU ml^{-1} of *S. aureus* with MAC-T cells after a 1 h

treatment with range of CPV conditions starting at 0.2% with half step dilutions down

to 0.025%. The control had an equivalent volume of DMSO added to the wells.

Associated bacteria include those invaded or attached to MAC-T cells. Significant

differences were found (P<0.05) in log CFU ml^{-1} of associated bacteria in *S. aureus*

when compared among groups. Concentrations significantly different are marked

with asterisks. There was a significant CPV concentration effect (P<0.05).

Figure 5: Inhibition of S. aureus Invasion of MAC-T cells by Cold-Pressed, Terpeneless Valencia Orange Oil
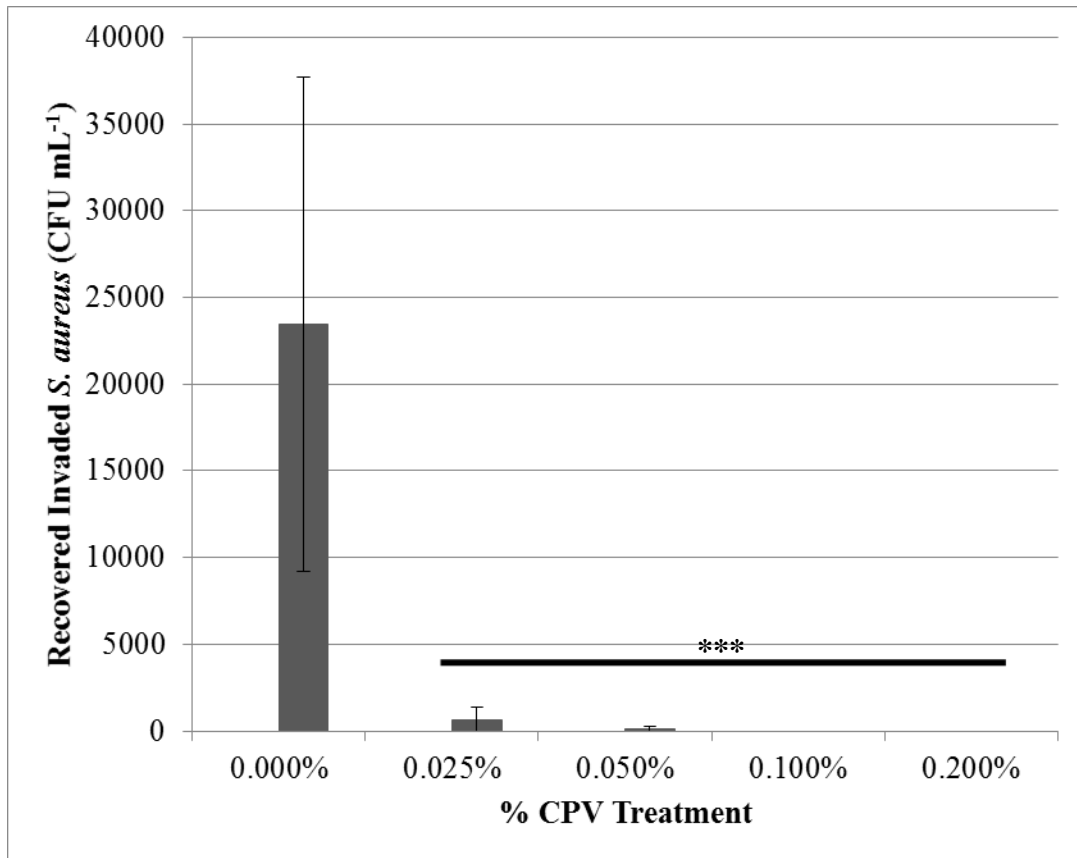
Invasion of MAC-T cells by *S. aureus* under a range of CPV conditions starting at 0.2% with half step dilutions down to 0.025%. These MAC-T cells were treated with their respective citrus oil condition for 1 h. A concentration of 0% CPV was used a control. Invasion of MAC-T cells was measured after 1 h of treatment. Concentrations significantly different are marked with asterisks. There was a significant CPV concentration effect ($P<0.05$) based on one-way ANOVA and Tukey's HSD.

Figure 6: Growth Inhibition Assay of Citral and Linalool on S. aureus over 72 h
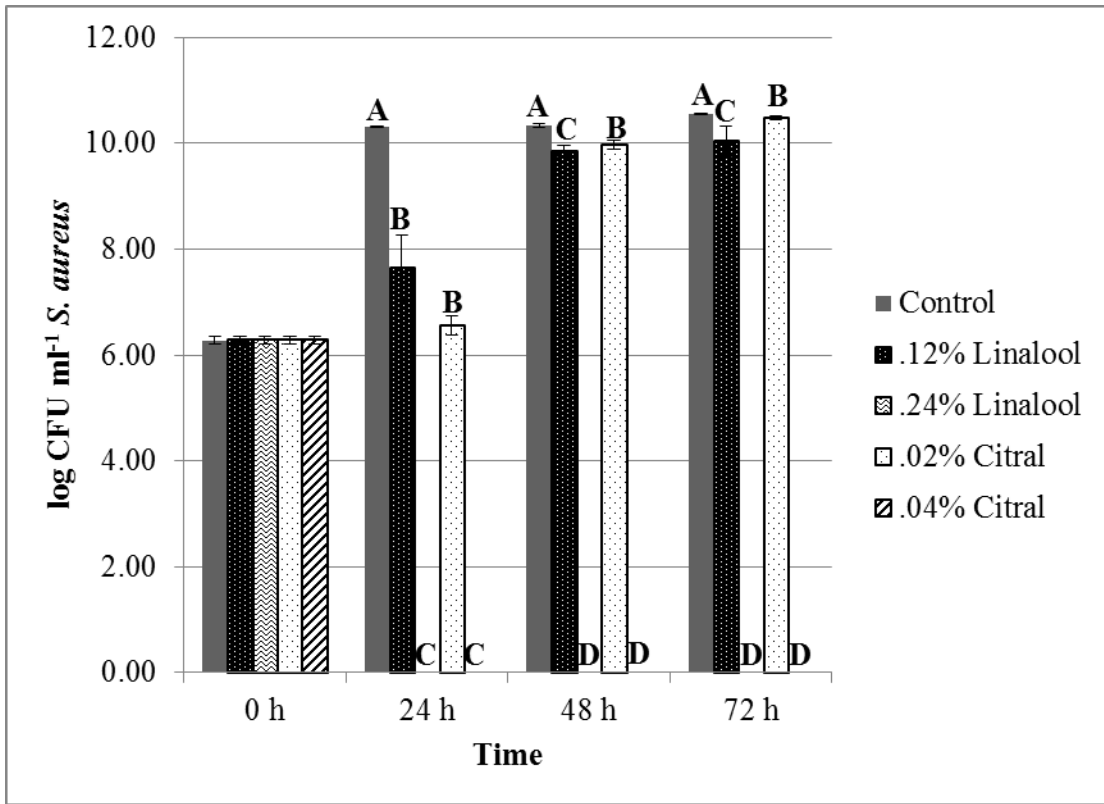

A growth inhibition assay of *S. aureus* after being treated with only LB broth (control), 0.02% citral, 0.04% citral, 0.12% linalool, or 0.24% linalool in LB broth. This assay was performed 72 h with bacterial enumeration occurring every 24 h. The starting inocula of *S. aureus* was 1.9×10^6 CFU mL^{-1}. The data illustrates the mean at each time point with bars representing the standard deviation. For each time point, statistical significance was determined by Tukey's HSD test. Statistically significant differences are denoted by different letters.

Figure 7: Growth Inhibition Assay of 0.04% Citral and 0.24% Linalool on S. aureus over 3 h
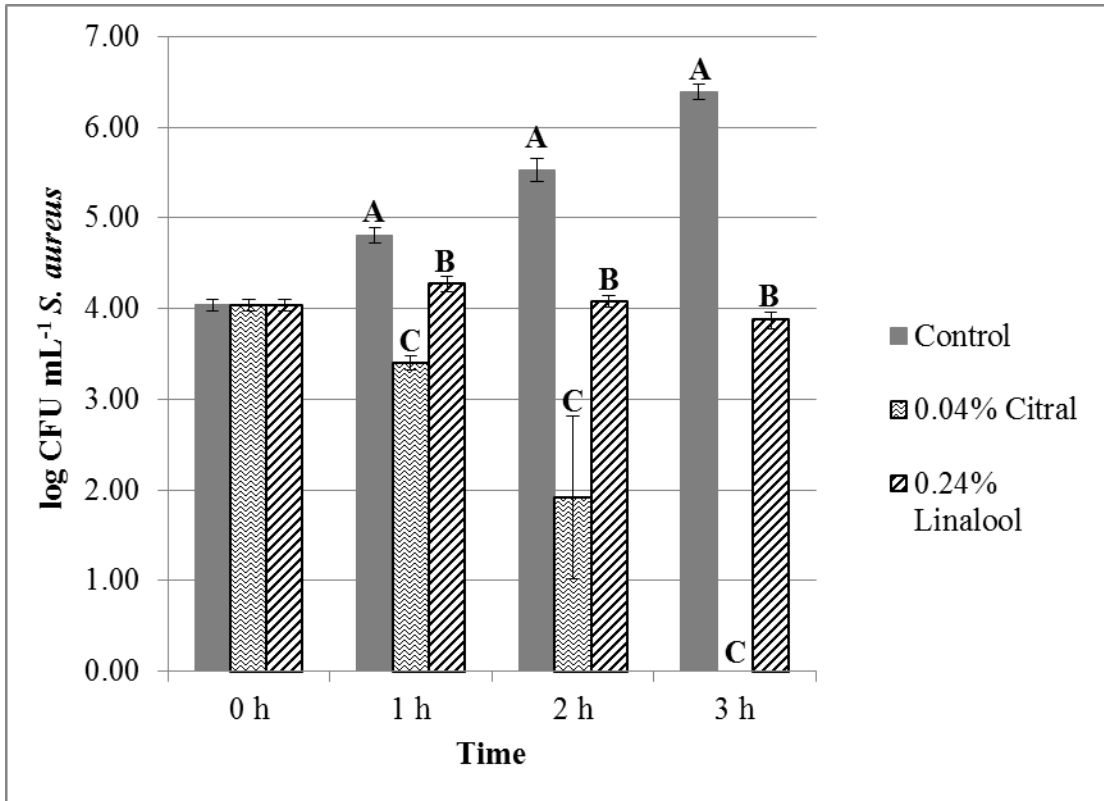
A growth inhibition assay of *S. aureus* after being treated with only LB broth (control), 0.04% citral, or 0.24% linalool in LB broth. This assay was performed 3 h with bacterial enumeration occurring every 1 h. The starting inocula of *S. aureus* was 1.9×10^4 CFU mL^{-1}. The data illustrates the mean at each time point with bars representing the standard deviation. For each time point, statistical significance was determined by Tukey's HSD test. Statistically significant differences are denoted by different letters.

Figure 8: Inhibition of Pre-Formed S. aureus Biofilms by Citral and Linalool
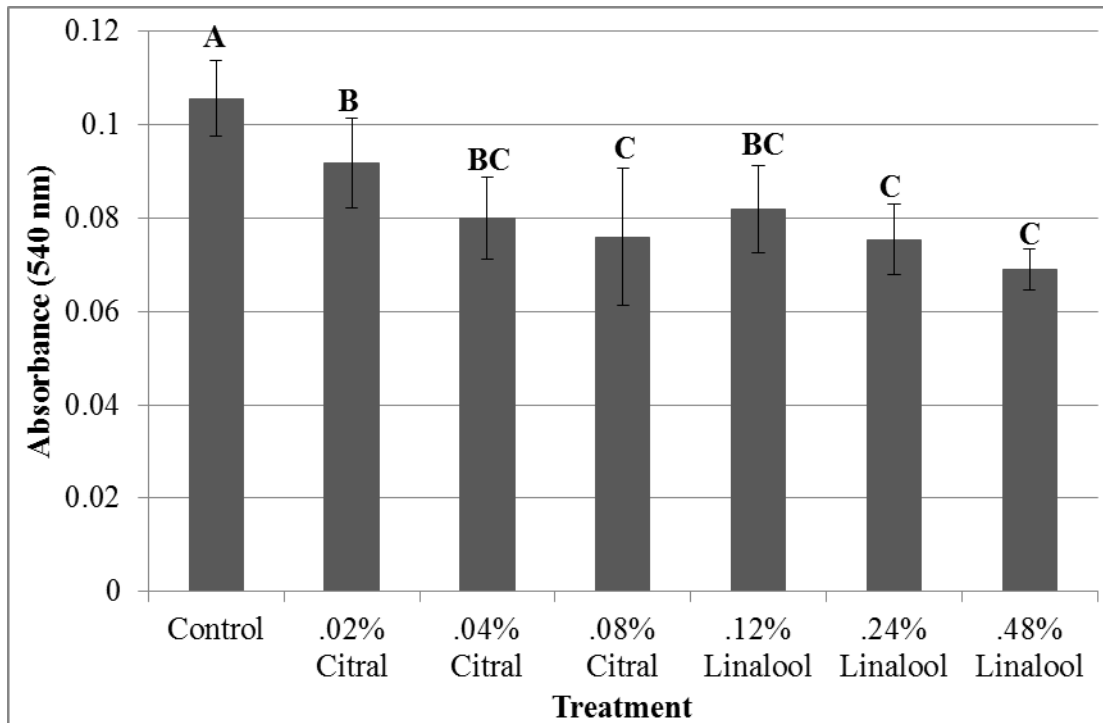
Inhibition of pre-formed *S. aureus* biofilms by citral and linalool. Absorbances of biofilms were measured after 24 h of growth and another 24 h of treatment at 540 nm. Biofilms were either treated with only LB broth, 0.02% citral, 0.04% citral, 0.08% citral, 0.12% linalool, 0.24% linalool, or 0.48% linalool in LB broth. The data illustrates the mean with bars representing the standard deviation. Statistically significant differences are denoted by different letters.
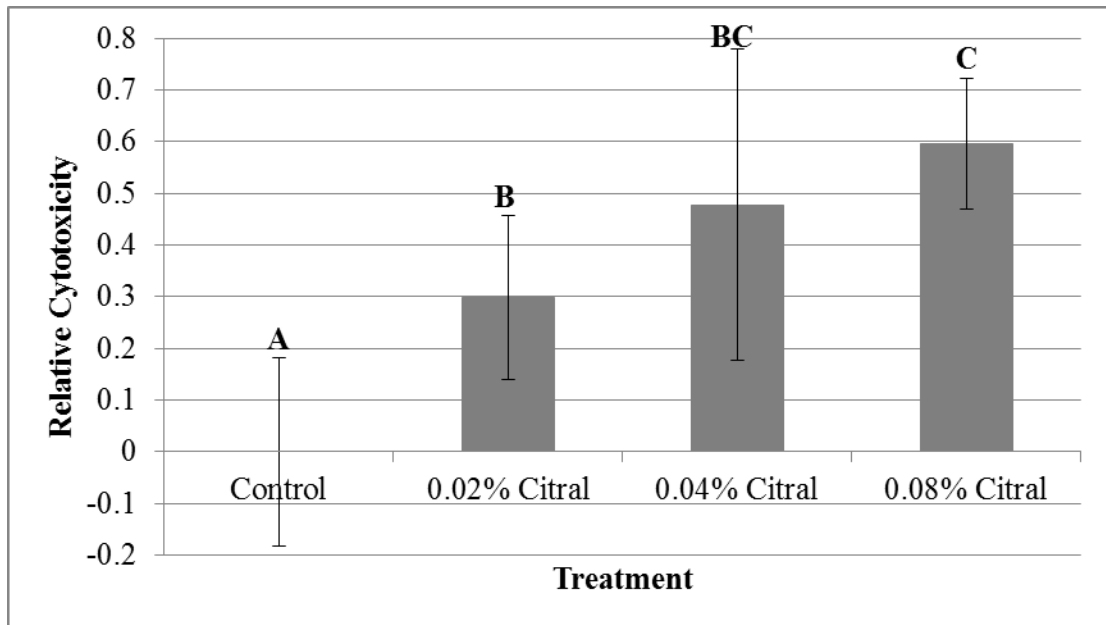
Detection of cytotoxicity of citral at concentrations of 0.02%, 0.04%, and 0.08% on MAC-T cells. Cytotoxicities were determined relative to live cells in an untreated control. The data illustrates the mean with bars representing the standard deviation. Statistically significant differences are denoted by different letters.

Figure 10: Relative Cytotoxicity of Linalool on MAC-T Cells
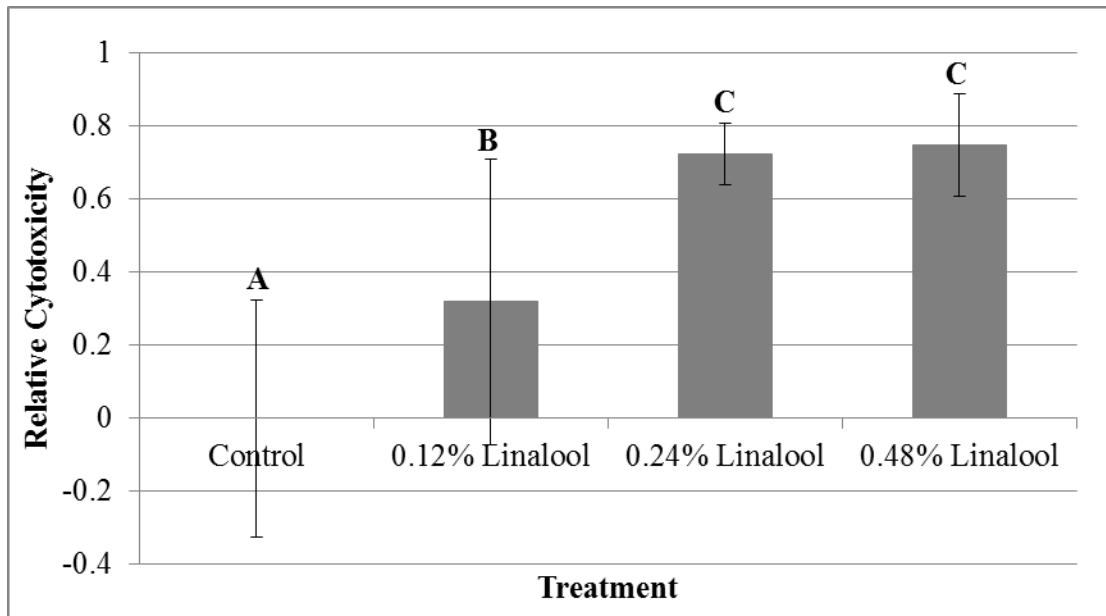
Detection of cytotoxicity of linalool at concentrations of 0.12%, 0.24%, and 0.48% on MAC-T cells. Cytotoxicities were determined relative to live cells in an untreated control. The data illustrates the mean with bars representing the standard deviation. Statistically significant differences are denoted by different letters.
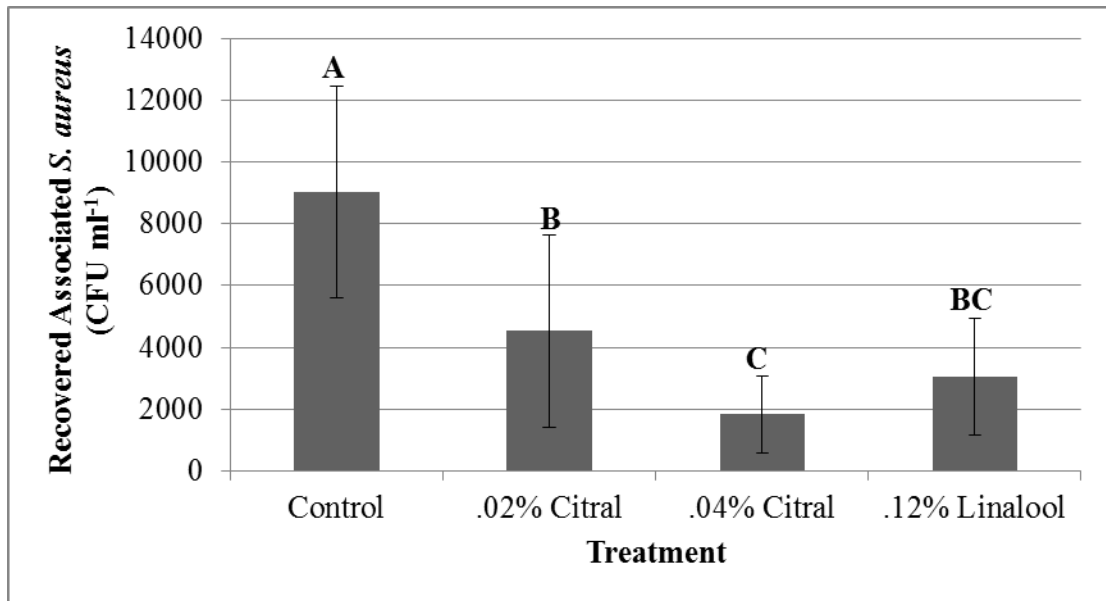
Figure 11: Inhibition of S. aureus Association to MAC-T cells by Citral and Linalool

Association assay of *S. aureus* on MAC-T cells following 1 h of infection and 1 h of treatment with either a control of DMEM supplemented with 10% FBS, 0.02% citral, 0.04% citral, or 0.12% linalool in DMEM supplemented with 10% FBS. The data illustrates the mean with bars representing the standard deviation. Statistically significant differences are denoted by different letters.

Figure 12: Inhibition of S. aureus Invasion of MAC-T cells by Citral and Linalool
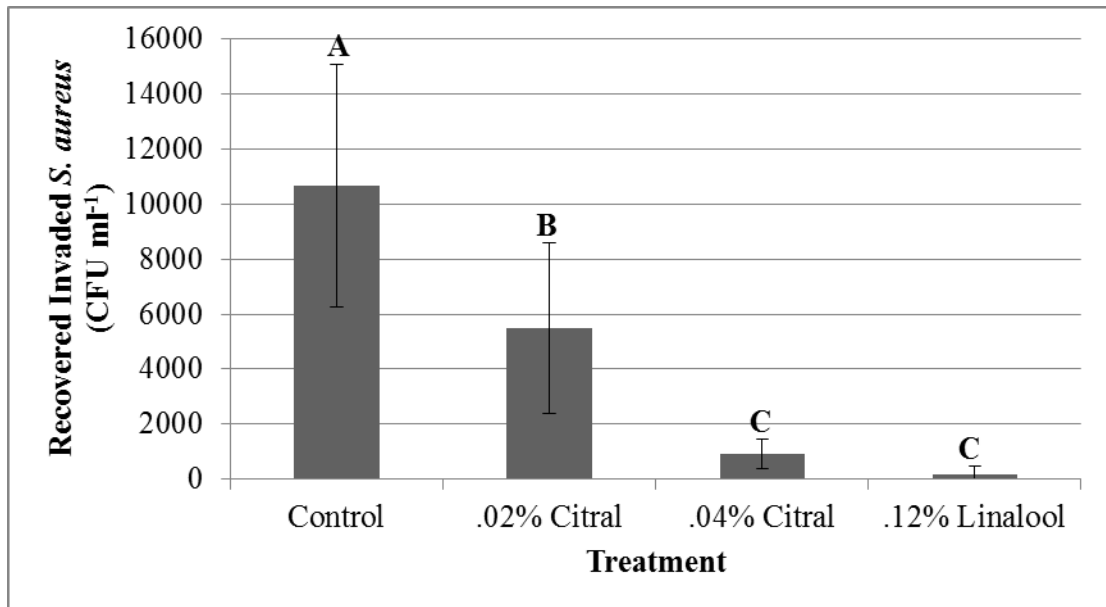
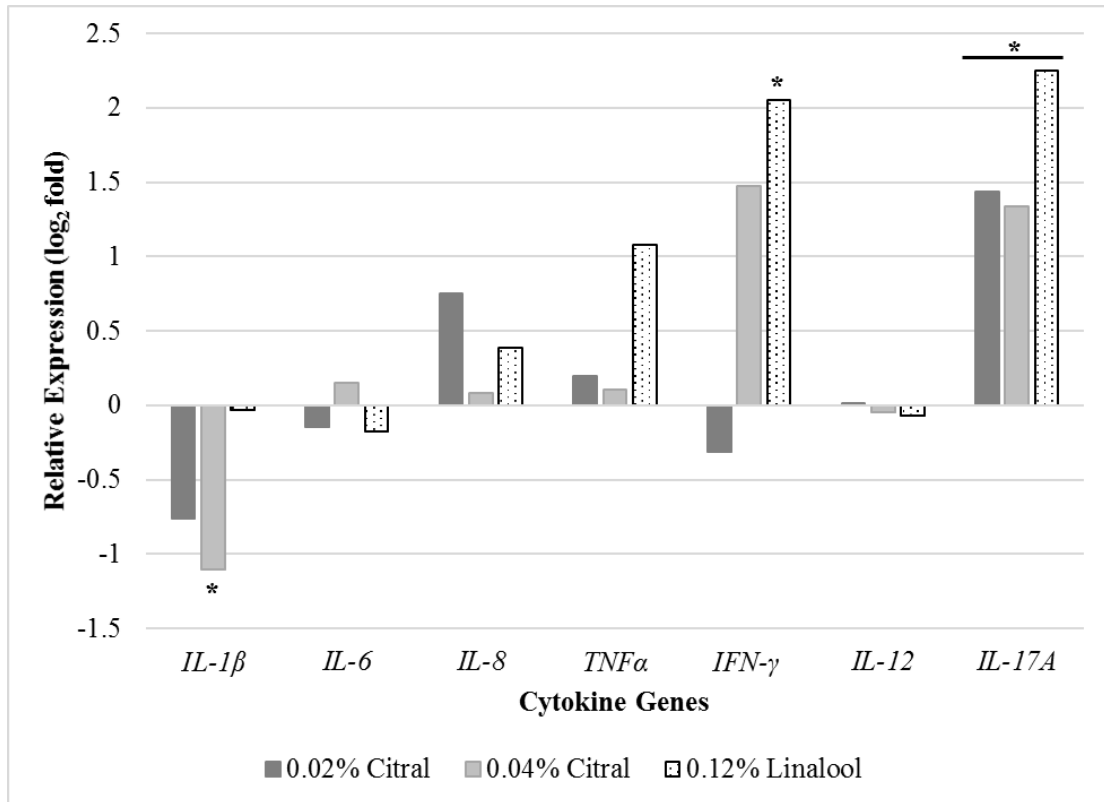Invasion assay of *S. aureus* on MAC-T cells following 1 h of infection and 1 h of treatment with either a control of DMEM supplemented with 10% FBS, 0.02% citral, 0.04% citral, or 0.12% linalool in DMEM supplemented with 10% FBS. The data illustrates the mean with bars representing the standard deviation. Statistically significant differences are denoted by different letters.

Gene expression of MAC-T cytokine genes after 1 h of infection with *S. aureus* and 1 h of treatment with either a control of DMEM supplemented with 10% FBS, 0.02% citral, 0.04% citral, or 0.12% linalool in DMEM supplemented with 10% FBS. Statistically significant differences from infected and untreated control are denoted by asterisks.

Figure 14: Relative Expression of S. aureus Virulence Genes During Infection of

MAC-T Cells After Being Treated with Citral and Linalool
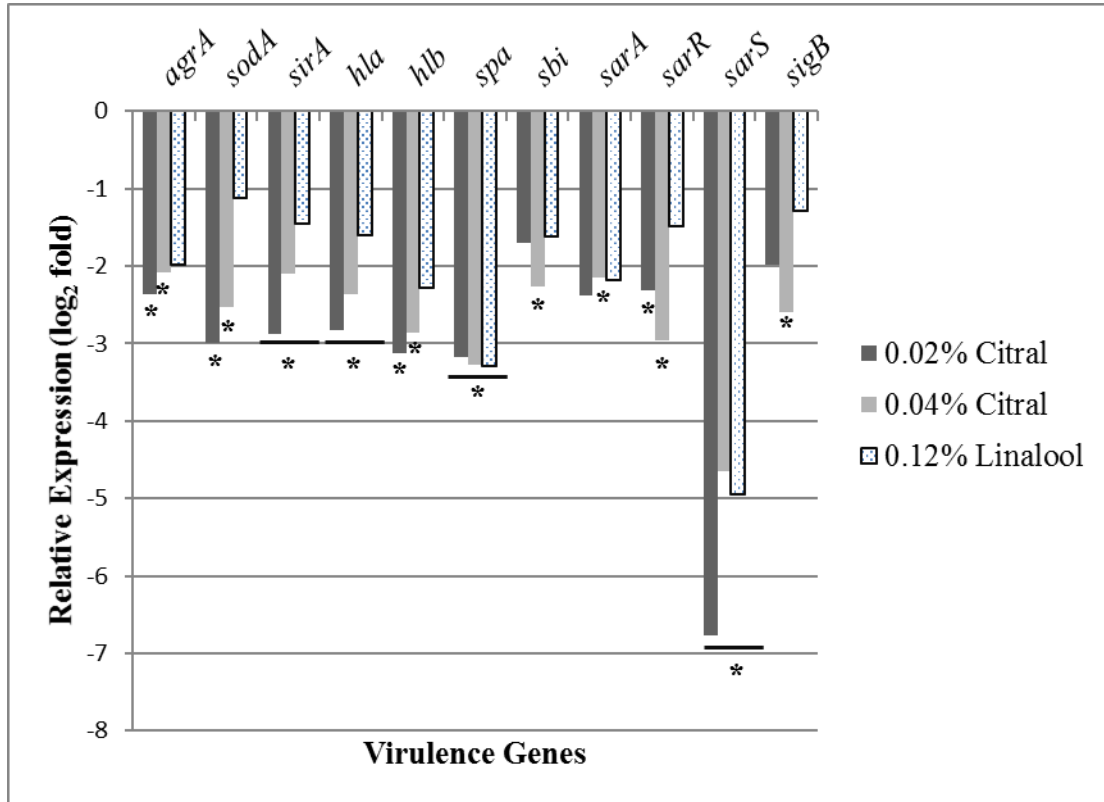
Gene expression of *S. aureus* virulence genes after 1 h of infection of MAC-T cells

and 1 h of treatment with either a control of DMEM supplemented with 10% FBS,

0.02% citral, 0.04% citral, or 0.12% linalool in DMEM supplemented with 10% FBS.

Statistically significant differences from baseline are denoted by asterisks.
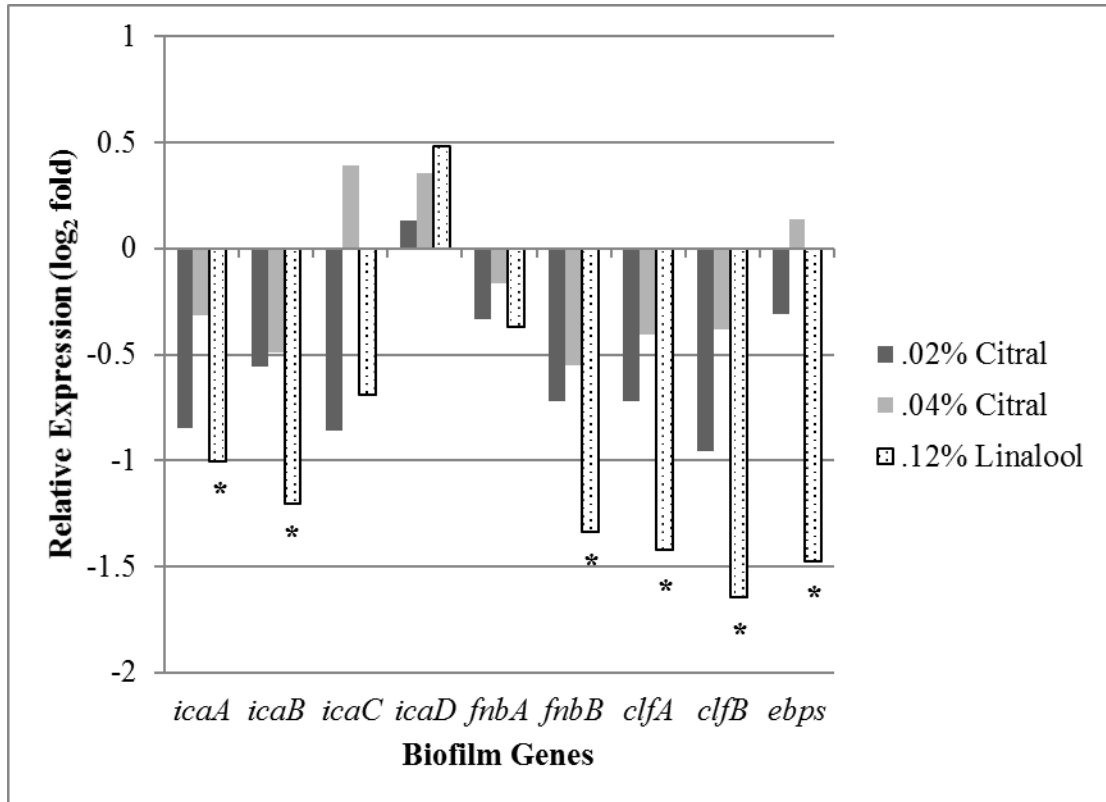
Gene expression of *S. aureus* biofilm genes after 24 h of growth and 24 h of treatment with either a control of LB broth, 0.02% citral, 0.04% citral, or 0.12% linalool in LB broth. Statistically significant differences from baseline are denoted by asterisks.

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