5-2015

Application of Essential Oil Compounds and Bacteriophage to Control Staphylococcus aureus

Anisha Ghosh
University of Arkansas, Fayetteville

Application of Essential Oil Compounds and Bacteriophage to Control *Staphylococcus aureus*

Application of Essential Oil Compounds and Bacteriophage to Control *Staphylococcus aureus*

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Food Science

By

Anisha Ghosh
Institute of Management Studies, C.C.S. University
Bachelor of Science (Hons) in Biotechnology, 2007
Bangalore University
Master of Science in Biotechnology, 2009

May 2015
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

Dr. Kristen E. Gibson
Thesis Director

Dr. Steven C. Ricke
Committee Member

Dr. John A. Marcy
Committee Member

Abstract

Staphylococcus aureus is one of the most important pathogens, causing various diseases in humans and animals. In addition, *S. aureus* is a common foodborne pathogen. As methicillin-resistant *S. aureus* (MRSA) becomes increasingly prevalent, controlling this pathogen in animals and humans with standard antibiotic treatment has become challenging. Combinations of different antimicrobial agents represent one of the most promising approaches for combating multidrug – resistant bacteria both for treatment of clinical disease as well as in food. Two such antimicrobials with potential application in the food industry include essential oils (EO) and host-specific bacteriophage (phage). The objectives of this study were 1) to determine the efficacy of varying concentrations of pure EOs compounds against *S. aureus* and 2) to evaluate the efficacy of a *S. aureus*-specific bacteriophage against 4 strains of *S. aureus*. The overall goal was to combine these antimicrobials to determine potential synergism and possible application for the control of *S. aureus* on raw chicken products. Four EO compounds were evaluated by disc diffusion assay to determine inhibitory effects against five strains of *S. aureus*. Next, a growth inhibition assay was performed using a 96-well plate bioassay to measure change in optical density over a 48-hour period. Phage adsorption assays were performed up to 120 h at 6, 13, and 37°C to determine lytic activity. The results from disc diffusion, growth inhibition, and phage adsorption assays indicate that EO compounds and bacteriophage can be used as antimicrobials against *S. aureus.* For application in the food industry, these antimicrobials were evaluated for their efficacy against *S. aureus* on raw chicken pieces at 6, 13, and 25°C. Results indicate that at 25°C phage K alone inhibits *S. aureus* growth better as compared to other antimicrobial combination. At 6 and 13°C, there was no significant effect of EO and phage alone or in combination against *S. aureus* when applied on the raw chicken pieces. Therefore, for these

antimicrobials to work *in vivo* such as raw meat products, a better delivery method should be employed for a uniform application on meat.

Acknowledgements:

The work on this thesis has been one of the most enriching educational experiences. The lessons, experience and knowledge would play a crucial part of my armor in facing the challenges in forthcoming professional and personal life. I can never seek to call out everyone who backed me in the countless turns in this road, but the next few lines are an attempt to acknowledge the efforts of some of the key names.

First and foremost, I would like to thank Prof. Kristen Gibson. She has been a true teacher in every aspect. There are countless lessons she has taught me on the subject and otherwise along the way. She has held tall standards to meet but also guided me to reach those standards and feel proud. She has been my guide and advisor in every aspect of the words. Prof Gibson, I am grateful for it all.

I would also like to thank my committee members, Prof. Steven Ricke and Prof. John Marcy. Their insightful inputs at critical junctions made it possible to ensure I was not lost in a sea of possibilities yet explore the right areas.

I would like to thank Giselle Almeida, our lab in-charge. She has helped me learn a lot of tricks of the trade in this research, both as a lab expert as well as a lab roomie.

Last but not the least I would like to thank my lab mates. You all have been my support system at every stage of the last two years.

Momsi, Popsi and Rahul, you know this is for you and because of you.

Contents

Chapter 1: Literature Review

Staphylococcus aureus

Introduction

Staphylococcus aureus is an aerobic Gram-positive bacterium that occurs in grape like clusters. They are non-motile, non-spore forming, spherical cells of 1 µm in diameter. It is a member of the *Micrococcaceae* family (Franklin, 1998). The organism was first discovered in 1880 by a surgeon named Sir Alexander Ogsten. In 1884, Rosenbach proposed two pigmented types of the cocci. The bacterium that produced yellow colonies was known as *S. aureus* and the one that produced non-pigmented or white colonies was known as *S. epidermidis.* It is an opportunistic pathogen found in humans and animals and is one of the most common sources of hospital and community-acquired infections (Alsaimary, 2012). The organism is a commensal colonizer of the skin and is common inhabitant of the nasal passage (25-30% of people), mucous membranes (e.g., throat, axilla, and rectum) and other anatomical locales on humans and other warm-blooded mammals (Chao *et al*., 2008).

Biochemical Characteristics

According to a review by Panneerseelan and Muriana (2009), *Staphylococcus aureus* are facultative anaerobes and they can grow by aerobic respiration or by fermentation producing lactic acid. The organism can grow at a wide temperature range (15°C to 45°C), a pH range of 4 to 11 and at NaCl concentrations as high as 15%. These characteristics enable *S. aureus* to grow in a wide variety of environments including foods. The bacteria are catalase-positive and

oxidase-negative. It is hemolytic on blood agar and almost all strains of *S. aureus* produce the enzyme coagulase. The organism ferments glucose and mannitol producing lactic acid.

Antibiotic Resistance

The widespread use of antibiotics has provoked an exponential increase in the incidence of antibiotic resistance in several bacterial groups in recent years. *Staphylococcus aureus* are notorious for their resistance to antibiotics. The occurrence of methicillin resistant *S. aureus* (MRSA) and the detection of a vancomycin resistance gene in *S. aureus* during the late 1950s and the early 1960s, has led to increased concern regarding this microorganism (Panneerseelan and Muriana, 2009).

Methicillin Resistance

Methicillin-resistant *S. aureus* is a special type of *S. aureus* that is resistant to the antibacterial activity of methicillin and other related antibiotics of the penicillin class (Alsaimary, 2012). *Staphylococcus aureus* infections are traditionally treated with penicillins. The organism developed resistance to penicillins via the BlaZ penicillinase (β-lactamase). In this mechanism, β-lactamase produced by the bacteria provide antibiotic resistance by breaking the β-lactam ring during hydrolysis, thus deactivating the antibiotic's antibacterial properties (Alsaimary, 2012). In 1959, Beecham developed a penicillinase-stable penicillin called methicillin to overcome this resistance mechanism (Wendlandt *et al.*, 2013). The first isolates of MRSA were documented in the late 1950s. By 1967, multidrug resistant MRSA were reported from Switzerland, India, France, Denmark, England and Australia (Grundmann *et al.,* 2006). Virulence and quorum-sensing mechanisms allow *S. aureus* to cause a broad variety of serious infections in humans (Miller *et al.,* 2001). The genetic diversity and ability to acquire exogenous genes allows MRSA

to modulate its pathogenicity by adapting to changing environmental conditions and thus acquiring resistance to multiple antibiotics (Wendlandt *et al*., 2013). The U.S. Centers for Disease Control and Prevention (CDC) have reported that the proportion of overall staphylococcal infections due to MRSA has risen from 2% in 1974 to 22% in 1995, and then to 65% in 2004 in the U.S. (Chao *et al*., 2008). Methicillin-resistant *S. aureus* are one of the most common nosocomial pathogens throughout the world causing a wide range of hospital-linked infections (Lee, 2003). Now concerns have intensified as MRSA has resulted in human infections linked to unexpected community settings: children in day-care centers, army recruits, athletes in contact sports, prison populations, and intra-venous drug users (Klevens *et al.*, 2007). Methicillin-resistant *S. aureus* also been detected in food animals and in food such as meat, milk and dairy products and fishery products (Lee, 2003).

Vancomycin Resistance

Vancomycin has been a reliable treatment for gram positive bacterial infection for more than 30 years. Injectable forms of vancomycin were introduced in 1991 and since then have been used for MRSA infection. The first report on a strain of *S. aureus* with reduced susceptibility to vancomycin was reported in 1996 (Hiramatsu *et al*., 1997). It was called Mu 50 (vancomycin minimum inhibitory concentration [MIC] 8 mg/L). The bacterial strain had an intermediate resistance to vancomycin and was known as vancomycin-intermediate *S. aureus* (VISA). By 2002, eight documented cases of infection with VISA were reported (Smith *et al*., 1999). A CDC report in 2002 reported on the first documented case of *S. aureus* infection caused by vancomycin resistant *S. aureus* (VRSA) (Smith *et al*., 1999).

Enterotoxins

Staphylococcus aureus produces enterotoxins that are the causative agents of foodborne intoxications. Enterotoxins are single chain polypeptides with a molecular weight of 26-28 kDa and contains 228-239 amino acid residues (Muller-Alouf *et al.*, 2001). There are twenty serological types of staphylococcal enterotoxins (SEs), the most common of which are SEA, SEB, SEC, SED and SEE. They are classified into classical and novel enterotoxins. The enterotoxin genes are present on regions of the chromosome known as staphylococcal pathogenicity islands (SaPIs) (Balaban and Rasooly, 2000). The genes expressed in one environment may not be expressed in another (Normanno *et al*., 2005). These enterotoxins are active in minute quantities and are resistant to conditions such as heat treatment and low pH that would normally kill the bacteria that produce them. They can remain potent in the digestive tract as they are also resistant to proteolytic enzymes (Argudin *et al*., 2010). As a result, toxins present before cooking may not be killed in most food preparation regimens (Panneerseelan and Muriana, 2009).

Staphylococcal enterotoxins belong to a large family of staphylococcal and streptococcal pyrogenic exotoxins, sharing common phylogenetic relationships, structure, function, and sequence homology. These toxins cause toxic shock-like syndromes and have been implicated in several allergic and autoimmune diseases along with food poisoning (Balaban and Rasooly, 2000). Duration of the illness usually lasts 6-24 h and prolonged illness or death can occur in infants, elderly, and severely immune compromised persons (Panneerseelan and Muriana, 2009). *Staphylococcus aureus* enterotoxins are also powerful super antigens (SAgs) that are able to stimulate polyclonal proliferation response of human T-lymphocytes (O'Hehir and Lamb, 1990). The T-cell proliferation results in a massive release of chemokines and pro-inflammatory

cytokines by bypassing conventional antigen recognition by T-cell receptors; thus, this T-cell mediated response can affect the immune system of infected individuals, leading to potential toxic shock syndrome and emesis in the case of food poisoning (Argudin *et al*., 2010). This helps in the early detection of the infection in the body as the body shows the symptoms of intoxication at an early stage (Panneerseelan and Muriana, 2009).

Transmission of *Staphylococcus aureus* in food

Food poisoning is a common form of illness, and it can vary in severity from being mild to fatal (Loir *et al*., 2003). Food poisoning caused by infectious agents (bacteria, viruses, parasites) can be broadly classified into two categories, namely foodborne infections and foodborne intoxications. Foodborne infections occur when food consumed is contaminated with pathogens. The pathogen causes inflammation, resulting in poor absorption of water and nutrients. Food intoxications on the other hand may result from ingesting food contaminated with preformed toxins produced by foodborne pathogens (Bergdoll *et al*., 1989). Hence even if the microorganisms are destroyed during cooking or processing, some of the toxins which are heat stable may persist in foods and may still cause intoxication (Panneerseelan and Muriana, 2009).

Staphylococcus aureus is a commensal and lives on the anatomical locales of humans and animals. Hence food handlers are the common source of contamination of foods (Gutierrez *et al.*, 2012). Foods such as salads including egg, chicken or potato salads and sandwiches which are hand-made and require no additional cooking are common sources of *S. aureus*. Other sources of food contamination include the equipment and surfaces on which food is prepared. The bacteria can multiply quickly at room temperature to produce SEs. The organism also enters the food during processing of animal products. Staphylococcal enterotoxins in suspect foods must be

identified quickly because they are potent even in very minute quantities. In dairy products a concentration of SEs as low as 0.5 ng/ml have resulted in 850 cases of foodborne illness in children (Panneerseelan and Muriana, 2009).

Staphylococcal food poisoning results from the consumption of food contaminated with one or more staphylococcal enterotoxins (SEs). The toxins of *S. aureus* are known as enterotoxins because they are able to promote water loss from the small intestine mucosa resulting in vomiting and diarrhea (Martin *et al*., 2003). It can also cause acute enteric responses such as nausea, abdominal cramps, and changes in blood pressure. Staphylococcal food poisoning is characterized by a short onset time (2 to 6 h) after ingestion of preformed toxins (Panneerseelan and Muriana, 2009). Staphylococcal enterotoxin A is the most common enterotoxin implicated in food-poisoning outbreaks in the US (77.8% of all outbreaks) followed by SED (37.5%) and SEB (10%) (Balaban and Rasooly, 2000). However, it is not clear if an outbreak is caused as a result of an individual toxin or if it is caused due to a combination of multiple toxins. Considerable research has been conducted in detection of enterotoxins in foods. Development of immunoassays has revolutionized the process; however, the need for improving the limit of detection is important as SEs are potent in minute quantities (Balaban and Rasooly, 2000).

Staphylococcus aureus is also known to form biofilms on food surfaces (Gutierrez *et al*., 2012). Biofilms are the most common bacterial lifestyle in nature. A biofilm is any group of microorganisms in which cells stick to each other on a surface (food products). After initial attachment of cells to a surface, they start to multiply and secrete a consistent matrix of extracellular polymeric substances in which cells are wrapped (Gutierrez *et al*., 2012). *S. aureus* is one of the major causative agents of food-borne diseases in humans. O'Brien *et al.* (2012) showed that *S. aureus* were prevalent in 64.8% pork samples (256 out of 395) collected across

the U.S. (36 stores) and both susceptible and methicillin-resistant *S. aureus* were detected. Overall, outbreaks caused by *S. aureus* in meat and poultry dishes caused 55% of the reported *S. aureus* outbreaks from 1998-2008 with pork and pork dishes such as ham being the most common (Bennett *et al.*, 2013).

Poor hygiene practices in food processing plants can result in the contamination of food products with pathogens potentially causing serious health issues for consumers. Since bacteria can attach to food contact surfaces and form biofilms, the complete elimination of pathogens from the food processing environment has been a difficult task as biofilms protect bacteria from the effects of antimicrobial agents (Savage *et al*., 2013). Moreover, pathogens can survive even after cleaning and disinfection. *S. aureus* can survive in hostile environments such as food industry surfaces through their ability to form biofilms on food surfaces (Gutierrez *et al*., 2012).

Essential oil Compounds as Antimicrobials

Introduction

An essential oil (EO) is a concentrated hydrophobic liquid containing volatile aroma compounds from plants. It has an oily consistency (Palmer *et al*., 1998). Essential oils are also known as volatile oils, ethereal oils or aetherolea. Most of them are liquid at room temperature though a few of them can be solid or resinous (Bassole *et al*., 2012). Their colors range from pale yellow to emerald green and from blue to dark brownish red (Balz, 1999). Most of the plant organs such as buds, flowers, leaves, barks, fruits, seeds, etc. synthesize essential oils and store them in secretory cells, cavities, canals and epidermic cells (Upadhyay *et al*., 2010).

Essentials oils do not form a distinctive category for any medical, pharmacological or culinary purpose. They are generally extracted by distillation, often by using steam. They are used in perfumes, cosmetics, soaps and other products, for flavoring food and drink and for adding scents to incense and household cleaning products (Ciocan *et al*., 2007).

Mode of Action

Most of the antimicrobial activity in EOs appears to derive from terpenoids particularly phenolic terpenes, phenylpropanoids and alcohols. Other constituents such as hydrocarbons show low activities but can be used in combinations to increase their bioactivities (Bassole *et al*., 2012). Interactions between these compounds may lead to antagonistic, additive or synergistic effects. The most common mechanism of antimicrobial interaction is by synergism (Ciocan *et al*., 2007). This includes the sequential inhibition of a common biochemical pathway, inhibition of protective enzymes, and the use of cell wall active agents to enhance the uptake of other antimicrobials. This mechanism involving a combination of components having synergistic effect will then reduce the concentration needed to yield the same microbial effect when compared with the sum of the purified components. There are many studies that have demonstrated that crude EOs have higher antimicrobial activity than the mixtures of their major components. When a combination of EOs is used, their synergistic activity can play a critical role in the overall antimicrobial activity (Palmer *et al*., 1997).

Application in food products

It is widely believed that EOs and other plant products have healing powers. There is evidence that Neanderthals living 60,000 years ago used a plant called hollyhock for medicinal purposes, and today, it is still used in ethno medicine around the world (Burt, 2004). Today around one-

half of all pharmaceuticals dispensed in the U.S. have plant origins; however, very few of them are used as antimicrobials (Ciocan *et al*., 2007). As drug resistance in microbes is becoming increasingly prevalent, plant origin herbal medicines are considered as alternatives to synthetic drugs and possess antimicrobial, anticancer, and antioxidant properties. There are some forms of traditional medicines such as Ayurveda, Homeopathy, and Unani, which utilize plant products for drug production (Upadhyay *et al*., 2010). Plant derived medicines have made large contributions to human health and well-being (Upadhyay *et al*., 2010). Plant derived products act either as natural blueprints for the development of new drugs or a phytomedicinals to be used for the treatment of disease. The primary benefits of using such products are that they are comparatively safer than the synthetic alternatives, thus offering therapeutic benefits and affordable treatment (Edwards-Jones *et al.,* 2004).

Food products may become easily contaminated with microbes such as bacteria, yeasts and fungi. These microorganisms cause undesirable effects such as deterioration of the flavor, odor, color, sensory, and textual properties of foods. In packaged foods, pathogenic microorganisms including *Listeria monocytogenes, S. aureus, Campylobacter, Salmonella, Escherichia coli,* and *Clostridium perfringens,* may continue to grow and survive, despite changes that may occur in intrinsic factors such as pH, water activity, and lower oxygen level or extrinsic factors such as temperature, time and humidity (Nazzaro *et al*., 2013). As a result, food products such as cheeses, meats, poultry, and baked products are susceptible to microbial spoilage even after packaging (Kuorwel *et al*., 2011). To prevent the growth of spoilage and pathogenic microorganisms on foods, new preservation techniques have been developed as consumers demand safe, fresh, and minimally processed foods. “Active packaging” (AP) technologies are the latest technique to provide safe food products with longer shelf lives (Kuorwel *et al*., 2011).

The AP technologies are primarily based on the use of synthetic or natural antimicrobial (AM) agents such as EOs. In food industries, food spoilage caused by microorganisms is reduced by the use of different AM agents that are incorporated directly into the food. According to Kuorwel *et al.* (2011), this method has some disadvantages including (i) consumers may prefer food products with no or minimal synthetic agents because of concerns about potential side-effects; (ii) incorporation of such AM agents in the bulk of the food may not be justified as food spoilage occurs primarily on the surface; (iii) these agents might possess a distinct flavor which may render the food flavor; and (iv) if AM agents are used in the food product, they have to be declared on the package which may deter some consumers from purchasing the product. In spite of these disadvantages, AM agents are still used as an additional protective barrier in food industries (Kuorwel *et al*., 2011). Also, in order to obtain antimicrobial activity against microorganisms on food products, the concentration of EOs is generally higher than the concentration applied for flavoring purposes. This may result in food tainting or adverse sensorial effects to certain food products (Bassole *et al*., 2012). In order to reduce adverse sensory issues in food products that may be caused due to the presence of EO, lower concentrations of EO must be used that can yield a similar antimicrobial activity (Bassole *et al*., 2012).

Potential as Anti-Staphylococcal Compounds

Due to the widespread use of antibiotics during 1960s in order to minimize the spread of *S. aureus* spread, resistant forms of *S. aureus* (MRSA and VRSA) were isolated soon. The public health impact of both widespread VRSA and MRSA can be quite significant. In short it became essential to find alternative, natural antimicrobials to treat staphylococcal infections (Li *et al*., 2011). Plant sources of antimicrobial compounds are good candidates for limiting MRSA

without doing any harm to humans since some oils have been used in the past for curative purposes (Li *et al*., 2011).

Methicillin resistant *S. aureus* is susceptible to tea tree oil but there are concerns about its toxicity (Chao *et al*., 2008). Tea tree oil obtained from the Australian tree, *Melaleuca alternifolia,* is a powerful antimicrobial agent against bacteria, fungi, and viruses and is used commercially in a wide range of products (Edwards-Jones *et al.,* 2004). According to Cox *et al*. (2000), tea tree oil has the ability to disrupt the permeability barrier of cell membranes of bacteria such as *E. coli* and *S. aureus* which results in the loss of chemiosmotic control in the organisms. Upadhyay *et al.* (2010) reported that Ajwaine oil is highly lethal to *S. aureus, Streptococcus pneumonia*, and *Lactobacillus acidophilus*. These authors also showed that bavchi oil and olive oil have antimicrobial activity against *S. aureus.* In addition, Chao *et al.*, (2008) reported inhibitory zones of 45 to 83 mm against *S.aureus* with EOs such as lemongrass, lemon myrtle, mountain savory, cinnamon bark and Melissa.

There are hundreds of other EOs available for use, many with known antimicrobial properties (Chao *et al.*, 2008). These oils contain numerous constituents that contribute to the /characteristic odor and medical effects. The presence and quantity of the various chemical compound components varies between oils and this determines the individuality of the oil. Due to the lack of scientific evidence of their efficacy as conventional antimicrobial treatments combined with their toxicity issues, medical teams rarely use EOs in spite of their proven antimicrobial properties (Edwards-Jones *et al*., 2004).

Bacteriophage therapy

Introduction

The widespread emergence of multiple antibiotic resistant pathogenic bacteria including strains of *E. coli, Staphylococcus sp., Streptococcus sp., Pseudomonas sp*., etc., has become a significant problem in treating bacterial infections of humans and animals. Scientists are now predicting a return to the pre-antibiotic era in order to find alternatives to the use of antibiotics to control bacterial pathogens (Rapson, 2002). A varied and large source of antibiotic agents specifically active against a wide range of bacterial infections (including MRSA) is the lytic bacteriophage population (Rapson, 2002). Bacteriophages are bacterial viruses that are able to infect specific bacterial species. Similar to other viruses, they are obligate parasites that are able to replicate only in living cells. A bacteriophage particle usually consists of a single nucleic acid molecule which may be single-stranded or double-stranded, linear or circular DNA, or single-stranded linear RNA. The three major morphological classes of the bacteriophages are icosahedral, icosahedral tailed, and filamentous (Platt, 2010). Bacteriophages have a specific host range because they require specific receptors on the host cell surface to bind and initiate an infection (Balasubramanian *et al*., 2007). They are nontoxic to humans, animals, and plants.

Bacteriophages were first discovered in 1915 from *Staphylococcus* sp. by a British bacteriologist, Frederick W Twort, and independently in 1917 from *Shigella dysenteriae* by a Canadian medical bacteriologist, Felix Hubert d'Herelle (Summers, 2001). D'Herelle named the invisible microbe that was an obligate parasite of living bacteria he found "a bacteriophage" or by the shortened term "phage". The therapeutic potential of bacteriophages was also realized early on by d'Herelle who laid the foundation for experimental phage work, and by 1940,

research involving bacteriophage therapy applications were being published in great numbers indicating the utility of bacteriophage as a promising antibacterial agent.

Mode of Action

Bacteriophages have two distinct life cycles, lytic and lysogenic. In the lytic cycle, bacteriophages utilize the host cells' replication machinery and precursors to produce many phage particles, and then end the cycle by lysing the host cells. In the lysogenic cycle, no progeny particles are produced and the bacteriophage DNA usually becomes part of the bacterial chromosome by site-specific recombination (Platt, 2010). Examples of lysogenic phage include λ phage of *E. coli* and P22 of *Salmonella* Typhimurium (Platt, 2010). The decision on whether to utilize lytic or lysogenic growth depends on several factors, including the expression of the bacteriophage repressor and the nutritional status of the host (Platt, 2010). A phage capable only of lytic growth is called a virulent phage while a bacteriophage capable of both lytic and lysogenic growth is called a temperate phage. The bacterial host that contains a complete set of bacteriophage DNA in the chromosome is called a lysogen. A bacteriophage whose genome is inserted and integrated into a bacterial DNA chromosome is called a prophage (Summers, 2001). Some prophages in a population of lysogens may switch to the lytic growth cycle and continue to release small amount of progeny bacteriophages into the environment. The progeny bacteriophages will then repeat the infection cycle in their susceptible host (Platt, 2010).

Limitations of Bacteriophage therapy

In spite of the successes in the bacteriophage therapy studies, it is not found routinely in the toolkits of infectious disease specialists, public health workers, and hospital infection control officers, because of its limitations (Summers, 2001). The causes of its limitations included the

narrow host range of a particular bacteriophage, and a specific bacteriophage is needed to treat a specific bacterial infection. If an infection is caused by a mix of bacterial pathogens, one bacteriophage could not stop the infection but rather a mixture of different bacteriophages specific to each bacterial pathogen would be required (Summers, 2001). The emergence of bacteriophage resistant bacteria is also possible through the selection of mutants (Platt, 2010). Furthermore, lysogen of a temperate bacteriophage can become immune to a super infection with the same bacteriophage and able to perpetuate the infection (Platt, 2010).

Staphylococcus aureus specific bacteriophage

Staphylococcus aureus bacteriophages encode proteins that target components of the DNA replication and RNA transcription machinery of *S. aureus* (Kwan *et al.*, 2005). Kwan *et al*. (2005) have reported the complete genomic sequences and predicted proteins of 27 bacteriophages of *S. aureus*. In dairy industry, mastitis caused by *S. aureus* is a major concern, and the most important source of milk contamination. The ability of the lytic *S. aureus* bacteriophage K to eliminate bovine *S. aureus* intramammary infection during lactation has been studied; however, Garcia *et al.* (2008) reported that the infused phage showed significant degradation or inactivation within the gland. Phage K inactivation was also reported in raw milk, likely due to the adsorption of whey proteins to the cell surface that interfere with phage attachment (Garcia *et al*., 2008). However, it has been documented that a cocktail of two lytic phages of dairy origin can successfully inhibit *S. aureus* in acid and enzymatic manufacturing processes (Garcia *et al*., 2008).

Detection of harmful pathogens such as *S. aureus* at low levels is vital in industry and has a huge environmental and economic impact. Balasubramanian *et al*. (2007) emphasized the use of bacteriophage immobilization technique to detect *S. aureus.* In this study, a lytic bacteriophage

was used as a highly specific and selective biorecognition element combined with a surface Plasmon resonance-based detection sensor. The authors found a detection limit of 10,000 colony forming units per ml, suggesting that lytic bacteriophage can be used as a probe for bacterial detection.

Application in food industry

There are various reliable techniques that are used in present day to kill or inactivate bacterial pathogens in food animals as well as food products including 1) antibiotics administered at production (Zaczek *et al*., 2015); 2) chlorine to inactivate pathogens on various food products including raw meat (Buncic and Sofos, 2012), fruits and vegetables (Beuchat, 2008); 3) thermal pasteurization of liquid foods (Corry *et al*., 1995); and 4) food irradiation (Farkas, 1998). However, due to continuous increase in several foodborne diseases, a possible alternative to chemical-based bactericides might be the use of natural antimicrobials such as bacteriophage.

Contaminating bacteria can enter the food supply during slaughter, milking, fermentation, processing, storage or packaging (Zaczek *et al*., 2015). Studies on bacteriophage molecular biology have shed light on its various applications including nanotechnology, vaccine development, therapeutic delivery, and bacterial detection systems (Summers, 2001). The application of bacteriophages as novel, natural antimicrobials in food to inhibit undesirable bacteria is also very promising (Garcia *et al*., 2008). Exploring bacteriophages as new biocontrol agent against antibiotic-resistant bacteria has become an important topic of research with products based on bacteriophage therapy are already available in the market (Zaczek *et al*., 2015). Moreover, several companies are using bacteriophages as tools for detecting pathogens in feed and foodstuffs. For example, the U.S. Environmental Protection Agency approved

OmniLytics, Inc to use their product 'Agriphage' against plant pathogenic bacteria. In the food manufacturing industry, Listex P100 has been approved by EBI Food Safety for controlling *Listeria* in meat and cheese products. The U.S. Food and Drug Administration (FDA) in 2006 approved the use of LMP 102 (Intralytix, Inc.) targeting *Listeria* in ready-to-eat meat and poultry products (Garcia *et al*., 2008).

The concept of fighting bacterial pathogens in food by means of bacteriophages can be addressed at all stages of production. This can be applied in the classic "farm to fork' concept. Bacteriophage is suitable for enhancing microbiological safety in food because of the following reasons: (i) it prevents or reduces the colonization of bacteria in livestock (bacteriophage therapy); (ii) it can decontaminate carcasses and raw products, such as fruits and vegetables; (iii) it can be used to disinfect equipment and contact surfaces (i.e. bacteriophage bio sanitation and bio control); and (iv) it can be used to extend the shelf life of perishable manufactured foods as natural preservatives (i.e. bio preservation) (Zaczek *et al*., 2015). Bacteriophage can also be used in hurdle technology in combination with other preservation methods (Martinez *et al*., 2008).

Despite the advantages offered by bacteriophages, they can be commercially damaging as well. For instance, in the fermentation industry, harmful bacteriophages are those which contaminate the process. These bacteriophages also became a major problem in the dairy industry, causing considerable economic losses by destroying starter cultures and disrupting fermentation (Lawrence *et al*., 1976). Other industries using bacteria for fermentation have experienced losses from bacteriophage contamination as well.

Strategies for Controlling *Staphylococcus aureus* in food

It is well known that many pathogenic microorganisms such as *S. aureus* form biofilms on food and food-contact surfaces under appropriate environments (Shi *et al*., 2009). In the food processing environment, biofilm formation is favored by many conditions (e.g., flowing water, suitable attachment surfaces, ample nutrients, and raw materials) (Shi *et al*., 2009). The time available for biofilm formation depends on the frequency of cleaning regimes. Product contact surfaces should typically be cleaned several times per day while environmental surfaces such as walls may be cleaned once per week (Gibson *et al*., 1999). An investigation by Sharma and Anand (2002) revealed that *S. aureus* could establish biofilms on the equipment surfaces of the production lines such as pasteurization lines of dairy processing plants. Pathogenic microorganisms can also attach to and grow on food and food contact surfaces under appropriate environments by forming biofilms (Shi *et al*., 2009). A number of studies have shown that bacterial pathogens can attach to food surfaces such as *L. monocytogenes* to beef surfaces (Dickson, 1990) and *Salmonella* spp. to chicken skin (Campbell *et al.,* 1987). However, humans are the main source of transmission of these pathogens from one place to another (e.g., food handlers contaminate food via manual contact or by coughing and sneezing). Nevertheless, in foods such as raw meat, sausages, raw milk, and raw milk cheese, contaminations from animal origins are more frequent and due to animal carriage or due to infections (e.g., mastitis) (Loir *et al*., 2003). Therefore, it is very important to develop cleaning and disinfection methods and control systems in food-processing plants and environments. Good Manufacturing Practice and Hazard Analysis and Critical Control Points have been established for controlling food quality and safety (Shi *et al.*, 2009). Moreover, cleaning-in-place (CIP) procedures have been employed in dairy processing lines. Some physical methods such as mechanical brushing, chemical agents

such as detergents and biological means such as enzymes are applied to CIP procedures in order to obtain a biofilm-free industrial environment (Shi *et al*., 2009). In addition, suitable contact precautions should be taken such as using antimicrobial soap for washing hands—particularly under fingernail area—vigorously before handling and preparing food. It is also suggested that gloves should be worn when touching any ready-to-eat food items though this is controversial (Herwadlt, 1999). The use of hand gloves has been much debated since it does reduce the likelihood of transmitting bacteria from a food worker's hand to food; however, the gloves may pick up bacterial pathogens from food or work surfaces touched, thus significantly reducing the effectiveness of gloves if they are worn for longer periods of time without changing (Lues *et al*., 2005).

Even after using an efficient cleaning procedure, microorganisms often remain and form biofilms on equipment surfaces (Shi *et al*., 2009). Residual microflora sometimes persists on food equipment surfaces after CIP treatment (Dunsmore, 1981; Sharma and Anand, 2002). Gibson *et al.* (1999) tested the resistance of biofilm to sanitizers, and they found that the commercial products Easyclean (an alkaline detergent) and Ambersan (an acidic cleaner) were not effective against *Pseudomonas aeruginosa* and *S. aureus* biofilms on stainless steel surfaces (Shi et al., 2009). Therefore, novel and innovative methods should be employed in order to battle this problem. The excessive use of chemical substances, some of which are suspect because of their potential toxicity, has resulted in increasing pressure on food manufacturers to either completely remove chemical preservatives from their food manufacturing systems or to adopt more "natural" alternatives for the maintenance or extension of a product' shelf life. There is considerable interest in the possible use of such natural alternatives which can prevent the growth of foodborne pathogens such as *S. aureus* on food products (Nychas, 2007). The

protective and therapeutic effects of bacteriophage against a wide range of bacterial pathogens have been well demonstrated (Monk *et al*., 2010). In addition, natural antimicrobials such as EOs from plant sources can be good candidates for limiting *S. aureus* infections (Li *et al*., 2011). More research should be done in this field so that a novel product can be developed which can utilize the antimicrobial properties of both EOs and bacteriophage to treat infections caused by pathogen such as *S. aureus*.

Research Objective

Since bacterial pathogens such as *S. aureus* have shown resistance against traditional chemotherapeutic agents such as antibiotics and other disinfectants, new natural approaches to control their growth in food and on food surfaces is pressing. In this research the possibility of using EOs and bacteriophage alone and in combination to prevent the growth of *S. aureus in vitro* has been evaluated. The hypothesis of this research is that combination of EOs and bacteriophage will control the growth of *S. aureus* more effectively than either treatment performed alone. To test this, two main objectives were set: 1) to determine the efficacy of varying concentrations of pure EOs compounds against *S. aureus* and 2) to evaluate the efficacy of a *S. aureus*-specific bacteriophage against *S. aureus.* The ultimate goal was to evaluate if the combination of these antimicrobials has potential synergism and possible application in food industry for controlling of *S. aureus* on raw chicken products.

References

Abuladze, T., Li, M., Menetrez, M.Y., Dean, T., Senecal, A. and Sulakvelidze, A. (2008) Bacteriophages reduce experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by *Escherichia coli* O157:H7. *Appl Environ Microbiol* **74**, 6230–6238.

Adams, J., Gibson, K.E., Martin, E.M., Almeida, G., Ricke, S.C., Frederick, N. and Carrier, D.J. (2014) Characterization and variation of essential oil from *Pinus Taeda* and antimicrobial effects against antibiotic-resistant and susceptible *Staphylococcus aureus*. *J Forest Prod* **64**, 161-165

Adukwu, E.C., Allen, S.C., Phillips, C.A. (2012) The anti-biofilm activity of lemongrass (*Cymbopogon flexuosus*) and grapefruit (*Citrus paradisi*) essential oils against five strains of *Staphylococcus aureus*. *J Appl Microbiol* **113**, 1217-1227.

Ait-Ouazzou, A., Cherrat, L., Espina, L., Lorán, S., Rota, C., and Pagán, R. (2011) The antimicrobial activity of hydrophobic essential oil constituents acting alone or in combined processes of food preservation. *Innovative Food Sci Emerg Technol* **12**, 320-329.

Alsaimary, I.E.A. (2012) Prevalence of β-lactamase-producing and non-producing *Staphylococcus aureus* associated with patients in intensive care units. *Med J Islamic World Academy Sci* **20**, 17-28.

Allahghadri, T., Rasooli, I., Owlia, P., Nadooshan, M.J., Ghazanfari, T., Taghizadeh, M., Astaneh, S.D. (2010) Antimicrobial property, antioxidant capacity, and cytotoxicity of essential oil from cumin produced in Iran. *J Food Sci* **75**, 54-61.

Argudin, M. A., Mendoza, M.C. and Rodicio, M.R. (2010) Food poisoning and *Staphylococcus aureus* enterotoxins. *J Toxicol* **2**, 1751-1773.Balaban, N. and Rasooly, A. (2000) Staphylococcal enterotoxins. *Int J Food Microbiol* **61**, 1-10.

Balasubramanian, S., Sorokulova, I.B., Vodyanoy, V.J., Simonian, A.L. (2007) Lytic phage as a specific and selective probe for detection of *Staphylococcus aureus* – A surface plasmon resonance spectroscopic study. *Biosens Bioelectron* **22**, 948- 955.

Bassole, I.H.N. and Juliani, H.R. (2012) Essential oils in combination and their antimicrobial properties. *J Molecules* **17**, 3989-4006.

Bennett, S.D., Walsh, K.A. and Gould, L.H. (2013) Foodborne disease outbreaks caused by *Bacillus cereus, Clostridium perfringens* and *Staphylococcus aureus* – United States, 1998-2008. *Clin Infect Dis* **57**, 425-433.

Bergdoll, M.S. (1989). *Staphylococcus aureus*. In: *Foodborne Bacterial Pathogens*. Marcel Dekker, Inc., New York, NY, USA, 463-523.

Beuchat, L.R. (2008). Surface decontamination of fruits and vegetables eaten raw. World Health Organization, Food Safety Unit. WHO/FSF/FOS/98.2. http://apps.who.int/iris/bitstream/10665/64435/1/WHO_FSF_FOS_98.2.pdf?ua=1 (accessed March 2008).

Bhargava, K., Wang, X., Donabedian, S., Zervos, M., Rocha, L.D. and Zhang, Y. (2011) Methicillin-Resistant *Staphylococcus aureus* in retail meat, Detroit, Michigan, USA. *Emerg Infect Dis* **17**, 1135–1137.

Bigwood, T., Hudson, J.A., Billington, C., Carey-Smith, G.V. and Heinemann, J. A. (2008) Phage inactivation of foodborne pathogens on cooked and raw meat. *J Food Micro* **25**, 400–406.

Bueno, E., García, P., Martínez, B. and Rodríguez, A. (2012) Phage inactivation of *Staphylococcus aureus* in fresh and hard-type cheeses. *Int J Food Microbio* **158**, 23–27.

Buncic, S. and Sofos, J. (2012) Interventions to control *Salmonella* contamination during poultry, cattle and pig slaughter. *Food Res Int* **45**, 641-655.

Burt, S. (2004) Essential oils: Their antibacterial properties and potential applications in foods—a review. *Int Food Microbiol* **94**, 223-253.

Cairns, B. J., Timms, A.R., Jansen, V.A., Connerton, I.F. and Payne, R.J. (2009) Quantitative models of in vitro bacteriophage–host dynamics and their application to phage therapy. *PLoS Pathogens* **5**, e1000253.

Callaway, T.R., Edrington, T.S., Brabban, A.D., Anderson, R.C., Rossman, M.L., Engler, M.J., Carr, M.A., Genovese, K.J., Keen, J.E., Looper, M.L. (2008). Bacteriophage isolated from feedlot cattle can reduce *Escherichia coli* O157:H7 populations in ruminant gastrointestinal tracts. *Food Pathog Dis* **5**, 154-159**.**

Campbell, S., Duckworth, S., Thomas, C., McMeekin, T. (1987) A note on adhesive of bacteria to chicken muscle connective tissue. *J Appl Bacteriol* **63**, 67-71.

Capparelli, R., Parlato, M., Borrielle, G., Salvatore, P. and Iannelli, D. (2007) Experimental phage therapy against *Staphylococcus aureus* in mice. *Antimicrob Agents Chemother* **51**, 2765-2773.

Chao, S., Young, G., Oberg, C. and Nakaoka, K. (2008) Inhibition of methicillin-resistant *Staphylococcus aureus* (MRSA) by essential oils. *Flavour Fragr J* **23**, 444-449.

Ciocan, I.D. and Bara, I.I. (2007) Plant products as antimicrobial agents. *Genetica Biol Mol* **8**, 151-156.

Corry, J.E.L., James, C., James, S.L. and Hinton, M. (1995) *Salmonella, Campylobacter and Escherichia coli* 0157:H7 decontamination techniques for the future. *Int J Food Microbiol* **28**, 187- 196.

Cox, S.D., Mann, C.M., Markham, J.L., Bell, H.C., Gustafson, J. E., Warmington, J. R. and Wyllie S.G. (2000) The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil). *J Appl Microbiol* **88**, 170-175.

Delaquis, P.J., Stanich, K., Girard, B., Mazza, G (2002) Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *Int J Food Microbiol* **74**, 101–109.

Dickson, J. S. (1990) Survival and growth of *Listeria monocytogenes* on beef tissue surfaces as affected by simulated processing condition. *J Food Safety* **10**, 165-174.

Edwards-Jones, V., Buck, R., Shawcross, S.G., Dawson, M.M. and Dunn, K. (2004) The effect of essential oils on methicillin-resistant *Staphylococcus aureus* using a dressing model. *J Burns* **2**, 772- 777.

El-Shibiny, A., Scott, A., Timms, A., Metawea, Y., Connerton, P. and Connerton, I. (2009)Application of a Group II *Campylobacter* bacteriophage to reduce strains of *Campylobacter jejuni* and *C. coli* colonizing broiler chickens. *J Food Protect* **72**, 733-740.

Farkas, J. (1998) Irradiation as a method for decontamination food: A review. *Int J Food Microbiol* **44,** 189-204.

Fu, Y., Zu, Y., Chen, L., Shi, X., Wang, Z., Sun, S. and Efferth, T. (2007) Antimicrobial activity of clove and rosemary essential oils alone and in combination. *Phytother Res*. **21**, 989-994.

Gallucci, M.N., Oliva, M., Casero, C., Dambolena, J., Luna, A., Zygadlo, J., Demo, M. (2009) Antimicrobial combined action of terpenes against the food-borne microorganisms *Escherichia coli, Staphylococcus aureus* and *Bacillus cereus*. *Flavour Fragr J* **24**, 348–354.

Garcia, P., Martinez, B., Obeso, J.M., Rodriquez, A. (2008) Bacteriophages and their application in food safety. *Lett Appl Microbiol* **47**, 479-485.

Gaysinsky, S., Taylor, T.M., Davidson, P.M., Bruce, B.D. and Weiss, J. (2007) Antimicrobial efficacy of eugenol microemulsions in milk against *Listeria monocytogenes* and *Escherichia coli* O157: H7. *J Food Protect* **70**, 2631–2637.

Gibson, H., Taylor, J.H., Hall, K.E., Holah, J.T. (1999) Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. *J Appl Microbiol* **87**, 41-48.

Goode, D., Allen, V.M. and Barrow, P.A. (2003) Reduction of experimental *Salmonella* and *Campylobacter* contamination of chicken skin by application of lytic bacteriophages *Appl Environ Microbiol* **69**, 5032–5036.

Greer, G.G. (1988) Effect of phage concentration, bacterial density, and temperature on phage control of beef spoilage. *J Food Sci* **53**, 1226-1227.

Grundmann, H., Aires-de-Sousa, M., Boyce, J., Tiemersma, E. (2006) Emergence and resurgence of methicillin-resistant *Staphylococcus aureus* as a public-health threat. *The Lancet* **368**, 874-881.

Gutierrez, D., Delgado, S., Vazquez-Sanchez, D., Martinez, B., Cabo, M.L., Rodriquez, A., Herrera, J.J. and Garcia, P. (2012) Incidence of *Staphylococcus aureus* and analysis of associated bacterial communities on food industry surfaces. *Appl Environ Microbiol* **78**, 8547-8554.

Guenther, S., Herzig, O., Fieseler, L., Klumpp, J. and Loessner, M.J. (2012) Biocontrol of *Salmonella Typhimurium* in RTE foods with the virulent bacteriophage FO1-E2. *Int J Food Microbio* **154**, 66–72.

Hagens, S. and Loessner, M.J. (2010) Bacteriophage for biocontrol of foodborne pathogens: calculations and considerations. *Curr Pharm Biotechnol* **1**, 58-68.

Hammer, K.A., Carson, C.F., Riley, T.V. (2001) Antimicrobial activity of essential oils and other plant extracts. *J Appl Microbiol* **86**, 985-990.

Hanson, B.M., Dressler, A.E., Harper, A.L., Scheibel, R.P., Wardyn, S.E., Roberts, L.K., Kroeger, J.S. and Smith, T.C. (2011) Prevalence of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) on retail meat in Iowa. *J Infect Public Health* **4**, 169-174.

Hao, Y.Y., Brackett, R.E. and Doyle, M.P. (1998) Efficacy of plant extract in inhibiting *Aeromonas hydrophila* and *Listeria monocytogenes* in refrigerated cooked poultry. *Food Microbiol* **15**, 367-378.

Helander, I.M., Alakomi, H.L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E.J., Gorris, L.G.M. and von Wright, A. (1998). Characterization of the action of selected essential oil components on Gram-negative bacteria. *J Agric Food Chem.* **46**, 3590e3595.

Herwaldt, L.A. (1999) Control of methicillin-resistant *Staphylococcus aureus* in the hospital setting. T*he Amer J Med* **106**, 11-18.

Hiramatsu, K., Aritaka, N., Hanaki, H., Kawasaki, S., Hosoda, Y., Hori, S., Fukuchi, Y. and Kobayashi, I. (1997) Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to Vancomycin. *The Lancet* **350**, 1670-1673.

Hoon, E.J., Na, K.J., Choi, K.C. and Jeung, E.B. (2004) Antibacterial and antifungal effects of essential oils from coniferous trees. *Biol Pharm Bull* **27**, 863-866.

Hudson, J.A., Billington, C., Wilson, T. and On, S.L.W. (2013) Effect of phage and host concentration on the inactivation of *Escherichia coli* O157:H7 on cooked and raw beef. *Food Sci Technol Int.* **1**, 1-6.

Hwang, S., Yun, J., Kim, K.P., Heu, S., Lee, S. and Ryu, S. (2009) Isolation and characterization of bacteriophages specific for *Campylobacter jejuni*. *Microbiol Immunol* **53**,559-566.

Hyun, J.E., Bae, Y.M., Song, H., Yoon, J.H. and Lee, S.Y. (2014) Antibacterial effect of various essential oils against pathogens and spoilage microorganisms in fresh produce. *J Food Safety* **10**, 51-62.

Intralytics (2014b) *Guidelines: Use of Bacteriophages (bacteria killing viruses) in Food (Ref: 70275202)*. Available at: http://intralytix.com/Intral_News_NFS.htm. (accessed 10 February 2014).

Jarvis, A.W., Lawrence, R.C. and Pritchard, G.G. (1973) Production of Staphylococcal enterotoxins A, B and C under conditions of controlled pH and aeration. *Infect Immun* **7**, 847-854.

Kavanaugh, N.L., Ribbeck, K. (2012) Selected antimicrobial essential oils eradicate *Pseudomonas* Spp. and *Staphylococcus aureus* biofilms. *Appl Environ Microbiol* **78**, 4057-4061.

Kim, J., Marshall, M.R., Wei, C.I. (1995) Antibacterial activity of some essential oil components against five foodborne pathogens. *J Agric Food Chem* **43**, 2839–2845.

Kleppen, H.P., Holo, H., Jeon, S.R., Nes, I.F. and Yoon, S.S. (2012) Novel Podoviridae family bacteriophage infecting *Weissella cibaria* isolated from Kimchi. *Appl Environ Microbiol* **78**, 7299–7308.

Kuorwel, K.K., Cran, M.J., Sonneveld, K., Miltz, J. and Bigger, S.W. (2011) Essential oils and their principal constituents as antimicrobial agents for synthetic packaging films. *J Food Sci* **76**, 164- 177.

Kwan, T., Liu, J., DuBow, M., Gros. P. and Pelletier, J. (2005) The complete genome and proteomes of 27 *Staphylococcus aureus* bacteriophages. *PNAS* **102**, 5174-5179.

Lee, J.H. (2003) Methicillin (Oxacillin)- Resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. *Appl Environ Microbiol* **69**, 6489- 6494.

Leuschner, R.G.K. and Lelsch, V. (2003) Antimicrobial effects of garlic, clove and red hot chilli on *Listeria monocytogenes* in broth model systems and soft cheese. *Int J Food Sci Nutri* **54**, 127-133.

Leverentz, B., Conway, W.S., Camp, M.J., Janisiewicz, W.J., Abuladze, T., Yang, M., Saftner, R. and Sulakvelidze, A. (2003) Biocontrol of *Listeria monocytogenes* on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. *Appl Enviro Microbiol* **69**, 4519-4526.

Li, M., Muthaiyan, A., O'Bryan, C.A., Gustafson, J.E., Li, Y., Crandall, P.G., Ricke, S.C. (2011) Use of natural antimicrobials from a food safety perspective for control of *Staphylococcus aureus. Curr Pharm Biotechnol* **12**, 1240-1254.

Lis-Balchin, M., Buchbauer, G., Ribisch, K. and Wenger, M.T. (1998) Comparative antibacterial effects of novel Pelargonium essential oils and solvent extracts. *Lett Appl Microbiol* **27**, 135–141.

Loir, Y.L., Baron, F. and Gautier, M. (2003) *Staphylococcus aureus* and food poisoning. *Genet Mol Res* **2**, 63-76.

Lowy, F.D. (1998) *Staphylococcus aureus* infections. *N Engl J Med* **339**, 520-532.

Lues, J.F.R., Van Tonder, I. (2005) The occurrence of indicator bacteria on hands and aprons of food handlers in the delicatessen sections of a retail group. *Food Control* **18**, 326-332.

Martínez, B., Obeso, J. M., Rodríguez, A. and García, P. (2008) Nisin-bacteriophage cross resistance in *Staphylococcus aureus*. *Int J Food Microbio* **122**, 253–258.

Miller, M.B. and Bassler, B.L. (2001) Quorum sensing in bacteria. *Annu Rev Microbiol* **55**, 165-199.

Monk, A.B., Rees, C.D., Barrow, P., Hagens, S. and Harper, D.R. (2010) Bacteriophage applications: where are we now? *Lett Appl Microbiol* **51**, 363-369.

Muller-Alouf, H., Carnoy, C., Simonet, M. and Alouf, J. E. (2001) Superantigen bacterial toxins: state of the art. *Toxicon* **39**, 1691-1701.

Mulyaningsih, S., Sporer, F., Zimmermann, S., Reichling, J., Wink, M. (2010) Synergistic properties of the terpenoids aromadendrene and 1,8-cineole from the essential oil of *Eucalyptus globulus* against antibiotic-susceptible and antibiotic-resistant pathogens. *Phytomedicine* **17**, 1061–1066.

Muthaiyan, A., Martin, E.M., Natesan, S., Crandall, P.G., Wilkinson, B.J. and Ricke, S.C. (2012) Antimicrobial effect and mode of action of terpeneless cold-pressed Valencia orange essential oil on methicillin-resistant *Staphylococcus aureus*. *J Appl Microbiol* **112**, 1020-1033.

Nazzaro, F., Fratianni, F., Martino, L. D., Coppola, R. and Feo, V. D. (2013) Effect of Essential Oils on Pathogenic Bacteria. *Pharm J* **6**, 1451-1474.

Normanno, G., Firinu, A., Virgilio, S., Mula, G., Dambrosio, A., Poggiu, A., Bolzoni, G. (2005) Coagulase-positive staphylococci and *staphylococcus aureus* in food products marketed in Italy. *Int J Food Microbiol* **98**, 73-79.

O'Brien, A.M., Hanson, B.M., Farina, S.A., Wu, J.Y., Simmering, J.E., Wardyn, S.E., Forshey, B.M., Kulick, M.E., Wallinga, D.B. and Smith, T.A. (2012) MRSA in conventional and alternative retail pork products. *PLoS ONE* **7**, e30092.

Palmer, A.S., Stewart, J., Fyfe, L. (1998) Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. *Lett Appl Microbiol* **26**, 118-122.

Panneerseelan, L. and Muriana, P.M. (2009) An immunomagnetic PCR signal amplification assay for sensitive detection of *Staphylococcus aureus* enterotoxins in foods. *J Food Protect* **72**, 2538-2546.

Puapermpoonsiri, U., Spencer, J. and van derWalle, C. (2009) A freeze-dried formulation of bacteriophage encapsulated in biodegradable microspheres. *Eur J Pharm Biopharm* **72**, 26e33.

Ratree Platt. (2010) Bacteriophage therapy: A novel method of lytic phage delivery.

Rapson, M.E. (2002) The biology of *Staphylococcus aureus* – specific bacteriophages and the assessment of their potential as therapeutic agents. *J Aerosol Med* **26**, 1-19.

Richter, S.S., Diekema, D.J., Heilmann, K.P., Dohrn, C.L., Crispell, E.K., Riahi, F., McDanel, J.S., Satola, S.W. and Doern, G.V. (2014) Activities of vancomycin, ceftaroline, and mupirocin against *Staphylococcus aureus* isolates collected in a 2011 National Surveillance Study in the United States. *Antimicrob Agents Chemother* **58**, 740-749.

Savage, V.J., Chopra, I., O'Neill, A,J. (2013) *Staphylococcus aureus* biofilms promote horizontal transfer of antibiotic resistance. *Antimicrob Agents Chemother* **57**, 1968-1970.

Sharma, M., Patel, J.R., Conway, W., Ferguson, S. and Sulakvelidze, A. (2009) Effectiveness of bacteriophages in reducing *Escherichia coli* O157: H7 on fresh-cut cantaloupes and lettuce. *J Food Protect* **72**, 1481-1485.

Shah, B., Ikeda, S., Davidson, P.M. and Zhong, Q. (2012) Nanodispersing thymol in whey protein isolate-maltodextrin conjugate capsules produced using the emulsion-evaporation technique. *J Food Eng* **113**, 79-86.

Shi, X., Zhu, X. (2009) Biofilm formation and food safety in food industries. *Trends Food Sci Tech* **20**, 407-413.

Skandamis, P.N. and Nychas, J.E. (2001) Effect of oregano essential oil on microbiological and physic-chemical attributes of minced meat stored in air and modified atmospheres. *J Appl Microbiol* **91**, 1011-1022.

Sofos, J.N., Beuchat, L., Davidson, P.M. (1998) Naturally occurring antimicrobials in food. *Council for Agricultural Science and Technology.*

Soni, K.A., Nannapaneni, R. and Hagens, S. (2010) Reduction of *Listeria monocytogenes* on the surface of fresh channel catfish fillets by bacteriophage Listex P100. *Foodborne Pathog Dis* **7**, 427–434.

Summers, W.C. (2001) Bacteriophage Therapy. *Annu Rev Microbiol* **55**, 437-451.

Synnott, A.J., Kuang, Y., Kurimoto, M., Yamamichi, K., Iwano, H. (2009) Isolation from sewage influent and characterization of novel *Staphylococcus aureus* bacteriophages with wide host ranges and potent lytic capabilities. *Antimicrob Agents Chemother* **75**, 4483-4490.

Tabla, R., Martínez, B., Rebollo J.E. (2012) Bacteriophage performance against *Staphylococcus aureus* in milk is improved by high hydrostatic pressure treatments. *Int J Food Microb* **156**, 209–213.

Traber, K.E., Lee, E., Benson, S., Corrigan, R., Cantera, M., Shopsin, B. and Novick, R.P. (2008) agr function in clinical *Staphylococcus aureus* isolates. *Microbiol* **154,** 2265-2274.

Tserennadmid, R., Takó, M., Galgóczy, L., Papp, T., Pesti, M., Vágvölgyi, C., Almássy, K., Krisch, J. (2011) Anti yeast activities of some essential oils in growth medium, fruit juices and milk. *Int J Food Microbiol* **144**, 480–486.

Upadhyay, R.K., Dwivedi, P. and Ahmad, S. (2010) Screening of antibacterial activity of six plant essential oils against pathogenic bacterial strains. *Asian J Med Sci* **2**, 152-158.

Vanderhaeghen, W., Cerpentier, T., Adriaensen, C., Vicca, J., Hermens, K. and Butaye, P. (2010). Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. *Vet Microbiol* **144**, 166-171.

Van Vuuren, S.F., Viljoen, A.M. (2007) Antimicrobial activity of limonene enantiomers and 1,8-cineole alone and in combination. *Flavour Fragr J* **22**, 540–544.

Viazis, S., Akhtar, M., Feirtag, J. and Diez-Gonzalez, F. (2011) Reduction of *Escherichia coli* O157: H7 viability on leafy green vegetables by treatment with a bacteriophage mixture and transcinnamaldehyde. *Food Microbiol*. **28**, 149-157.

Wang, Q. and Sabour, P.M. (2010) Encapsulation and controlled release of bacteriophages for food animal production. In *Bacteriophages in the Control of Food- and Waterborne Pathogens* eds. Sabour, P.M. and Griffiths, M.W. *Washington: ASM Press*, 237–255.

Waters, A.E., Contente-Cuomo, T., Buchhagen, J., Liu, C.M., Watson, L., Pearce, K., Foster, J.T., Bowers, J., Driebe, E.M., Engelthaler, D.M., Keim, P.S. and Price, L.B. (2011) Multidrug-resistant *Staphylococcus aureus* in US meat and poultry. *Clin Infect Dis* **52**, 1227-1230.

Weese, J.S., Reid-Smith, R., Rousseau, J. and Avery, B. (2010) Methicillin-resistant *Staphylococcus aureus* (MRSA) contamination of retail pork. *Can Ver J* **51**, 749-752.

Weiss, J., Gaysinsky, S., Davidson, M., McClements, J. (2009) Nanostructured encapsulation systems: Food antimicrobials. *Global Issues Food Sci Technol* **4,** 425–479.

Wendlandt, S., Schwarz, S., Silley, P. (2013) Methicillin-resistant *Staphylococcus aureus*: A foodborne pathogen? *Annu Rev Food Sci Technol* **4**, 1-23.

Ye, J., Kostrzynska, M., Dunfield, K. and Warriner, K. (2010) Control of *Salmonella* on sprouting mung bean and alfalfa seeds by using a biocontrol preparation based on antagonistic bacteria and lytic bacteriophages. *J Food Prot* **73**, 9–17.

Yoon, H., Yun, J., Lim, J.A., Roh, E., Jung, K.S., Chang, Y., Ryu, S., and Heu, S. (2013) Characterization and genomic analysis of two *Staphylococcus aureus* bacteriophages isolated from poultry/ livestock farms. *J Gen Virol* **94**, 2569-2576.

Zaczek, M., Weber-Dabrowska, B. and Gorski, A. (2015) Phages in the global fruit and vegetable industry. *J Appl Microbiol* **118**, 537-556.

Chapter 2: Application of Essential Oil Compounds and Bacteriophage alone and in combination to Control *Staphylococcus aureus in vitro.*

Abstract

The present study aimed to investigate the combination of essential oils (EO) and bacteriophages as alternative antimicrobials to control *S. aureus* in food. Four EO compounds were evaluated by disc diffusion assay to determine inhibitory effects against five strains of *S. aureus*. Next, 48-hour growth inhibition assays were performed using a 96-well bioassay. Phage adsorption assays were performed with phage K up to 120 h at 6, 13, and 37°C to determine lytic activity. Combinations of phage K and EOs against *S. aureus* were also evaluated at 37°C using a 96-well bioassay. Disc diffusion assays indicate that the zone of inhibition (IZ) of alpha-pinene (IZ=10-23mm) has a significantly higher inhibitory effect against *S. aureus* strains when compared to other EOs tested. Growth inhibition assay indicated that all four *S. aureus* strains showed significantly reduced growth ($p < 0.006$) when compared to the positive control over the 48 h period. Alpha-pinene at 1.5% and 3.28% showed the highest significant difference for inhibition of all *S. aureus* strains when compared to their respective positive controls. . Phage adsorption assays indicate that phage K has high lytic activity at 37°C with at least a 1.5 log increase in the number of PFU depending upon the strains when compared to 6 and 13°C, possibly impacting applicability in food industry. With phage and EO evaluated together, all four *S. aureus* strains showed a significant reduction in growth ($p < 0.05$) when compared to its normal growth curve. Results from the combined effect of EO and phage indicate that the phage alone inhibits *S. aureus in vitro* at 37°C as effectively as EO alone or the combination of EO and phage although there is variability between strains. The results from disc diffusion, growth inhibition, and phage adsorption assays indicate that EO compounds and bacteriophage can be used as antimicrobials against *S. aureus.* This study provides novel findings on the antibacterial properties of pure compounds found in crude pine oils and phage K against multiple strains of *S. aureus*. In

addition, the data presented here demonstrate the potential of these natural antimicrobials as a new approach for biocontrol of *S. aureus.*

Introduction

Staphylococcus aureus is considered one of the most important pathogens and can cause illnesses ranging from minor skin infections to life-threatening diseases such as pneumonia and bacteremia (Yoon *et al*., 2013). Food production animals such as dairy cows and broiler chickens can also be infected with *S. aureus* resulting in bovine mastitis and chicken arthritis (Vanderhaeghen *et al*., 2010). Although these ailments are harmful to the animals, these infections can also threaten food safety and cause food intoxications (due to production of enterotoxins) through contamination of foods such as salads including egg, chicken or potato salads and sandwiches which are made with hand contact and require no additional cooking. (Gutierrez *et al*., 2012) Unfortunately, management of *S. aureus* infections in humans has become compromised as a result of multiple antibiotic resistant strains including methicillin resistant *S. aureus* (MRSA) and vancomycin resistant *S. aureus* (VRSA). (Panneerseelan and Muriana, 2009).

Staphylococcus aureus is an aerobic, non-motile, non-spore forming Gram-positive bacterium. It is a commensal colonizer of the skin, nasal passage (25 to 30% of people), and mucous membranes (throat, axilla and rectum) of humans and warm-blooded mammals (Chao *et al*., 2008). *Staphylococcus aureus* has the ability to grow in wide temperature ranges (4°C to 45°C), pH levels (4 to 11), and salt concentrations (10 to 20%) allowing it to be a common foodborne pathogen (Panneerseelan and Muriana, 2009).

Due to the resistance of *S. aureus* to traditional chemotherapeutic agents, researchers are now evaluating other antimicrobial agents to fight against this pathogen such as essential oil (EO) compounds (Nazzaro *et al*., 2013). Essential oils are concentrated hydrophobic liquids derived

primarily from terpenoids (i.e. phenolic terpenes, phenylpropanoids, and alcohols) and have been recognized for their antimicrobial properties (Bassole and Juliani, 2012). Interactions between hydrocarbons present in EO compounds can lead to antagonistic, additive, or synergistic effects with synergism being the most common (Ciocan *et al*., 2007). Essential oils are capable of inhibiting or slowing the growth of bacteria, yeasts and molds by targeting the membrane and cytoplasm, and in some cases, they completely change the morphology of the cells. (Nazzaro *et al*., 2013). As a result, EOs and their components are widely used in medicine and food industry as they show promising activities against many food-borne pathogens, including *S. aureus*, when tested *in vitro* (Bassole and Juliani, 2012).

In contrast to EO compounds, lytic bacteriophage may be used as alternative antimicrobials with enhanced specificity to the microorganisms or pathogen of interest (Rapson, 2002). Bacteriophages are bacterial viruses that require specific receptors on the bacterial cell surface to bind and initiate an infection (Balasubramanian *et al*., 2007). Their specificity for a particular bacterial species and their lack of impact upon other micro flora make them efficient antibacterial agents (El-Shibiny *et al*., 2009). With respect to *S. aureus*, there are several well-characterized lytic bacteriophage. For example, bacteriophage K has been applied in the dairy cow industry for the elimination of bovine *S. aureus* intramammary infections during lactation (Garcia *et al*., 2008). In general, the application of bacteriophages to control foodborne pathogens such as *S. aureus, Listeria monocytogenes, Salmonella,* and *Escherichia coli* is steadily growing (Hudson *et al*., 2010; Hagens and Loessner, 2010). For instance, bacteriophage specific to Shiga toxin-producing *E. coli* (STEC) has been reported to reduce STEC on leafy green vegetables by 3 logs (Viazis *et al*., 2011). Moreover, bacteriophages are now accepted as natural food additives since they are naturally present in the digestive tract of humans and

throughout the environment (Monk *et al*., 2010). Here I propose combining *S. aureus*-specific bacteriophage with EOs as a way to inhibit the growth of *S. aureus*. Therefore, based on the reported antimicrobial potential of both EOs and host-specific bacteriophage, I investigated the efficacy of varying concentrations of EOs alone and in combination with *S. aureus* specific bacteriophage in order to determine potential synergistic effects. More specifically, I aimed 1) to determine the efficacy of varying concentrations of pure EOs compounds against *S. aureus*; 2) to evaluate the efficacy of a *S. aureus*-specific bacteriophage against 4 strains of *S. aureus* and 3) to combine these antimicrobials to determine potential synergism and possible application for the control of *S. aureus in vitro*. The overall goal is to utilize these antimicrobial compounds for control of *S. aureus* as both a nosocomial and foodborne pathogen with potential applications in the medical industry such as the treatment of cutaneous infections caused by *S. aureus* as well as the food industry.

Materials and methods

Bacterial culture

Five strains of *S. aureus* were maintained on tryptic soy agar (TSA, Acumedia, Lansing, MI, U.S.). The test strains included 3 MRSA (N315, COL, Mu50), 1 susceptible (ATCC 6538; American Type Culture Collection, Manassas, VA), and 1 susceptible clinical isolate (ASU 36) kindly provided by Dr. Dave Gilmore at Arkansas State University, Jonesboro, AR. For each experimental setup, one colony from each respective *S. aureus* strain was added to 5ml of tryptic soy broth (TSB, Acumedia, Lansing, MI, U.S.) and incubated at 37°C for 24 h with shaking at 150 rpm. Inocula were prepared as described below for the assay to be performed.

Disc diffusion assay

To evaluate the antimicrobial activity of four pure EO compounds against *S. aureus* a disc diffusion method previously described by Muthaiyan *et al.* (2012) was used with modifications. Four pure EO compounds commonly found in pine oils were used: alpha-pinene, 3-carene, (+)-limonene (ACROS Organics, Morris Plains, NJ, U.S.) and (1S)-(-)-β-pinene (referred to as beta-pinene for remainder of manuscript) (Alfa Aesar, Ward Hill, MA, U.S.). Alpha-pinene was evaluated alone and in combination with the three other EOs. Briefly, the overnight cultures of each *S. aureus* strain were diluted separately in TSB to achieve an optical density (OD) of 0.3 at 600 nm. A 100 µl aliquot of prepared inoculum for each *S. aureus* strain was evenly spread on TSA plates to create a lawn of bacteria. Sterile, blank, 6-mm paper discs (Becton, Dickinson and Company, Sparks, MD, U.S.) were aseptically placed on TSA plates containing *S. aureus* and 10 µl of EO were pipetted onto the discs and allowed to absorb for 5 min. For EOs in combination, 5 µl of each EO was added to the disc. Sterile distilled water was used as a negative control. After 24 h of incubation at 37°C, diameters (in millimeters) of zones of inhibition were measured and recorded. The same method was followed at 6 and 13°C except lawns of *S. aureus* strains were prepared at 37°C prior to addition of EO saturated discs.

Essential Oil Growth Inhibition Assay (GIA)

A growth inhibition assay was performed to evaluate the susceptibility of the *S. aureus* strains to varying EO concentrations (0.5, 1.5, 3.28, and 5%) at 6, 13 and 37°C. At 37°C, a 96-well plate bioassay and an Infinite M200 micro plate reader (TECAN, San Jose, CA, U.S.) with shaking was used and at 6 and 13°C, a refrigerated shaker and, a spectrophotometer (Beckman Coulter DU® 640, Brea, CA, U.S.) was used. Growth of *S. aureus* during exposure to the EOs was

monitored over a 48 h period with absorbance readings (at 600 nm) recorded every 30 min when using the micro plate reader and at 0 to every 30 min for 7 h and then at 24, 26, 28, 32, 48, 56 and 72 h when using the spectrophotometer. Essential oil solutions were prepared in four different concentrations in 0.5% Tween 80 (Amresco, Solon, Ohio, U.S.). For the 96-well plate bioassay, 100 µl of prepared inocula in TSB was dispensed into each well followed by 100 µl of the EO solutions. For each *S. aureus* inoculum, 500 µl of overnight culture was diluted in 25 ml of TSB to achieve an OD of 0.08 at 600 nm. Combinations of EOs were added in equal parts (50 µl + 50 µl) to achieve the same final concentration. Four controls were included in each 96-well plate including 200 µl of prepared inoculum without EOs; 200 µl of prepared inoculum + Tween 80; 200 µl of Tween 80; and 200 µl of TSB.

GIA – Essential Oils and Phage

To evaluate the susceptibility of *S. aureus* to varying concentrations of EOs in combination with phage, a 96-well plate bioassay as described above was used. All *S. aureus* strains were tested in three combinations: 1) EOs alone, 2) phage alone and 3) EOs + phage along with positive and negative controls as described above for GIA with EO only. Bacteriophage K was added at an MOI of 0.1.

At 6 and 13°C, the GIA described previously by Muthaiyan *et al.* (2012) was used with modifications. Inoculum for each *S. aureus* strain was prepared as described above. Individual *S. aureus* strains were mixed with varying concentrations of EO compounds in 50 ml Erlenmeyer flasks. The flasks were kept at 6 or 13°C with shaking. Growth inhibition of *S. aureus* at 6 and 13°C was monitored at regular intervals. A negative control flask of bacterial culture without EO was included.

Bacteriophage Characterization

Preparation of phage stock

Two strains of bacteriophage, bacteriophage K (ATCC 19685-B1) and bacteriophage 92 (ATCC 33741-B1) were initially used. A double overlay method was used to propagate the bacteriophage. Briefly, for propagation of bacteriophage K, 100 µl log phase *S. aureus* culture (ATCC 25923) was combined with 100 µl bacteriophage K in 5 ml of 0.7% TSA (soft agar). The soft agar was poured on TSA plates, allowed to solidify, and the plates were incubated at 37°C for 24 hours to allow for complete lysis of the bacterial lawn. After 24 hours, the soft agar was scraped off and placed in a 50 ml conical tube containing 15 ml of SM buffer (5.8 g NaCl, 1.2 g $MgSO_4$, 50 mL 1M Tris-HCl (pH 7.5), 0.1 g Gelatin) and vortexed. The bacteriophage suspension was then kept in an incubator at 37°C with gentle shaking for 4 hours. The suspension was centrifuged at 580 × *g* for 25 min at 4°C to sediment the cellular debris and agar. The clear supernatant containing bacteriophage K was collected and passed through a 0.22 µm pore size cellulose acetate filter (VWR, Radnor, PA, U.S.) to obtain a homogenous phage stock. The concentration of bacteriophage K stock was determined using the double agar overlay method described above. Following titration, the bacteriophage K stock was stored in small aliquots at −80°C. For bacteriophage 92, the above method was followed with the exception of using nutrient broth (NB, Becton, Dickinson & Company, Annapolis, MD, U.S.) with 400µg/ml of $CaCl_2$.

Phage adsorption

To evaluate adsorption properties of bacteriophage K and 92, phage adsorption assay as described previously by Hwang *et al.* (2009) was used with modifications. Briefly, *S. aureus*

strains were prepared in TSB with a final concentration of 10^8 CFU/ml. Bacteriophage K or 92 was added to the cell suspension at a MOI of 0.1 (10^7 PFU/ml) and incubated at 37°C to allow adsorption to the bacterial cells. At time 0 and every 10 min for 1 h, phage-bacteria suspensions were collected and centrifuged at 14,000 × *g* for 10 minutes. The supernatant was decanted and serially diluted. Plaque assay using the double overlay method as described above was used to determine the number of bacteriophage in the supernatant and reported as plaque forming units (PFU).

Inhibition of *S. aureus* in *vitro*

Each *S. aureus* inoculum was mixed with bacteriophage K at a MOI of 0.1. At the designated time points (e.g., time 0 followed by every hour for 6 h, then at 24, 48, 72, 96 and 120 h), the OD at 600 nm was measured, and the sample was processed for recovery and detection of bacteriophage as described above in bacteriophage-adsorption. Inactivation of *S. aureus* by bacteriophage K was performed at 6, 13, and 37°C.

Effect of essential oils on phage

The inhibitory effect of EOs on bacteriophage K was examined. Bacteriophage inhibition was measured at 6, 13, and 25°C for designated time points (0, 1, 6, and 24 h). Briefly, bacteriophage suspension was mixed with EOs at select concentrations and incubated at the appropriate temperature. A double overlay assay was performed to determine the effect of EOs against bacteriophage K using *S. aureus* host ATCC 25924.

Statistical Analysis

All experiments were repeated two times with duplicate plates. Before analysis, the mean of the duplicate plate counts from two replicates was converted to $\log_{10}$ PFU/ml. For disc diffusion assay, differences between means of zone of inhibitions were tested by least square difference, and statistical differences were examined using two factor factorial and randomized complete block diagram (RCBD). Population of *S. aureus* strains treated with bacteriophage-EOs cocktail were subjected to one-way analysis of variance (ANOVA) for a completely randomized design using JMP (Version 11.1.1, SAS Institute Inc., Cary, NC) to determine whether treatment with bacteriophage-EO significantly reduced the number of viable *S. aureus* cells. In all cases, the level of statistical significance was set at $P < 0.05$.

Results

Disc diffusion assay

The antimicrobial effects of EOs against five *S. aureus* strains based on the disc diffusion assay are shown in Table 1. All five *S. aureus* strains showed varied susceptibility to the EO compounds at 37°C with zones of inhibition (IZ) ranging from 8 to 23 mm in diameter. More specifically, alpha-pinene alone had significantly higher inhibitory effect against *S. aureus* strains (IZ = 10 to 23 mm) when compared to the use of the EO compounds in combination (Figure 1). This can probably be explained by the fact that 10 µl of each compound were used when evaluated alone whereas 5 µl of each compound was used when evaluated in combination. In addition, Mu50 strain was the least susceptible to the majority of EO compounds and thus was excluded from future experiments. With respect to EOs in combination, greater antimicrobial effects were observed with alpha-pinene + (+)-limonene (IZ =11 to 15 mm) when compared to

alpha-pinene used in combination with beta-pinene and 3-carene. Within *S. aureus* strains, COL had significantly different IZ with each of the four EO compounds (Table 1). Within EO compounds, all except alpha-pinene + (+)-limonene produced significantly different IZ across all five strains of *S. aureus* (Table 1).

In addition to 37°C, the antimicrobial properties of these EO compounds were also tested at 6 and 13°C. These temperatures were selected because it represents refrigeration temperature and temperature abuse of food products, respectively, in order to evaluate potential application in the food industry. However, the disc diffusion assay was not suitable for evaluation at these temperatures since lawns of *S. aureus* strains first had to be prepared at 37°C prior to addition of EO compounds which is counter indicative for this method (data not shown).

Efficacy of essential oils against *S. aureus*

To evaluate the inhibitory effect of EO compounds, growth inhibition assays were performed by exposing four *S. aureus* strains to various EO concentrations (0.5%, 1.5%, 3.28%, and 5%) over a 48-hour period at 37°C. All four *S. aureus* strains showed significantly reduced growth ($p < 0.006$) when compared to the positive control over the 48 h period (Figure 2). As expected, the lowest concentration of EO (0.5%) had the least inhibitory properties against *S. aureus* growth when compared to subsequently higher concentrations. Conversely, higher concentrations of EO (3.28% and 5%) showed similar results with inhibition to *S. aureus* up to 48 hours depending on the strain. Similar to the disc diffusion assay, alpha-pinene showed higher inhibition of *S. aureus* growth when compared to the EOs in combination. However, out of the EO combinations evaluated, alpha-pinene + 3-carene inhibited *S. aureus* growth better than the other combined EOs. Alpha-pinene at 1.5% and 3.28% showed the highest significant difference for inhibition of

all *S. aureus* strains with p-values ranging between 0.0006 to 0.001 when compared to their respective positive controls. Based on these results, 1.5% and 3.28% concentrations of alpha-pinene alone and alpha-pinene + 3-carene were selected for further evaluation. The results of the growth inhibition assay with alpha-pinene alone and alpha-pinene + 3-carene at concentrations of 1.5% and 3.28% against four *S. aureus* strains are shown in Figure 2.

Phage characterization

The lytic activity of the two *S. aureus* specific bacteriophage, bacteriophage K and 92 were tested against *S. aureus* strains. All five *S. aureus* were weakly susceptible towards bacteriophage 92 and thus the adsorption of the bacteriophage to the *S. aureus* strains was relatively low resulting in very few PFU (data not shown). Therefore, bacteriophage 92 was excluded from further experiments. Conversely, bacteriophage K showed high lytic activity against all *S. aureus* strains tested (Figure 3). For phage adsorption at 37°C, bacteriophage K showed a steady increase in the number of PFU over a period of 6 hours when incubated with all *S. aureus* strains (Figure 4C). After 6 hours at 37°C, *S. aureus* strains ASU 36 and ATCC 6538 had more than a 2 log increase in the number of PFU, and *S. aureus* strains N315 and COL showed more than a 1.5 log increase in the number of PFU. At 6 and 13°C, the lytic activity of bacteriophage K gradually decreased over the 6 h period against *S. aureus* strains (Figure 4A-B).

To investigate the antimicrobial activity of bacteriophage K over a prolonged period of time (24 to 120 hours), phage adsorption was performed at 6, 13, and 37°C (Figure 5A-C). At 6°C, the replication of bacteriophage K in all *S. aureus* strains showed fluctuations in number of PFU before decreasing after 120 hours. At 13°C, the number of PFU remained more or less constant after 72 hours, ranging between 4 and 6 $\log_{10}$ PFU/ml depending on the strain (Figure 5B). As

expected, at 37°C the number of PFU was 3 log higher after 24 hours as compared to 6 and 13°C (Figure 5). The lytic activity of bacteriophage K against *S. aureus* strain COL remained constant over a period of 120 hours. For other strains, the number of PFU slightly decreased after 72 hours. In addition to direct count of PFU, OD readings were also measured prior to centrifugation to collect PFU; however, the pattern of OD readings varied with different strains and did not often correspond with PFU/ml (data not shown).

Efficacy of combined phage and EO against *S. aureus*

To evaluate the inhibitory effect of EO compounds (α-pinene and 3-carene) combined with bacteriophage K, GIAs were performed by exposing four *S. aureus* strains to two concentrations (1.5% and 3.28%) of EO as well as bacteriophage K at a MOI of 1 over a 48-hour period at 37°C. All four *S. aureus* strains showed a significant reduction in growth ($p < 0.05$) when compared to its normal growth curve (Figure 6A-D). Both bacteriophage K alone and α-pinene alone at 1.5% and 3.28% showed similar inhibition trends as observed in phage adsorption and initial GIAs against all *S. aureus* strains, respectively. However, with bacteriophage K and EO combinations, bacteriophage K with 3.28% α-pinene inhibited *S. aureus* growth better than other combinations of EOs and bacteriophage depending upon the strain. For *S. aureus* strains N315 and ASU 36, there was no significant difference between the treatments. For COL and ATCC 6538, bacteriophage K had significantly higher inhibitory effect compared to other treatments such as 1.5% α-pinene and bacteriophage K + 1.5% α-pinene (p value ranging between 0.006 to 0.021) (Figure 6C). The results of the GIAs with α-pinene and bacteriophage K alone and in combination at concentrations of 1.5% and 3.28% against four *S. aureus* strains are shown in Figure 6A through 6D.

Discussion

Staphylococcus aureus is one of the major bacterial agents causing foodborne diseases in humans primarily through the production of enterotoxins. In addition to this, *S. aureus* is also the most common nosocomial pathogen according to the National Nosocomial Infections Surveillance (Wunderink *et al*., 2003). Humans are common asymptomatic carriers of enterotoxigenic *S. aureus* in the nose, throat, and skin. (Gutierrez *et al*., 2012). Since *S. aureus* is becoming universally resistant to traditional chemotherapeutic agents, there is more focus on the pre-antibiotic era for alternative antimicrobials effective against *S. aureus* including EOs and bacteriophage (Panneerseelan and Muriana, 2009).

In the present study, I evaluated the antimicrobial activity of four pure EO compounds that are common components of crude essential oils including those extracted from coniferous trees (e.g., loblolly pine or *Pinus taeda L.*), rosemary, citrus fruits, and cumin (Fu *et al*., 2007; Allahghadri *et al*., 2010). I focused on pure compounds from pine oils as a recent paper by Adams *et al.*, (2014) determined that α-pinene, β-pinene, and limonene were major components in crude oils extracted from the loblolly pine, a major forestry product of Arkansas in the U.S. The study by Adams *et al.* (2014) also indicated some antimicrobial activity of the crude essential oil extracts against *S. aureus*; therefore, in the present study, I aimed to evaluate some of the major components separately and in combination.

Numerous studies have evaluated the efficacy of a variety of EO compounds against bacterial pathogens *in vitro* including *S. aureus.* These EOs include bay, cinnamon, clove, lemongrass, lemon myrtle, mountain savory, Melissa, ajwaine oil, bavchi oil and oregano (Adukwu *et al.*, 2012; Hyun *et al.,* 2014; Palmer *et al.,* 1998 Upadhyay *et al.*, 2010; Chao *et al*., 2008 and

Leuschner and Lelsch, 2003). Palmer *et al.* (1998) investigated the antimicrobial activity of 21 plant essential oils against *S. aureus.* The authors reported that oils such as bay, cinnamon, clove, spearmint, thyme and eucalyptus had a high bacteriostatic and bactericidal effect against *S. aureus* with a zone of inhibition ranging from 8 to 11mm (disc diameter 4 mm) as compared to other plant essential oils tested. Another study by Adukwu *et al.* (2012) reported that low concentrations of lemongrass EO (0.03 to 0.06%, v/v) effectively inhibited the growth of *S. aureus* and was bactericidal at slightly higher concentrations (0.125%). Meanwhile, in the same study, the effective inhibitory and bactericidal concentrations of grapefruit EO against *S. aureus* were reported to be 10 to 100-fold higher than lemongrass. These results indicate that some EOs are effective at very low concentrations while others are effective at relatively higher concentrations. Therefore, in this study, I selected a range of concentrations (0.5, 1.5, 3.28 and 5%) to better understand the effect of the components of pine EO against *S. aureus*. To date, there are very few studies that have investigated the antimicrobial activity of pine EO and its individual components against bacterial pathogens.

Of direct relevance to the present study, Hong *et al.* (2004) reported that the EOs of pine needles exert antibacterial effects against *L. monocytogenes, S. aureus*, and *Klebsiella pneumoniae* with the zone of inhibition diameter in the range 11 to 14 mm. In the present study, the *S. aureus* strains showed varied zones of inhibition (IZ) to the EOs evaluated ranging from 8 to 23 mm in diameter. Palmer *et al*. (1998) studied the inhibitory effects of clove and rosemary EOs against *S. aureus* and reported IZ of 8 and 5.9 mm, respectively. Fu *et al*. (2007) investigated the same EOs for antimicrobial effects against *S. aureus* and observed zones of inhibition of 16.3 ± 0.7 and 18.5 ± 1.3 mm, respectively. Although these two studies used different diameter discs—4 mm and 6 mm—the differences in the outcomes of these studies show that the disc diffusion assay

can result in varied outcomes when the same EOs are evaluated. In this study, I also evaluated the inhibitory effect of *trans*-cinnamaldehyde on *S. aureus*. The *S. aureus* strains showed a higher susceptibility towards *trans*-cinnamaldehyde as compared to pine EO compounds with IZ ranging from 28-44 mm in diameter. In order to investigate differences in IZ that might result from the use of different media, disc diffusion assay using *trans*-cinnamaldehyde was also performed on CHROMagar *Staph aureus* (CHROMagar, Springfield, NJ, U.S.) plates and TSA plates using the same method. Comparison between TSA and CHROMagar plates indicated that the IZ produced by *trans*-cinnamaldehyde on CHROMagar plates were slightly larger than the IZ produced on TSA plates (data not shown). This is evidence of the variability the disc diffusion assay can have with only a change in media thus indicating that this is truly a crude screening method.

A novel aspect of the present study was the evaluation of combinations of pure EO compounds. Different terpenoid components of EOs can interact to either reduce or enhance antimicrobial efficacy (Delaquis *et al*., 2002). Synergism is observed when the effect of the combined substances is greater than the sum of the individual effects (Burt, 2004) while the absence of interaction is defined as indifference. Interestingly, phenolic monoterpenes and phenylpropanoid which typically show strong antimicrobial activities in combination with other components were found to increase the bioactivities of these mixtures. For instance, mixtures of menthol with geraniol yielded synergistic effects against *S. aureus* (Gallucci *et al*., 2009). Other monoterpenes have also been tested such as the oxide 1, 8-cineole in combination with aromadendrene and limonene and were found to have additive and synergistic effects against *S. aureus*, respectively (Mulyaningsih *et al*., 2010; van Vuuren and Viljoen, 2007). According to Tserennadmid *et al*.

(2011), monoterpene hydrocarbons (e.g., α-pinene) when combined with limonene or linalool exhibited synergistic effects against yeast.

Mixtures of crude EOs have also been shown to interact with each other acting as additive, synergistic, and antagonistic agents (Kim *et al*., 1995). The EOs of basil (*Ocimum basilicum*) and peppermint (*Mentha piperita*) were shown to have synergistic effect against *S. aureus, E. coli, and S.* Typhimurium when used in combination (Bassole *et al.,* 2012). In the same study, two lemon grass EOs, *Cymbopogon citratus* and *C. giganteus*, exhibited additive effects against *S. aureus, E. coli, L. monocytogenes,* and *S.* Typhimurium. Conversely, antagonistic effects have also been reported when antimicrobials are combined. Van Vuuren and Viljoen (2007) combined *Melaleuca alternifolia* EO (i.e. tea tree oil) and ciprofloxacin and reported an antagonistic effect against *S. aureus.* In the present study, the aim was to test the relationship (additive, synergistic or antagonistic) between pure EO compounds found in pine. The data indicate that EOs at different concentrations showed varied levels of inhibition against *S. aureus,* and in most cases, the combined EOs exhibited an additive effect against *S. aureus*. Here, the antimicrobial properties of *S. aureus*-specific bacteriophage were also investigated. Bacteriophage therapy is suggested as an alternative to treat bacterial infections due to its effectiveness against multidrug-resistant bacteria (e.g., MRSA), low cost, and self-replicative mode of action (Wang *et al*., 2010). Capparelli *et al*. (2007) investigated the effectiveness of a *S. aureus* specific bacteriophage against MRSA in a mouse model and reported that 100% of the mice receiving bacteriophage therapy for treatment of MRSA infection survived compared to 20% for the control group. Although bacteriophage therapy is most often associated with investigating treatments for clinical infections—human and animal (Capparelli *et al*., 2007.) One of the aims of the present study was to understand the potential application of bacteriophage for control of

pathogens in other settings such as the food industry. More specifically, bacteriophage may be used as bio control agents during industrial food processing to reduce colonization of pathogens by application to food surfaces such as meats and fresh produce and through mixing with processed foods or even raw milk. However, a study by Goode *et al.* (2003) observed that bacteriophages are very effective against actively growing bacteria, but lose effectiveness in metabolically inactive bacteria which may be problematic in the food industry. Conversely, Greer (1998) reported that bacteriophage may continue to display lytic activity at temperatures as low as 1°C. Moreover, once foods are moved to room temperature, bacteriophage are capable of further controlling bacterial proliferation (Bigwood *et al*., 2008). For instance, Abuladze *et al*. (2008) evaluated the effectiveness of a cocktail of bacteriophage in the reduction of *E. coli* O157:H7 on vegetables. The authors reported that the bacteriophage cocktail reduced bacterial contamination on broccoli samples by 99.5%, 99% and 97% during storage at 10°C for 24, 120, and 168 h, respectively. Additional evidence of bacteriophage application in the food industry include *Salmonella*-specific bacteriophage applied to raw meats, ready-to-eat (RTE) foods, and fresh produce (Guenther *et al*., 2012); *S. aureus*-specific bacteriophage use during cheese manufacturing (Bueno *et al.,* 2012); and commercially available bacteriophage cocktails (e.g., Listex™ P100 and ListShield™) for control of *L. monocytogenes* in raw and RTE foods (Soni *et al*., 2010).

In the present study, bacteriophage K was shown to effectively reduce *S. aureus* concentrations *in vitro* at various temperatures with the greatest antimicrobial effect shown at 37°C. Additive or synergistic effects of the combination of bacteriophage K and pine-derived EO compounds against *S. aureus* were also investigated. To my knowledge, only a single previous study has considered the potential synergism between bacteriophage and EO. Viazis *et al.* (2011)

investigated the potential synergism between *E. coli* O157:H7-specific bacteriophage and *trans*-cinnamaldehyde when applied to fresh produce. The authors observed at least a 1 log CFU reduction in the number of *E. coli* O157:H7 on organic baby spinach and baby romaine lettuce leaves after treatment with the combination of bacteriophage and EO suggesting a potential additive or even synergistic effect since the combination was more effective than either treatment alone. Additional studies have investigated the combination of bacteriophage with other antimicrobials such as nisin and bacterial strains exhibiting antagonistic activity towards pathogens (Martínez *et al.*, 2008; Guenther *et al.*, 2012; Table *et al*., 2012)

The primary aim of this study was to understand combined effects of *S. aureus* specific bacteriophages with various EO compounds of pine oil against the growth of *S. aureus*. The data presented in this study proved that *S. aureus* specific bacteriophages combined with pine EO compounds is a potential method to control *S. aureus in vitro*. Unfortunately, the antimicrobial activity of both bacteriophage and EO is temperature dependent. In this study, the antimicrobial activity of EO at low temperatures did not yield successful results. It was noted that application of pine EO compounds (α-pinene) and bacteriophage K together resulted in higher reduction of *S. aureus in vitro* as compared to when applied alone. Thus, using bacteriophages in combination with EO to reduce growth of bacterial pathogens such as *S. aureus*, has the potential to be a novel approach for inhibiting *S. aureus* in various settings including clinical and within the food industry.

List of Tables and Figures

References

Abuladze, T., Li, M., Menetrez, M.Y., Dean, T., Senecal, A. and Sulakvelidze, A. (2008) Bacteriophages reduce experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by *Escherichia coli* O157:H7. *Appl Environ Microbiol* **74**, 6230–6238.

Adams, J., Gibson, K.E., Martin, E.M., Almeida, G., Ricke, S.C., Frederick, N. and Carrier, D.J. (2014) Characterization and variation of essential oil from *Pinus Taeda* and antimicrobial effects against antibiotic-resistant and susceptible *Staphylococcus aureus*. *J Forest Prod* **64**, 161-165.

Adukwu, E.C., Allen, S.C., Phillips, C.A. (2012) The anti-biofilm activity of lemongrass (*Cymbopogon flexuosus*) and grapefruit (*Citrus paradisi*) essential oils against five strains of *Staphylococcus aureus*. *J Appl Microbiol* **113**, 1217-1227.

Allahghadri, T., Rasooli, I., Owlia, P., Nadooshan, M.J., Ghazanfari, T., Taghizadeh, M., Astaneh, S.D. (2010) Antimicrobial property, antioxidant capacity, and cytotoxicity of essential oil from cumin produced in Iran. *J Food Sci* **75**, 54-61.

Balasubramanian, S., Sorokulova, I.B., Vodyanoy, V.J., Simonian, A.L. (2007) Lytic phage as a specific and selective probe for detection of *Staphylococcus aureus* – A surface plasmon resonance spectroscopic study. *Biosens Bioelectron* **22**, 948- 955.

Bassole, I.H.N. and Juliani, H.R. (2012) Essential oils in combination and their antimicrobial properties. *J Molecules* **17**, 3989-4006.

Bigwood, T., Hudson, J.A., Billington, C., Carey-Smith, G.V. and Heinemann, J. A. (2008) Phage inactivation of foodborne pathogens on cooked and raw meat. *J Food Micro* **25**, 400–406.

Bueno, E., García, P., Martínez, B. and Rodríguez, A. (2012) Phage inactivation of *Staphylococcus aureus* in fresh and hard-type cheeses. *Int J Food Microbio* **158**, 23–27.

Burt, S. (2004) Essential oils: Their antibacterial properties and potential applications in foods—a review. *Int Food Microbiol* **94**, 223-253.

Capparelli, R., Parlato, M., Borrielle, G., Salvatore, P. and Iannelli, D. (2007) Experimental phage therapy against *Staphylococcus aureus* in mice. *Antimicrob Agents Chemother* **51**, 2765-2773.

Chao, S., Young, G., Oberg, C. and Nakaoka, K. (2008) Inhibition of methicillin-resistant *Staphylococcus aureus* (MRSA) by essential oils. *Flavour Fragr J* **23**, 444-449.

Ciocan, I.D. and Bara, I.I. (2007) Plant products as antimicrobial agents. *Genetica Biol Mol* **8**, 51-156.

Delaquis, P.J., Stanich, K., Girard, B., Mazza, G (2002) Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *Int J Food Microbiol* **74**, 101–109.

El-Shibiny, A., Scott, A., Timms, A., Metawea, Y., Connerton, P. and Connerton, I. (2009) Application of a Group II *Campylobacter* bacteriophage to reduce strains of *Campylobacter jejuni* and *C. coli* colonizing broiler chickens. *J Food Protect* **72**, 733-740.

Fu, Y., Zu, Y., Chen, L., Shi, X., Wang, Z., Sun, S. and Efferth, T. (2007) Antimicrobial activity of clove and rosemary essential oils alone and in combination. *Phytother Res*. **21**, 989-994.

Gallucci, M.N., Oliva, M., Casero, C., Dambolena, J., Luna, A., Zygadlo, J., Demo, M. (2009) Antimicrobial combined action of terpenes against the food-borne microorganisms *Escherichia coli, Staphylococcus aureus* and *Bacillus cereus. Flavour Fragr J* **24**, 348–354.

Garcia, P., Martinez, B., Obeso, J.M., Rodriquez, A. (2008) Bacteriophages and their application in food safety. *Lett Appl Microbiol* **47**, 479-485.

Goode, D., Allen, V.M. and Barrow, P.A. (2003) Reduction of experimental *Salmonella* and *Campylobacter* contamination of chicken skin by application of lytic bacteriophages *Appl Environ Microbiol* **69**, 5032–5036.

Greer, G.G. (1988) Effect of phage concentration, bacterial density, and temperature on phage control of beef spoilage. *J Food Sci* **53**, 1226-1227.

Gutierrez, D., Delgado, S., Vazquez-Sanchez, D., Martinez, B., Cabo, M.L., Rodriquez, A., Herrera, J.J. and Garcia, P. (2012) Incidence of *Staphylococcus aureus* and analysis of associated bacterial communities on food industry surfaces. *Appl Environ Microbiol* **78**, 8547-8554.

Guenther, S., Herzig, O., Fieseler, L., Klumpp, J. and Loessner, M.J. (2012) Biocontrol of *Salmonella Typhimurium* in RTE foods with the virulent bacteriophage FO1-E2, *Int J of Food Microbio* **154**, 66–72.

Hagens, S. and Loessner, M.J. (2010) Bacteriophage for biocontrol of foodborne pathogens: calculations and considerations. *Curr Pharm Biotechnol* **1**, 58-68.

Hoon, E.J., Na, K.J., Choi, K.C. and Jeung, E.B. (2004) Antibacterial and antifungal effects of essential oils from coniferous trees. *Biol Pharm Bull* **27**, 863-866.

Hudson, J.A., Billington, C., Wilson, T. and On, S.L.W. (2013) Effect of phage and host concentration on the inactivation of *Escherichia coli* O157:H7 on cooked and raw beef. *Food Sci Technol Int* **1**, 1-6.

Hwang, S., Yun, J., Kim, K.P., Heu, S., Lee, S. and Ryu, S. (2009) Isolation and characterization of bacteriophages specific for *Campylobacter jejuni*. *Microbiol Immunol* **53**,559-566.

Hyun, J.E., Bae, Y.M., Song, H., Yoon, J.H. and Lee, S.Y. (2014) Antibacterial effect of various essential oils against pathogens and spoilage microorganisms in fresh produce. *J Food Safety* **10**, 51-62.

Kim, J., Marshall, M.R., Wei, C.I. (1995) Antibacterial activity of some essential oil components against five foodborne pathogens. *J Agric Food Chem* **43**, 2839–2845.

Leuschner, R.G.K. and Lelsch, V. (2003) Antimicrobial effects of garlic, clove and red hot chilli on *Listeria monocytogenes* in broth model systems and soft cheese. *Int J Food Sci Nutri* **54**, 127-133.

Li, M., Muthaiyan, A., O'Bryan, C.A., Gustafson, J.E., Li, Y., Crandall, P.G., Ricke, S.C. (2011) Use of natural antimicrobials from a food safety perspective for control of *Staphylococcus aureus. Curr Pharm Biotechnol* **12**, 1240-1254.

Martínez, B., Obeso, J. M., Rodríguez, A. and García, P. (2008) Nisin-bacteriophage cross resistance in *Staphylococcus aureus*. *Int J Food Microbio* **122**, 253–258.

Monk, A.B., Rees, C.D., Barrow, P., Hagens, S. and Harper, D.R. (2010) Bacteriophage applications: where are we now? *Lett Appl Microbiol* **51**, 363-369.

Mulyaningsih, S., Sporer, F., Zimmermann, S., Reichling, J., Wink, M. (2010) Synergistic properties of the terpenoids aromadendrene and 1,8-cineole from the essential oil of *Eucalyptus globulus* against antibiotic-susceptible and antibiotic-resistant pathogens. *Phytomedicine* **17**, 1061–1066.

Nazzaro, F., Fratianni, F., Martino, L. D., Coppola, R. and Feo, V. D. (2013) Effect of Essential Oils on Pathogenic Bacteria. *Pharm J* **6**, 1451-1474.

Palmer, A.S., Stewart, J., Fyfe, L. (1998) Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. *Lett Appl Microbiol* **26**, 118-122.

Panneerseelan, L. and Muriana, P.M. (2009) An immunomagnetic PCR signal amplification assay for sensitive detection of *Staphylococcus aureus* enterotoxins in foods. *J Food Protect* **72**, 2538-2546.

Rapson, M.E. (2002) The biology of *Staphylococcus aureus* – specific bacteriophages and the assessment of their potential as therapeutic agents. *J Aerosol Med* **26**, 1-19.

Soni, K.A., Nannapaneni, R. and Hagens, S. (2010) Reduction of *Listeria monocytogenes* on the surface of fresh channel catfish fillets by bacteriophage Listex P100. *Foodborne Pathog Dis* **7**, 427–434.

Table, R., Martínez, B., Rebollo J.E. (2012) Bacteriophage performance against *Staphylococcus aureus* in milk is improved by high hydrostatic pressure treatments. *Int J Food Microb* **156**, 209–213.

Tserennadmid, R., Takó, M., Galgóczy, L., Papp, T., Pesti, M., Vágvölgyi, C., Almássy, K., Krisch, J. (2011) Anti yeast activities of some essential oils in growth medium, fruit juices and milk. *Int J Food Microbiol* **144**, 480–486.

Upadhyay, R.K., Dwivedi, P. and Ahmad, S. (2010) Screening of antibacterial activity of six plant essential oils against pathogenic bacterial strains. *Asian J Med Sci* **2**, 152-158.

Vanderhaeghen, W., Cerpentier, T., Adriaensen, C., Vicca, J., Hermens, K. and Butaye, P. (2010). Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. *Vet Microbiol* **144**, 166-171.

Van Vuuren, S.F., Viljoen, A.M. (2007) Antimicrobial activity of limonene enantiomers and 1,8-cineole alone and in combination. *Flavour Fragr J* **22**, 540–544.

Viazis, S., Akhtar, M., Feirtag, J. and Diez-Gonzalez, F. (2011) Reduction of *Escherichia coli* O157: H7 viability on leafy green vegetables by treatment with a bacteriophage mixture and transcinnamaldehyde. *J Food Microbiol* **28**, 149-157.

Wang, Q. and Sabour, P.M. (2010) Encapsulation and controlled release of bacteriophages for food animal production. In *Bacteriophages in the Control of Food- and Waterborne Pathogens* eds. Sabour, P.M. and Griffiths, M.W. *Washington: ASM Press*, 237–255.

Yoon, H., Yun, J., Lim, J.A., Roh, E., Jung, K.S., Chang, Y., Ryu, S., and Heu, S. (2013) Characterization and genomic analysis of two *Staphylococcus aureus* bacteriophages isolated from poultry/ livestock farms. *J Gen Virol* **94**, 2569-2576.

Wunderink, R.G., Rello, J.,Cammarata, S. K., Croos-Dabrera, R.V. and Kollef, M.H. (2003) Linezolid vs Vancomycin: Analysis of two double blind studies of patients with methicillin resistant *Staphylococcus aureus* nosocomial pneumonia. *Chest* **124**, 1789-1797.

Table 1: Zone of inhibition (mm) by essential oil compounds against *S. aureus* strains at 37°C.

	Zone of inhibition (mm)			
S. aureus strains	α-pinene	α-pinene + β-pinene	α-pinene + 3 carene	α- pinene + Limonene
ATCC 6538	23(3.22)^{a,x}	10(1.4)^{b,x}	14(1.96)^{c,x}	11(1.54)^{b,x}
ASU 36	18(2.52)^{a,y}	12(1.68)^{b,y}	14(1.96)^{c,x}	14(1.96)^{c,y}
COL	19(2.66)^{a,y}	9(1.26)^{b,x}	12(1.68)^{c,y}	14(1.96)^{d,y}
N315	18(2.52)^{a,y}	11(1.54)^{b,y}	12)(1.68)^{b,y}	15(2.1)^{c,y}
Mu50	10(1.4)^{a,z}	8(1.12)^{b,z}	9(1.26)^{b,z}	11(1.54)^{a,x}

$^{x-z}$Means in the same column are significantly different ($p< 0.05$).

$^{a-d}$ Means in the same rows are significantly different ($p< 0.05$).

Values in parenthesis are standard deviation observed.

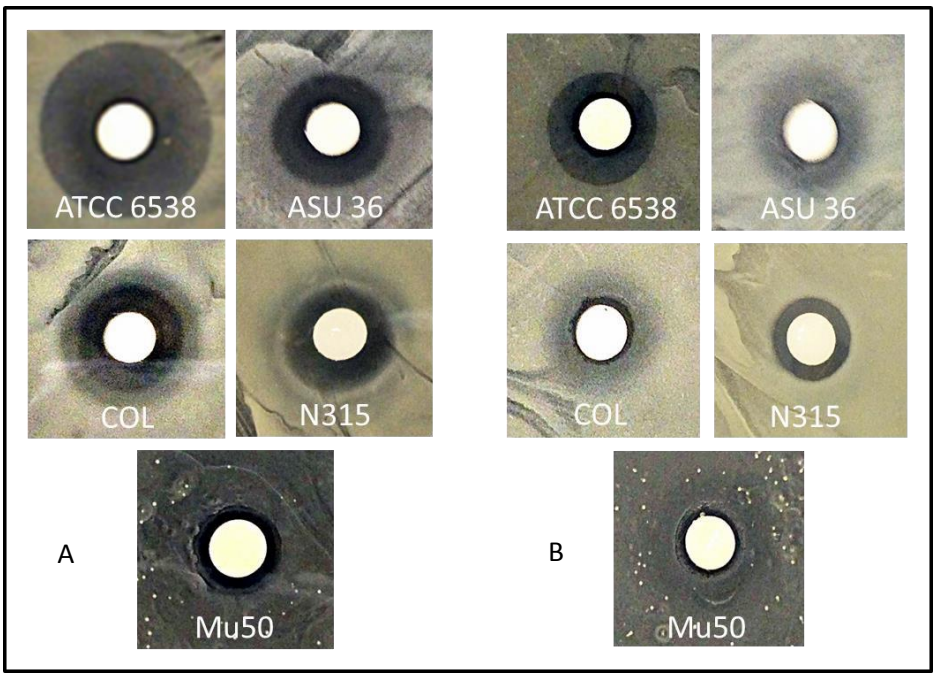


Figure 1. Disc diffusion assay. Susceptibility of *S. aureus* strains to (A) alpha-pinene and (B) alpha-pinene + 3-carene.

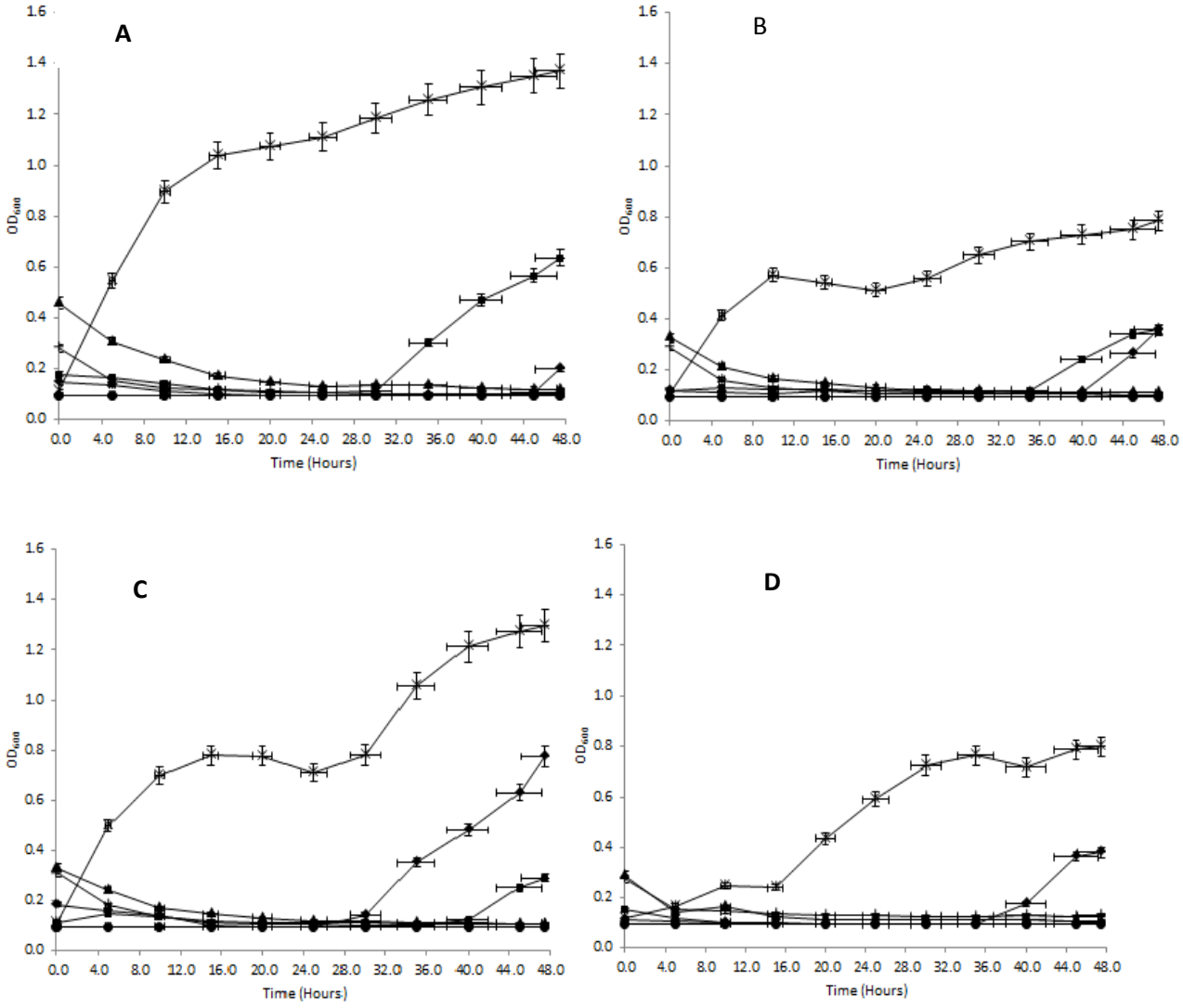


Figure 2. Growth inhibition assay with two essential oil compounds at 1.5% and 3.28% concentrations against four *S. aureus* strains (A) ASU 36, (B) ATCC 6538, (C) COL and (D) N315. Line styles used in the graph are indexed as follows:

1.5% α-pinene
1.5% α-pinene+1.5% carene
3.28 % α-pinene
3.28% α-pinene+ 3.28 % carene
S. aureus + *TSB*
TSB

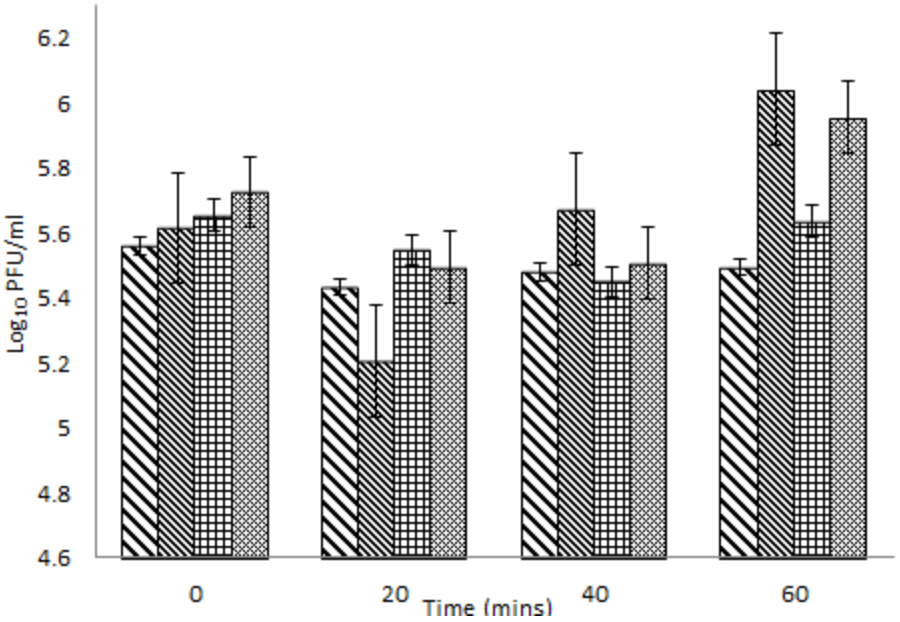


Figure 3. Phage adsorption assay with bacteriophage K and *S. aureus* strains at 37°C. Four strains are indicated by ASU36 ATCC 6538 COL N315

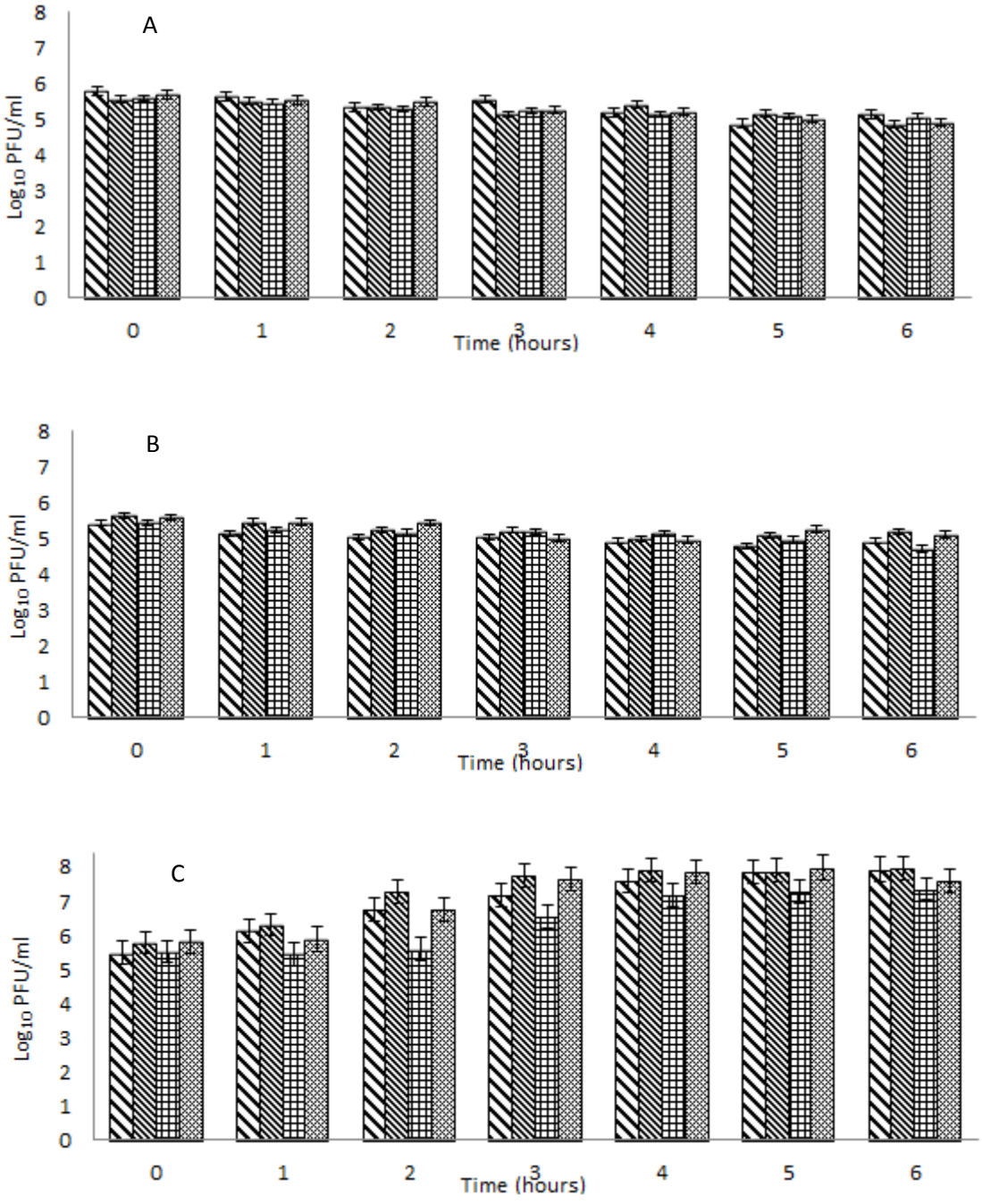


Figure 4. Phage adsorption assay of bacteriophage-bacteria suspension at various time points and three different temperatures; (A) 6°C (B) 13°C and (C) 37°C. Four strains are indicated by ASU36 ATCC 6538 COL N315

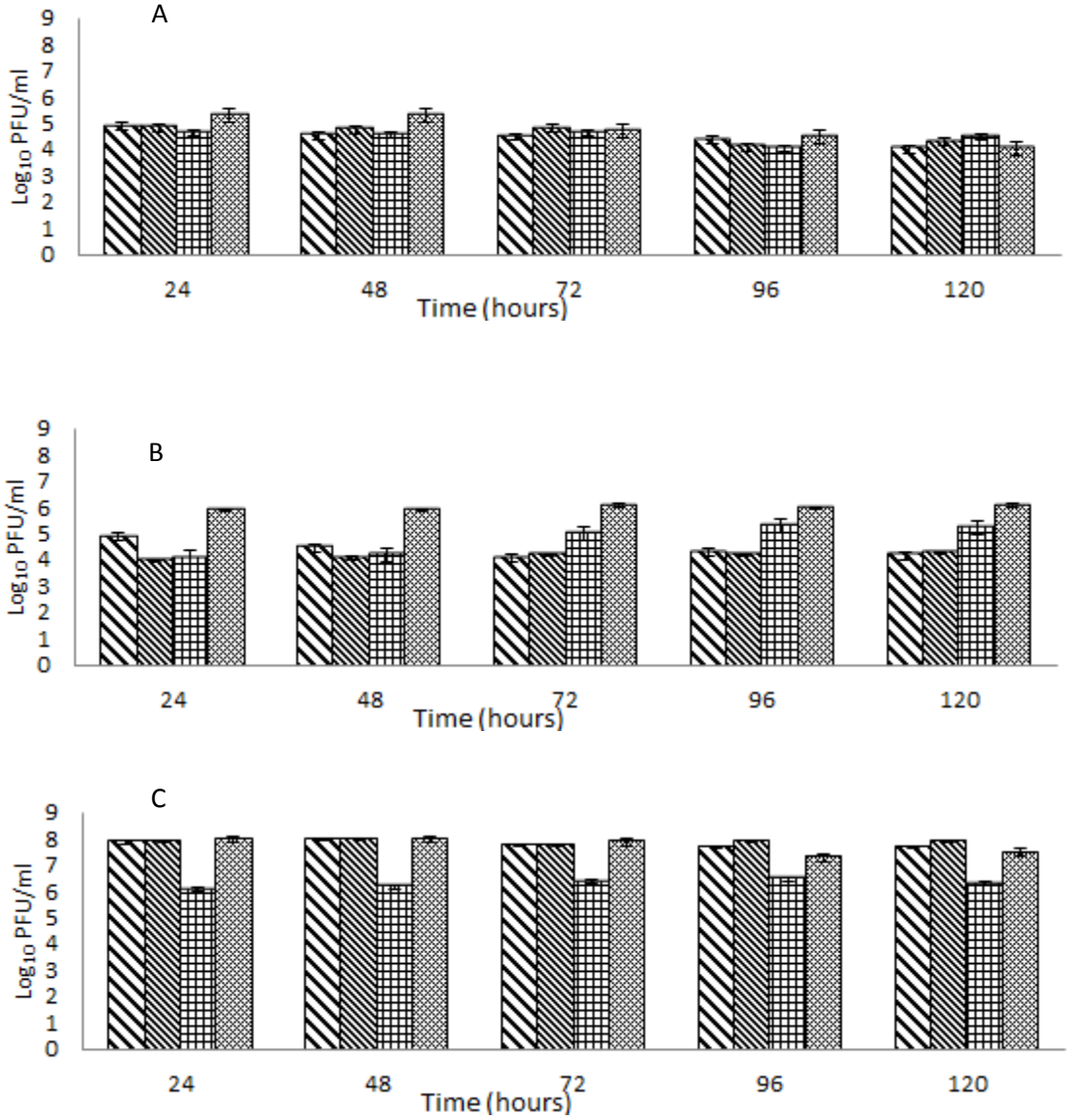


Figure 5. Phage adsorption assay with bacteriophage K and *S. aureus* strains at (A) 6°C, (B) 13°C and (C) 37°C. Four strains show up as ASU36 ATCC 6538 COL N315

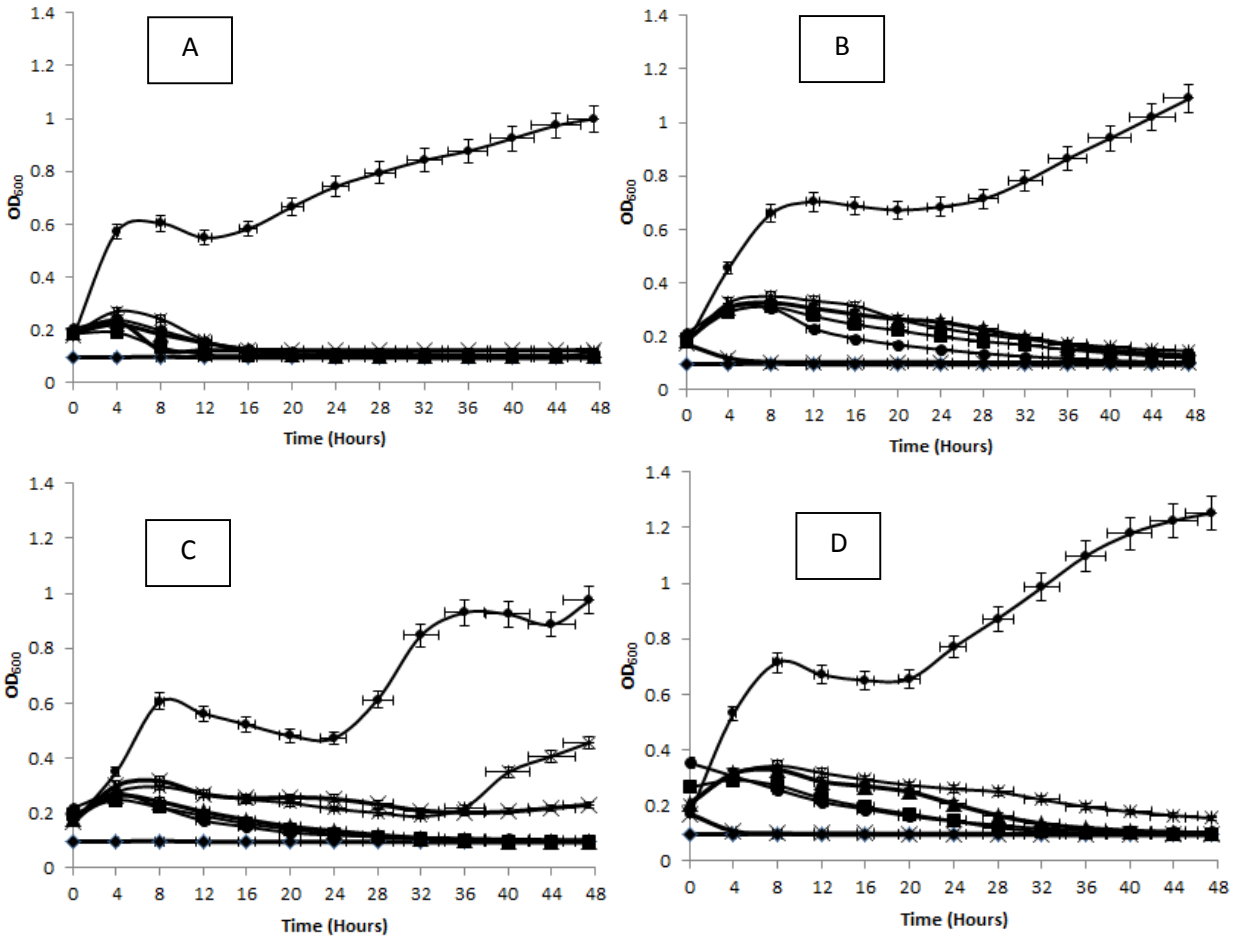
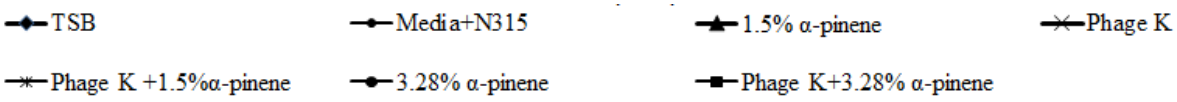


Figure. 6 Growth inhibition assay with bacteriophage K and two concentrations (1.5% and 3.28%) of essential oil compound α-pinene alone and in combination against four *S. aureus* strains (A) N315 (B) ASU36, (C) COL and (D) ATCC 6538. Line styles used in the graph are indexed as follows:



Chapter 3: Application of bacteriophage and essential oils for the control of *Staphylococcus aureus* on raw chicken products

Abstract

Multidrug-resistant (MDR) *Staphylococcus aureus* are increasingly prevalent and combinations of different antimicrobials represent an approach for combating MDR bacteria. Antimicrobials with potential food industry application include essential oils (EO) and bacteriophage. The aim of the present study was to investigate the combination of EOs and bacteriophages as alternative antimicrobials to control *S. aureus* on the surface of raw chicken products. To evaluate effectiveness on meat products, 2 cm × 2 cm pieces of chicken breast were inoculated with a 3×10^7 CFU of a four strain cocktail of *S. aureus* followed by addition of bacteriophage alone, EO alone, or bacteriophage and EO in combination. Chicken pieces were incubated at 6, 13, and 25°C, and samples were collected over time to measure reduction in *S. aureus* by bacterial spread plate method. Results indicate that at 25°C bacteriophage K alone inhibits *S. aureus* growth better as compared to other antimicrobial combination though these differences were not statistically significant. At 6 and 13°C, there was no significant effect of EO and bacteriophage alone or in combination against *S. aureus* when applied on the raw chicken pieces. Previous studies indicate that EOs and bacteriophage can be used as potential antimicrobials against *S. aureus in vitro*. However, for these antimicrobials to work *in situ* such as on raw meat products, a better delivery method should be employed for a uniform application on meat.

Introduction

Staphylococcus aureus is commensal on human skin and is an important human pathogen causing illnesses ranging from minor skin infections to life-threatening diseases such as pneumonia and bacteremia (Yoon *et al*., 2013). Food production animals such as chickens and pigs can also become infected with *S. aureus* resulting in bovine mastitis and chicken arthritis which can also threaten food safety and potentially cause food poisoning (Vanderhaeghen *et al*., 2010). Multiple antibiotic resistance is becoming a serious issue in the management of *S. aureus* infections. Since few new antibiotics have been placed on the market since 1998 and there are fewer prospects for new antibiotic agents, future treatments may depend on researching alternative therapies (Rapson, 2002). Research has revealed some promising novel antimicrobial candidates including plant essential oils (EO) and host-specific bacteriophage (Chao *et al*., 2008).

The application of bacteriophages to control the growth of foodborne pathogens such as *S. aureus, Listeria monocytogenes, Salmonella enterica,* and *Escherichia coli* is steadily growing (Hudson *et al*., 2010; Hagens and Loessner, 2010). For the purpose of the present study, the focus will be on bacteriophage specific to *S. aureus. Staphylococcus aureus* bacteriophages are grouped into three classes based on genomic size: class I (<20kb), class II (~40kb) and class III (>125 kb) (Kwan *et al*., 2005). Bacteriophages belonging to class III are obligate lytic bacteriophages with relatively wide host ranges. For example, bacteriophage K—the bacteriophage used in the present study—belongs to class III (127 kb) (Kwan *et al*., 2005). Obligate lytic bacteriophages have received renewed interest as potential therapeutic agents to replace or supplement antibiotics in the treatment of methicillin resistant *S. aureus* (MRSA) or other antibiotic-resistant *S. aureus* strains (Yoon *et al*., 2013). Bacteriophages have also been

developed as a food surface decontaminant for other foodborne pathogens including *Listeria*, *E. coli*, and *Salmonella* (e.g. Listex P100, EcoShield, and SalmoFresh) (Soni *et al.,* 2010). The acceptance of bacteriophages as natural food additives is based on the presence of naturally high levels of bacteriophages in the digestive tract of humans and throughout the environment. (Monk *et al*., 2010).

In vitro data indicate that many plant-derived EOs have antimicrobial activity. For instance, forestry by-products such as turpentine have been used topically as a medicinal for centuries for treating human parasites and even a modern day vapor rub (i.e. Vicks®) for treating congestion and coughs contains turpentine (Hoon *et al*., 2004). According to Dryden *et al*. (2004), topical tea tree oil is an effective therapy for reducing MRSA nasal colonization. Antimicrobial components of *Pelargonium* EOs (i.e. citral and linalool) have been found to be effective against *S. aureus* and could be used as a novel food or cosmetic antimicrobial agents (Lis Balchin *et al*., 1998). Adukwu *et al*. (2012) also reported that lemongrass EO at low concentrations (0.03 and 0.06% (v/v)) was effective at inhibiting the growth of *S. aureus* strains *in vitro*.

With respect to application of bacteriophage in food products, the use of bacteriophages for reduction of *Campylobacter* on meat products has been extensively investigated. For example, Bigwood *et al*. (2008) reported that *Campylobacter*-specific bacteriophage reduced *C. jejuni* inoculated on cooked and raw meat at 5°C and 24°C, by 2.4 $\log_{10}$ CFU/cm^2 and 1.5 $\log_{10}$ CFU/cm^2, respectively. Additional applications of bacteriophage in food products have also been reported (Zaczek *et al.,* 2015). Viazis *et al.* (2011) reported that *E. coli* O157:H7 specific bacteriophages significantly reduced the number of *E. coli* O157:H7 cells on baby romaine lettuce leaves. In addition, *Salmonella* bacteriophages (SSP5 and SSP6) have been shown to control *Salmonella* on alfalfa seeds (Kocharunchitt *et al*., 2009) while *Listeria* bacteriophages

were reported to suppress the growth of *L. monocytogenes* on honeydew melon (Leverentz *et al*., 2004).

Numerous studies have reported on the prevalence of various human pathogens including *S. aureus* on meat products. Waters *et al*., (2011) investigated the prevalence of *S. aureus* in U.S. meat and poultry samples (n = 136). *Staphylococcus aureus* contamination was most common among turkey samples (77%), followed by pork (42%), chicken (41%) and beef (37%). Similarly, Weese *et al*. (2010) reported the isolation of MRSA from 7.7% of pork samples tested (31/402). In another study by Hanson *et al*. (2011), *S. aureus* was isolated from 27 of 165 meat samples (i.e. turkey, pork, chicken, beef) from retail stores in Iowa with the highest prevalence in turkey (19.4%), pork (18.2%), and chicken (17.8%). Incidence of *S. aureus* on retail meat including MRSA have been reported in various other studies as well (Pu *et al*., 2008; Bhargava *et al*., 2011 and O'Brien *et al*., 2012). However, it is still unknown whether MRSA contamination of raw meats can play any role in the overall ecology and transmission of this organism (O'Brien *et al*., 2012).

To control the potential regrowth of pathogens in food products, more specifically meat and poultry products, a combined bacteriophage and EO treatment may offer a more practical and cost-effective approach. Since bacteriophages are present in almost every environment and EOs can be easily extracted from plants, these antimicrobials tend to be less expensive than many antimicrobials on the market (Viazis *et al.*, 2011). Both bacteriophages and EOs have been successfully applied to suppress the activity of food borne pathogens. For instance, Viazis *et al.* (2011) reported that the combination of *E. coli* O157:H7 specific bacteriophage BEC8 and the EO *trans*-cinnamaldehyde lead to complete inactivation (5 log CFU/leaf reduction) of *E. coli* O157:H7 within 10 minutes and 1 hour at all temperatures (4, 8, 23, and 37°C) for spinach and

lettuce, respectively. The bacteriophage treatment is a new and effective hurdle, which if applied with EOs may maximize the protection from food borne pathogens on both raw and processed food products.

Since EOs have the potential to be used as natural agents for food preservation due to their content of antimicrobial compounds (Helander *et al*., 1998) and bacteriophages offer a natural method to control bacterial contamination of foods (Callaway *et al*., 2008), using a combination of these treatments may prove more beneficial as it may produce additive or synergistic effects. The multiplicity of infection (MOI) is considered to be a key parameter in bacteriophage application to food surfaces (Viazis *et al*., 2011). Hudson *et al.,* (2013) reported that increasing bacteriophage concentrations result in greater reductions in the concentration of pathogenic bacteria. Therefore, with this proposed hurdle technology (i.e., bacteriophage combined with EO), lower concentrations of each antimicrobial could be employed in a specific sequence to get similar or better results than either alone and maximize protection from foodborne pathogens such as *S. aureus* on meat products can be achieved. Another advantage of using a combined treatment is overcoming the limitations of EO and bacteriophages when used separately. For instance, occurrence of bacteriophage resistance within bacterial populations can limit bacteriophage application in the food industry (Ye *et al*., 2010). Additionally, EOs can exhibit poor inhibitory effects against *S. aureus* at lower temperatures (6 and 13°C). To overcome the limited efficacy of bacteriophages and EO as antimicrobials used separately, this study investigates the effectiveness of their combined use against *S. aureus* on raw chicken products.

Material and Methods

Preparation of meat

Chicken breasts were purchased from a retail grocery store in Fayetteville, Arkansas. Packaging was removed using a razor blade sterilized with 10% bleach, and chicken breasts were transferred into individual zip lock bags and stored at -18°C for a maximum of 4 weeks and, before use, thawed and all excess liquid was removed. Raw chicken pieces were sliced aseptically into thin 2cm x 2cm squares using a sterile knife. The knife was sterilized with 70% ethyl alcohol followed by 10% bleach. The chicken pieces were then placed individually into pre-labeled Petri dishes and equilibrated to 6°C, 13°C, and 25°C for two hours prior to inoculation with *S. aureus* cocktail.

Preparation of bacterial stocks

Inocula of individual *S. aureus* strains (N315, ASU 36, ATCC 6538 and COL) were grown in tryptic soy broth (TSB, Acumedia, Lansing, MI, U.S.) overnight at 37°C with shaking at 150 rpm. Enumeration of each *S. aureus* strain was performed to determine the final concentration. A *S. aureus* cocktail was prepared by mixing 1 ml from each overnight grown culture strain. Serial dilution and spread plating on tryptic soy agar (TSA, Acumedia, Lansing, MI, USA) plates was performed to determine the overall stock concentration of the cocktail (1.5×10^9 CFU/ml).

Preparation of bacteriophage K

Propagation of high titer stocks of bacteriophage K was carried out using double overlay method with *S. aureus* ATCC 25923 as host as described in Chapter 2. Briefly, for propagation of bacteriophage K, 100 µl of a log phase *S. aureus* culture was combined with 100 µl bacteriophage K (10^{11} PFU/ml) in 5 ml of 0.7% TSA (soft agar). The soft agar was poured on

TSA plates, allowed to solidify, and the plates were incubated at 37°C for 24 h to allow for complete lysis of the bacterial lawn. After 24 h, the soft agar was scraped off and placed in a 50 ml conical tube containing 15 ml of SM buffer (5.8 g NaCl, 1.2 g $MgSO_4$, 50 ml 1M Tris-HCl (pH 7.5), 0.1 g Gelatin) and vortexed for 2 min. The bacteriophage-SM buffer suspension was then kept in an incubator at 37°C with gentle shaking for 4 hours. The suspension was centrifuged at 580 × *g* for 25 min at 4°C to sediment the cellular debris and agar. The clear supernatant containing bacteriophage K was collected and passed through a 0.22 µm pore size cellulose acetate filter (VWR, Radnor, PA, U.S.) to obtain a homogenous bacteriophage stock. The concentration of bacteriophage K stock was determined using the double agar overlay method. Following titration, the bacteriophage K stock was stored in small aliquots at −80°C. The bacteriophage K stock concentration was 1×10^{11} PFU/ml which was diluted to 1×10^{8} PFU/ml using sterile 1× phosphate buffered saline (PBS). The bacteriophage K was added at MOI 0.1 with a final concentration of 2×10^{6} PFU/20µl. Based on our previous bacteriophage adsorption studies (Chapter 2), bacteriophage K resulted in greater lytic activity against *S. aureus* strains at a MOI of 0.1 as compared to a MOI of 0.01 and 1.

Preparation of essential oil

Two EO compounds were selected for this study—alpha-pinene and 3-carene (ACROS Organics, Morris Plains, NJ, U.S.). Essential oil solutions were prepared in two different concentrations (1.5% and 3.28%) in 0.5% Tween 80 (Amresco, Solon, Ohio, U.S.). Based on our previous growth inhibition studies, 1.5% and 3.28% of alpha-pinene alone and alpha-pinene + 3-carene showed the highest inhibitory effect against all *S. aureus* strains compared to other treatments (Chapter 2).

Treatment of inoculated meat product with bacteriophage and/or EOs

For experimental samples, 20µl aliquots of exponential phase *S. aureus* host cocktail containing approximately 3×10^7 cells total were carefully pipetted onto the upper surface of the 2 cm × 2 cm raw chicken piece and allowed to dry for 10 min at room temperature for maximum adhesion to the food matrix. Each sample of chicken was then treated with a 20µl solution of either bacteriophage with a MOI of 0.1 or EO diluted (1.5 or 3.28%) suspension. To examine the combined effect of bacteriophage K and EO on chicken pieces inoculated with *S. aureus*, 10µl of each antimicrobial solution was pipetted onto the inoculated surface of the chicken to give the same final concentrations at the treatments alone. For control samples, a positive control with the same concentration of *S. aureus* cocktail was used without addition of bacteriophage and/or EO suspension. Chicken pieces inoculated with 20µl of PBS and/or 0.5% Tween 80 were used as negative controls. After adding *S. aureus* cocktail and the appropriate antimicrobial treatment (phage and/or EO), the individual Petri dishes were incubated at 6, 13, and 25°C for varying time periods depending upon the temperature.

Enumeration of bacteria on meat products

For enumeration of *S. aureus*, chicken pieces were individually transferred to sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI, U.S.). Five ml of PBS were added to the sample using a sterile serological pipet. Samples were then mixed vigorously by hand for two min. The liquid portion was transferred to a sterile 14 ml culture tube and vortexed for 20 s. After vortexing, 1 ml of the eluent was transferred to a micro centrifuge tube and centrifuged at 14,000 × *g* for 10 min. The supernatant was removed and the pellet was re-suspended in 1ml of PBS. The re-suspended pellet was then prepared in 10-fold dilutions in PBS and the appropriate dilution was applied to

CHROMagar *Staph aureus* plates (CHROMagar, Springfield, NJ, U.S.) using the spread plate method for enumeration of *S. aureus* at different time points (0, 1, 6, 24, 48, 72, 96 and 120 h). The plates were incubated at 37°C for 24 h. Each experiment was carried out in duplicate on two separate occasions.

Statistical Analysis

All experiments were repeated two times with duplicate plates. Population of *S. aureus* strains treated with bacteriophage and/or EOs solution was subjected to one-way analysis of variance (ANOVA) using JMP (Version 11.1.1, SAS Institute Inc., Cary, NC, U.S.) to determine whether treatment with bacteriophage and/or EO significantly reduced the number of viable *S. aureus* cells on raw chicken product. Results were considered significantly different at $p \leq 0.05$.

Results

The antimicrobial treatments alone and in combination were tested against a cocktail of four *S. aureus* strains on raw chicken breast pieces at three temperatures (6, 13, and 25°C) to simulate refrigeration, temperature abuse, and room temperature, respectively. At all three temperatures (6, 13, and 25°C), there was no significant reduction in the number of bacterial colonies by any antimicrobial treatment when compared to the positive control at any given time point (Figure 1). In addition, there was no significant difference between the treatments at any temperature. Results indicate that at 13 and 25°C 1.5% alpha-pinene and bacteriophage K alone, respectively, inhibit *S. aureus* growth slightly better as compared to the other antimicrobial combinations though this was not significant. At 6 and 13°C, there was no significant effect of EO and bacteriophage alone or in combination against *S. aureus* when applied on the raw chicken pieces (Figure 1).

Discussion

Foodborne pathogens can exist in raw and processed food, both in meat and vegetable products (Zaczek *et al*., 2015). The U.S. Food and Drug Administration stated that bacterial pathogens such as *S. aureus*, *Shigella* sp., and *E. coli* can be found in vegetables primarily leafy greens, tomatoes, and other fresh produce that make up salads and raw or ready-to-eat meat products (Zaczek *et al*., 2015). The concept of using natural antimicrobials such as bacteriophage and EO against spoilage bacteria and pathogens in foods has received increasing interest over time, and many studies support the value of this approach. For instance, *Salmonella* reduction after application of bacteriophages has been demonstrated for honeydew melon slices (Leverentz *et al*., 2001) and sprouts (Ye *et al*., 2010). Similarly, EO such as eugenol, coriander, clove, oregano, and thyme oils have been found to effectively inhibit *L. monocytogenes* and *Aeromonas hydrophila* in refrigerated, cooked poultry products (Skandamis and Nychas, 2001; Hao *et al.*, 1998).

Based on this, the purpose of this study was to determine the effect of pure EO compounds and a *S. aureus* specific bacteriophage, individually and combined, against *S. aureus* present on the surface of raw chicken pieces. One previous study has successfully demonstrated the application of bacteriophage and EO to control *E. coli* O157:H7 present on baby spinach leaves and lettuces (Viazis *et al.*, 2011). Other researchers have also reported that bacteriophages were able to effectively inactivate bacterial pathogens present on fresh produce and fruit such as lettuce (Sharma *et al*., 2009; Guenther *et al*., 2009), honeydew melon (Leverentz *et al*., 2004), tomatoes (Ye *et al*., 2009; Abuladze *et al*., 2008), spinach, and broccoli (Abuladze *et al*., 2008). However, these studies used high MOIs ranging from 1,000 to 100,000 and above. In this study, we applied bacteriophage at a relatively low MOI (0.1) to the chicken pieces inoculated with *S. aureus*.

Studies have shown that the degree of inhibition/killing of bacterial host directly correlates with the total bacteriophage concentration (Hudson *et al.*, 2013). Although the differences between the various treatments of bacteriophage and EOs were not statistically significant, there was an observed reduction of *S. aureus* counts with the bacteriophage treatment at 25°C which suggests that it may be possible to further improve the effectiveness of bacteriophage treatment by increasing the bacteriophage concentration and/or by using larger volumes of antimicrobial solution per unit of surface area. At 6 and 13 °C incubation, there was no significant decrease in the growth of *S. aureus* by any of the treatments. This effect can be explained by the immobilization of the bacteriophage particles on the chicken surfaces and matrix. Consequently, there was likely not an optimal distribution of the bacteriophage on the chicken surface in order to provide the highest probability for contact between bacteriophage and *S. aureus*. Our findings suggest that bacteriophage particles became immobilized on the chicken surfaces within the first few hours after addition, which in turn enabled survival of *S. aureus* in protected niches. Clearly, this is dependent on the surface properties and matrix of the raw chicken. Furthermore, bacteriophages can strongly bind to food molecules with hydrostatic interactions, resulting in loss of their lytic activity (Ye *et al*., 2009). A similar phenomenon has been observed with *Listeria* bacteriophage applied to contaminated surface-ripened soft cheese (Guenter and Loessner, 2011). The authors observed that the efficacy of bacteriophage treatment appeared to be highest at target cell concentrations at or below 100 cfu/cm^2. Also, repeated bactriophage application did not enhance bacterial inhibition when compared to a single dose. There has been exploration on different bacteriophage delivery systems. Puapermpoonsiri *et al*. (2009) reported that bacteriophages specific for *S. aureus* or *Pseudomonas aeruginosa* could be encapsulated into biodegradable polyester microspheres via a modified water/oil/water double emulsion

solvent extraction protocol resulting in only a partial loss of lytic activity. This type of delivery method can increase the solubility of bacteriophage in a food matrix such as raw chicken thus increasing the surface area for bacterial contact and improving the antimicrobial effectiveness (Shah *et al*., 2012). Despite the poor shelf-life of the formulation, the work is proof-of-concept for the formulation and controlled delivery of bacteriophages.

With respect to EOs, the limited water solubility of EOs reduces their effectiveness (Sofos *et al*., 1998), and the homogeneity of their distribution in food matrices (such as raw chicken) is required to ensure the inhibition of microbial growth throughout food products. The effectiveness of lipophilic EOs in foods may be further reduced because of the interaction with and/or solubilization by hydrophobic components of foods such as proteins and lipids. Challenges in dealing with such hydrophobic compounds can be overcome by dissolving them in a solvent with decreased polarity or dispersing them in emulsion droplets or biopolymer particles using nanoscale encapsulation systems (Shah *et al*., 2012). In a study by Gaysinsky *et al*. (2007), the antimicrobial activities of EO components such as eugenol and carvacrol against *L. monocytogenes* and *E. coli* O157:H7 were effectively improved by dissolving the EOs in surfactant micelles smaller than 100 nm.

Conclusions

Staphylococcus aureus are increasingly being reported with high prevalence on raw meat and ready-to-eat meat products. In the past few years, numerous research groups have evaluated natural antimicrobials as biocontrol agents for foodborne pathogens. Using *S. aureus* specific bacteriophages in combination with pure EO compounds to inhibit the growth of *S. aureus* on raw chicken products has the potential to be natural antimicrobials for reducing the incidence of

foodborne diseases. These natural antimicrobials have a long history and many areas linked with bacteriophage and EO application still need to be investigated. The data presented in this study emphasize the need for a more robust delivery technique of these antimicrobials on the meat surfaces in order to reach maximum inhibition of the target pathogen.

List of Figures

References

Abuladze, T., Li, M., Menetrez, M.Y., Dean, T., Senecal, A. and Sulakvelidze, A. (2008) Bacteriophages reduce experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by *Escherichia coli* O157:H7. *Appl Environ Microbiol* **74**, 6230–6238.

Adukwu, E.C., Allen, S.C., Phillips, C.A. (2012) The anti-biofilm activity of lemongrass (*Cymbopogon flexuosus*) and grapefruit (*Citrus paradisi*) essential oils against five strains of *Staphylococcus aureus. J Appl Microbiol* **113**, 1217-1227.

Bhargava, K., Wang, X., Donabedian, S., Zervos, M., Rocha, L.D. and Zhang, Y. (2011) Methicillin-Resistant *Staphylococcus aureus* in retail meat, Detroit, Michigan, USA. *Emerg Infect Dis* **17**, 1135–1137.

Bigwood, T., Hudson, J.A., Billington, C., Carey-Smith, G.V. and Heinemann, J. A. (2008) Phage inactivation of foodborne pathogens on cooked and raw meat. *J Food Micro* **25**, 400–406.

Callaway, T.R., Edrington, T.S., Brabban, A.D., Anderson, R.C., Rossman, M.L., Engler, M.J., Carr, M.A., Genovese, K.J., Keen, J.E., Looper, M.L. (2008). Bacteriophage isolated from feedlot cattle can reduce *Escherichia coli* O157:H7 populations in ruminant gastrointestinal tracts. *Food Pathog Dis* **5.**

Chao, S., Young, G., Oberg, C. and Nakaoka, K. (2008) Inhibition of methicillin-resistant *Staphylococcus aureus* (MRSA) by essential oils. *Flavour Fragr J* **23**, 444-449.

Gaysinsky, S., Taylor, T.M., Davidson, P.M., Bruce, B.D. and Weiss, J. (2007) Antimicrobial efficacy of eugenol microemulsions in milk against *Listeria monocytogenes* and *Escherichia coli* O157: H7. *J Food Protect* **70**, 2631–2637.

Goode, D., Allen, V.M. and Barrow, P.A. (2003) Reduction of experimental *Salmonella* and *Campylobacter* contamination of chicken skin by application of lytic bacteriophages *Appl Environ Microbiol* **69**, 5032–5036.

Guenther, S., Herzig, O., Fieseler, L., Klumpp, J. and Loessner, M.J. (2012) Biocontrol of *Salmonella Typhimurium* in RTE foods with the virulent bacteriophage FO1-E2. *Int J of Food Microbio* **154**, 66–72.

Hagens, S. and Loessner, M.J. (2010) Bacteriophage for biocontrol of foodborne pathogens: calculations and considerations. *Curr Pharm Biotechnol* **1**, 58-68.

Hanson, B.M., Dressler, A.E., Harper, A.L., Scheibel, R.P., Wardyn, S.E., Roberts, L.K., Kroeger, J.S. and Smith, T.C. (2011) Prevalence of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) on retail meat in Iowa. *J Infect Public Health* **4**, 169-174.

Hao, Y.Y., Brackett, R.E. and Doyle, M.P. (1998) Efficacy of plant extract in inhibiting *Aeromonas hydrophila* and *Listeria monocytogenes* in refrigerated cooked poultry. *Food Microbiol* **15**, 367-378.

Helander, I.M., Alakomi, H.L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E.J., Gorris, L.G.M. and von Wright, A. (1998). Characterization of the action of selected essential oil components on Gram-negative bacteria. *J Agric Food Chem.* **46**, 3590e3595.

Hoon, E.J., Na, K.J., Choi, K.C. and Jeung, E.B. (2004) Antibacterial and antifungal effects of essential oils from coniferous trees. *Biol Pharm Bull* **27**, 863-866.

Hudson, J.A., Billington, C., Wilson, T. and On, S.L.W.(2013) Effect of phage and host concentration on the inactivation of *Escherichia coli* O157:H7 on cooked and raw beef. *Food Sci Technol Int* **1**, 1-6.

Kwan, T., Liu, J., DuBow, M., Gros. P. and Pelletier, J. (2005) The complete genome and proteomes of 27 *Staphylococcus aureus* bacteriophages. *PNAS* **102**, 5174-5179.

Leverentz, B., Conway, W.S., Camp, M.J., Janisiewicz, W.J., Abuladze, T., Yang, M., Saftner, R. and Sulakvelidze, A. (2003) Biocontrol of *Listeria monocytogenes* on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. *Appl Enviro Microbiol* **69**, 4519-4526

Lis-Balchin, M., Buchbauer, G., Ribisch, K. and Wenger, M.T. (1998) Comparative antibacterial effects of novel Pelargonium essential oils and solvent extracts. *Lett Appl Microbiol* **27**, 135–141.

Monk, A.B., Rees, C.D., Barrow, P., Hagens, S. and Harper, D.R. (2010) Bacteriophage applications: where are we now? *Lett Appl Microbiol* **51**, 363-369.

O'Brien, A.M., Hanson, B.M., Farina, S.A., Wu, J.Y., Simmering, J.E., Wardyn, S.E., Forshey, B.M., Kulick, M.E., Wallinga, D.B. and Smith, T.A. (2012) MRSA in conventional and alternative retail pork products. *PLoS ONE* **7**, e30092

Puapermpoonsiri, U., Spencer, J. and van derWalle, C. (2009) A freeze-dried formulation of bacteriophage encapsulated in biodegradable microspheres. *Eur J Pharm Biopharm* **72**, 26e33.

Pu, S., Han, F. and Ge, B. (2008) Isolation and characterization of methicillin-resistant *Staphylococcus aureus* strains from Louisiana retail meats. *Appl Environ Micriol* **75**, 265-267.

Rapson, M.E. (2002) The biology of *Staphylococcus aureus* – specific bacteriophages and the assessment of their potential as therapeutic agents. *J Aerosol Med* **26**, 1-19.

Sharma, M., Patel, J.R., Conway, W., Ferguson, S. and Sulakvelidze, A. (2009) Effectiveness of bacteriophages in reducing *Escherichia coli* O157: H7 on fresh-cut cantaloupes and lettuce. *J Food Protect* **72**, 1481-1485.

Shah, B., Ikeda, S., Davidson, P.M. and Zhong, Q. (2012) Nanodispersing thymol in whey protein isolate-maltodextrin conjugate capsules produced using the emulsion-evaporation technique. *J Food Eng* **113**, 79-86.

Skandamis, P.N. and Nychas, J.E. (2001) Effect of oregano essential oil on microbiological and physic-chemical attributes of minced meat stored in air and modified atmospheres. *J Appl Microbiol* **91**, 1011-1022.

Sofos, J.N., Beuchat, L., Davidson, P.M. (1998) Naturally occurring antimicrobials in food. *Council for Agricultural Science and Technology*.

Soni, K.A., Nannapaneni, R. and Hagens, S. (2010) Reduction of *Listeria monocytogenes* on the surface of fresh channel catfish fillets by bacteriophage Listex P100. *Foodborne Pathog Dis* **7**, 427–434.

Vanderhaeghen, W., Cerpentier, T., Adriaensen, C., Vicca, J., Hermens, K. and Butaye, P. (2010). Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. *Vet Microbiol* **144**, 166-171.

Viazis, S., Akhtar, M., Feirtag, J. and Diez-Gonzalez, F. (2011) Reduction of *Escherichia coli* O157: H7 viability on leafy green vegetables by treatment with a bacteriophage mixture and transcinnamaldehyde. *Food Microbiol*. **28**, 149-157.

Wang, Q. and Sabour, P.M. (2010) Encapsulation and controlled release of bacteriophages for food animal production. In *Bacteriophages in the Control of Food- and Waterborne Pathogens* eds. Sabour, P.M. and Griffiths, M.W. *Washington: ASM Press*, 237–255.

Waters, A.E., Contente-Cuomo, T., Buchhagen, J., Liu, C.M., Watson, L., Pearce, K., Foster, J.T., Bowers, J., Driebe, E.M., Engelthaler, D.M., Keim, P.S. and Price, L.B. (2011) Multidrug-resistant *Staphylococcus aureus* in US meat and poultry. *Clin Infect Dis* **52**, 1227-1230.

Weese, J.S., Reid-Smith, R., Rousseau, J. and Avery, B. (2010) Methicillin-resistant *Staphylococcus aureus* (MRSA) contamination of retail pork. *Can Ver J* **51**, 749-752.

Weiss, J., Gaysinsky, S., Davidson, M., McClements, J. (2009) Nanostructured encapsulation systems: Food antimicrobials. *Global Issues Food Sci Technol* **4,** 425–479.

Wendlandt, S., Schwarz, S., Silley, P. (2013) Methicillin-resistant *Staphylococcus aureus*: A foodborne pathogen? *Annu Rev Food Sci Technol* **4**, 1-23.

Ye, J., Kostrzynska, M., Dunfield, K. and Warriner, K. (2010) Control of *Salmonella* on sprouting mung bean and alfalfa seeds by using a biocontrol preparation based on antagonistic bacteria and lytic bacteriophages. *J Food Prot* **73**, 9–17.

Yoon, H., Yun, J., Lim, J.A., Roh, E., Jung, K.S., Chang, Y., Ryu, S., and Heu, S. (2013) Characterization and genomic analysis of two *Staphylococcus aureus* bacteriophages isolated from poultry/ livestock farms. *J Gen Virol* **94**, 2569-2576.

Zaczek, M., Weber-Dabrowska, B. and Gorski, A. (2015) Phages in the global fruit and vegetable industry. *J Appl Microbiol* **118**, 537-556.

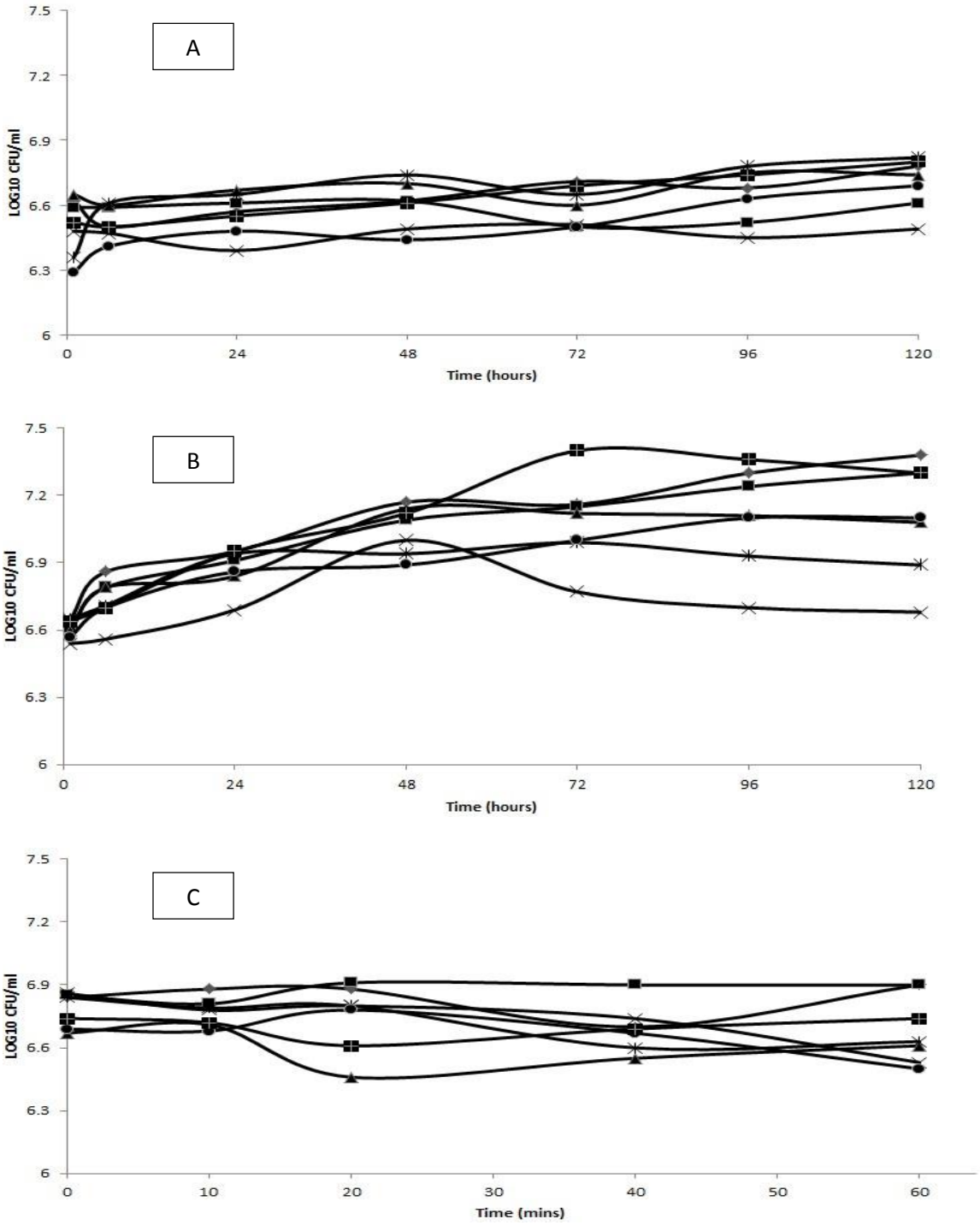


Figure 1. Effect of phage K and two essential oils at various time points on the survival of *S. aureus* at (A) 6°C, (B) 13°C and (C) 25°C. Line styles used in the graph are indexed as follows:

Chapter 4: Overall Conclusions

Staphylococcus aureus is an important human pathogen whose ability to acquire resistance mechanisms and other pathogenic determinants has added to its emergence in both acute and community healthcare settings (Adukwu *et al*., 2012). *Staphylococcus aureus* are notorious for their resistance to antibiotics. The occurrence of methicillin resistant *S. aureus* (MRSA) and the detection of a vancomycin resistance gene in *S. aureus* has led to increased concern regarding the bacteria (Panneerseelan and Muriana, 2009). Since *S. aureus* is a commensal and lives on the anatomical locales of humans, food handlers are often a common source of contamination of foods with *S. aureus* (Gutierrez *et al.,* 2012). Unfortunately, management of *S. aureus* infections in humans has become compromised as a result of multiple antibiotic resistant strains including MRSA and vancomycin resistant *S. aureus* (VRSA (Panneerseelan and Muriana, 2009). Numerous studies have discovered promising novel antimicrobial effects of plant-derived essential oils (EOs) and bacteriophages (Muthaiyan *et al*., 2012; Zaczek *et al*., 2015). Tea tree oil, ajwaine oil, citrus oil, olive oil, lemongrass, and cinnamon bark have been shown to be lethal to *S. aureus* (Chao *et al*., 2008; Upadhyay *et al.,* 2010; Edwards-Jones *et al.,* 2004). There are a number of host specific bacteriophages that are used in the food industry. For instance, *S. aureus*-specific bacteriophage use during cheese manufacturing (Bueno *et al.,* 2012) and commercially available bacteriophage cocktails (e.g., Listex™ P100 and ListShield™) are used for control of *L. monocytogenes* in raw and RTE foods (Soni *et al*., 2010). Moreover, bacteriophages are now accepted as natural food additives since they are naturally present in the digestive tract of humans and throughout the environment (Monk *et al*., 2010).

In the research presented here, I hypothesized that combining *S. aureus*-specific bacteriophage with EOs can inhibit the growth of *S. aureus* better than either treatment alone. This is a novel

approach as this is the first study to report on the use of these two antimicrobials against *S. aureus*. Similar research by Viazis *et al*. (2011) indicated that the combination of *E. coli* specific bacteriophage BEC8 and *trans*-cinnamaldehyde proved highly effective against *E.coli* O157:H7 on leafy greens. Therefore, based on the reported antimicrobial potential of both EOs and host-specific bacteriophage, I proposed to investigate the efficacy of varying concentrations of EOs alone and in combination with *S. aureus* specific bacteriophage in order to determine potential synergistic effects. More specifically, I aimed 1) to determine the efficacy of varying concentrations of pure EOs compounds against *S. aureus*; 2) to evaluate the efficacy of a *S. aureus*-specific bacteriophage against four strains of *S. aureus* and 3) to combine these antimicrobials to determine potential synergism and possible application for the control of *S. aureus* on raw meat product such as chicken. Chapter 2 focused on the investigation of the efficacy of pine oil compounds alone in combination with *S. aureus* bacteriophage K. For the screening of the pure oil compounds, disc diffusion assay was used and four EOs were chosen including alpha-pinene, beta-pinene, 3-carene, and limonene as well as five different *S. aureus* strains. Based on the DDA, I concluded that alpha-pinene showed significantly higher effect against all *S. aureus* strains. Further, growth inhibition assay was performed to analyze which concentration of EO would have the greatest inhibitory effect. It was concluded that alpha-pinene and alpha-pinene+3-carene at 1.5% and 3.28% showed subsequently higher inhibitory effect.

In order to evaluate the adsorption properties of bacteriophage K, a phage adsorption assay was used. I observed that at 37°C, all *S. aureus* strains were susceptible to bacteriophage K. Although, at 6 and 13°C, the lytic activity of bacteriophage K gradually decreased over a period of 6 hours against *S. aureus* strains. Phage adsorption assay was also performed over a prolonged period of time (24 to 120 hours) at 6, 13, and 37°C. At 6°C, the number of PFU against all *S.*

aureus strains showed fluctuations before decreasing after 120 hours. At 13°C, the number of PFU remained more or less constant after 72 hours. As expected, at 37°C the number of PFU was 3 log higher after 24 hours as compared to 6 and 13°C. Last, I tested the effect of both of these antimicrobials in combination *in vitro.* Over a period of time all four *S. aureus* strains showed a significant reduction in growth, though bacteriophage K with 3.28% α-pinene inhibited *S. aureus* growth better than other combinations of EOs and bacteriophage depending on the strain.

The overall goal of this research was to utilize these antimicrobial compounds for control of *S. aureus* as a foodborne pathogen with potential applications in the food industry such as inactivation of *S. aureus* of raw chicken products. Hence, these antimicrobials were evaluated against *S. aureus* on raw chicken pieces at 6, 13 and 25°C. Interestingly, the inhibitory effect of these antimicrobials were limited on food matrix. Results indicated that at 25°C bacteriophage K alone inhibited *S. aureus* growth better as compared to other antimicrobial combination. At 6 and 13°C, there was no significant effect of EO and bacteriophage alone or in combination against *S. aureus* when applied on the raw chicken pieces. This indicates that a better delivery method needs to be employed in order to get the desired inhibitory effect.

Overall, this research has increased our knowledge related to interactions between antimicrobials against *S. aureus*. There is a need for more in depth studies related to the inhibitory effect of bacteriophage and EO together and alone against bacterial pathogens such as *S. aureus*.

References

Adukwu, E.C., Allen, S.C., Phillips, C.A. (2012) The anti-biofilm activity of lemongrass (*Cymbopogon flexuosus*) and grapefruit (*Citrus paradisi*) essential oils against five strains of *Staphylococcus aureus. J Appl Microbiol* **113**, 1217-1227.

Chao, S., Young, G., Oberg, C. and Nakaoka, K. (2008) Inhibition of methicillin-resistant *Staphylococcus aureus* (MRSA) by essential oils. *Flavour Fragr J* **23**, 444-449.

Edwards-Jones, V., Buck, R., Shawcross, S.G., Dawson, M.M. and Dunn, K. (2004) The effect of essential oils on methicillin-resistant *Staphylococcus aureus* using a dressing model. *J Burns* **2**, 772- 777.

Garcia, P., Martinez, B., Obeso, J.M., Rodriquez, A. (2008) Bacteriophages and their application in food safety. *Lett Appl Microbiol* **47**, 479-485.

Gutierrez, D., Delgado, S., Vazquez-Sanchez, D., Martinez, B., Cabo, M.L., Rodriquez, A., Herrera, J.J. and Garcia, P. (2012) Incidence of *Staphylococcus aureus* and analysis of associated bacterial communities on food industry surfaces. *Appl Environ Microbiol* **78**, 8547-54.

Monk, A.B., Rees, C.D., Barrow, P., Hagens, S. and Harper, D.R. (2010) Bacteriophage applications: where are we now? *Lett Appl Microbiol* **51**, 363-369.

Muthaiyan, A., Martin, E.M., Natesan, S., Crandall, P.G., Wilkinson, B.J. and Ricke, S.C. (2012) Antimicrobial effect and mode of action of terpeneless cold-pressed Valencia orange essential oil on methicillin-resistant *Staphylococcus aureus*. *J Appl Microbiol* **112**, 1020-1033.

O'Brien, A.M., Hanson, B.M., Farina, S.A., Wu, J.Y., Simmering, J.E., Wardyn, S.E., Forshey, B.M., Kulick, M.E., Wallinga, D.B. and Smith, T.A. (2012) MRSA in conventional and alternative retail pork products. *PLoS ONE* **7**, e30092.

Panneerseelan, L. and Muriana, P.M. (2009) An immunomagnetic PCR signal amplification assay for sensitive detection of *Staphylococcus aureus* enterotoxins in foods. *J Food Protect* **72**, 2538-2546.

Upadhyay, R.K., Dwivedi, P. and Ahmad, S. (2010) Screening of antibacterial activity of six plant essential oils against pathogenic bacterial strains. *Asian J Med Sci* **2**, 152-158.

Zaczek, M., Weber-Dabrowska, B. and Gorski, A. (2015) Phages in the global fruit and vegetable industry. *J Appl Microbiol* **118**, 537-556.

Viazis, S., Akhtar, M., Feirtag, J. and Diez-Gonzalez, F. (2011) Reduction of *Escherichia coli* O157: H7 viability on leafy green vegetables by treatment with a bacteriophage mixture and transcinnamaldehyde. *Food Microbiol*. **28**, 149-157.

Appendix: Institutional Biosafety Committee Protocol Approval

UNIVERSITY OF ARKANSAS

Office of Research Compliance

August 14, 2012

MEMORANDUM

TO: Dr. Steven Ricke

FROM: W. Roy Penney
Institutional BioSafety Committee

RE: IBC Protocol Approval

IBC Protocol #: 13005

Protocol Title: "Enhanced Treatment of Highly Virulent Methicillin Resistant Staphylococcus aureus through a Novel, Combined Plant Essential Oil – Bacteriophage Therapy"

Approved Project Period: Start Date: August 9, 2012
Expiration Date: August 8, 2015

The Institutional Biosafety Committee (IBC) has approved Protocol 13005, "Enhanced Treatment of Highly Virulent Methicillin Resistant Staphylococcus aureus through a Novel, Combined Plant Essential Oil – Bacteriophage Therapy" You may begin your study.

If further modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199